Disruption of the *Staphylococcus aureus* biofilm by IsaA

by

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Abstract

Staphylococcus aureus, a human commensal and pathogen, is capable of forming biofilms on a variety of host tissues and implanted medical devices. Biofilm-associated infections resist antimicrobial chemotherapy and attack from the host immune system, making these infections particularly difficult to treat. To gain insight into environmental conditions that influence *S. aureus* biofilm development, we screened a library of small molecules for the ability to inhibit *S. aureus* biofilm formation. This led to the finding that the polyphenolic compound tannic acid inhibits *S. aureus* biofilm formation in multiple biofilm models without inhibiting bacterial growth. We present evidence that tannic acid inhibits *S. aureus* biofilm formation via a mechanism dependent upon the putative transglycosylase IsaA. Tannin-containing drinks like tea have been found to reduce methicillin-resistant *S. aureus* nasal colonization; we found that black tea inhibited *S. aureus* biofilm development and that an *isaA* mutant resisted this inhibition. We developed a rodent model for *S. aureus* throat colonization and found that tea consumption reduced *S. aureus* throat colonization via an *isaA*-dependent mechanism.
We also showed two distinct mechanisms by which *S. aureus* adapts to resist tannic acid stress. First, when the dedicated regulator of the Sigma B stress response system, *rsbU*, is mutated, the cells overproduce extracellular proteases, clearing IsaA from the extracellular milieu. Second, when the serine threonine kinase *pknB* is mutated, PIA (a major component of the biofilm matrix) is overproduced, which we hypothesize strengthens the overall structure of the matrix, conferring resistance to tannic acid. These findings provide insight into a molecular mechanism by which commonly consumed polyphenolic compounds, such as tannins, influence *S. aureus* surface colonization, as well as how bacteria can adapt to evade such antibiofilm treatments.

CHAPTER 1

General introduction

Staphylococcus aureus

In 1880, a Scottish surgeon named Alexander Ogston began investigating the cause of abscess wounds found on his patients. He hypothesized that the abscesses were the result of infecting microorganisms. Observing stained pus under a microscope, Ogston saw two different types of microbes [1,2]. One, growing in chains, was the already-described *Streptococcus*. The other was a novel bacterium that he described as appearing in clusters "like the roe of fish." He later named this second type *Staphylococcus* [3].

Among the *Staphylococci*, *Staphylococcus aureus* is of particular interest today. *S. aureus* is best known as a versatile pathogen, causing bacteremia, endocarditis, sepsis, skin and soft tissue infections, implanted device infections, and several other relevant human diseases [3–9]. The mortality of patients with *S. aureus* bacteremia in the pre-antibiotic era exceeded 80%, and over 70% developed metastatic infections [10]. Considering that *S. aureus* kills approximately 19,000 Americans annually (significantly more than AIDS) and the fact that *S. aureus* antibiotic resistance is rapidly increasing [11], there is an urgent need to better understand this pathogen and develop novel treatment strategies.

S. aureus is also able to colonize a host non-pathogenically. This sort of commensal colonization is very common in the human nose, throat, and on several other body sites [12–14].

The proportion of people who are colonized varies depending on the specific population under study, but it is commonly agreed upon that approximately 20-50% of the healthy adult population is colonized with *S. aureus* in their noses [12,15,16]. Colonization is a major risk factor for Staphylococcal disease [17]; for example, several studies have found surgical patients have a 7-10 fold increase in the likelihood of developing *S. aureus* surgical site infections if they are nasally colonized [18,19].

Biofilms

For a century and a half, scientists primarily studied bacteria shaking in rich liquid media. Although this allowed us to understand many facets of bacterial form, physiology, and metabolism, the picture we saw was incomplete because these conditions do not represent the spectrum of lifestyles that bacteria live. Whether we are discussing environmental bacteria growing on river rocks or pathogens growing on a damaged heart valve, many bacteria live much of their lives attached to surfaces. Figure 1 shows an electron micrograph of a surface-attached community (termed a biolfilm) of *S. aureus* growing on a catheter. The study of bacteria in biofilms, largely pioneered by Costerton, opened up completely novel ideas about how bacteria live and interact with their environment [20].

The biofilm matrix

Biofilms are found encased in a polymeric matrix. Although producing this matrix is costly, it allows the bacterial community to survive a wide array of insults and harsh environments. The exact composition of this matrix varies tremendously depending on both the environmental conditions and the specific bacteria that compose the biofilm. Despite this variability, there are a few classes of molecules that are typically represented in the matrix, and are generally agreed upon to be the major components. These classes are protein adhesins, extracellular DNA

(eDNA), polysaccharide, and amyloid fibers. A brief overview of these macromolecules and a few representatives from each class follows.

Protein adhesins

There are many different proteins involved in surface adhesion, and their roles are often highly redundant and poorly understood. Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) specifically recognize and interact with host factors to facilitate attachment of bacteria to surfaces within a host [21]. These include proteins that bind to fibronectin, fibrinogen, collagen, and other host proteins.

An example of a protein adhesin, biofilm-associated protein (Bap) was discovered in certain *S. aureus* strains as a cell wall-anchored protein mediating initial attachment to biotic and abiotic surfaces [22]. Further investigation revealed Bap homologs in *Enterococcus faecalis* [23], *E. coli* [24], *Salmonella enteritidis* [25], and other species.

eDNA

eDNA is a major structural component of biofilms formed by many species. The importance of this component in biofilms formed by many species can be seen in the fact that exogenous DNase addition can either inhibit or disperse biofilms of *E. coli* [26], *P. aeruginosa* [27], *N. gonorrhoeae* [28], *S. aureus* [29], and others.

Matrix eDNA can come from several sources. In many cases, it comes from autolysis of a subpopulation of cells in the biofilm. This autolysis can result from either an altruistic, suicidal mechanism [30] or through a coordinated fratricidal mechanism whereby the whole population produces a toxin, and all but a small subset also produce the antitoxin, thereby lysing a subpopulation to release eDNA [31].

eDNA can also be generated without lysis. DNA has been found to be released through membrane vesicles by several gram negative bacteria, including *P. aeruginosa*, *E. coli*, *S. typhimurium*, and *Y. pestis* [32,33]. These DNA-containing vesicles have been shown to promote biofilm formation, though the specific role of the DNA itself remains controversial. DNA can also be released from *N. gonorrhoeae* by a type IV secretion system [34].

Amyloids

Amyloids are incredibly resilient fibers that form from protein oligomers. Although amyloids were originally thought to be the result of protein folding error (and therefore associated only with various human disease states), work by Chapman and others showed that amyloids can be functional [35]. The most studied functional amyloids are those that stabilize the biofilm matrix.

Amyloids share many properties due to a unique fold, but the primary structure of the protein monomers is not conserved. This means that the amyloid components of biofilms formed by many species of bacteria are diverse. For example, *Bacillus subtilis* produces amyloids formed of the 261-residue TasA [36], while *S. aureus* makes its amyloids of the Phenol Soluble Modulin (PSM) peptides, which are as small as 20 residues long [37]. Some, like the PSMs, have multiple functions, while others, such as *E. coli*'s CsgA, appear to have the sole function of stabilizing the biofilm [35,38].

Polysaccharides

The polysaccharide component of the biofilm matrix is thought to be generally the most abundant. Although matrix composition is highly variable, it is estimated that on average 50-90% of the organic carbon in a biofilm is found in the polysaccharide [39]. Like the other components of the biofilm matrix, the polysaccharide component is diverse. Different bacteria

produce dramatically different polysaccharides. *E. coli* and *Salmonella typhimurium*, for example, produce the uncharged, homopolymeric cellulose [40]. *S. aureus*, by contrast, produces a polycationic homopolymer called Polysaccharide Intercelluar Adhesin (PIA) [41]. *Streptococcus thermophilus* produces three distinct heteropolymeric polysaccharides as part of its biofilm matrix [42].

These polysaccharides can play many roles in the biofilm, including aiding in initial adherence to a surface [43], adding structural stability [44], serving as a nutrient storage reservoir [45], retaining water to prevent desiccation [46], and protecting from the host immune system [47].

Biofilms in a human host

Bacterial biofilms are found in diverse environments, including industrial (such as *Pseudomonas aeruginosa* in the pipes in water treatment plants [48]), natural (such as *Legionella pneumophila* in freshwater lakes [49]), and clinical (such as *S. aureus* on a damaged heart valve [50]). Of particular interest to this work are biofilms that grow in and on a human host.

There is controversy in the field of biofilm study about whether or not bacteria are in a biofilm state when colonizing various host sites. Much of this controversy is a result of definitional arguments about what constitutes a biofilm. Resolving this conflict is not trivial. One problem is the lack of a single defining physiological marker of the biofilm state. Further, because biofilms are by definition a coordinated group lifestyle in response to physiological and environmental conditions, biofilms cannot easily be removed from the host and studied *ex vivo* without causing them to revert to a disorganized state. To simplify the discussion of biofilms in a human host, the term "biofilm" will be used somewhat loosely to encompass circumstances where cells are living in microcolonies attached to, or associated with, a surface.

The interactions of biofilms with their human hosts fall on a spectrum from mutualistic to parasitic. On the mutualistic end, we have interactions like the one occurring in the large intestine. There, a diverse population of microbes forms a complex, multispecies biofilm on the gut epithelial surface. The benefits to the bacteria include a safe, nutrient rich environment where temperature is constant and waste is removed by the host. The bacteria, in turn, benefit the host by aiding in digestion and excluding pathogens [51].

On the far end of the spectrum from this interaction, there are many examples of disease-associated biofilms. Dental caries are caused by acid produced by a multispecies biofilm growing on teeth [52]. Urinary tract infections involve *E. coli* and other species growing within epithelial cells of the urinary tract as biofilms [53]. *S. aureus* biofilms growing on damaged heart valves can cause infective endocarditis, leading to impaired heart function, bloodstream infections, and embolisms [54]. Lungs of cystic fibrosis (CF) patients are densely colonized with biofilms consisting primarily of *P. aeruginosa*. Although the lungs are typically sterile, CF patients are persistently colonized for the rest of their lives [55]. Indwelling devices rapidly become coated in a biofilm of *S. aureus* or *S. epidermidis*, which can only be cleared by surgically removing and replacing the device [56,57].

Between the two extremes of mutualism and parasitism, bacteria can also colonize host surfaces in a commensal fashion. *Streptococcus pneumoniae* colonizes the human nasopharynx asymptomatically [58]. *Clostridium difficile* is a common colonizer in human intestines, particularly in infants [59]. The human skin is teaming with diverse bacteria, notably *Staphylococcus epidermidis* [60]. *S. aureus* commensally colonizes the human nose, throat, and other body sites [12].

These categories of symbiosis are not fixed, but rather fluid. *S. pneumoniae* living as a commensal in the nasopharynx can, under certain circumstances, invade the inner ear to cause otitis media [61], the respiratory tract to cause pneumonia [62], or the blood to cause meningitis [63]. When antibiotics perturb the normal flora of the gut, *C. difficile* can transition from its commensal state to cause severe diarrhea.

Of particular interest to this work is *S. aureus*' transition from commensal to pathogen. Surgical patients who carry *S. aureus* in their nose have a 7-fold increase in their risk of *S. aureus* surgical site infection. In those who are infected, 30-100% are infected by the same strain that inhabited their nose prior to surgery, suggesting that the bacteria traveled from the nose to the surgical site, invaded, and colonized new surfaces there [18].

Biofilm Regulation

Biofilms form when planktonic bacteria attach to a surface, proliferate on that surface, and encase themselves in a polymeric matrix (Figure 2). These phases of the biofilm lifecycle are tightly regulated. However, because biofilms are so diverse, there is no single, ubiquitous biofilm regulatory system. Even within a specific species, the regulation can differ among different types of biofilm formed under different growth conditions. Therefore, a comprehensive review of all biofilm regulatory pathways is impractical for this document. Instead, this section will focus on biofilm regulation in *S. aureus*, as it is the most relevant organism to the rest of this work.

The best studied mode of biofilm regulation in *S. aureus* is regulation of the polysaccharide component of the biofilm matrix, the Polysaccharide Intercelluar Adhesin (PIA). PIA is produced by the enzymes encoded in the Intercellular Adhesin (*ica*) operon. The *ica* genes were

discovered in a transposon screen of *S. epidermidis*, looking for genes that were important for biofilm formation [64] and further investigation revealed homologues in *S. aureus* [65]. At the time of their discovery, the *ica* genes were thought to be indispensable for biofilm formation in *S. aureus*.

The *S. aureus ica* locus consists of five genes: the *icaADBC* operon and the divergently transcribed regulatory gene *icaR*. IcaA and IcaD work together to produce short oligomers of N-acetylglucosamine, about 20 sugars long [66]. IcaC is thought to be responsible for transporting the oligomers across the membrane to the outside of the cell. IcaB deacetylates the polymer, allowing it to be attached to the cell surface and to participate in biofilm formation [67]. IcaR is a *tetR* family transcriptional repressor [68].

PIA production (and therefore PIA-dependent biofilm formation) is controlled by several genetic regulatory systems. Spx regulates a number of cell functions in response to the redox state of the cell. When Spx is active, *icaR* expression is increased, decreasing PIA production [69]. Another global regulator, SarA (Staphylococcal Accessory Regulator), is required for *ica* transcription [70]. It is worth noting that, although SarA regulates expression of *agr* (another master regulator to be discussed later in this section), SarA's regulation of *ica* is *agr*-independent. σ^B, a master regulator of the *S. aureus* stress response, has been shown both to regulate PIA production [70] and not to regulate PIA production [71], indicating that σ^B involvement in biofilm regulation and PIA production is complex and likely dependent on strain and/or growth conditions.

In addition to these genetic regulation mechanisms, several environmental conditions have been shown to regulate PIA production. Subinhibitory concentrations of certain antibiotics, including tetracycline, increase *ica* expression through undefined mechanisms [72]. NaCl increases *ica*

expression both by directly promoting transcription of the *icaADBC* operon and indirectly by repressing transcription of the *icaR* repressor [73]. Anaerobic [74] and nutrient-limiting [75] conditions, such as those found deep within a biofilm, induce PIA production. This suggests that once the cells begin the process of biofilm formation, they enter a positive feedback loop, wherein more PIA leads to a denser, more hypoxic, less nutrient-dense biofilm, which in turn leads to more PIA production.

When PIA was discovered to be produced by *S. aureus*, it was thought to be absolutely essential for biofilm formation [65]. This was such a prevalent belief that "slime" and "PIA" were used interchangeably with "biofilm matrix." This was later shown to not be the case as PIA-independent biofilms were discovered [76]. This was particularly surprising because in *S. aureus*, unlike *S. epidermidis*, all sequenced isolates carry an intact *icaADBC* operon [77]. These PIA-independent biofilms share some regulatory pathways with PIA-dependent biofilms, but also involve unique regulators.

Several protein adhesins, including the previously mentioned Bap, have been shown to be sufficient to induce biofilm formation, even when the *ica* locus was mutated [22,78]. Shortly after this observation was made, Smeltzer showed that deletion of the *ica* locus in UAMS-1, a relevant clinical isolate, did not inhibit biofilm formation [79]. In both cases, deletion of *sarA* abrogated biofilm formation, demonstrating a role for SarA in both PIA-dependent and PIA-independent biofilm regulation. A likely role of SarA in PIA-independent biofilm regulation is its role in downregulating several extracellular proteases [80].

The major quorum-sensing system in *S. aureus* is the Accessory Gene Regulator (*agr*) system. Quorum-sensing is often associated with biofilm formation, so *agr* is heavily studied in

relationship to biofilm regulation in *S. aureus*. The results of these studies have been mixed, with different researchers showing that agr mutations inhibit, have no effect on, and promote biofilm formation [81,82]. This variability is typically explained as reflecting differences in strains and growth conditions [83].

Another sensory system implicated in biofilm regulation is the ArlRS (Autolysis Related Locus) two component system [84]. When the system is disrupted by transposon insertion, an *ica*-deficient strain forms a robust biofilm. Although little is written about this system's role in biofilm regulation in *S. aureus*, two points are worth noting. First, the biofilm phenotype of the *arlRS* mutant appears to be independent of autolysis. Second, an *arlRS* mutant has less extracellular protease activity than the wild-type [85].

Although there are diverse mechanisms of PIA-independent biofilm regulation, many of them have a common theme of either upregulating protein adhesins or downregulating extracellular proteases, which would effectively increase the abundance of extracellular protein adhesins. This underscores the importance of understanding the roles of the different molecules forming the biofilm matrix.

Antibiofilm strategies

The National Institutes of Health estimate that 80% of bacterial infections are biofilm-related [86]. Because biofilms can be 1000-fold more resistant to antibiotics, these infections are typically not able to be cleared by antibiotic treatment. To address this rising health problem, researchers are turning to alternative methods of biofilm inhibition.

Natural products, small molecule metabolites produced by plants and many forms of marine life, are a rich source of anti-biofilm compounds. Treatment with garlic extracts, for example, has

been shown both to clear pulmonary *P. aeruginosa* infections in a mouse model and to make *in vitro* biofilms more susceptible to antibiotic treatment [87]. Phloretin, an antioxidant found in apples, was able to inhibit biofilm formation in some *E. coli* strains (including enterohemorrhagic strain O157:H7) while leaving others (including commensal K12 strains) undisturbed [88]. Sea sponges are simple marine animals that produce a wide range of effective anti-biofilm compounds [89].

One specific way that natural products inhibit biofilms is by inhibiting quorum sensing. For example, flavonoids from various citrus species act as antagonists to the quorum sensing molecules homoserine lactone and AI-2 from *V. harveyi* [90]. Extract from grapefruit juice inhibits quorum sensing-mediated biofilm formation in *E. coli* [91]. Due to the success of natural products in this area, researchers have focused on finding other ways to inhibit quorum sensing, particularly ways to synthesize optimized inhibitors based on these natural compounds. [92,93].

Antimicrobial peptides (AMPs) are small peptides produced by the host to fight off bacterial infection, and are highly effective against both planktonic and biofilm cells. These peptides generally kill by permeabilizing the bacterial cell membrane, though other AMPs have different activities such as specifically destabilizing the biofilm matrix [94]. Lactoferrin and its semi-synthetic derivatives are effective at reducing *P. aeruginosa* biofilms, presumably through their ability to chelate iron [95]. At sub-growth-inhibitory concentrations, Cathelicidins (the class of AMPs that includes human LL-37) inhibit biofilm formation of several species, including *E. coli*, *S. epidermidis*, and *S. aureus* [96].

Much attention has been given to making various surfaces less hospitable for colonization and biofilm formation. For example, Sharklet is a company that sells items ranging from iPhone

cases to urinary catheters stamped with a "micropattern" that they claim makes the item resistant to biofilm colonization [97]. Others coat the surface with silver nanoparticles, which are toxic to bacterial cells [98]. A recent trend has been to impregnate surfaces with the antibiotics like triclosan, but evidence has shown that triclosan and many other antibiotics actually increase biofilm formation [99].

While there are many ways to inhibit biofilm formation, they all have limitations and drawbacks. Natural products are a treasure trove of bioactive compounds, but finding the ones that have a particular desired effect is searching for a needle in the proverbial haystack. Quorum inhibitors often modulate other processes in both the host and the bacterium, making their effects hard to predict. AMPs, while very powerful, have not evolved in a vacuum; as host organisms have evolved these peptides to fight infection, invaders have evolved proteases and other means of evading them. The surface-centered solutions are limited by several factors, notably that in any medical context, the surface would be coated with host proteins, blocking any interaction between the biofilm and the surface. Therefore new approaches to limit biofilm formation or disrupt established biofilms are urgently needed.

Conclusion

Biofilms are an important mode of bacterial growth, distinct from the planktonic state in which bacteria are often studied. Many human health problems are caused by biofilm-associated infections and exacerbated by their resistance to antimicrobial chemotherapies. Our understanding of how biofilm formation is regulated, and when and how biofilms form, is incomplete. Although advances have been made to address the problems that stem from biofilms, there remains a significant need for practical and effective interventions.

The research laid out in this work begins with addressing the problem of a lack of reliable anti-biofilm compounds for *S. aureus*.

