PSM small peptide toxins form functional bacterial amyloids in the *Staphylococcus aureus* biofilm matrix

by

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Dedicated with love to my friends and family - especially to my cousin Jess Tischler, my grandfather Joe Schwartz, Dr. Willow Wedemeyer, and to my colleagues in the Boles/Chapman Labs.

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Chapter 1

General Introduction

Staphylococcus aureus: background and impact

Staphylococcaceae are a family of Gram-positive bacteria within the phylum Firmicutes. Staphylococci are closely related to streptococci, as well as bacilli, lactococci, and clostridium, many of which are opportunistic pathogens. The Staphylococcal genome is approximately 2.8 Mb, with large variability within regions associated with human colonization and disease [1,2]. More than forty species of staphylococci have been described, several of which colonize humans [1]. Of these, two species particularly are relevant to human health: *Staphylococcus aureus*, and to a lesser extent, *Staphylococcus epidermidis*. Staphylococcal colonization is widespread. Nearly all humans are non-infectiously colonized by *S. epidermidis*, an opportunistic pathogen in immunocompromised individuals, and approximately ⅓ of the human population carries *S. aureus* in the anterior nares of the nose at any given time, with 10-20% displaying persistent colonization [3,4]. Noninfectious colonization can contribute to Staphylococcal disease under certain conditions

Staphylococcus aureus is a prominent pathogen based on its transmissibility, antibiotic resistance, and potential to cause invasive disease [3,4]. It is the primary source of hospital

acquired “nosocomial” infections that cost loss of life and financial expenditures well over US $1 billion per year [4]. Nosocomial infections may include devastating disorders like necrotizing pneumonia and septic shock resulting from surgical site contamination [5]. Toted as a “superbug”, *S. aureus* is a titan of antibiotic resistance, causing devastating and difficult to treat infections [6-8]. Methicillin and vancomycin antibiotic resistant strains are leading killers among nosocomial infections [2,5,8-10]. Antibiotic resistant community-acquired *S. aureus* strains cause hard-to-treat diseases like osteomyelitis, septic arthritis, and skin infections [8]. Certain subgroups, including children, prison inmates, diabetics, AIDS patients and other immunocompromised individuals, are more susceptible to infection [6-8, 11]. *S. aureus*’ contribution to human disease is complicated by its non-infectious colonization of a large portion of the human population. Under certain conditions, acute and chronic infections result from the invasion of specific tissues, resulting in pathogenicity [4]. The mechanisms responsible for *S. aureus*’ transition from commensal to deadly pathogen are the subject of investigation.

S. aureus colonization correlates with infection

S. aureus has a complicated relationship with its human hosts: it persists in the anterior nares as a commensal carried by 20 – 50% of the general population where it may never cause disease in the colonized individual [2]. Other regions commonly colonized by *S. aureus* include the nasopharynx, hands, skin, axilla, perineum, and vagina, where nasal-colonized individuals are more likely to carry at these other sites [3]. Colonization may be persistent or transient, and many individuals never host commensal *S. aureus* [3, 10-11]. However, *S. aureus* is highly transmissible, and inoculation occurs through contact with colonized individuals, and occurs

both in hospital settings, and more recently, through community acquired sources. Disease is often concurrent with colonization where invasive infection proceeds from damaged skin or mucosal membranes [12]. Staphylococcal nasal persistence correlates with an increased risk for infection from disseminated bacteria entering the body from an epithelial breach that are upregulating adherence factors [12-14]. Patients colonized with *S. aureus* are much more susceptible to infection, but are less likely to die as a result of the infection than non-colonized individuals [12,13]. The production of virulence factors is often very strain-dependent and relies on accessory gene regulatory systems that can be switched on and off rapidly to modulate interactions with the host environment.

Quorum sensing coordinates *S. aureus* virulence

Highly virulent strains of *S. aureus* show marked upregulation of the accessory gene regulator (agr) quorum sensing system, which is the major regulator of most staphylococcal virulence factors (Fig. 1.1). Quorum sensing is a form of social, growth-phase dependent bacterial communication that coordinates genetic expression and directs the construction and maturation of the biofilm extracellular matrix [15]. Like other Gram-positive bacterial quorum sensing systems, the agr system drives an autoinducing positive feedback loop using extracellular peptide signals as a means of communication within the community [14]. The agr regulatory system is encoded in two divergently transcribed transcripts called *RNAII* and *RNAIII*. RNAII encodes four genes, *agrBDCA* that produce the machinery of the quorum sensing system. AgrA and AgrC form a two-component system of a response regulator and receptor, respectively. AgrD encodes the auto inducing peptide (AIP) signaling component, which is

processed at the cell membrane by AgrB and signal peptidase SpsB [16-18]. The primary effector molecule of agr is the regulatory RNA molecule, RNAIII. Within RNAIII is a gene (*hld*) that codes for the peptide hemolysin delta-toxin. Delta-toxin expression does not affect the quorum sensing system, but has downstream implications for virulence [19]. Downstream virulence results from upregulation of factors like proteases, lipases, and toxins that target host tissues, as well as superantigens that cause widespread immunogenic shutdown [20]. Highly virulent strains of *S. aureus* can cause diseases ranging from relatively mild conditions, like skin and soft tissue infections, to more serious diseases like toxic shock syndrome, which can be complicated by septic shock [6]. Under certain conditions, these infections can result in the formation of biofilms.

S. aureus biofilm development

Invasive bacteria can attach to host extracellular matrix proteins or indwelling medical devices, forming structures called biofilms. A biofilm is a surface-associated community of microbes encased in a self-produced matrix of secreted biomolecules [21-24]. Biofilms range from thin, unstructured monolayers to more complex and specialized microcolony structures (like "mushrooms" or "pillars"), depending on the growth conditions and species [25-27]. The transition from free-living to surface-attached bacterial communities is an important component of microbial behavior. Biofilm related diseases that originate from non-infective colonization include osteomyelitis, vegetative growth on indwelling medical devices, endocarditis, and other chronic wound infections [14, 26]. Once embedded in the polymeric

biofilm matrix, bacterial cells are protected from antimicrobials and the host immune system making biofilms difficult to clear.

S. aureus biofilm lifecycle

Biofilms usually form in four transitional steps: 1) surface attachment by adherent cells, 2) microcolony assembly of attached cells, 3) growth of the matrix to form mature biofilm structures, and 4) detachment and dispersal of individuals or small clusters of cells [25] (Fig. 1.2). Surface attachment is mediated by adhesion to virtually any biotic or abiotic surface through electrostatic interactions and production of adhesion molecules. Proliferation along a surface occurs concurrent with production of the polymeric extracellular matrix, promoting intercellular aggregation and shaping of the biofilm. Shaping includes production of channels, stratification of genetic expression, and physioheterogeneous partitioning that ultimately produces the 3D structure of mature biofilms. Detachment is mediated by the expression of enzymes that degrade matrix materials, by the detergent-like activity of surfactant molecules, and by environmental stresses like mechanical shearing [6,23,-24]. Each step of the complex biofilm lifecycle is controlled by a variety of transcriptional regulators, including agr [28].

S. aureus biofilm lifecycle is influenced by agr expression

Agr quorum sensing elicits a phenotypic change in population behavior that coordinates growth-phase dependent genetic expression of many important virulence factors, affecting particular aspects of biofilm development [29,30]. Many bacteria, like *Pseudomonas aeruginosa* use quorum sensing signaling to build biofilms [31]. The reverse is true in *S. aureus*. Activation of quorum sensing negatively regulates biofilm formation by impeding attachment and production of certain matrix components, and by promoting the dispersal of attached cells [16, 32]. Other transcriptional regulators like SarA [33,48] alternate sigma factor SigB stress response [35,36] and SaeRS work in tandem with the other regulators to counter agr and promote biofilm formation [37]. For example, SarA upregulates genes that are important for biofilm formation while agr downregulates surface associated adherence factors and upregulates secreted virulence factors [38]. Many of the mechanisms *S. aureus* uses to disperse biofilms are virulence factors that are antagonistic against the host. Associated with active infection, agr disperses biofilms by upregulating the production of matrix degrading enzymes like proteases and nucleases [39-42]. Although agr is considered a negative regulator of biofilm formation, its contribution to the biofilm lifecycle is critical for its influence on the matrix composition.

Components of the *S. aureus* biofilm matrix

The biofilm matrix is defined by the production of secreted biomolecules that form the structure of the biofilm over the course of its lifecycle. Like many bacteria, Staphylococcal biofilms consist primarily of exopolysaccharides, eDNA, and proteins.

S. aureus produces exopolysaccharides called polysaccharide intercellular adhesin (PIA) formed primarily from beta-1,6-linked N-acetylglucosamine residues [43]. PIA is produced through the *icaABCDEFG* operon and functions as a structural component that self-associates and interacts with matrix proteins to modulate the viscosity of the biofilm [44]. Although exopolysaccharides are important for biofilms in many species, PIA is only necessary for *S. aureus* biofilm formation under certain growth conditions [45].

Extracellular DNA (eDNA) contributes to biofilm formation in many important respects [46-48]. eDNA release in *S. aureus* is mediated by the Cid/Lrg toxin/anti-toxin system which responds to extracellular cues like pH as a form of coordinated programmed cell death [49]. Low levels of beta-lactam antibiotics can also cause eDNA release that promotes biofilm formation [50,51]. eDNA promotes bacterial attachment in early biofilm adherence. Exogenous addition of DNase or induction of nuclease enzymes can cause dispersal [52-54]. However, mature biofilms appear to be resistant to DNase dispersal, suggesting that eDNA is more important in early biofilm persistence [52].

A diversity of proteins also modulate biofilm development. Adhesins like the fibronectin binding proteins (FnBPs), accumulation-associated protein (Aap), and biofilm-associated protein (Bap) promote PIA-independent biofilm formation [45, 55]. Enzymes like the major autolysin (AtlA) also drive biofilm structuring. AtlA is important for cell wall metabolism and cell separation in *S. aureus*, and can promote biofilm attachment or dispersal through coordinated autolysis within biofilms [14, 56--71]. Other proteins can form structural elements to stabilize biofilms, preventing dispersal. Amyloids are a common form of structural protein found in bacterial biofilms. Coordination of a novel protein interaction forming amyloids in the *S. aureus* biofilm matrix has been the primary focus of my graduate studies.

Functional amyloids are important for biofilm components

Amyloids are proteins that produce remarkably stable polymeric fibrils composed of folded β-sheets stacked perpendicular to the fibril access, forming structures with a central diameter of 3–12 nm [58]. Fibrillation is initiated by self-aggregation of protein monomers into oligomers, which accumulate over time. These oligomers nucleate the self-assembly cascade of fibril polymerization characterized by the stacking of parallel or anti-parallel β-sheet secondary structure. Hydrogen bonding between adjacent β-sheets provides additional fiber stability. Once formed, amyloid fibers are robust and can resist disassembly by enzymatic or chemical digestion [59].

Amyloids' inherent resistance to protease digestion and denaturation helps them reinforce and shield biofilms from harsh environmental stresses [60]. Chapman *et al.* first recognized the concept of functional bacterial amyloids in their groundbreaking 2002 *Science* article. Their work characterized curli fimbriae produced in *Escherichia coli* biofilms as being biochemically similar to disease-associated amyloid structures [61]. The production of curli greatly influences *E. coli* biofilm formation [62, 63]. *E. coli* producing curli fibers are often coated in exopolysaccharides and create flocculates that help to establish biofilms [64]. Curli have also been implicated in surface adhesion, immune evasion, and pathogenesis [65]. Curli-like systems have been described in numerous enteric bacterial species, and recently interspecies complementation between non-homologous *E. coli*, *Salmonella*, *Citrobacter*, and *Shewanella* curli subunits has been demonstrated [66]. Beyond curli systems, bacterial amyloids produced by gram-negative organisms that contribute to biofilm formation, like FapC in *Pseudomonas fluorescens*, continue to be elucidated (Table 1) [67]. The importance of amyloid formation in biofilms is supported by the abundance of microbial systems that utilize them.

S. aureus phenol soluble modulins connect virulence to biofilm persistence

Like many organisms, *S. aureus* produces proteins that aggregate to form robust fibril structures called amyloids (Fig. 1.3) [64]. My work has revealed that *S. aureus* produces amyloid structures composed of small peptides called phenol soluble modulins (PSMs) (Fig. 1.4). PSMs are most famously known as cytolytic toxins that are positively regulated by the agr virulence

network [32, 82-69]. PSMs are produced under direct control of the AgrA quorum sensing response regulator [82, 69). This discovery challenges the notion that agr is a purely negative regulator of biofilm development in *S. aureus*. Agr is generally considered to be downregulated in *S. aureus* biofilms under most conditions, although it can still remain active in certain areas of the biofilm [40,42]. Loss of agr function by repression or mutation produces strains with more robust biofilm growth [8, 16-17, 38-39, 70]. However, PSMs are still produced during biofilm growth, and their dispersal activity seems to vary depending on growth conditions [32, 71-72]. The co-functioning of PSMs as both an instrument of infection and as a building block of the biofilm matrix is particularly interesting as an intersection between the divergent bacterial lifestyles of persistence and active infection.

PSMs are virulent small peptides produced by *S. aureus*

The survival of *S. aureus* in the human body depends on the careful regulation of secreted virulence factors by adaptive or accessory genetic regulatory networks [30,67]. *S. aureus'* capacity for virulence is largely determined by its ability to produce a wide range of toxins, including factors that subvert the host immune system. Among these toxins are the phenol soluble modulins, which are highly conserved and produced by nearly all strains of *S. aureus* and *S. epidermidis.* PSMs are small amphipathic alpha helical peptides, none more than 5 kDa in size that are expressed from several discrete loci in the *S. aureus* genome that are positively regulated by agr quorum sensing [68-69,73]. Because the PSMs are so small, they fell beneath the threshold for detection of annotation algorithms and assignment during genome annotation and have only recently been characterized [73]. PSMs are primarily grouped

together by their biochemical properties and genomic location into the PSMα, PSMβ, and staphylococcal delta hemolysin classes (Fig 3). The smallest, PSMα 1-4 are encoded in an operon and are between 20 and 22 amino acids in length. The longer PSMs include PSMβ 1 -2, which are also encoded in an operon, and delta toxin, a 26 amino acid peptide divergently transcribed from within the RNA III effector molecule of the agr quorum sensing system [74]. Outside of this core family of peptides, additional PSM-like peptides have been described, including PSM-mec encoded in the mec pathogenicity island, and more recently, a cleavage fragment of the agr quorum sensing molecule AgrD [75-76]. PSMs are of particular concern due to the variety of functions they contribute to Staphylococcal infections. PSMs trigger inflammatory responses by inducing cytokine production, recruiting neutrophils, triggering cytolysis in host cells, and more recently they have been shown to play a role in the biofilm lifecycle [68,74-75, 77]. The discovery of PSMs as an amyloid protein opens many new and exciting avenues for exploration based on previous data concerning disease-associated amyloid behavior. The unique biochemical properties of PSMs and other microbial amyloids make them well suited to perform multiple roles in bacterial physiology [72].

Functional amyloids act as toxin repositories

Some amyloid proteins form into pre-fibrillar toxic oligomers that cause damage to lipid membranes [79]. These toxic oligomers also promote amyloid fibril formation by acting as nucleators for aggregation [78]. Some bacteria can utilize the toxicity associated with amyloid formation (Fig. 1.5). Microcin E492 (Mcc) is a small bactericidal peptide produced by *Klebsiella pneumoniae*. Mcc monomers and oligomers create cytotoxic pores that induce cell lysis in

niche-occupying enteric bacteria like *Enterobacteriaceae* [79]. Mcc oligomers can aggregate into amyloid fibers, effectively sequestering them as inert fibril structures [80]. Recent studies described environmental triggers, like pH and the ionic dissociation of salt, that induced fiber formation accompanied by a loss of toxic oligomeric species. Changing environmental conditions to favor fiber dissociation (high pH or low salt concentration) trigger Mcc amyloids to disassociate back into cytotoxic oligmers [80]. Similarly, *S. aureus* can use amyloid aggregation to control PSM toxicity (Fig. 1.5).

PSM toxicity is modulated by amyloid aggregation

PSMs contribute to staphylococcal pathogenesis in multiple ways. The innate immune system detects foreign bodies through leukocytes, like macrophage and neutrophils, and initiates a signaling cascade resulting in inflammation and recruitment of immune factors to clear bacteria and debris from the site of infection [76]. *S. aureus* is sensed by macrophages, triggering an inflammatory immune response which recruits neutrophils [68]. PSMs are described to be the major neutrophils chemoattractants in *S. aureus* supernatants [68, 81-82]. When *S. aureus* cells are engulfed by neutrophils, a rapid increase in PSM expression contributes to neutrophil lysis, effectively silencing the immune response [83]. PSMs, like many small peptide toxins, interact with cell membranes to create pores that induce cell lysis [84-86]. PSM-mediated lysis has been demonstrated for a wide range of cell types, including leukocytes and erythrocytes [68, 87]. In my thesis work, I present evidence that pro-inflammatory activity is silenced and cytotoxicity is reduced when PSMs form amyloid fibrils [88].

PSM amyloid fibrils promote biofilm stability

Our data provide new insight into biofilm structuring dynamics. It has been previously shown that soluble PSM species can contribute to the dispersal of established biofilms. PSMs are amphipathic molecules that have long been touted for their "surfactant-like" properties [32, 73]. Soluble PSM species have been shown to contribute to structuring and dispersal of established biofilms *In vitro* and *In vivo* [72, 64, 59,89]. Detachment and biofilm dispersal is an efficacious way for *S. aureus* to travel without motility organelles [68,65]. It was reported that the PSMβs seemed to help structure the biofilm by facilitating movement and dispersal within the matrix [72, 75,64]. Likewise, isogenic *S. aureus* Δ*agr* and Δ*PSM* mutants formed biofilms that were thinner and had fewer channels throughout the biofilm matrix as compared to the wild type parent [65]. Mutants that did not produce PSMs grew into robust biofilms with reduced detachment of cell clusters from the matrix [89]. In my work, I have demonstrated how the assembly of PSMs into amyloid fibrils correlates to a robust biofilm phenotype that resists dispersal by enzymes, surfactants and mechanical disruption [71]. The addition of synthetic soluble PSM peptide induced biofilm dispersal in a concentration dependent manner, while pre-aggregated fibrils did not, demonstrating the modulation of behavior before and after amyloid aggregation [71].

The N-terminal leader peptide of AgrD displays PSM-like amyloid formation and virulence

In studying amyloid fibril formation in a highly virulent USA300 *S. aureus* strain, I have also discovered that a cleavage fragment, the N-terminal leader sequence of AgrD (N-AgrD), is also capable of amyloid formation and PSM-like activity [88]. Our lab has characterized this peptide as an additional member of the PSM family of toxins based on its sequential homology, biophysical properties, and capacity for virulence. N-AgrD is able to self-seed its amyloid formation, as well as that of known amyloid protein PSMα1. We provide evidence that N-AgrD, like PSMα1, induces pro-inflammatory activity, neutrophil chemotaxis, and cytolysis. The virulence associated with N-AgrD and PSMα are inhibited when they are aggregated into amyloid fibrils, suggesting that aggregation can sequester toxins and modulate their activity.

PSMs aggregate in the presence of DNA

One imminent question that proceeds from our characterizations of PSM amyloid formation is, "what are the factors promoting PSM aggregation in biofilms". This question led me to investigate how other biofilm matrix components may influence aggregation in the biofilm. I had previously observed that a mutant deficient in autolysis, the primary method of DNA excretion in biofilms, did not produce extracellular fibrils (Fig. 4.1). From this observation, I proceeded and found that the absence of DNA in biofilms correlates with the absence of PSM fibrils. Further characterization demonstrated that PSMα1 can interact with DNA to form amyloid fibrils (Fig. 4.2-4.4). Once aggregated, PSMα1 showed reduced hemolysis, suggesting that the presence of DNA can diminish toxicity (Fig. 4.5).

Conclusions

When I began my thesis work, I was interested in learning how biochemistry connected living systems together from the microscopic to the macroscopic world. I chose to study microbial biofilms as a model for biochemical interactions because it relates to so many disparate fields of study – from organic chemistry to public health. *Staphylococcus aureus* is an important opportunistic pathogen that spends the majority of its life cycle persisting in human hosts undetected. *S. aureus* colonization is a fantastic example of how pathogenesis itself can be considered a novel form of ecology. What processes are responsible for *S. aureus*' switch from harmless commensal to deadly pathogen? In the Boles lab, we work through the paradigm of microbial ecology to gain new insights into how the human body environment affects bacterial physiology.

In my graduate work, I have characterized a novel function for a well-characterized class of small peptide toxins, the phenol soluble modulins. I have discovered that PSMs can form amyloid fibrils that stabilize biofilms (Fig. 2.1-2.8). Formation of amyloid fibrils modulates the roles of these peptides by inhibiting their dispersal activity, or the injury of host cells. The discovery of PSM-like function in N-AgrD suggests that *S. aureus* can utilize a wide arsenal of small peptide toxins, many of which may still be unknown (Fig.3.1-3.5). Finally, I have determined that the presence of DNA can promote PSM amyloid formation and abrogate toxicity, suggesting a link between matrix composition and impaired virulence (Fig. 4.1-4.5). These findings shed new light on the complexities of agr and virulence as both a positive and

negative regulator of biofilms. I also provide evidence that extracellular DNA can promote amyloid formation and effectively detoxify PSM activity. This finding connects fibril formation to the extracellular environment and illustrates a novel way in which the extracellular matrix environment can alter bacterial behavior through direct biochemical interactions.

Throughout my thesis, I have applied the notion that the physical environment can have a profound biophysical effect on the phenotypic behavior we observe in bacterial communities.

In my work, I have explored the following questions:

1. Are there novel conditions that promote biofilm resistance to dispersal?

2. How does amyloid formation contribute to *S. aureus* virulence?

3. Are there external factors that promote PSM aggregation?

Figures and Tables

Table 1.1, Characterized Bacterial Amyloid Systems.
Adapted from [90]

Table 1

Examples of known bacterial amyloids and their functions.

Organism	Amyloid protein(s)	Amyloid function/characteristics
Escherichia coli	Curli (CsgA)	Biofilm component; adhesion to surfaces
Salmonella ssp.	Curli/Tafi (CsgA)	Biofilm component; adhesion to surfaces
Mycobacterium tuberculosis	Mtp	Pili formation; binding to laminin
Klebsiella pneumoniae	MccE492	Amyloid formation proposed to regulate MccE492 antimicrobial activity
Pseudomonas fluorescens	FapC	Biofilm component
Streptomyces coelicolor	Chaplins (ChpA-H)	Spore surface protein
Staphylococcus aureus	Phenol soluble modulins	Biofilm component; amyloid formation proposed to regulate PSM biofilm dispersal activity
Bacillus subtillus	TasA	Biofilm component; spore surface protein
Xanthomonas axonopodis	Harpins (HpaG)	Amyloid formation proposed to regulate HpaG cytotoxic activity

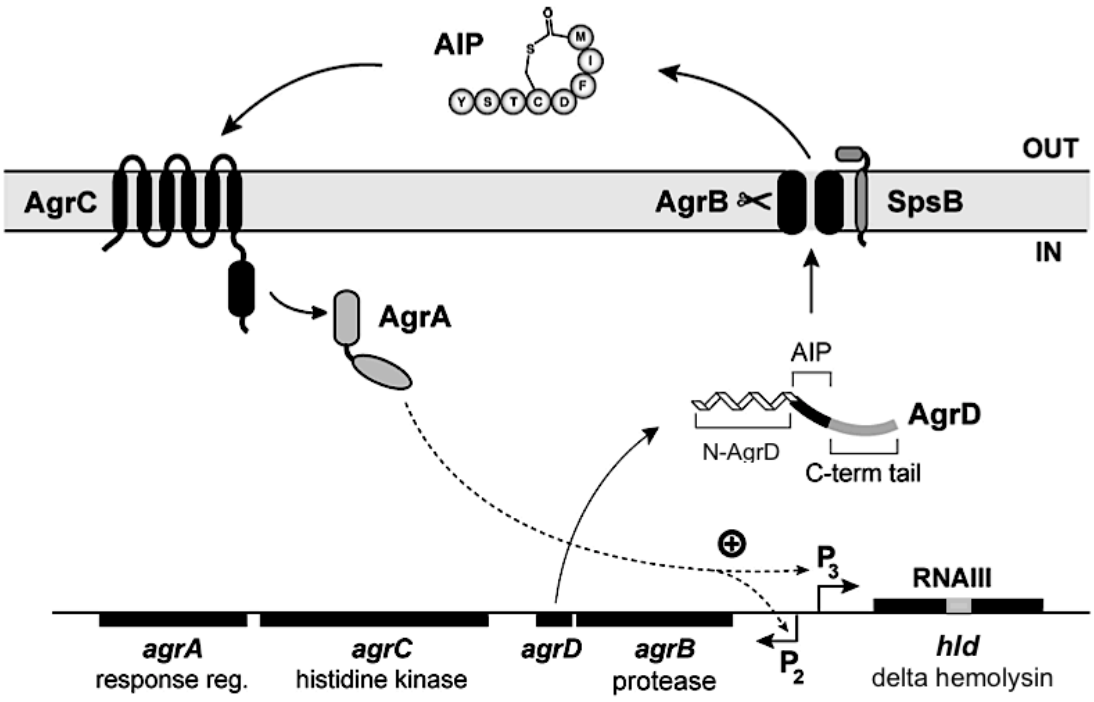

Figure 1.1, *Staphylococcus aureus* accessory gene regulator (agr) quorum sensing system,
The *agr* quorum sensing locus is encoded on two divergently expressed transcripts in the core region of the genome. Promoter P2 regulates expression of the *agrBDCA* operon encoding the quorum sensing machinery. The autoinducing peptide (AIP) signaling molecule AgrD is translated as a propeptide composed of three parts: an N-terminal leader peptide (N-AgrD) to direct the peptide to the membrane; a middle region that is modified to become AIP; and a charged C-terminal tail (*C-term*). The AgrD propeptide is processed during transport by integral membrane endopeptidase AgrB and SpsB type I signal peptidase. AgrC and AgrA form a two-component signaling system that senses AIP in the extracellular environment. AgrC is a membrane histidine kinase with an extracellular receptor for AIP binding., and AgrA is the response regulator. Activated AgrA induces transcription at P2 and P3 promoters, and the P3 promoter drives expression of RNAIII, the primary effector of the *agr* system and virulence. Delta hemolysin (hld) encoded within the RNAIII transcript does not affect agr, but has downstream virulence effects. Figure adapted from Thoendel & Horswill (2009) [91,92]

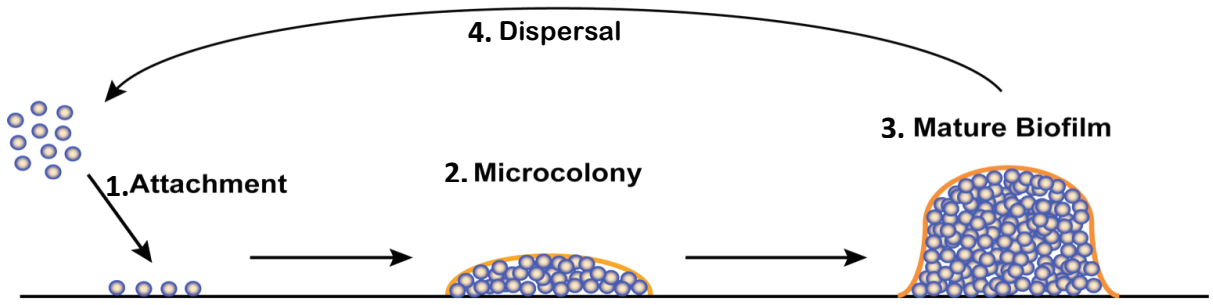

Figure 1.2 ,The *S. aureus* biofilm life cycle,
During the course of biofilm development, cells go through four main phases of growth. (1) Attachment is characterized by surface attachment by cells producing adhesion molecules; (2) Microcolony development involves repression of dispersal factors and initial production of extracellular matrix holding the community in place; (3) Mature biofilms display spatio-physical heterogeneity among cell types and environmental states with regions of motile or detaching cells that contribute to partial or total (4) Dispersal of the biofilm via enzymatic, chemical, or shearing stress. Adapted from [25].

