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Extracellular DNA facilitates the formation of functional amyloids in *Staphylococcus aureus* biofilms

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Summary

Persistent staphylococcal infections often involve surface-associated communities called biofilms. *Staphylococcus aureus* biofilm development is mediated by the co-ordinated production of the biofilm matrix, which can be composed of polysaccharides, extracellular DNA (eDNA) and proteins including amyloid fibers. The nature of the interactions between matrix components, and how these interactions contribute to the formation of matrix, remain unclear. Here we show that the presence of eDNA in *S. aureus* biofilms promotes the formation of amyloid fibers. Conditions or mutants that do not generate eDNA result in lack of amyloids during biofilm growth despite the amyloidogeneic subunits, phenol soluble modulin peptides, being produced. *In vitro* studies revealed that the presence of DNA promotes amyloid formation by PSM peptides. Thus, this work exposes a previously unacknowledged interaction between biofilm matrix components that furthers our understanding of functional amyloid formation and *S. aureus* biofilm biology.

Introduction

Staphylococcus aureus is Gram-positive bacterium that exists both as a commensal, commonly colonizing humans, and as a pathogen, being the causative agent of a diverse array of acute and chronic infections (Lowy, 1998; Wertheim *et al.*, 2005). Persistent *S. aureus* infections, including osteomyelitis and endocarditis, occur when *S. aureus* accumulates to form a biofilm at the infection site (Lowy, 1998). The challenge presented by *S. aureus* biofilm infections is their remarkable resistance to both host immune responses and available antibiotic chemotherapies (Patel, 2005; Boles and Horswill, 2008). A detailed understanding of the processes that allow *S. aureus* to colonize surfaces and persist in the biofilm state will facilitate the discovery of improved treatment strategies.

Biofilms are communities of bacterial cells encased in a polymeric matrix (Flemming and Wingender, 2010). Although the exact composition of the matrix varies greatly between strains and growth conditions, *S. aureus* biofilms often include extracellular DNA (eDNA), polysaccharides and proteins, including adhesins and amyloid fibers (Gotz, 2002; Rice *et al.*, 2007; Boles *et al.*, 2010; Schwartz *et al.*, 2012; Foster *et al.*, 2014; Foulston *et al.*, 2014). Recent studies indicate that biofilm matrix composition is modified in response to specific environmental cues, thus biofilm matrices from identical bacterial strains can vary depending on local conditions (Rohde *et al.*, 2001; Landini, 2009; Sharma-Kuinkel *et al.*, 2009; Boles *et al.*, 2010; Beenken *et al.*, 2012; Moormeier *et al.*, 2013). Interactions between matrix components within the biofilm are likely responsible for creating an adaptable structure during adherence, maturation and dispersal (Huseby *et al.*, 2010; Periasamy *et al.*, 2012; Ganesan *et al.*, 2013; Pavlovsky *et al.*, 2013).

eDNA is an important and abundant matrix component of many single- and multispecies cultured biofilms (Whitchurch *et al.*, 2002; Mann *et al.*, 2009; Flemming and Wingender, 2010). eDNA strengthens biofilms, helps confer antibiotic resistance, acts as a nutrient source during starvation, promotes colony spreading and structuring and serves as a gene pool for horizontal gene transfer (Whitchurch *et al.*, 2002; Molin and Tolker-Nielsen, 2003; Mann *et al.*, 2009; Dominiak *et al.*, 2011; Kiedrowski *et al.*, 2011; Chiang *et al.*, 2013; Gloag *et al.*, 2013). In *S. aureus*, eDNA is produced through the autolysis of a subpopulation of the biofilm cells (Thomas and Hancock, 2009), and this altruistic suicide behavior is mediated through the activity of a murein hydrolase, AtlA (Nedelcu *et al.*, 2011; Bose *et al.*, 2012). AtlA is a secreted enzyme thought to be responsible for maintaining cell wall metabolism during cell

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division and growth (Oshida *et al*., 1995; Baba and Schneewind, 1998; Biswas *et al*., 2006), and its upregulation results in increased lysis (Bose *et al*., 2012). Loss of AtlA activity results in the reduction of eDNA and decreased biofilm formation in some biofilm models (Heilmann *et al*., 1997; Rice *et al*., 2007; Mann *et al*., 2009; Houston *et al*., 2011).

Many extracellular proteins found in the *S. aureus* biofilm matrix contribute to biofilm development (Flemming and Wingender, 2010; Foulston *et al*., 2014). Several are enzymatic, like AtlA, and others are structural intra- and intercellular adhesins. Under some growth conditions, the *S. aureus* biofilm matrix includes remarkably stable, β-sheet-rich amyloid polymers. Amyloids are highly aggregative proteins that form ordered, self-templating fibers that can promote biofilm stability (Shewmaker *et al*., 2011; DePas and Chapman, 2012; Schwartz and Boles, 2013). Bacterial amyloids are an increasingly appreciated part of many biofilm matrices (Chapman *et al*., 2002; Bieler *et al*., 2005; Alteri *et al*., 2007; Dueholm *et al*., 2010; DePas and Chapman, 2012; Oli *et al*., 2012). Their inherent resistance to protease degradation and detergents helps amyloids to strengthen biofilms by reinforcing and protecting the matrix from destruction (Shewmaker *et al*., 2011). The amyloid fibers produced by *S. aureus* are composed of small peptides called phenol soluble modulins (PSMs) (Schwartz *et al*., 2012; 2014). In many biologically relevant systems, PSMs act as toxins influencing neutrophil chemotaxis and cytolysis and are reported to possess surfactant properties, influencing biofilm development and colony spreading (Wang *et al*., 2007; 2011; Tsompanidou *et al*., 2011; Periasamy *et al*., 2012), activities often associated with non-aggregated amyloid forming proteins (Soreghan *et al*., 1994; Zhou *et al*., 2012). Our previous findings revealed PSMs are capable of forming amyloid structures in biofilms, and this aggregation mediates their toxic activity (Schwartz *et al*., 2012; 2014). However, the *in vivo* relevance and environmental factors influencing the transition from soluble toxin to inert fibril are poorly understood in the biofilm environment.

