

**Staph Wars: How triclosan promotes nasal colonization with
Staphylococcus aureus, Phenol Soluble Modulins induce a
pro-inflammatory response from the skin, and how polymicrobial
interactions influence *S. aureus* motility**

by

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of the requirements for the degree of
Doctor of Philosophy
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THE SPRAY, THE SPRAY, THE ANTISEPTIC SPRAY
A.O. WOULD SHOWER IT MORNING, NIGHT AND DAY
FOR EVERY SORT OF SCRATCH
WHERE OTHERS WOULD ATTACH
A STICKING PLASTER PATCH
HE GAVE THE SPRAY

– THE STUDENTS OF SIR ALEXANDER OGSTON

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Chapter 1

Introduction

Staphylococcus aureus

Discovery

Sir Alexander Ogston, a Scottish surgeon in the late 19th century, was convinced that putrefaction, or inflammation and formation of pus, was not a necessary step of wound healing but was in fact due to a yet unknown factor. In 1880, Ogston analyzed pus from a patient's knee joint under a microscope and found that there were a large amount of small coccoid cells (1). He hypothesized that these microorganisms were the causative agent of putrefaction. To investigate this further, he injected the pus from his patients into mice and the mice developed abscesses similar to human patients (1). Furthermore, the pus recovered from injected mice contained the same cocci (1). To confirm that the microorganisms were the causative agents, he treated the pus with heat to kill any microorganism before injecting the mice and found that heat treated pus was insufficient to cause abscesses (1). Once he cultured these organisms he noticed that in contrast to the previously described Streptococci, these organisms did not form chains but rather formed grape like clusters. From this characterization he named these bacteria *Staphylococcus* after the Greek word for a bunch of grapes.

Soon after Ogston's discovery, the German surgeon Anton Rosenbach discovered that there were two predominant types of Staphylococci and named them after their color, *Staphylococcus aureus* (Latin for gold) and *Staphylococcus albus* (Latin for white) which was later renamed *Staphylococcus epidermidis* (2). The genus now contains at least 40 different species. Many of these species are commensal bacteria that live on the skin of not only humans, but other mammals, as well livestock such as chickens.

Clinical and economic impact

S. aureus is an opportunistic pathogen that colonizes the nares and skin of approximately 30% of the human population. *S. aureus* is a leading cause of nosocomial infections and was responsible for the death of approximately 19,000 people in the United States in 2005 (3). Colonization with *S. aureus* puts a person at a higher risk of post-operative infection (4). Infections range from relatively mild boils and abscess to much more severe diseases such as endocarditis, bacteremia, and toxic shock.

In the United States, it is estimated that each case of antibiotic resistant *S. aureus* costs society between \$7,070 and \$20,489 per patient depending on age. This means that annually \$1.4 – \$13.8 billion is used for treating *S. aureus* infections (5). This figure does not take into account the \$478 million – \$2.2 billion that third party payers, such as insurance companies, spend on *S. aureus* infections, bringing the total economic burden to \$1.8 – \$16 billion annually in the United States alone (5). To put this into perspective it cost \$2.5 billion for NASA to design, build, send, and operate the Curiosity rover on Mars.

S. aureus infections are a problem worldwide. In the European Union, *S. aureus* causes more than 150,000 infections annually (6). *S. aureus* infections result in €380 million in extra

hospital cost (6). In Canada alone, the cost of coping with *S. aureus* infections, hospital screening, and management is estimated to total \$42 – \$59 million annually. Clearly, *S. aureus* is a significant burden to human health and global economy (7).

***S. aureus* colonizes the human host**

S. aureus commonly colonizes mucus membranes of the nose (30% colonization rate), throat (40%), and vagina (9%) (8-10). Due to the ability of *S. aureus* to survive high salt and desiccation, it can also colonize drier areas of the body like the skin and can also survive the gastrointestinal tract (11, 12). The nasal passage is the most commonly sampled area and it is estimated that while 20% – 30% of individuals are persistently colonized, while up to 60% of the population are transient carriers (13). Upwards of 10% – 20% of the population is never colonized with *S. aureus* but the reason for this difference remains unknown.

Some populations are at a greater risk than others for nasal colonization with *S. aureus*. These include individuals with diabetes mellitus, intravenous drug users, HIV, living in a long-term care facilities, and those on hemodialysis (10, 11). Additionally, individuals of African descent or Mexican birth have lower rates of *S. aureus* colonization than Caucasians and Mexican-Americans (11). Men also have a higher rate of nasal colonization compared to women (11). Although epidemiologists have found these discrepancies between populations, few factors have been identified that can account for the differences in colonization frequency. Some genetic factors have been shown to be associated with *S. aureus* nasal colonization including mutations in glucocorticoid receptors, interleukin-4, and C-reactive protein (14, 15). Although mutations in these genes are associated with increased *S. aureus* colonization, none have been described as necessary or sufficient.

The ability of *S. aureus* to colonize the human nose is multifactorial and depends on its ability to adhere to the host nasal epithelia. *S. aureus* produces several factors to allow attachment and colonization including clumping factor B (ClfB), iron-regulated surface determinant A (IsdA), and wall teichoic acids (WTA) (16-18). Several major regulators of *S. aureus* virulence such as the exoprotein regulator (*sae*), the accessory gene regulator (*agr*), and the alternative sigma factor B (*sigB*) are repressed during *S. aureus* colonization, favoring attachment of cells rather than toxin production against niche competing bacteria and the host (19, 20).

***S. aureus* is an opportunistic pathogen**

S. aureus is not only a commensal bacteria, but can become a deadly pathogen when the opportunity presents itself. *S. aureus* is a versatile pathogen. Unlike many pathogens, *S. aureus* can cause a wide variety of diseases. It can cause minor skin lesions and abscesses but can cause many other severe diseases including endocarditis, sepsis, osteomyelitis, pneumonia, necrotizing fasciitis, and food poisoning among others. Furthermore, *S. aureus* is a major cause of hospital-acquired infections, specifically post-surgical infections (4). The highest risk is with those individuals that are colonized with *S. aureus* during surgery. Colonized individuals are at a 6-fold higher risk of a post-operative *S. aureus* infection (10). Because of this, many hospitals worldwide have begun to monitor incoming surgical patients for *S. aureus* colonization. Once colonization is found, these individuals undergo de-colonization treatments that include nasal mupirocin treatments as well as bath soaps containing triclosan (21, 22).

S. aureus success as a pathogen is due to the production of a large repertoire of molecules that can help it to evade the immune system. After invading host tissues, *S. aureus* produces

proinflammatory molecules, thus *S. aureus* must be equipped to protect itself against the immune response. *S. aureus* produces protein A, which binds the heavy chain of IgG antibodies and prevents recognition and phagocytosis by immune cells (23). In addition to preventing antibody recognition, *S. aureus* also can produce a large range of superantigen proteins. These superantigens trigger the non-specific activation of T-cells leading to a large, systemic release of cytokines, preventing the recruitment of macrophages to the specific site of infection (24).

When immune evasion is not sufficient, *S. aureus* can produce pore-forming toxins including hemolysin α , hemolysin β , and the phenol soluble modulins (PSMs). Hemolysin α is a pore forming toxin that binds the membranes of eukaryotic cells (25). Monomers oligomerize in the membrane, forming a pore complex that allows for cell depolarization and cell death (26). Hemolysin β can cause cell lysis by degrading sphingomyelin, a lipid found in eukaryotic membranes (27). The phenol soluble modulin (PSM) family is a group of eight small peptides that can lyse membranes by acting as biosurfactants (28). The PSMs will be discussed in further detail below.

Even with all of these mechanisms to evade the immune system, *S. aureus* can still be phagocytosed by neutrophils and macrophages. Once in the phagolysosome of neutrophils and macrophages, bacteria are exposed to a large variety of antimicrobials including reactive oxygen species, low pH, nitric oxide, antimicrobial peptides, proteases, and cell wall hydrolases (29). *S. aureus* has also evolved mechanisms to survive these stresses. Through the production of superoxide dismutase and catalase, *S. aureus* can neutralize superoxide and hydrogen peroxide, respectively (30). *S. aureus* detoxifies nitric oxide by production of flavohemoglobin (30-32). Furthermore, *S. aureus* has a modified peptidoglycan preventing it from being degraded by lysozyme (33). Not only does *S. aureus* survive in phagolysosomes, it is able to escape through

the production of PSM peptides that lyse the phagolysosome allowing *S. aureus* to enter the cytoplasm and thrive (34).

Bacterial biofilms

Biofilms are multicellular communities of bacteria that are surface-attached and encapsulated by an extracellular matrix on biotic and abiotic surfaces (35). Biofilm formation helps protect bacteria from the host defense, desiccation, and a myriad of other environmental conditions (36). Biofilms are highly resistant to antibiotics, making antibiotics an inefficient therapy, requiring long-term treatment with antibiotic concentrations over 200 times greater than the minimal bacteriocidal concentration of planktonic cells (37).

S. aureus biofilms

The *S. aureus* biofilm lifecycle involves four phases of development, beginning with surface attachment, followed by the formation of microcolonies, maturation, and detachment, bringing the biofilm cells back to a planktonic state (Figure 1.1). Attachment begins when cells use adhesins to adhere to biotic or abiotic surfaces, where they are able to proliferate. These cells form microcolonies, produce extracellular matrix components, and proliferate until they create a mature biofilm (Figure 1.1). Mature biofilm cells enter into a sessile state where the bacteria can remain until environmental signals trigger biofilm disassembly (38).

The accessory gene regulator (*agr*) quorum sensing system in *S. aureus* is one of the main regulators of *S. aureus* pathogenesis and biofilm formation (Figure 1.2). Under biofilm forming conditions, the *agr* system is mainly repressed (39). The *agr* system contains a two component system (AgrC/AgrA) that recognizes the self produced autoinducer peptide (AIP)

molecule (40). The cells respond to AIP by a positive feedback loop, increasing the amount of AIP produced by cells while also promoting the transcription of the effector molecule RNAIII and the PSM peptides (41). The AIP molecule is the mature product of the prepro-AgrD molecule. This AgrD molecule is targeted to the cell membrane by the N-terminal region of the peptide. The prepro-peptide is then secreted from the cell after processing of the C-terminus and formation of a thiolactone ring by AgrB and SpsB (42). Once on the exterior of the cell, the N-terminal region is then cleaved releasing the mature AIP molecule into the environment (42). Once the *agr* system is activated, the RNAIII molecule de-represses the translation of transcripts for proteases and toxins that help cells to degrade the extracellular matrix of the biofilm (43, 44). These proteases, in combination with upregulation of the PSM surfactant proteins, aid in the disassembly of biofilms, allowing for cells to escape and disseminate (45).

The biofilm matrix of *S. aureus* is composed mainly of polysaccharides, extracellular DNA (eDNA), and proteins, with different strains favoring some components over others (36, 46-48). It was long believed that *S. aureus* biofilms were dependent on polysaccharide intercellular adhesin (PIA) until it was shown that many clinical isolates of *S. aureus* form biofilms in the presence of the PIA degrading enzyme dispersin B (48). Subsequent research investigated the essential components of these polysaccharide-independent biofilms, and implicated proteins as essential structural components (47, 48).

Functional amyloids

One of the important protein components of the biofilm are amyloids. Functional amyloids are a recently identified class of amyloids that have been found in organisms from mammals to fungi to bacteria (49-52). Since the characterization of the bacterial functional

amyloid curli in *Escherichia coli* and other members of the Enterobacteriaceae family, bacterial functional amyloids have been discovered in other species, notably PSMs in *S. aureus*, chaplins in *Streptomyces coelicolor*, TasA in *Bacillus subtilis*, FapC in *Pseudomonas*, and microcin in *Klebsiella pneumonia* (49, 53-58). The large body of research on disease-associated amyloids has provided many tools for the investigation of functional amyloids.

Curli

Curli are the most well studied bacterial functional amyloid. Through a dedicated pathway, curli form amyloids on the surface of Enterobacteria, such as *E. coli* and *Salmonella*, where they aid bacteria in attaching to surfaces as well as defending the population from stress. Curli are made through a highly controlled master regulator CsgD, which induces the transcription of other curli specific genes (*csg*) to produce these amyloids (Figure 1.3) (59). The major functional subunit of curli, CsgA, is secreted from the cell in a soluble form, leaving the outer-membrane through the pore formed by nine CsgG subunits (Figure 1.3) (49, 60, 61). The minor fiber subunit CsgB is linked with the membrane and facilitates the nucleation of CsgA into amyloid fibers (Figure 1.3) (62-65). Proper assembly, localization, and regulation of curli fibers are modulated by CsgC, CsgE, and CsgF (66-70). Recently, CsgC has been demonstrated to possess an extremely efficient way to prevent the formation of CsgA amyloid fibers in the periplasm (68).

Curli fibers are important for *E. coli* surface colonization and biofilm formation (71, 72). The expression of curli is a tightly regulated process in regards to environmental signals (49, 73-75). The matrix components curli and cellulose help *E. coli* cells protect their community from predation (76). Recently, it has been shown that there is spatial regulation within an *E. coli*

rugose biofilms where curli producing cells are localized to the exterior of the biofilms, whereas cells on the interior of the community were not producing curli fibers (77). This bimodal growth allows for a protective shell of matrix-encased cells that contain a population of cells that are ready to disperse and disseminate when conditions become favorable (77).

Phenol Soluble Modulins

Phenol soluble modulins (PSMs) are a family of proteins that are found in Staphylococci, most notably the significant human pathogen *Staphylococcus aureus* and the human commensal *Staphylococcus epidermidis* (28, 78). *S. aureus* PSMs are able to form amyloid fibers under certain conditions that stabilize the biofilm and make it resistant to known dispersal agents (79). *S. aureus* has nine characterized PSM peptides that are all regulated by the *agr* quorum sensing system (41). There are four PSM α , two PSM β , and δ -toxin that are present in three separate regions of the chromosome (Figure 1.2) (28). The newest member to this family is the N-terminal signal sequence of the AgrD molecule N-AgrD (80). This sequence is critical for localization of AgrD to the membrane and once cleaved from the rest of the AgrD molecule has many structural and functional similarities to the other PSMs (80, 81). In addition, some strains of *S. aureus* contain a pathogenicity island that harbors a ninth PSM called PSM-mec (82). The PSMs are secreted from the cells by a dedicated, essential secretion system called phenol-soluble modulins transporter (PMT) (83). These PSM peptides are amphipathic alpha-helices, meaning that one face of the helix is hydrophobic while the other is hydrophilic (28). This shared property is thought to allow for them to form pores in the membranes of competing microbes and host cells to invade tissues and evade immune cells (28, 84).

PSMs have been shown to be important for the formation of biofilms (79, 85). Soluble PSMs are important biosurfactants that aid in the characteristic waves of dissemination of parts of the biofilms to colonize other areas (Figure 1.4) (86). The PSM peptides are also able to form amyloid fibers which stabilize *S. aureus* biofilms (79). This switch changes the soluble alpha-helical peptides into beta-sheet rich protein aggregates that have the characteristics of amyloid proteins (79). The aggregation of PSM peptides into amyloids is mediated through a self-templating mechanism that facilitates the transformation of other nearby proteins to adopt the amyloid fold. The PSMs form amyloid fibers in biofilms that were grown in a non-standard rich media containing peptone, glucose, and NaCl (79). These biofilms are completely resistant to known biofilm dispersing enzymes Proteinase K, DNase and Dispersin B, suggesting that the PSM amyloids are able to structurally stabilize the biofilm against enzymatic targeting of the previously characterized matrix components (Figure 1.4) (79). Importantly, PSMs were demonstrated to have bifunctional abilities to either strengthen biofilms or disperse them dependent on their secondary structure (Figure 1.4) (79). If monomeric PSMs were added to an established biofilm they exhibit surfactant like properties, dispersing the biofilms in a concentration dependent manner, whereas PSM fiber addition does not disperse biofilms (79).

In addition to their role in biofilms, the PSMs have been shown to have a large variety of uses. PSMs have been shown to be critical determinants of *S. aureus* to cause skin abscesses and wounds in murine models as well as aiding in the survival of *S. aureus* in murine bacteremia models (28). PSMs stimulate neutrophil chemotaxis through the Human Formyl Peptide Receptor 2 (FPR2), at nanomolar concentrations, independent of the formylation state of the peptides (Figure 1.4) (87). Once the neutrophils are in close proximity, PSMs are able to infiltrate cells and cause cell death (Figure 1.4) (28). Recently though, the field has shifted

towards the hypothesis that in the host, PSMs may be important in virulence once *S. aureus* is phagocytosed by neutrophils. This hypothesis is supported by data demonstrating that serum lipoproteins are able to bind to and inactivate PSMs, meaning that they would be unable to function in the presence of serum in the host (88). Secondly, once phagocytosed by neutrophils, *S. aureus* cells highly upregulate the production of PSM peptides which aid in escaping from the phagolysosome (Figure 1.4) (34).

PSMs are not only reported to be important for *S. aureus* pathogenesis and virulence against the host. PSMs have also been shown to be antimicrobial against potential competitors (84, 89). PSMs were first determined to have antimicrobial effects from *S. epidermidis* (90). PSMs share structural similarity to mammalian antimicrobial peptides such as LL-37, thus it was tested to determine if *S. epidermidis* PSMs were able to kill mammalian pathogens. Two PSMs from *S. epidermidis* were found to have antimicrobial effects against *S. aureus* as well as Group A Streptococcus (GAS) and work synergistically with LL-37 (91). Focus then turned to determine if and how *S. aureus* PSMs may act against niche competing bacteria. It was found that full length PSM peptides possessed little antimicrobial activity but derivatives of PSMs (dPSMs), PSMs that have been proteolytically processed to be missing the first few amino acids, have strong antimicrobial properties against *S. pyrogenes*, *S. epidermidis*, and GAS (Figure 1.4) (84). Furthermore, when a colony of *S. aureus* is grown in close proximity to *S. epidermidis* or GAS, dPSMs are localized to the zone of inhibition of the competing bacteria (84).

Along with their role as toxins, the biophysical properties of PSMs give them several unique properties in modulating *S. aureus* communities. First, PSMs confer the ability of *S. aureus* spread across a soft agar plate (92, 93). This suggests that some PSMs are able to act with surfactant-like properties lowering hydrophobicity and allowing for *S. aureus* to spread.

Further studies are needed to investigate the flexibility of the PSM peptides to switch from soluble peptides to amyloid fibers and if this change is irreversible or only temporary (94). Interestingly, where PSM α and PSM β peptides were shown to be essential for *S. aureus* colony spreading, it has been shown that colony spreading can be inhibited by δ -toxin (95). This may suggest a role in amyloid nucleation by δ -toxin on the other PSMs that inhibit their ability to act as surfactants. I suggest that PSM aggregates may be reservoirs of toxins that *S. aureus* can utilize to both defend itself while also promoting biofilm spreading and dispersal. It will also be interesting to determine if the aggregation of PSMs into amyloid fibers can abrogate neutrophil chemotaxis, thus acting as a way to hide from the immune system when forming biofilms in the host. Much more work is needed to fully understand how *S. aureus* and other Staphylococcal species utilize the fold of these PSM peptides to modulate their function.

Dissertation Goals

The goal of this dissertation is to investigate factors that influence the ability of *S. aureus* to colonize the human host and contribute to disease. Broadly speaking I have divided these factors into three categories – environmental factors, interactions with the host, and polymicrobial interactions. In chapter 2, I investigate how the biocide triclosan, that is present in many consumer products, is present in human nasal secretions and promotes nasal colonization with *S. aureus* (96). Chapter 3 investigates the interactions between *S. aureus* and the human skin. Here, I show that *S. aureus* PSMs are able to induce the lytic release of cytokines from keratinocytes and promote skin inflammation in a mouse model of cutaneous colonization (97). Chapter 4 demonstrates that the motile bacteria *Brevbacillus* is able to carry non-motile *S. aureus* across a soft-agar surface. I will also show that *S. aureus* contribute to this by producing

surfactants to promote *Brevibacillus* swarming. These findings contribute to the existing knowledge on *S. aureus* colonization and infection and provide novel avenues of exploration for *S. aureus* decolonization and treatment.

Figures

Figure 1.1: Diagram of the *S. aureus* biofilm lifecycle. Planktonic cells attach to a surface where they then proliferate to form small microcolonies. These microcolonies grow, producing matrix components accumulating into a mature biofilms. Environmental cues trigger dispersal from biofilms, returning to a planktonic state.

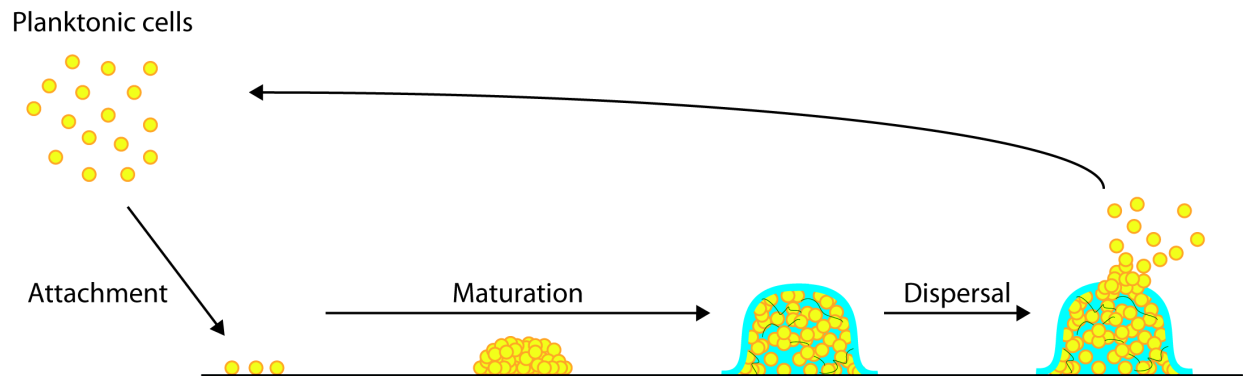


Figure 1.2: The genetic positions of the *S. aureus* Phenol Soluble Modulins (PSMs). The loci of the eight chromosomal PSM peptides with their amino acid sequence is listed near their name. All PSMs are under direct genetic regulation of the *agr* quorum sensing system.