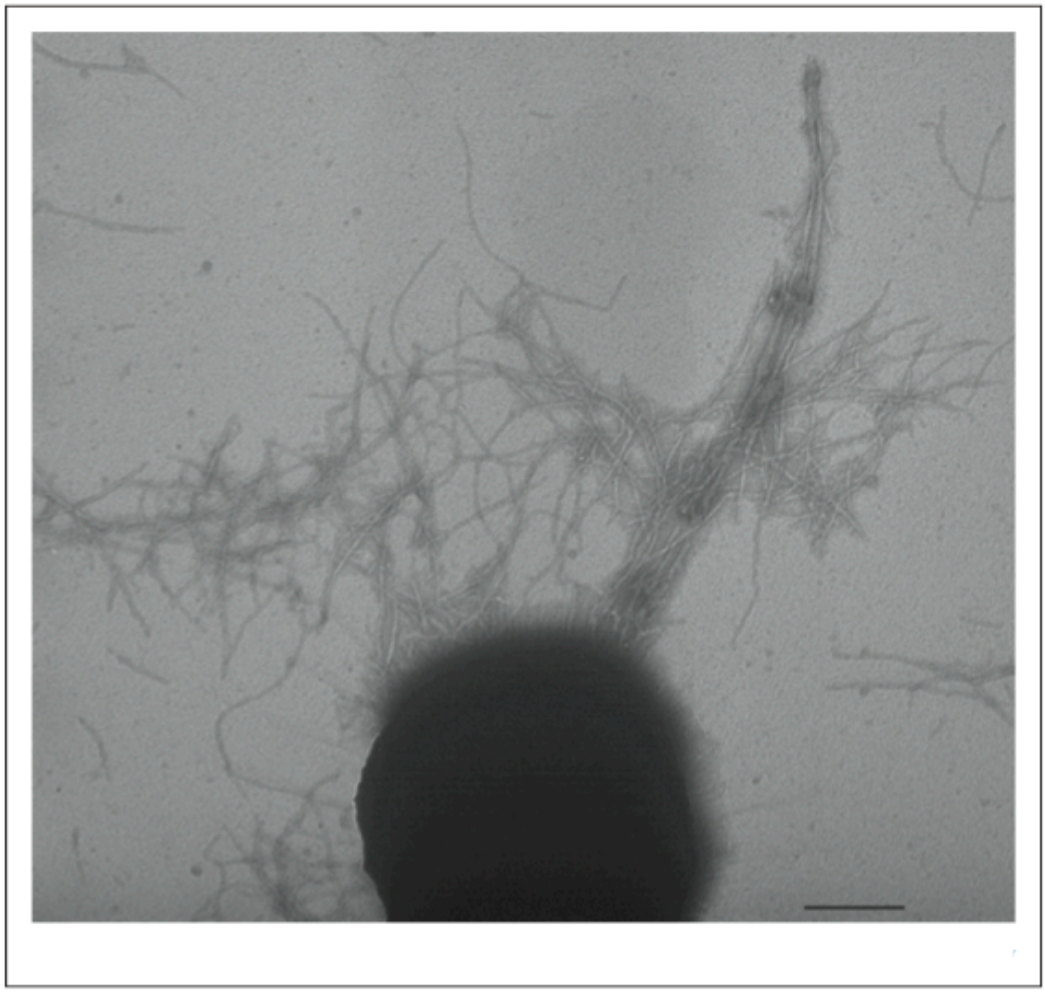

Figure 1.3, TEM micrograph of a *Staphylococcus aureus* cell after five days of biofilm growth, The extracellular fibers observed have amyloid properties and consist of small peptides called phenol soluble modulins (PSMs). Bar length indicates 500 nm. [90]

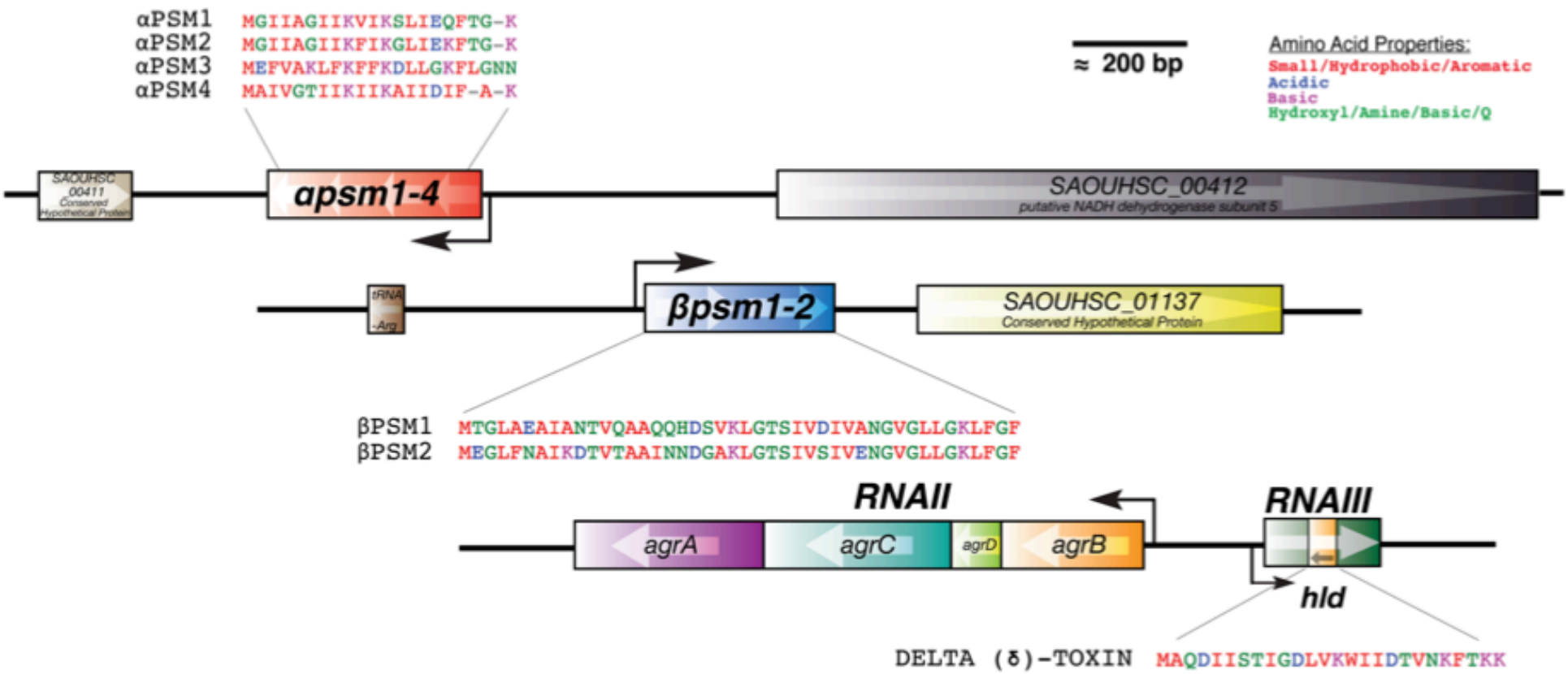

Figure 1.4

Phenol soluble modulins are small peptides expressed from three discrete regions of the *S. aureus* genome, Phenol soluble modulins (PSMs) are encoded in two operons, the alpha (aPSM1–4) and beta (bPSM1–2) operons, and d-toxin is encoded within the Agr regulatory RNA, RNAIII (*hld*).

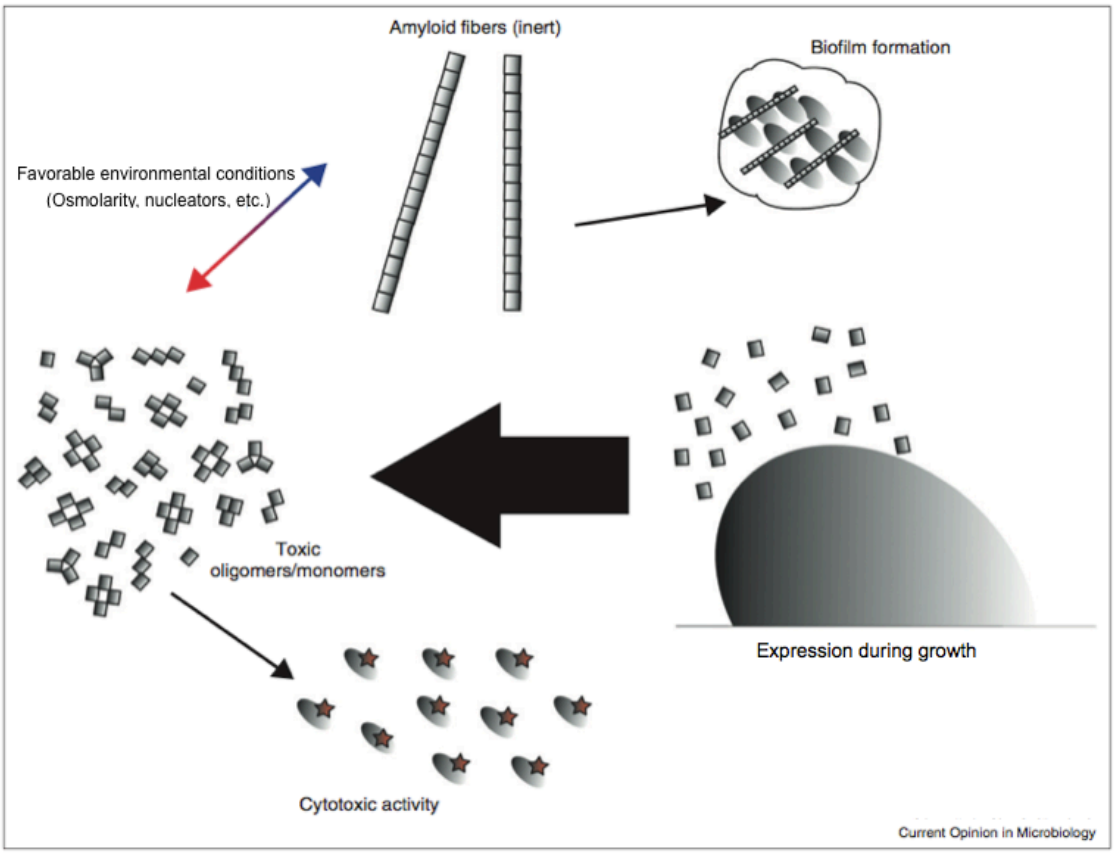

Figure 1.5, PSMs amyloids as toxin repositories

Staphylococcus aureus phenol soluble modulin amyloids act as toxic peptides forming amyloid fibrils under certain environmental conditions. PSMs' cytotoxicity is attributed to pore formation through aggregation on the outer membranes of mammalian cells and niche-occupying bacteria [68,71, 88, 93-97]. When PSMs autoaggregate into amyloid fibrils, they lose the cytotoxic activity associated with smaller oligomeric and monomeric species [71, 88]. Varying the growth conditions of *S. aureus* cultures can promote amyloid fibril aggregation in biofilms [71]. It is likely that this post-translational control mechanism serves to regulate the growth-phase dependent level of toxic activity. Amyloid fibrils stabilize the biofilm and impede dispersal, favoring a sessile lifestyle and reducing interactions with the host immune system [71, 88]. Adapted from [90].

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Chapter 2

Functional Amyloids Composed of Phenol Soluble Modulins Stabilize *Staphylococcus aureus* Biofilms

Abstract:

Staphylococcus aureus is an opportunistic pathogen that colonizes the skin and mucosal surfaces of mammals. Persistent staphylococcal infections often involve surface-associated communities called biofilms. Here we report the discovery of a novel extracellular fibril structure that promotes *S. aureus* biofilm integrity. Biochemical and genetic analysis has revealed that these fibers have amyloid-like properties and consist of small peptides called phenol soluble modulins (PSMs). Mutants unable to produce PSMs were susceptible to biofilm disassembly by matrix degrading enzymes and mechanical stress. Previous work has associated PSMs with biofilm disassembly, and we present data showing that soluble PSM peptides disperse biofilms while polymerized peptides do not. This work suggests the PSMs' aggregation into amyloid fibers modulates their biological activity and role in biofilms.

Introduction:

Staphylococcus aureus is the causative agent of numerous diseases ranging from relatively benign skin conditions to fatal systemic infections. Formation of bacterial biofilms on host

tissues and implanted materials contributes to chronic *S. aureus* infections, as biofilms are exceptionally resistant to host immune response and chemotherapies [1]. Biofilms are multicellular structures encased in a matrix of proteins, polysaccharides, extracellular DNA, and other environmental factors [1,2]. Biomolecules that digest matrix components (e.g., proteases, DNases, and glycoside hydrolases) can disrupt established biofilms and render detached cells susceptible to antimicrobials [3,4,5,6,7].

The precise composition of the biofilm matrix varies greatly by strain, physiological state, and nutrient availability [5,8,9,10,11,12]. In this study, we examined how growth media affects the composition of the biofilm matrix. This led to the discovery of an extracellular fibril structure in *S. aureus* biofilms grown in a non-standard rich media. These fibers share morphological and biophysical characteristics with functional bacterial amyloids such as curli in *Escherichia coli* biofilms, TasA of *Bacillus subtilis*, and the Fap fimbriae in *Pseudomonas aeruginosa* [13,14,15,16]. Biochemical and genetic analysis revealed that these fibril structures are composed of small peptides called phenol soluble modulins (PSMs). Mutants incapable of producing PSMs formed biofilms that were susceptible to disassembly by enzymatic degradation and mechanical stress.

Previous work has demonstrated that PSMs are surfactant-like peptides that promote biofilm disassembly [17,18,19,20,21]; exhibit antimicrobial activity against niche bacteria [22,23,24]; hinder host immune response by recruiting and lysing neutrophils; and are abundant virulence factors produced by community-associated MRSA strains (CA-MRSA) [18,25,26,27]. The genes

encoding the core family of PSM peptides are highly conserved across *S. aureus* strains: four are expressed from the alpha (*αpsm*1-4) operon, two are expressed from the beta (*βpsm*1&2) operon, and the delta hemolysin (*hld*) is encoded within the regulatory RNA, *RNAIII* [28,29,30]. The significance of the PSMs has only recently been investigated because the coding sequences of the *αpsm* & *βpsm* peptides are small enough to have eluded detection by conventional gene annotation programs, and they are still poorly annotated in public databases [29,30].

We have found that ordered aggregation of PSM peptides into amyloid-like fibers can abrogate the biofilm disassembly activity ascribed to monomeric PSM peptides [12,17,18,19,20,21]. Our findings suggest that PSMs can modulate biofilm disassembly using amyloid-like aggregation as a control point for their activity. This is the first report to identify and characterize extracellular fimbriae in the *S. aureus* biofilm, and our research could lead to new approaches in treating persistent biofilm associated infections.

Materials and Methods:

Bacterial strains and growth conditions, The bacterial strains and plasmids used in this study are listed in Table 1. All DNA manipulations were performed in *Escherichia coli* strain DH5α. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Plasmids were transformed into *Staphylococcus aureus* RN4220 by electroporation, purified, and moved to indicated *S. aureus* strains by electroporation. Deletion mutants were generated via allelic replacement using the vector pKFC as described previously [58]. To create the *αpsm* mutation, a region upstream of *αpsm* was amplified from SH1000 genomic DNA using primers

alphaPSM221 (CGC GAG CTC GTT GAG GCA CGC GCC ACT CGC CAG) and alphaPSM162 (GCT AGC GGT ACC ACG CGT GAT GCC AGC GAT GAT ACC CAT TAA) and a downstream region was amplified using alphaPSM163 (ACG CGT GGT ACC GCT AGC TTA AAA TTC TCA GGC CAC TAT ACC) and alphaPSM164 (TAT CCC GGG GAT GGT GGG GGA CTA TCG CGC ACA G). The resulting PCR products were gel purified, digested with KpnI, ligated with T4 DNA ligase and the ligation was used as a template in a subsequent PCR reaction with the primers alphaPSM221 and alphaPSM164. The resulting PCR product was gel purified and digested with SacI and XmaI and ligated with pKFC plasmid digested with the same enzymes to create pKFC-*αpsm*. The resulting plasmid construct was used to create an allelic *Δαpsm* deletion in the SH1000 background following the protocol outline by Kato et al [58].

To create the *βpsm* mutation, a region upstream of *βpsm* was amplified from SH1000 genomic DNA using primers BetaUpF (CCC GGA TCC GGT GTA GTG TTG GTG TAG TTC AGG) and BetaUpR (ACG CGT GGT ACC GCT AGC GCG TTA AAT AAA CCT TCC ATT G) and a downstream region was amplified using primers BetaDownF (5'GCT AGC GGT ACC ACG CGT GGC ACA AGT ATC GTA GAC ATC G) and BetaDownR (5'GCG GTC GAC GGC GTC TGA TTT AAC CTT CTC). The resulting PCR products were gel purified and used as a template in a subsequent PCR reaction with the BetaUpF and BetaDownR primers. The resulting PCR product was gel purified and digested with BamHI and SalI and ligated with pKFC plasmid digested with the same enzymes to create pKFC-*βpsm*. The resulting plasmid was used to create an allelic *Δβpsm* deletion in the SH1000 *Δαpsm* background following the protocol outline by Kato et al. to create the double knockout *αβPSM* mutant [58].

Complementation vectors were created as follows: the *βpsm* locus with its native promoter was amplified from *S. aureus* SH1000 genomic DNA using primers GAC GAA TTC AGG CAA CTT AAT TGT G and GAC AAG CTT GCT TCC CAA TGT TGG TG. The resulting PCR product was digested with HindIII and EcoRI and ligated with pAH8 [4], that had been digested with the same enzymes to create pRS*βpsm*. The *αpsm* locus was amplified from SH1000 genomic DNA using primers ACT GAG GTA CCA GAC TCA CCT CAC ATC AAT AA and ACT AGG AGC TCC AAA GGA GGT AAT CTT AAT GGG T. The resulting PCR product was digested with KpnI and SacI and ligated with pALC2073 [59], then digested with the same enzymes to create pALC2073*αPSM*.

Biofilm experiments, Flow cell and drip biofilms were grown as previously described [5,60]. Biofilm growth medium was either 0.6 g/L tryptic soy broth and 1.5 g/L glucose (TSBg) or 3.3 g/L peptone, 2.6 g/L NaCl, 3.3 g/L glucose (PNG).

For biofilm disassembly experiments performed in flow cells, enzymes proteinase K, DNaseI and dispersin B were suspended in water and added to the media reservoir at a final concentration of 0.2 μg/mL. Confocal scanning laser microscopy and image analysis was performed as described previously [5]. Strains contained pAH9 expressing mCherry or were stained with propidium iodide as previously described [5].

Test tube biofilms forming at the air-liquid interface of glass culture tubes were grown in 3 mL of TSBg or PNG for 2 days at 37°C shaking at 200 rpm. Liquid media was removed and exchanged with either 10 mL sterile ddH_2O containing 1% SDS or sterile ddH_2O alone. Tubes

were vortexed for 5 seconds and all liquid was removed. The remaining biofilm biomass was visualized by staining with 0.1% crystal violet and quantified by solublizing the stain in acidified ethanol and measuring the optical density at A_{595}.

Transmission electron microscopy (TEM) was performed using a Philips CM12 transmission electron microscope. Samples prepared for TEM imaging were spotted onto formvar-coated copper grids, incubated for 5 minutes, washed with sterile ddH_2O, and negatively stained with 2% uranyl acetate for 60 seconds.

Isolation of fibers from biofilm cultures, Drip bioreactor biofilms were grown as previously described [60]. Fibers were collected after 5 days growth by scraping biofilms into 3 mL of potassium phosphate buffer (50mM, pH 7). The biofilm suspensions were homogenized using a tissue homogenizer (TissueMiser, Fisher) to shear fibers free from the cell walls. Supernatants were clarified by repeated centrifugation at 13,000 RPM for 2 minutes to remove cells. The cell-free supernatant was incubated in 200mM NaCl and the fibers were isolated using Millipore Amicon Ultra Centrifugal Filter Units with a pore size of 100 kDa. Presence of fibers was confirmed via TEM imaging.

Identification of aggregative peptides, Fibril subunits were identified by harvesting drip biofilms after 5 days of growth in PNG and suspending them in 15 mL 10 mM Tris HCl, pH 8.0 (Tris buffer), supplemented with 0.1 mg of RNase A (bovine pancreas; Sigma Chemical Co., St. Louis, Mo.) and 0.1 mg of DNaseI (bovine pancreas; Boehringer, Mannheim, Germany) per mL.

Cells were lysed by repeated sonication and the addition of lysostaphin (1 mg/mL, Sigma) and 1 mM $MgCl_2$ prior to incubation at 37°C for 20 min. Lysozyme (Sigma) was added to 1 mg/mL, and the samples were incubated with shaking for 40 min at 37°C, after which they were adjusted to 1% sodium dodecyl sulfate (SDS) and incubated further (30 min, 37°C). The remaining insoluble material was collected by centrifugation (12,100 x g, 15 min, 25°C), washed and suspended in 10 mL 10mM Tris buffer. The pellet was digested again with RNase, DNaseI, and lysotaphin as described above, washed twice with Tris buffer, and suspended in 2 mL SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (10% glycerol, 5%-mercaptoethanol, 1% SDS, 62.5 mM Tris HCl [pH 6.8]). The sample was boiled for 5 min, loaded onto a 12% polyacrylamide gel (3% stacking gel), and subjected to electrophoresis at 20 mA for 5 h. The material retained in the stacking gel was excised, washed three times in ddH_20, extracted twice with 95% ethanol, and dried in a speedvac. The desiccated sample was resuspended in ddH_20 and sonicated to break up any clumps. Half of this material was incubated with formic acid (90%) for 20 min then dried in a speedvac. Both the formic acid treated and untreated samples were resuspended in SDS-PAGE sample buffer and run into a fresh 12% PAGE gel. Bands that appeared in the formic acid treated sample were excised and analyzed via LC-MS/MS (MS Bioworks, Ann Arbor, MI).

Fiber protein components were also identified by incubating fiber isolates in pepsin for 24 h before subsequent LC-MS/MS analysis (MS Bioworks, Ann Arbor, MI). The value for the abundance measurement is the Normalized Spectral Abundance Factor (NSAF).

PSM polymerization experiments, Non-formylated PSM peptides were synthesized by Peptide 2.0 and assayed to be >90% pure by HPLC. Synthetic peptides were prepared and assayed as previously described [15,34] to eliminate large aggregates from lyophilization prior to assay. Each dry peptide stock was dissolved to a concentration of 0.5 mg/mL in a 1:1 mixture of trifluoroacetic acid (TFA) and hexafluoroisopropanol (HFIP). Peptides were then sonicated for 10 minutes and incubated at room temperature for 1 h. Solvent TFA/HFIP was removed by speedvac at room temperature. Dried peptide stocks were stored at -80°C. All assays were performed with equal stoichiometric ratios of 0.1 mg/mL peptide unless otherwise noted.

All polymerization assays were performed in 96-well black opaque, polystyrene, TC-treated plates (Corning). Prior to assay, treated peptides were thawed and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL immediately prior to assay. Freshly dissolved peptides were diluted into sterile ddH_2O containing 0.2 mM thioflavin T (ThT) and assayed at room temperature. Fluorescence was measured every 10 minutes after shaking by a Tecan Infinite M200 plate reader at 438 nm excitation and 495 nm emission. ThT fluorescence during polymerization was corrected by subtracting the background intensity of an identical sample without ThT.

Additionally, ThT fluorescence and Congo red (CR) absorbance scans were performed on polymerized peptides that were allowed to polymerize for 48 h in ddH_2O. Samples were incubated in 0.2 mM ThT or 0.001% (w/v) CR in ddH_2O for 15 minutes prior to assay on the

Tecan plate reader. CR and ThT scans were corrected by subtracting the background intensity of an identical sample without dye.

Circular dichroism spectroscopy, Treated peptide stocks were thawed and dissolved in hexafluoroisopropanol (HFIP) to a concentration of 10 mg/mL immediately prior to assay. Triplicate samples consisting of 0.1 mg/mL of each freshly dissolved peptide diluted together in 500µL sterile ddH_2O were incubated with shaking at room temperature for 48 h. Samples were then pelleted at 15,000 RPM for 30 minutes to isolate any aggregated species. The supernatant was carefully removed from the pellet by aspiration and transferred to a clean, sterile eppendorf tube. The remaining pellet was resuspended in 200 µL ddH_2O by bath sonication for 10 minutes. The supernatant and pellets of each sample were assayed separately. Far UV circular dichroism (CD) measurements were performed with a Jasco-J715 spectropolarimeter using quartz cells with 0.1 cm path length. CD spectra between 190 and 250 nm were recorded in millidegrees and converted to molar ellipticity using an average MRW of 113 for αPSM1-4, βPSM1&2, and δ-toxin. The average of five scans was recorded at 25°C using a 2 nm bandwidth with a 20 nm min^{-1} scanning speed. All triplicate samples showed similar ellipticity patterns.

Biofilm dispersal assay, Synthetic PSM peptides were allowed to polymerize overnight, and fibril formation was verified by TEM imaging. Equivalent concentrations of either polymerized or freshly diluted peptides were added to 24 hours SH1000 biofilms grown in 66% TSB + 0.2% glucose and incubated at 37˚C for 6 hours. Biofilms were washed to remove non-adherent cells

then stained with 0.1% crystal violet, dried, and solublized with acidified ethanol and spectroscopically quantitated at A_{595}.

Statistics were performed using a 1-way analysis of variance (ANOVA). Results are expressed as mean ± standard deviation.

Results

Biofilms grown in PNG media resist biofilm disassembly

Biofilms that persist in the human body are often resistant to conventional antimicrobial treatment prior to dispersal. To gain insight into how the *S. aureus* biofilm matrix affects disassembly under different growth conditions, we grew *S. aureus* flow cell biofilms with various lab media. Next we used enzymes known to target primary matrix components in order to test biofilm resistance (Fig. 1A & 1B). These enzymes include proteinase K (protein), DNaseI (DNA), and dispersin B (polysaccharide). By using a variety of degradative enzymes, we expected to observe complete biofilm eradication. Biofilms grown in tryptic soy broth supplemented with glucose (TSBg) rapidly disassembled after enzymatic treatment (Fig. 1A). However, biofilms grown in peptone-NaCl-glucose (PNG) media did not disassemble after the same enzymatic treatment (Fig. 1B).

We also assessed the ability of biofilms attatched at the air-liquid interface of glass culture tubes to withstand exposure to an anionic surfactant, sodium dodecyl sulfate (SDS). Again, biofilms grown in TSBg were more sensitive to surfactant-mediated disassembly than those

grown in PNG (Fig. 1C). We interpreted these results to be an indication that growth in PNG alters the matrix composition, increasing the biofilm's resistance to enzymatic degradation and surfactant dispersal. We hypothesized that a new, previously unaccounted for matrix component was influencing *S. aureus* biofilm integrity under these growth conditions.

Biofilms resistant to dispersal contain extracellular fibers

To investigate how biofilms grown in PNG media are able to resist disassembly, we grew biofilms in drip bioreactors under sensitive (TSBg) or resistant (PNG) conditions for five days. Biofilms were harvested and disrupted by vortexing and sonication. Transmission electron microscope (TEM) imaging of cells revealed the presence of extracellular fibers in enzyme-resistant biofilms (Fig. 2B), but not in enzyme-sensitive biofilms (Fig. 2A). The fibers had a central diameter of ~12 nm and were closely associated with bacterial cells (Fig. 2C). *S. aureus* has never before been shown to produce large, extracellular structures. Additionally, we observed identical fibers associated with biofilm cells in several lab strains (LAC, UAMS, MN8) and six clinical isolates (three nasal isolates and three blood isolates) by TEM, demonstrating that fiber formation is not specific to strain SH1000. Of note, we found that an *agr* quorum sensing mutant (SH1001) was unable to produce fibers (Fig. 2D).