In this study, we demonstrate a novel mechanism for amyloid formation in *S. aureus*. We found that the presence of eDNA in the biofilm matrix promotes the formation of PSM amyloid fibers. Biofilms lacking eDNA do not assemble extracellular fibers in drip biofilm reactors, even when PSM peptides are produced. Additionally, *in vitro* assays demonstrate a pronounced interaction between DNA and PSMs that promotes amyloid formation. PSMs mixed with DNA are less cytotoxic than soluble PSM peptides, indicating that DNA may be able to sequester these toxins by favoring aggregation of free peptides. Our findings reveal a previously unappreciated interaction between biofilm matrix components that furthers our understanding of *S. aureus* biofilm biology.

Results

The influence of media conditions on PSM production and polymerization

Staphylococcus aureus biofilms are encased in a matrix composed primarily of polysaccharides, proteins and eDNA (Gotz, 2002; Rice *et al*., 2007; Schwartz *et al*., 2012; 2014). The overall composition can vary depending on growth conditions, leading to a highly variable biofilm architecture and biofilms displaying different tolerances to perturbations. Previously, we observed biofilm growth in different media types resulted in altered biofilm matrix compositions; ie, growth in a peptone-based medium-termed PNG-generated biofilms containing an amyloid composed of PSMs that promoted resistance to matrix degrading enzymes (proteinase K, DNase, dipsersin B) and physical disruption, whereas growth in tryptic soy broth produced no detectable fibers (Schwartz *et al*., 2012). To better understand how the PNG growth condition resulted in amyloid generation, we first tested the hypothesis that altered PSM expression was resulting in PSM amyloid production. To test this hypothesis, *S. aureus* drip biofilms were grown in TSBg or PNG medium, and PSM production was monitored (Fig. 1). Under both fiber producing conditions (PNG Fig. 1C and D) and fiber non-producing conditions (TSBg Fig. 1A and B), no significant difference was observed in transcription of the *psmα* promoter throughout biofilm growth (Fig. 1E). In addition, western blot analysis revealed similar levels of PSMα1 from both biofilm growth conditions (Fig. 1F). Taken together, these results suggest that PSMs are produced at similar levels in both growth conditions, but PSM amyloids are only formed in the PNG media condition. These observations led us to hypothesize that amyloid formation may be controlled by external factors.

We next sought to determine if a component of the biofilm growth media influenced PSM amyloid polymerization. We used Thioflavin T binding assays to determine whether the presence of DNA can alter PSM polymerization kinetics. 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 (LeVine, 1999). We observed that synthetic PSMα1 peptide polymerized with similar kinetics when resuspended in either TSBg or PNG (Fig. 2A). Examination of the resulting fibers from both conditions via transmission electron microscopy did not reveal any gross changes in fiber morphology (Fig. 2B and C). These results suggest that the biofilm growth medium was not influencing PSM polymerization (Fig. 2) or expression (Fig. 1) and that another factor was responsible for the observed differences in amyloid fiber formation in different growth environments.

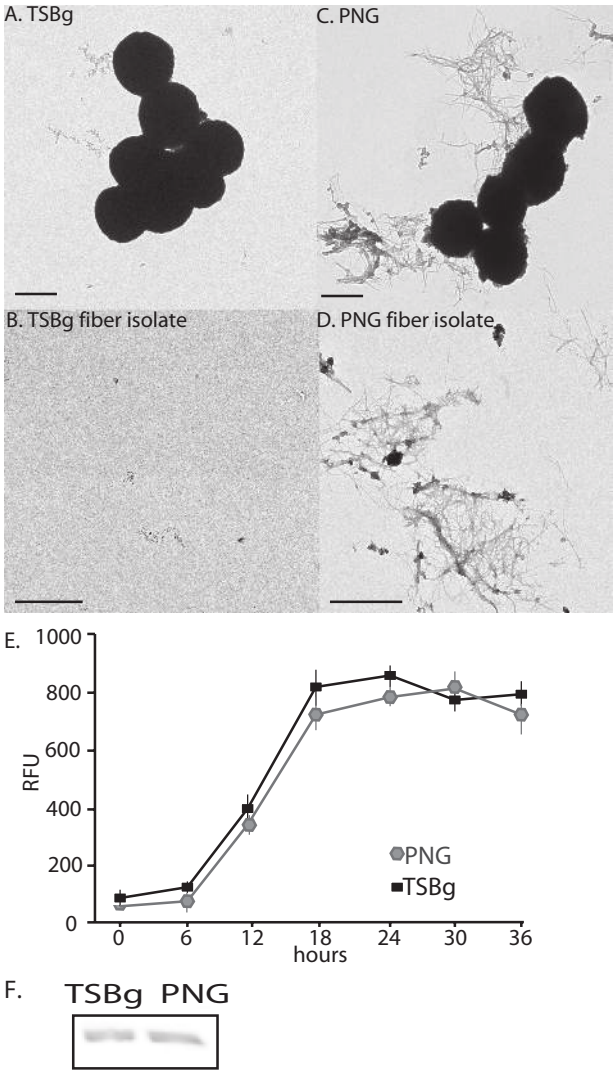

Fig. 1. PSMs are produced in both fiber producing and fiber non-producing biofilm growth conditions. (A–D) TEM micrographs of wild-type *S. aureus* biofilm cells grown for 3 days in TSBg or PNG media: (A) cells grown in TSBg, (B) amyloid fiber preparation from cells grown in TSBg, (C) cells grown in PNG, (D) amyloid fiber preparation from cells grown in PNG. (E) Measurement of the *psmα1* -YFP reporter activity in wild-type *S. aureus* grown in drip reactors in either TSBg or PNG. Error bars show standard error of the mean (SEM). (F) Western blot with anti-PSMα1 antibody from biofilms grown for 72 h in either TSBg or PNG.