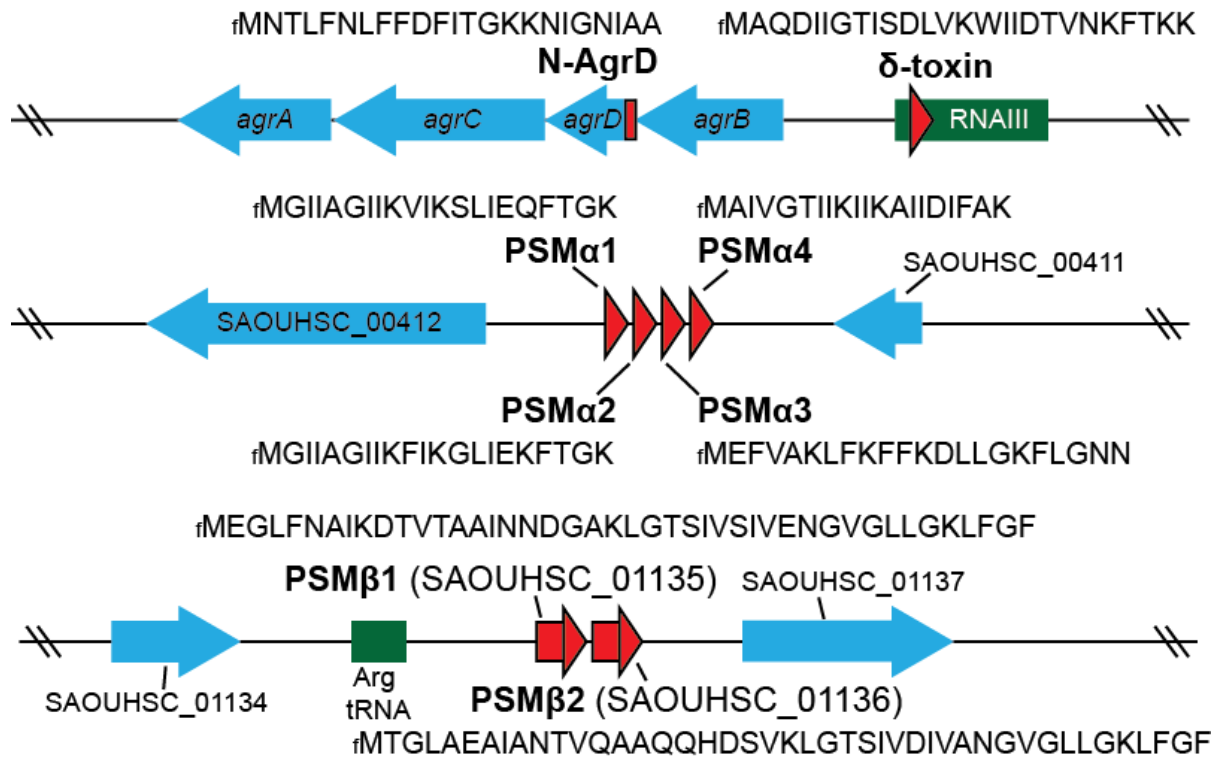


Figure 1.3: Curli biogenesis model. The curli system in *E. coli* is a highly controlled process that only expressed the curli amyloid under conditions that promote biofilm formation. The system is transcriptionally controlled by the master regulator CsgD which increases the transcription of the major and minor subunits CsgA and CsgB. All Csg proteins other than CsgD are secreted through the Sec secretion pathway into the periplasm where CsgA, CsgB, and CsgF are then translocated outside of the cell through the CsgG pore complex. CsgE and CsgF aid in proper export and localization of the structural components while CsgC has potent activity in the periplasm to prevent premature amyloid formation. Figure adapted from (94).

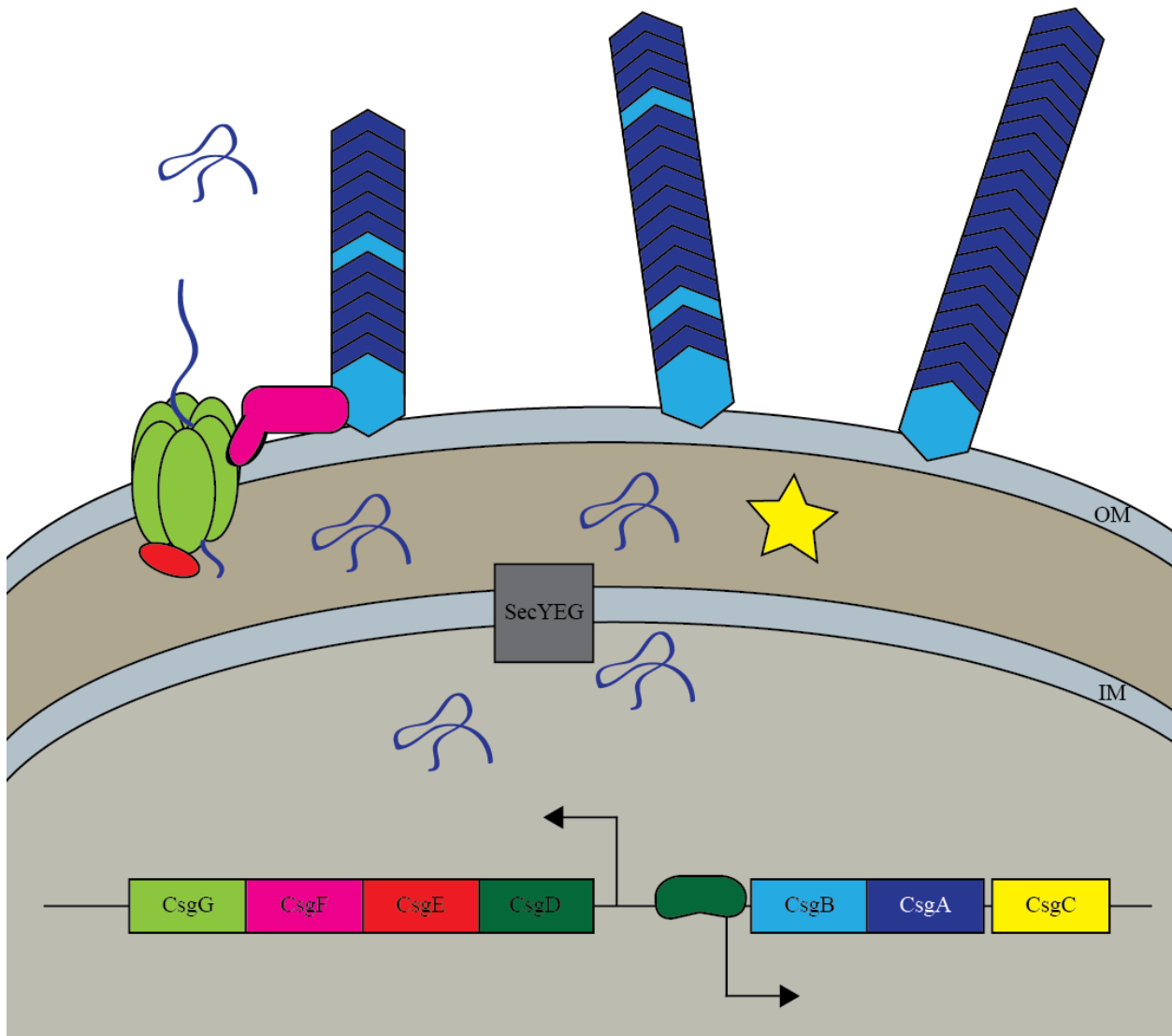
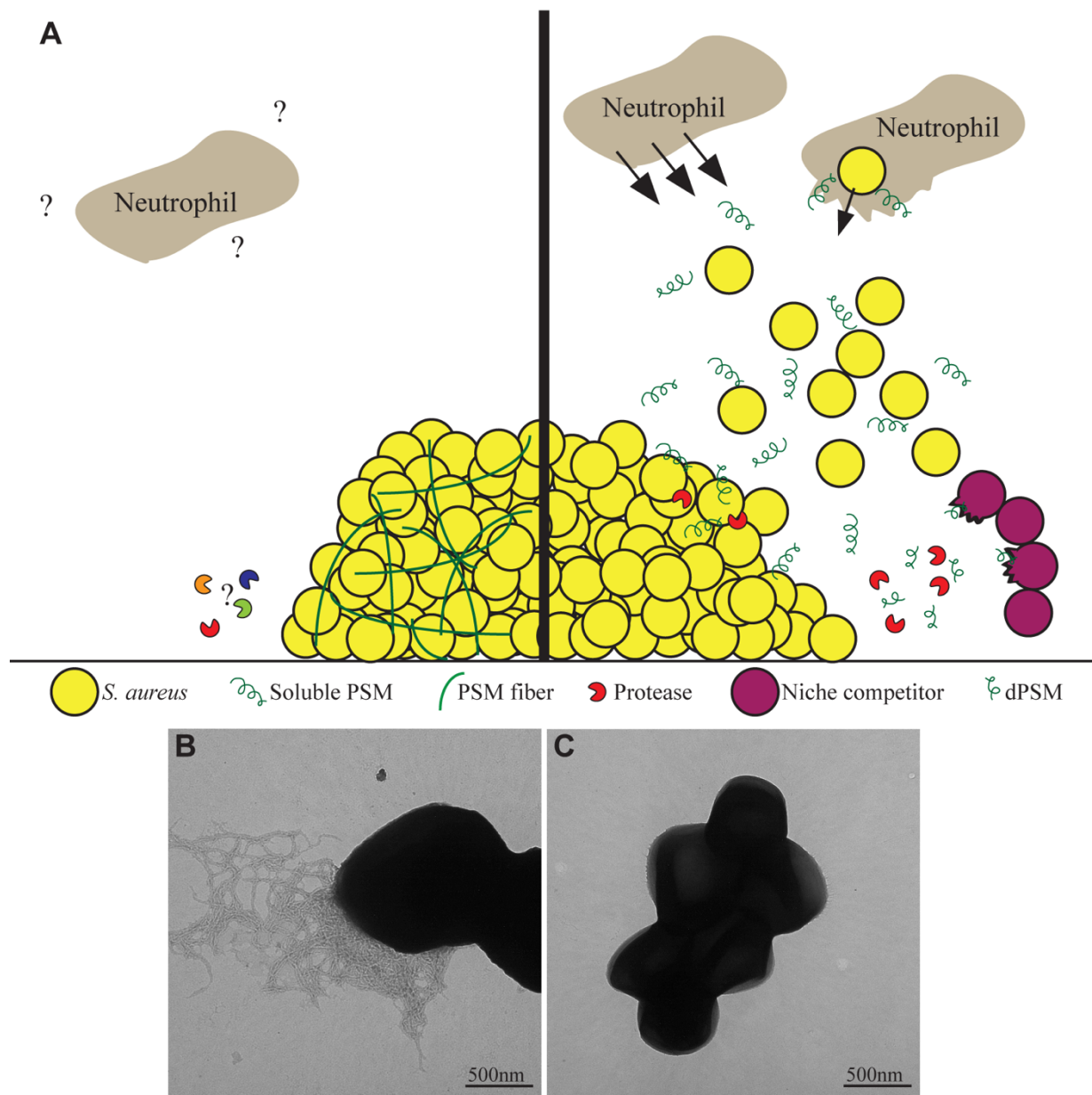


Figure 1.4: Phenol Soluble Modulins as bifunctional proteins. **(A)** *S. aureus* produces PSM proteins that have been found to have many diverse functions. As soluble molecules, they are able to stimulate chemotaxis of neutrophils as well as aid *S. aureus* escape from the phagolysosome upon phagocytosis. Additionally, soluble PSMs can disperse biofilms as well as be proteolytically processed into PSM derivatives (dPSMs) that can kill niche competing bacteria. Once polymerized into amyloid fibers, PSMs provide functional support to the biofilm community that prevents degradation by biofilm dispersing enzymes. The interactions between PSM fibers and immune cells have yet to be characterized. **(B)** Transmission electron microscopy of a *S. aureus* biofilm that is producing PSM amyloid fibers. **(C)** *S. aureus* biofilm cells under conditions where amyloid fibers are not detected. Figure from (94).



Notes

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Chapter 2

Triclosan Promotes *Staphylococcus aureus* Nasal Colonization

Abstract

The biocide triclosan is used in many personal care products including toothpastes, soaps, clothing, and medical equipment. Consequently it is present as a contaminant in the environment and has been detected in human fluids including serum, urine, and milk. *Staphylococcus aureus* is an opportunistic pathogen that colonizes the nose and throat of approximately 30% of the population. Colonization with *S. aureus* is a risk factor for several types of infection. Here I demonstrated that triclosan is commonly found in the nasal secretions of healthy adults and the presence of triclosan trends positively with nasal colonization by *S. aureus*. Triclosan can promote the binding of *S. aureus*, *P. aeruginosa*, and *E. coli* to surfaces and promotes biofilm formation. Furthermore, triclosan exposure changes the lipid profile and proteome of *S. aureus*. Lastly, triclosan exposed rats are more susceptible to nasal colonization with *S. aureus*. These data reveal a novel factor that influences the ability of *S. aureus* to bind surfaces and alters *S. aureus* nasal colonization.

Introduction

Staphylococcus aureus is a human commensal and pathogen that colonizes the nares and throat of approximately 30% of the human population and was responsible for the death of approximately 19,000 people in the United States in 2005 (1). *S. aureus* infections range from relatively mild abscesses to more severe diseases such as endocarditis and bacteremia (1). *S. aureus* nasal colonization is a significant risk factor for several infections including bacteremia, post-operative infections, diabetic foot ulcer infections, and contributes to the spread of this pathogen in nosocomial environments (2). *S. aureus* nasal colonization is influenced by host and bacterial factors with carriage rates differing among ethnic groups and families (3). The binding of host proteins such as keratin, fibrinogen, and collagen promotes *S. aureus* adherence and human colonization (4). Despite this knowledge, the reasons *S. aureus* successfully colonizes some individuals while others remain non-colonized remains an enigma.

The biocide triclosan is used in a vast number of personal care products including soaps, toothpastes, kitchen surfaces, clothes, and medical equipment (5). Despite recent reviews of triclosan's potential impact on public health it is still used widely and faces minimal regulation in the U.S.; as a result, triclosan is accumulating in the environment as well as in human bodies (4). Triclosan is readily absorbed by the gastrointestinal tract and oral mucosa leading to the detection of sub-minimum inhibitory concentrations (sub-MIC) of triclosan in human serum, urine, and milk (4-6). In a recent study, the concentration of triclosan in stream sediments was found to increase with urbanization and correlated with a significant change in the streambed microbial ecology (7). Triclosan targets the enoyl-acyl carrier protein reductase of the bacterial type II fatty acid synthesis (FASII) pathway, FabI, therefore disrupting fatty acid biosynthesis (8). At higher concentrations, triclosan is thought to have broad antimicrobial effects through the

disruption of cell membranes and less specific interactions with other proteins (9). In mammals, triclosan has been shown to disrupt the endocrine system by antagonizing estrogen and androgen receptors as well as elevate resting cytosolic $[Ca^{2+}]$ in primary skeletal myotubes (10). Further analysis on the effect of triclosan on mammalian muscle function found that the presence of triclosan impairs the excitation-contraction coupling of cardiac and skeletal muscles (13). Strikingly, mice exposed to triclosan exhibited up to a 25% decrease in cardiac output and an 18% mean decrease in grip strength demonstrating a striking effect of triclosan on muscle function (13).

Triclosan is embedded into many medical devices such as sutures and catheters for the prevention of infections (11, 12). Triclosan baths are also a recommended method for decolonizing an individual that is colonized with *S. aureus* prior to surgery (16). Using triclosan in medical devices may lead to unexpected complications by affecting microbes exposed to sub-MIC levels of the biocide. It has been shown that long-term exposure of *S. aureus* to triclosan results in the induction of small colony variants that are more resistant to antimicrobials (13). Furthermore, resistance to triclosan can be gained by *S. aureus* through mutations in the FabI gene as well as other unknown Fab-independent ways (14). There is also concern about the essentiality of the Type II fatty acid biosynthesis pathway that triclosan targets. In the presence of triclosan in human serum, *S. aureus* can modify and incorporate exogenous host fatty acids into cells, strengthening the argument against the essentiality of FASII in this host (19).

Results

Triclosan can be detected in nasal secretions and trends with S. aureus nasal colonization.

I first set out to investigate if triclosan can be detected in human nasal secretions and if its presence increases the risk for *S. aureus* nasal colonization. I hypothesized that triclosan would be present in the nasal secretions of some individuals because it has been detected in serum, a major component of nasal secretions (15). Nasal secretions were collected from 90 healthy adults and ELISA's were performed to detect the levels of triclosan present. 37 out of 90 (41%) individuals of our sample population had detectable levels (≥ 1.75 nM) of triclosan in their nasal secretions (Figure 2.1A). To investigate if the presence of triclosan in nasal secretions relates to colonization with *S. aureus*, concurrent nasal swabbing of our sample population was performed to detect *S. aureus* colonization using a selective and differential medium for *S. aureus* isolation, mannitol salt agar. A positive trend was observed between the presence of triclosan in nasal secretions and colonization with *S. aureus* (Figure 2.1B). Individuals without triclosan or with levels less than 175 nM had *S. aureus* carriage rates of 32% and 27%, respectively, which were in line with previous studies; however 64% of individuals with greater than 176 nM triclosan in their nasal secretions were colonized with *S. aureus* (Figure 2.1B). Analysis of variance revealed that *S. aureus* nasal colonization in individuals with greater than 176 nM triclosan was significantly higher ($p < 0.01$) than individuals without and with less than 175 nM triclosan.

Triclosan increases S. aureus adhesion to surfaces.

Previous work has shown that attachment to host proteins is important for *S. aureus* nasal colonization (16). Therefore we performed attachment assays to determine if the presence of triclosan induced the binding of *S. aureus* to host proteins (17). *S. aureus* grown in the presence

of triclosan displayed significantly increased attachment (1.5 to 2 times higher) to all tested host proteins compared to non-triclosan exposed cells (Figure 2.1C). Along with host proteins, we also tested the ability of triclosan to induce the attachment of *S. aureus* to plastic (vinyl) and glass surfaces. We determined that triclosan exposed *S. aureus* had increased attachment (1.5 to 2 times higher) to both plastic and glass surfaces compared to the controls (Figure 2.1C).

Triclosan gavaged rats are more susceptible to S. aureus nasal colonization

To further investigate the role that triclosan may be playing in nasal colonization we used a cotton rat model of *S. aureus* nasal colonization (23). Rats were gavaged with 1ml of triclosan resuspended in corn oil at a concentration of 100 mg/kg/day or an oil control for three consecutive days. On day five rats were nasally inoculated with either a low inoculum (10^5 CFU) or a high inoculum (10^8 CFU) of *S. aureus*. On day twelve the noses were removed and enumerated for *S. aureus*. We observed that triclosan exposed rats were significantly more susceptible to colonization with *S. aureus* than rats not exposed to triclosan (Figure 2.2). The triclosan-exposed rats were significantly more susceptible to colonization with the low inoculum (10^5) whereas the control rats needed a higher inoculum (10^8) to become stably colonized.

Triclosan promotes surface attachment and biofilm formation of other significant pathogens.

In order to determine how widespread triclosan induced cell attachment was, the model organisms and opportunistic pathogens *Pseudomonas aeruginosa* (PA14) and *Escherichia coli* (UTI89) were chosen for analysis. When *P. aeruginosa* was exposed to sub-MIC levels of triclosan, there was a dose dependent increase in the amount of cells that attached to PVC surfaces (Figure 2.3A). Furthermore, *E. coli* formed more robust pellicle biofilms upon triclosan

exposure (Figure 2.3B). Importantly, the levels of triclosan that were capable to induce *E. coli* pellicles was within the lower range of triclosan found in 75% of urine samples in the U.S. (4).

Fatty acid profiles change upon triclosan exposure in S. aureus cells.

In order to investigate the mechanism by which sub-MIC triclosan affects bacteria, I chose to study the effect that triclosan exposure has on the fatty acid profile of *S. aureus*. Because it is known that triclosan can disrupt FABII, I hypothesized that the fatty acid profile would change upon triclosan exposure. Thus, *S. aureus* cells were exposed to various levels (0-100 nM) of triclosan and Fatty Acid Methyl Ester analysis (FAME) was performed to determine changes in the fatty acid profile. FAME analysis revealed an increase in the abundance of 3-hydroxy fatty acids and a decrease in the amount of saturated fatty acids present in the cells (Table 2.1).

Triclosan exposure alters the proteome of S. aureus.

Since adhesive proteins are necessary for *S. aureus* attachment to surfaces, I hypothesized that upon triclosan exposure, there would be an increase in the abundance of *S. aureus* adhesions. To investigate this, two complementary approaches were taken to investigate the proteome upon triclosan exposure – whole cell proteomics and 2D gel electrophoresis. For whole cell proteomic analysis, *S. aureus* was grown in the presence or absence of sub-MIC triclosan (100 nM). After 24 hours of shaking incubation, whole cells were taken, washed, and sent for LC-MS proteomic analysis. As expected, some adhesions such as the Fibronectin-binding protein A (FnbA) was five fold higher in the cells exposed to triclosan. FnbA is known to be an important protein for attaching to surfaces as well as invading human skin (Table 2.2) (18-20). Additionally, the Serine/threonine-protein Kinase (prkC) was also found at 4.5 higher levels in triclosan exposed

cells. PrkC has been implicated to be important for biofilm formation through a yet unknown mechanism (21). Interestingly, the other proteins that were found to be more abundant upon triclosan exposure were cytoplasmic proteins that are not known to have extracellular roles. Recently though, cytoplasmic proteins are being appreciated as having secondary roles as biofilm matrix proteins (22).