***Staphylococcus aureus* fibers are composed of phenol soluble modulins (PSMs)**

The novel extracellular fibers isolated from robust biofilm matrices share morphological similarities with the bacterial functional amyloids curli in *E. coli* and TasA in *B. subtilis* [15,16]. Amyloid proteins form highly stable polymerized aggregates that exhibit well-defined

biochemical and biophysical characteristics [14,31,32]. We hypothesized that our fibers were also functional bacterial amyloids. To identify the protein composing these fibers, we used two approaches to take advantage of the biophysical characteristics of functional bacterial amyloids.

Amyloid fibrils from bacterial biofilms were previously shown to be poorly soluble in sodium dodecyl sulfate (SDS) and do not migrate through polyacrylamide gels [33]. We therefore employed SDS-PAGE to isolate large insoluble structures. *S. aureus* biofilm samples were grown in drip bioreactors for five days with PNG media or TSBg. These biofilms were harvested, homogenized, and lysed, and the lysates were run into a 12% SDS-polyacrylamide gel. Lysates from biofilms grown in PNG media retained insoluble material in the wells of the stacking gel while TSBg-grown lysates did not (Fig. 3A). The insoluble material retained within the wells of the stacking gel was recovered, treated with 100% formic acid (FA), then separated once more by SDS-PAGE alongside an untreated control (Fig. 3B). We observed protein enrichment in the FA-treated sample, and the four dominant bands were excised and analyzed via mass spectrometry (MS). Surprisingly, MS analysis identified the same peptides as being abundant in each sample, regardless of the band's migration through the gel matrix (Fig. 3B & 3D). These proteins were the alpha (αPSM) phenol soluble modulins and the *S. aureus* delta hemolysin (δ-toxin) (Fig. 4A & 4B).

An additional approach to identify the fiber subunit was to isolate fibers from biofilm cells using a tissue homogenizer (Fig. 3C), incubate fiber isolates for 48 hours at pH 2 with pepsin, and

analyze the sample with MS. Again, we detected the same αPSM peptides present in the SDS-PAGE isolation plus two beta PSMs (βPSM) (Fig. 3D). αPSM3 was not identified in either preparation, but it should be noted that αPSM3's sequence contains several trypsin cleavage sites, so it is likely that it would not be detected after extensive digestion. The same fiber isolation procedure revealed no visible fibers by TEM when biofilms were grown in TSBg.

We generated an Δ*αβPSM* double-knockout mutant and assessed fiber production. TEM analysis of biofilm cells revealed that this mutant did not produce fibers after five days of growth in PNG media (Fig. 5B) compared to the wildtype parent strain grown under the same conditions (Fig. 5A). In addition, fibers could be isolated from wildtype (Fig. 5D) but not mutant biofilms (Fig. 5E). Fiber production was complemented by expression of the *αpsm* and *βpsm* operons *in trans* (Fig. 5C & 5F).

PSM peptides form fibers similar to bacterial functional amyloids

We assayed synthetic PSMs peptides for their capacity to form fibers *in vitro*. To minimize the prevalence of polymeric "seed" complexes, all synthetic peptides were treated with HFIP/TFA and dried *in vacuo* prior to assay [34]. Incubation of the seven previously identified PSM peptides (αPSMs1-4, βPSMs1-2, and δ-toxin) demonstrated their capacity to self-assemble into fibers (Fig. 6B). We used the amyloid-specific dye thioflavin T (ThT) to observe amyloid formation over time [34,35]. When we assayed the PSMs for polymerization in the presence of ThT at room temperature, we observed a robust increase in normalized fluorescence (Fig. 6A). Greater peptide concentration increased ThT fluorescence and showed rapid binding kinetics

similar to an amyloid-nucleator system (Fig. 6A) [35,36,37]. PSM fibers exposed to ThT exhibited an emission spike near 490 nm that is also observed in other amyloid fibrils (Fig. 6C) [36,38,39]. Incubation of PSM fibers with the dye Congo red (CR) resulted in a characteristic absorbance "red shift", indicative of cross β structure conserved in all amyloid fibers (Fig. 6D) [40]. Furthermore, PSM fibers isolated from solution through centrifugation displayed increased β-sheet content (Fig. 6E), which is consistent with data published from other bacterial amyloids [13,16]. These *in vitro* observations compliment our genetic and physiological data, further supporting the notion that PSMs can form amyloid fibrils.

Mutants unable to synthesize PSMs produce biofilms susceptible to matrix-degrading enzymes and mechanical stress

Because biofilms grown in PNG media resist disassembly by matrix-degrading enzymes and surfactants (Fig. 1), we challenged Δ*αβpsm* mutant biofilms grown under the same conditions. In contrast to its isogenic parent strain, an Δ*αβpsm* mutant biofilm readily disassembled after exposure to proteinase K, DNaseI, and dispersin B (Fig. 7A). Complementation of the *αβPSM* mutant *in trans* restored the resistant biofilm phenotype (Fig. 7B). We also examined the effects of mechanical stress (vortexing) on biofilms attatched at the air-liquid interface of glass culture tubes. An Δ*αβpsm* mutant biofilm readily disassembled with exposure to mechanical stress, while biofilms of the isogenic parent and complemented strains both remained intact (Fig. 7C). Taken together these data do suggest that PSM fibers enhance biofilm integrity.

Fibrilation modulates PSM activity

Previous work has demonstrated that soluble PSMs assist biofilm disassembly [19,21,27]. Based on our findings that PSM fibers improve biofilm integrity (Fig. 1 & Fig. 7), we hypothesized that sequestration of PSMs into extracellular fibers could alter their activity. Synthetic αPSM1 peptides readily formed fibers that bind CR and ThT after 24 hours of incubation in solution (Fig. 8C, 8D, 8E). To test whether or not fibrillation alters peptide activity, we exposed 24-hour *S. aureus* biofilms to either freshly solublized αPSM1 peptides (Fig. 8B) or αPSM1 that had been allowed to polymerize overnight (Fig. 8C). Exposure to soluble αPSM1 significantly reduced the amount of adherent biofilm; however, exposure to αPSM1 fibers had no discernable effect on the biofilms (Fig. 8A). This finding suggests that the aggregation of PSMs into amyloid-like fibers can modulate their ability to disassemble biofilms.

Discussion

Biofilm formation and disassembly are carefully coordinated with the production and breakdown of matrix components. The biofilm lifecycle of attachment, maturation, and disassembly participates as a mechanism of virulence in many persistent *S. aureus* infections [1,2,4,5,11,12,19]. A better understanding of the dynamic *S. aureus* matrix environment may inspire new, innovative techniques for controlling biofilm infections.

Previous studies have shown that the *S. aureus* biofilm matrix contains polysaccharides and DNA that interact with structural and enzymatic proteins [3,4,5,7,41]. In this work we demonstrate that under certain growth conditions, *S. aureus* produces amyloid-like fibers that contribute to biofilm integrity (Fig. 1, 2, 7). Purification and analysis of fibers revealed that

several small peptides of the phenol soluble modulin family were present (Fig. 3 & Fig. 4). Fibers were not detected in biofilms of an Δ*αβpsm* deletion mutant under the same conditions that favor their production in wildtype strains (Fig. 5). Δ*αβpsm* mutant biofilms were further demonstrated to be significantly more susceptible to disassembly with matrix degrading enzymes and mechanical stress than their isogenic parent (Fig. 7).

To the best of our knowledge, this is the first report describing an extracellular fibril structure in *S. aureus* biofilms. We refer to these matrix components as being amyloid-like because they possess some of the characteristics often attributed to amyloid proteins: fibril morphology (Fig. 2, 5, 6, 8), relative SDS insolubility (Fig. 1 & Fig. 3), binding to the amyloid-specific dyes thioflavin T and Congo red (Fig. 6 & Fig. 8), and they display β-sheet structure [14,31]. The observation that PSM peptides not only self-assemble, but contribute to the biofilm's structural integrity is intriguing in light of recent work describing the PSMs' involvement in biofilm disassembly [11,20,27].

It is well-documented that the PSMs are regulated by the agr quorum-sensing network [11,12,28,42], and we similarly have found that an *agr* deficient strain did not produce fibers (Fig. 2D). This contributes to a growing body of evidence which implicates the agr system to have wide-ranging effects beyond heightened pathogenicity and biofilm dispersal [4,20,21,43]. It is tempting to speculate that the media-dependant fiber production is somehow influencing *agr* regulation, perhaps through metabolism or through other signaling cues.

PSMs were first isolated from *Staphylococcus epidermidis* cultures as a polypeptide complex, and have since been shown to interact biochemically [22,29]. We have demonstrated that synthetic *S. aureus* PSM peptides are capable of self-assembling into amyloid-like fibers *in vitro* (Fig. 6A & Fig. 6B). These fibers demonstrate CR and ThT binding capacities similar to known amyloid proteins. PSMs, including δ-toxin have been previously characterized as amphipathic α helices [22,30,44,45]. Our data indicate that soluble PSMs have a helical structure in solution, but transition to adopt a more β-rich structure after aggregation (Fig. 6E). The assembly of αPSM1 into fibers prevents the biofilm disassembly activity attributed to soluble peptides species (Fig. 7). We interpret these results as evidence that aggregation into amyloid fibers can regulate PSM activity in the microenvironment of the biofilm.

Our findings demonstrate that *S. aureus* PSMs can be found in biofilms as fibrils, and may implicate fibril formation as a means of altering their activity and function. It is not known at this time what mechanisms influence the PSMs' ability to switch from a monomeric to fibril state, nor is it clear how this affects the formation and disassembly of biofilms. It is possible that PSM fibrillation is synchronized *in vivo* by a nucleator protein, similar to CsgB in *E. coli* [34]. Formylation may also play a role; the PSMs and δ-toxin are detected at the protein level both with and without with a formylated methionine modification [29,30,46,47], and the PSMs identified in our MS analysis contained primarily deformylated N-methionines. Recent work demonstrates that non-N-formylated PSMs are strong activators of FPR2 receptors, which also respond to amyloidogenic peptides like $A\beta_{1-42}$ and serum amyloid A [48,49,50], and may implicate a role for deformylation in fibril construction. We speculate that there are numerous

other environmental cues (such as pH and osmolarity) driving the PSMs commitment to the fibrillation pathway, and this is currently under investigation.

This study builds upon an emerging paradigm emphasizing that amyloid fibers are common in the biofilm matrices of many bacterial species. Curli fibers produced by pathogenic *E. coli* and other enterics were the first functional amyoloids to be characterized [15,32,33]. The gram-positive bacteria *Streptomyces coelicolor* produces several small peptide species, which have been shown to polymerize *in vitro* and *in vivo* to facilitate sporulation at the air-liquid interface [51,52,53,54]. Recent work in *B. subtilis* has shown that the antimicrobial and spore coat protein TasA can assemble into amyloid-like fibrils during biofilm growth [16]. Even natural biofilms collected from a variety of environmental niches appear to contain amyloid-like fibers [55], indicating that the production of bacterial amyloids may be a shared feature of biofilm matrices from many different bacterial communities.

We propose that amyloid-like aggregation of toxic proteins is an under appreciated form of posttranslational regulation utilized throughout nature, and even more examples continue to emerge. The antimicrobial activity of the *Klebsiella pneumonia* bacteriocin microcin E492 can be turned off through their assembly into amyloid-like fibers [56]. Recent work by Maji *et al.* has demonstrated that even human peptide hormones can form amyloid-like structures for storage [57]. Likewise, PSMs may be stored as inert fibrils in a sessile biofilm until conditions arise that favor their dissociation to promote biofilm disassembly, antimicrobial activity, or virulence. This work presents evidence that *S. aureus* PSMs can be found in biofilms as large fibril structures

providing new insight into how quorum sensing and virulence play into the complexity of the biofilm lifecycle.

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Figures and Tables:

Table 2.1, Strains and plasmids used in the present study.

Strain or plasmid	Relevant Genotype	Resistance	Source or reference
Escherichia coli			
DH5α-E	Cloning strain		Invitrogen
Staphylococcus aureus			
RN4220	Restriction modification deficient		[61]
SH1000	Lab strain- σ^{B+} derivative of NCTC8325-4		[62]
SH1001	SH1000 agr::tet		[62]
UAMS-1	Osteomylitus isolate		[63]
MN8	Toxic shock isolate		[64]
LAC	CA-MRSA USA300-0114		[4]
AH500	SH1000 / pAH9	Erm	[4]
BB606	Blood isolate		This work
BB607	Blood isolate		This work
BB608	Blood isolate		This work
BB862	Nasal isolate		This work
BB863	Nasal isolate		This work
BB864	Nasal isolate		This work
BB2388	SH1000 Δ*αβPSM*		This work
BB2407	Δ*αβPSM* + pALC2073 & pAH8	Erm, Cm	This work
BB2408	Δ*αβPSM* + pALC2073- *αPSM* & pRS*βPSM*	Erm, Cm	This work
Plasmids			
pAH8	*agr* promoter P_3-RFP	Amp, Erm	[4]
pALC2073	*agr* promoter P_3-RFP	Cm	[4]
pALC2073 - αPSM	*αPSM* locus under control of tet promoter	Cm	This work
pRS - βPSM	*βPSM* locus under control of native promoter in pRS10	Amp, Erm	This work
pKFC	Gene replacement vector	Amp, Tet	[58]
pKFC- αPSM	*αPSM* knockout vector	Amp, Tet	This work
pKFC- βPSM	*βPSM* knockout vector	Amp, Tet	This work

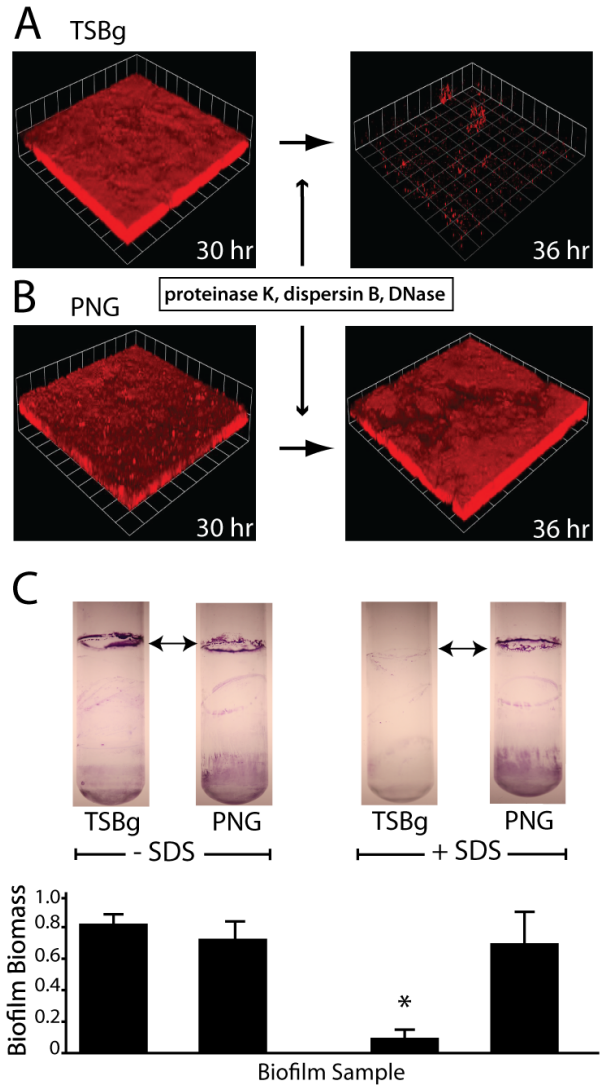


Figure 2.1, Growth media influences biofilm disassembly

Confocal micrographs of *S. aureus* SH1000 biofilms grown in TSBg media (A) for 30 hours readily disassemble upon exposure to biofilm matrix degrading enzymes proteinase K, dispersin B, and DNaseI at 0.2 μg/mL each. *S. aureus* biofilms grown in PNG media (B) for 30 hours fail to disassemble upon exposure to matrix-degrading enzymes. Images are representative of three separate experiments and each side of a grid square represents 20 μm. (C) Biofilms at the air-liquid interface of test tube cultures withstand 1% SDS exposure when grown in PNG media but disassemble when grown in TSBg. Top images show stained test tube biofilms; graph below is quantification of biofilm biomass. * $P<0.002$ compared to no SDS treatment.

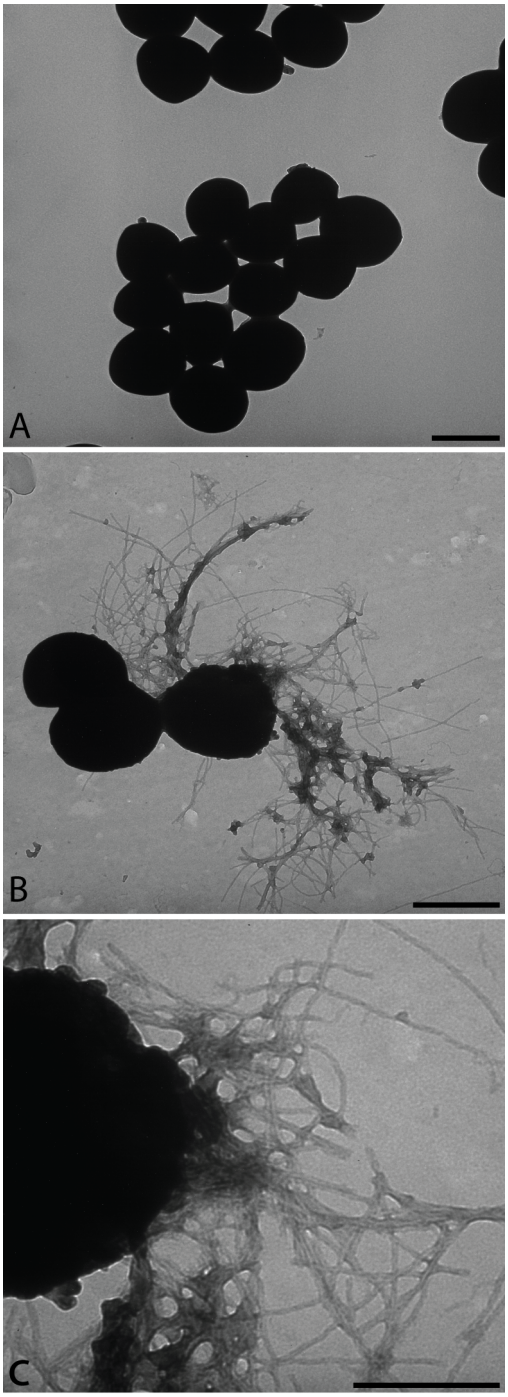
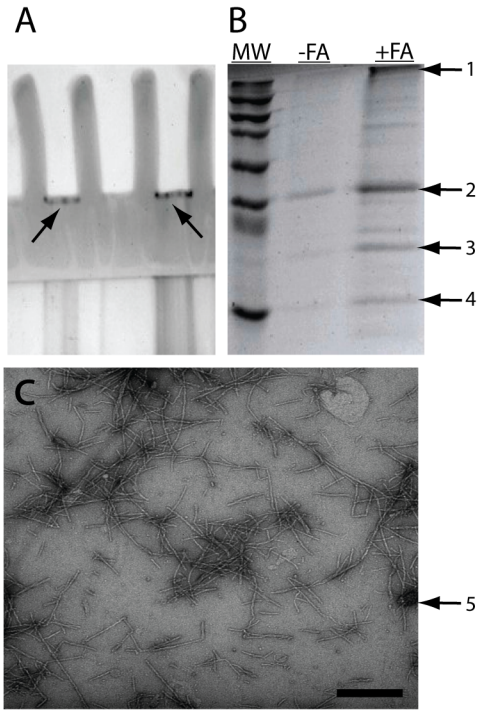


Figure 2.2 , *S. aureus* produces extracellular fibers during biofilm growth in PNG media.
TEM micrographs of cells from *S. aureus* SH1000 biofilms grown in TSBg medium (A) versus cells from biofilms grown in PNG media (B). High magnification reveals fibers are associated with the cell wall and approximately 12 nm in width (C). An *agr* mutant does not produce extracellular fibers (D). Bar length indicates 1 µm in A, B, and D, and 250 nm in C.



D

	Identified Proteins	Accession #	NSAF
Band 1	δ-toxin	Q8NVK6	29.30
	αPSM1	A8Z0V1	13.52
	αPSM4	P0C827	11.29
	αPSM2	A8Z0V0	3.58
Band 2	δ-toxin	Q8NVK6	22.51
	αPSM1	A8Z0V1	16.89
	αPSM4	P0C827	8.32
	αPSM2	A8Z0V0	2.21
Band 3	αPSM1	A8Z0V1	22.73
	αPSM4	P0C827	12.87
	δ-toxin	Q8NVK6	8.58
	αPSM2	A8Z0V0	1.83
Band 4	αPSM1	A8Z0V1	27.23
	αPSM4	P0C827	16.08
	δ-toxin	Q8NVK6	6.87
	αPSM2	A8Z0V0	6.79
Fiber Isolate 5	αPSM4	P0C827	36.29
	αPSM1	A8Z0V1	34.03
	αPSM2	A8Z0V0	7.94
	βPSM1	A5IS59	7.37
	βPSM2	A5IS60	5.67

Figure 2.3, Fibers are composed of phenol soluble modulins.

(A) *S. aureus* biofilm cells were lysed and run into a 12% SDS-PAGE gel (TSBg first lane or PNG second lane); protein that did not migrate through the gel (indicated by arrow) was extracted from the staking gel, treated with formic acid to break up aggregated proteins, and finally run on a new 12% SDS-PAGE gel (B). Bands that appeared after formic acid treatment (1-4) were excised and analyzed via LC-MS/MS. (C) TEM micrograph of purified fiber sample that was then exposed to extensive pepsin digestion and analyzed via LC-MS/MS. Bar indicates 250 nm. (D) Peptides identified by mass spectrometry analysis and their relative abundance factors in the sample (NSAF).

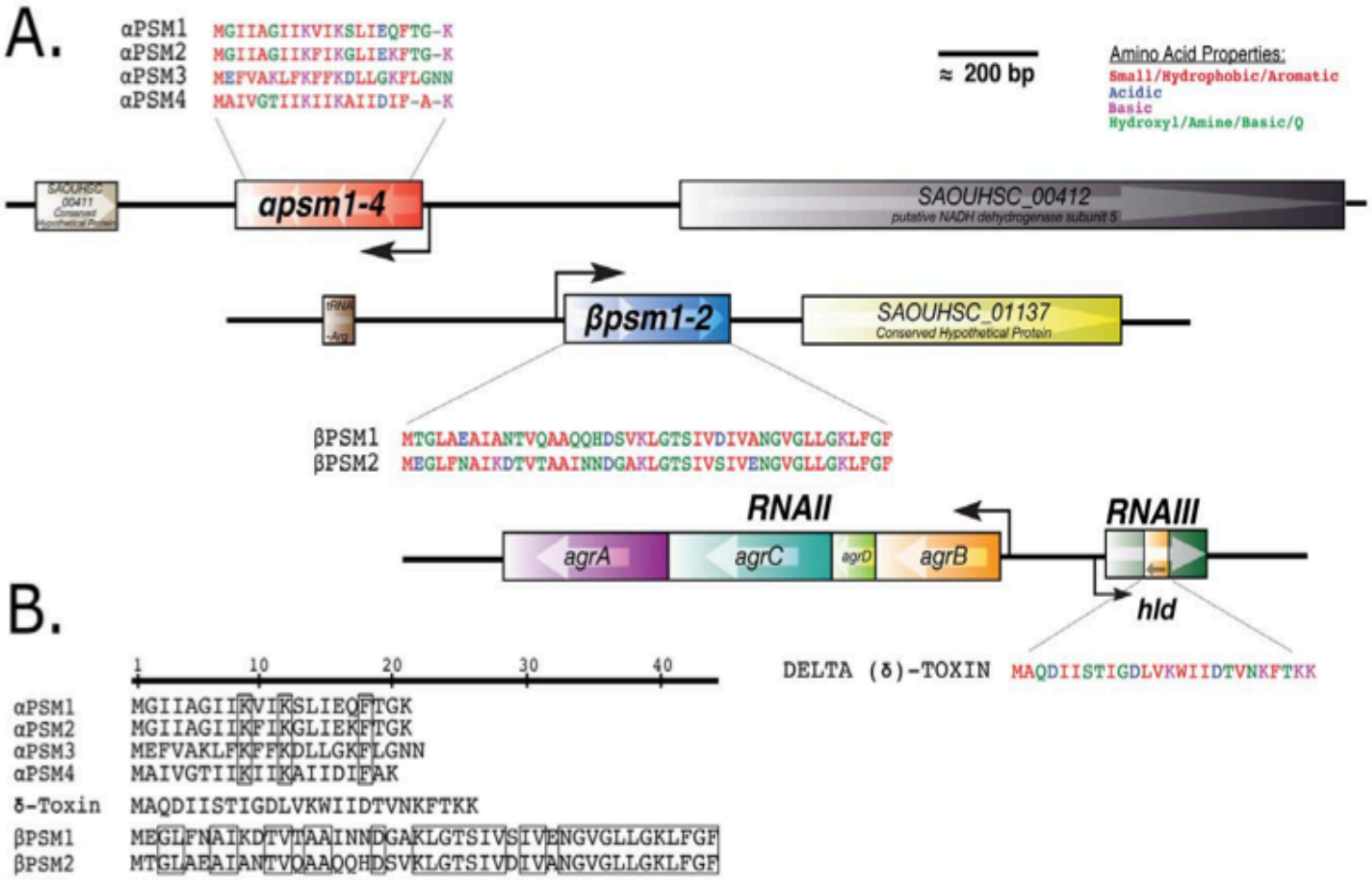


Figure 1.4

Phenol soluble modulins are small peptides expressed from three discrete regions of the *S. aureus* genome, Phenol soluble modulins (PSMs) are encoded in two operons, the alpha (aPSM1–4) and beta (bPSM1–2) operons, and d-toxin is encoded within the Agr regulatory RNA, RNAIII (*hld*).

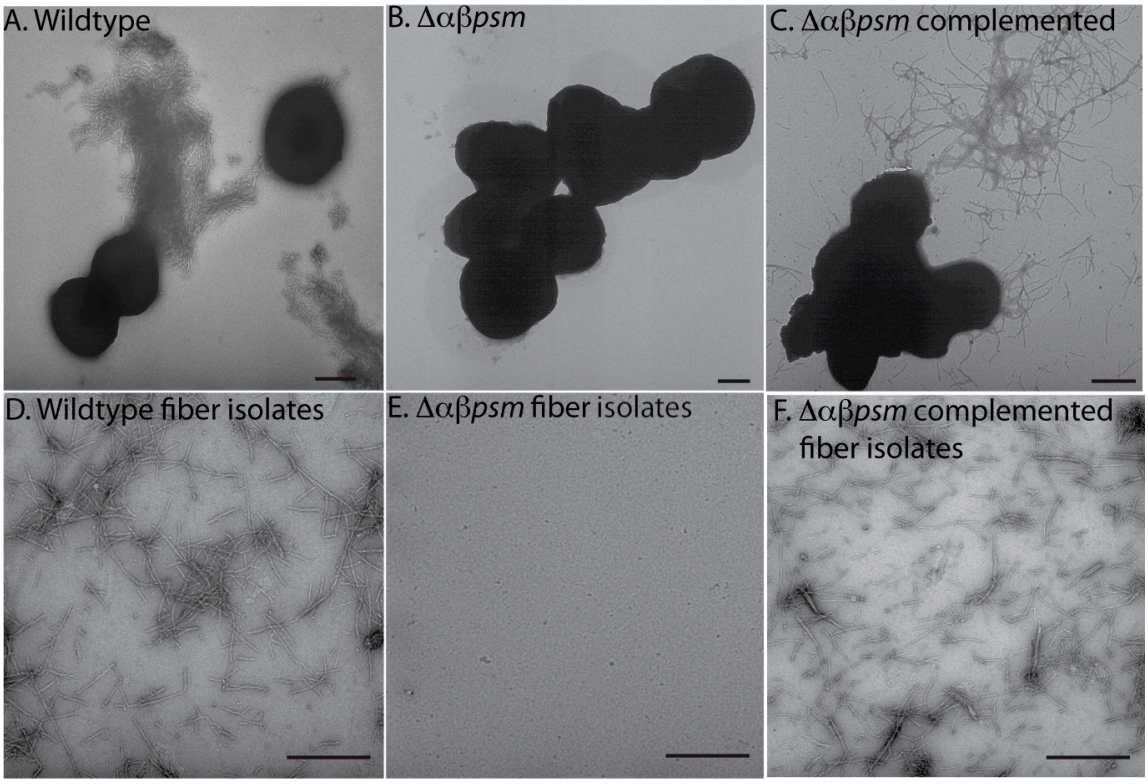


Figure 2.5, Mutants unable to produce α and βPSMs fail to form fibers during biofilm growth. TEM micrographs of *S. aureus* biofilm cells grown for five days in PNG media. (A) wildtype (strain SH1000), (B) Δαβpsm (strain BB2388), (C) Δαβpsm complemented (strain BB2408). (D-F) TEM micrographs of fiber preparations from wildtype (D), Δαβpsm (E), and Δαβpsm complemented (F). Bars indicate 500 nm.