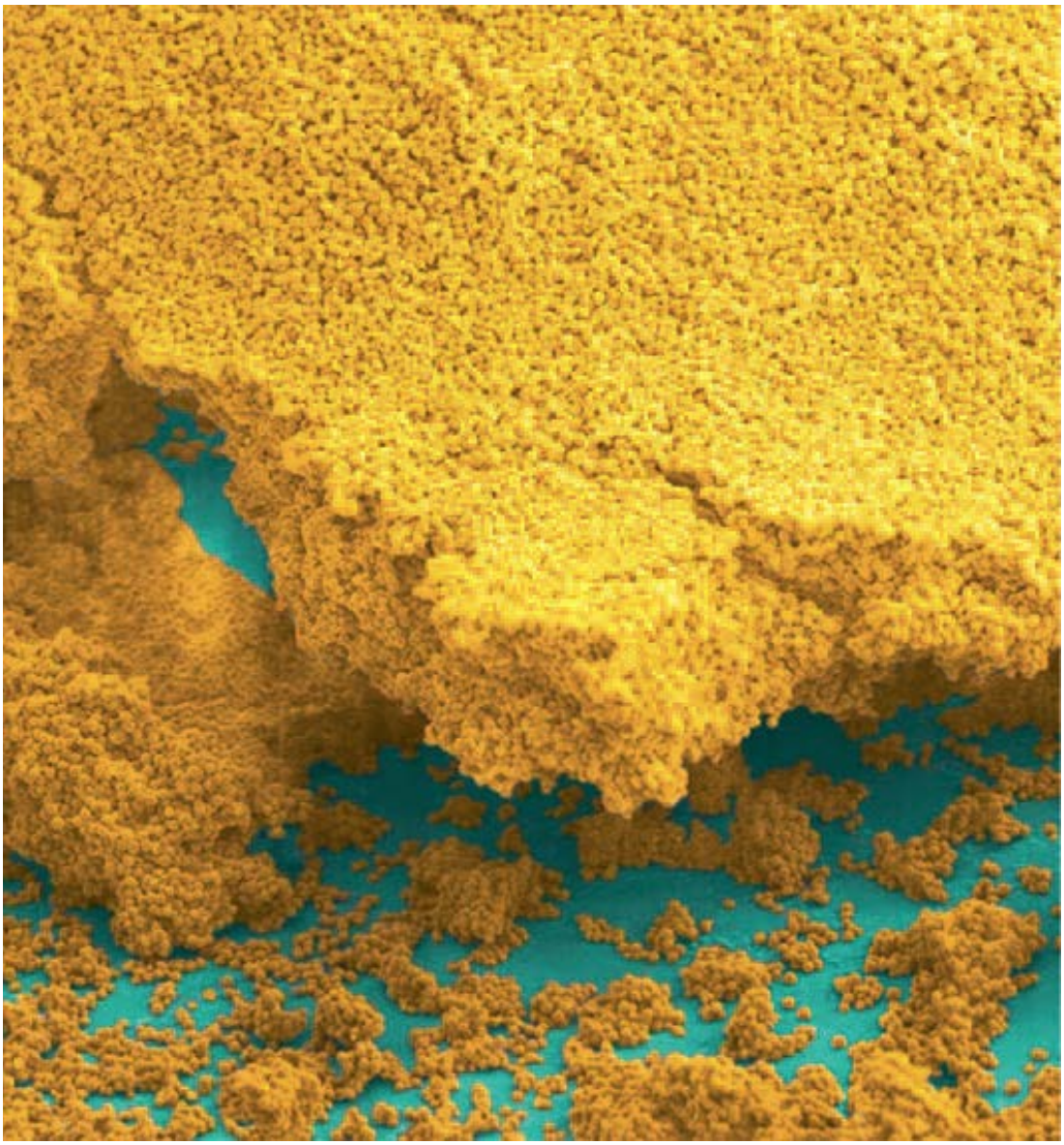

Figure 1. *S. aureus* biofilm.
SEM image of *S. aureus* biofilm grown on catheter. Reproduced from Boles and Horswill [100].

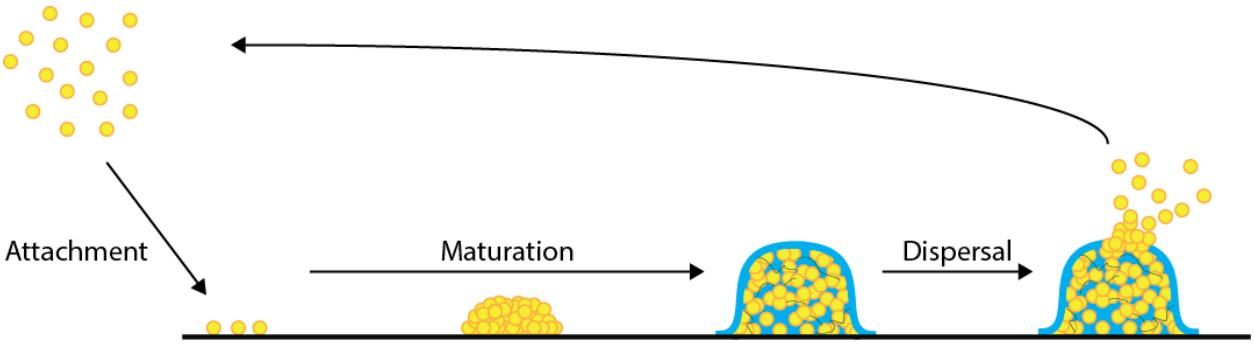

Figure 2. The biofilm lifecycle.
Biofilms form when planktonic cells attach to a surface, proliferate on that surface, and encase themselves in a protective matrix. Under certain conditions, cells can disperse from the biofilm, reverting to a planktonic state.

CHAPTER 2

IsaA inhibits biofilm formation

Introduction

Staphylococcus aureus is a gram-positive bacterium that exists both as a commensal, commonly colonizing humans, and as a deadly pathogen, possessing the ability to cause a multitude of infections [13,14,101]. The ability of *S. aureus* to colonize surfaces contributes to its lifestyle as both a commensal and a pathogen [102]. When colonizing a surface, *S. aureus* forms a structured community called a biofilm, in which cells are encased in a polymeric matrix. Although the exact composition of this matrix varies greatly from strain to strain and between different growth conditions, its components include extracellular DNA, polysaccharides, proteins, and amyloid fibers [37,100]. The variability among biofilms formed by *S. aureus* contributes to its ability to colonize humans and cause many different kinds of biofilm-associated infections including osteomyelitis [103], endocarditis [7], and implanted device infections [104].

Management of biofilm infections is extremely difficult due to their inherent resistance to both antimicrobial chemotherapies and the host immune response [102,105]. New approaches are needed to overcome the challenge of antimicrobial resistance. Enzymatic disruption of the biofilm matrix and altering gene expression to induce biofilm disassembly are currently among the alternatives being investigated [100]. In addition, much research has focused on understanding the environmental conditions and bacterial molecular mechanisms that influence *S. aureus* biofilm development. Exposure to glucose, osmolarity, ethanol, hemoglobin, temperature, and anaerobiosis, have been reported to affect biofilm formation and disassembly [41,100,106,107].

Beyond these examples, little is known about the contribution of other environmental conditions and the molecular mechanisms that respond to them to control biofilm development. A deeper

understanding of these environmental cues may lead to innovative treatments for *S. aureus* biofilm infections. Therefore, we set out to look for novel environmental factors that could influence *S. aureus* biofilm development by screening a small chemical library.

The data presented in this chapter demonstrate that the polyphenolic compound tannic acid inhibits *S. aureus* biofilm formation by increasing the abundance of the lytic transglycosylase IsaA. We show that, although tannic acid is a known antimicrobial, its antibiofilm effect is not due to growth inhibition. Further, we show that increasing IsaA abundance by other means (such as through use of an overexpression vector) has the same effect. Finally, we show that tannic acid's antibiofilm effect can be seen *in vivo*, as black tea eliminates *S. aureus* throat colonization in a cotton rat model.

Materials and Methods

Strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1. Strains of *Escherichia coli* were cultured in Luria-Bertani broth or on Luria agar plates. For selection of chromosomal markers or maintenance of plasmids, *E. coli* antibiotic concentrations were (in µg/ml): ampicillin (Amp) 100; chloramphenicol (Cam) 10. Except where noted, *S. aureus* strains were cultured in tryptic soy broth (TSB) or on tryptic soy agar (TSA). For selection of chromosomal markers or maintenance of plasmids, *S. aureus* antibiotic concentrations were (in µg/ml): erythromycin (Erm) 10; chloramphenicol (Cam) 10. All reagents were purchased from Fisher Scientific (Pittsburg, PA) or Sigma (St. Louis, MO) unless otherwise indicated. pKP1 was created by PCR amplifying the *isaA* ORF from SH1000 using primers o82 (5' – ATGCGGTACCCTTGCACTACGACATTCAAATTC – 3') and o83 (5' – ATGCGAATTCCTCTCCCCAATTTCTATGGG – 3'), and ligating this fragment into the multiple cloning site of pALC2073. pKP1-IsaA.EQ was created by PCR amplifying the pKP1 vector with overlapping primers o172 (5' – TCATCGCTCGTCAATCA – 3') and o173 (5' – TGACCATTTGATTGACGAG – 3'), both of which contain the desired point mutation. PCR product was treated with DpnI to remove the template plasmid and transformed. Mutated plasmids were verified by DNA sequencing.

Growth assays

SH1000 cultures were incubated at 37°C in TSBg (66% TSB with 0.2% glucose). OD_{600} was measured every 30 minutes, and ODs from log phase were used to calculate doubling time. Doubling times presented are averages from three biological replicates. To calculate population density in stationary phase, cultures were incubated for 24 hours, washed in PBS, bath sonicated

for 4 minutes, serially diluted, and plated on TSA. CFUs were counted the following day. CFU counts presented are averages from four biological replicates.

Biofilm assays

Microtiter plate biofilms were grown as previously described [106]. Briefly, late-log phase *S. aureus* cultures were diluted 1:200 in a final volume of 200 µl 66% TSB in wells of a 96-well microtiter plate (Nunc 164688). Glucose was added to a final concentration of 0.2% to induce biofilm formation. Plates were incubated overnight at 37°C with shaking at 200 RPM. After incubation, medium was removed by pipetting and wells were washed gently with 150 µl sterile water. 100 µl of 0.1% crystal violet was added and allowed to sit for 10 minutes. Crystal violet was removed by pipetting and wells were again washed with 150 µl sterile water. Plates were air dried and photographed. To quantitate crystal violet staining, 150 µl of 40 mM HCl in EtOH was added to each well, pipetted to mix, and allowed to sit for 5 minutes. Wells were again mixed, and 100 µl of stain was moved to a new plate and the absorbance at 595 nm was measured. All microtiter plate quantitations with multigroup comparisons were analyzed by ANOVA and found to have p-values of <0.05. Data sets were analyzed by a Dunnett's test, and the p-values of these tests are listed in the applicable figure legends.

Drip flow biofilms were set up and grown as described previously [37] with the growth medium being 2% tryptic soy broth (0.6 g/L) with 0.2% glucose (2 g/L). After 5 days of growth, stainless steel coupons were removed with sterile tweezers and biofilm cells were harvested into 10 mL sterile phosphate buffered saline (PBS). Samples were bath-sonicated for 10 minutes, serially diluted, and plated in plate count agar. Colonies were counted the following morning.

Flow cell biofilms were grown in 2% tryptic soy broth (0.6 g/L) with 0.2% glucose (2 g/L),

supplemented with tannic acid as indicated in the figure legends. Confocal scanning laser microscopy and image analysis was performed as described previously (11). Biofilms were treated with 330 nM Syto9 (LIVE/DEAD BacLight Bacterial Viability Kit; Invitrogen, Carlsbad, CA) 15 min prior to visualization.

Cotton rat oropharyngeal colonization model

To assess the ability of *S. aureus* to colonize the throat, a cotton rat throat colonization model was developed, based on previous studies done in mice studying *Streptococcus pyogenes* throat colonization [108]. Animal work was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on Use and Care of Animals (UCUCA) of the University of Michigan (Permit Number:10394). All efforts were made to minimize pain and discomfort during the procedure. Female cotton rats (age 6-8 weeks) were obtained from Harlen laboratories and housed 3 per cage in a room kept at 23°C ± 2°C with 50-60% relative humidity and a 12-h light-dark cycle. Rats were given tap water and rodent chow *ad libitum* and were acclimated to the laboratory environment for a minimum of 6 days before inoculation. *S. aureus* (BB2146 or BB2497) was cultured overnight in TSB, harvested by centrifugation, washed and resuspended in phosphate buffered saline (PBS). Female cotton rats were anesthetized, and a 100-µl aliquot containing 1×10^5 CFU was instilled into the throat of anesthetized animals via gavage. Throat cultures were taken using an alginate swab inserted into the oropharynx of anesthetized rats at indicated time points. The swab was streaked onto Mannitol Salt Agar containing spectinomycin at 100 µg/ml and these plates were incubated for 24 hours at 37°C. The growth of *S. aureus* colonies on these plates was interpreted as the animal being colonized. Tea was given to indicated animals at 2, 5, and 8 days after the initial *S. aureus* colonization by

slowly delivering 200 µl of room temperature tea (prepared as described below) into the anesthetized animal's throat via gavage. Control animals were given the same volume of water. Animals were held upright during gavage and monitored closely to avoid pulmonary aspiration.

Protein gels and Western blots

Cultures were incubated overnight at 37°C with shaking in 66% TSB + 0.2% glucose. Cultures were normalized by OD_{600} and cells removed by centrifugation. Culture supernatants were concentrated by TCA precipitation, boiled for 10 minutes in SDS running buffer, run on a 14% polyacrylamide gel, and stained with Coomassie. Western blotting was performed by boiling normalized culture supernatants for 10 minutes in SDS running buffer and separating on a 14% polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with a polyclonal anti-IsaA antibody. The IsaA antibody was generated in rabbits using the peptide DQLNAAPIKDGAYD, which corresponds to amino acids 48-61 of the IsaA protein.

Tea

Black tea was brewed by adding 100 ml boiling water to one bag of Twinings® English Breakfast tea and steeped for 7 minutes. Tea was cooled to room temperature and filter sterilized. Milk was made from powder to a concentration of 100 mg/ml (approximately the same concentration as is used to make milk from powder for consumption). To precipitate tannins from tea, 5 µl milk was mixed with 25 µl tea and 20 µl water. Mixture was allowed to sit at room temperature for 1 hour before use.

Results

Screen for biofilm-modulatory compounds

To identify chemicals and environmental conditions that influence *S. aureus* biofilm formation, we screened a collection of compounds contained in Biolog plates PM1-PM20 (Biolog, Inc., Hayward, CA) for the ability to inhibit or enhance surface colonization by SH1000, a common laboratory strain of *S. aureus* that descends from NCTC8325-4 [109]. Briefly, the contents of each well of the Biolog plates were resuspended in 102 µl distilled water, and 50 µl of this resuspension was added to microtiter plate assays under both conditions that are typically biofilm-inducing (66% TSBg), and not biofilm-inducing (66% TSB). Of the 1,920 conditions tested, 41 inhibited and 20 induced *S. aureus* biofilm formation (for a complete list, see Table 2).

Tannic acid inhibits *S. aureus* biofilm formation in multiple biofilm models

Among the compounds found to inhibit biofilm formation was tannic acid, a common component in teas and other plant-derived foods. Because *S. aureus* colonizes the oropharynx and oral cavity [13,110,111] and is likely to encounter this compound during colonization, we focused our efforts on tannic acid. We first confirmed and expanded the result from the screen, showing that tannic acid inhibited *S. aureus* biofilm formation in the microtiter plate biofilm assay. This activity was seen at low micromolar concentrations in a concentration-dependent manner (Figure 1A).

To ensure that this effect was not unique to one particular *in vitro* assay, we also tested tannic acid's ability to inhibit biofilm formation in flow cells and drip biofilm reactors. Tannic acid inhibited surface colonization in both of these established biofilm model systems (Figure 1B, C).

Tannic acid is a mix of plant-derived polyphenolic compounds, the specific composition of which varies tremendously from manufacturer to manufacturer, as well as from lot to lot. Two of the most abundant and consistently isolated components of commercially available tannic acid are gallic acid and pentagalloyl glucose [112] (Figure 2B). Therefore, we also tested the ability of these compounds alone to inhibit *S. aureus* biofilm formation. Gallic acid failed to inhibit *S. aureus* biofilm formation whereas pentagalloyl glucose inhibited at concentrations similar to those observed with tannic acid (Figure 2A).

20 μM tannic acid does not inhibit growth of *S aureus*

Because tannic acid is known to have antimicrobial activity, we investigated whether it affected *S. aureus* growth at the concentrations where we see anti-biofilm activity. We incubated cultures in 66% TSBg supplemented with up to 20 μM tannic acid and observed growth (Figure 1D, Table 3). In log phase, the doubling times of cultures grown with and without tannic acid were not statistically different. We also allowed the cultures to grow to stationary phase and counted CFUs in each culture. There was no statistical difference between final CFU counts.

Tannic acid's effect is not strain-specific

To ensure that tannic acid's biofilm-inhibitory activity was not specific to SH1000, we tested its effect on biofilms of 15 other *S. aureus* strains in microtiter plate assays (Figure 3). These strains included both clinical isolates and established lab strains. TSBg is a common medium for *in vitro* biofilm studies, but many of the strains we tested did not grow robust biofilms in this medium. To better assay these strains, as well as to see if the phenotype was robust to different growth media, we also tested these 15 strains in a peptone-based medium (PNG). Research in the Boles lab has shown that biofilms grown in PNG medium have altered matrix that contains

amyloid fibers [37]. The overwhelming majority of strains tested in both media formed less robust biofilms in the presence of tannic acid, suggesting that this effect is broadly applicable.

Tannic acid inhibits biofilm formation only if added early in development

To begin elucidating how tannic acid exerts its antibiofilm effect, we asked whether tannic acid could be added late in biofilm growth to disperse an already-grown biofilm, or if it needed to be added early. We grew biofilms in the standard microtiter plate biofilm assay, adding tannic acid at different timepoints. When tannic acid was added 1.5 hours after inoculation, the biofilm was inhibited. When tannic acid was added 2.5 hours after inoculation, the biofilm was not inhibited (Figure 4).

***S. aureus* supernatants display increased levels of IsaA in the presence of tannic acid**

We grew planktonic cultures of *S. aureus* in the presence of various concentrations of tannic acid. We examined gross changes in the extracellular protein profile of culture supernatants by SDS-PAGE. One band, migrating at approximately 26 kilodaltons, became more pronounced with increasing concentrations of tannic acid (Figure 5A). This protein band was excised and identified by mass spectrometric analysis as the Immunodominant Staphylococcal Antigen A (IsaA). IsaA is a putative lytic transglycosylase that has previously been implicated in cleaving peptidoglycan [113]. A polyclonal antibody was generated against IsaA and subsequent western blot analysis confirmed increased levels of IsaA present in culture supernatants supplemented with tannic acid (Figure 5B).

Tannic acid does not regulate transcription of *isaA*

We hypothesized that the increased extracellular abundance of IsaA was due to increased transcription of *isaA* in the presence of tannic acid. We obtained reporter fusions of β-

galactosidase to the *isaA* promoter and saw no increased β-galactosidase activity when the cells were grown with tannic acid (Figure 6A). We were concerned that the tannic acid itself might be interfering with the β-galactosidase assay, so we also constructed a reporter fusion of YFP to the *isaA* promoter. We saw no increase in YFP fluorescence when the cells were grown with tannic acid (Figure 6B).

To ensure that our reporter fusions accurately reflected levels of transcription, we performed qRT-PCR to directly compare IsaA transcript levels in cells with and without tannic acid. No significant difference was observed between the two conditions (Figure 6C). Taken together, these data strongly indicate that tannic acid does not cause increased transcription of *isaA*.

An *isaA* mutant resists tannic acid mediated biofilm inhibition

Since tannic acid affected IsaA abundance, we asked whether IsaA has a role in tannic acid biofilm inhibition. An isogenic *isaA* mutant was assessed for its ability to form biofilms in the presence of tannic acid (Figure 7A and B). In contrast to what we observed with the wildtype, increasing tannic acid concentration up to 20 μM had no effect on the ability of an *isaA* mutant to form a biofilm. However, complementation of the *isaA* mutant by expressing *isaA* from its native promoter on a plasmid restored the susceptibility of this strain to the antibiofilm effects of tannic acid. Taken together these results suggest that the antibiofilm effects of tannic acid are dependent upon the presence of IsaA.

IsaA expression prevents *S. aureus* biofilm formation

Since tannic acid increases the level of IsaA found in culture supernatants and results in reduced biofilm formation, we hypothesized that over-expression of IsaA would inhibit biofilm formation. To test this hypothesis we cloned the *isaA* gene behind an inducible promoter and

assessed biofilm formation. Induction of IsaA expression did not interfere with growth (data not shown) and resulted in no biofilm formation, whereas non-inducing conditions or the empty vector allowed biofilm formation (Figure 8A).

The overexpression vector we used, pALC2073, is known to be leaky [114], and therefore IsaA levels in the absence of inducer were higher than in the empty vector controls (Figure 8B). This increase in IsaA concentration in the non-inducing conditions did not affect biofilm formation. Taken together, these results suggest that it is possible to increase IsaA levels somewhat without having an effect, but that once IsaA levels hit a certain threshold, biofilm formation is inhibited.

The putative transglycosylase active site is necessary for IsaA's antibiofilm activity

To investigate if the putative transglycosylase activity was required for the antibiofilm activity of IsaA, we constructed a point mutant in the conserved transglycosylase active site that would be expected to abolish activity. Family 1 lytic transglycoslases, including IsaA, share a conserved ES motif, with the glutamyl residue being essential for catalysis [115,116]. In *Salmonella enterica*, the peptidoglycan-digesting activity of two lytic transglycosylases was dramatically reduced by replacing the conserved glutamyl residue with a glutamine [117]. We made the analogous mutation in IsaA's conserved active site (E183Q) and expressed it from the plasmid pKP1.IsaA.EQ. Western blot analysis revealed that this construct produced a protein consistent with the size of the wildtype (Figure 8B). However, overexpression of pKP1.IsaA.EQ did not result in biofilm inhibition (Figure 8A), suggesting that the IsaA putative transglycoylase activity is responsible for the protein's antibiofilm effects.

Spontaneous mutants that resist tannic acid biofilm inhibition fail to produce IsaA.

Although tannic acid inhibits biofilm formation in drip reactors grown for 3 days (see Figure 1), we noticed that extending the drip biofilm growth period to 5 days allowed a significant biofilm to form in the presence of tannic acid. A single tannic acid resistant biofilm was broken up by sonication and plated onto nutrient agar to isolate single clonal colonies. In testing 11 of these isolates, we found that three strongly resisted tannic acid biofilm inhibition (Figure 9A). Western blot analysis revealed that the three tannic acid resistant isolates also lacked IsaA in their culture supernatants (Figure 9B), further strengthening the link between IsaA and tannic acid mediated biofilm inhibition.