The second approach to identify proteins differentially expressed upon triclosan exposure was 2D gel electrophoresis. *S. aureus* was grown for 24 hours in the presence or absence of triclosan (50 nM) and the proteome was separated by isoelectric point and size. Interestingly, this approach only identified four protein spots that were differentially regulated between control and triclosan exposure, three upregulated, and one downregulated (Figure 2.4). These four spots were excised and sent for LC-MS analysis. The triclosan induced spots contained more than one highly abundant protein and were identified as follows: (A) Transglycosylase IsaA/ABC transporter, ATP-binding protein NWMN_2353, (B) Elongation factor Ts (Tsf)/Lactate dehydrogenase (Ldh1), (C) Glyceraldehyde-3-phosphate dehydrogenase 1 (GapA1) (Figure 2.4, Table 2.3). The spot that was found to be significantly reduced upon triclosan exposure was (D) Enolase (Eno) (Figure 2.4, Table 2.3).

Discussion

Together, our data demonstrates that triclosan is commonly present in the nasal secretions of healthy human adults and trends positively with *S. aureus* nasal colonization (Figure 2.1A,B). Additionally triclosan can influence a broad range of bacteria (*S. aureus*, *P. aeruginosa*, *E. coli*) to attach to surfaces and form biofilms (Figures 2.1C, 2.3). Furthermore, in an *in vivo* model of nasal colonization, triclosan exposed rats are more susceptible to colonization with *S. aureus*

(Figure 2.2). We have also found that exposure to triclosan can alter the fatty acid profile and proteome of *S. aureus* (Figure 2.4, Tables 2.1, 2.2, 2.3).

Triclosan has been widely used in consumer products for over 40 years. This has led to worldwide spread of this compound in soil, natural water bodies, and even the human body (4-7). The data presented here demonstrates the effect that triclosan has on *S. aureus* nasal colonization. Combined with previous studies showing the impact of triclosan on the endocrine system and muscle function as well as tumor promotion suggest that the reevaluation of triclosan in consumer products is urgently needed (10, 23-25).

Since triclosan is known to interact with bacterial fatty acid biosynthesis and lipid membranes, we investigated how lipids are changed upon triclosan exposure in *S. aureus*. Our data suggests that there is a decrease in saturated fatty acids upon triclosan exposure (Table 2.1). This change in saturation would suggest that membranes are becoming more fluid, perhaps in response to triclosan incorporation into the membrane. Unexpectedly, we also detected a large increase in 3-hydroxy fatty acids. Interestingly, 3-hydroxy fatty acids are a component of Gram-negative lipopolysaccharide (LPS). Additionally, in other microorganisms, 3-hydroxy fatty acids have been implicated as quorum sensing (QS) molecules. The opportunistic pathogen, *Candida albicans* has been shown to use 3(R)-hydroxy-tetradecaenoic acid (3(R)-HTDE) as a QS molecule (26). *C. albicans* produces 3(R)-HTDE as a metabolite of β -oxidation of linoleic acid, a fatty acid abundant on human skin (26). At high cell densities 3(R)-HTDE promotes biofilm formation by *C. albicans* (26). Bacteria have also been shown to use 3-hydroxy fatty acids for QS. The plant pathogen *Ralstonia Solanacearum* used 3-hydroxypalmitic acid methyl ester (3-OH PAME) as a volatile extracellular factor that controls virulence factor production (27). In the absence of these fatty acids, the bacteria no longer express the genes that are necessary for

pathogenesis (27). Further investigation is needed to determine if *S. aureus* also uses 3-hydroxy fatty acids as quorum sensing molecules to induce biofilm formation or to regulate pathogenesis.

Proteins are critical for the attachment of *S. aureus* to surfaces as well as for host colonization and biofilm formation (22, 28-32). Our proteomics approaches revealed an increase in some proteins suggested to be involved in colonization and biofilm formation including FnbA, PrkC, and IsaA (Tables 2.2, 2.3). There was also an unexpected abundance of intracellular proteins that may have a role in cellular attachment, a function just beginning to be understood (22). Because many of these proteins are essential for cell survival, they have not been found to be important in previous studies that screened for factors involved in attachment, biofilm formation, nasal colonization or infection. The tools to study the role of these proteins outside the cell are currently being developed (22, 33).

Our rat nasal colonization study demonstrated that triclosan gavage made rats more susceptible to colonization with *S. aureus*. Furthermore, attachment assays showed that triclosan exposure made *S. aureus* cells better able to adhere to surfaces. This led to the conclusion that in the nose, *S. aureus* is better able to adhere to the nasal epithelia. An interesting alternative hypothesis is that through triclosan gavage, the nasal flora that may protect from *S. aureus* colonization is disrupted, allowing for easier colonization by *S. aureus*. Previous studies have reported that the presence of low amounts of triclosan in stream ecosystems significantly changes streambed ecology by eliminating up to 80% of the biodiversity (7). This allows for the surviving bacteria to become the dominant species in the environment. Further studies investigating the effect that triclosan has on the normal flora of the host are needed to determine microbiome changes in individuals with high levels of triclosan in their body.

Methods

Nasal colonization model and human nasal secretion collection

The cotton rat nasal colonization model described by Kokai-Kun was utilized in this study (23). Briefly, rats were gavaged with 1 ml of triclosan resuspended in corn oil at a concentration of 100 mg/kg/day or an oil control for three consecutive days. On day five rats were nasally inoculated with either a low inoculum (10^5 CFU) or a high inoculum (10^8 CFU) of *S. aureus* strain SH1000. On day twelve the noses were removed and enumerated for *S. aureus*. All animal experiments were conducted 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 University of Michigan University Committee on Use and Care of Animals (UCUCA) approval number 10394. Significance was determined using a T-test.

Nasal secretions were collected from a convenience sample of 100 adult volunteers in Ann Arbor, MI. Written informed consent was provided by study participants.

(University of Michigan Institutional Review Board approval number IRB00001996). The anterior nares were swabbed and the swab was streaked onto Mannitol Salt Agar to determine colonization by *S. aureus*. Secretions were collected by thoroughly swabbing the anterior and posterior nasal passageways with a sterile cotton swab (Remel BactiSwab). The tip of the swab was cut from the shaft, placed in a ridged eppendorf tube and centrifuged to obtain secretions. Volumes of secretions obtained varied from ~50 μ l to ~200 μ l. Collected secretions were vortexed and sonicated to break up clumps then passed through a 0.22 μ m syringe filter. Secretions were stored at -20°C until they were analyzed by ELISA (Abraxis, Warminster PA). Significance was determined using ANOVA (Macanova version 5.05).

S. aureus attachment assay

The binding of *S. aureus* to surfaces coated with human serum, collagen, fibronectin and keratin was tested as previously (22). All reagents were purchased from Sigma Aldrich, St. Louis, MO) unless otherwise indicated. Briefly, untreated 96 well plates (Nunc 265301) were incubated with 100ul 1% Human serum, 0.2% Type IV collagen from human placenta, 0.01% Fibronectin from human plasma, 0.2% Keratin from human epidermis for 24 hours shaking at 4°C. The plate was then washed three times with 1% BSA. 100ul of *S. aureus* strain SH1000 cells grown for 24 hours in the presence or absence of sub-MIC levels (50 nM) of triclosan or control DMSO was incubated in plates for one hour static at 37°C. At the end of the exposure, wells were washed three times with PBS to remove non-adherent cells. Attached cells were fixed using 2.5% glutaraldehyde in PBS for 1 hour static at 37°C. Attached cells were then stained with 0.1% crystal violet for 30 minutes at RT, washed three times with water then quantified by resuspending in acidified ethanol and measuring absorbance at 570 nm. Significance was determined using a T-test.

To assay for *S. aureus* binding to surfaces, glass or plastic coverslips were placed in twelve well plates (Costar 3513). 3 ml of triclosan exposed or control *S. aureus* were incubated in plates for one hour static at 37°C. At the end of the exposure, wells were washed three times with PBS to remove non-adherent cells. Attached cells were fixed using 2.5% glutaraldehyde in PBS for 1 hour static at 37°C. Attached cells were then stained with 0.1% crystal violet for 30 minutes at RT, washed three times with water then transferred to a clean container and quantified by resuspending in acidified ethanol and measuring absorbance at 570 nm. Significance was determined using a T-test.

Pseudomonas aeruginosa attachment assay

Attachment assays were performed as previously described (34). Briefly, *P. aeruginosa* (PA14) was grown overnight in M63 media. After 24 hours of growth, the cells were washed with M63 and resuspended in M63 or M63 containing different amounts of triclosan. Cells were immediately transferred to round bottom PVC microtiter plates and incubated for 30 minutes at 37°C. Attachment was measured by adding 1% crystal violet to the cells for 15 minutes followed by three washes with water. Crystal violet was dissolved in acidified ethanol and absorbance was read on a spectrophotometer for quantification.

Escherichia coli Pellicle formation

Pellicles were grown as previously described (35). Briefly, *E. coli* (UTI89) was grown overnight at 37°C in YESCA. 2 ml of overnight culture was used to inoculate 2 ml of fresh YESCA media in wells of a 24 well plate. Various amounts of sub-MIC levels of triclosan were added to the wells at the time of inoculation. The plates were then incubated static for three days at 26°C. Pellicle formation was analyzed by staining with crystal violet and photographing of the pellicle.

Fatty Acid Methyl Ester (FAME) analysis of triclosan exposed S. aureus

S. aureus cells were grown for 24 hours at 37°C in the presence or absence of 10 nM, 50 nM, or 100 nM triclosan. Cells were pelleted and 3 mg cells were sent for fatty acid identification to Microbial ID (Newark, DE).

Whole cell proteomic analysis of triclosan exposed S. aureus

S. aureus cells were grown with or without 100 nM triclosan for 24 hours at 37°C. Cells were normalized then washed with PBS. Cell pellets were sent for identification by MS Bioworks (Ann Arbor, MI). Proteomic analysis was performed using Scaffold software (Proteome Software Inc, Portland OR).

2D gel electrophoresis

2D gels were performed as previously described (36). Briefly, *S. aureus* was incubated in the presence of absence of 50 nM triclosan for 24 hours. Cells were then TCA precipitated and resuspended in denaturing alkylation buffer (6 M Urea, 100 nM Tris pH 8.5, 10 mM EDTA, 0.5% (w/v) SDS). Protein concentration was determined using Protein Dc Kit (Bio-rad) and 115 ug of protein was removed and TCA precipitated. Proteins were resuspended in 2D lysis buffer and 2D gel electrophoresis performed. Analysis of gels was performed using DECODON Delta2D software. Significant changes between proteins were then removed from the gel and sent for identification to MS Bioworks (Ann Arbor, MI).

Figures and Tables

Figure 2.1: Triclosan influences interactions with the human nares. **(A)** Nasal secretions were collected from 90 healthy individuals and ELISA was performed to determine the concentration of triclosan in each secretion. **(B)** The same individuals were also swabbed for the presence of *S. aureus* colonization in the nares. The differences in *S. aureus* nasal colonization between ≤ 175 nM triclosan and >175 nM triclosan is statistically significant by ANOVA ($p < 0.01$). **(C)** Attachment assays were performed with human serum, collagen, fibronectin (Fbn), and keratin, which are found in nasal secretions as well as plastic and glass surfaces. *S. aureus* exposed to 50 nM triclosan bound all host proteins and artificial surfaces to a greater extent than control cells ($p < 0.05$ by T-test). Figure from (24).

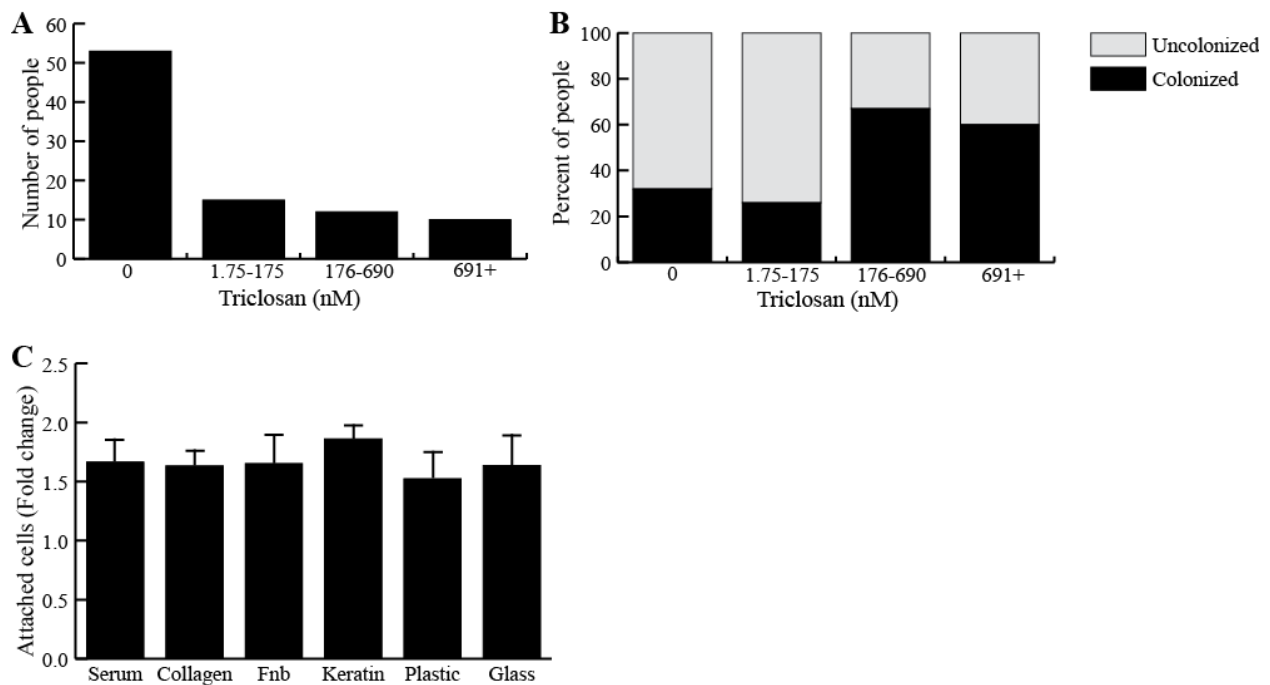


Figure 2.2: Triclosan gavaged rats are more susceptible to *S. aureus* nasal colonization. Cotton rats were gavaged with triclosan or an oil control for three days. On day 5 they were challenged with either a low inoculum (10^5 CFU) or a high inoculum (10^8 CFU) of *S. aureus*. On day twelve, the noses were removed and enumerated for *S. aureus*. Rats gavaged with triclosan were unable to clear the low inoculum whereas the oil gavaged rats were able to clear the bacteria from the nose. * $p < 0.01$ by T-test. Figure from (24).

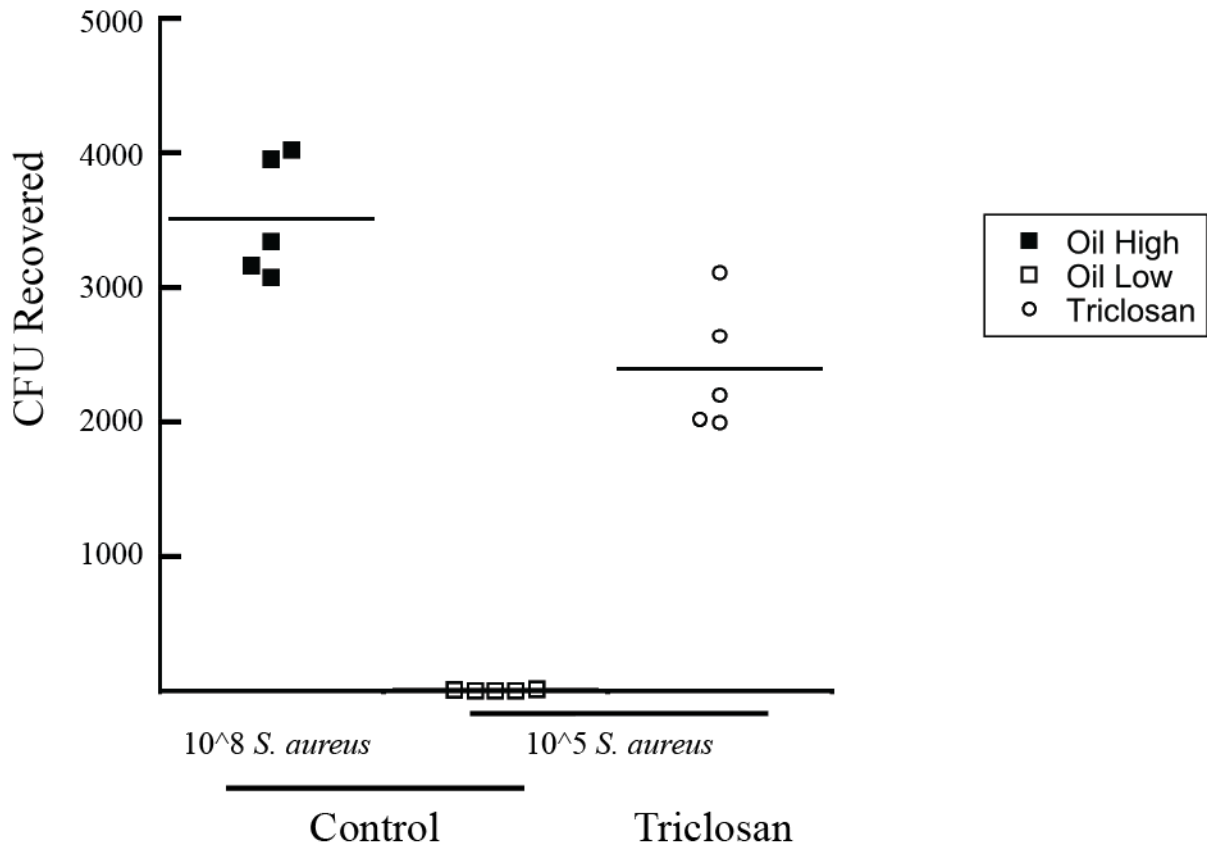


Figure 2.3: Triclosan promotes attachment and biofilm formation of significant pathogens. **(A)** Triclosan promotes the surface attachment of *P. aeruginosa* (PA14) to PVC plates. No concentrations had any affect on the growth of the bacteria. **(B)** Triclosan induced pellicle biofilm formation of *E. coli* (UTI89). Pellicles were stained using crystal violet.

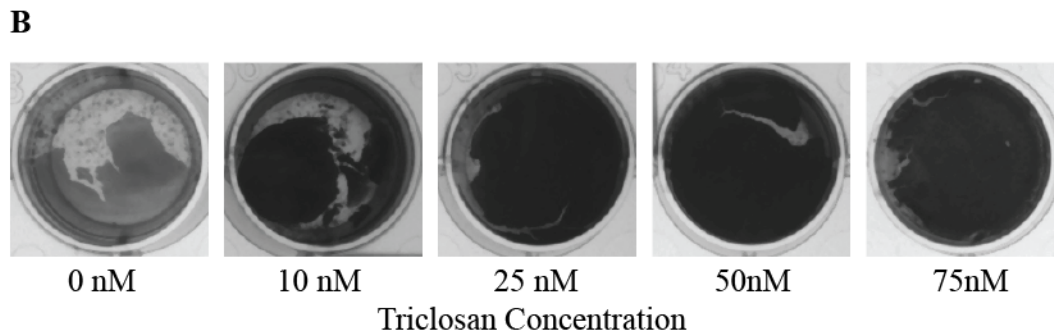
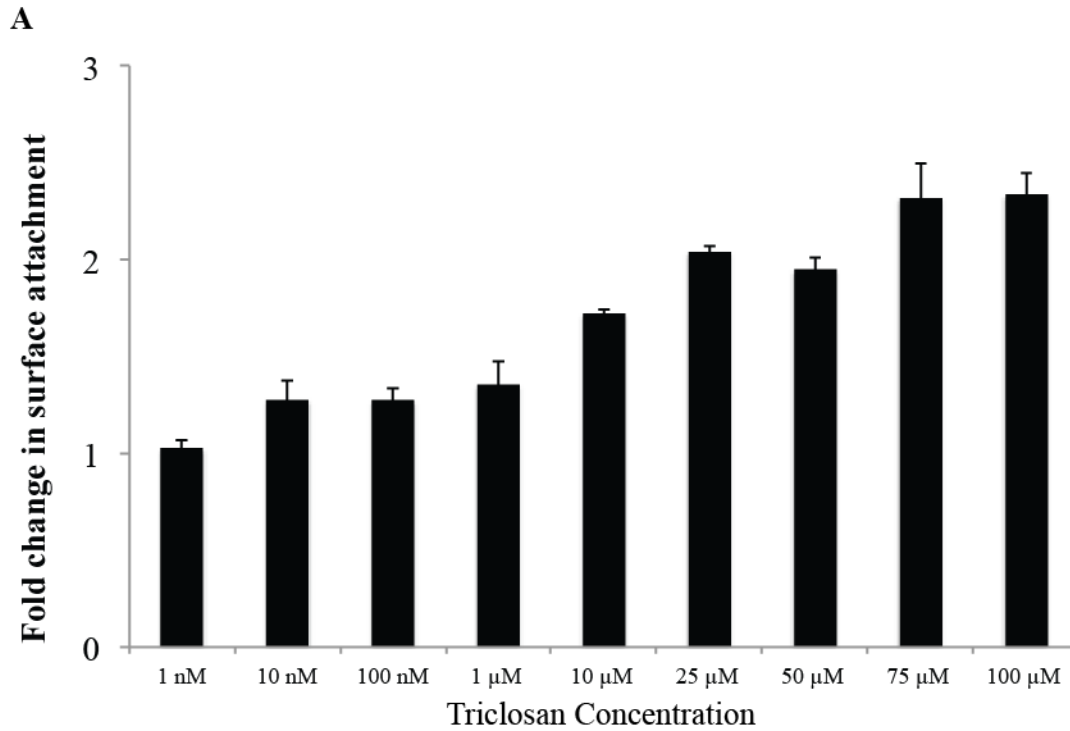


Table 2.1: *S. aureus* fatty acids changed upon triclosan exposure. Fatty acids are all represented as fold change to cells grown in the absence of triclosan. Fatty acids labeled in blue represent those that are enriched upon triclosan exposure while red represents those that are depleted by triclosan exposure.