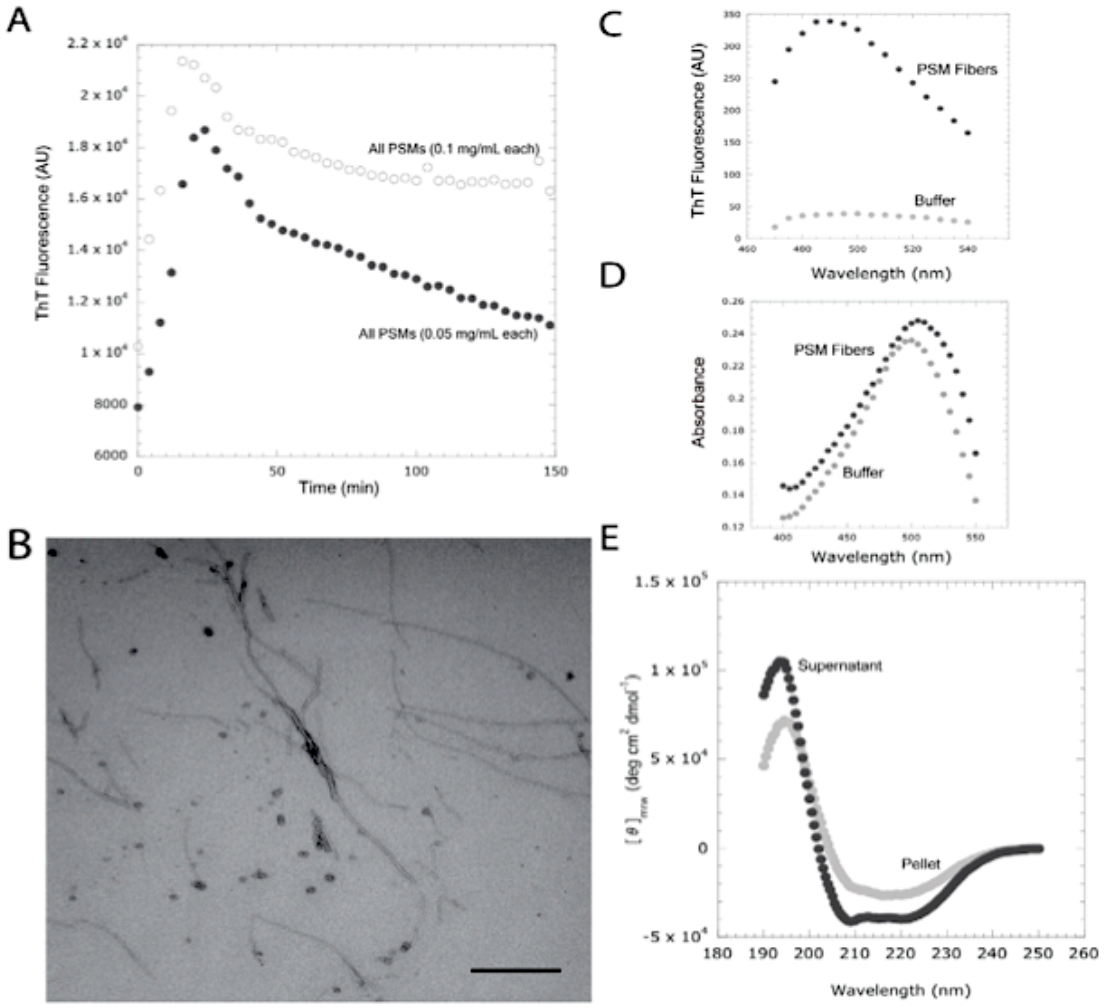


Figure 2.6, Synthetic phenol soluble modulin peptides bind ThT and polymerize into amyloid-like fibers.

(A) Normalized fluorescence intensity of [white circle] 0.1 mg/mL of each PSM peptide or [black circle] 0.05 mg/mL of each PSM peptide in 2 mM ThT. Fluorescence emission was measured at 495 nm after excitation at 438 nm. Assays were repeated in triplicate and all demonstrated a similar trend. (B) 48 hours after mixing 100 μg/mL each of the seven PSM peptides (α1-4, β1-2, and δ-toxin), fibril structures are readily observed by TEM. (C) PSM fibers [black circle] display a ThT fluorescence peak around 482nm compared to a ThT-only blank [grey circle]. (D) PSM fibers [black circle] produce a characteristic Congo red (CR) absorbance "red-shift" associated with amyloid binding compared to a CR-only blank [grey circle]. (E) Pelleted PSM fibers [grey circle] display a greater β-sheet content than the remaining supernatant [black circle]. Assays were repeated in triplicate and displayed similar trends. Bar indicates 500 nm.

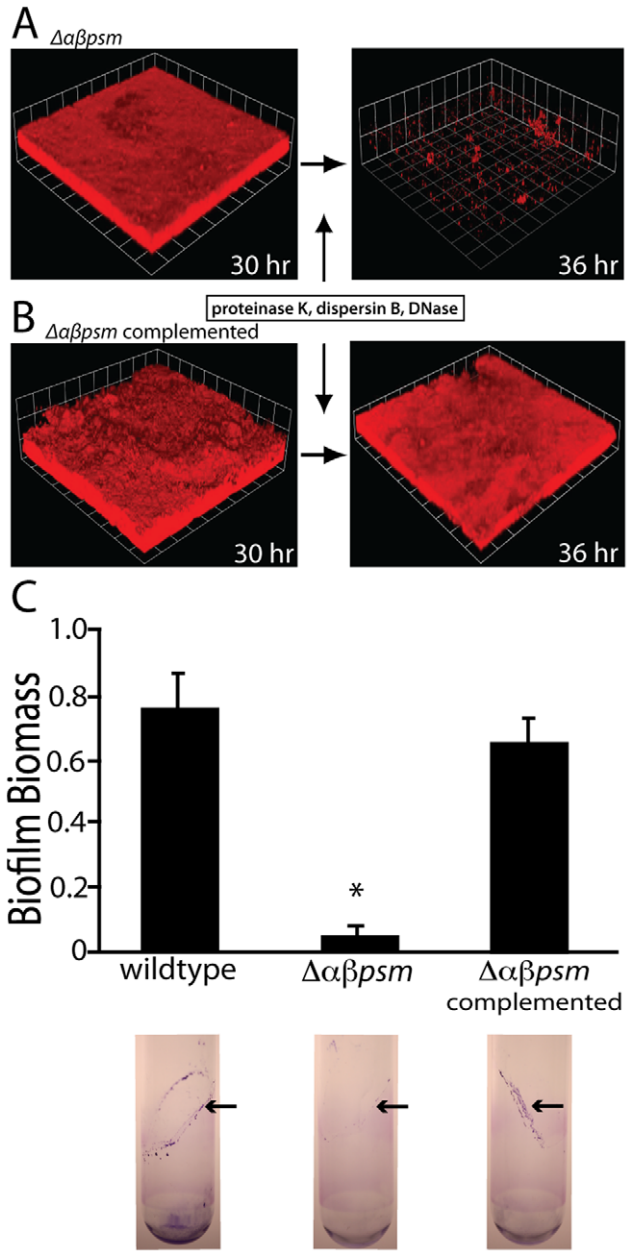


Figure 2.7, An *αβPSM* mutant forms biofilms susceptible to disassembly by matrix degrading enzymes and mechanical stress.

Confocal micrographs of Δ$\alpha\beta$*psm* mutant (A) (strain BB2388) versus complemented mutant expressing α and β*psm* operons *in trans* (B) (strain BB2408) flow cell biofilms grown for 30 hours prior to proteinase K, dispersin B, and DNaseI exposure (at 0.2 μg/mL each). Images are representative of three separate experiments and each side of a grid square represents 20 μm. (C) Analysis of biofilm development at the air-liquid interface of test tube cultures in PNG media after vortexing. Graph shows quantification of biofilm biomass (OD A_{595}) and images below show stained test tube biofilms. * P< 0.005 compared to wildtype.

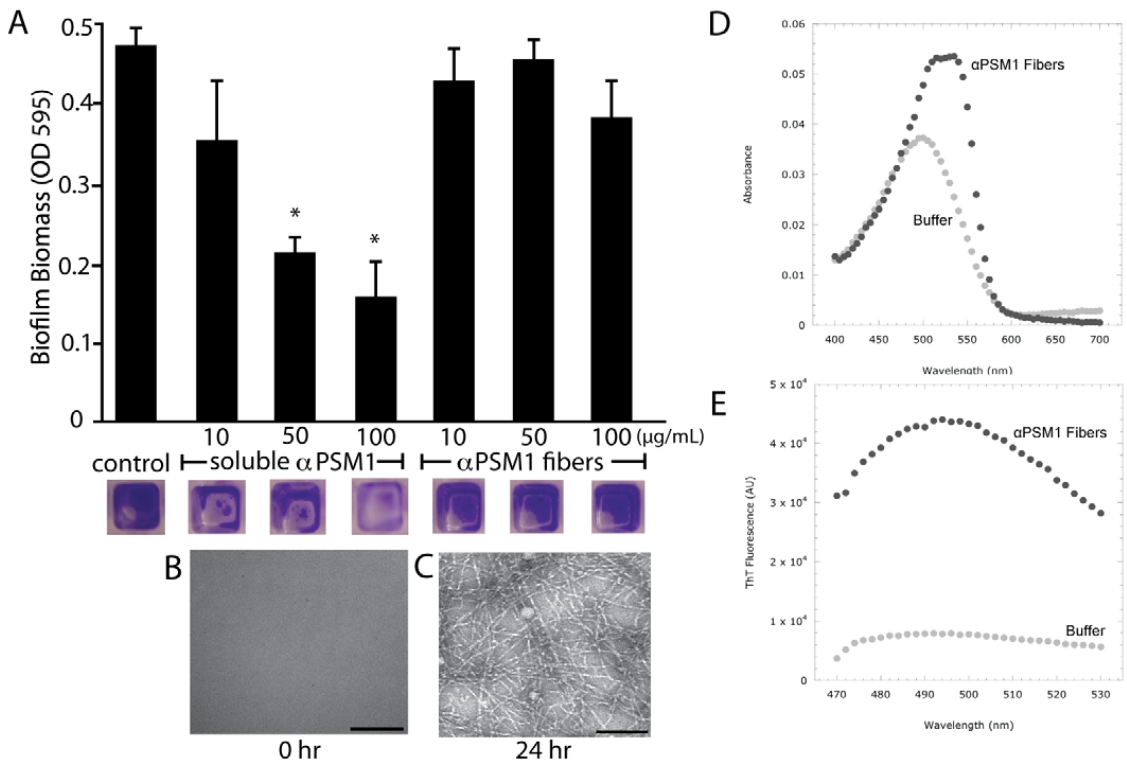


Figure 2.8 , Amyloid fiber formation modulates PSM activity.

(A) *S. aureus* wildtype biofilms were grown in microtiter plates for 24 hours then washed and exposed to increasing concentrations of soluble αPSM1 or αPSM1 fibers at concentrations of 10, 50 or 100 μg/mL for six hours. Biofilms were then washed, stained and remaining biofilm biomass was visualized (images of wells below graph) and quantitated (OD at A_{595}). (B & C) TEM micrographs of αPSM1 samples used in the experiment demonstrate the absence (B) and presence (C) of fibers. * P<0.002 compared to control no αPSM1 treatment. We verified that αPSM1 fibers bind CR (D) and ThT (E) similar to amyloid fibers.

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Chapter 3

The AgrD N-terminal leader peptide of *Staphylococcus aureus* has cytolytic and amyloidogenic properties.

Abstract

Staphylococcus aureus virulence is coordinated through the Agr quorum sensing system to produce an array of secreted molecules. One important class of secreted virulence factors is the phenol soluble modulins (PSMs). PSMs are small peptide toxins that have been recently characterized for their roles in infection, biofilm development, and subversion the host immune system. In this work, we illustrate that the signal peptide of the *S. aureus* quorum-sensing signal, AgrD, shares structural and functional similarities with the PSM family of toxins. Efficacy of this peptide (termed "N-AgrD") outside of AgrD propeptide trafficking has never before been described. We observe that N-AgrD, like the PSMs, is found in amyloid fibrils of *S. aureus* biofilms, and is capable of forming and seeding amyloid fibrils *in vitro*. N-AgrD displays cytolytic and pro-inflammatory properties that are abrogated after fibril formation. These data suggest that the N-AgrD leader peptide affects *S. aureus* biology in a manner similar to that previously described for the PSM peptide toxins. Taken together, our findings suggest that peptide cleavage products can affect cellular function beyond their canonical roles, and may represent a class of virulence factors warranting further exploration.

Introduction

Staphylococcus aureus is a common constituent of the human microflora, living commensally on the skin or in the anterior nares of approximately one third of the population (1, 2). Despite this typically innocuous relationship, *S. aureus* can cause diseases ranging from benign skin conditions to fatal systemic infections (3-6). The severity of a *S. aureus* infection is partially attributed to toxins produced by the infecting strain, which include superantigens, α-toxin, leukocidins and other small peptide toxins like phenol-soluble modulins (PSMs) (7-12). PSMs are of particular interest because they perform multiple roles in pathogenesis. These short peptides compound pathogenicity by activating host receptor-mediated inflammatory responses (12-17), lysing red and white blood cells (16, 18, 19), altering biofilm development (20-23), and also by acting as antimicrobial agents against niche-occupying organisms in the host (24-27).

Our group has described previously that PSMs enhance *S. aureus* biofilm structures, likely through coordinated aggregation into amyloid structures (22). Amyloid aggregates are robust fibril structures produced through the self-seeded auto-aggregation of monomeric units. Mature amyloid fibrils display a characteristic cross β-sheet structure and are strongly resistant to chemical and enzymatic degradation (28, 29). Biofilms that produced PSM fibrils were resistant to dispersal by matrix degrading enzymes, surfactants, and mechanical disruption. Soluble PSMs dispersed biofilms, while pre-aggregation of PSMs into amyloid fibrils abolished this dispersal activity (22).

The *S. aureus* accessory gene regulatory (Agr) quorum sensing system is associated with toxin production during aggressive acute infections, while in several animal models *agr* mutants are less virulent (3, 5, 10, 30-32). Agr directly regulates the expression of all three characterized PSM types (*PSMα1–4, PSMβ1–2, and* δ-toxin) (13, 33). The *agrBDCA* operon encodes the core components of the quorum-sensing system, while the divergently transcribed RNAIII is a regulatory RNA and the primary effector of the *Agr* regulon. Briefly, the Agr quorum sensing circuit functions through the AgrCA two-component sensory complex, which detects and responds to the autoinducing peptide (AIP) signal derived from AgrD (30, 31, 34). AgrA binds to promoters P2 and P3 to upregulate production of Agr machinery and the RNA III response element. It was thought that P2 and P3 were AgrA's exclusive targets until it was demonstrated that AgrA binds to promoters upstream from the PSMα and PSMβ operons (33). Thus, Agr and the PSMs are linked through their shared transcriptional regulation.

AgrD is translated as a propeptide composed of three parts: an N-terminal amphipathic leader, a middle region of eight residues that is processed into the final AIP structure, and a charged C-terminal tail (Fig. 1F) (31). The N-terminal region of AgrD is essential for directing the propeptide to the cell membrane (35) , where the integral membrane endopeptidase AgrB removes the C-terminal tail, catalyzes thiolactone ring formation, and transports the AgrD-AIP intermediate across the cell membrane (34, 36). Once this intermediate peptide is secreted from the cell, the SpsB signal peptidase cleaves the N-terminal peptide to release the active form of AIP, leaving the leader peptide associated with the cytoplasmic membrane (37, 38). Thus, the current model of AIP processing suggests that the N-terminal leader fragment is

essential for targeting the propeptide to the cell membrane, but the fate of the peptide after AIP processing is unknown.

Here we provide evidence for the first time that the N-terminal amphipathic leader of the AgrD propeptide (termed N-AgrD) has multiple properties that are similar to the PSM peptides. N-AgrD is present in *S. aureus* amyloid fibrils during biofilm growth, and is capable of autoaggregation and of seeding amyloid polymerization of PSM peptides *in vitro*. Soluble N-AgrD is cytolytic against human cells and displays pro-inflammatory activity. These findings demonstrate a novel biological role for a leader peptide beyond trafficking, and may provide additional context into staphylococcal PSM biological function.

Materials and Methods

Bacterial Strains and Growth Conditions. *S. aureus* strain, LAC (MRSA USA300-0114) was the wildtype strain used in this study (39). Construction of the pAgrBD plasmid was described previously (40). Plasmids pAgrBD and empty vector pEPSA5 were transformed into a *S. aureus LAC agr- mutant* by electroporation to create strains BB2933 (LAC *agr*$^-$ + pAGRBD) and BB2945 (LAC *agr*$^-$ + pEPSA5).

Strains were routinely grown in trypic soy broth (TSB) incubated at 37 °C with 200 rpm shaking unless otherwise noted. Carbon limited growth media (CLM) for detection of N-AgrD in culture supernatants consisted of: glucose (75 mM), ammonium sulfate (7.5 mM), potassium phosphate (33 mM), di-potassium phosphate (60 mM) supplemented with NaCl (11 mM), KCl (2

mM), Casamino Acids (BD Biosciences) (0.5%), $MgSO_4$ (0.1 mM), and the vitamins nicotinamide (500 μg/liter), thiamine (500 μg/liter), pantothenate (500 μg/liter), and biotin (0.3 μg/liter) (40).

Biofilm Experiments, amyloid fibril isolation, mass spectrometry and microscopy. Drip-flow biofilms were grown in 3.3 g/L peptone, 2.6 g/L NaCl, 3.3 g/L glucose (PNG media) as previously described (22, 41). Amyloid fibrils were collected after 5 days of growth. Briefly, biofilms were scraped into 3 mL of potassium phosphate buffer (50 mM, pH 7) and homogenized (TissueMiser, Fisher) to shear fibrils from the cell walls. Supernatants were clarified by repeated centrifugation at 13,000 RPM for 2 minutes to remove cells. The cell-free supernatant was incubated in 200 mM NaCl and the fibrils were isolated using Millipore Amicon Ultra Centrifugal Filter Units with a pore size of 100 kDa.

Fibril protein components were identified by LC-MS/MS after digestion with pepsin (MS Bioworks, Ann Arbor, MI) as previously described (22). The value for the abundance measurement is the Normalized Spectral Abundance Factor (NSAF).
Transmission electron microscopy (TEM) was performed using a Philips CM12 transmission electron microscope. Samples prepared for TEM imaging were spotted onto formvar-coated copper grids, incubated for 5 minutes, washed with sterile ddH_2O, and negatively stained with 2% uranyl acetate for 60 seconds.

To detect the presence of N-AgrD in culture supernatants, cultures were grown in 100 ml CLM for 24 hours at 37°C and cells were removed by centrifugation and passage of supernatant

through a 0.22 micron filter. The supernatant was then concentrated 1000 fold by TCA precipitation and resuspended in water. Following trypsin digestion samples were analyzed by nano-LC-MS/MS (MS Bioworks, Ann Arbor, MI).

PSM Polymerization Experiments. N-AgrD and PSMα1 peptides were synthesized by Lifetein (South Plainfield, NJ) and assayed to be >90% pure by HPLC. Synthetic peptides were prepared and assayed as previously described to eliminate large aggregates from lyophilization prior to assay (22, 42). The amino acid sequence of the N-AgrD peptide was MNTLFNLFFDFITGILKNIGNIAA ; the scrambled N-AgrD peptide was LGAAFNMNLINFDFTGIFNKLTII, and the sequence of the PSMα1 peptide was MGIIAGIIKVIKSLIEQFTGK. Each lyophilized peptide stock was dissolved directly into hexafluoroisopropanol (HFIP) to a concentration of 0.5 mg/mL and briefly vortexed. Peptide stocks were aliquoted into microcentrifuge tubes and the HFIP solvent was removed by speedvac at room temperature. Dried peptide stocks were stored at −80°C.

All amyloid dye-binding assays were performed in 96-well black opaque, polystyrene, TC-treated plates (Costar 3603, Corning). Immediately prior to assay, dried peptide stocks were thawed and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL. Freshly dissolved peptides were diluted into sterile ddH_2O containing 0.2 mM Thioflavin T (ThT) and assayed at room temperature. Fluorescence was measured every 10 minutes after shaking by a Tecan Infinite M200 plate reader at 438 nm excitation and 495 nm emission. ThT fluorescence

during polymerization was corrected by subtracting the background intensity of an identical sample without ThT. Assays were repeated at least twice.

Congo red (CR) absorbance scans were performed on polymerized peptides that were allowed to polymerize for 24 h in ddH_2O. Samples were incubated in 0.001% (w/v) CR in ddH_2O for 30 minutes prior to assay on a Tecan Infinite M200 plate reader. CR scans were corrected by subtracting the background intensity the identical sample before addition of dye. Assays were repeated at least twice.

Circular dichroism spectroscopy. Treated peptide stocks were thawed and dissolved in hexafluoroisopropanol (HFIP) to a concentration of 10 mg/mL and incubated on ice for 20 minutes. Samples 15 μM peptide were made by diluting dissolved peptide into 500 μL sterile ddH_2O immediately prior to assay. Far UV circular dichroism (CD) measurements were performed with a Jasco-J810 spectropolarimeter using quartz cells with 0.1 cm path length. CD spectra between 190 and 260 nm were recorded in millidegrees and converted to molar ellipticity. The average of five scans was recorded at 25°C using a 2 nm bandwidth with a 20 nm min^{-1} scanning speed. CD scans were performed over the course of 20 hours. Triplicate samples showed similar ellipticity patterns.

Hemolysis Assays. The collection of blood from human subjects was approved by the University of Michigan Institutional Review Board, approval number IRB00001995. Human whole blood was washed in PBS and diluted to a final concentration of 1:25 (v/v) in PBS. 100 μL of blood was

then placed into individual wells of a flat-bottom 96-well microtiter plate (Costar 3596, Corning).

PSMα1 and N-AgrD synthetic peptides resuspended in DMSO were added directly to the wells at indicated concentrations, and the mixture was incubated for 60 min at 37°C. After incubation, plates were centrifuged at 500 × g for 10 min, and an aliquot of supernatant (100 μL) was transferred to a separate microtiter plate to measure hemoglobin absorbance at 450 nm on a Tecan Infinite M200 plate reader. Assays were repeated in triplicate

Overnight cultures of *S. aureus* containing plasmids encoding pAgrBD or pESPA5 were diluted 1:100 in 5 ml of TSB containing indicated levels of xylose. Cells were grown by shaking at 37°C to an optical density of 2.00 at 600 nm. Supernatants were then prepared by passing through 0.22-μm filters. 10 μL of supernatant was then added to 100 μL of blood and incubated for 60 min at 37°C. After incubation, plates were centrifuged at 500 *× C* for 10 min, and an aliquot of supernatant (100 ical density of 2.00 at separate microtiter plate to measure hemoglobin absorbance at 450 nm on a Tecan Infinite M200 plate reader.

Lysis of human neutrophils. Venous blood was collected from healthy human volunteers (approved by the University of Michigan Institutional Review Board, approval number IRB00001995) using 0.2% EDTA as an anticoagulant. Neutrophils were isolated by sequential centrifugation in Ficoll Paque Plus (GE Healthcare), and hypotonic lysis of erythrocytes, as previously described (12, 18). Briefly, 3.7 μM of either PSMα1 or N-AgrD synthetic peptide was added to wells of a 96-well microtiter plate containing 10^6 neutrophils, and plates were

incubated at 37°C for 2 h. Neutrophil lysis was determined by quantitating the release of lactate dehydrogenase (LDH) (Cytotoxicity Detection Kit, Roche Applied Sciences). Assays were repeated in triplicate.

Measurement of IL-8 production and neutrophil chemotaxis. Neutrophil chemotaxis in the presence of 2 μM N-AgrD or PSMα1 synthetic peptide was analyzed using a QCM 3 μm chemotaxis assay kit (Millipore #ECM504). Briefly isolated human neutrophils were subjected to a brief hypotonic shock with pyrogen-free water (Sigma), washed, and suspended at 5×10^6 cells/ml in HBSS containing 0.05% human serum albumin. Chemotaxis of neutrophils was determined by using fluorescently-labeled neutrophils that migrated through a membrane fitted into an insert of a 24-well microtiter plate transwell system containing a prewetted 3-μm-pore-size polycarbonate filter. Measurement of IL-8 production in human neutrophils after exposure to 6 μM N-AgrD or PSMα1 synthetic peptide was performed as described using a commercial ELISA assay kit (R&D systems) according to the manufacturer's instructions. Statistics were performed using a 1-way analysis of variance (ANOVA).

RESULTS

N-AgrD peptide is present in purified amyloid fibrils

Our previous work demonstrated that *S. aureus* laboratory strain SH1000 produced amyloid fibrils composed primarily of PSMs (22). In the current study, we sought to investigate whether a clinical isolate produced amyloid fibrils of a different composition than our common lab strain. For this work we utilized CA-MRSA USA300 strain LAC, which exhibits robust Agr activity

producing high levels of PSMs (12, 43). When viewed by transmission electron microscopy (TEM), LAC biofilms also produced substantial quantities of extracellular fibrils that were readily isolated from cells (Figs. 1A & 1B). LC-MS/MS analysis of LAC biofilm fibrils confirmed that PSM species were associated with the amyloid fibrils but we also detected substantial quantities of the AgrD N-terminal leader peptide in our fibrils isolations (Figs. 1C & 1F)(22). This finding prompted our investigation to determine the relevance of the N-terminal AgrD peptide in *S. aureus* biofilms.

N-AgrD displays amyloid-like characteristics

An alignment of the N-AgrD amino acid sequence to PSMα1 showed distinct similarity to PSMα1 (Fig. 1D). N-AgrD has also been predicted to adopt an amphipathic α-helical structure that is similar to the PSMs (12, 38) (Fig. 1E). These striking primary and secondary structural similarities, combined with the observation that N-AgrD is associated with fibrils in biofilms, led us to speculate that N-AgrD may contribute to amyloid formation.