The three resistant isolates were white in appearance, in contrast to the other 8 which had the golden coloring typically seen in *S. aureus*. White coloring in *S. aureus* is due to a lack of the golden pigment staphyloxanthin, and is generally indicative of dysfunction in the Sigma B stress response system [118]. Sigma B mutants also characteristically have overabundant extracellular proteases [119]. As a preliminary test for protease activity, we cultured these isolates on milk-agar plates and observed a large zone of clearing around them (Data not shown). These data suggested to us that the lack of IsaA in these isolates' supernatants could be attributed to overabundant proteases due to a mutation in the Sigma B stress response system. This hypothesis is further explored in Chapter 3.

Tea inhibits *S. aureus* biofilm formation in an *isaA*-dependent manner.

Tannic acid is a variable mixture of plant-derived polyphenols, consisting primarily of gallotannins [112], that has historically been used to precipitate proteins from solution [120]. Tannins are an abundant component of vascular plant tissue and help protect plants against bacterial and fungal infection [121]. In addition, tannins are thought to be partially responsible for the astringent taste of red wines and tea [122,123]. We therefore hypothesized that a tannin-containing drink, such

as tea, would directly affect biofilms by the same *isaA*-dependent mechanism as tannic acid. We added freshly brewed black tea at various dilutions to a biofilm formation assay. In the wildtype background, very low concentrations of tea (0.2% v/v or a 1:500 dilution) significantly inhibited biofilm formation (Figure 10A). However, an *isaA* mutant formed a biofilm in the presence of tea at any concentration tested. This phenotype could be complemented by expression of *isaA* from its native promoter on a plasmid (Figure 10C).

Since tannins and proteins readily co-precipitate [124], we tested whether the addition of milk (a relevant source of protein) to tea would affect the antibiofilm properties of tea. Milk was added to freshly brewed black tea at a concentration of 5 mg/ml, a concentration approximating an amount of milk a person might reasonably add to tea for consumption. The mixture was vortexed briefly and allowed to sit at room temperature for 1 hour before adding directly to microtiter plate biofilm assays. Unlike tea alone, the tea-milk mixture failed to inhibit biofilm formation (Figure 10B). The lack of biofilm inhibition by the milk/tea mixture corresponded to the removal of polyphenols from the tea [125,126].

Tea inhibits *S. aureus* throat colonization in an *isaA*-dependent manner.

Emerging evidence suggests that, in addition to the nasopharynx, *S. aureus* commonly colonizes the oropharynx and oral cavity [13,110,111]. Since tea is a commonly consumed beverage that inhibits surface colonization in our *in vitro* models, we tested if tea could impact *S. aureus* throat colonization *in vivo*. The cotton rat has previously been used to study *S. aureus* nasal colonization [127], and to use it as a model for *S. aureus* throat colonization, we first assessed if *S. aureus* would colonize the cotton rat oropharynx. Oral inoculation with 1×10^5 *S. aureus* CFU resulted in reproducible oropharynx colonization, with 100% of animals being initially colonized, and reliable colonization remaining for over 2 weeks (Figure 11A).

To determine if tea ingestion influenced *S. aureus* oropharynx colonization, 200 μl of freshly brewed, room temperature black tea was administered via gavage to colonized rats at 2, 5, and 8 days after initial colonization. Tea ingestion reduced the number of animals colonized with wildtype *S. aureus* in the oropharynx, with 5 out of 6 animals not colonized after tea ingestion versus 1 out of 6 animals not colonized in a water gavage control group (Figure 11B). An *isaA* mutant maintained higher levels of colonization upon tea ingestion, with 5 out of 6 animals remaining colonized post-treatment (Figure 11C).

Given that the *isaA* mutant resisted tea treatment, we hypothesized that the one rat that remained colonized with wildtype *S. aureus* would be enriched for spontaneous mutants that have less IsaA in their culture supernatant. We tested six colonies from that rat for IsaA production. Three of the six isolates had no detectable IsaA in their culture supernatants, and one had dramatically less IsaA than the wildtype (Figure 12A). Similar to what was observed with spontaneous mutants from the drip reactors (Figure 9), we found that the four rat-throat isolates that had reduced or undetectable IsaA in their supernatants were resistant to tannic acid in the microtiter plate assay (Figure 12B). The mechanism of these isolates' resistance to tannic acid will be the focus of work discussed in Chapter 3.

These results suggest that consumption of polyphenolic compounds, like those in tea, may reduce *S. aureus* oropharynx colonization in an *isaA*-dependent manner, and that mutations that lead to reduced IsaA abundance can arise spontaneously to confer resistance.

Discussion

The ability of *S. aureus* to colonize surfaces and form biofilms contributes to its success as a commensal and pathogen. *S. aureus* lives as a commensal attached to surfaces such as the skin, nasopharynx and oropharynx [13]. As a pathogen, *S. aureus* can attach to internal tissues such as bone, heart valves, or implanted medical devices [7,103,104]. Colonization by *S. aureus* increases the incidence of infection, and biofilm infections represent a serious clinical situation based on their recalcitrance to antibiotics, their persistence, and the propensity of organisms to detach and colonize new sites [100]. Relatively little is known regarding how natural products that are common in the human diet can influence *S. aureus* colonization and biofilm development. Therefore, understanding how *S. aureus* responds to natural products and different environmental conditions is an important issue that warrants further investigation.

In this chapter we demonstrate that the polyphenolic compound tannic acid can inhibit *S. aureus* surface colonization in a multitude of biofilm models (Figure 1). Analysis of liquid culture supernatants revealed increased levels of the protein IsaA when strains were cultured in the presence of tannic acid (Figure 5). An *isaA* mutant was not susceptible to the biofilm inhibition effects of tannic acid and this phenotype was complemented by expressing *isaA* under control of its native promoter *in trans* (Figure 7). Expression of IsaA from an inducible promoter inhibited biofilm formation and this was dependent upon a catalytic residue at the putative IsaA transglycosylase active site (Figure 8). Black tea, a common source of tannic acid in the human diet, inhibited biofilm formation *in vitro* in an *isaA*-dependent manner (Figure 10). We developed an animal model for *S. aureus* throat colonization and found that tea reduced throat colonization in an *isaA*-dependent manner (Figure 11). After prolonged tannic acid or tea

treatment either *in vitro* or *in vivo*, isolates that were resistant to the antibiofilm effect of tannic acid appeared and these isolates failed to produce IsaA (Figures 9 and 12).

Tannic acid has long been known to have antibacterial properties [128], bacteria are known to actively modulate gene expression in response to tannins [129,130], and recently it has been suggested that tannic acid has antibiofilm properties [131]. Pentagalloyl glucose (one of the major components of commercial tannic acid) and ellagic acid (another plant-derived polyphenolic compound) have also been shown to inhibit biofilm formation in *S. aureus* [132,133]. To the best of our knowledge, no genetic mechanism has been proposed for the antibiofilm properties of tannic acid or related polyphenols in *S. aureus*. Our work shows that tannic acid causes an increase in extracellular IsaA levels, and that increased levels of IsaA are able to inhibit biofilm formation in *S. aureus*. At this time, the molecular mechanism leading to increased extracellular IsaA levels is not known, although we demonstrate here that it does not involve increasing transcription of *isaA* (Figure 6).

One potential mechanism by which IsaA abundance could be increased is by tannic acid modulation of IsaA stability. It is known that extracellular IsaA abundance trends with cell density, increasing exponentially during exponential growth and leveling off during stationary phase [134]. Our observation has been that in late stationary phase, IsaA abundance drops dramatically as the extracellular proteases become more abundant and more active. It is possible that addition of tannic acid prevents the degradation of IsaA in stationary phase, which would appear as a dramatic increase when compared with untreated cultures where IsaA has been degraded. At the time of the writing of this document, this hypothesis remains to be tested.

Lytic transglycosylases have been extensively studied in *E. coli*, where they have been shown to

cleave peptidoglycan at the β-1,4-glycosidic bond between N-acetylmuramic acid (MurNAc) and N-acetyl glucosamine (GlcNAc) [135]. By virtue of their ability to cleave the polysaccharide backbone of the peptidoglycan layer, lytic transglycosylases are thought to play a role in synthesis and degradation of the peptidoglycan. It has been proposed that lytic transglycosylases play important roles in cellular elongation, septation, recycling of muropeptides, and pore formation [136].

To the best of our knowledge, this is the first report describing a specific function of the lytic transglycosylase IsaA outside of cell wall maintenance in *S. aureus.* The mechanism by which IsaA leads to biofilm inhibition remains unclear, but the evidence we present demonstrates that this activity depends on IsaA's catalytic function as a lytic transglycosylase. There are several ways in which cleavage of peptidoglycan could lead to a reduction in biofilm formation. For example, peptidoglycan cleavage could change the composition of proteins and teichoic acids displayed on the cell wall, cleaving away factors necessary for surface colonization. An alternate theory, discussed in Chapter 3, is that peptidoglycan cleavage could release a signaling molecule [137], leading to modulation of biofilm-related gene expression.

S. aureus nasal colonization is a significant risk factor for several infections including bacteremia, post-operative infections, and diabetic foot ulcer infections and contributes to the spread of this pathogen in hospital environments [17,138–140]. Many hospitals employ rigorous *S. aureus* infection control policies, including active surveillance of nasal colonization for patients and personnel, contact precautions, and isolation of colonized patients [141]. Current decolonization strategies involve applying topical agents such as mupirocin to the nose. Several recent studies have identified the oropharynx as another common site of *S. aureus* colonization [13,110,111]. Because hospital efforts to track and eliminate *S. aureus* colonization focus primarily

on the nasal cavity, these approaches are not likely to affect throat carriage and therefore this reservoir for future infection may persist.

Our finding that tea inhibited *S. aureus* biofilm development *in vitro* and reduced throat colonization in an animal model may have important consequences. In addition to helping us understand the function and role of IsaA, it gives us a safe, effective tool for decolonizing a second common site of *S. aureus* colonization, aiding in hospitals' efforts to reduce the risk of infection and spread of this deadly pathogen. Hot tea or coffee has previously been associated with reduced methicillin resistant *S. aureus* nasal colonization, suggesting that our results may translate to human colonization [142]. Understanding the effects of tannic acid and tea in decolonizing this reservoir, as well as the genetic mechanism underlying this effect, could lead us to more effective treatments for *S. aureus* colonization and infection.

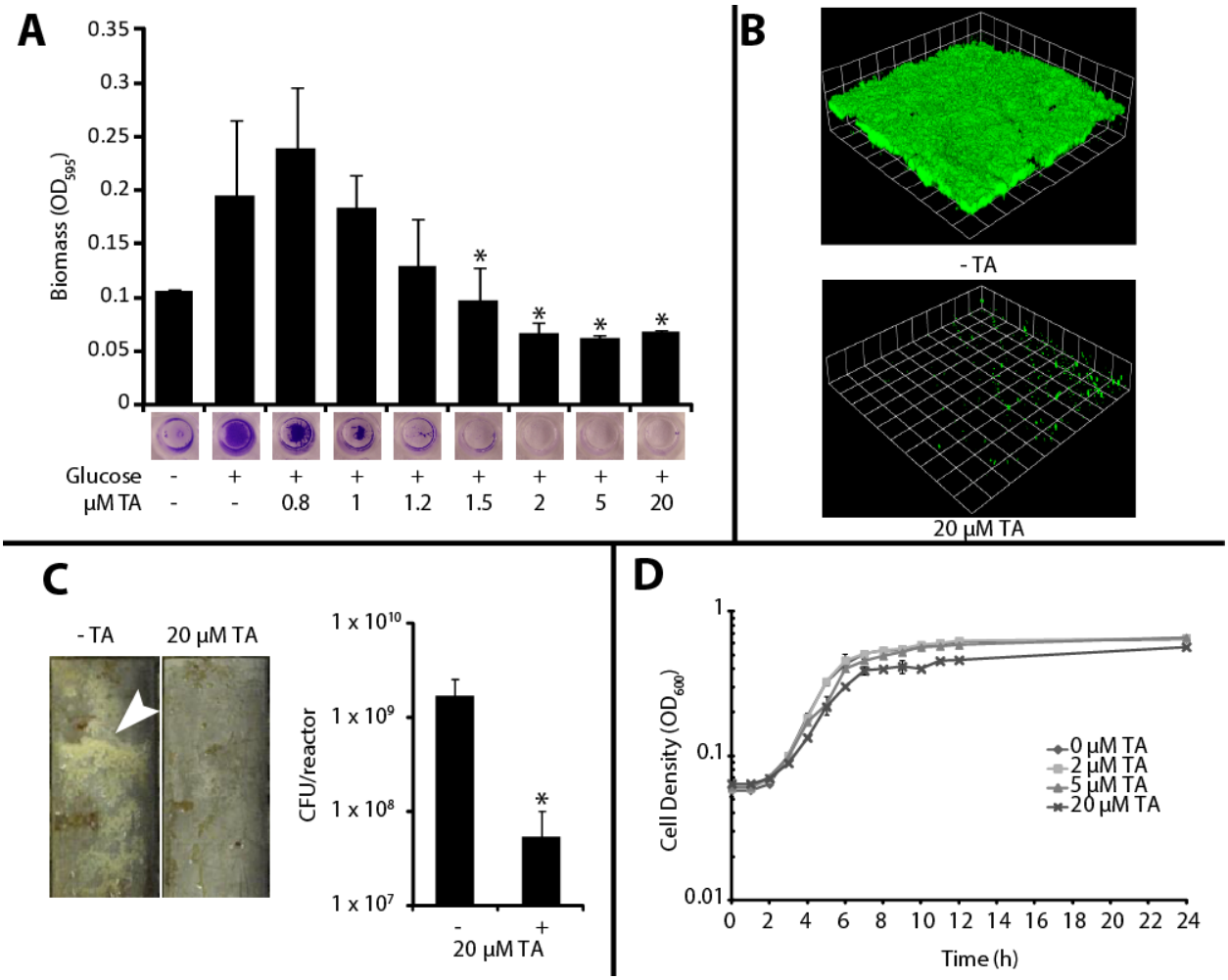

Figure 3. Tannic acid inhibits biofilm formation in *S. aureus*.
(A) *S. aureus* does not form a biofilm in microtiter plate assay when cultured with micromolar concentrations of tannic acid (TA). Biofilm formation was induced by supplementing the growth medium with 0.2% glucose. Error bars represent standard deviation. * indicates $p<0.005$ compared to + glucose, -TA control. (B) *S. aureus* biofilm formation in a flow cell is dramatically reduced by treatment with 20 μM TA. Shown are three dimensional image reconstructions of a z series created with Velocity software. Confocal laser scanning microscopy images are representative of three separate experiments and each side of a grid square represents 15 micrometers. (C) *S. aureus* forms significantly less biofilm in drip reactors when grown with 20 μM TA. Arrow indicates biofilm growth. Drip reactor biofilms were grown for three days and photographed before harvesting. Cells were harvested from four replicate drip reactors and colony forming units were counted. Error bars represent standard deviation. * indicates $p<0.01$ compared to untreated control. (D) *S. aureus* growth is not affected by TA at concentrations up to 20 micromolar.

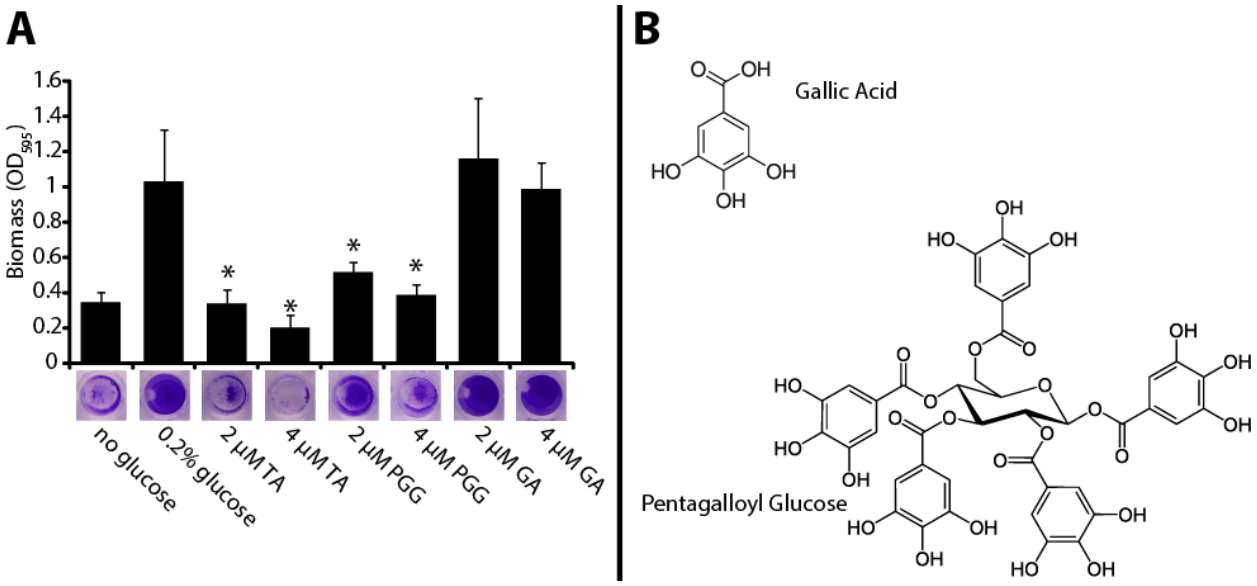

Figure 4. Pentagalloyl glucose inhibits biofilm formation in *S. aureus*.
(A) Two major components of commercial tannic acid are compared for anti-biofilm activity. Pentagalloyl glucose (PGG) significantly inhibits biofilm formation. Gallic acid (GA) causes no significant inhibition. Error bars represent standard deviation. * indicates $p<0.01$ compared to + glucose, -TA control. (B) Chemical structures of gallic acid and pentagalloyl glucose.

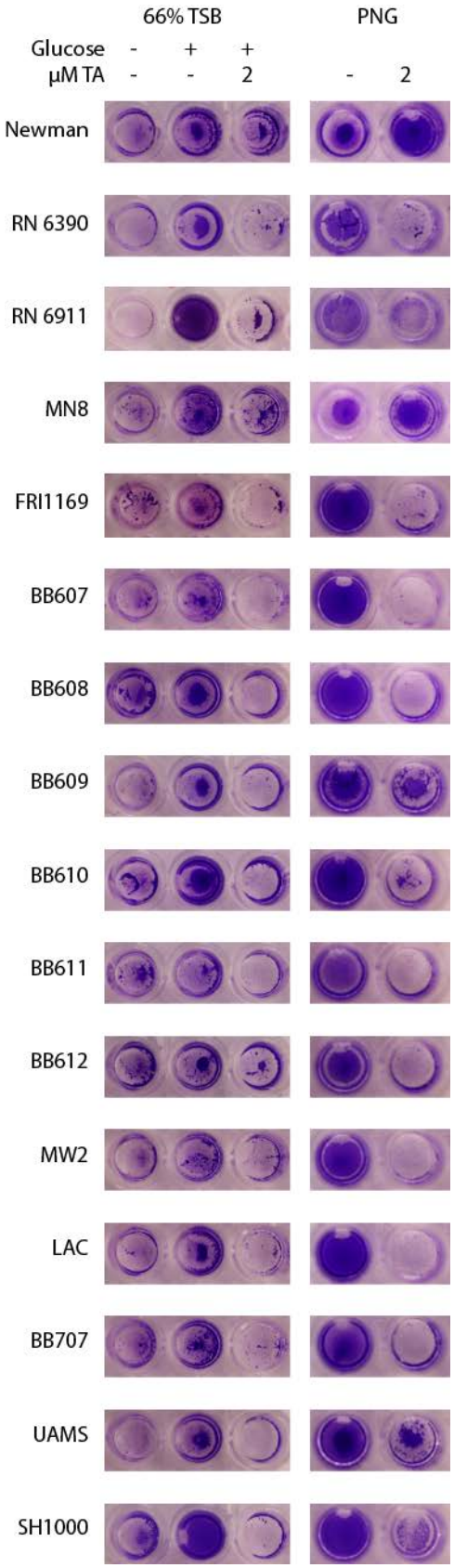

Figure 5. Tannic acid inhibits biofilm formation in multiple strains of *S. aureus.*
Sixteen clinical isolates and lab strains of *S. aureus* grow weaker biofilms in the presence of tannic acid as compared to controls. Biofilms were grown in 66% tryptic soy broth (66% TSB) or peptone medium (PNG).

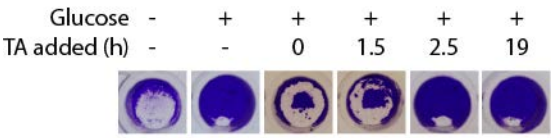

Figure 6. Tannic acid inhibits biofilm formation only if added early.
S. aureus biofilms grown in 66% TSB + 0.2% glucose with tannic acid added at indicated times after inoculation. TA added at 1.5 hours post-inoculation or earlier inhibits biofilm formation, while TA added 2.5 hours post-inoculation or later does not.