eDNA levels vary in different growth conditions and influence amyloid polymerization

The possibility that the growth media could alter the composition of the biofilm matrix, leading to the promotion or inhibition of PSM amyloid formation was also examined. Because extracellular DNA (eDNA) is known to be an important biofilm matrix component that is generated through cell autolysis (Mann *et al.*, 2009; Jakubovics *et al.*, 2013), we first assessed whether levels of autolysis and eDNA differed between the two biofilm growth conditions (TSBg versus PNG). To test for this, we assayed for autolysis as a function of β-galactosidase release into culture supernatants. Significantly higher β-galactosidase activity was observed in effluents of *S. aureus* biofilms grown in PNG media compared with TSBg grown biofilms. This result demonstrates increased autolysis under PNG conditions (Fig. 3A). To determine whether differences in autolysis correlated with differences in eDNA levels, eDNA was isolated from biofilms and quantitated. In both 48 h and 72 h old biofilms, the PNG grown biofilms contained more eDNA than TSBg-grown biofilms (Fig. 3B).

Previous research on human disease amyloids has demonstrated that nucleic acids are capable of modulating amyloid assembly (Calamai *et al.*, 2006; Di Domizio *et al.*, 2012a,b). Amyloidogenic proteins, including alpha synuclein, prions and amyloid-beta, are all known to interact with nucleic acids *in vitro* (Cordeiro *et al.*, 2001; Suram *et al.*, 2002; 2007; Hegde and Rao, 2007). Amyloids have even been found associated with DNA *in vivo* (Suram *et al.*, 2002; Camero *et al.*, 2013). Because of this precedent, and our finding that significantly more eDNA was present when PSMs formed amyloids, we hypothesized that eDNA could modulate the assembly of PSMs into amyloid fibrils within the biofilm matrix. To determine

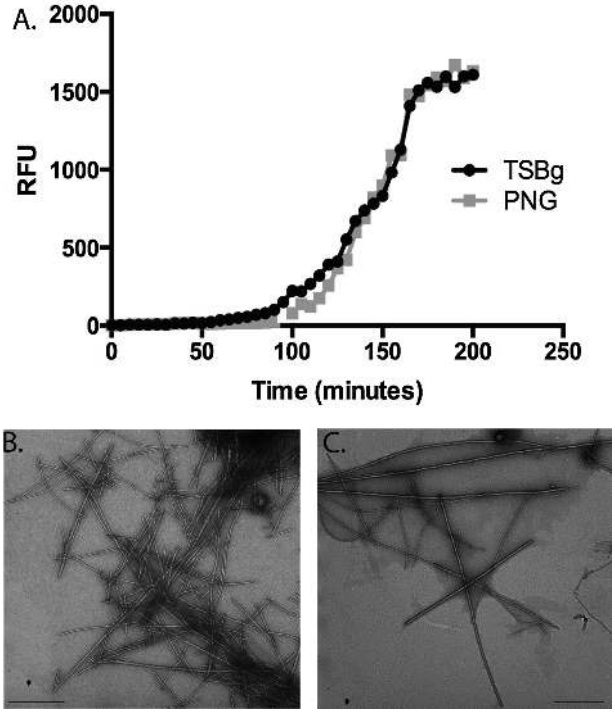

Fig. 2. Amyloid polymerization in different biofilm growth mediums.
A. ThT assay monitoring amyloid polymerization kinetics of PSMα1 peptide (500 μg ml^{-1}) resuspended in TSBg or PNG biofilm media. B and C. TEM micrographs of PSMα1 peptide (500 μg ml^{-1}) after 24 h in TSBg (B) or PNG (C) biofilm growth media. Bars indicate 500 nm.

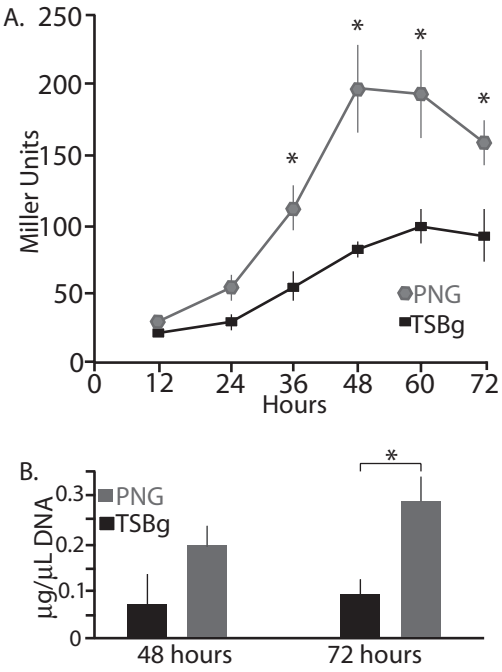

Fig. 3. Comparison of autolysis and eDNA levels from *S. aureus* grown in TSBg versus PNG.
A. Biofilm cultures of wild-type *S. aureus* harboring plasmid pAJ22, which expresses cytoplasmic β-galactosidase, were grown for 72 h in either TSBg or PNG. Every 12 h during the time course, samples were removed, and β-galactosidase activity in cell free supernatants was measured (reported in Miller units). *$P < 0.01$ by *t* test (B) Quantitation of eDNA in biofilms. Wild-type *S. aureus* was grown for 48 or 72 h in either TSBg or PNG and eDNA isolated and quantitated. Results shown were the average of three independent experiments done in triplicate and error bars show standard deviation. *$P < 0.01$ by *t*-test.