The cross β-sheet architecture of mature amyloid fibrils can be detected using several biochemical assays, including amyloid specific dyes and circular dichroism (28, 29, 44, 45). To test whether N-AgrD could form amyloids, we assayed synthetic N-AgrD peptide for its capacity to autoaggregate using an *in vitro* Thioflavin T (ThT) fluorescence assay (46). Amyloid aggregation generally occurs in two phases — 1) a lag phase, during which monomers associate into oligomeric nuclei, and 2) an exponential polymerization phase, during which mature amyloid fibrils are formed (a shift in ThT fluorescence occurs as it binds to amyloid fibrils, and

fluorescence levels will increase over time as more monomers adopt the amyloid fold in solution) (46, 47). Over a 12 hour time course, synthetic N-AgrD displayed an initial lag phase followed by a concentration-dependent increase in ThT fluorescence indicative of amyloid assembly (Fig. 2A)(46). We verified the presence of amyloid aggregates at the end of the time course by taking transmission electron microscopy (TEM) images of N-AgrD 24 hours post-inoculation. We observed fibrils with a diameter of ~12 microns, consistent with amyloid fibril morphology (Fig. 2B). A scrambled peptide control (sN-AgrD) did not display ThT fluorescence, and did not form fibrils, indicating that amyloid aggregation is sequence-dependent (Figs. 2C & 2D).

To further demonstrate that the observed fibrils were amyloids, we incubated N-AgrD fibrils with another amyloid-binding dye, Congo Red (CR). A characteristic absorbance "red shift" at ~ 515 nm was observed relative to a buffer control, which is indicative of the cross-β structure of amyloid fibrils (Fig. 2E)(48). To confirm that N-AgrD was adopting a β-sheet conformation after aggregation, we used circular dichroism (CD) to assay the secondary structural changes of synthetic N-AgrD over time. As predicted, freshly dissolved peptide displayed a change in peak absorbances consistent with a transition to increased in β-sheet content over time (Fig. 2F).

Considering the sequence similarity shared between N-AgrD and PSMα1 (Fig. 1), these observations suggest a potential role for both peptides in biofilms. These biochemical and biophysical data were consistent with our hypothesis that N-AgrD is capable of forming amyloid

fibrils (Fig. 2). We next aimed to investigate the biological relationship between N-AgrD and the PSM amyloids.

N-AgrD is capable of seeding PSM amyloid polymerization

Autoaggregation of amyloid proteins is rate-limiting, but aggregation is accelerated with the addition of oligomers or preformed fibrils called "seeds" (49, 50). We asked whether polymerized N-AgrD could seed its own amyloid formation or seed the aggregation of PSM peptides (Fig. 3). Addition of 5% (w/v) N-AgrD fibrils (seeds) to freshly resuspended soluble N-AgrD reduced the lag phase and increased the relative ThT fluorescence intensity (Fig. 3A). 5% N-AgrD seeds alone displayed negligible fluorescence throughout the duration of the experiment. A similar lag-reduction was observed when 5% N-AgrD seeds were added to freshly resuspended PSMα1 (Fig. 3B). These findings demonstrate that N-AgrD can seed *in vitro* amyloid formation of both itself and PSMα1.

Soluble N-AgrD has cytolytic activity against human cells

PSMs are cytolytic against several host cell types – a quality contributing to *S. aureus* infection (12, 18). Cytotoxicity is attributed to PSM peptides found as soluble species found in the supernatants of liquid cultures (51, 52). Thus, and we used LC-MS/MS to verify that N-AgrD is also found in culture supernatants. Indeed, we detected N-AgrD (full length: MNTLFNLFFDFITGILKNIGNIAA, and two fragments: FITGILKNIGNIAA and ILKNIGNIAA) in cell-free supernatants from *S. aureus* LAC grown in a minimal media to late stationary phase (data not shown).

We next tested synthetic N-AgrD peptide for cytolytic activity (Fig. 4). Using an LDH assay as an indicator of cell lysis, we found that exposure of neutrophils to 3.7 μM of N-AgrD or PSMα1 induced neutrophils lysis after a 90 minute incubation (Fig. 4A). N-AgrD, also lysed human red blood cells, resulting in 64% hemoglobin release (Fig. 4B). To determine whether amyloid formation abrogated this effect, we compared N-AgrD and PSMα1 fibrils lytic activity. Interestingly, we observed these fibrils displayed a pronounced reduction in cytolysis against neutrophils and red blood cells relative to freely soluble peptides (Figs. 4A & 4B). Taken together, these findings suggest that N-AgrD may act as a virulence factor by lysing human cells, and that this activity can be modulated by the ordered aggregation of N-AgrD and PSMs into amyloid fibrils.

To test N-AgrD's cytotoxicity in a biological context, we overexpressed AgrD from a plasmid containing an *agrBD* construct on a xylose-inducible promoter in an *agr* mutant background (strain BB2933). *agrB* was included in this construct because it is necessary for proper processing and secretion of the AgrD propeptide to release the N-terminal leader peptide (34, 53). Compared to the empty vector control, expression of *agrBD* resulted in increased lysis of red blood cells (Fig. 4C).

N-AgrD displays pro-inflammatory properties

PSMs can induce neutrophil pro-inflammatory responses like neutrophil chemotaxis and IL-8 cytokine release (12-14). Since N-AgrD displayed cytolytic activity against host cells (Fig. 4), we

examined the pro-inflammatory response to N-AgrD peptides. Human neutrophils displayed a similar chemotactic response upon exposure to soluble N-AgrD and PSM peptides, including both PSMα1 and δ-toxin (Fig. 5A). However, polymerization of N-AgrD and PSMα1 peptides prior to assay abrogated this neutrophil chemotactic activity (Fig. 5A).

Next, we assayed the production of cytokine IL-8 in human neutrophils after exposure to N-AgrD and PSM peptides. We observed a robust induction of IL-8 production in response to soluble N-AgrD and PSMα1 that was significantly reduced in polymerized peptide samples (Fig. 5B). These findings suggest that N-AgrD promotes a pro-inflammatory response similar to that induced by known PSMs, and further promotes the idea that N-AgrD should be considered a PSM-like peptide in structure and activity.

Discussion

PSM peptides are major determinants of *S. aureus* virulence that possess cytolytic and immune modulating activities (12, 14, 15, 52). The high levels of PSMs produced by CA-MRSA strains like USA300 is thought to contribute to the increased virulence of these strains (43). We previously demonstrated that, under certain growth conditions, PSMs are capable of forming amyloid fibrils that protect *S. aureus* biofilms from disassembly (22). In the present study, we identify the N-terminal secretion signal peptide of AgrD as a PSM-like peptide and a supplementary component of the amyloid fibrils produced in clinically relevant CA-MRSA biofilms.

The N-AgrD peptide displays structural and functional similarity to the canonical PSM family of peptides (Figs. 1 & 2). We have found that N-AgrD can autoaggregate to form fibrils, and that pre-aggregated N-AgrD can seed amyloid formation of both soluble PSMα1 and N-AgrD (Figs. 2 & 3). N-AgrD is present in both biofilms and the planktonic supernatants of *S. aureus* LAC bacterial cultures, and also shares many biophysical characteristics with the PSMs and other amyloid proteins (Figs. 1-3) (22, 28). Amyloid toxicity is often attributed to the presence of reactive intermediate species (54, 55) and here we present evidence that the cytotoxic activity of N-AgrD and PSMα1 can also be modulated through amyloid aggregation (Fig. 4). We observed high levels of cytolytic and pro-inflammatory activity from soluble forms of N-AgrD and PSMα1 peptides that was inhibited after aggregation (Figs. 4 & 5). Because of the striking similarities between PSMα1 and N-AgrD in form and function, we propose that N-AgrD should be considered a new member of the PSM family.

Aside from its role in cellular trafficking, no other activity has been associated with N-AgrD. Like many signal peptides, it was considered a waste product that is simply discarded into the extracellular milieu, or associated with the cellular membrane (38, 56, 57). We provide evidence that the N-terminal signal peptide is not a waste product derived from AIP processing, but instead appears to possess qualities similar to other peptide virulence factors known to be beneficial for the *S. aureus* lifecycle.

Functions of other N-terminal leader peptides have been described in the literature. The N-terminal region of the Pantone Valentine Leukocidin (PVL) was found to promote *S. aureus*

cellular adhesion to extracellular matrix components like heparan (58). *Enterococcus* spp. utilize a distinctive set of sex signaling pheromones that are derived from the signal peptides of lipoproteins (59, 60). In eukaryotic systems, signal peptides processed at the endoplasmic reticulum promote calmodulin signaling (61) and promote the inflammatory response (62). Many viruses, including the Hepatitis C virus, cytomegalovirus, and foamy virus, utilize cleaved signal peptides to assist in cellular targeting and virus maturation (63-65). Signal peptides are being considered as novel targets for vaccine development, owing to their unique biology (66). There is even evidence that signal peptides can sequester misfolded proteins at the ER membrane to prevent toxicity (67), suggesting a potential role for N-AgrD in seeding amyloid fibril aggregation at the *S. aureus* cellular membrane.

Based on these published accounts, we speculate that N-AgrD, like other leader peptides previously thought only to direct protein trafficking, may also serve additional roles outside of the organism after processing at the cellular membrane.

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Figures

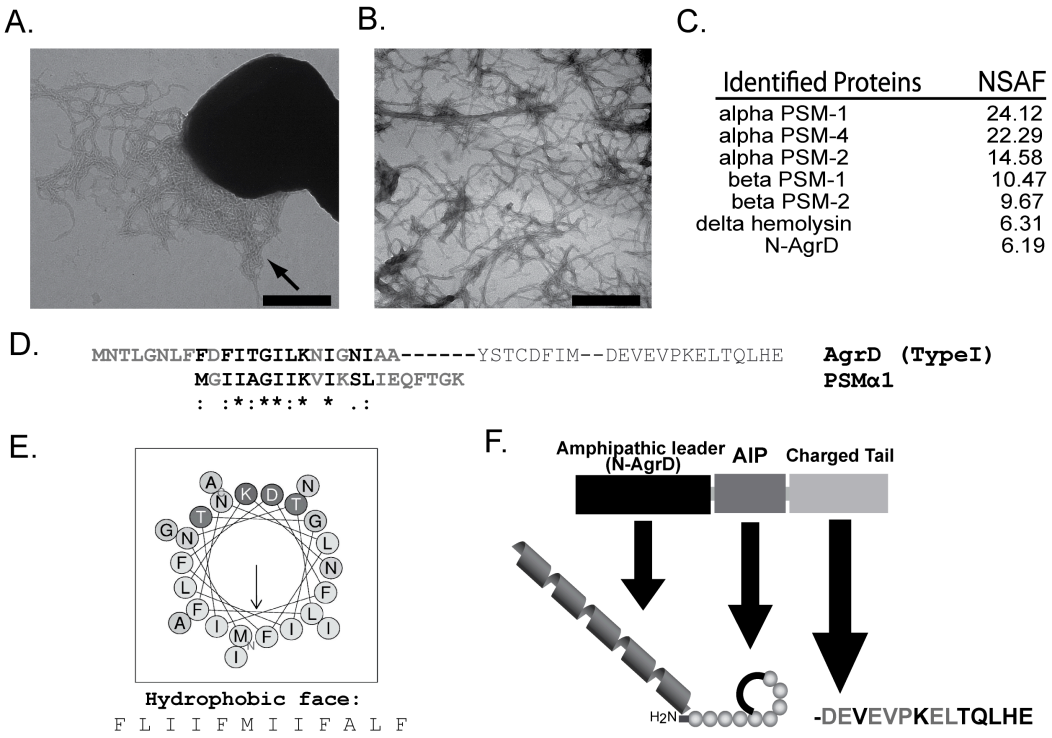

Figure 3.1 , *S. aureus* USA300 strain LAC displays robust amyloid fibril formation.
Fibrils extracted from biofilms contained high levels of phenol soluble modulins the N-terminus region of AgrD (N-AgrD). (A) Transmission electron micrograph (TEM) illustrating fibril formation (indicated by arrow) of *S. aureus* strain LAC after 5 days of biofilm growth. Bar indicates 500 nm. (B) TEM of purified fibrils that were next subjected to mass spectrometry (MS) analysis. Bar indicates 500 nm. (C) Peptide species identified from duplicate fibril isolations via MS analysis and their relative abundance factors within the sample (NSAF). (D) ClustalW sequence alignment illustrating the similarity of primary structure shared between the N-terminal region of AgrD and PSMα1 (E) Predicted arrangement of N-AgrD in an α-helical wheel showing amphipathy that is typical of PSMs (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py) (F) Graphic illustration of AgrD propeptide domains, including the amphipathic leader sequence, the autoinducing peptide (AIP), and the C-terminal charged tail (figure illustrates Agr Type I with residues conserved amongst Agr types lightened).

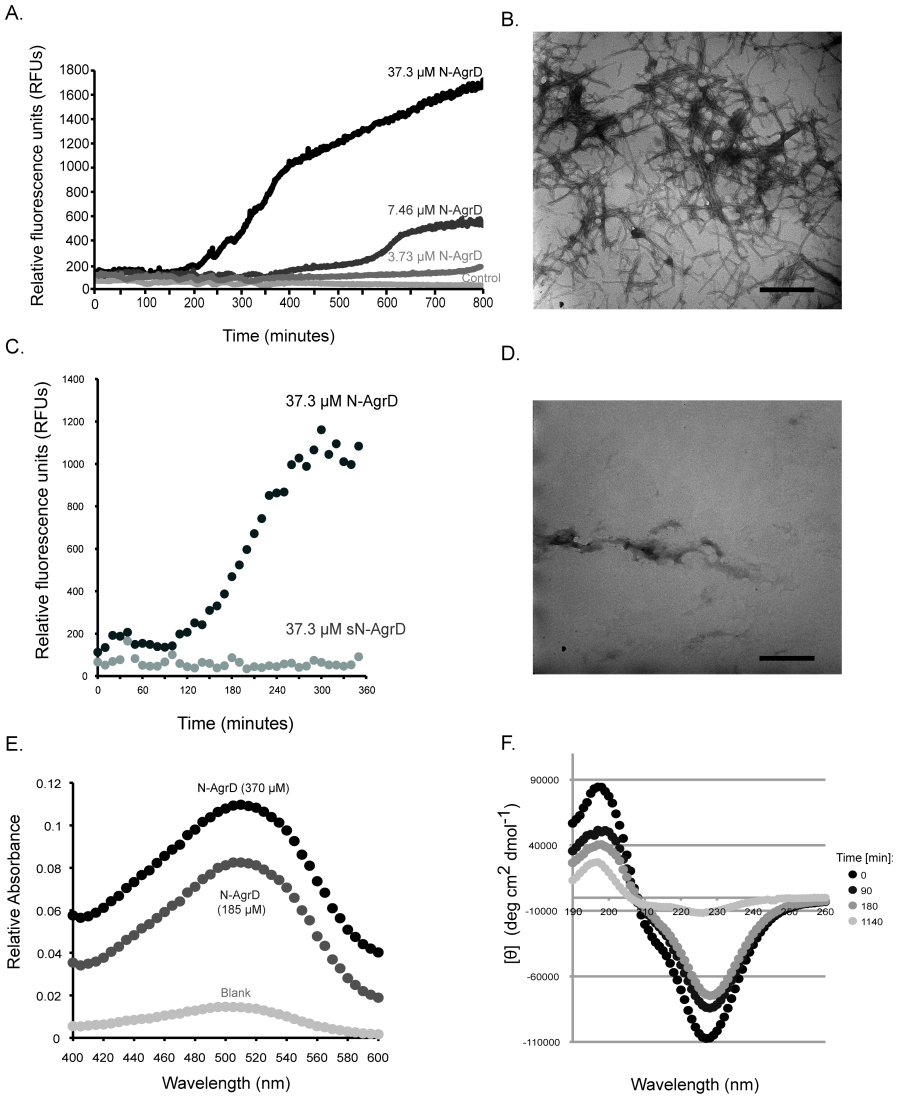

Figure 3.2, N-AgrD possesses amyloid-like characteristics.

A) Thioflavin T (ThT) assay which monitors amyloid polymerization kinetics with synthetic N-AgrD peptide. Normalized fluorescence intensity is shown for N-AgrD at indicated concentrations incubated with 2 mM ThT (•) 37.3 µM; (•) 7.46 µM; (•) 3.73 µM ; (•) no peptide control. B) TEM of 37.3 µM N-AgrD after 24 hours in solution. Bar indicates 500 nm. (C) A scrambled version of the N-AgrD peptide (labeled "sN-AgrD") (•) does not display amyloid ThT fluorescence relative to intact N-AgrD (•). Peptides were assayed at a concentration of 37.3 µM. (D) TEM of 37.3 µM scrambled N-AgrD after 24 hours in solution. Bar indicates 500 nm. (E) Congo Red (CR) absorbance of N-AgrD peptide displays a concentration-dependant characteristic absorbance increase 515 nm at (•) 370 µM and (•) 185 µM relative to (•) a CR only. (F) CD spectroscopy of 15 µM N-AgrD peptide transitioning to β-sheet conformation over time.

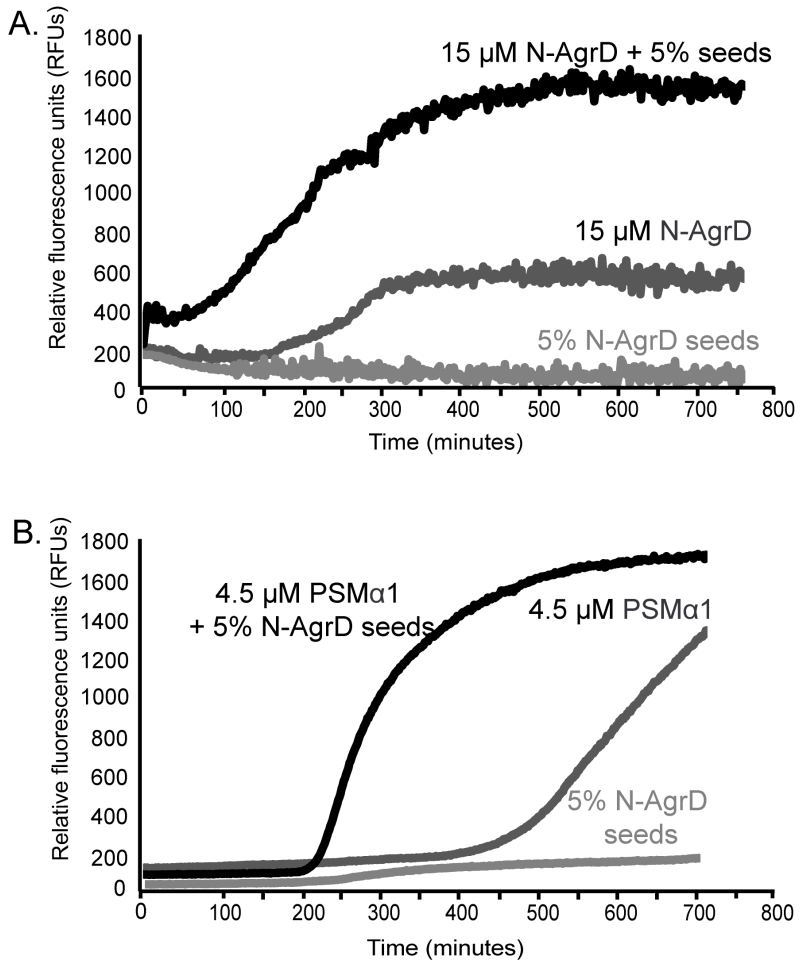

Figure 3.3, N-AgrD amyloid fibrils are capable of seeding amyloid formation.

(A) ThT polymerization assay of 15 μM N-AgrD synthetic peptide in the presence (•) or absence (•) of 5% (w/w) sonicated N-AgrD seeds (•) (B) ThT polymerization assay of PSMα1 peptide at 4.5 μM in the presence (•) or absence (•) of 5% (w/w) sonicated N-AgrD seeds (•).

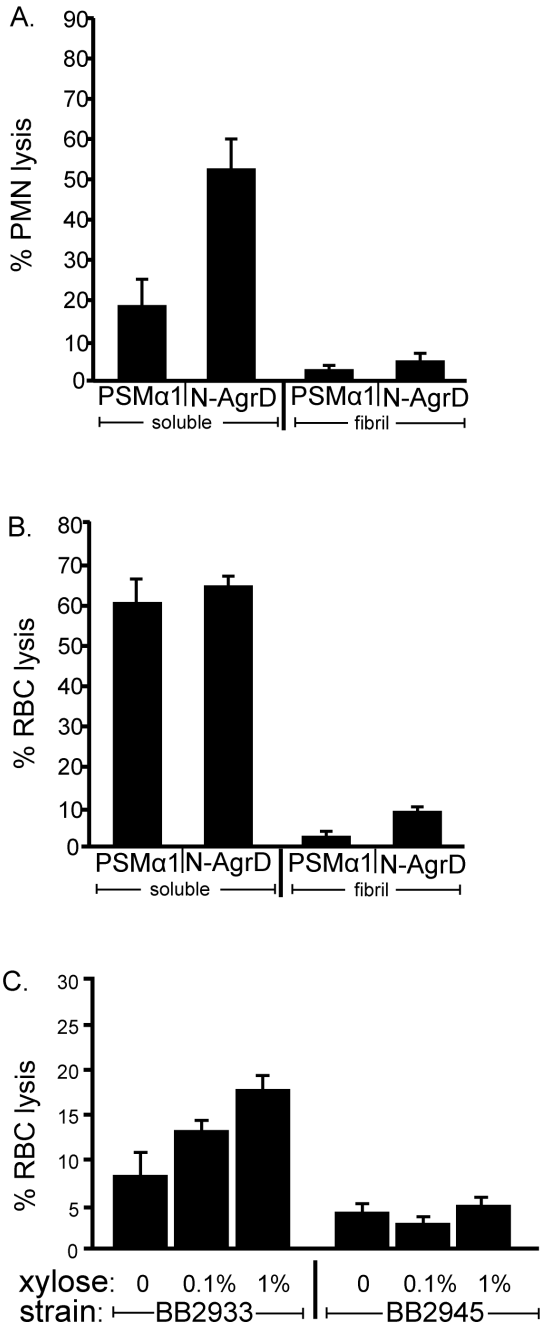

Figure 3.4, Soluble N-AgrD possesses cytolytic activity against human cells.
(A) Neutrophil lysis by 3.7 μM N-AgrD or PSMα1 after 90 minutes exposure. (B) Red blood cell lysis by 3.7 μM N-AgrD or PSMα1 for 90 minutes. For both assays N-AgrD and PSMα1 were analyzed in both their soluble versus polymerized states. (C) Lysis of red blood cells after exposure to cell free *S. aureus* supernatants from strains BB2933 (*agr*$^-$ + pAgrBD) and BB2945 (*agr*$^-$ + pEPSA5 (empty vector)).

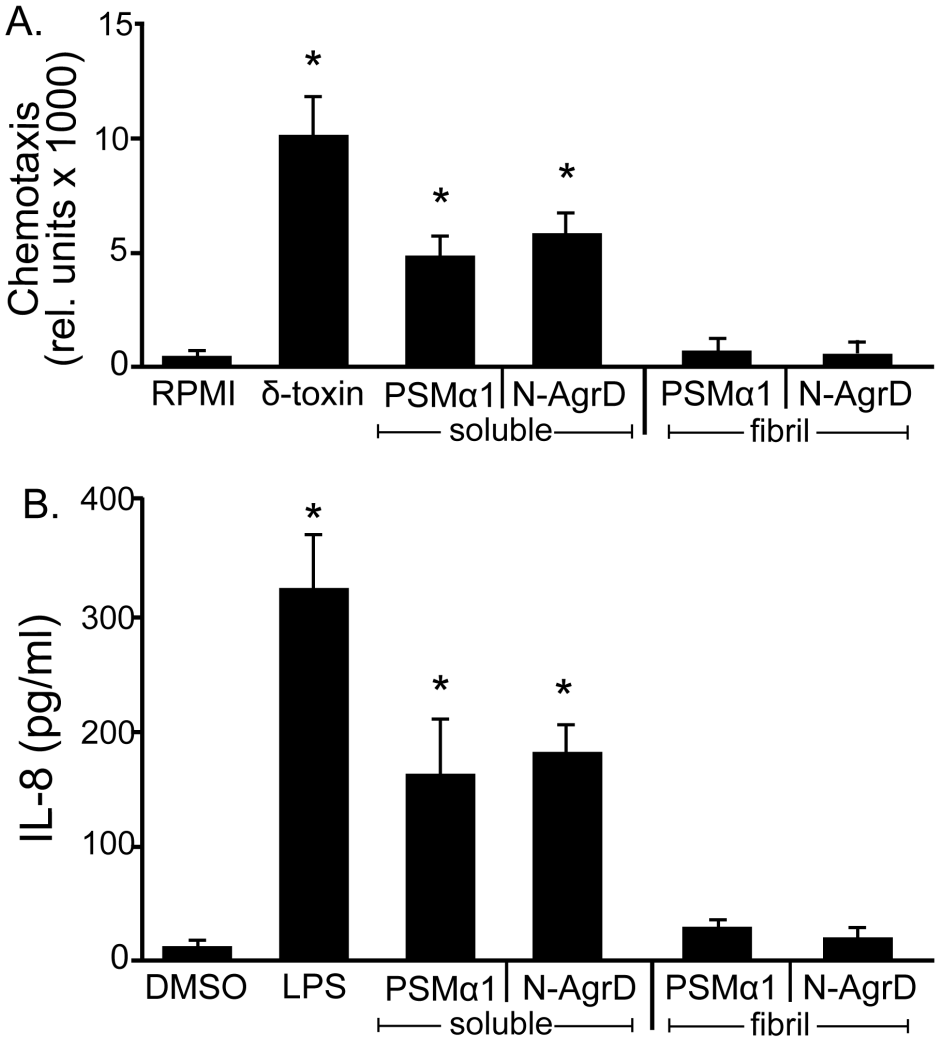

Figure 3.5. N-AgrD and PSMα1 display pro-inflammatory activity.

(A) Chemotaxis of human neutrophils. Peptides were applied at a concentration of 2 μM; sN-AgrD refers to the scrambled peptide LGAAFNMNLINFDFTGIFNKLTII. Error bars show standard error of the mean; * P<0.01 comparing samples to RPMI media control (B) Neutrophil secretion of IL-8 after treatment with N-AgrD or PSMα1 at a concentration of 6 μM. LPS was used at 10 ng/ml. For both assays N-AgrD and PSMα1 were analyzed in both soluble versus polymerized states. Error bars show standard error of the mean; * P<0.01 comparing samples to DMSO control.

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Chapter 4

DNA mediates *Staphylococcus aureus* PSM amyloid aggregation

Abstract

Invasive *Staphylococcus aureus* strains produce large concentrations of phenol soluble modulin (PSM) toxic peptides. PSMs serve to recruit, activate and subsequently lyse human neutrophils. Amyloid fibril formation inhibits PSM toxicity, demonstrating that autoaggregation of small peptide species can switch off virulence associated with freely diffusible peptides. The mechanisms that regulate PSM amyloid aggregation *in vivo* are not known. Autoaggregation of PSMs into amyloid fibers was previously shown to abrogate biofilm dispersal activity caused by soluble peptides. We speculated that extracellular DNA produced during biofilm formation may promote amyloid assembly. Using a combination of techniques, we demonstrate that *PSMα1*, a main component of *S. aureus* biofilm fibrils, interacts with DNA to form large aggregates. These aggregates were confirmed to be amyloids, and have fibril morphology. Finally, we demonstrate that PSMα1 displays reduced toxicity when incubated with DNA, which supports prior evidence suggesting amyloid aggregation is control point for virulence in *S. aureus* biofilms. These findings demonstrate that amyloid formation modulates PSM activity in the microenvironment of the biofilm and beyond.

Introduction

Microorganisms can survive in dynamic environments as multicellular communities, altering their environment through the coordinated secretion of biomolecules to resist environmental stressors. Multicellular communities encased in a self-produced matrix are called biofilms [1]. Biofilm structure and composition varies greatly. The progression of biofilm development and the components used to construct these structures also change over time, and are largely dependent on cues received from the external environment. We observe this phenomenon in the diversity of macrostructures associated with variable biofilm phenotypes [1]. The variability observed in biofilm construction and composition highlights the fact that biofilms can form through numerous mechanisms. The biophysical interactions that occur within the biofilm matrix help to create an adaptable structure during adherence, maturation, and dispersal [2].