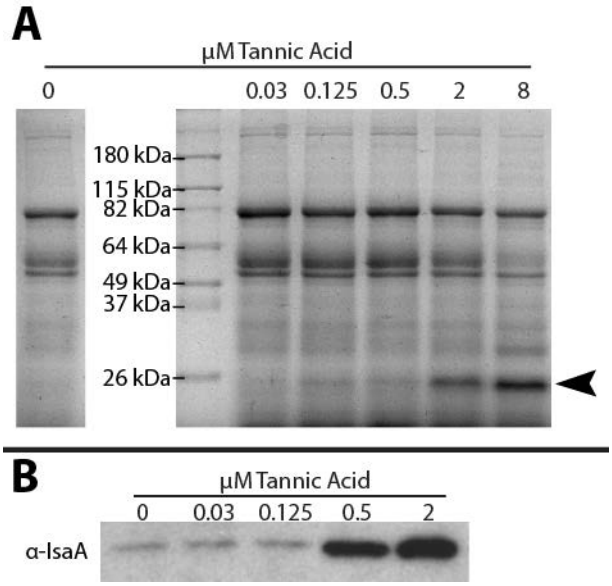

Figure 7. Tannic acid increases levels of IsaA in *S. aureus* culture supernatants.
(A) TCA-precipitated supernatants from overnight cultures of *S. aureus* grown in 66% TSB, supplemented with 0.2% glucose and with the indicated concentrations of tannic acid. When cultures are grown with higher concentrations of tannic acid, a band (indicated with an arrow) appears with an apparent molecular weight slightly below 26 kDa. Band was excised and protein was identified by mass spectrometry as IsaA. (B) Western blot of unprecipitated supernatants from overnight cultures probed with polyclonal anti-IsaA antibody. IsaA levels in culture supernatants increase when culture is grown with tannic acid.

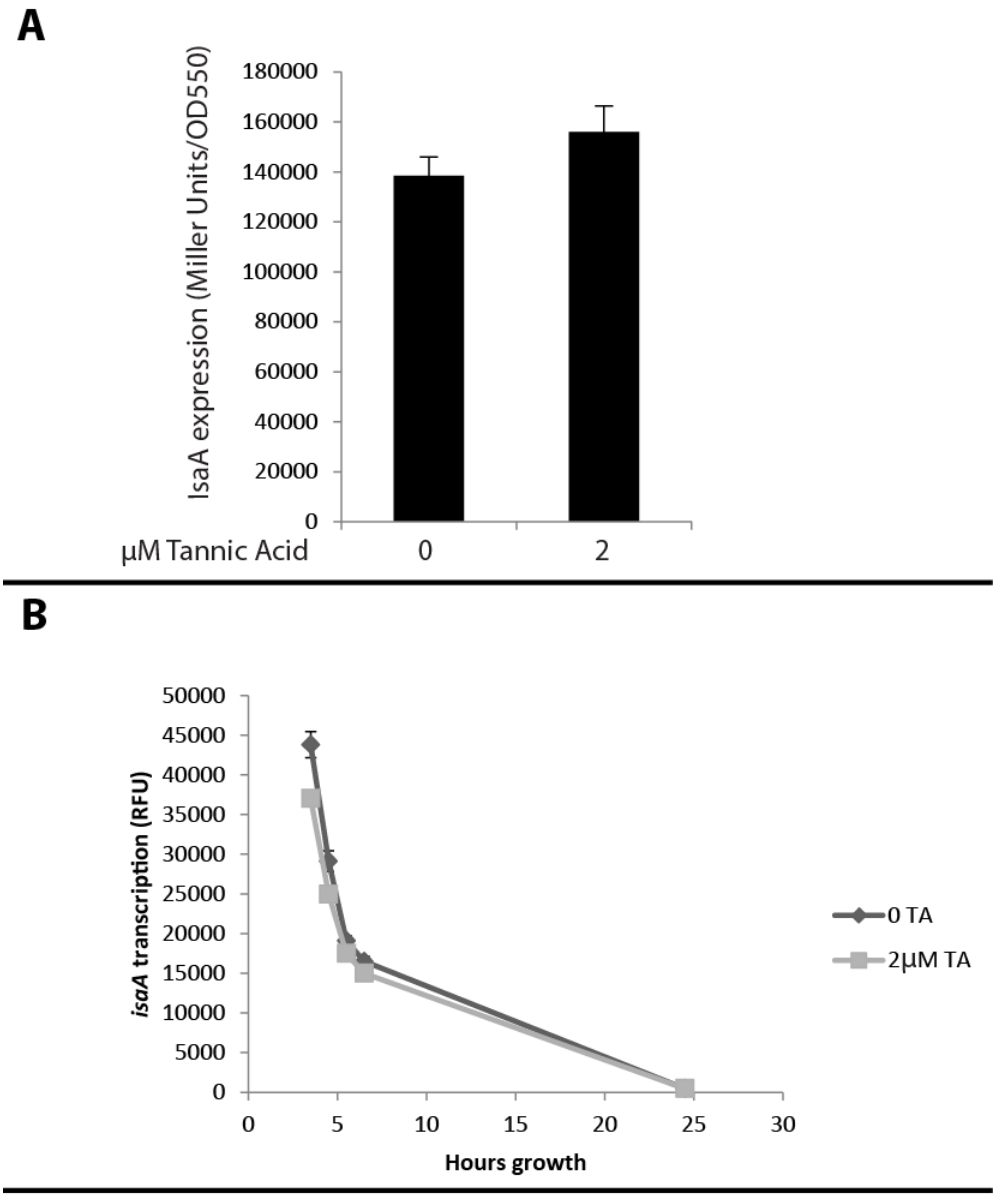

Figure 8. Tannic acid does not increase *isaA* transcription. (A) SH1000 *isaA::lacZ* (BB2186) grown 16 hours with and without tannic acid. Beta galactosidase activity was measured by MUG assay and normalized to OD. No significant difference was observed. (B) SH1000 + pIsaA-YFP (BB2332) was cultured 25 hours in 66% TSBg + 2 uM TA. 100 µl was removed to read YFP fluorescence and OD in a Tecan infinite reader. Data presented is fluorescence/OD. (C) SH1000 grown for 16 hours in 66% TSBg with indicated concentration of tannic acid. qRT-PCR was performed to observe levels of IsaA mRNA compared to 16s rRNA. Error bars represent standard deviation.

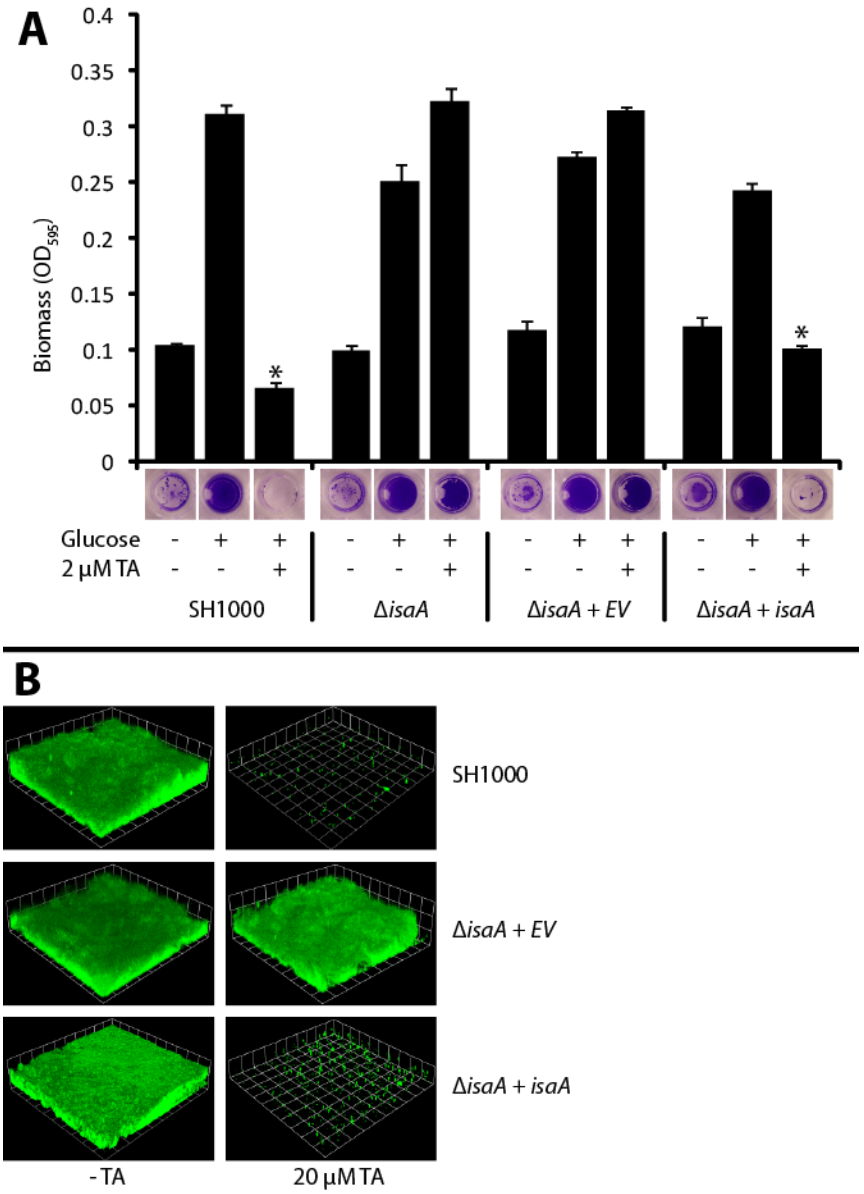

Figure 9. *isaA* is necessary for tannic acid-induced biofilm inhibition.
(A) SH1000 (BB386), *isaA*::tetR (BB2183), *isaA*::tetR + empty vector (BB2184), and *isaA*::tetR + *isaA* complement (BB2185) were assayed in microtiter plate for tannic acid-induced biofilm inhibition. Strains lacking functional *isaA* were resistant to inhibition. * indicates $p<<0.001$ compared to isogenic untreated control. (B) SH1000 (BB386), *isaA*::tetR + empty vector (BB2184), and *isaA*::tetR + *isaA* complement (BB2185) were assayed in a flow cell for tannic acid-induced biofilm inhibition. *isaA* mutant was resistant to inhibition.

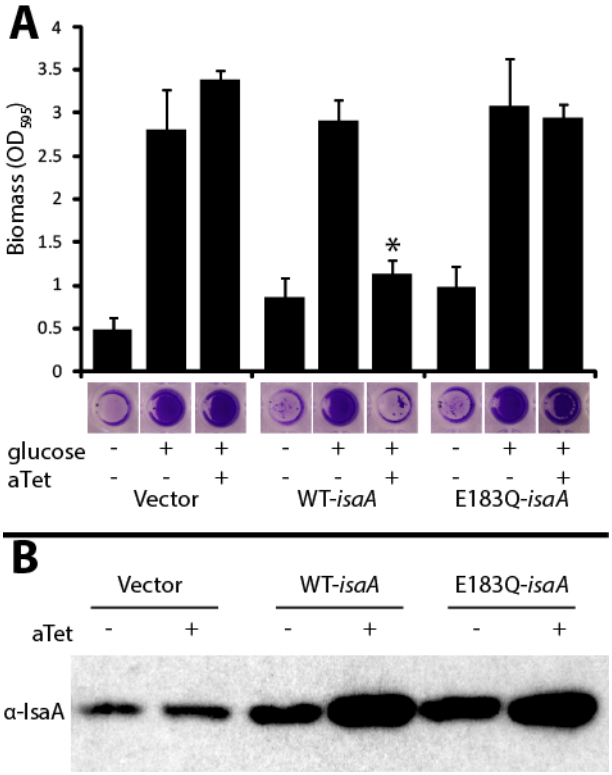

Figure 10. Induced expression of IsaA inhibits biofilm formation.
(A) SH1000 harboring empty vector (1209), *isaA* (2242), or E183Q-*isaA* (2333) under a tet-inducible promoter was cultured with 0.2% glucose and 250 ng/ml anhydrotetracycline (aTet). No biofilm formed when IsaA was overexpressed. A biofilm formed when E183Q IsaA was overexpressed. Error bars represent standard deviation. * indicates $p<<0.001$ compared to + glucose, - aTet isogenic control. (B) Wild-type IsaA and E183Q IsaA are overexpressed when induced with 250 ng/ml aTet. Western blot of SH1000 harboring empty vector (1209), *isaA* (2242), or E183Q-*isaA* (2333) under a tet-inducible promoter, with and without aTet induction, probed with polyclonal anti-IsaA antibody.

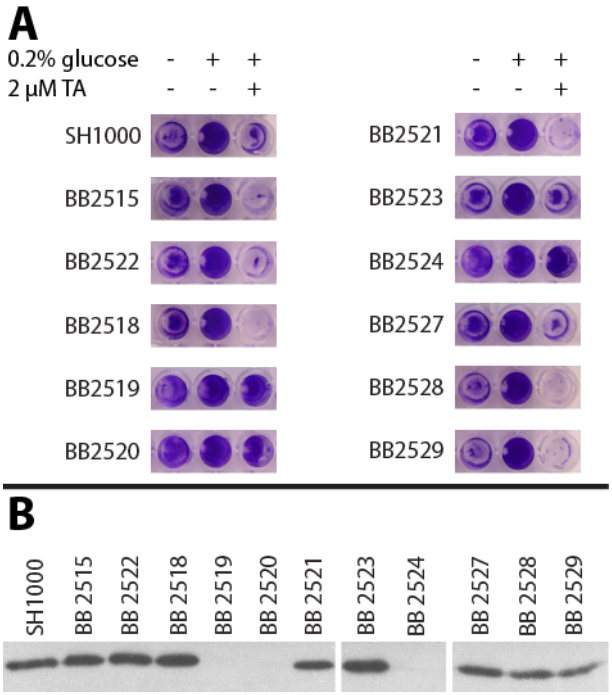

Figure 11. Biofilm resistance to tannic acid in *in vitro* isolates is coincident with a reduction in IsaA expression. Strains derived from 11 *S. aureus* colonies isolated from a tannic acid-treated biofilm were tested for resistance to tannic acid-induced biofilm inhibition, as well as for IsaA production. (A) 3 of 11 strains (2519, 2520, and 2524) are robustly resistant to tannic acid-induced biofilm inhibition in a microtiter plate biofilm assay. (B) The same 3 strains also do not have detectable IsaA in their culture supernatants. Western blot of culture supernatants from the 11 strains isolated from tannic acid-resistant biofilm (along with wild-type) probed with polyclonal anti-IsaA antibody.

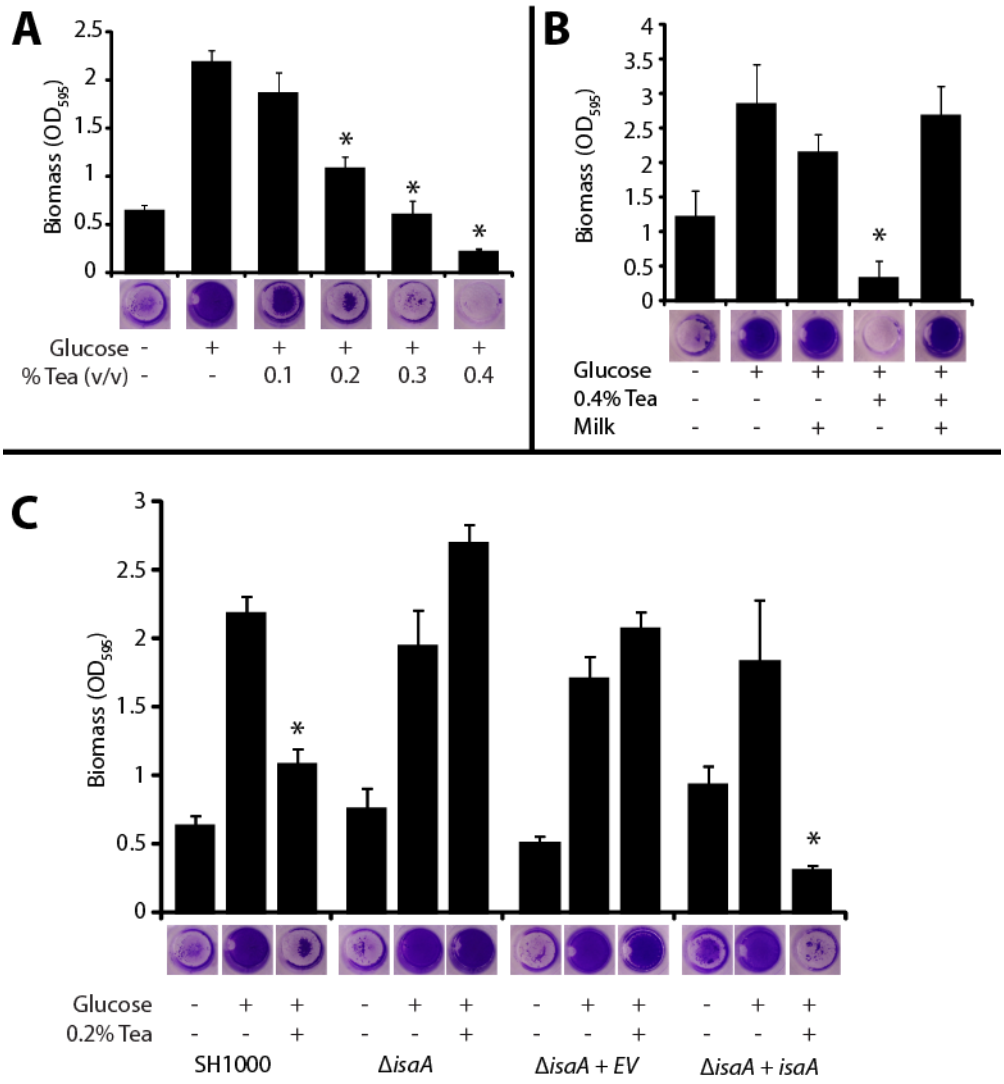

Figure 12. Black tea inhibits biofilm formation in *S. aureus*.
(A) Black tea inhibits biofilm formation in a dose-dependent manner. Biofilm formation was induced in a microtiter plate biofilm assay by supplementing the medium with 0.2% glucose. Freshly brewed black tea was diluted into biofilm medium as indicated. Error bars represent standard deviation. * indicates $p<<0.001$ compared to + glucose, - tea control. (B) When black tea is mixed with milk, the tea loses its biofilm-inhibitory effect. Error bars represent standard deviation. * indicates $p<<0.001$ compared to + glucose, - tea, - milk control. (C) *isaA* is necessary for tea-induced biofilm inhibition. SH1000 (BB386), *isaA*::tetR (BB2183), *isaA*::tetR + empty vector (BB2184), and *isaA*::tetR + *isaA* complement (BB2185) were assayed in microtiter plate for tea-induced biofilm inhibition. Strains lacking functional *isaA* were resistant to inhibition. Error bars represent standard deviation. * indicates $p<<0.001$ compared to isogenic + glucose, - tea control.

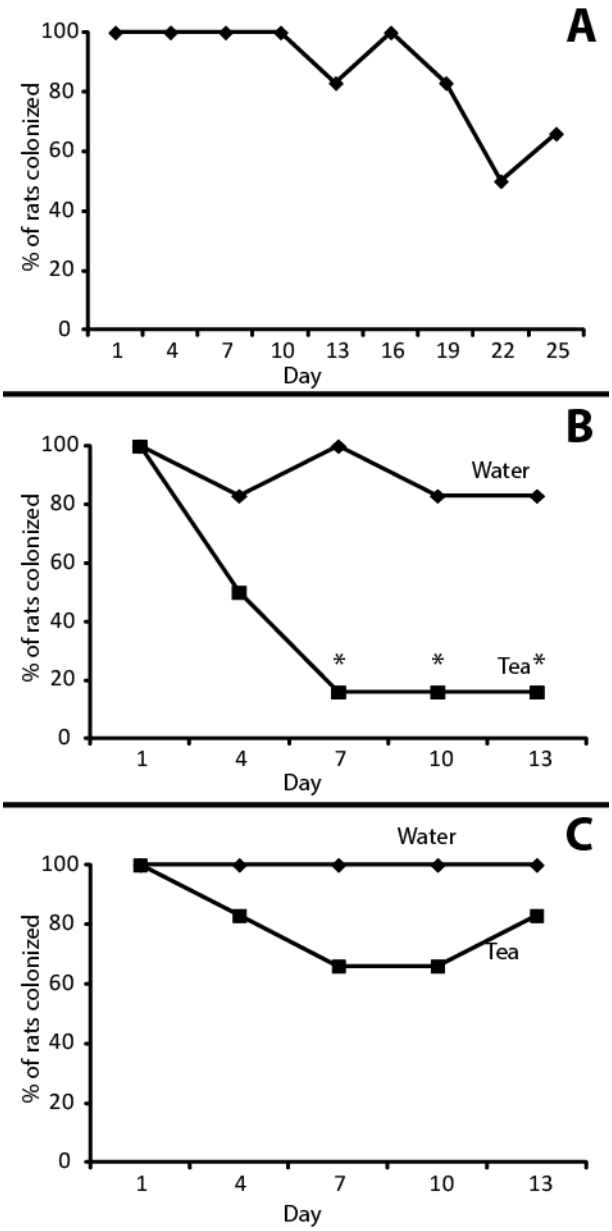

Figure 13. *S. aureus* throat colonization is reduced by tea in an *isaA*-dependent manner.
(A) Cotton rats colonized with *S. aureus* via gavage to the oropharynx remain consistently throat colonized for 19 days (N=6). (B) Tea ingestion (square) via gavage at days 2, 5, and 8 reduces the number of animals colonized with wildtype *S. aureus* in the oropharynx to 1 out of 6 animals at days 7, 10, and 13 (N=6). Control water ingestion (diamond) resulted in oropharynx colonization of 6 out of 6 animals at day 7 and 5 out of 6 animals at days 10 and 13 (N=6). Data were analyzed by Fisher Exact Test. *= p-value < 0.05. (C) Colonization of cotton rats with an *isaA* mutant. All animals remained colonized after water ingestion (diamond) via gavage at days 2, 5, and 8, and tea ingestion (square) via gavage resulted in 4 out of 6 animals colonized at days 7 and 10 and 5 out of 6 colonized at day 13.