whether eDNA influenced PSM amyloid formation in biofilms, we grew the autolysis deficient mutant Δ*atlA* in biofilm drip reactors with PNG media (fiber producing conditions, Fig. 4). Strains unable to produce the major murein hydrolase AtlA produced biofilms with biomasses comparable with a wild-type strain in the drip reactor biofilm (data not shown). We observed that the Δ*atlA* biofilms did not produce extracellular fibril structures (Fig. 4A). Western blot analysis with anti-PSMα1 antibody verified the presence of PSMα1 in fibril isolates of a wild-type parent strain, but not in Δ*psm* or Δ*atlA* strains, lacking the fibril structures detected via TEM (Fig. 4B). We also confirmed that PSMα1 was produced in whole cell lysates of the Δ*atlA* mutant, demonstrating that PSMα1 was produced in Δ*atlA* biofilms, but not assembled into fibrils (Fig. 4C). eDNA levels in these biofilms were quantified, and it was confirmed that Δ*atlA* mutant biofilms did not produce detectable amounts of eDNA as compared with a wild type and a Δ*psm* mutant (Fig. 4D). These findings substantiate the hypothesis that autolytic eDNA release prompts PSM amyloid assembly in biofilms.

Next, we examined whether exogenous addition of eDNA to an *atlA* mutant could complement biofilm amyloid assembly during biofilm growth in PNG (Fig. 5). As anticipated, the addition of eDNA to a Δ*psm* mutant did not result in the generation of fibers after biofilm growth (Fig. 5A). However, the addition of eDNA to the Δ*atlA* mutant resulted the production of fibers (Fig. 5B). In addition, biofilm growth of wild-type *S. aureus* in TSBg with eDNA added exogenously, resulted in the generation of fibers (Fig. 5C).

PSMα1 and other *S. aureus* PSM peptides autoaggregate to form amyloid fibril structures in a concentration-dependent manner (Schwartz *et al.*, 2012; 2014). Utilizing a low concentration of PSMα1 peptide below the threshold for autoaggregation, we observed that ThT fluorescence increased over time in samples containing both PSMα1 and DNA as compared with PSMα1 only (Fig. 6A). *S. aureus* genomic DNA alone did not show increased fluorescence above baseline (Fig. 6A). In addition, by TEM analysis, we observed that the co-incubation of PSMα1 with DNA yielded fibril structures that were not present in DNA alone or PSMα1 alone conditions (Fig. 6B–D). Taken together, these data suggest that PSMα1 forms ordered amyloid structures in the presence of DNA and that the addition of DNA stimulates amyloid formation at peptide concentrations that do not typically auto-aggregate. Furthermore, this suggests that DNA can lower the critical concentration threshold necessary for the spontaneous aggregation of PSM peptides.

eDNA-mediated PSM aggregation reduces PSM cytotoxity

Finding that PSMα1 forms amyloid aggregates in the presence of DNA led us to consider the role that DNA might play in virulence. PSMs are potent toxins, contributing to infection in part by facilitating lysis of multiple host cell types (Wang *et al.*, 2007; Li *et al.*, 2009). However, the formation of amyloid fibers by PSMs can significantly reduce their cytotoxicity (Schwartz *et al.*, 2014). Therefore, we hypothesized that the addition of DNA could facilitate the conversion of soluble PSMα1 into an aggregated fibril form, thus abrogating its cytotoxic activity. Incubation of red blood cells (RBCs) with freshly resuspended soluble PSMα1 peptides resulted in significant lysis (Fig. 7A). However, addition of DNA to PSMα1 reduced hemolysis activity. These results indicate that PSMα1 interacts with DNA over the course of at most 1 h to form amyloid complexes that display reduced cytotoxic activity compared with the same concentration of non-aggregated PSMα1.

We next sought to determine whether PSMs and eDNA physically interact using an *in vitro* gel shift experiment. *S. aureus* bacterial genomic DNA was mixed with freshly