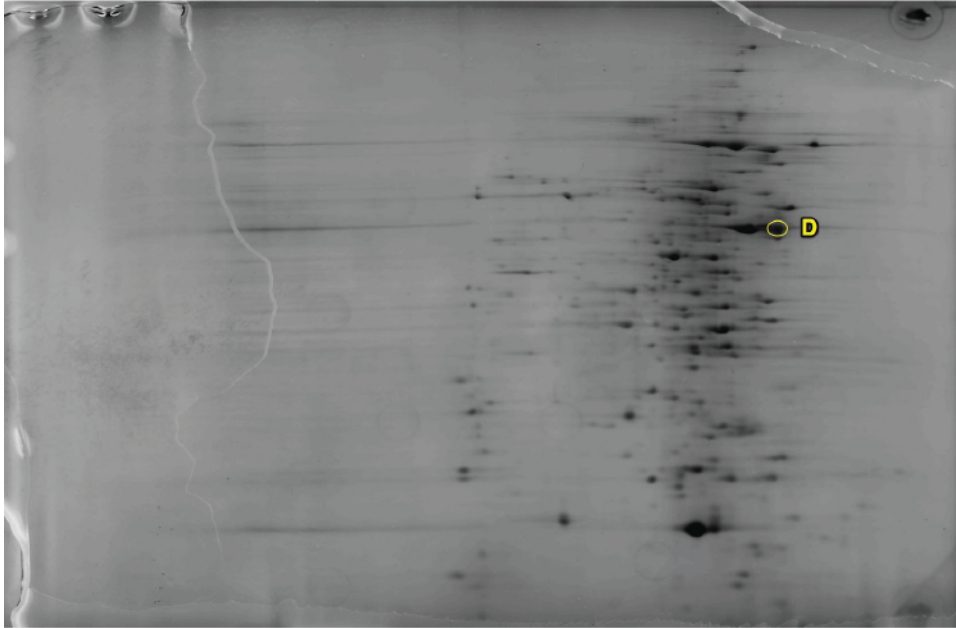
Fatty acid	10 nM/0 nM	50 nM/0 nM	100 nM/0 nM
13:0 iso	0.96	1.14	2.84
13:0 anteiso	2.16	3.07	1.80
14:0	0.83	1.11	3.08
15:1 iso G	0.92	1.30	2.14
15:0 iso 3OH	1.11	3.96	14.10
16:0 3OH	0.00	3.13	11.35
18:0 3OH	1.98	4.20	12.62
unknown 14.502	0.79	0.42	0.00
15:0	0.67	0.45	0.13
16:1 w9c	1.01	0.16	0.00
19:0 anteiso	0.77	0.96	0.23
19:0	1.11	0.76	0.17

Table 2.2: Whole cell proteomic analysis of triclosan exposed *S. aureus*. Presented are the most highly increased proteins upon triclosan exposure.

Identified Proteins	NSAF TSB	NSAF TSB Tric	Tric/TSB
30S ribosomal protein RpsF	0.00	0.27	-
Glutamine-fructose-6-phosphate transaminase GlmS	0.00	0.18	-
Putative uncharacterized protein SaurJH9_1139	0.00	0.11	-
CHAP domain containing protein SaurJH9_2327	0.00	0.07	-
ATP-dependent Clp protease ATP-binding subunit ClpL	0.00	0.06	-
Ornithine--oxo-acid transaminase RocD	0.00	0.05	-
Fibronectin-binding protein A FnbA	0.04	0.22	5.72
Serine/threonine-protein kinase PrkC	0.04	0.17	4.54
Peptide methionine sulfoxide reductase MsrA1	0.21	0.79	3.70
Glutamate synthase GltB	0.03	0.10	3.03
Peptidase, membrane zinc metallopeptidase SaurJH9_1519	0.17	0.52	3.03
ROK family protein SaurJH9_0299	0.09	0.27	3.03
MazG nucleotide pyrophosphohydrolase SaurJH9_1518	0.24	0.72	3.03
Isochorismate synthase SaurJH9_1101	0.05	0.17	3.03

Figure 2.4: 2D gel electrophoresis of *S. aureus* proteins changed upon exposure with triclosan. (A) Proteins that were present in *S. aureus* cells that were not exposed to triclosan (TSB). (B) Proteins that were present in *S. aureus* cells that were exposed to triclosan (50 nM). Circled spots were significantly changed between the two treatments and sent for MS identification.

A



B

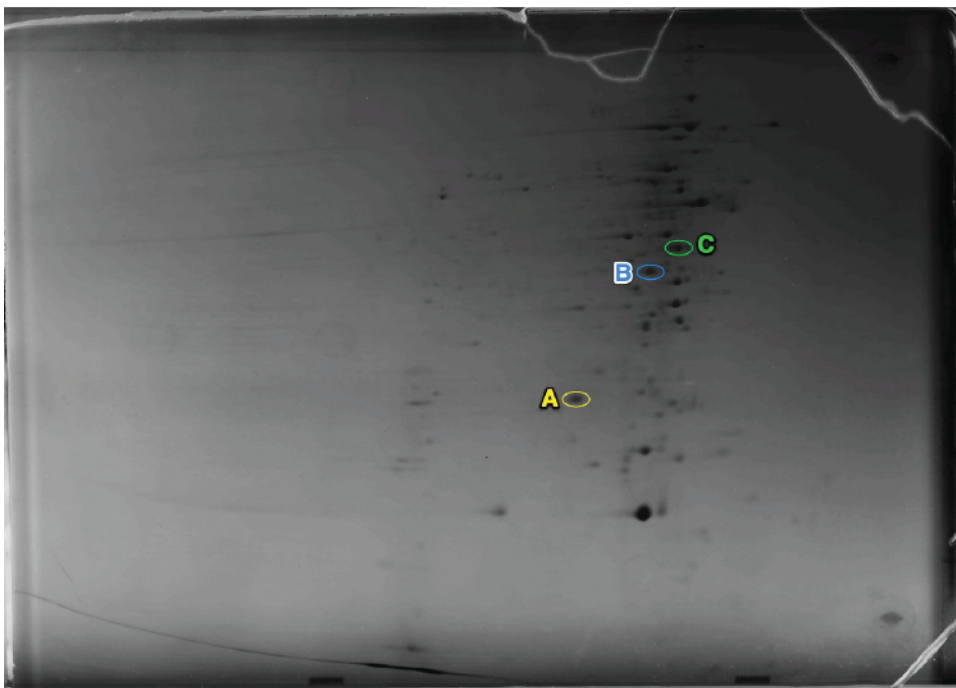


Table 2.3: Proteomic identification of spots changed between control and triclosan exposed *S. aureus*. Proteins labeled in blue are those that were increased upon triclosan exposure while red signifies proteins that were significantly decreased upon triclosan exposure.

Identified protein	Spot	Spectral Abundance
Probable transglycosylase - IsaA	A	90
ABC transporter, ATP-binding protein - NWMN_2353	A	48
Elongation factor Ts - Tsf	B	184
L-lactate dehydrogenase 1 - Ldh1	B	117
Glyceraldehyde-3-phosphate dehydrogenase 1 – GapA1	C	249
Enolase - Eno	D	402

Notes and Acknowledgments

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Chapter 3

***Staphylococcus aureus* phenol soluble modulins stimulate the release of pro-inflammatory cytokines from keratinocytes and are required for induction of skin inflammation**

Abstract

Staphylococcus aureus is a human commensal that colonizes the skin. While it is normally innocuous, it has strong associations with atopic dermatitis pathogenesis, and has become the leading cause of skin and soft tissue infections in the USA. The factors that dictate the role of *S. aureus* in disease are still being determined. In this work, we utilized primary keratinocyte culture and an epidermal murine colonization model to investigate the role of *S. aureus* phenol soluble modulins (PSMs) on pro-inflammatory cytokine release and inflammation induction. We demonstrated that many species of *Staphylococcus* are capable of causing release of IL-18 from keratinocytes and that *S. aureus* PSMs are necessary and sufficient to stimulate IL-18 release from keratinocytes independent of caspase-1. Further, after seven days of epicutaneous exposure to wild type *S. aureus*, but not Δ *psm* *S. aureus*, we saw dramatic changes in gross pathology as well as systemic release of pro-inflammatory cytokines. This work demonstrates the importance of PSM peptides in *S. aureus*-mediated inflammatory cytokine release from keratinocytes *in vitro* and *in vivo* and further implicates PSMs as important contributors to pathogenesis.

Introduction

Staphylococcus aureus is a human commensal that lives in the nose, skin and throat of approximately 30% of the human population (1, 2). While *S. aureus* is usually harmless, it is an opportunistic pathogen that has become a leading cause of nosocomial infections in the USA (3), and can manifest as skin and soft tissue infections, infective endocarditis, osteomyelitis, and sepsis (1). Further, it has a role in exacerbation of atopic dermatitis (AD), a chronic inflammatory disease of the skin that affects up to 20% of children and up to 3% of adults (4, 5). Chronic cutaneous inflammation during AD results in increased production of extracellular matrix components which permit attachment of *S. aureus* (6). Subsequently, *S. aureus* promotes AD by stimulating a strong pro-inflammatory response (7). *S. aureus* proteins such as hemolysin α (HLA), Staphylococcal protein A (SPA) and lipoteichoic acids (LTA) promote pro-inflammatory cytokine production from keratinocytes (7-11). However, this stimulation requires the concurrent presence of surfactant such as SDS (11).

Keratinocytes serve as the first line of defense against cutaneous pathogens and are able to stimulate an immune response by releasing cytokines, defensins and antimicrobial cationic peptides such as LL-37 to fight off pathogens (12). One cytokine that is produced by keratinocytes and has a role in promoting AD is IL-18 (13, 14). IL-18 is a pro-inflammatory cytokine that is cleaved and activated primarily by caspase-1 (15). Caspase-1 activation occurs following exposure to danger associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) which stimulate the activation of the inflammasome (16). Caspase-1 activation can also result in the activation of the pro-inflammatory cytokine IL-1 β (17). These mature cytokines are then released from the cell and have varied effects such as leukocyte

recruitment, promotion of T and B cell activation and upregulation of other inflammatory cytokines (18).

The phenol soluble modulins (PSMs) of *S. aureus* have been shown to have many characteristics including the ability to recruit leukocytes, lyse erythrocytes and neutrophils, act as antimicrobial peptides, modulate biofilm development, and exhibit surfactant like properties (19-28). PSMs are small peptides ranging from 22-44 amino acids in length that are predicted to form amphipathic helices and in some cases can form amyloid fibers to abrogate their toxic properties and stabilize biofilms (19, 23). Several Staphylococcal species have been reported to produce PSM proteins including *S. aureus*, *S. epidermidis*, and *S. lugdunensis* (19, 29, 30).

Here, we set out to investigate Staphylococcal factors that contribute to the release of pro-inflammatory cytokines from keratinocytes. We found that some, but not all species of Staphylococci examined produce compounds that are able to cause the lytic release of active IL-18 from human keratinocytes independent of caspase-1 activation. We also showed that PSMs are necessary and sufficient for this release. Further, we utilized a mouse model of cutaneous colonization (31), to demonstrate that neutrophil recruitment and systemic inflammatory response are dependent on *S. aureus* PSMs.

Results

Some Staphylococci produce cytotoxic factors that affect human keratinocytes

To investigate the role that secreted factors from different Staphylococci have on human keratinocytes, we exposed human keratinocytes to bacterial supernatants grown in tryptic soy broth (TSB). Keratinocytes were monitored for the release of IL-18 because of its constitutive production in keratinocytes and its pro-inflammatory activity in the skin (11). We found that

supernatants from several Staphylococci species including *S. aureus*, *S. lugdunensis*, *S. epidermidis*, *S. capitis*, *S. warneri*, and to a lesser extent but still significant *S. hominis* were able to trigger the release of IL-18 from keratinocytes (Figure 3.1A). Supernatants from *S. saprophyticus* and *S. haemolyticus* did not induce release of IL-18 (Figure 3.1A). Importantly, release of LDH, a marker of cell lysis, aligned with IL-18 release from the cells with the exception of *S. hominis*, which showed no significant increase in LDH release (Figure 3.1B).

S. aureus phenol soluble modulins induce IL-18, IL-1 β , and LDH release from human keratinocytes

Because of its prominent role in skin infections and AD, we chose to focus the rest of our study on *S. aureus*. Using a candidate approach, we found that the quorum sensing system in *S. aureus* known as the accessory gene regulator (*agr*) was necessary for cytotoxicity, IL-18, and IL-1 β release (Figure 3.1C, 3.1D, 3.1E). Because the *agr* system is known to regulate the expression of many virulence factors produced by *S. aureus*, we investigated mutants of two well-studied cytotoxic factors: hemolysin α (HLA) and the phenol soluble modulin family (PSM). *S. aureus* supernatants without PSMs (Δ PSM) showed a significant decrease in their ability to stimulate the release of IL-18, IL-1 β , and LDH from keratinocytes, and the release was restored when the strains were complemented with PSM α and PSM β operons (Figure 3.1C, 3.1D, and 3.1E). Notably, lack of HLA, which has been reported to lyse human cells and activate the inflammasome (32), did not impact the release of IL-18, LDH, or IL-1 β when compared to our WT *S. aureus* supernatant (Figure 3.1C, 3.1D, and 3.1E). These data suggest that PSMs are the primary *S. aureus* factors which induce lysis of keratinocytes.

To further investigate which PSMs contribute to IL-18 release and keratinocyte lysis, synthetic PSMs were used. PSM peptides were sufficient to stimulate the release of IL-18 from keratinocytes (Figure 3.2A). Interestingly, all of the PSM α peptides as well as δ -toxin but neither of the PSM β peptides induced release of LDH from the keratinocytes (Figure 3.2B). As PSMs are thought to have a helical structure similar to the known lytic antimicrobial peptide LL-37 (reviewed in (33)), we also compared a dose response of the ability of PSM α 1 to induce IL-18 release as compared with LL-37. As shown in figure 3.2C, both peptides induce IL-18 release at 5 μ g/mL, but LL-37 appears to be more potent. Taken together, these data demonstrate that the PSM peptides of *S. aureus* are necessary and sufficient for the release of IL-18, likely through lytic release from human keratinocytes.

The release of mature IL-18 is independent of caspase activity

Several *S. aureus* toxins have been reported to activate the inflammasome, so we next investigated whether inflammasome activation participated in the release of IL-18 from keratinocytes following *S. aureus* exposure (34). As shown in figure 3.3A, we confirmed that stimulation of keratinocytes with WT *S. aureus* supernatant resulted in detectable active caspase-1 within the keratinocytes. Western blot confirmed that the IL-18 released was in its active form (Figure 3.3B). To see if caspase-1 activation was required for the release of IL-18 or IL-1 β , we pretreated primary keratinocytes with various cell-permeable caspase inhibitors followed by stimulation with WT *S. aureus* supernatant. Inhibition of caspase-1 (YVAD), caspase-3 (DEVD), caspase-5 (WEHD), caspase-6 (VEID), caspase-8 (IEHD) or all caspases (ZVAD) did not impact *S. aureus*-induced lytic cytokine release (Figure 3.3C, 3.3D, 3.3F). However, identical concentrations of both YVAD and ZVAD repressed inflammasome activation induced by LPS in

PBMCs (Figure 3.3E). Elevated extracellular potassium, another condition which inhibits NLRP3 inflammasome activation, did not inhibit *S. aureus*-mediated cytokine release (data not shown). These data suggest that release of active IL-18 by PSMs is specific to disruption of cell membranes and is not due to specific activation of the inflammasome.

Phenol Soluble Modulins are required for neutrophilic infiltration following epidermal S. aureus exposure.

Given that PSMs induce lysis of and inflammatory cytokine release from keratinocytes, we hypothesized that they may be required for initiation of inflammation in response to cutaneous colonization by *S. aureus*. We thus challenged mice epicutaneously with WT or Δpsm strains of *S. aureus*. As shown in figure 3.4A, application of WT *S. aureus* to the surface of the skin resulted in infectious changes including erythema and ulceration whereas Δpsm strains produced minimal skin changes. Flow cytometry of the colonized skin demonstrated a significant decrease in neutrophilic infiltration of the skin when comparing WT to Δpsm strains. Other cell types, including macrophages, dendritic cells, B-cells and T-cells were not significantly altered following *S. aureus* exposure (Figure 3.4B). Using C57Bl/6 IL-18 $-/-$ mice, we found that IL-18 was not required for *S. aureus*-induced neutrophil infiltration into the skin (Figure 3.4C). Absence of IL-18 enhanced macrophage, CD11b⁺ DC, and CD4⁺ T cell migration suggesting that keratinocyte release of IL-18 may have repressive effects on chronic inflammation (Figure 3.4C). These data suggest that PSMs are required for invasive infection when *S. aureus* is exposed to intact keratinocyte layers via epicutaneous application and that IL-18 is not required for this process.

Others have demonstrated that epicutaneous application of *S. aureus* results in upregulation of pro-inflammatory cytokines (31). In order to determine whether PSMs play a role in modulating inflammatory gene expression in the skin, we performed RT-PCR on skin sections following exposure to WT or Δpsm strains. As shown in figure 3.5A, both WT and Δpsm were able to dramatically upregulate proinflammatory genes within the skin, including interferon (IFN) α , IFN γ , IL-18, IL-1 β , TNF α and IL-6 when compared to skin exposed to PBS. Both were also able to upregulate the antimicrobial peptide, CAMP, the murine LL-37 ortholog. Chemokines, such as CCL4, were also upregulated by both strains. Given that the skin phenotype following exposure to WT vs. Δpsm strains was so dramatically different, we then chose to examine systemic cytokine concentrations in mice exposed to both strains. As shown in figure 3.5B and table 3.3, serum concentrations of inflammatory cytokines such as G-CSF, GM-CSF, IL-1 β , IL-6, and IL-17 are significantly diminished in Δpsm when compared to WT exposed mice. Together, these data suggest that PSMs are not required to stimulate inflammatory gene upregulation, but are required to initiate release of pro-inflammatory cytokines into circulation and permit the subsequent inflammatory response.

Discussion

S. aureus colonization is known to be highly prevalent in the US population and also contributes to chronic skin conditions such as AD. In this paper, we demonstrate that the PSM α family, including δ -toxin, are necessary and sufficient for lytic activity on keratinocytes, which results in the release of inflammatory cytokines, such as IL-18 and IL-1 β (Figures 3.1 and 3.2). Additionally, we demonstrate that PSMs, while not required for transcriptional upregulation of

pro-inflammatory genes within the skin, are required for instigation of skin inflammation and systemic cytokine changes in an epicutaneous colonization model (Figures 3.4 and 3.5).

Several groups have investigated mechanisms by which *S. aureus* can elicit an immune response from keratinocytes; however, these factors have required the use of surfactants to promote membrane permeability (11). Our data presented here demonstrate that keratinocyte mediated inflammation can be stimulated by *S. aureus* PSMs alone. Because of their amphipathic nature, PSMs have surfactant like properties which allow for membrane intercalation and pore formation similar to the human cathelicidin LL-37 (Figure 3.2C) (21). Interestingly, not all species of Staphylococci induce keratinocyte lysis (Figure 3.1). We observe strong lytic abilities by species known to contain PSMs including *S. aureus*, *S. epidermidis*, and *S. lugdunensis* but there has been no study, to our knowledge, investigating the presence of PSMs in other Staphylococcal species. Further investigation of *S. hominis*, which is able to induce release of IL-18 without inducing cell lysis will be of interest. Additionally, the PSM β peptides were able to induce the release of IL-18 from human keratinocytes without causing a release of LDH (Figure 3.2). It is possible that the PSM β peptides are able to activate a receptor mediated cytokine release or possibly trigger slow, non-lytic inflammasome activation.

Keratinocyte activation and release of IL-18 and IL-1 β by caspase-1 as part of an active inflammasome has been reported (35). Additionally, an inflammasome-independent role for caspase-1 in apoptosis of keratinocytes has been described (36). Interestingly we observe that in the presence of *S. aureus* supernatants, we detect activation of caspase-1, but this activation is not required for IL-18 release. This suggests that PSMs are promoting cytokine release via lysis (Figure 3.3) and that other enzymes, such as cathepsin activation or granzyme B may be important for IL-18 activation in keratinocytes (37) This PSM-induced IL-18 release may

resolve the conundrum between the role of increased IL-18 signaling in atopic dermatitis and recent data which describes repressed inflammasome activity in AD (38, 39). Chronic colonization with PSM-producing *S. aureus* may circumvent the need for inflammasome-mediated IL-18 release.

In our epicutaneous model of *S. aureus* exposure, we found that absence of IL-18 did not impact neutrophil recruitment but enhanced chronic inflammatory cell infiltration. This is an interesting finding as the actual function of IL-18 in the skin has not been systematically investigated. Many studies examining the role of IL-18 in cutaneous disease report up or down regulation of this cytokine without reporting the consequence of these changes (40). Our data suggest that following *S. aureus* exposure, IL-18 may function to modulate the acute vs. chronic inflammatory response. Given that absence of IL-18 promoted a large increase in CD4⁺ T cells in our study, further investigation into the Th1 vs. Th2 skewing of these populations is warranted. Additionally, determining whether the effects of IL-18 are different in AD models exposed to *S. aureus* is an important area for future research.