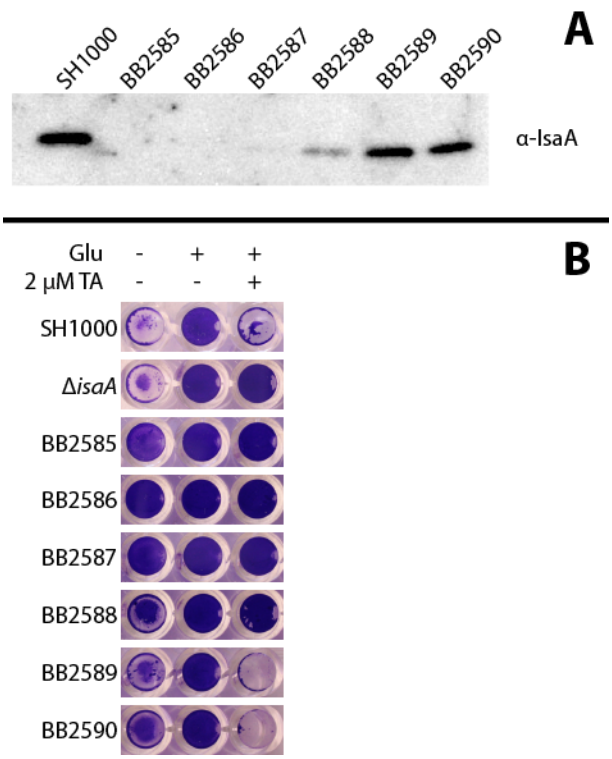

Figure 14. Biofilm resistance to tannic acid in *in vivo* isolates is coincident with a reduction in IsaA expression. (A) Western blot with anti-IsaA antibody on culture supernatants from 6 colonies isolated from the one rat that remained colonized after tea ingestion. Three of the six colonies tested did not produce detectable levels of IsaA in culture supernatants. (B) Six isolates were assayed in microtiter plate for biofilm resistance to tannic acid. Isolates that reduced IsaA (2585-2588) were resistant while those with wild-type levels of IsaA were not.

Table 1. Strains and plasmids used in Chapter 2

Strain	Description	Resistance	Source
BB1209	SH1000/pALC2073	Cm	[143]
BB2146	SH1000 Spectinomycin resistant	Spec	[107]
BB2183	*isaA*::*tet*	Tet	[113]
BB2184	*isaA*::*tet* / pSK5630	Cm	[113]
BB2185	*isaA*::*tet* / pMEL4	Cm	[113]
BB2242	SH1000 / pKP1	Cm	This work
BB2333	SH1000 / pKP1.IsaA.EQ	Cm	This work
BB2515	Dripper isolate	None	This work
BB2518	Dripper isolate	None	This work
BB2519	Dripper isolate	None	This work
BB2520	Dripper isolate	None	This work
BB2521	Dripper isolate	None	This work
BB2522	Dripper isolate	None	This work
BB2523	Dripper isolate	None	This work
BB2524	Dripper isolate	None	This work
BB2527	Dripper isolate	None	This work
BB2528	Dripper isolate	None	This work
BB2529	Dripper isolate	None	This work
BB2585	Rat isolate	Spec	This work
BB2586	Rat isolate	Spec	This work
BB2587	Rat isolate	Spec	This work
BB2588	Rat isolate	Spec	This work
BB2589	Rat isolate	Spec	This work
BB2590	Rat isolate	Spec	This work
BB204	Newman		[144]
BB206	RN6390		[145]
BB207	RN6911		[146]
BB248	FRI1169		[147]
BB607	Blood isolate		[37]
BB608	Blood isolate		[37]
BB609	Blood isolate		This work
BB610	Bone isolate		This work
BB611	Bone isolate		This work
BB612	Bone isolate		This work
BB687	MW2		[148]
BB1263	LAC		[149]
BB707	Nasal isolate		This work
BB759	UAMS		[150]
Plasmids			
pSK5630		Cm	[113]
pMEL4	*isaA* under control of native promoter	Cm	[113]
pALC2073		Cm	[143]
pKP1	*isaA* under control of tet promoter	Cm	This work
pKP1.IsaA.EQ	*isaA*-E183Q under control of tet promoter	Cm	This work

Table 2. Compounds from screen of Biolog small molecule library that either promoted or inhibited biofilm formation.

Biofilm inhibitory compounds:	Biofilm promoting compounds:
D-Serine	Capric acid
D-Sorbitol	Itaconic acid
Tween 20	Sodium chloride
Tween 80	Ethylene glycol
Mono Methyl Succinate	Potassium chloride
Inosine	Sodium lactate
D,L-Carnitine	Sodium Benzoate
Chondroitin Sulfate C	5-fluorouracil
Putrescine	Manganese II Chloride
Guanosine	EGTA
Xanthine	Cefmetazole
D,L-α-Amino Caprylic Acid	Cinoxacin
Sodium Sulfate	Sulfanilamide
Ethylene Glycol	Chloroxylenol
Sodium Formate	Sodium selenite
Urea	Sodium salicylate
Sodium Lactate	5-fluoro 5 deoxyuridine
Sodium Phosphate	Pentachlorophenol
Sodium Nitrate	Tinidazole
Sodium Nitrite	Sodium caprylate
Chloramphenicol	
Neomycin	
Rolitetracycline	
Cupric Chloride	
Boric Acid	
Piperacillin	
Promethazine	
Cefmetazole	
Nordihydroguaiaretic Acid	
5,7-Dichloro-8-hydroxyquinoline	
Rifamycin SV	
Ferric Chloride	
Tannic acid	
Lidocaine	
Sodium Bromate	
Myricetin	
2-Phenylphenol	
Phenyl-methylsulfonyl-fluoride	
Sodium caprylate	
4-hydroxycoumarin	
Pridinol	

Table 3. Growth rate and final culture density of *S. aureus* cultured in the presence of tannic acid.

µM TA	Doubling Time (min)	CFU/ml after 24 hr (*10^9)
0	35.6 ± 0.4	1.35 ± 0.77
5	34.6 ± 2.6	1.4 ± 0.66
20	36.2 ± 0.8	1.65 ± 1.03

Notes

Much of the work in this chapter was published in the February, 2013 issue of Infection and Immunity[151]. It is reproduced here with permission.

CHAPTER 3

Analysis of resistant isolates

Introduction

Staphylococcus aureus is a major pathogen, responsible for 19,000 deaths per year in the United States [152]. *S. aureus* is also a commensal, asymptomatically colonizing roughly 30% of healthy adult humans [12]. In many cases of hospital infection, the colonizing strain appears to be responsible for the infection [18]. Because of this interplay between commensal and pathogenic lifestyles, hospitals make efforts to decolonize patients prior to invasive surgery. Although this effort is valuable, *S. aureus*'s ability to adapt and evade treatments makes it vital that we expand our understanding of how *S. aureus* colonizes hosts and how it evades our efforts to fight it.

The ability of *S. aureus* to form biofilms is central to both its pathogenic and commensal lifestyles. A biofilm is a colony of bacterial cells that grows attached to a surface and is held together by a polymeric matrix. Although the exact composition of this matrix varies greatly depending on species, strain, and growth conditions, it typically contains some combination of DNA, adhesive proteins, polysaccharide, and amyloid fibers [21,35,153,154].

In chapter 2, we showed that tannic acid inhibited biofilm formation in *S. aureus*. This effect was found to be due to an increased abundance of IsaA, a *S. aureus* lytic transglycosylase that cleaves peptidoglycan. Rats colonized with *S. aureus* could be decolonized by treatment with

black tea, which is rich in tannic acid. A small number of *S. aureus* colonies were isolated from one rat that resisted decolonization [151].

In this chapter, we explore how that resistance to decolonization arose. We sequenced the genomes of the isolates from the rat throat, as well as tannic acid resistant isolates from an *in vitro* biofilm. We studied one of these isolates in depth, showing first that it formed a robust biofilm both in the presence of tannic acid and when IsaA was overexpressed. This resistance was found to be due to a missense mutation in *pknB*, a eukaryotic-like serine threonine kinase that is conserved in all sequenced strains of *S. aureus*. The mutation in *pknB* caused upregulation of the *ica* operon and thereby increased production of PIA. The research presented in this chapter demonstrates how, even in the face of very effective treatments, bacteria are able to adapt and evade clearing.

Materials and Methods

Strains and plasmids

Strains and plasmids used in this chapter are shown in Table 4. *S. aureus* strains were cultured in tryptic soy broth unless otherwise indicated. Plasmids were maintained by growth in 10 μg ml^{-1} chloramphenicol.

Plasmids pJB38-ΔpknB and pJB38-pknB-P208R were both created by ligating different PCR products into pJB38 after digestion with EcoRI-HF and SalI HF. The PCR fragment for pJB38-Δ*pknB* was created by SOEing PCR using primers o437, o438, o452, and o440. The PCR fragment for pJB38-pknB-P208R was created by SOEing PCR using primers o437, o493, o492, and o440.

Strains BB2753 and BB2827 were created by allelic replacement using pJB38-ΔpknB and pJB38-PknB-P208R, respectively, as described [155]. All mutations were confirmed by Sanger sequencing.

BB3016 was created by phage transduction. The *ica*::*tet* mutation was moved from BB595 into BB2753 using ϕ11, as described [156].

Biofilm assays

Biofilm assays in the SH1000 background were performed as previously described [106]. Briefly, overnight cultures of *S. aureus* were diluted 1:200 in fresh 66% TSB (20g/l) + 0.2% glucose. 200 μl final volume was added to wells of a 96 well microtiter plate (164688; Nunc). Plates were incubated at 37°C shaking at 250 RPM for 16 hours.

For biofilms in the JE2 background, the assay was modified for stronger biofilm growth, as previously described [37]. Overnight cultures were diluted 1:200 in fresh PNG medium (20g peptone, 20g NaCl, 20g glucose per liter). 200 µl final volume was added to wells of a 96 well microtiter plate (164688; Nunc). Plates were incubated at 37°C shaking at 250 RPM for 48 hours.

For the transwell assay, biofilms were grown as above with the following changes. Growth medium was split between the upper and lower chambers for initial inoculation. Medium containing BB2242 was added to the upper chamber, and the lower chamber was inoculated with different strains as indicated. Anhydrotetracycline (aTet) was added to all wells at a concentration of 250 ng/ml.

For the spent supernatant assay, biofilms were grown as above with the following changes. Overnight cultures were diluted in fresh 66% TSB (20g TSB per 1 l H2O) + 0.2% glucose and cultured in microtiter plate biofilm conditions for times indicated (2-6 hours). At the time indicated, entire wells were mixed by vigorous pipetting to dislodge as much biofilm as possible. Cells were removed by centrifugation, supernatant was sterilized by passing through a 0.22 µm filter, and sterile supernatants were kept on ice until ready to use. Overnight culture of BB1209 was diluted 1:100 in 2x 66% TSB (40g/l) + 0.4% glucose. 100 µl sterile culture supernatant was added to bring final volume to 200 µl. This was added to wells of a microtiter plate and grown as above.

Following incubation, the biofilms were stained. Medium was removed from the wells by pipetting, then wells were washed with 200 µl sterile water. 100 µl of 0.1% crystal violet was added and allowed to stain for 10 minutes. Crystal violet was removed, then wells were washed

twice with 200 µl sterile water. Plates were dried and photographed. For quantification, stain was dissolved in 150 µl of destain solution (40 mM HCl in 95% ethanol). After 10 minutes, the dissolved stain was diluted in destain solution to bring it into the linear detection range. OD595 was measured in a Tecan Infinite reader.

Western blots

Western blots were performed as previously described [151]. Cultures were normalized to OD_{600}, then cells were removed by centrifugation. Supernatants were boiled with SDS loading buffer and separated on 15% polyacrylamide gels. Proteins were transferred to PVDF membranes and probed with a polyclonal rabbit-anti-IsaA antibody [151].

High-throughput sequencing, alignment, and variant calling

Genomic DNA was purified by phenol chloroform extraction. DNA sequencing library preparation and sequencing on an Illumina HiSeq 2000 were performed by the University of Michigan DNA Sequencing Core. Fifty base single end reads were aligned to the *S. aureus* NCTC 8325 reference genome (Accession number NC_007795) using bwa aln and samse, version 0.7.8-r455, allowing reads to align only once to the reference sequence [157]. Aligment files were generated using samtools and duplicate reads were marked using picard-tools MarkDuplicates version 1.118 with the following command line parameters: VALIDATION_STRINGENCY=LENIENT, REMOVE_DUPLICATES=true [158]. Variants were called and VCF files generated using freebayes version 0.9.14-12-g88f4c76 with the command line parameters --standard-filters --ploidy 1 --min-alternate-fraction 0.8 --min-cov 10 [159]. Variants found in wild type control alignments were removed from the variants found in all other isolates using the vcf-isec command from vcf-tools version 0.1.12 with the command line parameters -f –c [160].

PIA dotblot

Polysaccharide Intercellular Adhesin (PIA) was prepared and analyzed as described [161]. Briefly, biofilms were grown in a microtiter plate. Multiple wells were homogenized by vigorous pipetting, then pooled. Cells were pelleted at 6,000 x g for 5 minutes. Pellet was resuspended in 0.5 M EDTA and normalized by OD600. Cells were boiled 5 minutes and spun at 6,000 x g for 5 minutes. 40 µl of supernatant was mixed with 10 µl 20 mg/ml Proteinase K and incubated 60 minutes at 37C. Samples were boiled 5 minutes, then dotted onto a PVDF membrane and allowed to dry. Membrane was probed with rabbit anti-PIA antibody graciously provided by Paul Fey [161], followed by Licor goat anti-rabbit secondary. Blot was scanned on Licor Odyssey for visualization and quantitation.

Germination assay

B. subtilis spores were produced and purified as described [162]. Briefly, cells were grown 48 hours in Difco Sporulation Medium (8g Difco nutrient broth, 1 ml 1M $MgSO_4$, 10 ml 10% KCl, 500 µl 1N NaOH in 1 L water. After autoclaving and immediately before use, add 100 µl 1M $CaNO_3$, 100 µl 10 mM $MnCl_2$, and 100 µl 1 mM $FeSO_4$). Culture was centrifuged at 10,000 x *g* for 10 minutes, then washed three times with cold water. Pellet was resuspended in cold water and shaken gently overnight at 4C. Every day for 10-14 days, suspension was centrifuged 20 minutes at 20,000 x *g*, resuspended in cold water, and returned to shake in the cold.

Germination was measured by loss of optical density [162]. Briefly, spores were diluted in 10 mM Tris HCl (pH 8.4) and either germinants or peptidoglycan fragments were added. OD_{580} was read every 10 minutes in a TECAN plate reader. Graphs are presented as inverted (to show increase in germination rather than loss of OD) for the convenience of the reader.

Results

Whole genome sequencing of tannic acid-resistant isolates

In Chapter 2, we observed that when a drip reactor was treated with tannic acid for an extended period, a biofilm arose that resisted treatment (See Chapter 2, Figure 9). We also observed that one rat of six remained colonized with *S. aureus* despite treatment (See Chapter 2, Figure 12). To gain better understanding of how tannic acid resistance arose, we analyzed isolates from these two tannic acid-resistant biofilms (See Table 5 for an overview of all isolates).

As discussed in Chapter 2, we isolated 11 colonies from the *in vitro* system and 6 from *in vivo*. Although all were isolated from biofilms grown with inhibitory concentrations of tannic acid, only 3 of 11 and 4 of 6 were resistant to tannic acid's antibiofilm effect when grown in pure culture. We sequenced the genomes of the three resistant isolates from the drip reactor, as well as all six isolates taken from the rat throat. The results of this sequencing are found in Table 6. Some notable results are discussed below.

Lack of IsaA in *rsbU* mutants is due to a secreted factor

Of the seven sequenced isolates that resisted biofilm inhibition by tannic acid, six had mutations in *rsbU*, while neither of the sensitive isolates did. These six isolates all displayed phenotypes characteristic of inactive SigB, namely lack of the golden pigment staphyloxanthin and increased extracellular protease activity (See Chapter 2). They also displayed reduced IsaA in their culture supernatants. We hypothesized that the lack of IsaA in the culture supernatants of these isolates was due to breakdown by extracellular proteases.

To simplify experimentation, we selected a representative non-resistant isolate (BB2515) and a representative resistant isolate (BB2519), both isolated from a drip reactor biofilm. We had

previously performed our Western blots on these isolates using horseradish peroxidase-conjugated antibodies, but by the time of the experiments in this chapter, we had access to the much more sensitive Licor Odyssey system. Using this system, we observed extracellular IsaA abundance in wild type and the two isolates (Figure 13A). As we saw previously, BB2515 has similar IsaA levels to wildtype, while BB2519 has dramatically less. Interestingly, the more sensitive system clearly detects a set of bands at higher molecular weight corresponding to Surface Protein A (Spa) in the wildtype and BB2515. In BB2519, these protein A bands appear farther down the blot, indicating degradation.

We grew mixed cultures of wildtype with either BB2515 or BB2519 and probed their supernatants for IsaA. When wildtype is cultured with BB2515, IsaA is present at levels similar to those observed in the wildtype, but when grown with BB2519, IsaA is severely diminished (Figure 13B), indicating that BB2519 is not simply deficient for IsaA production, but actively removes it from the supernatant.

Further, we mixed cell-free supernatant from wildtype with cell-free supernatants from BB2515 or BB2519. When wildtype supernatant is mixed with supernatant from BB2515, IsaA is present at normal levels. When mixed with supernatant from BB 2519, IsaA is diminished (Figure 13C).

Because many of the *S. aureus* proteases are dependent on divalent cations, we cultured BB2515 and BB2519 in TSB with and without EDTA to reduce extracellular protease activity, then Western blotted the culture supernatants for IsaA. EDTA partially restored IsaA to the supernatant of BB2519, but did not affect levels in BB2515 (Figure 13D). Taken together, these results suggest that the lack of IsaA in these six isolates is likely due to increased extracellular protease activity.

Rat throat isolate forms robust biofilm despite tannic acid treatment or IsaA overexpression

Isolate BB2588 is the only isolate that both resists biofilm inhibition by tannic acid and does not have a mutation in *rsbU*. In a microtiter plate biofilm model, it resisted tannic acid's biofilm inhibitory effect, mimicking the phenotype of an *isaA* mutant (Figure 14A). We showed in Chapter 2 that when wild-type *S. aureus* is grown with tannic acid, there is more IsaA found in the culture supernatant. One explanation for this isolate's resistance, and its phenotypic similarity to the *isaA* mutant, would be that it does not respond to tannic acid by increasing extracellular IsaA abundance. To test this, we grew the isolate in 66% TSBg supplemented with tannic acid and western blotted the cell-free culture supernatants for IsaA (Figure 14B). The trend seen in this Western blot is similar to what we had previously seen in the wild-type, with both strains demonstrating increased IsaA abundance in spent medium when grown with tannic acid.

It is possible that, although IsaA abundance increases in this isolate, it does not increase to the same degree as in the wild-type. To address this possibility, we transformed pKP1 into isolate BB2588 and induced IsaA production in our microtiter plate biofilm model. When induced with anhydrotetracycline (aTet), IsaA levels are similar in the wild-type and the isolate (Figure 14C). Despite the increased levels of IsaA, the isolate forms a robust biofilm in the microtiter plate (Figure 14D).

Variant in PknB leads to resistant biofilm

We hypothesized that one or more of the variants found in the isolate was responsible for the biofilm phenotypes that we observed in Figure 14. To narrow our list of candidates, we wanted to sort out which variants were likely to be deleterious to protein function from those that would

likely be benign. Two web-based tools exist for such analysis, and we used both to reduce our chances of ignoring a relevant variant due to a false negative. These two tools use different algorithms to perform essentially the same task; they both use a wide array of sequence information (including conservation across homologs and annotated active sites) to determine how likely a given amino acid substitution is to impede protein function. First, we used SNAP2 (Screening for Non-Acceptable Polymorphisms) [163], which scored two of the variants (those found in *pknB* and *arlS*) as being likely to affect protein function. We also used SIFT (Sorting Intolerant From Tolerant) [164]. SIFT flagged those two variants, as well as two more (those found in *ftsH* and *gluD*). These results allowed us to narrow our search to these four genes.

To see if one of our four candidate genes was involved in biofilm resistance to tannic acid, we used transposon mutants from the Nebraska Transposon Mutant Library (NTML) [165]. Transposon mutants for each of the four candidate genes, along with JE2 wildtype, were cultured in a microtiter plate biofilm assay. The *pknB*::*tn* strain was the only strain that resisted tannic acid's biofilm-inhibitory effect (Figure 15A). This result suggested that PknB may play a role in the isolate's resistance to tannic acid.

To further demonstrate PknB's role, we complemented the isolate by expressing PknB from a xylose-inducible promoter and challenged this strain with tannic acid in a microtiter plate biofilm. Expression of PknB restores tannic acid sensitivity. We also recreated the *pknB* variant in a clean SH1000 background, which was sufficient to give this new strain tannic acid resistance. Resistance in this strain could also be complemented by expressing wild-type PknB (Figure 15B). These results demonstrate that the *pknB* variant is responsible for the tannic acid resistant phenotype of the isolate.