The Gram-positive opportunistic pathogen *Staphylococcus aureus* has been studied in biofilm communities that contribute to colonization and disease in humans [1,3]. To survive in biofilms, *S. aureus* produces a variety of secreted virulence factors. Prominent among these secreted factors are the phenol soluble modulins, or PSMs [4]. PSMs are small, amphipathic peptides characterized to have profound impacts in *S. aureus* persistence and disease [4]. PSMs can attract neutrophils, lyse host cells, induce inflammation, and promote colony spreading through surfactant-like activity [4-9]. Strains capable of producing PSMs demonstrate an increased ability to cause disease in mouse bacteremia, skin abscess and peritonitis models of infection [4]. Beyond the PSMs role in virulence, our lab previously demonstrated that the assembly of

extracellular amyloid fibers composed of PSMs correlates with resistance to biofilm dispersal [10].

Staphylococcal biofilms form in a variety of ways, but most often contain polysaccharides called PIA (polysaccharide intracellular adhesions) extracellular DNA (eDNA), and a variety of proteins including enzymes, adhesions, and structural amyloids fibers. These components work together synergistically to shape the biofilm matrix. Selective degradation or modulation of key components can have profound effects on biofilm lifecycle [11].

The methods by which PSM amyloid fibers assemble *in situ* are not known. When PSMs are produced during biofilm growth and take on a soluble form, they can induce dispersal through surfactant-like activity [9,12]. However, PSM amyloid assembly inhibits this PSM-mediated dispersal and furthermore stabilizes the biofilm against enzymatic and mechanical dispersal [10]. When PSMs form amyloid fibrils they not only resist dispersal, but are less toxic to red blood cells and neutrophils, suggesting that biofilms producing amyloid fibers are less deleterious than those that do not [10, 13]. Factors that dictate if PSMs take on a soluble or amyloid form are unknown. We hypothesized that other biofilm matrix components could play a role in PSM aggregation.

One of the primary candidates for a potential amyloid interacting partner is extracellular DNA, (eDNA), an important component of many biofilms [14]. In other systems, it has been noted that nucleic acids modulate amyloid assembly. Amyloidogenic proteins including alpha

synuclein, prions, and amyloid-beta are all known to interact with nucleic acids to regulate amyloid assembly *in vitro*, [15-17]. DNA-amyloid hybrids have long been implicated in the pathology promoting neurodegenerative amyloidosis, and DNA has even been found inside plaques isolated from brains of Alzheimer's patients [18-21].

Here, we provide evidence that phenol soluble modulin aggregation is enhanced in the presence of DNA. Biofilms lacking eDNA do not produce extracellular fibers, and *in vitro* assays demonstrate the interaction between DNA and PSMs and that DNA can promote amyloid formation of PSMs. PSMs mixed with DNA are less cytotoxic solule peptides, indicating DNA may act as a "chelating" agent against toxicity. These findings reveal a previously unappreciated interaction between biofilm matrix components that furthers our understanding of *S. aureus* biofilm biology.

Materials and Methods

Bacterial Strains and Growth Conditions, *S. aureus* strain, SH1000 was the wildtype strain used in this study. Construction of mutant strains is described elsewhere for the Δ*PSM* [10] and Δ*atlA* [22]. Liquid cultures were routinely grown in trypic soy broth (TSB) incubated at 37 °C with 200 rpm shaking unless otherwise noted.

Biofilm Experiments and amyloid fibril isolation, Drip-flow biofilms were grown in 3.3 g/L peptone, 2.6 g/L NaCl, 3.3 g/L glucose (PNG media) as previously described [10, 23]. After 5 days of growth, biofilms were scraped into 3 mL of potassium phosphate buffer (50 mM, pH 7)

and homogenized (TissueMiser, Fisher). Cell densities were measured and samples were normalized to OD_{600} of 0.1.

Amyloid fibrils were collected by shearing fibrils from the cell walls by tip sonication of cells on ice. Supernatants were clarified by repeated centrifugation at 13,000 RPM for 2 minutes to remove cells. The cell-free supernatant was incubated in 200 mM NaCl and the fibrils were isolated using Millipore Amicon Ultra Centrifugal Filter Units with a pore size of 100 kDa. The retentate fractions (fiber isolates) were collected and used for further experimentation.

Extracellular DNA was quantitated using a protocol used by Jones et al. [24] and adapted for Qubit using a kit and fluorometer (Invitrogen) according to the manufacturer's protocol. The supernatants of homogenized biofilms scraped into TE buffer (10 mM Tris-Cl (pH 8.0), 0.1 mM NaCl) or high salt TE buffer (TE buffer + 1 M NaCl) with the cellular fraction spun out at 5000K RPM.

Transmission Electron Microscopy (TEM), Biofilm cells, fibril isolates, and synthetic peptide fibrils were prepared and imaged via TEM as described previously [10]. Transmission electron microscopy (TEM) was performed using a Philips CM12 transmission electron microscope. Samples prepared for TEM imaging were spotted onto formvar-coated copper grids, incubated for 10 minutes, washed with sterile ddH_2O, and negatively stained with 2% uranyl acetate for 60 seconds.

Production of PSMα1 antibody, Rabbit polyclonal antibodies against PSMα1 were generated by Abgent (San Diego, CA) against a PSMα1 epitope peptide sequence aa 7-21(NH_2-IKVIKSLIEQFTGKC-$CONH_2$, wherein a cysteine was added to C-terminus of peptide sequence to provide for conjugation to KLH carrier) generated by Abgent (San Diego, CA) making the antigen more readily soluble in aqueous environments than the full length PSMα1 peptide. Rabbits were immunized with purified peptide epitopes (Abgent) and the resulting sera were tested by enzyme-linked immunosorbent assay (ELISA) before Protein A affinity purification (Abgent).

Western Blot Protocol, Cell Fractions were prepared as follows: Biofilms cells from drip bioreactor cultures were harvested, washed in once with filter sterile HPLC grade water, and normalized by cell density to an OD_{600} of 0.1 in a total of 200 µL filter sterile HPLC grade water.

Fibril isolates were prepared as previously described (Schwartz et al.). Proteins from 1 mL of pooled fibril isolates isolated with 250 µL 100% TCA and incubated at 4° for 2 hours. Precipitated protein samples were resuspended in 40 µL SDS loading buffer was added. Samples were bath sonicated for 20 min, vortexed, and boiled for 10 min.

For cell lysate fractions, 1 mg/mL lysostaphin was added to each culture and samples were incubated for 1 hour at 37°C with shaking. These samples were then pelleted and the supernatant transferred to a fresh tube. 40 µL SDS loading dye was added, and samples were boiled for 10 min, bath sonicated for 20 min, vortexed, and finally boiled 10 min.

20 µL of each cell fraction was loaded into a 16.5% SDS PAGE gel. Gels were run in duplicate and in the same electrophoresis tank (BioRad Mini-Protean Tetra) for wet-transfer. After denaturation in sample buffer (BioRad – 1x Biorad Tris-Tricine SDS PAGE loading dye plus 200 mM BME), 20 µL of each sample was loaded into pre-cast 16.5% Biorad Tris-Tricine acrylimde gels and run 100V/65mA for 100 min at room temperature. These gels were transferred onto 0.22 µM polyvinylidene fluoride (PVDF) membrane run at 70V/250mA for 80 min at 4°C.

Western blotting procedure was performed for use with the LiCor Odyssey imaging system according to LiCor protocols. 10X TBS (25mM Tris-Base, 150mM NaCl, 2mM KCl, pH 7.40) was stored at 4°C and diluted just prior to use for 1X TBS and 1X TBST. 1X TBST (100 mL 10x TBS + 900 mL MQ H2O + 1 mL Tween-20) was stored at 4°C between washes. Blocking Buffer was made fresh using 200 mL 1X TBS 8.5 g powdered skim milk and used to dilute antibodies. Membranes were incubated with 5% milk blocking buffer (Li-Cor) prior to incubation with rabbit anti-PSMα1 (1:1000, Abgene) and goat anti-rabbit IRDye 800 (1:15000, Li-Cor) secondary antibody rocking at RT for 1 hr, washed between antibodies with 1X TBST. Imaging was carried out using the LI-COR Odyssey® scanner and software (LI-COR Biosciences). Experiments performed at least two times showed similar results.

Peptide Preparations, Lyophilized peptide stocks (10 mg, LifeTein) were mixed with ice cold HFIP and transferred to sterile silicone coated tubes (Fisherbrand™ Siliconized Low-Retention Microcentrifuge Tubes) at 0.5 mg per tube, and dried via speed vac (2 hrs) and further dried to completion under N_2 stream (2 hr). Immediately prior to assay, dried peptide stocks were

thawed and dissolved into filtered HPLC-grade dimethyl sulfoxide, and allowed to solubilize for at least 30 min rocking at room temperature.

Gel Shift Assay, PSMα1 peptide stock was resuspended in 50 µL filtered HPLC-grade DMSO, vortexed well to solubilize, and incubated with shaking at room temperature for 20 minutes prior to assay. Care was taken to ensure that no protein was stuck to the sides of the wells.

Staphylococcus aureus and *Escherichia coli* genomic DNA were isolated using Gentra Puregene Yeast/Bact. Kit (Quigen). *S. aureus* or *E. coli* gDNA (100 ng/µL), 1000 µg/mL PSMα1 stock, preformed fibrils, or 1000 µg/mL BSA (Invitrogen, 1:10 dilution) were dissolved into a total volume of 20 uL in sterile HPlC-grade H2O and incubated rocking at room temperature. Samples were separated by electrophoresis at 1h 30min at 150V/400mA on a 1% agarose gel. Fresh ethidium bromide was mixed into ddH2O and the gel was stained for 30 min, and soaked in ddH2O for 30 min before visualization.

For time course assays, samples were prepared and incubated for the stated duration of time rocking at room temperature. PSMα1 stock was diluted into equal volumes of filter sterile HPLC-grade DMSO when stated. TEM imaging was performed on samples containing DNA incubated 24 hrs with or without 1 mg/mL PSMα1. All assays were run in triplicate and showed similar trends.

Dynamic light scattering (DLS), DLS was performed on a compact goniometer system (ALV CGS-3, ALV, Langen Germany) equipped with a multi-tau digital correlator (ALV 7004, Langen, Germany) and a laser light source of wavelength λ= 632.8 nm (He-Ne, JDS Uniphase Corp, USA). All measurements were done at T = 298 ± 0.5 K. The solvents and buffers used to make the DNA and protein solutions were first sterilized, filtered through 0.2 μm Whatman Anotop syringe filters (Whatman, USA). The samples were prepared in siliconized microcentrifuge tubes (Fisherbrand™ Siliconized Low-Retention Microcentrifuge Tubes) to prevent sample from binding to the walls of the tube. The hydrodynamic radii, R_H (nm), of the samples were obtained using relaxation times, τ (ms), measured at a fixed scattering angle of $\theta = 90^\circ$ and the Stokes – Einstein relation. Peptides were prepared as described above for gel shift assay. Salmon Sperm DNA (sDNA) was purchased from Invitrogen (Carlsbad, Ca).

Thioflavin T Assays, All amyloid dye-binding assays were performed in 96-well black opaque, polystyrene, TC-treated plates (Costar 3603, Corning). Freshly dissolved peptide stocks in DMSO were inoculated with or without DNA as stated, and diluted into sterile HPLC-grade H_2O containing 0.2 mM Thioflavin T (ThT) prior to assay. Fluorescence was measured every 10 minutes after shaking by a Tecan Infinite M200 plate reader at 438 nm excitation and 495 nm emission. ThT fluorescence during polymerization was corrected by subtracting the background intensity of an identical sample without ThT. Samples were imaged via TEM upon completion of timecourse. Assays were repeated at least twice and showed similar trends.

Preparation of RBC stock solutions, 5 mL of mechanically defibrinated rabbit blood (Hemostat Laboratories, Dixon CA) were pelleted to isolate red blood cells (RBCs) and washed twice with 9 mL PBS by centrifuging at 2000 rpm for 5 min. The RBCs were diluted into a total of 10 mL of PBS and gently resuspended by inversion. Cells were diluted 1:1000 and visually inspected in a hemacytometer (0.1 mm3 volume) under a microscope for counting. Based on cell counts, a working stock was diluted such that a 100 µL reaction volume contained 3.0×10^8 cells/mL in each microplate well.

Hemolysis of Red Blood Cells, Red blood cell preparation and heme absorbance assay performed according to Kuroda Lab protocol [25]. Summarily, PSMα1 synthetic peptide was resuspended in 25 µL filtered HPLC grade DMSO (20 mg/mL). Invitrogen salmon sperm DNA (10 mg/mL) was mixed 100 µL into 900 µL filtered HPLC water, and this working stock (1 mg/mL) was aliquoted for all DNA samples in fresh, sterile microcentrifuge tubes.

Either 1 µL or 2 µL of a 10 mg/mL PSMα1 stock was added to filter sterile HPLC water or DNA working stock to make a 100 µL volume and flicked to mix. 10 µL sample was added to 90 µL of 3.0×10^8 rabbit red blood cells (RBCs). Positive control was 1% Triton X in PBS, negative was PBS only. DNA only has no DMSO, and DMSO has 2 µL of filtered DMSO. Samples were set to rock on a shaker at RT until assay (24 hrs). Sample averages are background subtracted of PBS alone. After addition of freshly inoculated PSMα1 samples or incubated samples were added to RBCs and were incubated 1 hour shaking at 180 rpm at 37°C. Plates were spun down to pellet RBCs and 6 µL supernatant was added to 94 µL PBS and absorbance was read at 480 nm to calculate

heme release. Triplicates were averaged and experiments performed a minimum of 3 times showed identical trends.

Peptide stock solutions or control solutions were added as annotated to a 96 well microplate (PP, round bottom, not treated for cell culture) containing 90 µL RBC working stock. Plates were parafilmed with their lids and incubated for 1 hr at 37°C at 180 rpm in an orbital shaker. After incubation, the microplate was centrifuged for 5 minutes at 1000 rpm at 4°C. The supernatant (6 µL) was diluted into 96 µL PBS buffer and the absorbance measured at 415 nm was read as the hemoglobin concentration. 1% (v/v) Triton X-100 and PBS buffer were used as positive (100%) and negative (0%) control samples in every experiment.

Percentage of hemolysis (H) was calculated from the equation:

$$H = \frac{OD_{415}(polymer) - OD_{415}(buffer)}{OD_{415}(TritonX) - OD_{415}(buffer)}$$

RESULTS

S. aureus biofilms are encased in a self-produced matrix composed primarily of polysaccharides, proteins, and eDNA. The overall composition can vary depending on growth conditions, leading to a highly variable biofilm architecture. Unlike many bacterial species, including *Staphylococcus epidermidis*, *S. aureus* does not depend on the production of polysaccharides to maintain biofilm structural integrity [26]. Previous research suggests that in the absence of polysaccharide production, *S. aureus* relies on alternative components to maintain biofilm

structure [22, 26]. Among these "protein-dependent" biofilm phenotypes described for strain SH1000, it was noted that an Δ*atlA* mutant was deficient in autolysis and displayed a reduced ability to form biofilms [22,27].

To determine whether the presence of eDNA would influence PSM amyloid formation in biofilms, we grew the autolysis deficient mutant Δ*atlA* in biofilm drip bioreactors under fiber producing conditions (Fig. 1). We observed that these biofilms did not produce extracellular fibril structures (Fig. 1A). Western blots verified the presence of PSMα1 in fibril isolates of a wildtype parent strain, but not in Δ*PSM* or Δ*atlA* strains lacking the fibril structures detected via TEM. (Fig. 1B). We also confirmed that PSMα1 was produced in whole cell lysates of the Δ*atlA* mutant, demonstrating that PSMα1 was produced by these biofilms, but not assembled into fibrils (Fig. 1C). We quantitated the eDNA levels in the matrices of these biofilms, and confirmed that Δ*atlA* mutant biofilms did not produce detectable amounts of eDNA as compared to a wild type and a Δ*PSM* mutant, in both high and low salt TE buffers (Fig. 1D). Taken together, these findings led us to speculate that PSMs and eDNA may both be necessary for fibril formation in biofilms. To test this hypothesis, we grew co-cultured biofilms of Δ*PSM* and Δ*atlA* mutant strains, and found that this was sufficient to compliment the fibril forming phenotype (Fig. 1A). Taken together, these data suggest that eDNA induces PSM fibril formation in *S. aureus* biofilms.

We next sought to determine whether or not PSMs and eDNA physically interact using a *In vitro* gel shifting experiment. *S. aureus* bacterial genomic DNA (SA gDNA) was isolated from

planktonic cultures and purified. This gDNA was mixed with freshly soluble PSMα1, PSMα1 that had already polymerized, or a BSA negative control, and was incubated for 24 hours. Samples were loaded into an agarose gel and separated using gel electrophoresis to detect protein-DNA interactions that elicit a "shift" in band migration. gDNA incubated with a control protein (bovine serum albumen, BSA) did not elicit a shift, while gDNA incubated with soluble PSMα1 demonstrated shifted band migration relative to a gDNA-only sample, indicating formation of a protein-DNA complex (Fig. 2A). gDNA incubated with PSMα1 fibrils produced a less intense band shift, and may be partially due to physical restraint from molecular crowding (Fig. 2A). To determine PSMα1-DNA interaction was specific to *S. aureus* low GC content DNA, we also tested PSMα1 samples incubated with *E. coli* genomic DNA (EC gDNA). Again, we observed that incubation in the presence of non-polymerized PSMα1 produced a shifted banding pattern. PSMa-DNA gel shifting can be observed over time, and by 4 hr post-inoculation, SA gDNA is no longer able to migrate through the gel matrix (Fig. 2B). Finally, we found that gel shifting was peptide concentration dependent in samples 100 μg/mL DNA, indicating a critical peptide concentration around 1000 μg/mL was necessary for DNA interaction in this assay. (Fig. 2C). Taken together, these data suggest that soluble, non-polymerized form of PSMα1 is capable of interacting with gDNA and PSM fibrils display reduced interaction with gDNA.

If PSMα1 and DNA interact, they may form large complexes where DNA acts as a kind of scaffold. PSMs are cationic peptides that should interact with negatively charged anions. To help us determine the relative size of the PSMα1-DNA complexes *In vitro*, we employed dynamic light scattering (DLS). DLS is a sensitive technique used to measure the size

distribution of heterogeneous particles in solution. PSMα1 associates with bacterial DNA isolated from Gram-positive and Gram-negative organisms, and is likely to be caused by nonspecific intermolecular force interactions (Fig. 2a). Thus, in order to eliminate the concern of contamination from cellular debris in gDNA samples, we utilized molecular biology-grade salmon sperm DNA (sDNA). sDNA has been extensively studied using DLS, and our average size distribution of sDNA-only samples agreed with previously published results (data not shown). Samples containing soluble PSMα1 with or without DNA were incubated overnight, to allow ample time for complete protein-DNA interaction. As expected, freshly soluble "aging" PSMα1 formed aggregates spontaneously in a heterogeneous distribution of sizes that increased over time (Fig. 3A). PSMs, like many amphipathic toxic peptides, are known to aggregate and oligomerize, sometimes forming amyloid structures [10, 28]. When sDNA was incubated with freshly soluble "aging" PSMα1, we detected a uniform distribution of complexes in solution that were larger than those produced by PSMα1 alone (Fig. 3B). A comparison between samples containing DNA only, PSMα1 fibrils with or without DNA, and freshly soluble "aging" PSMα1 showed that after 24 hours of incubation, soluble PSMα1 incubated with DNA formed a distinctive and large complex as compared to PSMα1 fibrils or DNA only (Fig. 3C). PSMα1 fibrils incubated with DNA showed size distribution very different from soluble PSMα1 incubated with DNA. This suggested that soluble species were forming complexes with DNA. Size distribution of pre-aggregated PSMα1 fibrils and DNA correlated to those observed when each component was assayed separately (Fig. 3C). This suggested to us that the fibrils were not interacting with the DNA and each component stayed separate in solution, whereas soluble PSMα1 seemed to interact with DNA to form a distribution of complexes that was larger than each individual

component. To verify that soluble PSMα1 was interacting with DNA in solution, we ran a time course assaying samples that contained soluble PSMα1 only or soluble PSMα1 with sDNA. As expected, PSMα1 "aging" in the presence of DNA formed larger complexes than PSMα1 alone over time (Fig. 3D). From the results of our gel-shift and DLS experiments, we concluded that not only can PSMα1 interact with DNA, but also creates complexes that are larger than fibrils or DNA by itself.

PSMα1 and other *S. aureus* PSM peptides autoaggregate to form amyloid fibril structures [10,13]. To determine whether the large structures formed by PSMα1 and DNA are amyloid like in nature, we assayed synthetic PSMα1 and SA gDNA 's interactions in a Thioflavin T binding assay. Thioflavin T (ThT) is an amyloid specific dye that fluoresces when bound to amyloid aggregates, eliciting an increase in intensity as amyloid structures form in solution. We observed that ThT fluorescence increased over time in samples containing both PSMα1 and DNA as compared to PSMα1 only, while *S. aureus* genomic DNA alone did not show increased fluorescence above baseline (Fig. 4A). Addition of DNA stimulated amyloid formation at a low concentration of PSMα1, indicating that DNA lowers the critical peptide concentration necessary for spontaneous aggregation (Fig. 4B). Interestingly, we determined that ThT binding increases logarithmically at 200 minutes, around the same time frame that we observed DNA gel shifts and an increase in the aggregate dynamic radius via DLS (Figs 2-4). To confirm that the amyloid-formation effects we were observing were not specific to PSMα1, we assayed a synthetic prep of the newly discovered PSM-like peptide N-AgrD for its ability to interact with DNA. Again, the addition of DNA promoted amyloid formation (Fig. 4C). Finally, we observed

that over time, PSMα1 formed fibril structures that were morphologically distinct from PSMα1 that was incubated with DNA (Fig. 4D). DNA alone did not produce these structures (Fig. 4D). Taken together, these data suggest that PSMα1 is forming ordered amyloid structures in the presence of DNA.

Finding that PSMα1 forms amyloid aggregates in the presence of DNA lead us to consider the role that DNA might play in virulence. PSMs are potent toxins, contributing to infection by facilitating lysis of multiple host cell types [4]. However, our lab recently discovered that formation of amyloid fibers by PSMs can significantly reduce their toxicity in a red blood cell hemolysis assay [13]. Similarly, we speculated that the addition of DNA could push free PSMα1 into the fibril form, thus abrogating its toxic activity. A Δ*PSM* mutant shows a reduced zone of hemolysis as compared to WT on rabbit blood plates, indicating that PSMs readily lyse rabbit blood cells and that loss of PSM activity correlates to reduced toxicity (Fig. 5A). We then sought to determine whether the addition of sterile salmon sperm DNA could abrogate the toxicity of synthetic PSMα1. Samples were prepared 1 hour prior to hemolysis assay and were inoculated simultaneously to ensure aggregation timeframes were synchronized. We observed that samples that containing only PSMα1 were not aggregated at the time of assay, but PSMα1 in the presence of DNA showed considerable aggregation via TEM (Fig. 5B). As predicted, significant lysis occurred when we exposed red blood cells to non-aggregated PSMα1, while this effect was mitigated by the addition of DNA (Fig. 5C). Similarly, pre-formed PSMα1 fibrils incubated with DNA did not display the toxicity attributed to soluble PSMα1 (Fig. 5C). These results indicate that PSMα1 interacts with DNA rapidly to form amyloid complexes that are

therefore observed to be less toxic than the same concentration of non-aggregated PSMα1 under the same conditions.

Discussion

The extracellular matrix produced during biofilm development plays a critical role in the formation and persistence of biofilm communities. However, there is little understanding of potential interactions between matrix components. This work demonstrates eDNA's role in functional bacterial amyloid formation within the biofilm environment. DNA is important for phosphate cycling, sequestration of divalent cations, and subversion of host antimicrobial responses [14,29]. In biofilms, DNA promotes intracellular and surface adhesion, modulation of visoelastic relaxation by interacting with matrix components in response to mechanical stress [29-31]. The production of extracellular DNA helps to stabilize the biofilm structure against dispersal [14]. Expression of extracellular nucleases or endogenous addition of DNase can disrupt bacterial biofilms, especially early in development (20). The production of nuclease is known to have a profound effect on biofilm structuring and dispersal in *S. aureus*, which produces two thermostable nuclease enzymes that are active under variable conditions (32-34). In this report, we describe a new contribution of eDNA to biofilm physiology and virulence.

AtlA is a bifunctional peptidoglycan hydrolase that plays a role in *S. aureus* cellular division, daughter cell separation, and autolysis (35-34). Coordinated autolysis of discreet subpopulations within *S. aureus* biofilms lyse is considered to a form of programmed cell death, contributing DNA to bifilm environments [34, 38]. Previous research demonstrated that *S.*

aureus strains lacking AtlA showed reduced autolytic activity and impaired biofilm formation [22,27]. An Δ*atlA* mutant does not produce extracellular fibrils under fiber-inducing growth conditions (Fig 1A,B). We verified that an Δ*atlA* mutant biofilms produce PSMα1, but lacks extracellular DNA (Fig 1B-D). Fibril production can be complimented by co-culture with a Δ*PSM* isogenic mutant, which lacks fibrils but produces eDNA in biofilms (Fig. 1). We conclude that the presence of eDNA is required for PSM fibril assembly in the biofilm environment.

Staphylococcal PSMs form amyloid structures in biofilms under certain growth conditions [10]. Amyloid aggregation has been shown to promote biofilm stability and reduce PSM toxicity, implicating that amyloid formation serves to modulate PSM activity [10,13]. We demonstrate in this report that DNA helps to push PSMα1 down the amyloid formation pathway by promoting aggregation *In vitro* (Fig. 2-4). We confirm that PSMα1 directly interacts with DNA to form large complexes with relatively consistent size distributions (Fig. 3). These complexes appear to be amyloid-like in nature, as the presence of DNA stimulates ThT binding to amyloid fibers (Fig. 4).

In addition to electrostatic interactions between positively charged cationic peptides and negatively charged polyanionic DNA, is possible that DNA could acting as a molecular "crowding" source, increasing the effective molarity of the sample. DNA is a polyanionic compound, and many other polyanions are present in the environmental conditions outside of and throughout the host environment. Heparin and closely related proteoglycans (like heparan and chondroitin) are found ubiquitously throughout the extracellular matrix environment in the human body. Heparin has been previously shown to promote biofilms *in vitro* and in catheter

lock solutions [39]. It is possible that biofilms forming in these environments are at least partially influenced by PSM amyloid formation, which may further modulate immunogenicity of these kinds of infections. eDNA plays a role in several biofilm diseases including Candida pneumonia, otitis, pertussis, and endocarditis [24, 40-41]. It is tempting to speculate that other bacterial toxins or amyloids form macrostructures in the presence of DNA. Similarly, the interactions between PSMs and DNA could likely reflect other interactions between host cationic antimicrobial peptides (CAMPS) and DNA in the biofilm.

Once aggregated into amyloid fibrils PSMs lose the cytotoxicity associated with the soluble forms (Fig. 5B-C). Although staphylococcal α-toxin is known to be the dominant hemolysin, the PSMs also display an observable hemolytic phenotype that is inhibited by interaction in the presence of. (Fig. 5A)[6]. Another toxin in the biofilm matrix that was characterized to interact directly with DNA is staphylococcal neutral sphingomyelinase, β-toxin, which can also bind single and double stranded DNA to create matrix interactions that are shown to be important for endocarditis [42].

Taken together, these data support the claim that virulence can be modified in the biofilm environment, and that sequestration of toxins in an aggregation dependent manner can occur through many different methods. DNA, and perhaps other polyanionic compounds, may act as an environmental “switch” to sequester PSM toxins out of the environment. It is unknown why DNA would induce this switch, or what benefit it gives to the bacterial community. Perhaps the DNA serves as an extracellular signal that promotes bacterial adhesion to protect the colony

from environmental stressors and cell death. Further research is needed to understand how this finding fits into the larger picture of Staphylococcal biofilm development.