PSMs produced by *S. aureus* have been shown to be critical determinants of pathogenesis in murine models of abscess and bacteremia (19). They are recognized by the human formyl peptide receptor 2 which stimulates neutrophil chemotaxis (24). Not only can they recruit neutrophils, but PSMs have been shown to intercalate into cell membranes resulting in lysis and death (19). In our *in vivo* model, transcriptional changes of cytokines in the dermis of our mice did not differ between mice exposed to WT and Δ *psm* *S. aureus* (Figure 3.5A). This likely reflects intact toll-like receptor (TLR) ligands, such as peptidoglycan, present on each strain. Keratinocytes express TLR2 which results in robust inflammatory gene upregulation after exposure to peptidoglycan (41). Thus, it is not surprising to see robust gene expression changes

in the skin after exposure to WT and Δpsm strains. Importantly, however, while a robust systemic inflammatory response was noted with WT *S. aureus*, the circulating concentrations of inflammatory cytokines were dramatically and significantly reduced after exposure to Δpsm *S. aureus* (Figure 3.5B). The large difference in granulocyte colony stimulating factor (G-CSF) agrees with previously reported effects of one of the PSMs, δ -toxin, on mast cell degranulation and G-CSF release (42). Differences in circulating IL-6 and IL-1 β , both of which are released from keratinocytes were also noted (Figure 3.3 and (35, 43)). Systemic levels of IL-18 were not altered after *S. aureus* exposure (Table 3.3), but this does not rule out important localized effects as other important cytokines in AD have been noted to be increased in the skin but not in serum (44). Taken together with our *in vitro* data which demonstrate PSM-mediated release of IL-18 and IL-1 β from keratinocytes (Figures 3.1C, 3.1E, and 3.5B), these results suggest that PSM-mediated keratinocyte lysis may be a required factor to convert *S. aureus* colonization into infection.

In summary, we have described a novel role for Staphylococcal PSMs which induce keratinocyte lysis and release of inflammatory cytokines. This translates to a role of PSMs in infection and systemic inflammatory response *in vivo*. We propose that PSMs are important for initiating pathogenesis after colonization and may serve as important targets for treating infection or modulating inflammatory cutaneous disease.

Methods

Bacterial strains and growth conditions

The bacteria used in this study are summarized in Table 3.1. *Staphylococcus aureus* strain LAC (45) was used as the wild type strain for this study. To create a Δpsm deletion strain,

PSM α and PSM β operons were deleted using allelic replacement as previously described (23) and the start codon of δ -toxin was mutated to prevent translation while not affecting the function of RNAlIII as done previously (19). Genetic complementation of the PSM α and PSM β operons in the Δpsm strain was done as previously described using pALC2073-PSM α & pRS-PSM β (23). Bacterial supernatants were collected after 24 hrs of growth in tryptic soy broth (TSB) (MP Biosciences, Santa Ana, CA) at 37°C orbital shaking at 200 rpm. The cells were centrifuged at 7000 \times g for 10 min followed by filter sterilization of the supernatants through 0.22 μ m filters. Supernatants were stored at -20°C until use. Bacteria used for infection were grown overnight in TSB at 37°C orbital shaking at 200 rpm. Before use, they were diluted 1:500 in fresh TSB and grown for 4 h to obtain cells in exponential growth phase. The cells were resuspended in phosphate buffered saline (PBS) prior to use (Gibco, Grand Island, NY).

Peptides

PSM peptides were synthesized by LifeTein (South Plainfield, NJ) and assayed to be >90% pure by HPLC. Synthetic peptides were prepared as previously described (46). Briefly, peptides were resuspended in hexafluoroisopropanol (HFIP) and 0.5 mg was aliquoted into microcentrifuge tubes. HFIP was then removed using a SpeedVac system at room temperature. Dried peptides were stored at -20°C. Immediately before use, peptides were suspended in PBS to a concentration of 10 mg/ml and then diluted further in PBS to desired concentration. LL-37 was purchased from AnaSpec Inc (Fremont, CA).

Cell culture

Primary human keratinocyte cultures were obtained from Dr. Johann Gudjonsson, University of Michigan. Cells were cultured in EpiLife medium (Gibco) supplemented with

Human Keratinocyte Growth Supplement (Gibco), 1% Pen-Strep (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco), and 0.25 µg/ml Fungizone (Gibco). For all experiments, cells were used at passage 2-6 and 2×10^4 cells/well were plated in 24 well plates.

Cells were exposed to bacterial supernatants or peptides at confluence. Cells were pretreated for 30 min with 10 µM caspase inhibitors (Enzo) where indicated prior to addition of indicated concentrations of bacterial supernatants or peptides. Cells were treated for 1 h prior to harvesting. The extracellular media was removed after 1 h and the remaining cells were lysed using RIPA buffer (300 mM NaCl, 50 mM Tris, 6.4 mM EDTA, 0.5% Triton X-100) containing protease inhibitor cocktail (Complete Mini, EDTA-free, Roche, South San Francisco, CA) by incubation for 10 min on ice. Cell debris was removed by centrifugation at $13,000 \times g$ for 5 min and lysates were frozen at -20°C until use.

For caspase-1 activation studies, keratinocytes were plated 1×10^4 cells/well of a 96 well plate. The following day, cells were exposed to with *S. aureus* supernatants for one hour followed by addition of FITC-conjugated YVAD for one hour (ImmunoChemistry Technologies) and processed per manufacturer's recommendations. Cells were counterstained with DAPI before fixation. Images of active caspase-1 were acquired on an Olympus IX70 inverted microscope (Olympus; Center Valley PA) using a 40x objective at the Center for Live Cell Imaging (CLCI) at the University of Michigan Medical School.

To control for caspase inhibitor effectiveness, human PBMCs were isolated via Ficoll gradient. 1×10^6 cells were plated per well of a 12 well plate. 10 µM YVAD or ZVAD was added 30 min prior to stimulation with 1 µg/mL LPS for 24 h. Media was collected and IL-1β release was measured via ELISA.

Mice

All animal studies were performed according to protocols approved by the University of Michigan Committee on the Use and Care of Animals protocol #01823. Animals were obtained from Jackson Labs and housed at the University of Michigan in specific pathogen free housing followed by biohazard containment after treatment with bacterial strains. To test the effects of WT vs. Δpsm *S. aureus* exposure, the dorsal skin of 8-10 week old C57BL/6 female mice was shaved and depilated with Veet. The next day, the stratum corneum barrier was disrupted via mild stripping using three applications of Tegaderm (3M, St. Paul, MN). This technique exposes the keratinocyte layers without creating a wound (31). 1×10^7 CFU *S. aureus* or PBS were placed with sterile gauze on the stripped skin and occluded with Tegaderm (3M). 1 week after *S. aureus* exposure, animals were euthanized. Serum was collected via cardiac puncture. Skin was removed for cell count analysis via flow cytometry (see below) and RNA analysis.

Cytokine production

In vitro quantification of IL-18 and IL-1 β was determined by ELISA (eBioscience, Vienna, Austria) according to the manufacturer's instructions. Active IL-18 release was assessed via Western blot (primary antibody rabbit anti-human IL-18, Santa Cruz), secondary antibody goat anti-rabbit-HRP (Abcam). To investigate changes in serum cytokine profiles of mice exposed to *S. aureus*, a Milliplex assay was performed (EMD Millipore, Darmstadt, Germany).

Cytotoxicity

To test for cell lysis following peptide treatments of keratinocytes, release of lactate dehydrogenase (LDH) was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Flow Cytometry

Lymphocytes were isolated from skin biopsies as follows: immediately after euthanasia, skin samples were removed, diced into 1-2 mm pieces using sterile razor blades, and then placed into a GentleMacs C Tube with 10 ml RPMI-10 (RPMI + 10% fetal bovine serum) with 2 ml enzyme solution (100 mg/ml DNase type I Sigma #DN-25, 100 mg/ml Hyaluronidase type V Sigma #H6254, 500 mg/ml Collagenase Sigma #C5138 in HBSS). GentleMacs tissue dissociation was performed for 1 min followed by a 2 h incubation at 37°C. After incubation, dissociation was repeated for 1 min. Cells were strained and pelleted by centrifugation at 800 \times g for 7 min at 4°C. Supernatant was discarded and cells were washed once with 15ml RPMI-10. After wash, cells were divided into tubes for FACS analysis. To determine inflammatory cellular infiltrate following *S. aureus* exposure, purified cells were stained for one hour on ice with the following antibodies (all from BioLegend, San Diego, CA): CD19-PE-Cy7, CD3-APC, CD4-FITC, CD8-PE, Ly6G-FITC, F4/80 Pacific Blue, CD11b-APC, CD11c-PE-Cy7, CD11c-PE, and Ly6C-PE-Cy7. Flow data was collected on a BD LSR II and analyzed via FlowJo V10 (Tree Star).

RT-PCR

Skin biopsies were homogenized in TriPure (Roche) and RNA was isolated via Direct-zol mini RNA prep (Zymo). 100 ng of RNA was transcribed into cDNA and real-time PCR analysis was completed on an ABI PRISM 7900HT (Applied Biosystems) using Sybr Green (Life Technologies). See Table 3.2 for primers. Cycle times were normalized to β -actin and fold change ($2^{-\Delta\Delta ct}$) was calculated vs. PBS exposed mice.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Comparisons between data were made via 2-sided student's unpaired t test or Mann-Whitney *U* tests for non-normalized data. A *p* value <0.05 was considered significant.

Figures and Tables

Table 3.1: Bacterial strains used in this study

Strain	Description	Source or reference
<i>S. aureus</i>		
AH1181	LAC <i>hla::erm</i>	This study
AH1263	LAC (WT)	(45)
AH1292	LAC <i>agr::tet</i>	(47)
BB3047	LAC Δ <i>psm</i> - pALC2073 & pAH8	This study, (23)
BB3048	LAC Δ <i>psm</i> - pALC2073-PSM α & pRS-PSM β	This study, (23)
Other Staphylococci		
BB2153	<i>S. saprophyticus</i>	This study
BB2164	<i>S. epidermidis</i>	This study
BB2191	<i>S. lugdunensis</i>	This study
BB2201	<i>S. warneri</i>	This study
BB2203	<i>S. hominis</i>	This study
BB2205	<i>S. haemolyticus</i>	This study
BB2207	<i>S. capitis</i>	This study

Table 3.2: Primers used for Real-Time PCR.

Gene	Forward (5'->3')	Reverse (5'->3')
<i>Interferon alpha (IFNα)</i>	ATGGCTAGRCTCTGTGCTTTTCCT	AGGGCTCTCCAGAYTTCTGCTCTG
<i>Interferon beta (IFNβ)</i>	AGCTCCAAGAAAGGACGAACAT	ATTCTTGCTTCGGCAGTTAC
<i>Interferon gamma (IFN-γ)</i>	AGCGGCTGACTGAACTCAGATTGTA	GTCACAGTTTTTCAGCTGTATAGGG
<i>IL-18</i>	ACTGTACAACCGCAGTAATACGC	AGTGAACATTACAGATTTATCCC
<i>IL-1β</i>	CCCTGCAGCTGGAGAGTGTGGA	CTGAGCGACCTGTCTTGGCCG
<i>IL-6</i>	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
<i>IL-1α</i>	CGGGTGACAGTATCAGCAAC	GACAACTTCTGCCTGACGA
<i>Tumor necrosis factor alpha (TNFα)</i>	CCCCTCTGACCCCTTTACT	TTTGAGTCCTTGATGGTGGT
<i>Cathelicidin antimicrobial peptide (CAMP)</i>	TCAACCAGCAGTCCCTAGAC	AAGGCACATTGCTCAGGTAG
<i>NLR Family, Pyrin Domain Containing 3 (NLRP3)</i>	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
<i>Chemokine (C-C motif) receptor 1 (CCR1)</i>	TGGGCAATGTCCTAGTGATT	GCATCACCAAAAATCCAGTC
<i>Chemokine (C-C motif) ligand 4 (CCL4)</i>	AGCAACACCATGAAGCTCTG	CTGTCTGCCTCTTTTGGTCA
<i>Chemokine (C-C motif) ligand 5 (CCL5)</i>	CAATCTTGCAGTCGTGTTTG	GGAGTGGGAGTAGGGGATTA
<i>C-X-C motif chemokine 10 (CXCL10)</i>	CAAAAGTAACTGCCGAAGCA	CTGAGCTAGGGAGGACAAGG
<i>C-X-C motif chemokine 13 (CXCL13)</i>	AGAGGTTTGCGAGATGGACT	GAGCCTGGACCTTTAAGCTG
<i>β-Actin</i>	TGGAATCCTGTGGCATCCTGAAAC	TAAAACGCAGCTCAGTAACAGTCCG

Table 3.3: Cytokine concentrations (pg/ml) from mice exposed to WT *S. aureus*, Δ *psm S. aureus* or vehicle control (PBS).

	WT	<i>Δpsm</i>	PBS	Significant difference between WT vs. <i>Δpsm</i> (p value)
G-CSF	3443.4	287.9	434.1	Yes (0.0011)
GM-CSF	34.5	12.1	14.8	Yes (0.00540)
IFNγ	2.5	2.0	2.1	No (0.2000)
IL-1α	249.6	266.4	275.4	No (0.0827)
IL-1β	16.6	7.9	22.7	Yes (0.0009)
IL-2	3.6	2.7	3.7	Yes (0.0256)
IL-4	3.0	3.0	3.4	No (1.0000)
IL-5	8.4	5.6	17.8	No (0.2887)
IL-6	71.6	9.0	23.5	Yes (0.0070)
IL-7	3.4	1.0	18.5	Yes (0.0002)
IL-10	3.8	2.1	3.0	Yes (0.0020)
IL-12p40	4.9	3.7	3.5	No (0.2822)
IL-12p70	10.2	3.7	13.0	Yes (0.00480)
IL-13	84.7	45.7	58.7	Yes (0.0047)
IL-15	6.8	2.5	2.5	Yes (0.0256)
IL-17	11.7	3.0	3.3	Yes (0.00280)
IL-18	193.8	201.1	190.9	No (0.769)
IP-10	142.7	101.9	80.4	Yes (0.0379)
KC	116.3	81.5	104.1	No (0.3754)
MCP-1	63.9	25.4	24.4	No (0.0692)

MIP-1a	39.5	13.6	19.5	Yes (0.0006)
MIP-1b	18.7	9.0	13.1	No (0.2124)
MIP-2	88.0	75.1	60.4	No (0.3958)
RANTES	14.9	9.4	7.1	No (0.2247)
TNFα	5.2	3.4	3.6	No (0.0816)

Figure 3.1: Various Staphylococcal species induce lytic release of IL-18 from human keratinocytes. Human keratinocytes were exposed to supernatants from several species of *Staphylococcus* and tested for release of (A) IL-18 and (B) Lactate Dehydrogenase (LDH). Human keratinocytes were also exposed to supernatants from several strains of *S. aureus* and tested for the release of (C) IL-18, (D) LDH, and (E) IL-1 β . (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$) $n=2$ in duplicate for each study. Error bars represent standard error of the mean.

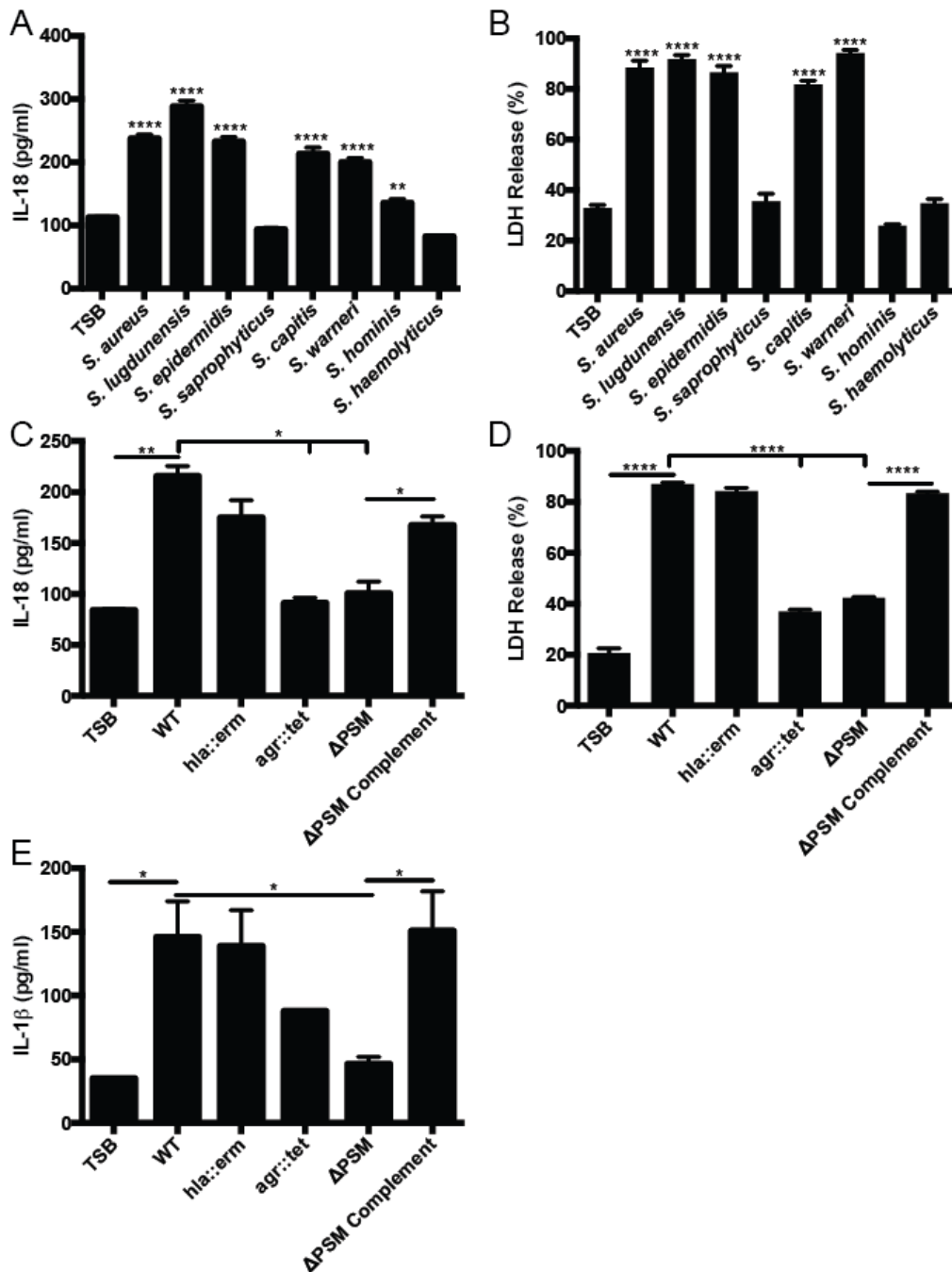


Figure 3.2: *S. aureus* PSMs are sufficient for lytic release of IL-18 from human keratinocytes. Human keratinocytes were exposed to synthetic PSM peptides and tested for release of (A) IL-18 and (B) LDH. PSMs induce release of IL-18 in a dose dependent mechanism similar to LL-37 (C). (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) $n=3$ in duplicate for each study. Error bars represent standard error of the mean.

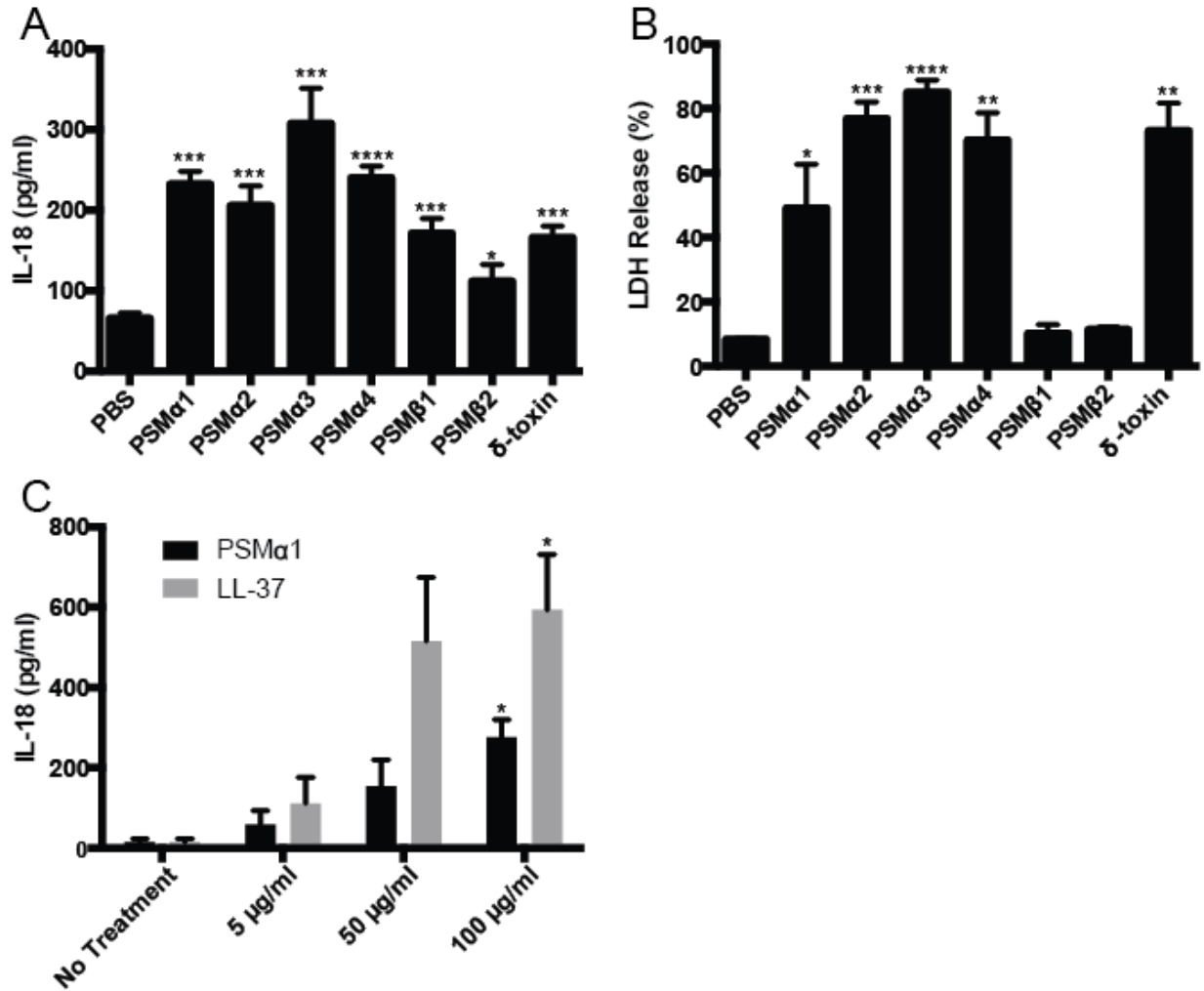


Figure 3.3: The release of mature IL-18 from human keratinocytes in response to *S. aureus* is independent of caspase-1 function. (A) Human keratinocytes were exposed to TSB or WT *S. aureus* supernatants for one hour followed by addition of fluorescent-labeled peptide that binds active caspase-1 and visualized by fluorescent microscopy (n=3). (B) Representative western blot for IL-18 released from keratinocytes exposed to TSB or WT supernatants. Recombinant active IL-18 (rIL-18) was run as a positive control. DMSO or caspase inhibitors (10 mM) were added to human keratinocytes prior to treatment with *S. aureus* supernatants and then tested for the release of (C) IL-18 (n=3), (D) IL-1 β (n=3), and (F) LDH (n=1). (E) Human PBMCs were incubated with 10 mM YVAD or ZVAD for 30 min prior to overnight incubation with 1mg/mL LPS. Released IL-1 β was detected via ELISA. Error bars represent standard error of the mean. (** $p < 0.01$, **** $p < 0.0001$).