PknB deletion is phenotypically equivalent to variant

Because the biofilm phenotype of the isolate could be complemented by expressing wild-type PknB, we hypothesized that the *pknB* variant in the isolate rendered PknB non-functional and that this would be functionally equivalent to a clean *pknB* deletion. We created a Δ*pknB* strain and observed its response to tannic acid in the microtiter plate biofilm assay. The deletion strain was resistant to tannic acid, and sensitivity was restored when PknB was expressed from a plasmid (Figure 15B).

To further demonstrate this, we also overexpressed IsaA in a Δ*pknB* strain. When overexpressed, IsaA was found in the culture supernatant at similarly elevated levels in both the wild type and the Δ*pknB* strain (Figure 16B). As in the isolate, the Δ*pknB* strain formed a robust biofilm even when IsaA was overexpressed (Figure 16A).

Spent medium does not inhibit biofilm formation

The exact role of PknB in *S. aureus* is unknown, though a model has been put forward by Dworkin's group by analogy to PrkC, a homolog found in *B. subtilis* [137]. Briefly, it was demonstrated by Shah *et al*. that *S. aureus* PknB binds extracellular fragments of *S. aureus* peptidoglycan that diffuse away from dividing cells. If PknB is heterologously expressed in *B. subtilis* spores, the spores will germinate in response to *S. aureus* peptidoglycan fragments. Maximal germination was achieved by adding concentrations as low as 1 ng/ml and as high as 100 mg/ml of digested peptidoglycan. Based on these data, they propose that PknB is activated by binding a fragment of peptidoglycan, and that this activation will have some effect on signaling within the cell.

Based on this model and on our own results that PknB was involved in biofilm inhibition, we hypothesized that PknB binds a fragment of peptidoglycan released by IsaA, and that as a result of that binding, biofilm formation is inhibited. We set out to see whether such signaling occurred in *S. aureus*, and if so, to identify the signal.

The simplest way to assay for this signal's existence was to add spent medium from a culture overexpressing IsaA to a culture growing in a microtiter plate biofilm assay. Contrary to our expectation, sterile spent medium from cells overexpressing IsaA had no effect on biofilm formation (Figure 17). This suggested that no signaling molecule is released by IsaA or that such a signal is released but is unstable, making such a static addition of spent medium ineffective.

Exogenous IsaA inhibits biofilm

Given that spent medium failed to inhibit biofilm formation, we wanted to determine if IsaA's effect could be communicated from one cell to another, or if the cells of a biofilm had to be producing IsaA themselves in order to be dispersed. To test this, we used a transwell apparatus, which is essentially a microtiter plate divided into two chambers. The chambers are separated by a 0.45 μm filter, allowing proteins, metabolites, and other small molecules to pass through, but preventing the passage of cells. We grew cells overexpressing IsaA in the upper chamber and assayed biofilm formation of various strains in the lower chamber (Figure 18).

Wildtype *S. aureus* overexpressing IsaA did not form a biofilm. Wildtype *S. aureus* without IsaA overexpression has a distinct clearing in the middle of the well, corresponding to the part of the well directly below the filter. A Δ*pknB* mutant formed a robust biofilm, even when IsaA was

overexpressed in the top chamber. These results indicate that overabundance of IsaA inhibits biofilm formation, even when the IsaA is produced by cells outside the biofilm.

SceD also inhibits biofilm formation

Based on the hypothesis that PknB recognizes a specific fragment of peptidoglycan released by IsaA, we wondered if other peptidoglycan digesting enzymes could have similar effects on biofilm formation. We overexpressed *sceD, lytN, and ssaA* in the microtiter plate biofilm assay. SceD is the other lytic transglycosylase in *S. aureus*, and is predicted to have the same substrate specificity as IsaA [113]. LytN and SsaA are amidases, which cleave between the glycan chain and the peptide stem. Overexpression of *sceD* inhibited biofilm formation similar to *isaA*. Overexpression of either amidase had no effect (Figure 19). The fact that the two lytic transglycosylases inhibit biofilm formation, as well as the fact that neither of the amidases we tested has this effect, suggests that SceD functions in the same way as IsaA to inhibit biofilm formation.

Peptidoglycan fragments do not activate PknB

We wanted to use Shah *et al.*'s *B. subtilis* heterologous expression system to develop a more sensitive assay to look for the signal that PknB recognized. To do this, we first tried to replicate their results showing that *B. subtilis* spores heterologously expressing *S. aureus pknB* could be germinated by *S. aureus* peptidoglycan [137]. Spores of *B. subtilis* Δ*prkC* heterologously expressing *pknB* were successfully germinated by incubation with alanine alone or a mix of alanine and aspartic acid (Figure 20A). Spores of the same strain were not germinated by addition of purified peptidoglycan that had been digested with mutanolysin (Figure 20B). The concentrations of peptidoglycan shown are between 0.2 and 20 µg/ml, several orders of

magnitude higher than the minimum concentration shown by Shah *et al.* to induce maximal germination, and well within the effective range they tested.

To rule out the possibility that the failure to germinate was due to PknB not being properly heterologously expressed, we attempted to germinate wildtype *B. subtilis* spores with *B. subtilis* peptidoglycan. Spores were not germinated by incubation with purified peptidoglycan digested with mutanolysin (Figure 20C).

PknB mutant's resistance is a result of increased PIA

In addition to searching for a diffusible signaling molecule, we were also interested in elucidating how PknB affects resistance to tannic acid and IsaA. One way that the Δ*pknB* mutant could resist IsaA's antibiofilm effect is by modulating its matrix composition. We hypothesized that the *pknB*-dependent biofilm phenotypes we have observed could be due to overabundant Polysaccharide Intercellular Adhesin (PIA), which would lead to a sturdier matrix and a more robust biofilm [44]. This is consistent with our data showing that the Δ*pknB* mutant forms a thicker biofilm than the wildtype (Figure 4). We created and tested a Δ*pknB*, *ica*::*tet* strain for tannic acid resistance. The *ica* locus encodes the enzymes responsible for PIA synthesis, rendering this strain unable to produce PIA. Where the Δ*pknB* strain is resistant to tannic acid, the double mutant is sensitive, similar to the wild-type (Figure 21A). We also grew biofilms of the wildtype and Δ*pknB* strains, prepped PIA from those biofilms, and performed a dot blot with α-PIA antibody. The Δ*pknB* strain's biofilm had more PIA (Figure 21B). Taken together, these results indicate that PIA production is increased in the Δ*pknB* strain, leading to a more robust biofilm that can withstand tannic acid stress.

Discussion

In Chapter 2, we demonstrated that tannic acid effectively inhibited biofilm formation by *S. aureus*. We also showed that, given time, resistance to tannic acid can occur both *in vitro* and *in vivo*. Understanding how resistance arises can help us understand more about the mechanism by which tannic acid and IsaA inhibit biofilm formation. In this chapter, we investigated how these biofilms developed resistance to treatment.

We sequenced the genomes of nine isolates from tannic acid resistant biofilms, and found that six of them shared the common theme of *rsbU* mutations, leading to decreased extracellular IsaA (Figure 13) and robust biofilm formation (Chapter 2, Figure 9). Another of the isolates had a mutation in *pknB*, which caused its resistance to tannic acid. Complementing *pknB* restored sensitivity similar to the wild-type (Figure 15), and a Δ*pknB* mutant shared the resistant phenotype of the isolate (Figure 16). The Δ*pknB* mutant had increased PIA, and knocking out PIA production in a Δ*pknB* mutant background restored sensitivity (Figure 21).

RsbU (Regulator of Sigma factor B) has only one described function in *S. aureus*: to activate the alternative sigma factor SigB. Because of this, *rsbU* mutations and *sigB* mutations lead to the same phenotypes and are typically considered to be functionally interchangeable [166,167]. The three sequenced isolates from a drip reactor biofilm demonstrated phenotypes consistent with *rsbU* or *sigB* mutation, namely lack of golden pigment and increased extracellular protease activity [119,168]. This protease activity leads to a decrease in extracellular IsaA abundance, which could explain how these cells are resistant to tannic acid's antibiofilm effect.

Interestingly, these *rsbU* mutants also appear to confer resistance on their neighbors, as they are the only ones of the 11 dripper isolates to have resistance when cultured individually.

Overproduction of extracellular proteases could be considered a communal good, and a way for a small subset of cells to adapt in a way that allows a larger community to thrive in the face of harsh conditions.

The role of SigB and RsbU in *S. aureus* biofilm formation is controversial [119,169–172], with some labs finding that *sigB* mutants are unable to form a biofilm and others finding that they form very robust biofilms. Our findings indicate that under our conditions, *rsbU* mutants do, in fact, form robust biofilms that resist inhibition. Further research is needed to understand the underlying reasons for the phenotypic discrepancies reported in the literature.

Three of the four rat-throat isolates that were resistant to tannic acid were also *rsbU* mutants. The other one owed its resistance to a mutation in *pknB*. PknB is the only annotated serine/threonine kinase in *S. aureus* [173]. It spans the cell membrane, with an intracellular kinase domain and an extracellular PASTA (Penicillin binding And Serine Threonine kinase Associated) domain.

The PASTA domain is known to bind peptidoglycan [174], but how that binding affects the cell has been debated. The model that most *S. aureus* researchers have adopted comes from research by Shah *et al*. in *B. subtilis* [137]. In that organism, PrkC (the PknB homolog) is found on the surface of spores. When neighboring cells divide, they emit specific fragments of peptidoglycan that are unique to growing cells. PrkC binds these fragments, and in response to this binding, the spores germinate and enter an active growth phase. They also show that the *S. aureus* PknB, when expressed in a Δ*prkC* mutant of *B. subtilis*, is able to cause germination in response to *S. aureus* peptidoglycan.

We found this model to be very promising, and we envisioned a role for PknB in a transition from a less active lifestyle (biofilm) to a more active one (planktonic). We hoped to use the tools developed in the Dworkin lab to help us understand PknB's contribution to biofilm regulation in *S. aureus*. Specifically, we wanted to search for a signaling molecule that would be released by IsaA cleavage of peptidoglycan and would be detected by PknB. Unfortunately, we were unable to replicate the results presented by Shah *et al.*, as purified peptidoglycan did not germinate *B. subtilis* spores.

Spent medium from cells overexpressing IsaA did not inhibit biofilm formation (Figure 17), but overexpression of IsaA did cause biofilm inhibition in neighboring cells (Figure 18). When added to our inability to replicate Shah *et al*.'s data, this has caused us to reevaluate our hypothesis that a signaling molecule is released by IsaA and detected by PknB. A competing hypothesis is that PknB threads through peptidoglycan to sense peptidoglycan rigidity. This would be supported by our data, and will be discussed in Chapter 4.

Polysaccharide Intercellular Adhesin (PIA) is a major component of the biofilm matrix [65]. PIA is produced and processed by genes found in the *ica* operon. The regulation of this operon is complex and responds to many factors (See Chapter 1). To the best of our knowledge, this is the first report directly linking PknB to PIA production. Although the mechanism by which increased PIA confers tannic acid resistance to a biofilm is unknown, we would speculate that PIA is lending structural support to strengthen the biofilm matrix, counteracting the destabilizing effect of IsaA overexpression.

How PknB affects PIA production is an open question that we are actively investigating. PknB inactivates the catabolite control protein CcpA by phosphorylating two threonine residues on

CcpA's DNA-binding surface [175]. CcpA, in turn, is known to regulate the *ica* operon [176]. It stands to reason that PknB could be regulating PIA through inactivation of CcpA.

Our findings demonstrate two unique ways in which *S. aureus* biofilms can adapt to evade tannic acid treatment. The first demonstrates how a subset of cells can adapt to provide resistance to their entire community. The second method (increasing PIA production) could also have a community effect, but that remains to be tested. Because *S. aureus* colonization is a major risk factor for surgical-site and other infections, hospitals make rigorous efforts to decolonize patients. Understanding how the bacteria adapt to evade decolonization efforts is an important step to improving such treatments.

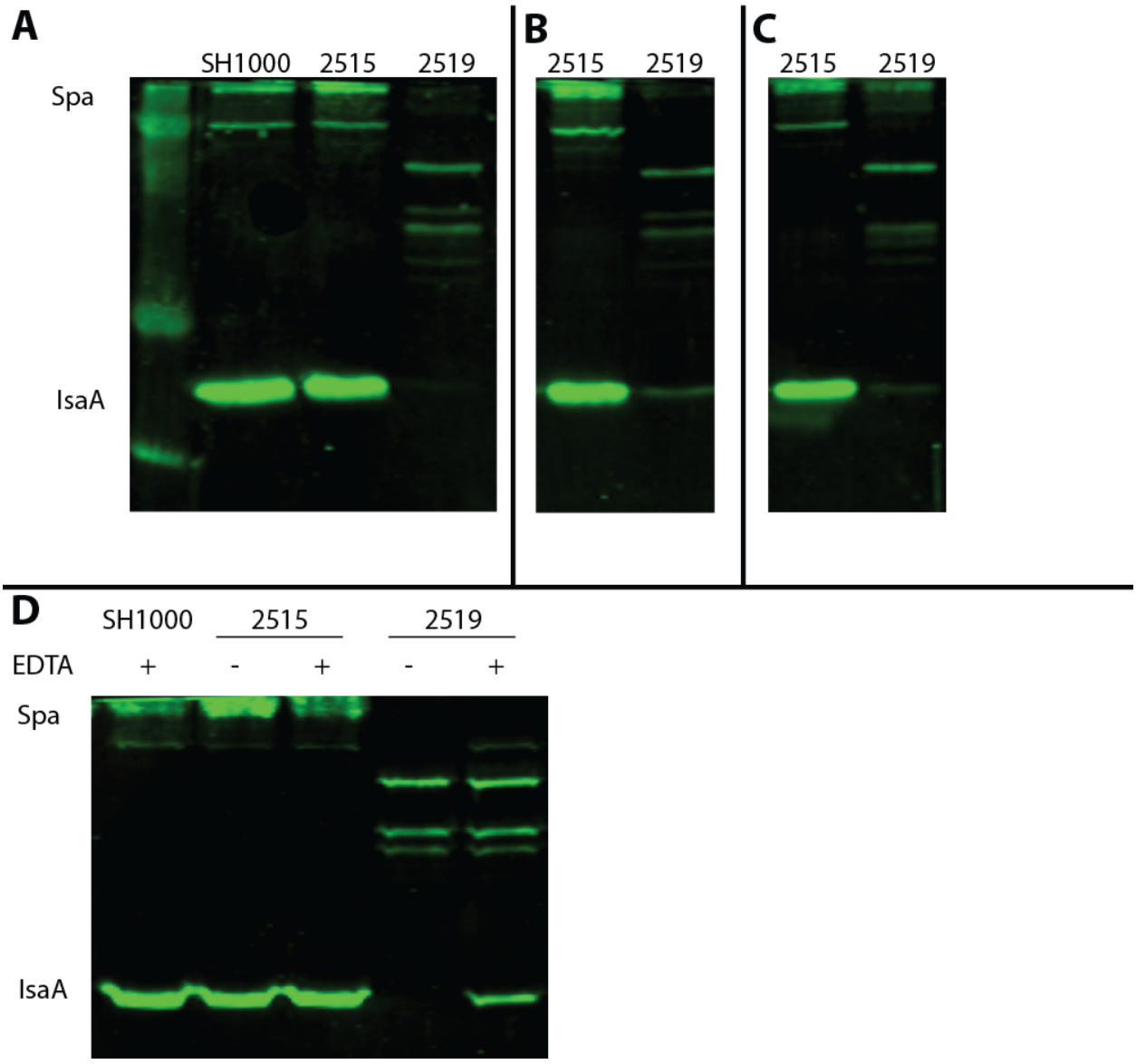

Figure 15. Tannic acid resistant dripper isolate degrades IsaA from supernatant.
α-IsaA Western blots of culture supernatants from mid-log phase SH1000 (BB386) and two isolates from dripper biofilm grown with tannic acid. IsaA and Surface Protein A (Spa) are marked. (A) Overnight cultures backdiluted and grown 4 hours. IsaA does not accumulate in supernatant from isolate BB2519. (B) Overnight cultures from two isolates mixed 1:1 with overnight culture of wildtype, then back diluted and grown 4 hours. IsaA does not accumulate in supernatant from coculture of isolate BB2519 with wildtype. (C) Cell-free supernatants from 1A mixed 1:1 (2515:386 and 2519:386). A factor in 2519 supernatant eliminates IsaA from wildtype supernatant. (D) Overnight cultures backdiluted in TSB containing 0.1 mM EDTA and grown 4 hours. Growth in EDTA restores IsaA in BB519 supernatant.

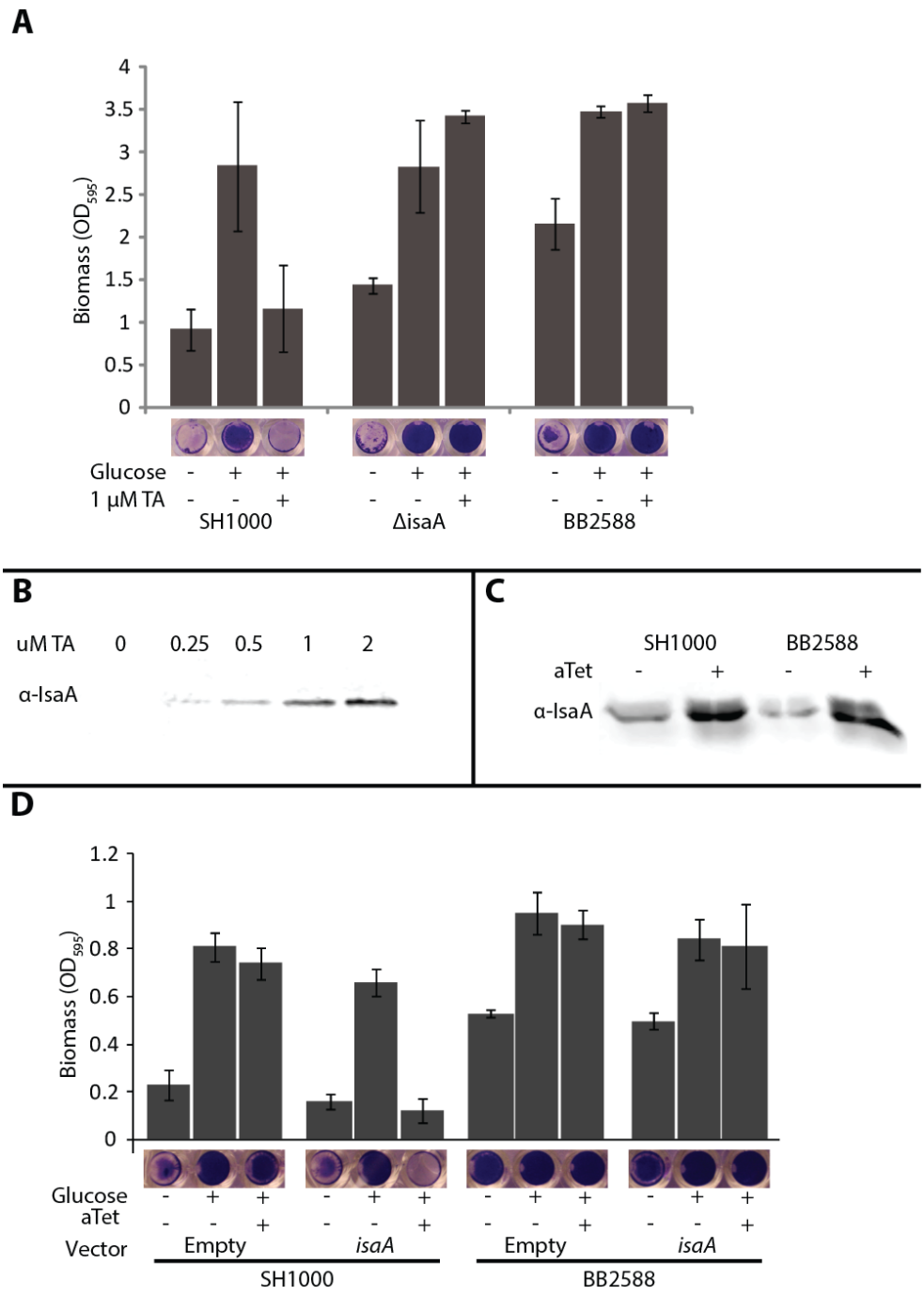

Figure 16. Rat throat isolate resists biofilm inhibition.
(A) *S. aureus* isolate BB2588 forms robust biofilm in the presence of tannic acid in the microtiter plate biofilm assay. Error bars represent standard deviation. (B) α-IsaA Western blot of cell-free supernatants from cultures of BB2588 grown overnight in 66% TSBg + tannic acid. Supernatant volume normalized to OD600 of culture. IsaA level increases when grown with tannic acid. (C) Microtiter plate biofilm assay of strains BB1209 ("WT + EV"), BB2242 ("WT + IsaA"), BB2768 ("2588 + EV"), and BB 2769 ("2588 + IsaA") with aTet induction. Isolate BB2588 forms a biofilm when IsaA is induced. (D) α-IsaA Western blot of IsaA induction in BB2242 and BB2769. IsaA is induced to similar levels by aTet.