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Figures

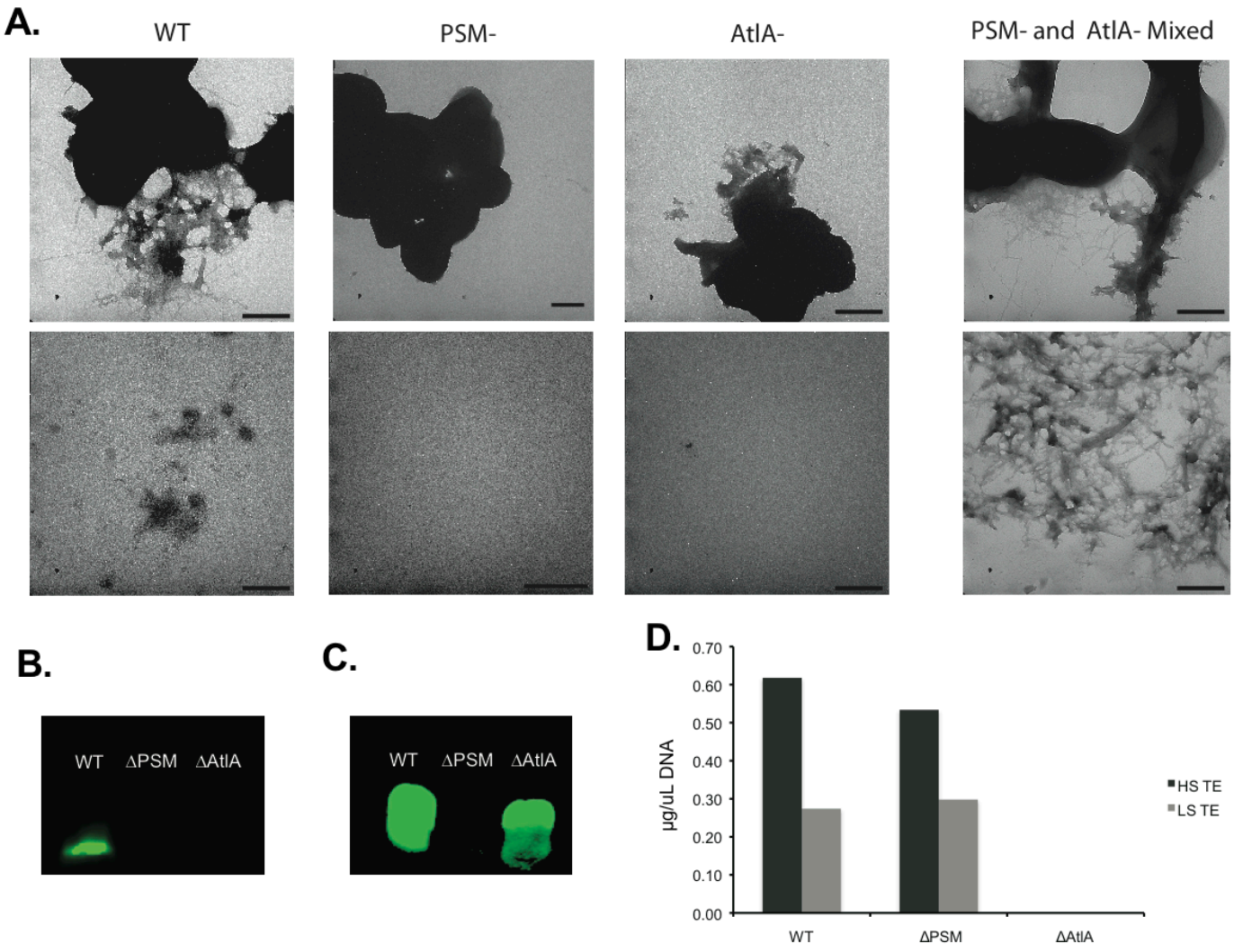

Figure 4.1, An autolysin mutant lacking eDNA does not form PSM amyloids in biofilms,
(A) Δ*atlA* mutant biofilms do not produce extracellular fibrils like the wlldtype parent. Coculture of a Δ*PSM* and an Δ*atlA* strain compliments fibril production, presumably through interaction of eDNA produced by the Δ*PSM* mutant and PSMs produced by the Δ*altA* mutant. Scale bar indicates 500 nm. (B) PSMα1 is detected in fiber isolates from wild type, but not Δ*PSM* or Δ*atlA* biofilms. (C) PSMα1 is detected in whole cell lysates of wild type and Δ*atlA* mutant biofilm cells, showing that PSMα1 is produced, but not forming fibril structures in the biofilm. (D) Quantitation of eDNA in biofilms. Wild type and Δ*PSM* mutant, but not an Δ*atlA* mutant, showed detectable amounts of eDNA when extracted and assayed using TE or high salt TE from biofilm supernatants. Results are representative of at least three independent trials.

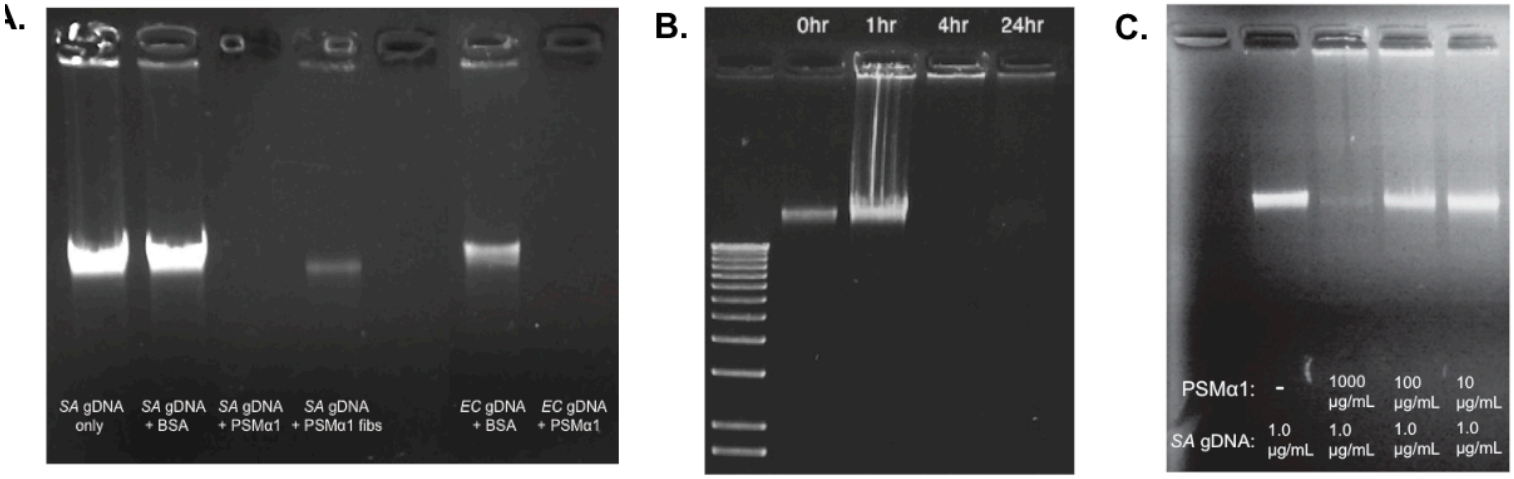

Figure 4.2, PSMα1 interacts with bacterial genomic DNA in a time and concentration-dependent manner,

(A) *Staphylococcus aureus* (SA) genomic DNA samples were incubated with a BSA control, soluble PSMα1 (1000 µg/mL), or PSMα1 pre-aggregated fibrils (1000 µg/mL) for 24 hours before DNA separation on an agarose gel. Incubation with PSMα1 prohibited DNA from migrating through the gel matrix. PSMα1 fibrils showed a weaker shift, while BSA showed no difference from DNA only. The effect was not specific to *S. aureus* DNA as *Escherichia coli* (EC) genomic DNA also elicited a shift when incubated overnight with PSMα1. (B) The interaction between PSMα1 and SA DNA can be observed over time. Initial association is observed as a smear at 1 hr and by 4 hrs DNA is no longer able to run through the gel matrix. (C) PSMα1 concentration of ≥ 1000 µg is necessary for SA the gDNA band shift.

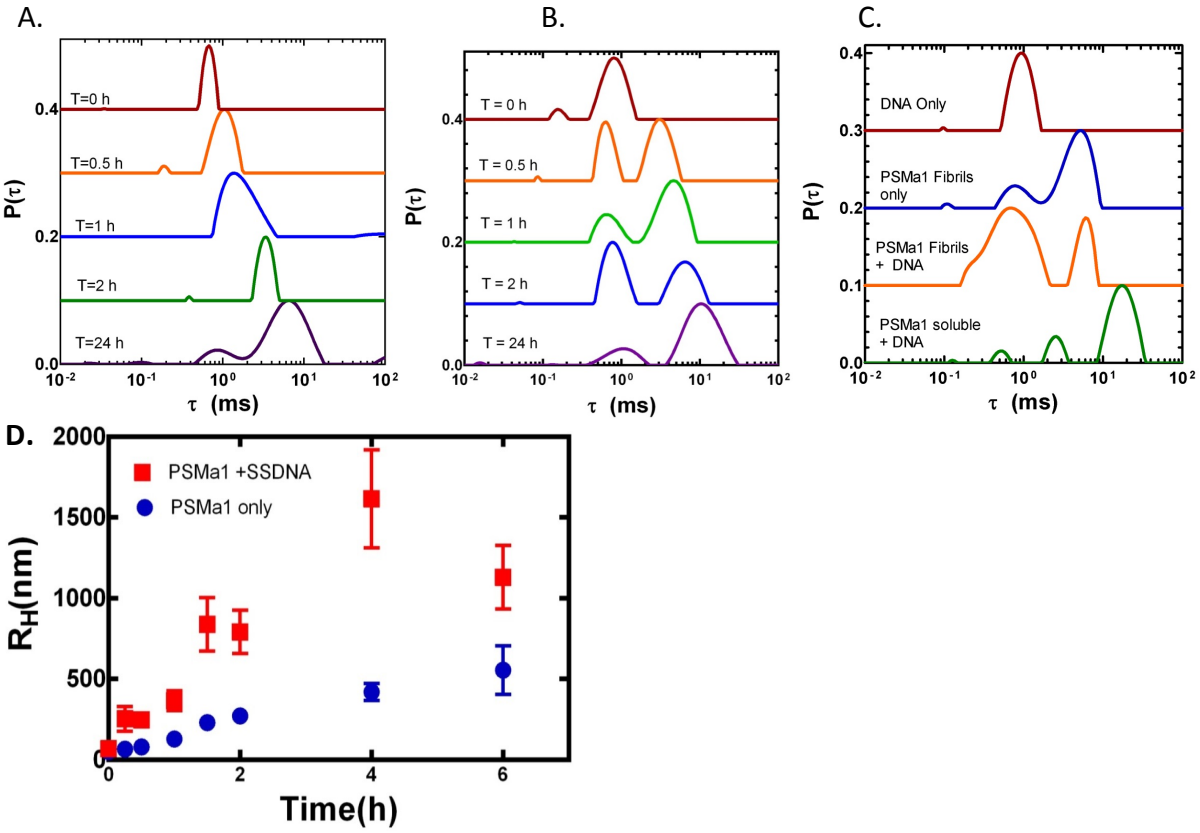

Figure 4.3 PSMα1 peptides interact in the presence and absence of DNA using DLS,
(A) The evolution of the probability distribution of DLS relaxation times, P(τ), with reaction time, T (h) for a dilute solution (200 ug/mL) of PSMα1 monomers. After 24 h, a weak bi-modal distribution is seen, interpreted as un-polymerized and polymerized peptides. The fast-mode peaks, seen at $\tau \leq 0.4$ ms are probably due to dust or artifacts arising from the CONTIN analysis of DLS correlation data 13. These modes were not re-producible. (B) We observed the growth of DLS relaxation time distribution with reaction time for a solution containing soluble PSMα1 and SDNA. At all reaction times observed, the fast mode peak coincides with that of bare SS DNA, indicating the presence of un-reacted DNA in the solution. In the presence of DNA, the self-polymerization of PSMα1 is prevented in the favor of a PSMα1 – DNA complex formation. (C) Comparison of the P(τ) of PSMα1 fibrils with and without DNA is compared with that of naked DNA and PSMα1 – DNA complex in Fig 3C. When SS DNA is added to PSMα1 in its monomeric state, we obtain a complex of larger RH, and when the former is added to a polymerized PSMα1, no increase in the sample size is observed.(D) the growth in effective hydrodynamic radius, RH (nm), of PSMα1 monomer with and without SS DNA as a function of polymerization time. It is seen that in the presence of DNA, PSMα1 monomers bind with the DNA molecules to form complexes that are about 3 times larger in RH than the polymerized PSMα1 molecules. This also indicates that the polymerization process in both the solutions approaches a steady state after a reaction time of 2 h.

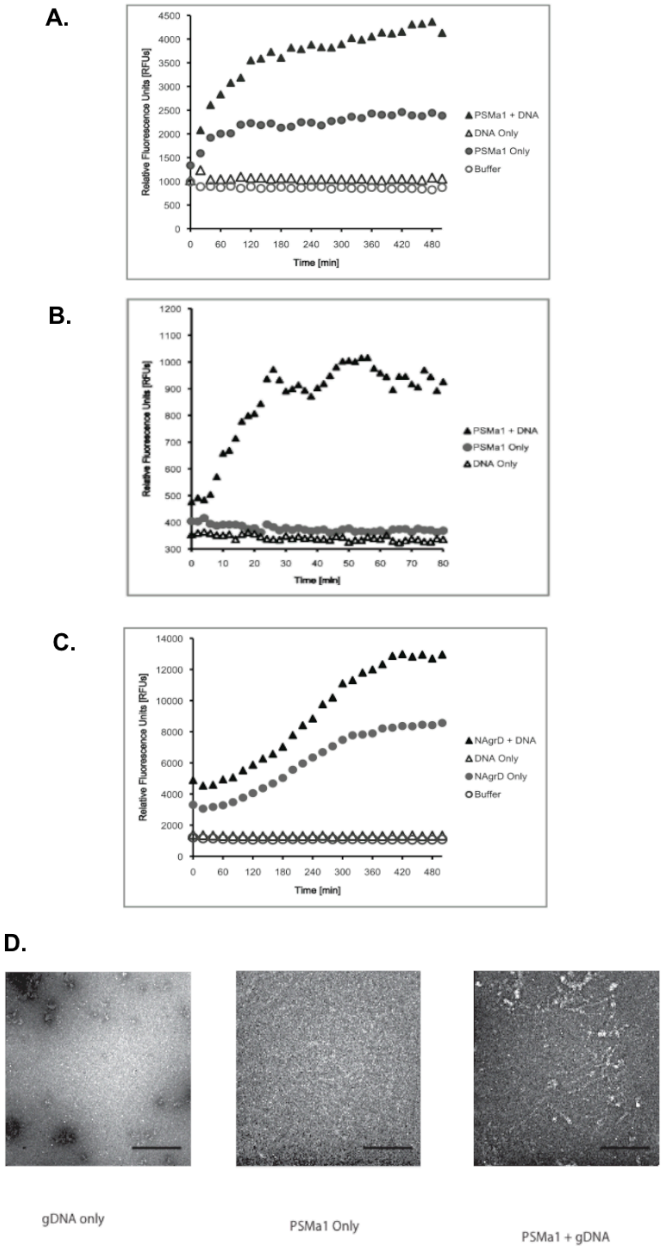

Figure 4.4, PSMα1 forms amyloid fibrils in the presence of DNA,
(A) Incubation of 1000 µg/mL PSMα1 with 0.1 µg/mL DNA shows increased ThT relative to PSMα1 alone, indicating increased amyloid formation. DNA alone did not appreciably increase ThT fluorescence relative to a buffer control. (B) DNA stimulates PSMα1 amyloid formation in sub-critical peptide concentrations (100 µg/mL) under assay conditions, indicating that DNA can induce rapid amyloid formation when peptide concentration is too low to favor aggregation. (C) DNA also enhances amyloid formation of PSM relative N-AgrD, indicating DNA-PSM interactions are not specific to PSMα1. (D) Amyloid fibrils can be visualized via TEM from samples of PSMα1 incubated with DNA after 24 hours. Data is representative of ≥ 3 independent experiments.

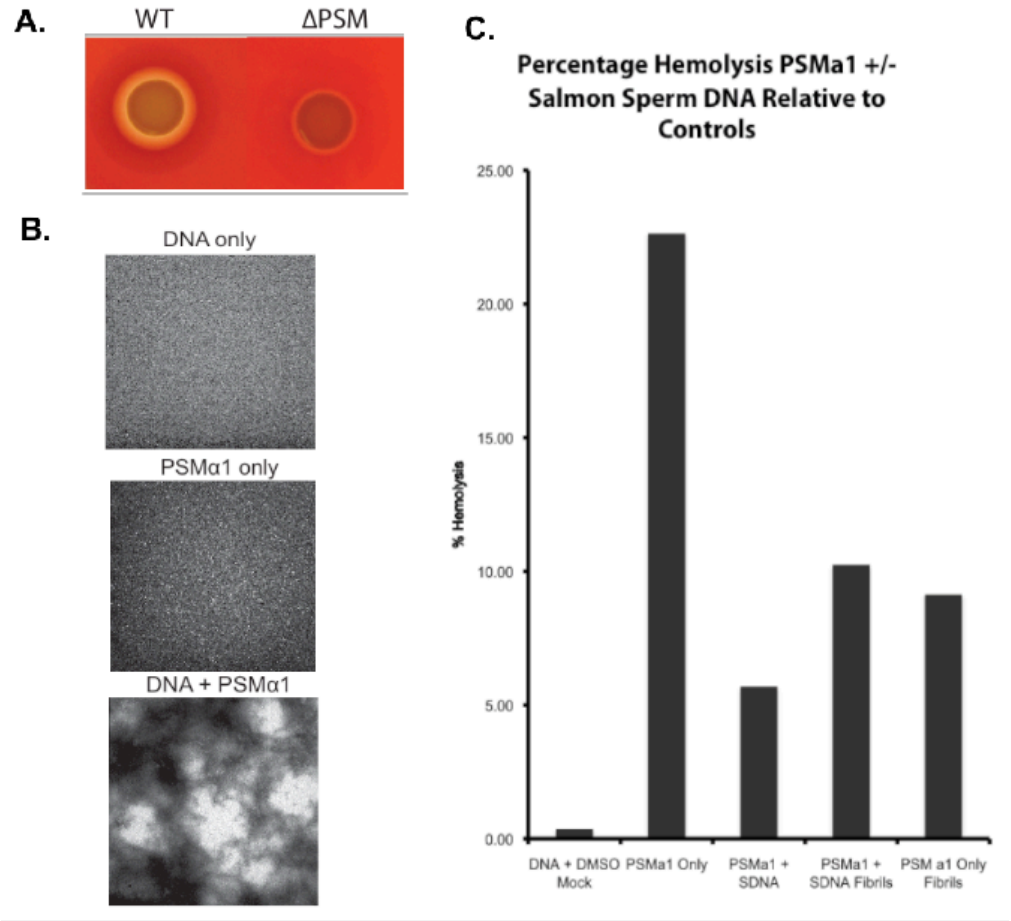

Figure 4.5, Interaction with DNA inhibits PSM toxicity,
(A) Cultures that are unable to produce PSMs show reduced zone of clearing on rabbit blood agar plates, indicating PSMs are import for hemolysis. (B) After 1 hr incubation, samples containing both DNA and PSMα1 had started to form large fibril aggregations relative to PSMα1 only and a DNA control. (C) PSMα1 hemolysis is greatly reduced in the presence of DNA or in pre-aggregated fibrils. Co-incubation of pre-aggregated fibrils and DNA did not reduce hemolysis relative to a fibrils only control. % hemolysis was calculated from the average of three replicates. Each assay was repeated at least twice and showed similar results.

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Chapter 5

Discussion

My work stems from the characterization of small *S. aureus* peptides that are capable of forming polymeric amyloid fibrils during biofilm growth. These fibrils prevent biofilm dispersal that would otherwise occur from peptide surfactant activity. The persistence of these biofilms

ultimately leads to altered colony behavior and reduction of virulence associated with soluble peptides.

1. Are there novel conditions that promote biofilm resistance to dispersal?

Biofilm communities form highly variable structures. For a long time, biofilm research has focused on the polysaccharide and glycocalyx slime layers as the defining characteristic of the biofilm matrix. [1,2]. In recent years, additional components have been defined as crucial for biofilm formation under a variety of environmental conditions promoting polysaccharide-independent growth [1-5]. In Staphylococcal biofilms, these factors include extracellular DNA and proteinaceous matrix material, like amyloid fibrils [6,7]. Depending on the specific biofilm model system or the growth conditions being investigated, biofilm communities can display a wide range of phenotypes. As a result of their variable matrix composition, biofilms show adaptable adherence, structural heterogeneity, resistance to dispersal, and protection from antimicrobial killing [2-4,7-8]. The importance of matrix constituents in biofilm formation can be examined by enzymatic degradation of key matrix compounds to induce dispersal [4]. By investigating the changeability of the *S. aureus* matrix, we found that biofilms can resist conventional dispersal under conditions promoting amyloid fibril formation.

Amyloids often form as an alternative to ordered protein-folding conformations, following a folding pathway driven by self-templating assembly from small nucleators to large fibril structures [5]. The polypeptide sequences of amyloid proteins often bear no direct homology in

in primary sequence, but all amyloid fibrils share the characteristic cross-β structure where β-sheet strands stack perpendicular to the fibril axis. [5]. Assembly is driven by peptide backbone interactions and coordinated side chain interactions of alternating hydrophobic burying and hydrophilic residues. The biophysical characteristics that define the amyloid fold have been extensively studied, and methodology to test for them is well defined [5].

Amyloid formation can be studied using a variety of *In vitro* and *In vivo* techniques. Assays like circular dichroism (CD) can be used to assess structural variation as amyloids transition from their monomeric form to large β-rich aggregates. Amyloid specific dyes like Congo Red and Thioflavin T (ThT) can be used to quantitate amyloid aggregation *in vitro* or to stain amyloid plaques from *In vivo* biological samples. Amyloid fibrils can resist degradation from detergents, urea, salt, and enzymatic digestion due to the strong protein interactions. In *S. aureus* biofilm systems, we have shown that amyloid structures stabilize the matrix and promote resistance to dispersal [7]. Using assays previously defined for determining bacterial amyloids, my work has shows that *S. aureus* produces amyloids in its biofilm matrix [6-8].

In Chapter 2 of this work, I have demonstrated that *Staphylococcus aureus* biofilms form amyloid fibrils composed of phenol soluble modulins (PSMs) when grown under non-standard laboratory conditions. Under these conditions, *S. aureus* biofilms are resistant to enzymatic and mechanical dispersal. Loss of PSM production restores the dispersal-sensitive phenotype correlating with loss of extracellular fibril production [7]. The correlation of fibrils with dispersal-resistant phenotype echoes the resilient biofilm phenotype that has been previously

observed in other biofilm systems producing functional amyloids [9]. PSMs have been previously associated with biofilm dispersal, and I present evidence that aggregation inhibits this dispersal activity [7,10]. This work suggests that the biofilm matrix displays variable phenotypes differently depending on growth conditions. Although the exact mechanism causing increased fibril formation in PNG media is unknown, this work reinforces the understanding that researchers should pay careful attention to growth and media conditions to avoid relying on confined laboratory conditions when studying bacterial behavior.

2. How does amyloid formation contribute to *S. aureus* virulence?

My work demonstrates that PSMs, which are known virulence factors, exhibit a fascinating propensity for amyloid fibril formation. Our discovery contributes to a growing body of knowledge showing amyloid formation is a ubiquitous and useful process . Many functional bacterial amyloids have been characterized, some of which contribute to disease in host systems [9]. Our findings add functional amyloids to a growing list of PSM functions that includes virulence, immunomodulation, and biofilm structuring [7-8, 10-13].

The agr quorum sensing system has a multitiered role in *S. aureus* virulence. Activation of quorum-sensing negatively regulates biofilm formation, impeding attachment and development [4,15-16]. Supporting this is work by Yarwood et al. who demonstrated that *S. aureus* biofilm detachment correlates with increased agr activity and virulence [17,18]. Agr quorum sensing coordinates RNA III regulated virulence mechanisms, including superantigen, toxin, and tissue degrading enzyme production while downregulating production of cellular adhesions [18-24].

We have found that agr may promote biofilm stabilization under fibril inducing conditions through PSM aggregation [7,8]. In addition to agr's contribution to PSM production, I have also discovered that *S. aureus* strains with highly upregulated agr expression produce fibrils that are composed of the AgrD signaling molecule cleavage fragment, N-AgrD in addition to the canonical PSM peptides [8].

In Chapter 3 of this work, I provide evidence that N-AgrD is an amyloidogenic PSM-like cytotoxin [8]. The discovery of N-AgrD in biofilm fibrils demonstrates a novel function for what was previously considered to be a byproduct of AIP propeptide processing. N-AgrD is N-terminal leader peptide and a cleavage fragment from the agr quorum sensing autoinducer propeptide. The AgrD propeptide is processed at the cell surface by AgrB and SpsB, which catalyze the thiolactone ring formation and peptide cleavage that releases the N-terminal leader peptide as an amphipathic helix outside of the cell [25-28]. We first discovered that N-AgrD was incorporated into *S. aureus* fibrils in a highly virulent strain, USA300. N-AgrD was also detected in earlier fibril preps from lab strain SH1000, albeit at lower levels than USA300 [7,8]. Others have shown a unique biochemistry of other small leader fragments that can contribute to cellular processes like DNA transport, cell signaling, and even virus maturation [29-38]. I speculate that there is likely an undiscovered realm of cellular activity and regulation associated with these kinds of leader peptides that has been overlooked. N-AgrD's incorporation into fibrils and ability to form and seed amyloid fibrils led us to investigate whether it showed the characteristic PSM virulence activity.

The host immune response relies on the efficient sensing and clearing of microorganisms, both of which are subverted by the expression of *S. aureus* PSMs [11-14]. Staphylococci evade innate immunity by secreting an array of immunomodulatory peptides, like PSMs, to hinder molecular recognition. While freely soluble PSMs recruit and lyse neutrophils, and this activity is inhibited when PSMs are aggregated into fibrils [8]. We show that N-AgrD displays cytolytic and immunomodulatory activity similar to the well characterized PSM toxin, PSMα1. PSMs' toxicity is attributed to its amphipathic helix structure and capacity to interact with cell membranes, causing cell lysis [39-45]. We have demonstrated that N-AgrD, like PSMα1, causes significant lysis of red and white blood cells. Furthermore, it is able to recruit neutrophils in a chemotaxis assay and induces IL-8 expression, correlated with TLR2 sensing of PSM peptides [11-13]. Finally, we demonstrate for the first time that amyloid aggregation of these toxic peptides inhibits both cell lysis and neutrophil recruitment. This suggests that PSMs may act as a kind of toxin repository when aggregated into amyloids. This is a first hint that amyloid fibril formation can modulate *S. aureus'* interactions with host cells, constituting a genuine switch from pathogen to non-invasive lifestyles.

S. aureus is sensed by macrophages, triggering an inflammatory immune response which recruits neutrophils [11-13]. PSMs are described to be the major neutrophils chemoattractants in *S. aureus* supernatants, and our work with PSMs and N-AgrD supports this [82, 102-103]. PSMs and their proteolytic derivatives are strong activators of neutrophil formyl-peptide receptor 2 (FPR2), and FPR1 to a lesser extent [46,47]. Interestingly, FPR2 binds a wide variety of amyloid-like ligands [47], including LL-37, serum amyloid A [47], brain amyloid precursor [48],

and annexin 1 [49], indicating a link between immune response to PSMs and other amyloids. Remarkably, amyloid aggregation of PSMs impairs neutrophil chemotaxis and cytokine production [46] and efficiently reduces the PSMs' impact on the immune system and fundamentally changes the way *S. aureus* interacts with its host.