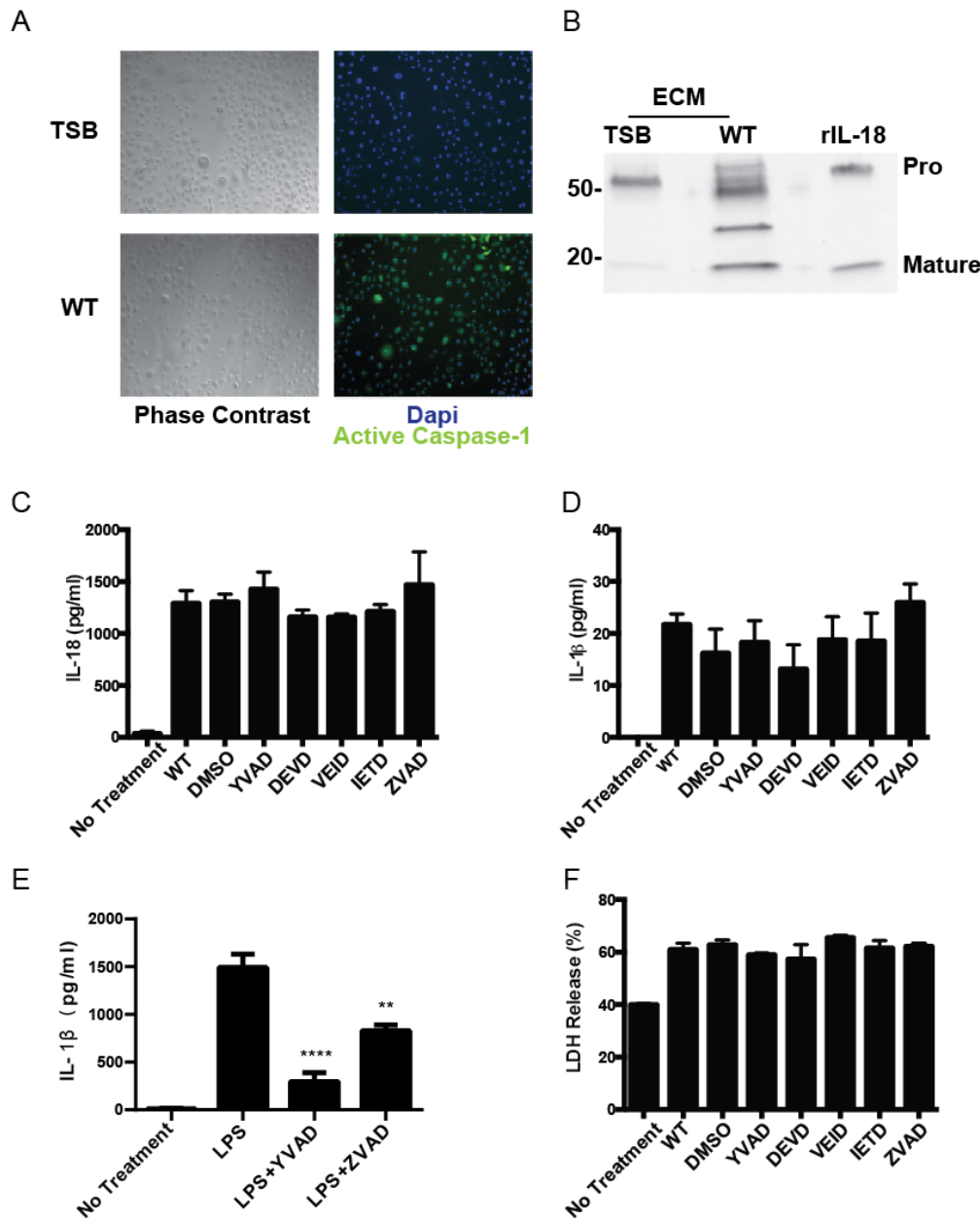


Figure 3.4: PSMs are critical for the recruitment of neutrophils into the skin during *S. aureus* epidermal exposure. (A) Representative photographs of dorsal surface of mice taken after 7 days of exposure. (B) FACS analysis of leukocytes obtained from skin samples of mice seven days post-inoculation with *S. aureus*. (* $p < 0.05$) $n = 8$ for each group. (C) FACS analysis of skin leukocytes from WT or IL-18 KO mice exposed ($n = 5$) to WT *S. aureus* as in B. Neutrophils=Ly6G+, Macrophages=F4/80+,CD11b+, dendritic cells=Ly6c+CD11c+, T cells=CD3+, B cells=CD19+. Error bars represent standard error of the mean. (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

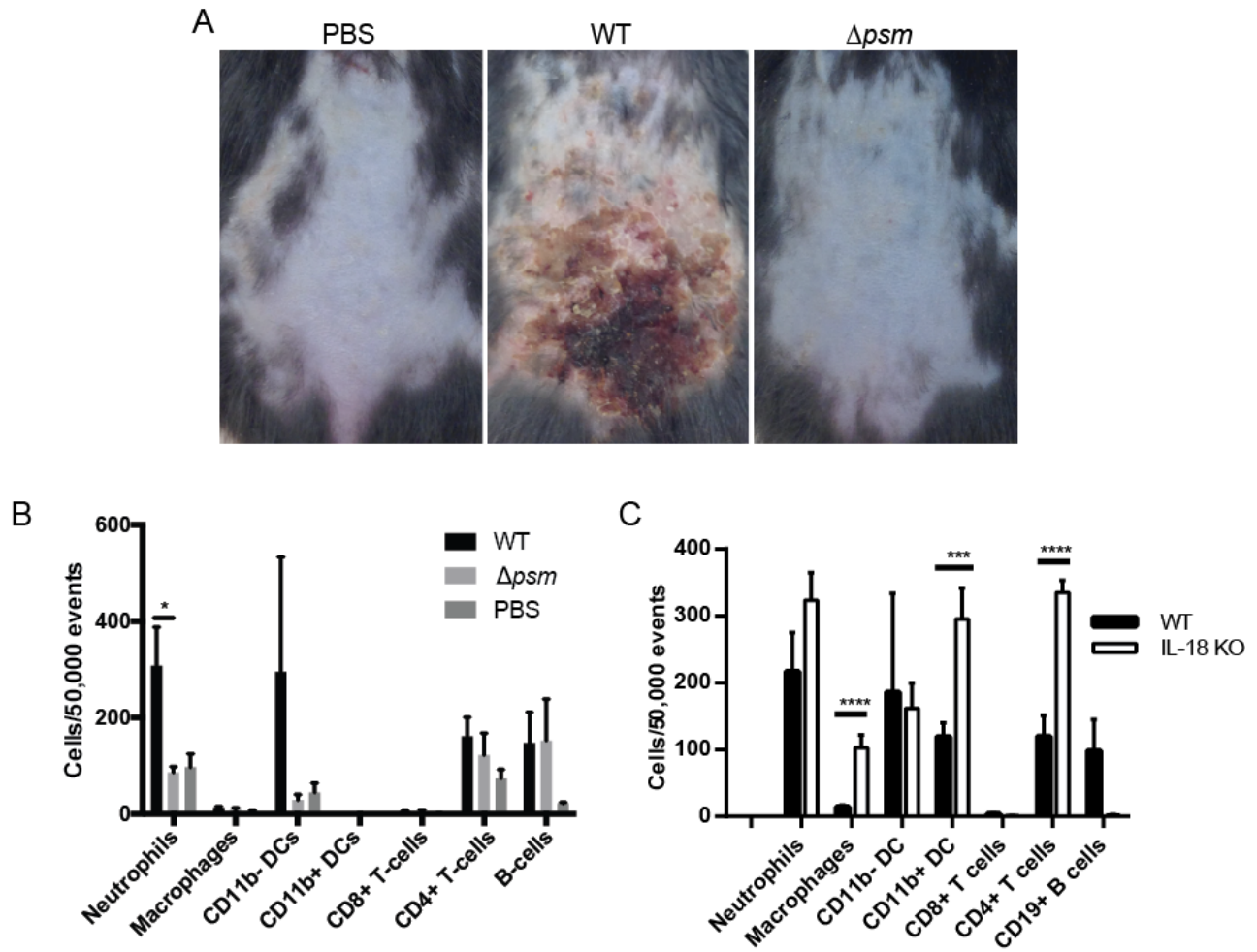
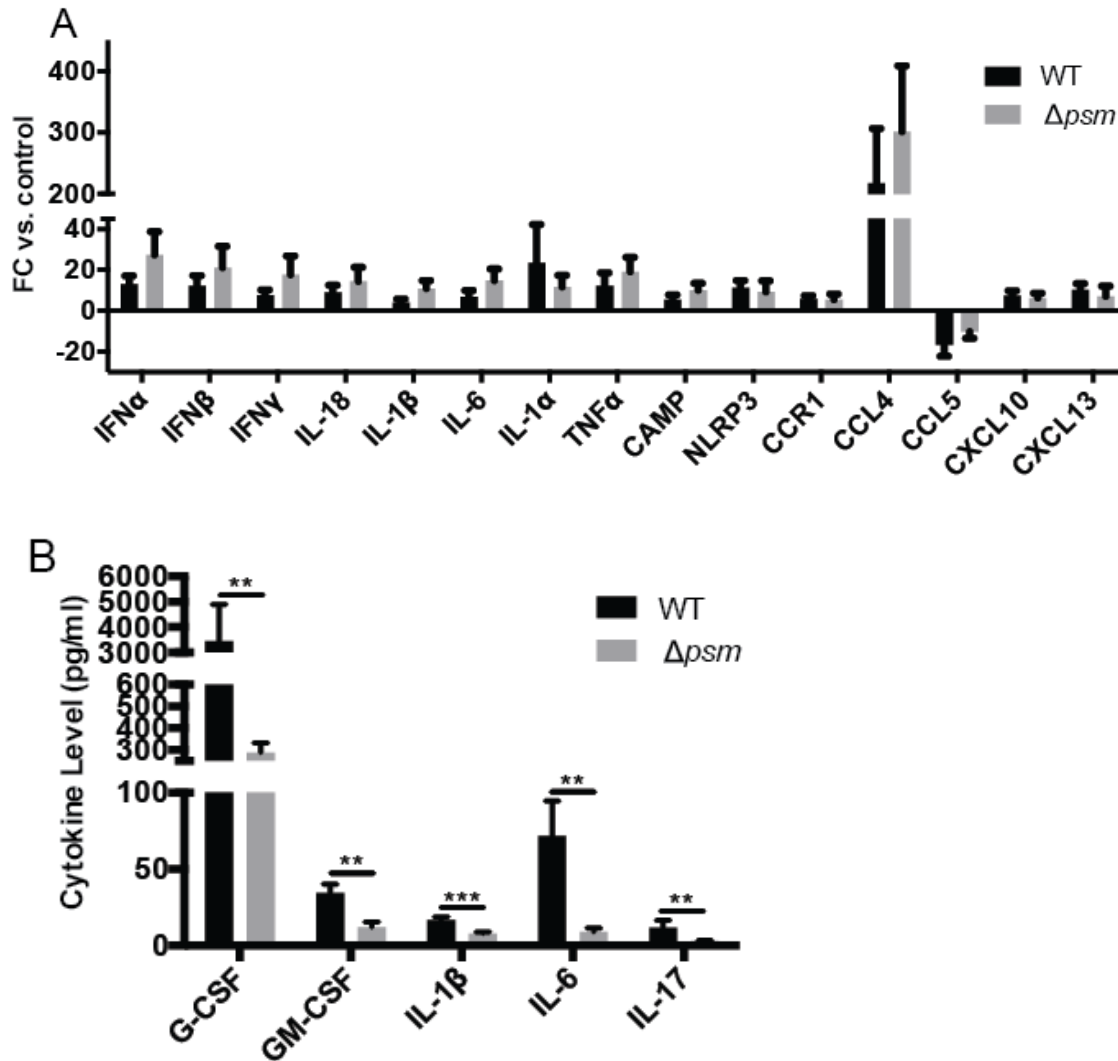


Figure 3.5: Systemic inflammatory response, but not inflammatory gene upregulation, requires *S. aureus* PSMs. (A) Graph represents fold change in cutaneous expression of listed genes when compared to PBS exposed mice as calculated by real-time PCR. (B) Graph represents serum concentrations of indicated cytokines as determined by Milliplex in WT vs. Δpsm exposed mice. (** $p < 0.01$, *** $p < 0.001$) (n=8 for each group). Error bars represent standard error of the mean.



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Chapter 4

***Staphylococcus aureus* hitchhikes on *Brevibacillus* and induces its swarming**

Abstract

Staphylococcus aureus is a non-motile opportunistic pathogen that colonizes an estimated 30% of the population and causes a wide range of diseases. Because *S. aureus* colonizes many people without causing disease, it is of great interest to determine which factors induce the change from commensal to pathogen. Many pathogens require motility to cause disease. Despite the importance of motility, some bacteria like *S. aureus* have no known genes that code for motility. I hypothesize that *S. aureus* may use other bacteria as a means of movement. To identify motile bacteria that may carry *S. aureus* I investigated the ability of environmental bacteria to migrate across agar surfaces in the absence or presence of *S. aureus*. I found that *Brevibacillus* can carry viable *S. aureus* across an agar surface. Additionally *S. aureus* can use the surfactant-like activity of the phenol soluble modulins (PSM) to induce *Brevibacillus* swarming prior to when *Brevibacillus* alone begins to swarm. This work demonstrates that non-motile bacteria can not only move with other bacteria, but that they can aid in motility by production of extracellular factors.

Introduction

Bacteria are frequently found in complex microbial communities. These communities influence natural ecosystems as well as human health, agriculture, and bioremediation (1). Advancements in DNA sequencing technology has led to metagenomic studies identifying the microbes present in various environments (2-9). The microbial community of the human nose has been of particular interest because of its role as a reservoir for the opportunistic pathogen *S. aureus* (10-13). *S. aureus* colonizes the nose and skin of approximately 30% of the human population (14). Interestingly, since *S. aureus* only colonizes 30% of the population, there are factors that make individuals resistant or amenable to colonization.

The ability of *S. aureus* to colonize the human nose is multifactorial and depends on its ability to adhere to the host and integrate into the microbial community (15). *S. aureus* produces several factors necessary for attachment and colonization of the nasal epithelia including clumping factor B (ClfB), iron-regulated surface determinant A (IsdA), and wall teichoic acids (WTA) (15-17). Several major regulators of *S. aureus* virulence including the exoprotein regulator (*sae*), the accessory gene regulator (*agr*), and the alternative sigma factor B (*sigB*) are repressed during *S. aureus* colonization, favoring attachment of cells rather than toxin production against niche competing bacteria and the host (18).

In addition to *S. aureus* interacting with host proteins, previous studies have investigated the role that commensal bacteria have on *S. aureus* colonization. For example, some, but not all, strains of *Staphylococcus epidermidis* produce a protease that can detach *S. aureus* from surfaces as well as eradicate *S. aureus* (19). Importantly, not all polymicrobial interactions negatively affect the ability of *S. aureus* to colonize the nose. Different species of *Corynebacterium* correlate with the presence or absence of *S. aureus* nasal colonization and can have positive or

negative metabolic affects on *S. aureus* (13). Additionally, *Propionibacterium*, a genus abundant in the human nose, can produce compounds that promotes surface attachment and aggregation of *S. aureus*, presumably promoting its incorporation into the community (20). Outside of these studies, metagenomics have demonstrated remarkable diversity within the nasal community, as well as suggests corollary evidence for bacterial interference with *S. aureus* colonization (10, 12, 21).

While considering other factors that influence nasal colonization, it is interesting that non-motile bacteria such as *S. aureus* can navigate the viscous mucus produced in the nose and attach to the epithelial cells. Many bacteria utilize motility to chemotax towards food, escape from predators as well as to disseminate in the environment (22). Traditionally, bacteriologists focused on motility by looking at a singular bacterium swimming through liquid (22).

Swarming on the other hand is a community behavior where cells move together over a soft surface (22). Swarming bacteria often produce a biosurfactant to reduce surface tension, allowing for easier movement (22). With so many diverse bacteria utilizing these large and complex systems for motility, it is of interest that some bacteria have no known genes coding for movement, including *S. aureus*.

Many bacteria use flagella to move through mucus to reach and attach to epithelial cells (23). In the urinary tract, pathogens such as *Escherichia coli* and *Proteus mirabilis* require flagella to ascend the urinary tract and establish an infection (24, 25). In contrast, *Staphylococcus saprophyticus* is able to ascend the urinary tract and cause an infection without any known means of motility (26). Similarly, *Pseudomonas aeruginosa* flagella are critical for the initial stages of pulmonary infections (27).

Non-motile *S. aureus* can cause severe infections similar to motile bacteria. Because of this, I hypothesize that *S. aureus* can use other bacteria as a means of motility. I investigated interactions between motile bacteria and *S. aureus* to determine if other bacterial species could serve as vehicles for *S. aureus*. Here, I demonstrated that a *Brevibacillus* species is able to carry viable *S. aureus* across a soft agar surface. Additionally, *S. aureus* contributes to swarming by producing Phenol Soluble Modulin (PSM) surfactants to promote *Brevibacillus* motility. Lastly, this ability is not unique to *S. aureus* but is shared by some, but not all Staphylococcal species. Together this data suggests that non-motile bacteria such as *S. aureus* employ a novel and alternative strategy for movement.

Results

S. aureus can hitchhike on *Brevibacillus*

To investigate if *S. aureus* can hitchhike on motile bacteria, we isolated bacteria from the nose, skin, and environment. First, these bacteria were tested for their ability to swarm alone on 0.7% tryptone agar (TA). 15 bacteria displayed swarming motility on 0.7% TA. To test if these bacteria allowed for *S. aureus* to migrate with them, the unlabeled motile bacteria were mixed with *S. aureus* expressing GFP and migration was monitored overtime by measuring GFP fluorescence distribution over the plate. This analysis was performed using an *in vivo* imaging system (IVIS) measuring GFP signal across the entire plate. Epifluorescent microscopy was used in addition to look for GFP fluorescence on the cellular level. Of the 15 bacteria tested, only two bacteria, an environmental *Brevibacillus* (species unknown), and *Paenibacillus vortex* were found to carry *S. aureus* across the agar surface (Figure 4.1 A, 4.1B, *P. vortex* data not shown). In order to determine the viability of the bacteria, samples were taken every 5 mm starting in the

center of the plate towards the edge of the plate and patched onto MSA (Mannitol salt agar, selects for *S. aureus*) and TA with 2 µg/ml mupirocin (Selects for *Brevibacillus*). Viable and culturable *S. aureus* and *Brevibacillus* were recovered from every distance tested from the center to the edge of the plate (Figure 4.1C). When grown by itself, *S. aureus* is unable to move from the original spot on the center of a 0.7% TA plate (Data not shown).

S. aureus can induce *Brevibacillus* swarming

In order to determine if *S. aureus* has any effect on *Brevibacillus* swarming, we performed a time course analysis of the bacterial swarms. *Brevibacillus* was spotted alone or in the presence of *S. aureus* and images were acquired over time. The amount of the plate covered by bacteria was then quantified (Figure 4.2B). Surprisingly, *Brevibacillus* began to swarm at an earlier time point (<10h) in the presence of *S. aureus* compared to *Brevibacillus* alone (>19h) (Figure 4.2A). The migration of each species was monitored by sampling every 5 mm and replica plating on MSA and TA with 2 µg/ml mupirocin.

S. aureus PSMs act as a surfactant to aid *Brevibacillus* swarming

To investigate the mechanism by which *S. aureus* can induce *Brevibacillus* swarming, a biochemical approach was employed, followed by a candidate approach. Firstly, cell free supernatants of wild-type (WT) *S. aureus* were added to 0.7% TA plates to investigate the effect of secreted factors on *Brevibacillus* swarming behavior. Cell free supernatants from *S. aureus* were able to induce *Brevibacillus* to swarm across the plate within 24 hours of incubation (Figure 4.3A). Further analysis of cell-free supernatant demonstrated that the secreted factor was heat and protease resistant and could be isolated in the >100 kDa fraction of centrifugal filter

units (Data not shown). One factor that has all of these characteristics are the Phenol Soluble Modulin (PSM) peptides produced by *S. aureus*. The addition of supernatants from *S. aureus* Δpsm strain in 0.7% TA were then tested for their ability to induce *Brevibacillus* swarming, but failed to do so (Figure 4.3A).