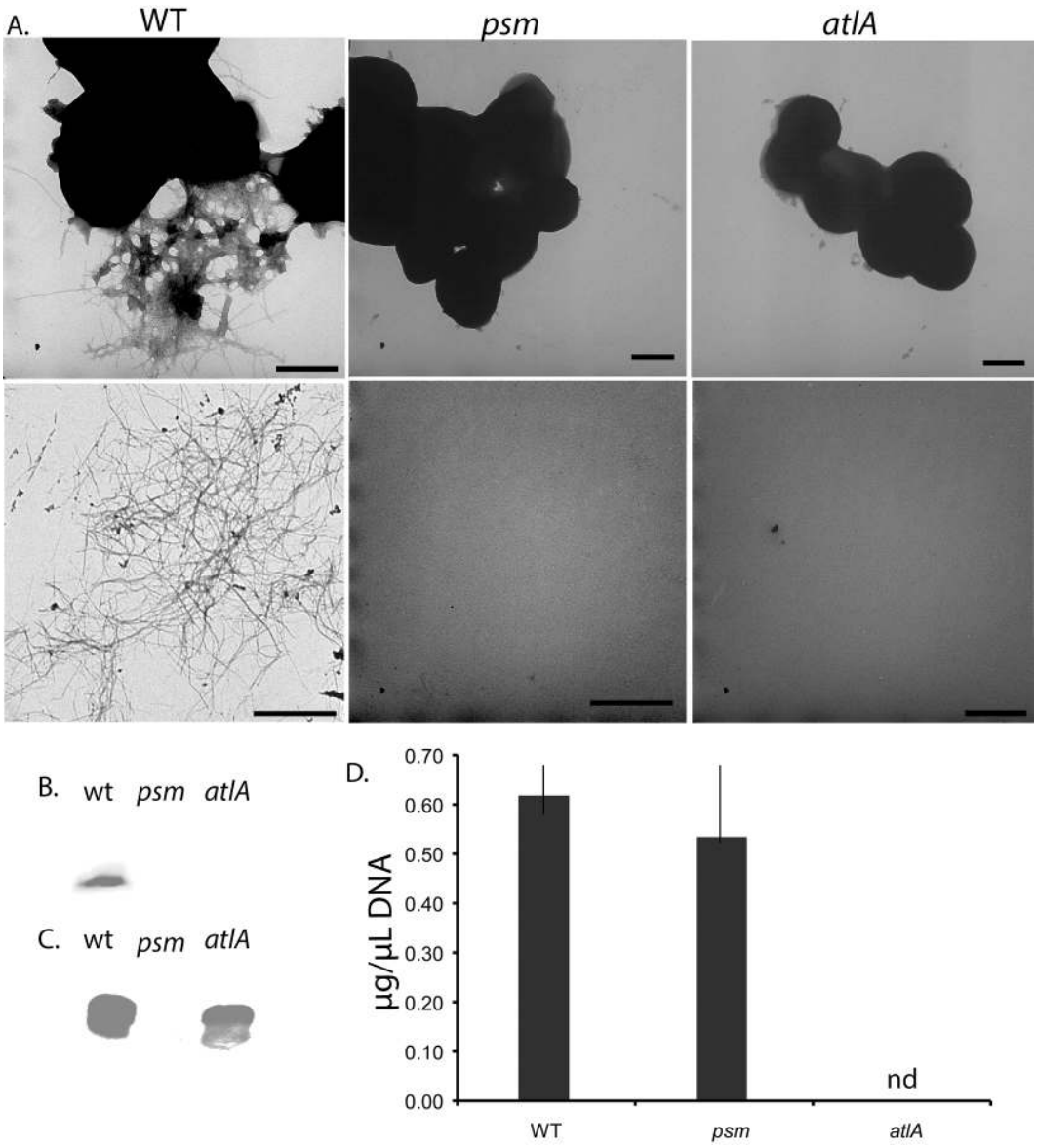

Fig. 4. An autolysin mutant lacking eDNA does not form PSM amyloids in biofilms.
A. TEM micrographs of *S. aureus* biofilm cells (top row) or amyloid fiber prepartions (bottom row) of wild type, Δ*psm* and Δ*atlA* mutants, demonstrating that these mutant strain biofilms do not produce extracellular fibrils like the wild type parent. Scale bar indicates 500 nm.
B. Western blot using anti-PSMα1 antibody against fiber preparations from wild type, Δ*psm* and Δ*atlA* biofilms.
C. Western blot using anti-PSMα1 antibody against whole cell lysates of wild type Δ*psm* and Δ*atlA* biofilms, showing that PSMα1 is produced in an Δ*atlA* mutant.
D. Quantitation of eDNA in biofilms. Wild type and Δ*psm* mutant showed comparable amounts of eDNA, whereas the Δ*atlA* mutant had none detectable. Results are the average of three independent experiments and error bars show standard deviation.

solubilized (non-aggregated) PSMα1 and incubated over a 24 h time course (Fig. 7B). Samples that were mixed immediately prior to loading onto the gel resulted in no retardation of the DNA, whereas samples incubated 1 h produced DNA migration patterns that appeared to be impeded, as indicated by smearing. DNA incubated with PSM peptide for 4 and 24 h did not migrate into the gel, being largely retained in the loading well area. We hypothesized that amyloid formation in the presence of DNA formed a large macromolecular structure around the nucleic acid. To determine the relative size of the PSMα1-DNA complexes *in vitro*, we employed dynamic light scattering (DLS). DLS is a sensitive and non-destructive technique used to measure the effective hydrodynamic radius, R_H, of macromolecules in solution (Berne, 2000). Particularly, it has also been used to track interaction and complex formation between biopolymers (Orberg *et al.*, 2007). As done for the gel shift experiment, DNA was

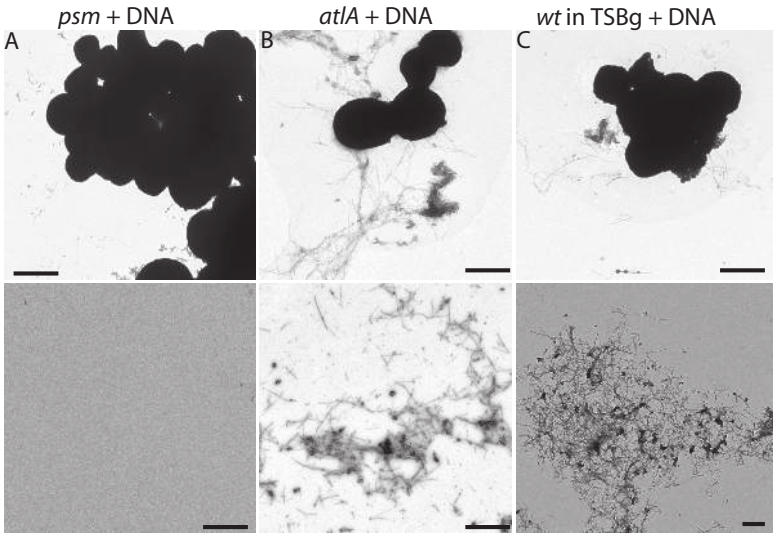

Fig. 5. The presence of eDNA restores fiber formation in an autolytic mutant. TEM micrographs of *S. aureus* biofilm cells (top row) or amyloid fiber prepartions (bottom row) in (A) a Δ*psm* mutant grown in PNG with salmon sperm DNA added exogenously, (B) an *atlA* mutant biofilm grown in PNG with salmon sperm DNA added exogenously, (C) wild-type *S. aureus* biofilm grown in TSBg with salmon sperm DNA added exogenously restores fibril formation. Scale bar indicates 500 nm.