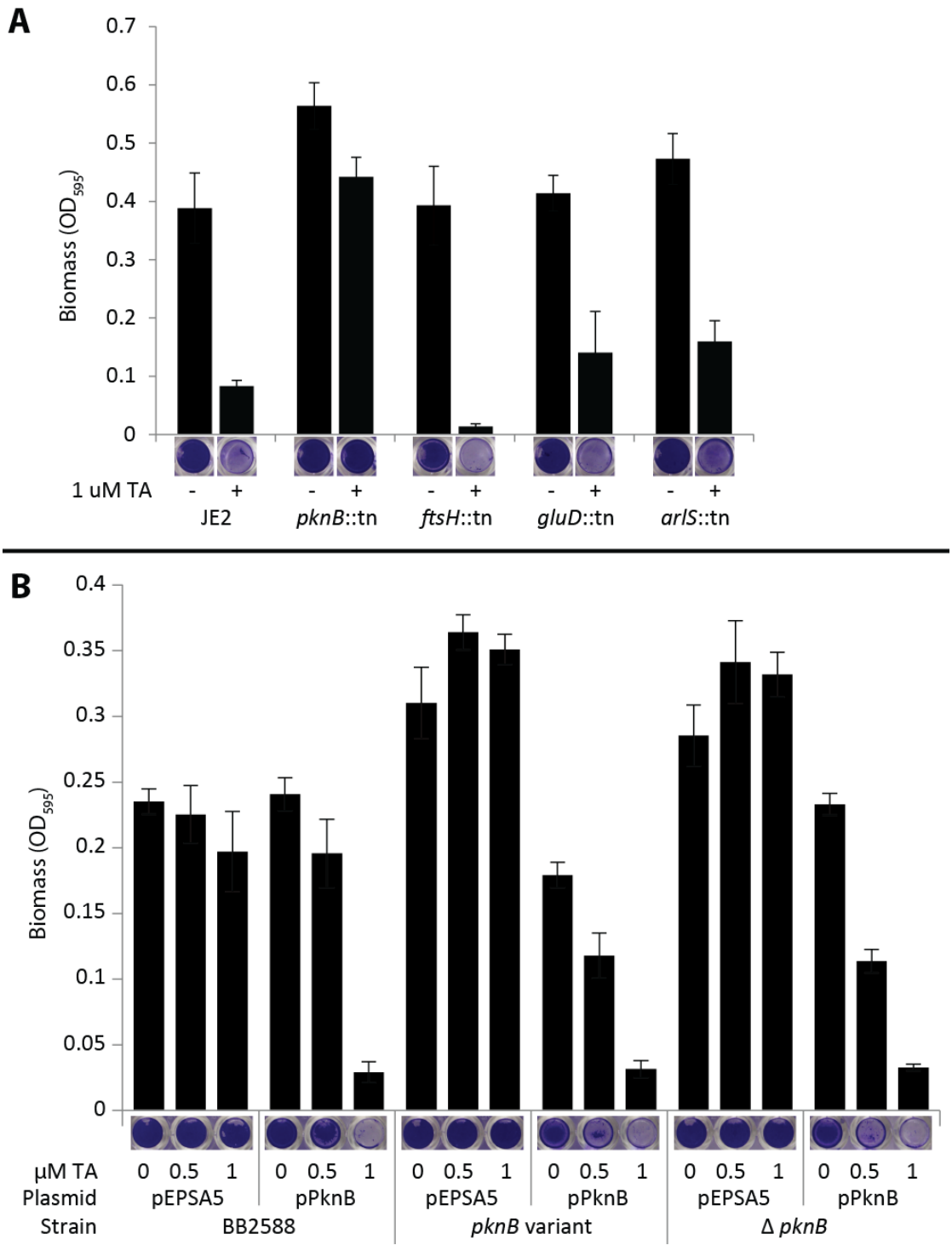

Figure 17. Variant in *pknB* causes resistance in isolate.
(A) Four transposon mutants from NARSA library tested for tannic acid resistance in microtiter plate biofilm assay, compared to JE2 wildtype. Biofilms grown in PNG medium. Error bars represent standard deviation. *pknB*::tn resists inhibition. (B) Induced expression of *pknB* from a plasmid in three strain backgrounds tested for resistance to tannic acid in microtiter plate biofilm assay. Error bars represent standard deviation. *pknB* expression restored sensitivity in all three strains.

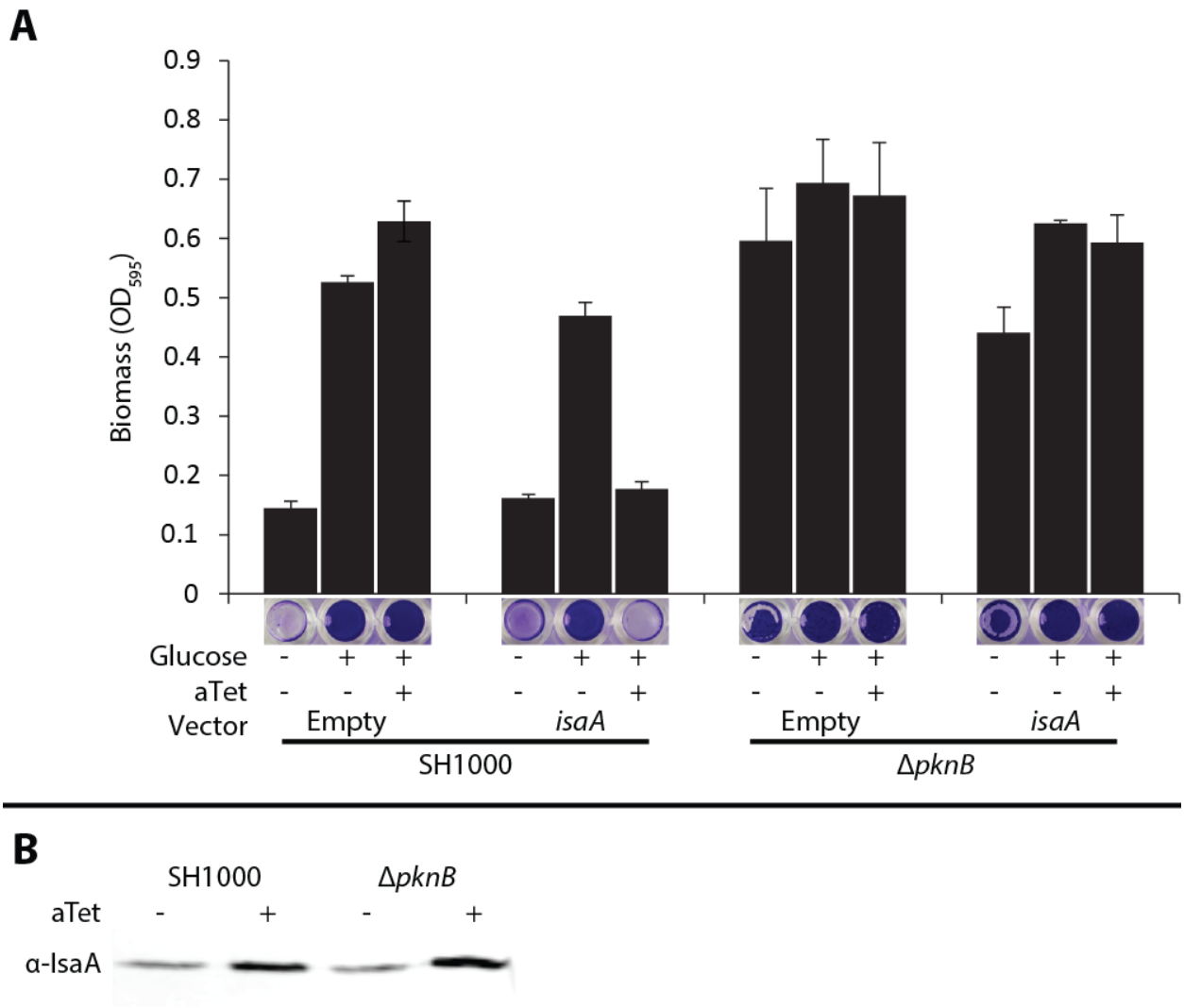

Figure 18. Δ*pknB* mutant recapitulates isolate biofilm phenotype.
(A) SH1000 and Δ*pknB* with IsaA overexpressed by aTet induction in microtiter plate biofilm assay. Error bars represent standard deviation. Δ*pknB* mutant's biofilm formation is not inhibited by IsaA overexpression. (B) α-IsaA Western blot of IsaA induction in BB2242 and BB2767. IsaA is induced to similar levels by aTet.

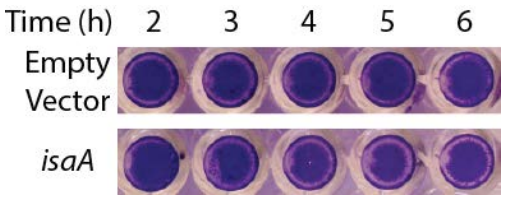

Figure 19. Cell-free supernatant from IsaA-overexpressing cultures does not inhibit biofilm formation. Biofilm cultures of BB1209 and BB2242 were grown in TSBg + aTet. At times indicated, supernatants were harvested. Sterile supernatants were added 1:1 to fresh biofilm cultures of BB1209. Following incubation, there is no difference between biofilms grown with supernatant where IsaA was overexpressed and where it was not.

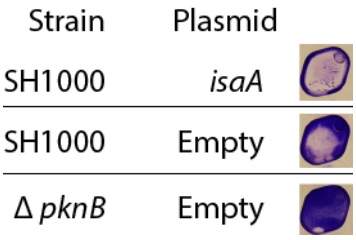

Figure 20. Exogenously expressed IsaA inhibits biofilm formation.
BB1209, BB2242, and BB 2766 were grown in the bottom chamber of a transwell apparatus with BB2242 in the upper chamber. Cultures were grown in 66% TSBg + aTet to induce IsaA expression and biofilm formation was assayed in the bottom chamber. BB1209's biofilm formation was inhibited by a diffusible factor released by BB2242 in the upper chamber.

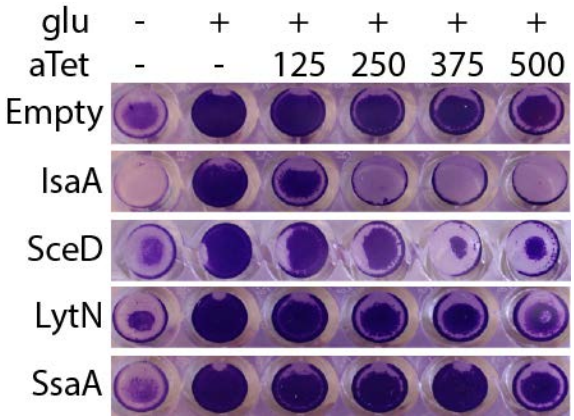

Figure 21. Overexpression of either *S. aureus* lytic transglycosylase inhibits biofilm formation.
SH1000 harboring an empty vector ("EV", BB1209), or overexpression vector for IsaA (BB2242), SceD (BB2664), LytN (BB2701) or SsaA (BB2702) was assayed for biofilm formation in the microtiter plate biofilm assay. Biofilm formation was inhibited in strains overexpressing IsaA and SceD, but not LytN or SsaA.

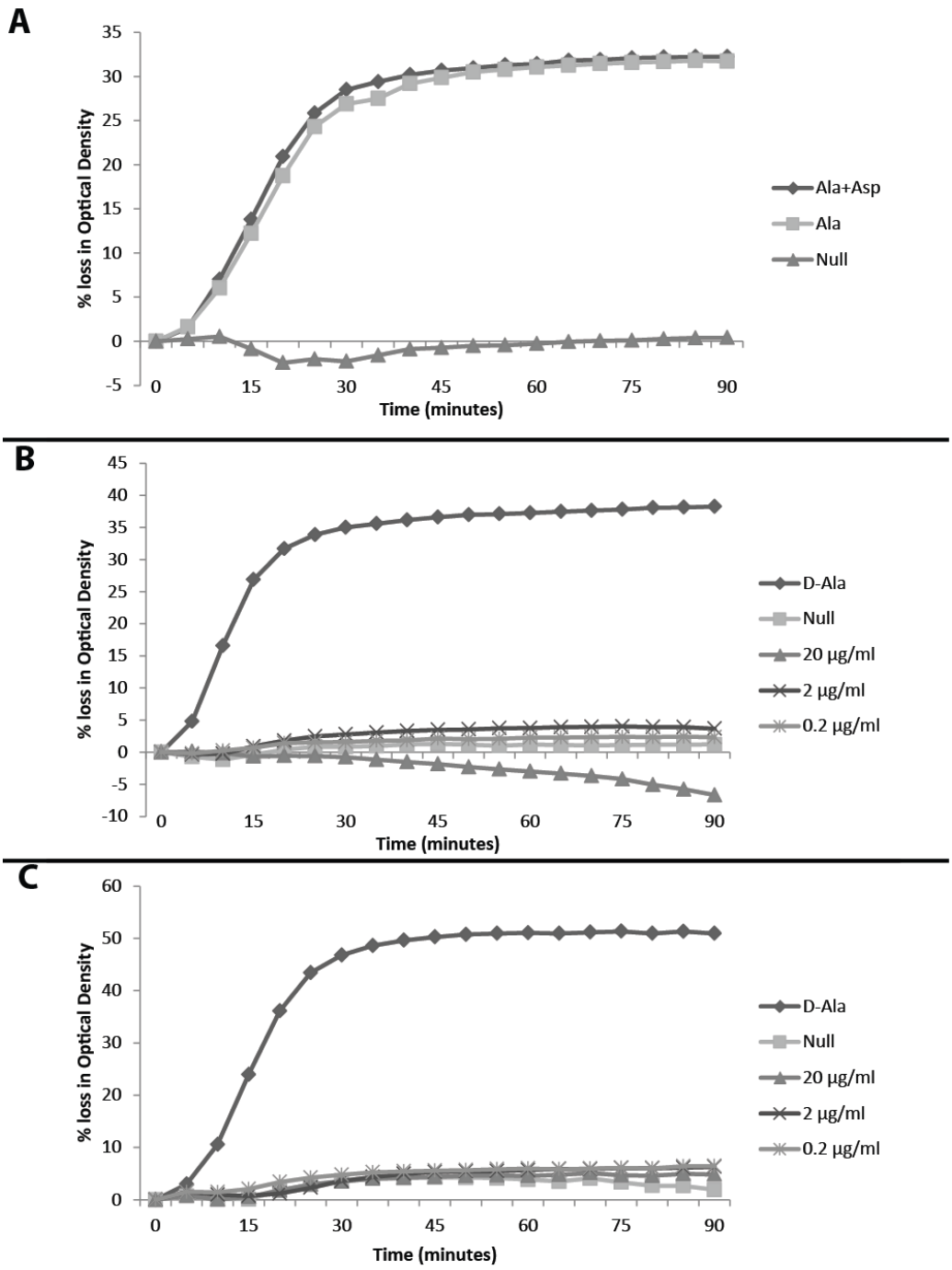

Figure 22. Peptidoglycan fragments do not cause germination of *B. subtilis* spores.
(A) *B. subtilis* spores germinate in response to alanine or alanine plus aspartic acid. Percent loss in optical density is proportional to fraction of spores germinated. (B) *B. subtilis* Δ*prkC* + *pknB*$_{(Sa)}$ does not germinate when incubated with noted concentrations of mutanolysin-digested *S. aureus* peptidoglycan. (C) Wildtype *B. subtilis* 168 does not germinate when incubated with noted concentrations of mutanolysin-digested *B. subtilis* peptidoglycan.

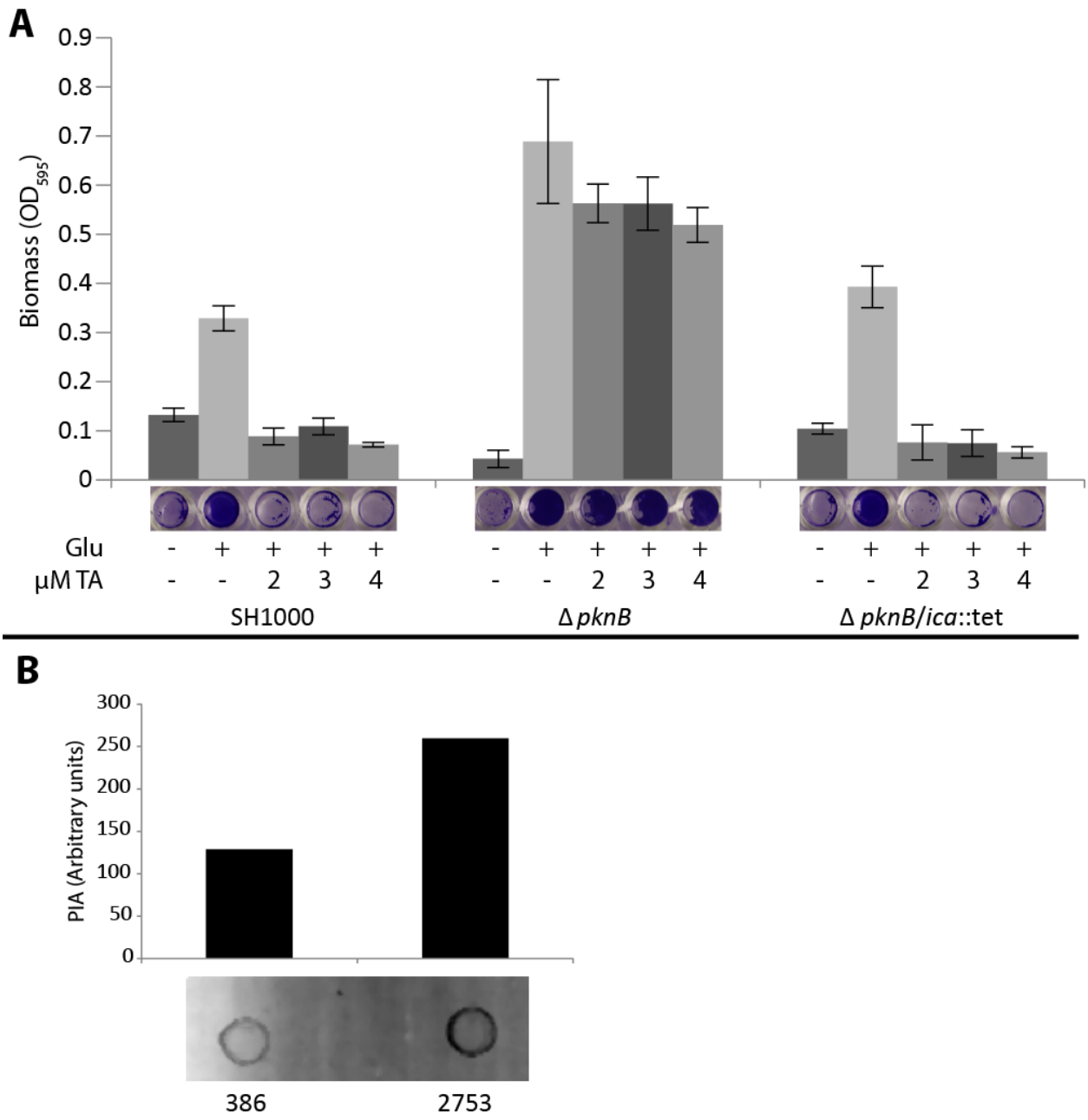

Figure 23. Δ*pknB* mutant's tannic acid resistance is due to overproduction of PIA.
(A) Microtiter plate biofilm assay of SH1000 (BB386), Δ*pknB* (BB2753), and Δ*pknB, ica*::tet (BB3016) grown in 66% TSBg + tannic acid. Error bars represent standard deviation. Δ*pknB* resists biofilm inhibition while Δ*pknB, ica*::tet is sensitive. (B) PIA is more abundant in Δ*pknB* than in wildtype SH1000. Bars represent quantitation of dot minus local background as measured by Licor Odyssey.

Table 4. Strains and plasmids used in Chapter 3.

Strain	Description	Resistance	Source
S aureus strains			
BB386	SH1000		[177]
BB595	SH1000 *ica*::tet	tet	[106]
BB1209	SH1000/pALC2073	cm	[143]
BB1415	SH1000/pEPSA5	cm	[178]
BB2183	SH1000 *isaA*::tet	tet	[113]
BB2242	SH1000/pKP1	cm	[151]
BB2268	JE2	erm	[165]
BB2519	Drip reactor isolate	spec	[151]
BB2520	Drip reactor isolate	spec	[151]
BB2524	Drip reactor isolate	spec	[151]
BB2546	JE2 *pknB*::Tn	erm	[165]
BB2585	Rat throat isolate	spec	[151]
BB2586	Rat throat isolate	spec	[151]
BB2587	Rat throat isolate	spec	[151]
BB2588	Rat throat isolate	spec	[151]
BB2589	Rat throat isolate	spec	[151]
BB2590	Rat throat isolate	spec	[151]
BB2664	SH1000/pALC2073-sceD	cm	This Work
BB2701	SH1000/pALC2073-lytN	cm	This Work
BB2702	SH1000/pALC2073-ssaA	cm	This Work
BB2753	SH1000 Δ*pknB*		This Work
BB2766	SH1000 Δ*pknB*/pALC2073	cm	This Work
BB2767	SH1000 Δ*pknB*/pKP1	cm	This Work
BB2768	BB2588/pALC2073	cm	This Work
BB2769	BB2588/pKP1	cm	This Work
BB2827	SH1000 *pknB*-P208R		This Work
BB2974	JE2 *ftsH*:tn	erm	[165]
BB2976	JE2 *gluD*::tn	erm	[165]
BB2978	JE2 *arlS*::tn	erm	[165]
BB3016	SH1000 Δ*pknB*, *ica*::tet	tet	This Work
BB3030	SH1000 *pknB*-P208R/pEPSA5	cm	This Work
BB3032	SH1000 *pknB*-P208R/pEPSA5-pknB	cm	This Work
BB3036	BB2588/pEPSA5	cm	This Work
BB3037	BB2588/pEPSA5-pknB	cm	This Work
BB3039	SH1000 Δ*pknB*/pEPSA5	cm	This Work
BB3040	SH1000 Δ*pknB*/pEPSA5-pknB	cm	This Work
B. subtilis strains			
BB2955	168 Δ*prkC*, amyE:pSpac-his6-pknB		[137]
BB2992	168		[179]
Plasmids			
pALC2073		Cm	[143]
pKP1	*isaA* under control of tet promoter	Cm	[151]
pEPSA5		Cm	[180]
pEPSA5-PknB	PknB under control of xylose promoter	Cm	[181]

Table 5. Overview of isolates discussed in Chapter 3. Indicated in rows is whether a given isolate has a variant in *rsbU*, a variant in *pknB*, whether a given isolate is resistant to tannic acid's antibiofilm effect, and whether or not an isolate was subjected to whole genome sequencing.