The PSM peptides are known to possess many properties, including acting as bio-surfactants (28). Because surfactants are known to be produced by bacteria while swarming, I hypothesized that the surfactant-like activity of the PSMs is able to promote *Brevibacillus* swarming. To test this, Tween-20, a widely used surfactant, was added to agar plates in increasing concentrations to determine if this alone could promote *Brevibacillus* swarming. We found that indeed, Tween-20 could induce the swarming of *Brevibacillus* in a dose-dependent manner (Figure 4.3B). Furthermore, to test if there are any additional roles of the PSMs in promoting *Brevibacillus* swarming, we also placed *Brevibacillus* with WT or a Δpsm strain of *S. aureus* on plates containing various concentrations of Tween-20. As expected we saw that tween could complement swarming of *Brevibacillus* with *S. aureus* Δpsm (Figure 4.3B). Additionally, there is no observable difference between the WT and Δpsm suggesting that the surfactant-like property of the PSMs is the main contributor to this phenotype (Figure 4.3B).

Some but not all Staphylococci can swarm with Brevibacillus

PSM peptides are unique to Staphylococci (29). Therefore, I wanted to determine which Staphylococcal species could induce the swarming of *Brevibacillus*. I found that some, but not all Staphylococcal species were able to promote *Brevibacillus* swarming. *S. lugdunensis*, *S. saprophyticus*, *S. capitis*, and *S. warneri* were able to promote *Brevibacillus* swarming, whereas *S. hominis* and *S. haemolyticus* seem to have no effect (Figure 4.4). Interestingly, a commensal

strain of *S. epidermidis* as well as *S. aureus* (UAMS-1) appear to inhibit *Brevibacillus* swarming (Figure 4.4). Further analysis of these species are needed, to determine if they produce PSM peptides under the conditions tested.

Discussion

Together, the data presented in this chapter demonstrate a novel mode of transportation that non-motile bacteria, such as *S. aureus*, have evolved. We show that *S. aureus* can hitchhike on *Brevibacillus* (Figure 4.1). Not only does *S. aureus* utilize *Brevibacillus* for motility, but *S. aureus* produces PSM surfactants to promote *Brevibacillus* swarming (Figures 4.2, 4.3). Interestingly, neither all strains of *S. aureus* nor all species of Staphylococci are able to promote the swarming of this *Brevibacillus* species (Figure 4.4). With the data presented here the reevaluation of the idea of bacterial immobility is needed.

Motility is a complex and expensive trait for bacteria to have, thus its conservation through many different species of bacteria should not be underappreciated (30). As discussed in the introduction, many species swarm across surfaces as a community behavior (22). Other bacteria have found interesting ways of motility. The intracellular pathogen *Listeria monocytogenes* has developed a novel way to move from cell to cell during infection. *L. monocytogenes* uses host cell actin which it polymerizes at one end of the cell to produce force, moving the bacteria through the cells (31). This force is used by *L. monocytogenes* to move from cell to cell without leaving the host cytoplasm (31). Since movement is vital for so many bacteria, it is fascinating that bacteria such as Staphylococci do not have any known means of movement. The findings here suggest that these “non-motile” bacteria have evolved alternative methods of movement, such as hitchhiking, to avoid this expensive trait.

Other groups have observed similar phenotypes in other systems. For example, the non-motile plant pathogen *Xanthomonas perforans* is able to hitchhike on the motile plant commensal *Penibacillus vortex* (32). This suggests that hitchhiking may have evolved by some bacteria in various environments. *X. perforans* produces a gaseous compound that induces *P. vortex* swarming (32). Interestingly *P. vortex* was identified in my screen as a species that can also carry *S. aureus* across a plate (Data not shown). We have not observed a gaseous signal in our system (Data not shown) but we do see that *S. aureus* contributes surfactants to promote *Brevibacillus* swarming (Figures 4.2, 4.3). Because *S. aureus* contributes to swarming, it suggests that this interaction may be mutualistic rather than parasitic but further experiments are needed to investigate this.

Furthermore, Dr. Mark Shirtliff's group has demonstrated that *S. aureus* can bind to *Candida albicans* hyphae during polymicrobial infections (33). They show that *S. aureus* binds to the Als3p protein on *C. albicans* hyphae and as these hyphae invade mammalian tissues, they can bring *S. aureus* with them, helping *S. aureus* invade tissues that it normally cannot (33). Interestingly, this Als3p protein is an amyloid-like protein (34). Further analysis needs to be conducted to determine if the *S. aureus* PSM amyloid fibers are also needed for this interaction or if a yet uncharacterized amyloid anchor on *S. aureus* may be necessary for this phenotype.

Since *Brevibacillus* is ubiquitous in soil, it is disregarded by clinical microbiologists and is regarded as a contaminant in infections. Interestingly, it has been reported to be found in several sites on the human body that overlap with places that *S. aureus* is commonly found. A metagenomic study of the microbiome of nasopharynx of children found *Brevibacillus* species present in the nose (35). Importantly, this study also found that *Brevibacillus* in the nose was only abundant during the spring season (35). This suggests that *Brevibacillus* may be a transient

nasal bacterium, which could explain its absence in other studies investigating the microbiome of the nasopharynx (11, 13). Additionally, *Brevibacillus* has been found in low levels in the vaginal cavity (36). As discussed previously, *S. aureus* is found in about 9% of vaginal samples (37). These studies suggest that even though we do not think of *Brevibacillus*, and many other bacteria, as a part of the human microbiome, they can be found as transient members of microbial flora.

Recently *Brevibacillus agri*, a clinically insignificant soil-microbe, was found to be the cause of a urinary tract infection (UTI). Interestingly, preliminary urine analysis tested negative for a UTI and it took more intensive analysis, after the patient developed pyelonephritis, to determine the agent causing this UTI (38). As mentioned earlier, the clinically significant urinary pathogen, *S. saprophyticus* has no means of motility to ascend the urinary tract, a trait essential for disease progression. We have shown that *S. saprophyticus* is able to induce the swarming of our *Brevibacillus* species *in vitro* (Figure 4.4). As these soil-microbes may not be identified using conventional clinical techniques, studies investigating possible polymicrobial infections of *S. saprophyticus* and motile soil-bacteria would be of considerable interest.

Future clinical studies as well as metagenomic studies investigating microbial communities from both a commensal and pathogen perspective should take these transient colonizers into consideration. Here, we have shown that the clinically relevant pathogen, *S. aureus* is able to interact with *Brevibacillus* *in vitro*. Ongoing studies will investigate how these bacteria interact in the context of nasal colonization and disease. If bacteria such as *Brevibacillus* interact with *S. aureus* in the host, this presents a novel therapeutic target to reduce *S. aureus* colonization and infection.

Methods

Bacterial strains and growth conditions

All reagents were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated. All bacteria were grown in a 1% Tryptone broth (TB) shaking at 200 rpm at 37°C unless otherwise stated. 2% Nobel Agar was added to TB for growth on plates, preventing swarming, unless otherwise stated. *Staphylococcus aureus* strain LAC was chosen as the wild type strain for this study as well as is the parent of all mutant strains used (39). Other Staphylococcal species were used in previous studies (40). To create a Δpsm deletion strain, PSM α and PSM β operons were deleted using allelic replacement as previously described (41) and the start codon of δ -toxin was mutated to prevent translation while not affecting the function of RNAIII as done previously (42). The GFP strain contains a plasmid with constitutive production of a GFP (pCM12) variant generously provided from the lab of Dr. Alex Horswill (43). *Brevibacillus* sp. was isolated from the environment. 16s sequencing identified it in the *Brevibacillus* genus, but there was not sufficient information to determine the species.

Bacteria were discerned after mixing by plating on mannitol salt agar (MSA) and TA + 2 $\mu\text{g/ml}$ mupirocin. MSA selects for Staphylococcal species but cannot support *Brevibacillus* growth. *S. aureus* strains used in this study are sensitive to the 2 $\mu\text{g/ml}$ mupirocin, while *Brevibacillus* is resistant. These allow us to separate growth of the two bacteria after they have been mixed.

Swarming analysis

Bacteria were grown overnight shaking at 37°C in TB. The following day the Staphylococcal species were diluted 1:500 while *Brevibacillus* was diluted 1:250 in fresh TB and

grown for 4 h to obtain exponentially growing cells. After 4 hours, the bacteria were normalized to an $OD_{600}=1$ in PBS. A final volume of 4ul of bacteria was then spotted on the center of a 0.7% TA (tryptone agar) plate either individually or mixed. Plates were then incubated in a high humidity incubator until time of analysis. *S. aureus* expressing GFP was analyzed using a Xenogen IVIS (Alameda, CA). Quantification of swarms was performed using Adobe Photoshop CS6 (San Jose, CA) where the amount of pixels covered by bacteria was determined in regards to the amount of pixels of a plate. This was then converted into a ratio of plate coverage.

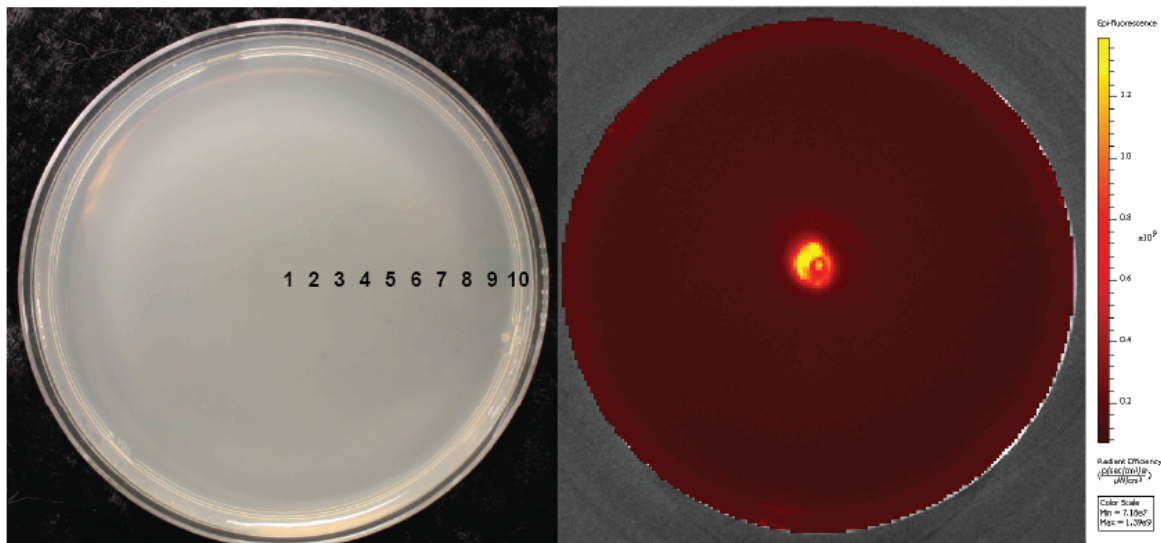
Surfactant plates

0.7% TA was used for surfactant plates. Sterile Tween-20 was added to autoclaved TA in various concentrations (0.05%, 0.1%, 0.15%). Bacteria were grown and inoculated as discussed above. Plates were grown for 16 h prior to analysis. Pictures were acquired using a Canon EOS Rebel XSi.

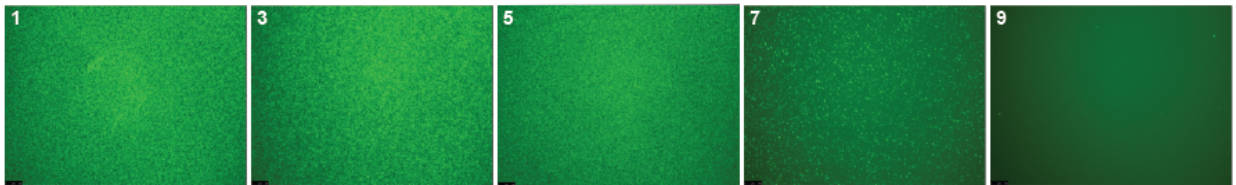
Figures

Figure 4.1: *S. aureus* hitchhikes on swarming *Brevibacillus*. **(A)** *Brevibacillus* and fluorescently labeled *S. aureus* were spotted in the center of a 0.7% TA plate and grown for 2 days at 37°C. Fluorescence was imaged using IVIS and a heat map produced. Red demonstrates GFP detection to the edge of the plate. **(B)** The same plates were analyzed using an epifluorescent microscope. Fluorescent *S. aureus* is colored in green and *Brevibacillus* is not visible because no fluorescence is emitted. Images are taken at 10x magnification. The numbers correspond to the position on the plate labeled in (A). **(C)** In order to determine the viability of the bacteria, samples of bacteria were taken and patched onto TA + 2 µg/ml mupirocin and MSA. *Brevibacillus* grows on 2 µg/ml mupirocin while MSA selects for *S. aureus* growth. The numbers on the patches correspond to the number indicated on the plate in (A).

A



B



C

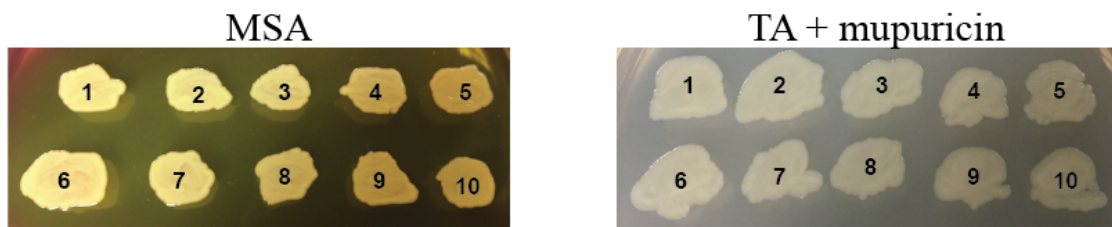


Figure 4.2: *S. aureus* promotes *Brevibacillus* swarming. **(A)** Time course analysis of *Brevibacillus* alone and *Brevibacillus* in the presence of *S. aureus* (WT) on 0.7% TA plates at 37°C. **(B)** Quantification of plate coverage by the bacterial swarms.

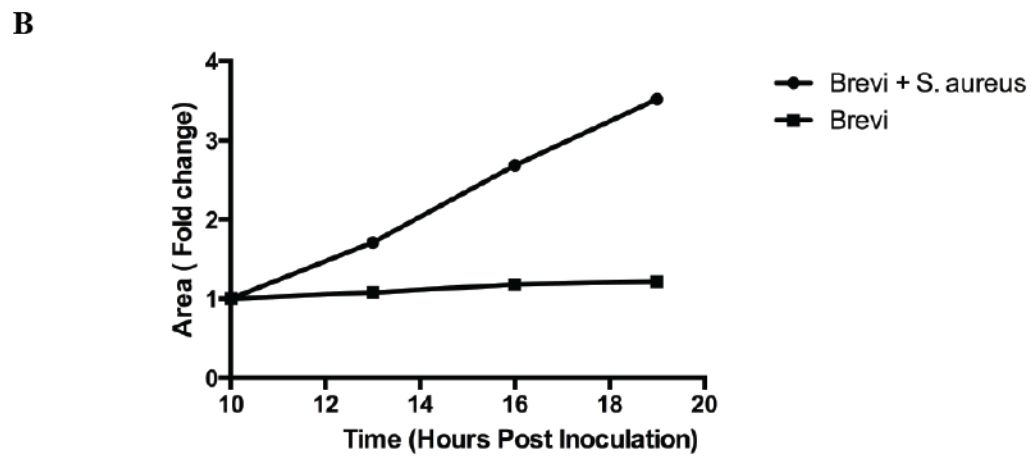
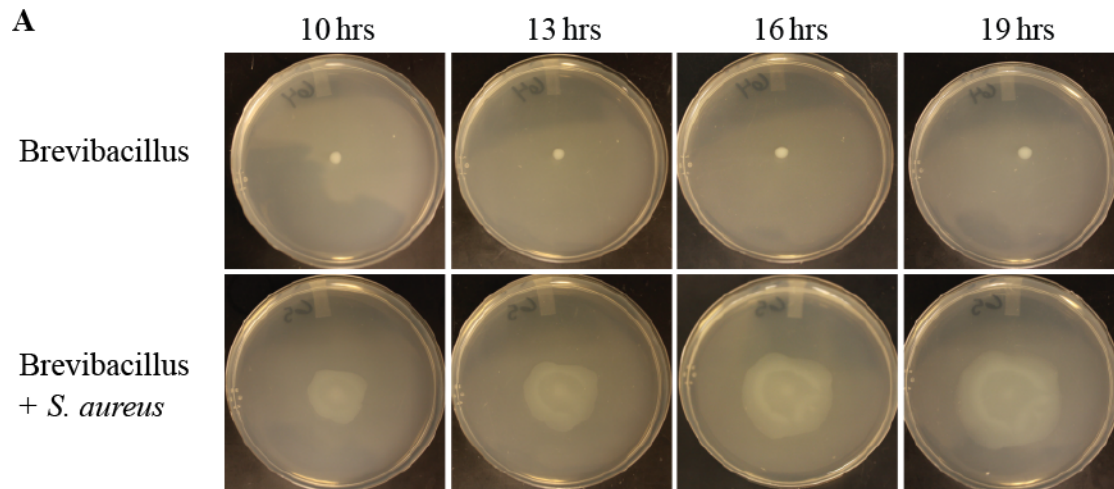


Figure 4.3: *S. aureus* PSMs act as surfactants to induce Brevibacillus swarming. **(A)** After 1 day of growth, Brevibacillus in the presence of wild type (WT) *S. aureus* supernatant swarms across a surface, but in the absence of PSMs (Δ PSM) Brevibacillus does not swarm at this time point. **(B)** The presence of Tween-20, an exogenously added surfactant, can induce Brevibacillus swarming. Additionally, Tween can complement the ability of a PSM mutant to induce swarming.

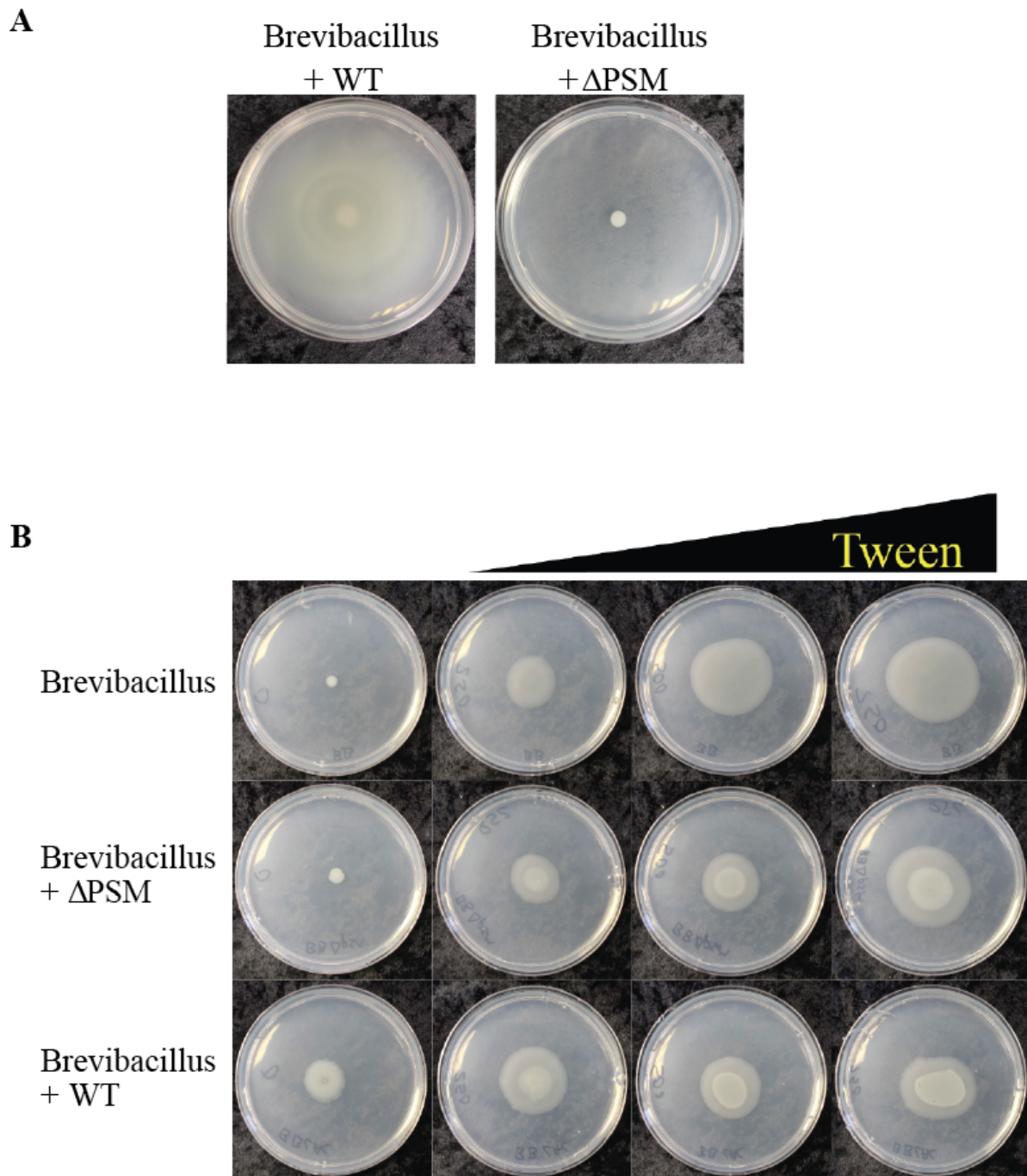
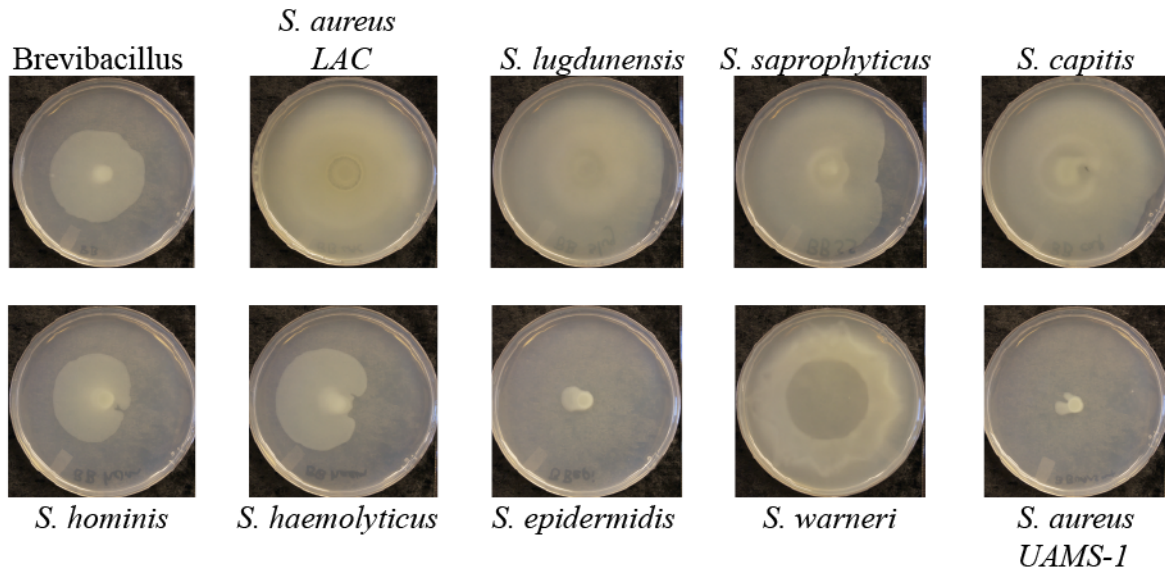


Figure 4.4: Not all strains of *S. aureus* or Staphylococcal species can induce *Brevibacillus* swarming. Staphylococci such as *S. lugdunensis*, *S. saprophyticus*, *S. capitis*, and *S. warneri* are all able to induce the swarming of *Brevibacillus* similar to *S. aureus* strain LAC. *S. hominis* and *S. haemolyticus* do not affect *Brevibacillus* swarming while *S. epidermidis* seems to impair *Brevibacillus* swarming. Unlike LAC, *S. aureus* UAMS-1 seems to repress *Brevibacillus* swarming.