incubated with non-aggregated PSMα1 and samples were retrieved after different incubation times for R_H measurement. We observed that over the 24 h time course, the effective size of the complexes formed by PSMα1 alone were about fourfold smaller than those formed by PSMα1 in the presence of DNA (Fig. 7C). From these findings, we concluded that PSMα1 and DNA are capable of forming large complexes through direct interactions.

Discussion

The extracellular matrix is one of the defining features of biofilms, providing a means for microorganisms to control their local environment. This matrix plays a critical role in the formation and persistence of biofilm communities. For example, matrix components such as eDNA, polysaccharides and functional amyloids are known to have a profound influence on biofilm development and resistance to antimicrobials (Flemming and Wingender, 2010). Despite the biological and clinical significance of the *S. aureus* biofilm matrix, only a limited understanding of its components, generation and interactions exist.

In this work, evidence is provided, demonstrating a role for eDNA in functional amyloid formation within the biofilm environment. The presence of amyloid fibers composed of PSMs in *S. aureus* biofilms was associated with the ability of the strain and growth condition to allow autolysis and the release of eDNA, rather than the production of PSMs

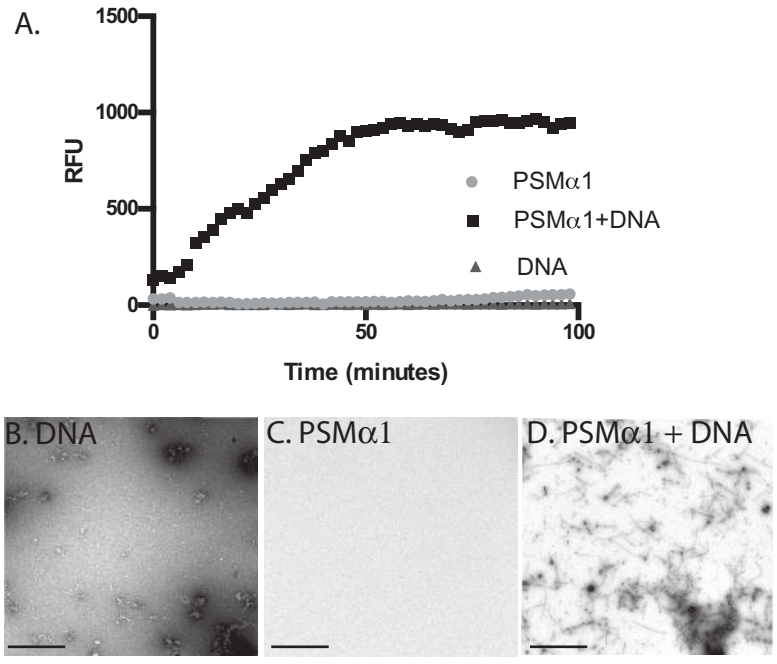

Fig. 6. DNA promotes PSMα1 amyloid fiber formation. (A) ThT assay monitoring amyloid polymerization kinetics of 5 μg ml^{-1} PSMα1 in the presence and absence of 0.1 μg ml^{-1} DNA or DNA alone. TEM micrographs of DNA (B), PSMα1 alone (C) and PSMα1 + DNA (D) samples after 12 h or incubation. Bar indicates 500 nm.

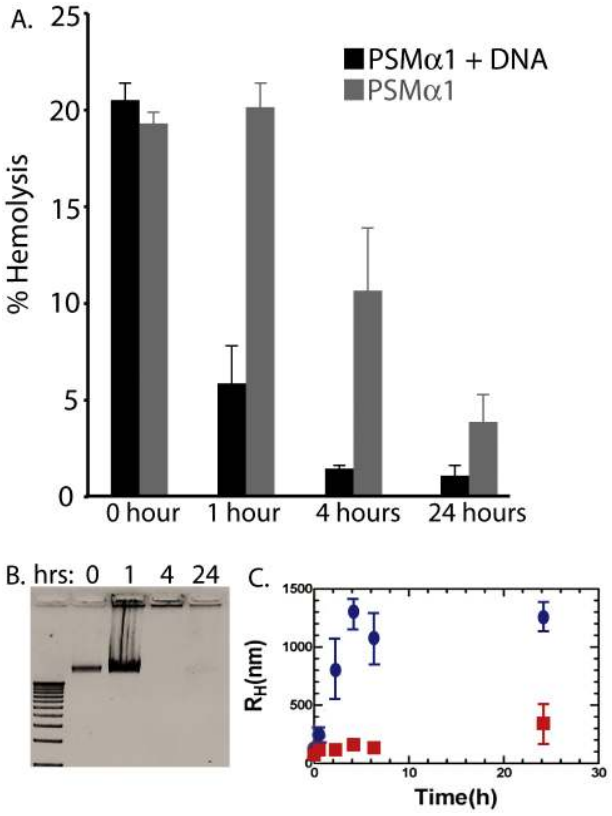

Fig. 7. Interaction with DNA reduces PSMα1 cytolytic activity.
A. PSMα1 (100 μg ml^{-1}) hemolysis is greatly reduced in the presence of DNA (100 ng μl^{-1}). % hemolysis was calculated from the average of three replicates.
B. Incubation of DNA (100 ng μl^{-1}) and PSMα1 (100 μg ml^{-1}) for different times (0, 1, 4, and 24 hours) reveals interaction between PSMα1 and DNA in a DNA migration assay. Initial association is observed as a smear at 1 h and by 4 h DNA is no longer able to run through the gel matrix.
C. Dynamic light scattering measuring the change in effective hydrodynamic radius, RH (nm), of PSMα1 peptide with (•) and without DNA (■) as a function of time. It is seen that in the presence of DNA, PSMα1 peptides bind with the DNA molecules to form complexes that are significantly larger in RH than the PSMα1 peptide alone.