PSMs are detergent like peptides that aggregate into multimeric complexes at lipid membranes [50]. Many small peptide toxins interact with host cell membranes to cause pores ultimately induce cell lysis in what is likely to be a receptor independent process with no specificity for cell type [40-45]. Indeed, PSMs were found to associate with phophatidylcholine vesicles. *In vitro*, demonstrating their ability to associate with membranes [42,44]. PSMα peptides are much more toxic to neutrophils than PSMβs, but all PSMs show synergistic lysis with other staphylococcal hemolysins both from native expression and exogenous addition of synthetic peptide [44]. PSM-mediated lysis is abrogated in the presence of lipoproteins found in human serum, suggesting that their toxicity is largely mediated by the extracellular environment in which they are produced [51,52]. It is possible that the presence of these lipoproteins is somehow enhancing amyloid fibril sequestration of toxins, although this theory is yet to be tested. When PSMs form fibrils, they are less cytotoxic, presumably due to interference of membrane-interaction as large aggregates [7,8].

Taken together, Chapter 3 of this work provides evidence that N-AgrD, like PSMα1, can modulate its activity through amyloid fibril formation. By forming amyloid aggregates, these peptides show a drastic decline in virulence capacity. *S. aureus* colonies may use amyloid

formation to modulate their interactions with the host using this unusual post-translational regulatory mechanism. The signals or accessories that facilitate the switch from soluble peptide to inert aggregate are unknown, but a potentially noteworthy mechanism is described in Chapter 4.

3. Are there external factors that promote PSM aggregation?

To further investigate how *S. aureus* uses PSMs to control its pathogenicity, I looked to other biofilm matrix components. The mechanism driving PSM aggregation *in situ* is not known, but some initial hints suggest that the presence of extracellular DNA could stimulate fibril formation. Amyloid-DNA interactions have been characterized elsewhere [53-60]. Using prototypic prion fragment and amyloid-beta as model for aggregation, Domizio et al. complexed amyloid proteins with the oligonucleotide CpGB fixed to a surface. They found that not only did nucleic acids promote amyloid formation, but produced species that were more immunologically active than with protein alone, suggesting that the immune system can recognize nucleic acids as part of the amyloid complex [53,54]. Other studies show that poly (A) RNA is critical for infectious prion protein conversion [61,62]. DNA has been described as an osmotic chaperone that can either promote or discourage amyloid aggregation of Parkinson's disease agent α-synuclein and Alzheimer-associated amyloid-beta and τ peptides, by stabilizing intermediate protein conformations [55]. Amyloid peptide and nucleic acid interactions may even have implications for early Darwinian evolution by promoting nucleic acid hybridization to create a "scaffold" for sequestration of nucleic acids for replication and division [63,64]. Based

on previous observations, there is substantial support for the hypothesis that extracellular DNA induces amyloid fibril formation.

Extracellular DNA is produced by autolysis in Staphylococcal biofilms. Autolysins are required cell wall metabolism by making specific cuts in the peptidoglycan cell wall to enable insertion of new muropepide strands during cellular growth and daughter cell separation. This cutting activity is believed to also cause breaches in the cell wall under carefully regulated conditions to induce cellular lysis of small subpopulations of bacteria in biofilms. Atl is a bifunctional peptidoglycan hydrolase composed of an amidase domain and a glucosaminidase domain and the predominant peptidoglycan hydrolase in staphylococci [65-67]. The importance for Atl-mediated autolytic release of eDNA in early biofilm development was demonstrated in closely related species *Staphylococcus epidermidis* [65]. Qin et al. demonstrate that the major autolysin Atl is the primary mediator of eDNA release assisting in initial attachment to glass or plastic surfaces and that DNase I treatment prevents attachment and biofilm formation *S. aureus* cells lacking AtlA form large clusters consistent with impaired cell-separation. Planktonic growth in complex media is largely unaffected by loss of AtlA function, but adhesion and biofilm formation was impaired [66].

In *S. aureus*, eDNA release is mediated by additional autolysins controlled by the *cid*/*lrg* operons, which function through a toxin/antitoxin “programmed cell death” process that respond to changes in carbohydrate metabolism and acetate accumulation [67]. AtlA is also

known to contribute to cell lysis in biofilms, so to remove any ambiguity associated with *cid*/*lrg* regulation, we chose to investigate the major autolysin AtlA's role in fibril formation.

Under fibril inducing conditions, an Δ*atlA* mutant biofilm did not produce PSM fibrils (Fig. 4.1). This was determined both by TEM microscopy and via Western blot of PSMα1 in fibril isolation preps (Fig. 4.1A-D). The Δ*atlA* mutant still produced PSMs, but they did not form fibrils outside of the cell (Fig. 4.1C). I confirmed that DNA was not present in Δ*atlA* biofilms, but was present in both wildtype and ΔPSM mutants (Fig. 4.1D). Co-culturing of a Δ*PSM* mutant, which produces eDNA, and an Δ*atlA* mutant, which produces PSMs, complemented for fibril formation, suggesting that both PSMs and DNA are necessary for PSM aggregation in *S. aureus* biofilms (Fig. 4.1A).

My recent work presented in Chapter 4 suggests that PSM-DNA interactions contribute to fibril formation and can inhibit virulence. DNA gel shift and dynamic light scattering assays demonstrate that non-aggregated PSMα1 interacts with DNA to form large aggregates. Aggregation in the presence of *S. aureus* genomic DNA promotes amyloid formation, again supporting DNA's role in promoting biofilm amyloid assembly. Furthermore, DNA-promoted aggregation inhibits PSM cytolytic activity, suggesting that DNA can detoxify PSMs by promoting amyloid assembly. Taken together, these data provide basic evidence that biofilm matrix components, like DNA, can modulate PSM activity to promote biofilm formation, or reduce interactions with the host immune system.

My graduate work has shown that bacterial amyloid formation is a complex, and oftentimes incidental, occurrence of bacterial physiology. The discovery that PSM peptides can form amyloid structures changes the way we should view their pathology and bacterial infection models. The elucidation of a novel function for the N-AgrD peptide demonstrates the complexity of *S. aureus* virulence and its reliance on small peptide toxins as a form of that is both recycling for economical and efficacious in its effects. The discovery that eDNA promotes PSM amyloid assembly adds further detail to our understanding of how extracellular factors modulate virulence. The fact that PSMs interact with DNA, similar to other known amyloid proteins also ties together disparate concepts and techniques, bridging between pathology, biochemistry, and microbiology. Taken together, I have defined how this recently discovered class of *S. aureus* toxins play multiple roles in bacterial physiology and lifestyle.

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Chapter 6

Future directions

The work presented in my thesis is just the beginning of a new and exciting field of *S. aureus* research. Among the many remaining questions, I find the following to be most critical:

1. How do PSMs aggregate/disaggregate *In vivo*?

2. How does PSM aggregation affect the biofilm lifecycle?

3. What is the functional role of PSM aggregation *in situ*?

How do PSMs aggregate/disaggregate *In vivo*?

We hypothesize that PSMs form "toxin repositories" that sequester toxins from the environment under a certain set of conditions (See Fig. 1.5). To define this as a biologically relevant impact, our next step will be to find specific conditions under which PSMs aggregate and conversely, disaggregate. Bacterial amyloids that aggregate to inhibit toxic activity have been described in other systems [1]. These include MccE492 in *Klebsiella pneumoniae* [2] and listerioloysin O (LLO) in *Listeria monocytogenes* [3]. LLO has been shown to aggregate under alkaline conditions to form inert fibrous structures that bind amyloid specific dyes while forming cytolytic dimers in acidic conditions (similar to that of the phagolysosome). As PSMs are known to be expressed after neutrophil engulfment, it would be useful to determine whether PSMs can aggregate/disaggregate in a similar manner [4]. Other factors that should be

explored include salts, trace metals, and osmolytes, all of which are known to affect the polymerization kinetics of amyloid formation [5]. Understanding how PSMs behave in solution will be especially important to guide our research into understanding how PNG media promotes fibril formation.

Little is known about how individual PSM peptides interact to form amyloid fibrils. The *In vitro* assay conditions I have used in my work test only a very limited set of environmental conditions to answer basic questions about amyloid formation. Multiple PSM species, including PSMβs and δ-toxin, were detected in our initial MS screen, and many of these have not been assayed for virulence or function in our lab. Some of my early assays using synthetic peptides suggested that some PSMs form amyloids while others do not, despite their structural similarity (Figs. 1.4, 6.1). A fundamental understanding of how each PSM interacts and folds to adopt amyloid structure is critical. We also know little about the basic stoichiometry of each component in fibril development. A more specific understanding of PSM interactions in amyloid formation will be critical to understanding how amyloid aggregation happens *In vivo*.

A confounding factor in my work has been reliance on synthetic peptides to assay fibril formation. While synthetic peptides have the advantage of being pure and free of cellular contaminants, lot-to-lot variation yields a product that can alter assay results, making comparison difficult over time. To correct this, it would be helpful to design a method to purify or extract PSMs in-house. Purification or isolation of aggregative proteins, like amyloids, is problematic and requires troubleshooting to optimize yield. Although methods have been

published to isolate or detect PSMs in culture supernatants, I have thus far been unsuccessful to do so [6-8]. These methods include phase separation with phenol or butanol. Even after following exact protocols published using HPLC and electrospray MS to detect and quantitate PSMs from culture supernatants, I was unable to get usable results, even with the help of an HPLC expert consult (Ray Barbahan, UM Dept. of MCDB). One method I had initially attempted to set up to purify PSMs used SUMO tagging to express and purify peptides from an *E. coli* expressions system. The plasmids were created, but purification was not attempted due to time constraints. Future researchers following up on my work may want to explore this as a possible avenue for generating PSMs in-house.

Another potentially interesting aspect of PSM fibril formation is the effect of methionine oxidation on peptide behavior. All PSMs have N-terminal methionines, and in the cellular environment pools of formylated and non-formylated PSMs exist (7,9-10). Our analysis *of S. aureus* fibril composition did not detect formylation, but the oxidative state of this methionine residue may have implications for protein folding. For example – amyloid- beta contains a methionine residue (M35) that can inhibit amyloid formation when oxidized [11,12]. The oxidation state of methionine can be affected by many factors, including: atmospheric oxygen, oxygen radicals (like H_2O_2), buffers that are not de-gassed, metal ions, desiccation, freeze/thaw cycles, photo reactions, and even DMSO. Any one of these factors can create variable amounts of oxidized/reduced peptide if not carefully controlled. If methionine oxidation does affect PSM amyloid formation, the environmental factors may provide biologically relevant triggers to initiate aggregation. One example of a biologically relevant oxidation event may occur during

ROS exposure inside neutrophils. By utilizing the ThT assay, peptides oxidation states should be tested for inhibition or promotion of amyloid formation.

How does PSM aggregation affect the biofilm lifecycle?

PSMs may contribute to biofilm organization by affecting cellular movement. It is well-established that the agr quorum sensing system promotes biofilm dispersal through several mechanisms. Dispersal occurs largely from the expression of proteases that degrade proteinaceous adhesins of the biofilm [13]. Agr also promotes dispersal by upregulating production of PSMs that function as "detergent-like" molecules to stimulate spreading and separation of the biofilm matrix [13-18]. Staphylococci do not produce specialized motility structures like flagella, and are described as being non-motile. Indeed, it has been shown that PSMs display a unique form of "colony spreading" motility when bacterial cultures are spotted on low agar plates [14]. This colony spreading is agr and PSM dependent [17,18] and the effect of each individual PSM's contribution to spreading on low agar TSB has already been well characterized and it was seen that smaller PSMs including PSMα3 and delta toxin promoted spreading [17,18]. Initial evidence suggests that PSMs influence spreading on low agar plates under fibril inducing media. I have observed that a form of agar "cracking" occurs only in the presence of PSMs (Fig. 6.2). It is unknown whether this is related to spreading motility or an artifact observation of the technique. Exogenous addition of PSMα1 promotes colony expansion in *S. aureus* culture spots (Fig. 6.2a). Virulent strains that are known to have highly up-regulated agr activity display distinct spreading and cracking on low agar PNG media relative to a PSM mutant (Fig. 6.2b). The effects of fibril formation should be studied to determine

whether it can also inhibit spreading, as it appears to inhibit biofilm dispersal activity also associated with surfactant-like behavior (Figs. 2.7-2.8). Another factor that influences spreading is eDNA. Kaito et al. found that extracellular nuclease activity is necessary to degrade eDNA for colony spreading [19]. The mechanism by which eDNA interacts with PSMs is unknown. I have also observed that loss of nuclease activity correlates to reduced movement, likely due to the presence of DNA interfering with movement (Fig. 6.2b). My current research implies that the presence of DNA can trigger PSM amyloid formation, inherently changing the biochemistry the amphipathic peptides, and inhibiting their surfactant-like activity. If so, DNA and PSMs may be interacting in such a way that movement is inhibited. On the other hand, DNA may guide bacterial motility similar to that described by Gloag et al. wherein DNA provides a sort of "rail system" for bacterial motility [20]. Endogenous addition of PSMs to Δ*PSM* bacterial cultures spotted onto low-agar plates with DNA may inform us whether DNA inhibits spreading in our strains under these conditions.

Finally, one big question that is unanswered for me is simply: are biofilms that produce PSM fibrils less toxic to cells than biofilms not producing PSM fibrils? I had attempted to test this in my last manuscript using DNA as a sort of "chelater" for toxic peptides. I found that the hemolysis assay protocol I was using did not show different toxicity between supernatants of biofilms grown in conventional media (TSBg) or fiber inducing media (PNG). One reason for this may be the presence of α-hemolysin, another agr-regulated toxin known for its robust hemolytic activity. Creation of α-hemolysin null mutants with inducible PSM expression could

be used to generate PSM-rich supernatants in an assay similar to that described in Chapter 3 (Fig. 3.4c), which could then be mixed with DNA to test the "chelater" hypothesis.

What is the functional role of PSM aggregation *in situ*?

If PSM aggregation has a role in modulating virulence, what other conditions favor amyloid formation? One way to address this question would be to determine whether other polyanions, like heparin or polyphosphate, can induce amyloid formation. DNA is a polyanionic compound, and many other polyanions are present in the environmental conditions outside of and throughout the host environment. Heparin, and closely related proteoglycans like heparan and chondroitin, can be found throughout the Animalia kingdom. Heparin is commonly used as an anticoagulant to keep blood from clotting in catheter lock solutions. Hospital patients are at great risk from hospital acquired *S. aureus* infections, and it is known that *S. aureus* can persist in catheters. Heparin has been previously shown to promote biofilms *In vitro* and in catheter lock solutions [21]. *S. aureus* is also known to attach to prosthetic joints and fixtures coated in proteoglycans used to stimulate growth and reassimilation into the body [22]. It is possible that biofilms forming in these environments are at least partially influenced by PSM amyloid formation, which may further modulate immunogenicity of these kinds of infections. My initial work suggests that PSMα1 aggregation is enhanced by heparin salts (Fig. 6.3). Previous efforts to detect amyloid fibrils in *S. aureus* – positive patient catheters were unsuccessful, mostly because TEM is not sensitive enough on its own to detect amyloid fibrils. Staining fixed biofilms with ThT, or using immunocytochemistry to detect PSMα1 in fibril structures, may prove to be more useful techniques. Beyond the body environment, there is evidence that polyphosphate,

polymers of charged phosphate monomers that are important phosphate sink for aquatic and soil dwelling organisms, may function as a type of molecular chaperone during amyloid aggregation [23]. It would be interesting to see if polyphosphate plays a role in PSM amyloid aggregation, similar to heparin.

Finally, an unexplored but potentially fruitful field of study involves PSM interaction with neutrophil DNA. It has been shown that neutrophils rapidly eject extracellular DNA nets in a process called NETosis (Neutrophil Extracellular Traps) to sequester and kill invasive bacteria. NETosis is signaled by sensing of PSMs, suggesting they may interact [24]. If PSMs form amyloids in the presence of neutrophil DNA, it may serve to detoxify the bacterial presence, and reduce inflammatory response by containing the infection. Immunostaining in abscess models may show the presence of PSM fibrils in chronic infections. Looking to interactions between PSMs and host cells, like neutrophils, will help us to understand what role they play in the body environment of humans suffering from chronic and acute *S. aureus* infections.

The future directions outlined here are just a small sample of the many directions my research could take. As previously stated, the fundamental discovery of functional bacterial amyloids in *S. aureus* biofilms opens doors to fields including medicine, biophysics, bacterial genetics and physiology, environmental ecology, and so on. The annotated aims stated here will help to fulfill our basic understanding of how PSMs aggregate *In vitro* (for assay optimization) and *In vivo* (for understanding their role in the bacterial lifecycle).

Figures

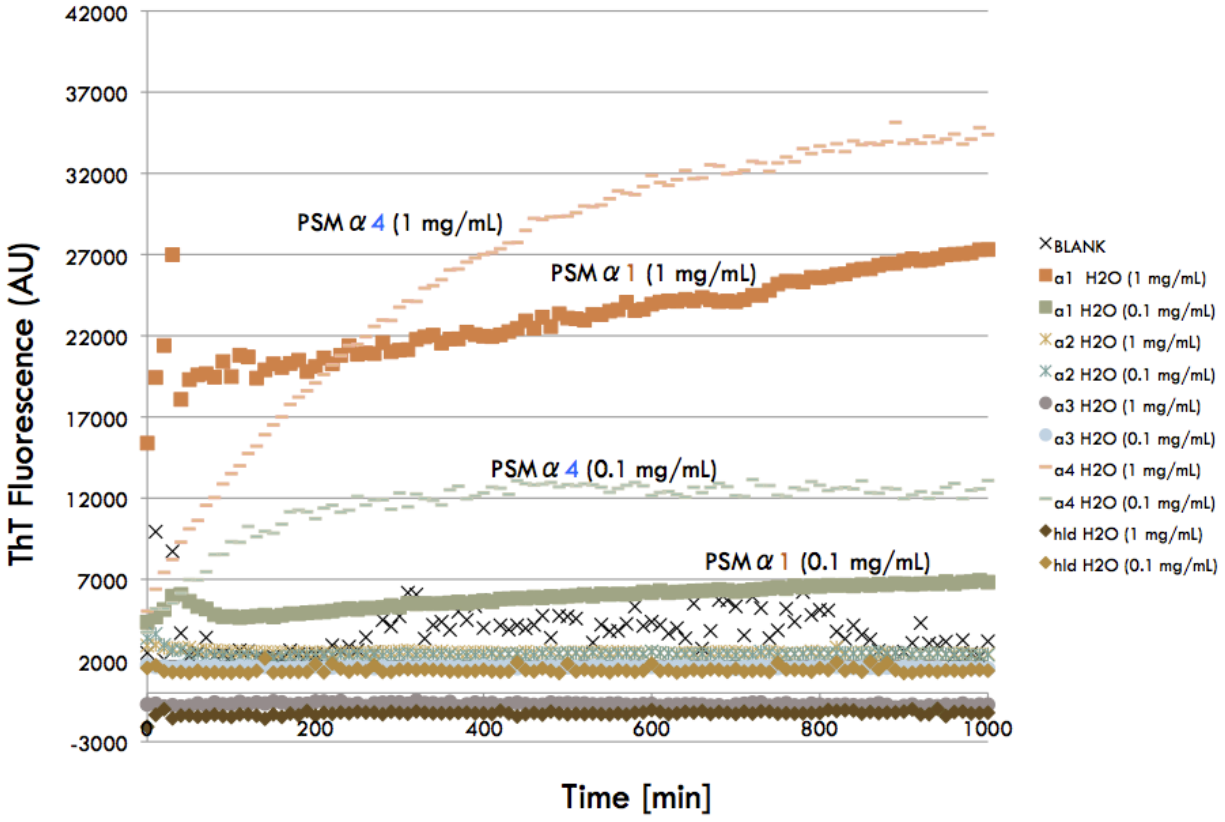
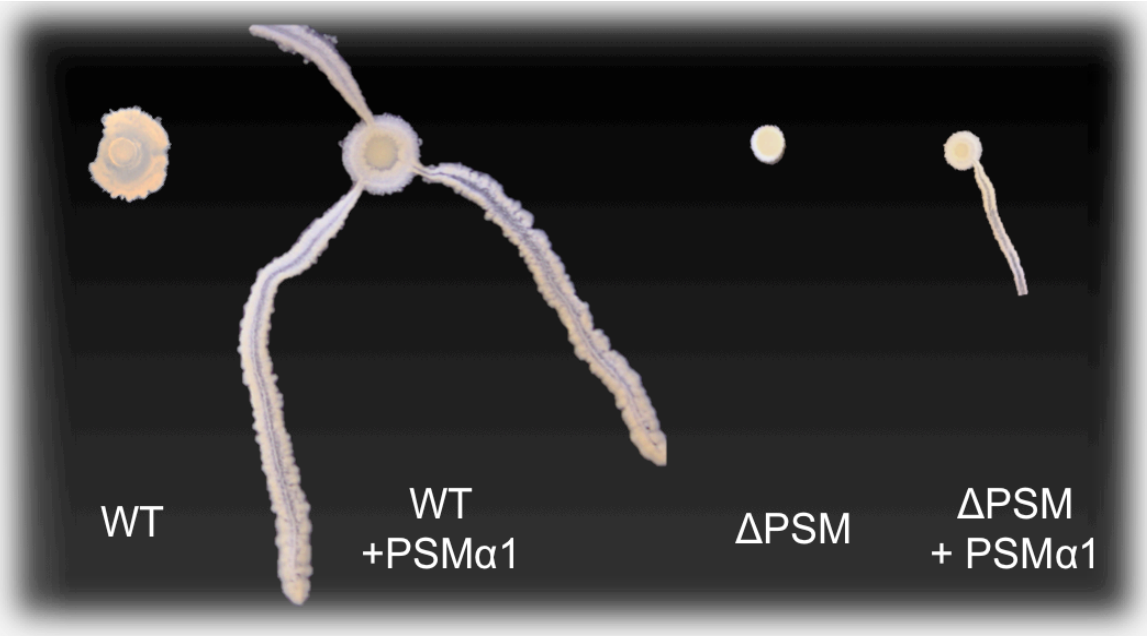


Fig. 6.1, Polymerization of synthetic PSMs *In vitro*,

In our original MS analysis of *Staphylococcus aureus* extracellular fibrils (summarized in Chapter 2, Fig. 2.3D), we detected the presence of several PSM species (PSMα1,2,4, PSMB1,2, and δ-hemolysin). Of these, I found that some polymerize readily in a concentration-dependent manner under standard assay conditions (Described in detail in Chapter 2). These include PSMα1 and PSMα4. Results were repeatable within peptide lot (Peptide 2.0).

A.



B.

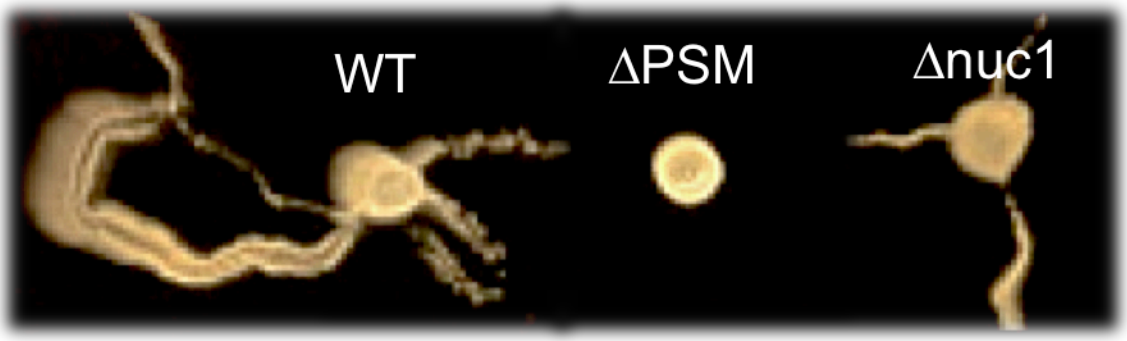


Fig. 6.2, *S. aureus* colony spreading depends on PSM and nuclease activity,
A) Exogenous addition of 1mg/mL PSMα1 to *S. aureus* culture prior to spotting induces spreading and cracking on low agar PNG plates in lab strain SH1000. B) *S. aureus* USA300 strain with up-regulated agr shows distinct spreading and cracking while a PSM mutant does not. Loss of nuclease activity disrupts gross movement, while some cracking is still visible.

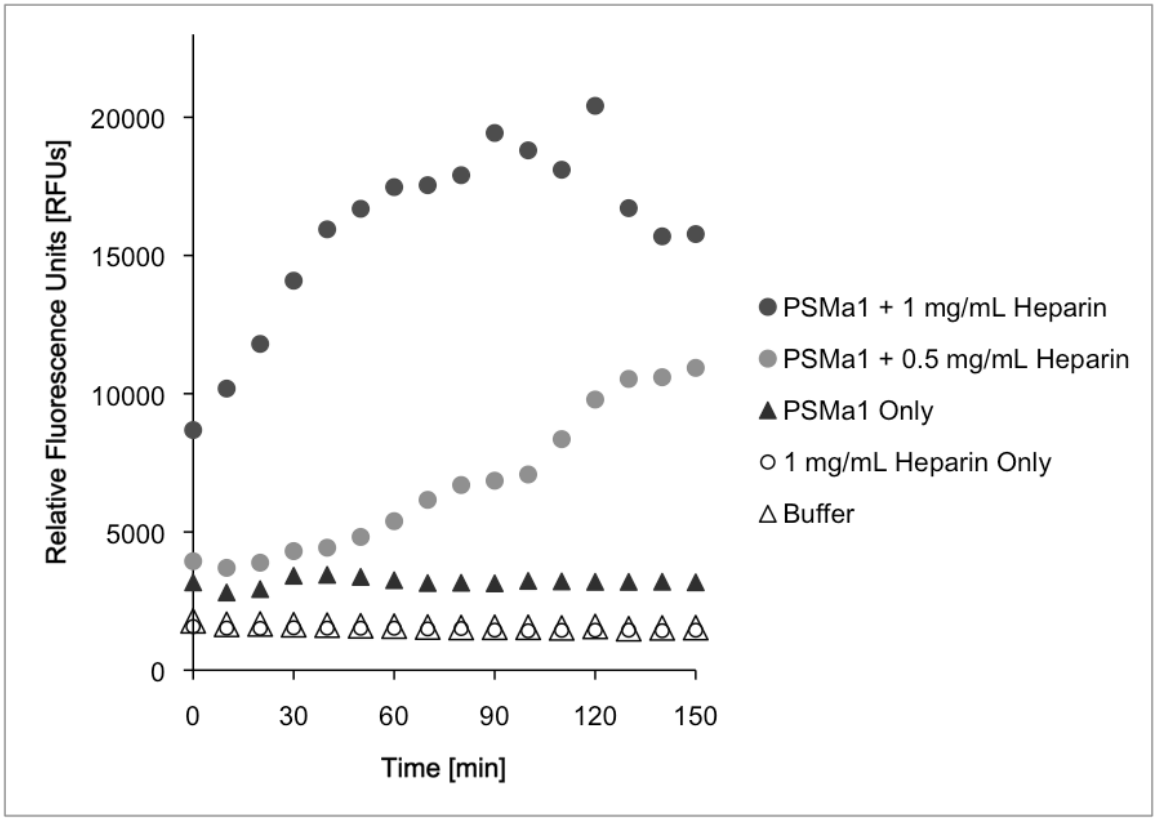


Fig. 6.3, PSMα1 interacts with heparin to induce amyloid formation,
PSMα1 (1 mg/mL) was incubated with heparin sulfate salt (Sigma) under conditions described in Chapter 4. Heparin increased ThT levels in a concentration-dependent manner. From this data, it may be understood that heparin, like DNA, can promote amyloid formation, and may have implications for human health.

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