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The idea for this project arose from early conversations with Blaise when I joined the lab and evolved throughout my tenure in the lab. Many thanks to Tom Moninger at the University of Iowa for his technical guidance on the imaging used in this chapter. Also thanks to Dr. Matt Brown in the Boles lab for helpful discussion. Also many thanks to my former undergraduate researcher Ellen Wiitala for her help during this project.

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Chapter 5

Discussion, Perspectives, and Future Directions

S. aureus is a clever and complex organism. It is often a harmless commensal bacteria living on 30% of the population, but as a pathogen it possesses many diverse and redundant mechanisms for causing infections. These mechanisms range from immune evasion and biofilm formation to the production of an array of toxins that can attack human cells in a variety of ways. Currently, antibiotics are the standard treatment for *S. aureus* infections. Widespread antibiotic use over the last century has left us with multidrug resistant strains of *S. aureus* that are exceedingly difficult to treat (1-3). The overuse of vancomycin, a last-resort antibiotic for *S. aureus* infections, has left us with the recent global development of vancomycin resistant strains of *S. aureus* (4-7). As we reach the tipping point of the post-antibiotic era, the need for alternative treatments for *S. aureus* is critical (8). As previously discussed, *S. aureus* colonization increases the risk of getting a *S. aureus* infection (9). Because of this, I have investigated different factors that influence the ability of *S. aureus* to colonize the host. Overall, in this work I have characterized environmental factors (Triclosan), immunomodulatory proteins (PSM), and polymicrobial interactions (Hitchhiking) that may affect the ability of *S. aureus* to colonize and disseminate (10, 11).

In Chapter 2, I discussed how the commercially used biocide triclosan promotes nasal colonization with *S. aureus* (10). The past ten years have revealed that low levels of many antibiotics can induce biofilm formation of various pathogens, but our study was the first to investigate the role that these sub-minimum inhibitory concentration (sub-MIC) of antibiotics may have in host colonization (12-14). We demonstrated that triclosan, a common component of consumer products, makes one more susceptible to *S. aureus* nasal carriage (Figures 2.1, 2.2) (10). Furthermore, triclosan is able to induce the surface attachment and biofilm formation of other significant pathogens including *P. aeruginosa*, *E. coli*, and *Streptococcus mutans* (Figure 2.3) (15). Not only does triclosan affect pathogens, but in the environment triclosan can devastate biodiversity (16, 17). Additionally, in the environment, triclosan has been shown to act as an endocrine disrupter and impair muscle contraction in fish (18, 19). Recently, it has been found that these effects are not limited to aquatic systems but triclosan can disrupt endocrine pathways in human cells and also promotes the formation of tumors (20-23). All of this evidence begs for the reexamination of the use of triclosan and other antibiotics in personal care products as well as cleaning supplies, as many of these will be diluted to levels that promote bacterial attachment.

Due to pressure on companies to stop using triclosan, many have begun to replace triclosan with other similar chemicals. Chlorohexidine, like triclosan, has been demonstrated to be effective in the prevention of gingivitis (24, 25). Because of this, chlorohexidine is beginning to replace triclosan in personal products such as soaps and mouthwash (26-28). Since chlorohexidine has not been a major additive in consumer products until recently, there is little research investigating the effects that chlorohexidine may have on bacteria, the endocrine system, and tumor promotion. Importantly, there is no evidence that soap containing antimicrobials are

more effective than plain soap (29, 30). This area of research is needed quickly before these alternative over-the-counter biocides become as widespread as triclosan.

Chapter 3 characterizes the PSMs as being necessary and sufficient for *S. aureus* induced cytokine release from keratinocytes (Figures 3.1, 3.2, 3.3) (11). This is of great interest because of the known associations between skin inflammatory diseases, such as Atopic Dermatitis (AD), and *S. aureus* colonization (31). This work shown here demonstrates that the PSMs can contribute to skin inflammation and provides a novel therapeutic target for AD (Figure 3.4). It has been previously shown that AD severity can be decreased by topical antibiotic treatment targeting *S. aureus*, but the mechanism by which *S. aureus* contributed to this disease was not characterized (32). Current treatments for AD revolve around disruption of immune signaling, decreasing inflammation (33). We have shown that in the absence of PSMs, *S. aureus* is no longer able to trigger an immune response in a mouse model of AD (Figure 3.4) (11). Similarly, *in vitro* we demonstrated that PSMs are necessary and sufficient for the release of active cytokines from human keratinocytes (Figures 3.1, 3.2, 3.3) (11). In these experiments, we focused on the soluble form of the PSM peptides.

As discussed in the introduction, PSMs are also able to form amyloid fibers (34). It is unknown if the PSMs in their amyloid form interact with human keratinocytes. In the future, it should be investigated if the amyloid form of the PSMs are able to induce a release of pro-inflammatory cytokines similarly to their soluble forms. If PSM amyloids are not able to induce this pro-inflammatory response, this would open the door for a novel, non-antibiotic based therapy to target *S. aureus* enhanced AD. Preliminary studies have found small molecules that promote the aggregation of PSMs into amyloid fibers (Figure 5.1). An important line of future research would be to investigate if these small molecules can abrogate *S. aureus* induced AD.

Additionally, since the soluble PSM peptides have been shown to be critical determinants for a variety of *S. aureus* diseases, these small molecules should also be investigated for their use as anti-virulence treatments during *S. aureus* infections (35, 36).

In addition to AD, there are other diseases that are characterized by an aberrant inflammation of the skin. The autoimmune disease systemic lupus erythematosus (SLE), is a diverse disease that is able to target most organs, where the cause and progression of the disease are poorly understood (37). In addition, SLE often remains dormant for long periods of time and manifests as disease flares (37). Thus, little is known about the factors that induce lupus, as a patient seeks medical attention only after the symptoms of the disease have appeared (37). As we have shown that *S. aureus* can induce the lytic release of active pro-inflammatory molecules in an model of cutaneous colonization, it would be interesting to investigate if *S. aureus* colonization is sufficient to induce lupus flares. This can be achieved by using a novel model of cutaneous lupus developed by the Kahlenberg lab (38). This model is in the mouse NZM2328 line and is ideal because unlike other commonly used models of lupus, this model does not spontaneously develop cutaneous lupus (38). We can colonize these mice with *S. aureus* and monitor them for the development of lupus by checking for proteinuria, a marker of kidney failure. Additional markers of lupus in these mice are deposition a high titer of dsDNA antibodies and the deposition of immune complexes in the kidneys (38). If *S. aureus* is indeed able to induce lupus, this could prove critical for understanding the causes of lupus. Additionally, *S. aureus* decolonization could be investigated as a potential treatment of lupus.

Another autoimmune disease of the skin, psoriasis, is triggered by physical damage to the epidermal keratinocyte layer (39). Once damaged, these keratinocytes play a large role in a persistent inflammation response driving psoriasis (39). Keratinocytes, in the presence of

cytokines, are able to detect cytosolic DNA and respond with a large increase in pro-inflammatory molecules that stimulate the innate immune system (40). Recently, it was discovered that the presence of the human antimicrobial peptide LL-37 was able to bind the DNA and shield it from recognition which normally drives an innate immune response to the cytoplasmic DNA (41). Fascinatingly, the *S. aureus* PSM molecules have a very similar structure to LL-37, sharing the characteristics of being small cationic amphipathic alpha helical proteins (35, 42). The PSMs and LL-37 also share the ability to associate with DNA (41, 43). This brings the tempting speculation that the PSM peptides may also be able to associate with DNA to modulate the response of keratinocytes to cytosolic DNA.

What makes this even more fascinating is that it has been shown that the *E. coli* functional amyloid curli can also bind DNA (44). When curli is bound to DNA it triggers a massive cytokine release by immune cells (44). Furthermore, when curli-DNA composites were added systemically to mice there was a large production of autoantibodies characteristic of an autoimmune disease (44). Also, infecting lupus-prone mice with curli expressing bacteria led to an increase in autoantibody response compared to the curli deficient cells (44). Since the PSM peptides are known to form amyloids in the presence of DNA, this brings an interesting paradox to the previous hypothesis that the PSMs may act like LL-37 to reduce the immune response in the skin. With these two compelling, and seemingly opposing hypotheses, much research needs to be conducted to investigate the interactions between PSMs and DNA and how this complex interacts with the immune response in keratinocytes.

Chapter 4 investigates the interactions that *S. aureus* has with other bacteria, specifically the ability of *Brevibacillus* to carry *S. aureus* across an agar surface. Fascinatingly, *S. aureus* promotes the ability of *Brevibacillus* to swarm by the production of PSM peptides (Figure 4.3).

These peptides act as surfactants to help *Brevibacillus* move across the surface (Figure 4.3). Because of this, I hypothesize that non-motile bacteria such as *S. aureus* can hitchhike on other bacteria to disseminate. Although this is a fascinating phenotype, it is unknown if this plate-based motility is relevant *in vivo*. It is tempting to speculate that movement across a soft surface can be similar to a mucus environment like the nose, or the surface of a wound.

An alternative hypothesis would be that these bacteria interact *ex vivo*. There is little known about the life cycle of *S. aureus* when it is not on a mammalian host. It is generally thought that *S. aureus* in the environment does not readily proliferate, but rather waits to be picked up by a suitable host. It could be in the environment that *S. aureus* utilizes *Brevibacillus* as a mode of transport until it comes upon a suitable host. This hypothesis can be investigated by measuring bacterial migration through soil. This can be done by creating a tube of soil by stacking a series of washers, filling the inside with soil (45). Once autoclaved, soil inoculated with the bacteria of interest, *Brevibacillus*, *S. aureus*, or a mix of the two, can be placed on the top layer (45). After incubation in a high humidity incubator, the washers can be removed sequentially and the bacteria present in the soil can be cultured to determine how far each bacterium migrated (45). This assay could demonstrate if these bacteria migrate together through an *ex vivo* environment such as soil.

Additionally, there are many facets by which bacteria can interact beyond hitchhiking. Preliminary experiments suggest that *Brevibacillus* and *S. aureus* have a mutualistic effect on each others growth in defined media. When grown on plates made of synthetic nasal mucus (SNM), *Brevibacillus* and *S. aureus* cultured separately take several days before they begin to produce a visible colony on the plates (Figure 5.2) (46). In contrast, when the two bacteria are inoculated together, they grow into a dense colony overnight (Figure 5.2). Microscopic

observation of the mixed colony showed large amounts of both bacteria suggesting that they both proliferate (Data not shown). Interestingly, this mutualistic behavior does not occur in liquid SNM, suggesting that the bacteria need to be in close proximity for this metabolic sharing to occur (Data not shown). Future experiments will further investigate this mutualistic phenotype in SNM between *Brevibacillus* and *S. aureus*. Recently, a systems biology approach investigating the amino acid cross-feeding in bacterial communities has found that amino acid biosynthesis is optimized at a community level so as to reduce the metabolic burden on each species while promoting community synergy (47). This leads to the hypothesis that *S. aureus* and *Brevibacillus* may share metabolites in this defined environment to reduce the burden on each species.

Throughout this dissertation I have characterized many diverse factors regulating *S. aureus* colonization. Interestingly, since *S. aureus* colonizes 30% of the population and only causes disease in a fraction of those individuals, it is of interest as to what factors allow it to persist and infect. As we have seen, PSMs are critical factors in eliciting an immune response from the skin and other systems. Additionally, PSMs as amyloids promote biofilm formation, a state where cells are mostly hidden from the immune response. I hypothesize that these peptides have developed this bifunctionality to modulate its ability to transfer from a commensal to a pathogen. When *S. aureus* is a commensal, the production of PSMs may be repressed or present as fibers, helping the bacteria to attach to surfaces and evade the immune response. Upon an unknown environmental cue, *S. aureus* produces soluble PSM peptides, which then facilitate the transition to a pathogen where *S. aureus* can now lyse human cells and fight against niche competing bacteria.

Here, I have investigated how environmental factors, interactions with the host, and polymicrobial interactions affect the way that *S. aureus* interacts with the human host. Now that

we are entering the post-antibiotic era, the need for alternative treatments is critical. By beginning to understand the factors that can aid in *S. aureus* colonization and infections, translational medicine can begin to investigate novel targets for decolonization therapies. The data here provides important insight into the variety of factors that influence *S. aureus* behavior.

Methods

Thioflavin T assay for amyloid formation

PSM α 1 was synthesized from Peptide 2.0 to be >90% pure by HPLC. The peptide was prepared as previously described (34). Briefly, to eliminate protein aggregates, peptide was dissolved in hexafluoroisopropanol (HFIP) for 10 minutes then vacuum dried using a SpeedVac (Sigma-Aldrich, MO). Peptides were stored at -80C until use. Prior to use, the protein was resuspended in DMSO and used immediately. FN071 was kept at room temperature in DMSO and was a kind gift from the lab of Dr. Matt Chapman. Polymerization was performed in a black 96-well plate (Corning, NY). 20 μ M Thioflavin T (Sigma-Aldrich) was present in each well to measure the formation of amyloid fibers. Fluorescence was detected every 10 minutes, after shaking, by a Tecan Infinite M200 plate reader with an excitation at 438 nm and emission at 495 nm.

Growth on synthetic nasal mucus (SNM)

In order to study interactions within the nasal cavity *in vitro*, we used a medium that has been developed to contain the average amount of all of the nutrients and co-factors that are present in nasal mucus (46). To test growth on plates, SNM media was made 2X concentrated, sterile filtered then mixed with warm, autoclaved Noble Agar (BD, NJ) prior to pouring. Bacteria

were grown overnight in Tryptone Broth (1% Tryptone), the following day, *S. aureus* were diluted 1:500 while *Brevibacillus* was diluted 1:250 in fresh TB and grown for 4 h to obtain exponentially growing cells. After 4 hours, the bacteria were normalized to an $OD_{600}=1$ in SNM. A final volume of 4 μ l of bacteria was then spotted on the center of the plate either individually or mixed and incubated at 37°C for 24 hours.

Figures

Figure 5.1: FN071 accelerates PSM α 1 amyloid formation. PSM α 1 (20 μ M) alone or in the presence of different amounts of FN071 (20, 100, or 200 μ M) were incubated in the presence of the amyloid specific dye Thioflavin T. Fluorescence was monitored overtime for the formation of amyloid fibers, measured by an increase in fluorescence. 1:5 and higher ratios of PSM α 1:FN071 decrease the lag phase of amyloid polymerization compared to PSM α 1 alone or in the presence of equal molar FN071.

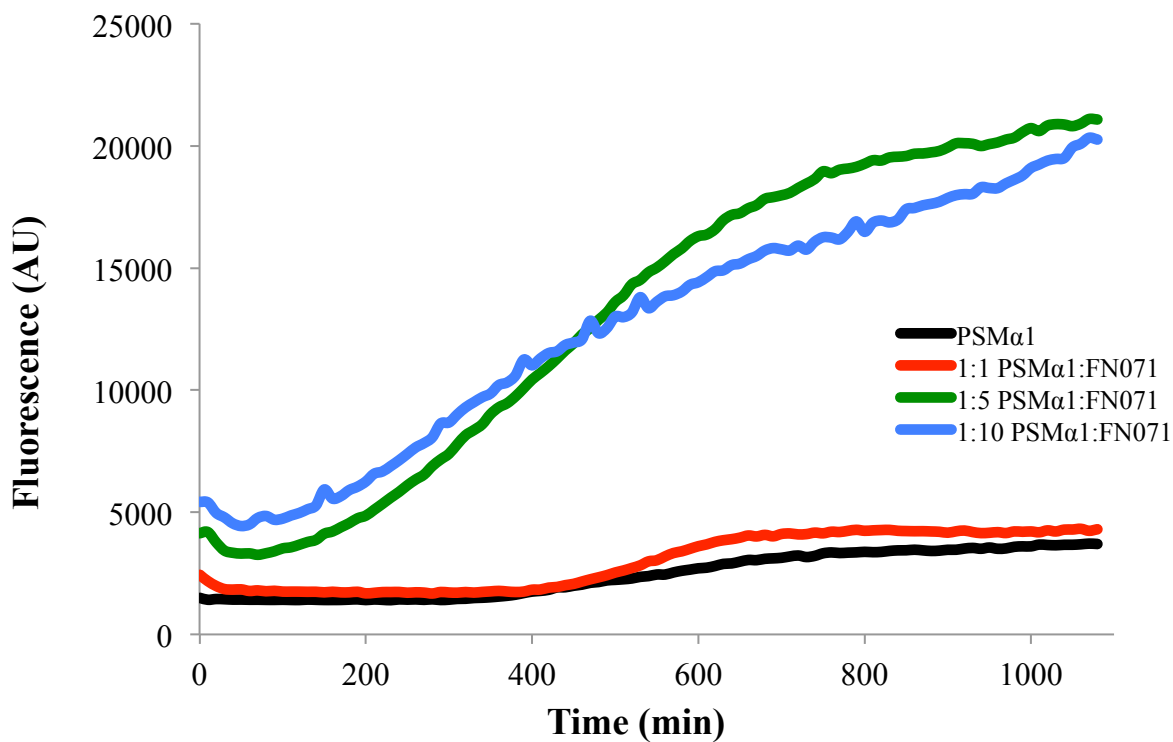
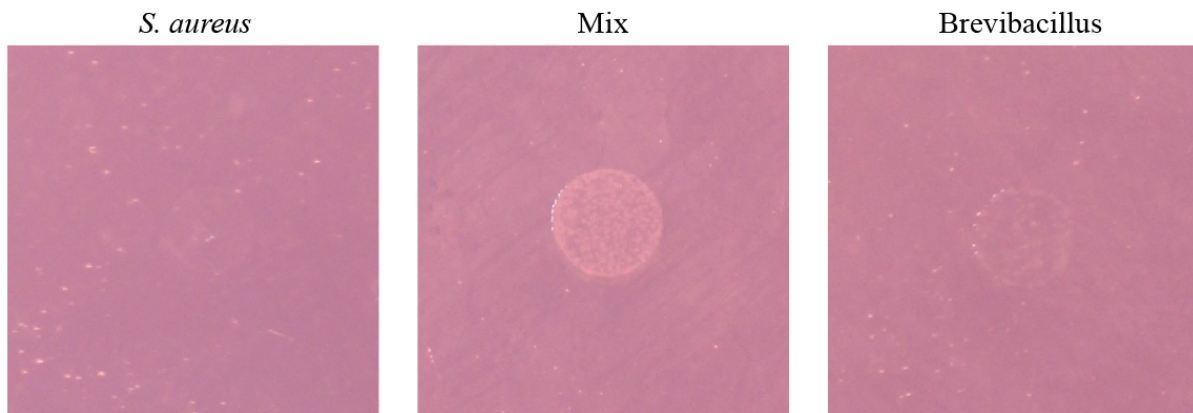


Figure 5.2: Mixed cultures of *S. aureus* and *Brevibacillus* grow better on defined synthetic nasal mucus (SNM) media than they do separately. Exponentially growing bacteria in TB were washed in SNM media and normalized to an $OD_{600}=1.4$ μ l of each bacteria were spotted separately on SNM plates. For the mixed culture, 100 μ l of the normalized cultures were mixed and 4 μ l of this mix was spotted on the plate. After 24 hours of growth *S. aureus* and *Brevibacillus* show little growth, but when mixed together produce a much larger colony of bacteria.



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