(Figs 1–5). The presence of DNA promotes the polymerization of PSMα1 at concentrations that PSMα1 alone does not readily polymerize (Fig. 6). We propose that this is a result of DNA attracting the positively charged PSM and raising the local peptide concentration, therefore resulting in polymerization. Finally, it was found that presence of DNA reduces the cytolytic activity of PSMα1, likely via a formation of a DNA/PSM complex (Fig. 7).

eDNA is an important component of many bacterial biofilms. It is known to be involved in clinically relevant settings including in the sputum of cystic fibrosis patients (Pressler, 2008), during otitis media infection (Jones *et al.*, 2013), in whooping cough caused by *Bordetella petussis* (Conover *et al.*, 2011) and during exposure to neutrophil NETS (Brinkmann and Zychlinsky, 2007). Many bacteria are capable of producing functional amyloids that can act as a biofilm matrix component. Amyloids have even been detected in naturally occurring bacterial populations of *Proteobacteria*, *Bacteriodetes*, *Chloroflexi*, *Actinobacteria* and *Firmicutes* (Otzen and Nielsen, 2008; DePas and Chapman, 2012). However, the identification of specific amyloidogenic proteins in many bacterial species has not been trivial, and our work suggest that additional factors like eDNA may be necessary to promote the conversion of these proteins into an amyloid state. It will also be of interest to determine how other polyanions influence the polymerization of other functional and disease-associated amyloids. Numerous amyloids have been documented for their interactions with polyanions like nucleic acid or glycosaminoglycans (Calamai *et al.*, 2006). For example, prion conversion of proteins into amyloid fibrils is modulated by the presence of nucleic acids like DNA and RNA (Cordeiro *et al.*, 2001; Deleault *et al.*, 2007). The cannonical amyloid model Amyloid Beta is known to interact with DNA *in vitro* to cause nicking and structural changes (Barrantes *et al.*, 2007; Hegde and Rao, 2007; Suram *et al.*, 2007; Yu *et al.*, 2007) and is frequently associated with DNA in amyloid plaques of Alzheimer patients (Suram *et al.*, 2002). Interest is also emerging in using nucleic acid – amyloid scaffolding for nanomaterials (Gour *et al.*, 2012). Interestingly, much of this research is based on speculation into the pre-DNA world where small peptide amyloids may have acted as scaffolds for nucleic acid assembly in the absence of cellular machinery (Carny and Gazit, 2005).

Examples of biofilm matrix interactions are beginning to emerge in recent years. eDNA was found to colocalize with polysaccharides in *Myxococcus xanthus*, increasing the mechanical strength, surface adhesion and stress resistance of the extracellular matrix against DNaseI disassembly (Hu *et al.*, 2012). In *Eschericia coli*, the functional amyloid component CsgA has been shown to bind to DNA, promoting curli amyloid assembly (Fernandez-Tresguerres *et al.*, 2010) and the resulting DNA/amyloid complex acts to stimulate autoimmunity (Gallo *et al.*, 2015). In *Pseudomonas aeruginosa*, two main biofilm matrix components (eDNA and the polysaccharide Psl) cooperate by physically interacting in a biofilm to form the web of Psl–eDNA fibers, which functions as a skeleton to allow bacteria to adhere and grow (Wang *et al.*, 2015). Finally, in *S. aureus*, it was recently shown that the neutral sphingomyelinase Beta toxin, can bind single and double stranded DNA to create matrix interactions that are shown to be important for endocarditis (Huseby *et al.*, 2010). Similarly, we demonstrate here that small peptide toxins, like PSMα1, can also interact with DNA. This interaction could have implications in virulence as PSM peptides bound to DNA are less toxic than freely soluble PSMs.

Taken together, our results underscore the notion that the formation of biofilm matrix is a complex, dynamic process with contribution of multiple factors, including bacterial cell death, the release of eDNA, the secretion of protein and the interaction between the matrix components. We speculate that the presence of DNA or other negatively charged polymers at infection sites like the cystic fibrosis lung likely promotes biofilm formation and reduces the cytolytic activity of virulence factors.

Experimental procedures

Bacterial strains, plasmids, and growth conditions

Staphylococcus aureus strain, SH1000, was the wild-type strain used in this study (Horsburgh *et al.*, 2002), and the Δ*psm* (alpha and beta PSM mutant) and Δ*atlA* mutants have been previously described (Boles *et al.*, 2010; Schwartz *et al.*, 2012). Previous work has shown the absence of PSMα1-4 and PSMB1-2 in strain SH1000 do not produce fibers when grown in drip biofilm reactors with PNG as the media despite the presence of other PSMs (delta toxin and N-AgrD) encoded on the genome (Malone *et al.*, 2009; Schwartz *et al.*, 2012). The *psmα*1::YFP transcriptional fusion reporter plasmid was created by cloning a 600 bp region upstream of the *psmα1* transcriptional start site into the HindIII and Kpn1 sites of pAH16 (Malone *et al.*, 2009). Liquid cultures were routinely grown in tryptic soy broth (TSB) incubated at 37°C with 200 r.p.m. shaking unless otherwise noted.