Source	Dripper											Rat Throat					
Isolate	2515	2518	2519	2520	2521	2522	2523	2524	2527	2528	2529	2585	2586	2587	2588	2589	2590
RsbU variant?	N	N	Y	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	N	N
PknB variant?	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	N	N
TA resistant?	N	N	Y	Y	N	N	N	Y	N	N	N	Y	Y	Y	Y	N	N
WGS?	N	N	Y	Y	N	N	N	Y	N	N	N	Y	Y	Y	Y	Y	Y

Table 6. Results from whole genome sequencing analysis

Genome position	Reference sequence	Variant sequence	Ref AA	Variant AA	Locus tag	Gene symbol	Protein function
				BB2519			
1447592	C	T	V	I	SAOUHSC_01492	engA	GTP-binding protein EngA
2134154	C	A			SAOUHSC_02301	rsbU	sigmaB regulation protein RsbU
2464717	G	T	A	D	SAOUHSC_02681	narG	nitrate reductase subunit alpha
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
2782815	ACCCCCCT	ACCCCCT	frameshift		SAOUHSC_03008	hisF	multifunctional imidazole glycerol phosphate synthase subunit/phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase
2805726	G	A	D	N	SAOUHSC_03033	nixA	high affinity nickel transporter
				BB2520			
2134530	TAT	TAAT	frameshift		SAOUHSC_02302	rsbU	sigmaB regulation protein RsbU
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
				BB2524			
2134530	TAT	TAAT	frameshift		SAOUHSC_02302	rsbU	sigmaB regulation protein RsbU
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
				BB2585			
1442555	C	A	G	V	SAOUHSC_01485	ndk	nucleoside diphosphate kinase
1447592	C	T	V	I	SAOUHSC_01492	engA	GTP-binding protein EngA
1921335	C	T	S	N	SAOUHSC_02012	mgt	glycosyltransferase
2134154	C	A			SAOUHSC_02301	rsbU	sigmaB regulation protein RsbU
2310015	C	T	G	D	SAOUHSC_02494	rpsE	30S ribosomal protein S5
2464717	G	T	A	D	SAOUHSC_02681	narG	nitrate reductase subunit alpha
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein

Genome position	Reference sequence	Variant sequence	Ref AA	Variant AA	Locus tag	Gene symbol	Protein function
2805726	G	A	D	N	SAOUHSC_03033	nixA	high affinity nickel transporter
				BB2586			
122322	A	T	N	I	SAOUHSC_00117	cap5D	capsular polysaccharide biosynthesis protein
418517	C	G	G	R	SAOUHSC_00415		hypothetical protein
1705465	C	A	L	I	SAOUHSC_01803	aapA	hypothetical protein
1860179	G	A	S	L	SAOUHSC_01955	lukEv	leukotoxin LukE
2079361	G	T	D	Y	SAOUHSC_02244	dapE	succinyl-diaminopimelate desuccinylase
2091181	C	A	A	E	SAOUHSC_02258		hypothetical protein
2134530	TAT	TAAT	frameshift		SAOUHSC_02302	rsbU	sigmaB regulation protein RsbU
2182641	T	G	E	D	SAOUHSC_02361	rpmE2	50S ribosomal protein L31 type B
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
2782815	ACCCCCCT	ACCCCCT	frameshift		SAOUHSC_03008	hisF	multifunctional imidazole glycerol phosphate synthase subunit/phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase
				BB2587			
944248	G	A	E	K	SAOUHSC_00968		hypothetical protein
2134530	TAT	TAAT			SAOUHSC_02302	rsbU	sigmaB regulation protein RsbU
2310017	T	A	K	N	SAOUHSC_02494	rpsE	30S ribosomal protein S5
2518792	G	C	A	G	SAOUHSC_02739		2-dehydropantoate 2-reductase
2518799	T	C	K	E	SAOUHSC_02739		2-dehydropantoate 2-reductase
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
2782815	ACCCCCCT	ACCCCCT	frameshift		SAOUHSC_03008	hisF	multifunctional imidazole glycerol phosphate synthase subunit/phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase
				BB2588			

Genome position	Reference sequence	Variant sequence	Ref AA	Variant AA	Locus tag	Gene symbol	Protein function
484434	C	A	A	D	SAOUHSC_00486	ftsH	hypothetical protein
852577	C	G	E	Q	SAOUHSC_00889	mnhA1	monovalent cation/H+ antiporter subunit A
859989	C	G	R	G	SAOUHSC_00895	gluD	glutamate dehydrogenase
1138825	C	G	P	R	SAOUHSC_01187	pknB	hypothetical protein
1360474	C	G	G	A	SAOUHSC_01419	arlS	hypothetical protein
2170507	C	G	G	A	SAOUHSC_02345	atpA	F0F1 ATP synthase subunit alpha
2310012	C	T	G	D	SAOUHSC_02494	rpsE	30S ribosomal protein S5
2569367	T	A	S	C	SAOUHSC_02797		hypothetical protein
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
2782815	ACCCCCCT	ACCCCCT	frameshift		SAOUHSC_03008	hisF	multifunctional imidazole glycerol phosphate synthase subunit/phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase
BB2589							
484434	C	A	A	D	SAOUHSC_00486	ftsH	hypothetical protein
2310017	T	A	K	N	SAOUHSC_02494	rpsE	30S ribosomal protein S5
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein
2782815	ACCCCCCT	ACCCCCT	frameshift		SAOUHSC_03008	hisF	multifunctional imidazole glycerol phosphate synthase subunit/phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase
BB2590							
1695507	G	T	A	D	SAOUHSC_01797		DNA polymerase I
2310015	C	T	G	D	SAOUHSC_02494	rpsE	30S ribosomal protein S5
2514397	A	T	L	I	SAOUHSC_02733		hypothetical protein
2746906	G	A	A	V	SAOUHSC_02980		hypothetical protein

Notes

The whole genome sequence analysis in this chapter was performed by Jeremy Schroeder at the University of Michigan. The Bacillus germination work was performed in collaboration with Tony Martini at the University of Iowa.

CHAPTER 4

Future perspectives

Introduction

S. aureus is an important pathogen, causing thousands of deaths and many more hospitalizations every year [152]. Its ability to form a biofilm is central to both its pathogenic and commensal lifestyles. Because of its genetic tractability, it is also an attractive model organism for studying community behaviors and dynamics.

As we approach the post-antibiotic era, it is more important than ever for the scientific and medical communities to discover novel treatments for biofilm-associated infections. Much research in recent years has focused on finding such treatments in natural products, such as tannic acid [87–89]. Unfortunately, this research is often exploratory, and it does not delve deep into mechanisms. Many groups only go as far as the initial screen and confirmation, finding a compound that can inhibit biofilm formation without investigating the mechanism of inhibition.

As has long been known, most antibacterial treatments select for their own failure. After decades of widespread antibiotic use, for example, more and more bacteria are becoming antibiotic resistant. Our own tannic acid treatment was no exception; we also saw resistance arise over a short period of time. This highlights the importance of understanding not only the treatments we discover, but also the mechanisms by which they work. As we understand the mechanisms, we can find both more effective ways to create the same effect (reducing the possibilities for

resistance) and we are better equipped to understand how bacteria will adapt to evade treatment.

In this work, we describe a novel antibiofilm compound, as well as parts of the mechanism by which it operates. In chapter 2 we describe a screen for antibiofilm compounds that lead us to find tannic acid. We show that tannic acid increases extracellular abundance of the lytic transglycosylase IsaA, and that this increase is responsible for its biofilm-inhibitory effect. We further show that this effect can be seen with dietary sources of tannic acid, such as tea, and that it is effective *in vivo* as well as *in vitro*. Finally, we show that resistance can arise to tannic acid treatment both *in vivo* and *in vitro*.

In chapter 3, we explore the mechanisms of resistance. We show that most of the resistant isolates have mutations in *rsbU*, which likely gives them resistance by increasing production of extracellular proteases. The only resistant isolate that did not have a mutation in *rsbU* had a mutation in *pknB*, which we show to be responsible for its resistance. We show that this resistance is due to the *pknB* mutant producing more PIA than the wildtype, likely leading to a stronger biofilm matrix.

Implications and Future Experiments

Biolog screen

Our first experiment in Chapter 2 was a screen of a small molecule library for compounds that could inhibit or promote *S. aureus* biofilm formation. We found many more compounds that inhibited than promoted, which was not surprising given that disrupting a complex behavior is much simpler than accidentally turning it on. Our initial ventures into tannic acid blossomed and turned into a very interesting, self-contained story. However, there were many other hits in the

screen that were left unexplored. A particularly interesting area for future work is the list of biofilm promoting compounds.

Synergy with antibiotic treatment

One exciting avenue of research is using biofilm inhibitory compounds in conjunction with antibiotics to increase efficacy of both. This sort of treatment is very appealing because biofilms are much more resistant to antibiotics than planktonic cells (up to 1000x more resistant).

A useful experiment for the future would be to pair antibiofilm compounds found in the Biolog screen (particularly tannic acid, about which we know the most) with various antibiotics, to see if they can act synergistically to more effectively clear biofilms. This could be done in any of our *in vitro* models, the rat throat colonization model, or several other relevant *in vivo* models of biofilm infection or colonization.

It is important to note that the bacterial adaptation to one treatment may affect susceptibility to the other. For example, *pknB* mutants (which arose naturally in the rat throat model) are known to be more susceptible to a wide array of antibiotics [173,181,182].

Is decolonization a good idea?

In the rat throat model, we presented an extremely rapid, effective treatment for eliminating *S. aureus* colonization from the throat. The mere fact that it is effective, however, does not answer the more important question of whether it is wise to use such treatments.

Our data focus solely on *S. aureus* colonization in the throat, and we make no attempt to discern any effect on the other members of the normal flora occupying that microenvironment. It is

possible that perturbing the delicate balance in this niche could lead to explosive growth of undesirable flora (as happens with *C. difficile* in the gut after antibiotic treatment).

Another potential unintended consequence is selection for more virulent *S. aureus*. We showed that resistance arose to tea treatment in the throat via mutation of two high-level genetic regulatory systems (*pknB* and *rsbU*). *pknB* mutants have been shown to have increased expression of α-toxin, form larger abscesses, and cause a greater response by the host immune system [181,183]. In other strains and different models of infection, the opposite trend has been reported; *pknB* mutants of SH1000 and its parent 8325-4 cause significantly less severe bacteremia than their wildtype counterparts [184]. *sigB* mutants (and by extension, phenotypically equivalent *rsbU* mutants) are much less adept at survival within the host, and are quickly cleared by the host immune system [185].

These factors together demonstrate the importance of understanding how resistance to tannic acid treatment arises. If *pknB* mutations are a common adaptation, then we would need to weigh the benefits of decolonization against the potential costs of remaining colonized with a more virulent strain. If *rsbU* mutations are common they would make any cells that resist treatment more sensitive to host immunity, making this treatment doubly effective.

Off-pathway resistance

We began our analysis of the resistant isolates hoping to find "on-pathway" mutations. That is, mutations in genes that were directly involved in tannic acid's inhibition of biofilm formation. The intent was to fill in some of the black boxes in our model and flesh out our knowledge of the pathway. However, both of the genes we investigated appear to be "off-pathway," having indirect effects to counteract the antibiofilm effects of tannic acid and IsaA.

This fact means that there are still avenues ripe for research within the model presented in this work. How does tannic acid increase IsaA abundance? How does IsaA inhibit biofilm formation?

As for the question of how tannic acid increases IsaA abundance, we have hypothesized in Chapter 2 that it involves slowing or preventing IsaA's degradation. This would be feasible to test with a pulse/chase experiment looking at IsaA production and degradation throughout stationary phase, or by using chloramphenicol to halt protein synthesis, thereby following only degradation throughout stationary phase.

There is not such a simple experimental avenue to find an answer to the second question. How does IsaA inhibit biofilm formation? We know that IsaA must be catalytically active, that it must accumulate, and that it can be counteracted by hyperproduction of PIA. Beyond those facts we can only speculate.

If overproduced PIA means a more stable, robust biofilm matrix [44], then it seems most reasonable to hypothesize that IsaA's antibiofilm effect is a result of destabilizing the matrix. How this happens, however, remains unclear. It is possible that it cleaves the peptidoglycan in such a way to release one or more of *S. aureus*'s many surface-associated adhesins.

Another possibility is that peptidoglycan plays an unappreciated role in stabilizing the biofilm matrix. Although peptidoglycan is not described as playing such a role, it is very possible that it could. Because eDNA is commonly released into the matrix through cell lysis [30], there would be a large amount of cell debris present in the biofilm. This would include eDNA, various intracellular proteins, and a massive amount of peptidoglycan. It stands to reason that such a

resilient, sturdy polymer would be used in some fashion to give strength to the biofilm, and that degrading it would destabilize the matrix.

An interesting experiment in this area would be to put PIA under our direct control (through the use of inducible promoters) rather than relying on a *pknB* mutation to upregulate its production. We could then grow biofilms with overexpressed IsaA and overexpressed PIA to see if overabundant PIA is sufficient to counteract IsaA's inhibitory effect. We speculate that it would be.

Common mutation in all sequences

In all 9 sequenced isolates, a variant appeared in SAOUHSC_02980, a hypothetical gene that has homology to an isochorismatiase-like protein (See Table 6). The variant caused a single amino acid substitution. This protein has no annotated function in any *S. aureus* strain we could find and has, to the best of our knowledge, never been studied in *S. aureus.*

The isolates that were sequenced came from two independent experiments (an *in vitro* biofilm grown in a drip reactor and an *in vivo* biofilm grown in a rat throat). These experiments were performed months apart with independent cultures grown from the same -80ºC freezer stock.

Because our sequences were aligned and variants called compared to *our* wild-type *S. aureus* (as opposed to simply aligning to a database), we know that this is not simply a variant that is present in the freezer stock of our wildtype. If these results were from only one experiment, the simple explanation for the ubiquity of this variant would be that it was present in the colony chosen to begin the experiment. However, it is astronomically unlikely that both of these independent experiments would begin with cultures that spontaneously contained the same variant.

It seems apparent that this variant is strongly selected for. What is not apparent is whether it is selected for by growth in a biofilm generally or specifically by growth in a biofilm with tannic acid stress. Unfortunately, given the data we have, we cannot make this distinction. Our sequencing was a *post hoc* experiment, and we did not save any samples from drippers grown without tannic acid or from rats that were not treated with tea.

To answer this question, we propose that the dripper experiment be repeated. Biofilms would be grown in drip reactors for 5-7 days with and without tannic acid treatment. When a resistant biofilm appears, cells would be harvested from both treated and untreated reactors. Because we would only be interested in this particular variant (and not the entire genomes of the isolates), dozens of isolates could be sequenced cheaply and easily by Sanger sequencing.

Contradictions with previous *rsbU* data

Six of the seven resistant isolates had mutations in *rsbU*, and these isolates appeared to be resistant to IsaA simply by virtue of their overproduction of extracellular proteases. This contradicts the dogma that protease production is associated with biofilm dispersal [169–172]. However, Atwood *et al.* showed that this is dependent on growth conditions, with *sigB* mutant forming a much better biofilm than wildtype under some conditions, despite elevated protease levels. This serves as a reminder that phenotypes can vary dramatically depending on strain variation and different growth conditions. Some conditions and some strains appear to favor a biofilm matrix that is more dependent on one or another component. In our strains, under the conditions we tested, *rsbU* mutants both produce more extracellular proteases and form very robust biofilms, suggesting that their biofilm does not depend as heavily on the protein adhesins as on other components.

Community goods – proteases

A surprising result from Chapter 1 was that only a subset of cells isolated from tannic acid resistant biofilms was resistant to tannic acid when grown in pure culture. This points to an interesting community dynamic, where some cells gain resistance by their own action and other cells gain resistance through the actions of their neighbors.

The *rsbU* results presented in Chapter 2 suggest that extracellular proteases become a community good. Proteases produced and exported by *rsbU* mutants degrade self-produced IsaA, as well as IsaA produced by wildtype neighbors. Presumably, this would give resistance to their neighbors by creating an extracellular environment for all cells wherein IsaA does not accumulate to high, biofilm inhibitory levels.

A simple extension of these results would be to coculture these *rsbU* mutants with wildtype cells in the microtiter plate biofilm assay to see if they do, in fact, confer tannic acid resistance to their neighbors. Because the *rsbU* mutants are white while the wildtype cells are golden, it would be very simple to assay afterward how many cells of each type are present in the biofilm after tannic acid treatment.

PIA in the *S. aureus* biofilm

The *pknB* mutant produces more PIA than the wildtype, likely increasing overall biofilm integrity. It is notable that, while *S. aureus* does produce some PIA, polysaccharide is a much less integral component of its matrix than its in, for example, the *S. epidermidis* matrix [77]. While PIA is higher in the *pknB* mutant, we observed in control blots that it is much lower in either strain than it is in *S. epidermidis*.

It is curious why this operon remains intact and functional in *S. aureus* if it is not necessary for biofilm formation and thought to be nearly always repressed. It is possible that *ica*-activating mutations, such as the *pknB* mutation we observed here, are frequent enough in biofilms to make it advantageous to the bacteria to maintain a functional *ica* operon.

Community goods – PIA

We observed that the *pknB* mutant isolate was able to withstand both tannic acid and IsaA overproduction, and that this resistance was a result of PIA overproduction. Because PIA is a major structural component of the biofilm matrix, it is possible that this stabilizing component could act as a community good, conferring resistance on PIA overproducers and non-overproducers alike.

It would be enlightening to perform biofilm assays where *pknB* mutants and wild type cells were mixed at different ratios, then harvested from the biofilm to see if the *pknB* mutant is able to confer resistance on neighboring cells. Like the coculture experiment proposed for the *rsbU* mutant, this experiment would give us insight into how communities adapt to stress.

Models of PknB function

When discussing PknB's function in *S. aureus*, there is near-unanimous acceptance of Shah *et al.*'s model [137]. Although hundreds of papers cite their work on PknB, very little has been published building on or further expanding their model. To our knowledge, the only research that expands on this finding is a paper by Mir *et al.* which finds that *Mycobacterium tuberculosis* PknB binds to fragments of peptidoglycan, but that this binding has no effect on resuscitating cells from dormancy [186]. These results from *M. tuberculosis* are consistent with our observations that peptidoglycan fragments do not germinate *B. subtilis* spores.

According to this mycobacterial model, PknB binding to peptidoglycan fragments helps localize PknB to the midcell, but does not activate PknB. In *Mycobacterium*, the specific activating signal has not been found, but it is thought to be related to peptidoglycan. Adding certain peptidoglycan digesting enzymes (termed RPFs or Resuscitation Promoting Factors) has been shown to revive mycobacterial cells from dormancy. It is possible that PknB threads through peptidoglycan, with its PASTA domains bound to intact peptidoglycan. As peptidoglycan is degraded by RPFs or experiences other stress, it would become less rigid and more flexible, allowing PknB to also be more flexible.

PknB regulates SigB activity

It is worth noting that PknB positively regulates activity of the alternative sigma factor SigB [181]. It seems likely, then, that SigB activity would be lowered in the *pknB* mutant, as well as isolate BB2588 which harbored a variant in *pknB*. It is unlikely, however that the resistance of this isolate can be attributed to reduced SigB activity. The isolate is golden in color, demonstrating that SigB is active enough to promote production of the pigment staphyloxanthin. It also did not demonstrate increased protease activity as was seen in the isolates with *rsbU* mutations. Inactivation of the *ica* locus was sufficient to restore sensitivity to the isolate, indicating that PknB's effects on SigB alone could not explain the isolate's resistance. The conditions under which PknB activates SigB are not defined. In order to understand how the two might interact in a biofilm, more research into their interplay must be done.

Conclusions

In this dissertation, we demonstrate how tannic acid, a compound common in the human diet, can modulate biofilm formation by *S. aureus*, a pathogen and commensal commonly found in the human throat. We begin to show a genetic mechanism by which this inhibition takes place, as

well as two distinct mechanisms that independently evolved for resisting inhibition. We also propose some potential future directions for research that will further the findings presented here. Taken together, this provides us with important insights into how this relevant pathogen and model organism functions in a community to interact with its environment and adapt to tolerate new challenges.

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