Biofilm experiments

Drip-flow biofilms were grown in 3.3 g l^{-1} peptone, 2.6 g l^{-1} NaCl, 3.3 g l^{-1} glucose (PNG media) or 0.6 g l^{-1} tryptic soy broth and 1.5 g l^{-1} glucose (TSBg) as previously described (Schwartz *et al.*, 2010; 2012). 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 ('fiber preparations') as previously described (Schwartz *et al.*, 2012). Biofilm cells, fibril isolates and synthetic peptide fibrils were prepared and imaged via TEM as described previously (Schwartz *et al.*, 2012). Extracellular DNA was quantitated using a protocol used by Jones *et al.* (2013) and adapted for Qubit using a kit and fluorometer (Invitrogen) according to the manufacturer's protocol. Autolysis assays using B-galactosidase activity measurements were performed as previously described (Boles *et al.*, 2010), and samples were obtained by collecting 5 ml of effluent from drip biofilms at indicated time points. In experiments that supplemented biofilms with DNA, salmon sperm (sDNA) DNA (1 mg ml^{-1} in 1 ml of PBS, sterilized by heating to 95°C for 20 min then cooling at room temp for 2 h) was added to drip biofilm reactors by injection into the flow port at the following time points after the initiation of media flow: 1 h, 12 h, 24 h.

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. Rabbits were immunized with purified peptide epitopes (Abgent), and the resulting sera were tested by enzyme-linked immunosorbent assay before Protein A affinity purification.

Western blot sample prep and 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.*, 2012). Proteins from 1 ml of pooled fibril isolates were concentrated by precipitation with 250 μl 100% TCA and incubated at 4° for 2 h. Precipitated protein samples were resuspended in 40 μl SDS loading buffer (Bio-Rad – 1× Bio-Rad Tris-Tricine SDS PAGE loading dye plus 200 mM BME). Samples were bath sonicated for 20 min, vortexed and boiled for 10 min prior to loading.

For cell lysate fractions, 1000 μg ml^{-1} lysostaphin was added to each culture and samples were incubated for 1 h at 37°C with shaking. These samples were then pelleted, and the supernatant transferred to a fresh tube. Forty microliters of SDS loading dye (Bio-Rad) was added, and samples were boiled for 10 min, bath sonicated for 20 min, vortexed and finally boiled 10 min. Twenty microliters of each cell fraction was loaded into a 16.5% SDS PAGE gel. Gels were run in duplicate and in the same electrophoresis tank (Bio-Rad Mini-Protean Tetra) for wet-transfer. After denaturation in sample buffer (Bio-Rad), 20 μl of each sample was loaded into pre-cast 16.5% Bio-Rad Tris-Tricine acrylimde gels and run at 100V/65 mA for 100 min at room temperature. These gels were transferred onto 0.22 μM polyvinylidene fluoride membrane run at 70V/250 mA for 80 min at 4°C.

Western blotting was performed for use with the LiCor Odyssey imaging system according to LiCor protocols. 10X TBS (25 mM Tris-Base, 150 mM NaCl, 2 mM KCl, pH 7.40) was stored at 4°C and diluted just prior to use for 1× TBS and 1× TBST. 1× TBST (100 ml 10× TBS + 900 ml MQ H_2O + 1 ml Tween-20) was stored at 4°C between washes. Blocking Buffer was made fresh using 200 ml 1× 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 h, washed between antibodies with 1× TBST. Imaging was carried out using the LI-COR Odyssey® scanner and software (LI-COR Biosciences).

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 h) and further dried to completion under N_2 stream (2 h). 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.

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 or indicated medium condition containing 0.2 mM Thioflavin T (ThT) prior to assay. Fluorescence was measured every 10 min 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 time course.

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 min prior to assay. Care was taken to ensure that no protein was stuck to the sides of the wells. *S. aureus* genomic DNA was isolated using Gentra Puregene Yeast/Bact. Kit (Qiagen). *S. aureus* gDNA (0.1, mg ml^{-1}) and PSMα1 stock (1.0, g ml^{-1}) were dissolved into a total volume of 20 µl in sterile HPLC-grade H_2O and incubated rocking at room temperature. 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 h with or without 1.0 mg ml^{-1} PSMα1. Samples were separated by electrophoresis for 1 h 30 min at 150V/400 mA on a 1% agarose gel. Fresh ethidium bromide was mixed into ddH2O, and the gel was stained for 30 min, and soaked in ddH_2O for 30 min before visualization.

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 $\lambda = 632.8$ nm (He-Ne, JDS Uniphase Corp, USA). All measurements were done at $T = 298 \pm 0.5$ 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 $\theta = 90°$ 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).

Hemolysis of RBCs

Red blood cell preparation and heme absorbance assay performed as previously described (Schwartz *et al.*, 2014). Summarily, PSMα1 synthetic peptide was resuspended in 25 µl filtered HPLC grade DMSO to make a 10 mg ml^{-1} stock solution. Salmon sperm DNA (10 mg ml^{-1}) was mixed 100 µl into 900 µl filtered HPLC water, and this working stock (1 mg ml^{-1}) was aliquoted in sterile microcentrifuge tubes. PSMα1 was added to filter sterile HPLC water or DNA working stock to make a 100 µl volume, and incubated for indicated times on a rocker at room temperature. Ten microliters of these samples was added to 90 µl of 3.0×10^8 rabbit RBCs and were incubated 1 h shaking at 180 r.p.m. at 37°C. Unlysed RBCs were pelleted by centrifugation and 6 µl supernatant was added to 94 µl PBS and absorbance was read at 480 nm to calculate heme release.

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