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# Do amyloid structures formed by *Staphylococcus aureus* phenol-soluble modulins have a biological function?

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ABSTRACT

Phenol-soluble modulins (PSMs) are alpha-helical, amphipathic peptides that have multiple functions in staphylococcal physiology and virulence. Recent research has suggested that PSMs form amyloid fibrils and amyloids are involved in PSM-mediated phenotypes such as cytolysis and biofilm stability. While we observed PSM amyloid formation using electron microscopy and dye assays, there were no apparent differences in the production of extracellular fibrous material between a PSM-deficient strain and the isogenic wild-type strain. Furthermore, we detected no correlation between cytolytic or pro-inflammatory activities with the propensity of PSM derivatives to form amyloids. In addition, we propose a model based on our finding of non-specific attachment of PSMs to DNA, which we here report results in resistance to DNase digestion, explaining previous findings on PSM-mediated biofilm stability without the necessity to assume amyloid involvement. Collectively, our results indicate that PSM amyloid formation may not be of major relevance for known key biological functions of PSMs. Intriguingly, however, we found that amyloid-forming capacity of PSMalpha3 allows almost no amino acid exchanges, suggesting importance of amyloid formation in possibly yet unknown functions of PSMs.

## 1. Introduction

Phenol-soluble modulins (PSMs) have first been described in *Staphylococcus epidermidis* by the group of Seymour Klebanoff, who coined the name for a group of three pro-inflammatory peptides he named PSMα, PSMβ, and PSMγ, which participated into the phenol phase during hot phenol extraction (Mehlin et al., 1999). One of those peptides, PSMγ, is identical to the *S. epidermidis* δ-toxin, a homologue of the well-known *Staphylococcus aureus* δ-toxin.

In the following years, we performed systematic investigations to analyze the PSM composition of staphylococcal pathogens. Most staphylococci appear to produce PSMs, as judged by chromatographic and mass spectrometric (MS) analyses, with the pattern being characteristic for, but different in every species (Rautenberg et al., 2011). Notably, a complete analysis of the PSM pattern of a given species requires purification efforts in addition to high-pressure liquid chromatography (HPLC)/MS analyses. To date, the list of PSMs in *S. epidermidis* has been completed (Otto, 2009; Vuong et al., 2004; Yao et al., 2005); and we analyzed the PSMs produced by *S. aureus* and *Staphylococcus haemolyticus* (Da et al., 2017; Wang et al., 2007).

*S. aureus* is by far the most important pathogenic species among the staphylococci. It is a widespread and leading human pathogen, causing many thousands of deaths per year in the U.S. alone. *S. aureus* causes a wide spectrum of diseases, ranging from chronic diseases such as atopic dermatitis and other skin diseases, to acute and often fatal conditions such as sepsis (Lowy, 1998). Together with *S. epidermidis*, *S. aureus* is also a premier cause of infections associated with indwelling medical devices, in which the propensity to form biofilms plays a major role for pathogenesis (Otto, 2008).

PSMs have multiple functions in staphylococcal physiology and pathogenesis (Cheung et al., 2014a). They have been implicated in major types of staphylococcal infections, including skin, blood, and bone infections (Cassat et al., 2013; Peschel and Otto, 2013; Wang et al., 2007). Many PSMs, in particular those of the smaller α-type (~20–25 amino acids, as opposed to β-type PSMs, ~44–45 amino acids), lyse leukocytes and erythrocytes, among other cell types, and

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thus have a major function in acute *S. aureus* infection (Peschel and Otto, 2013; Rasigade et al., 2013; Wang et al., 2007). All PSMs structure biofilms and cause dissemination of biofilm-associated infection to the bloodstream and organs in vivo (Periasamy et al., 2012; Wang et al., 2011). In addition, there is recent evidence for the δ-toxin representing a major factor exacerbating atopic dermatitis (Nakamura et al., 2013). Finally, PSMs activate the formyl peptide receptor 2 (FPR2) (Kretschmer et al., 2010), and in an indirect fashion the toll-like receptor 2 (TLR2) (Hanzelmann et al., 2016), an interaction probably serving for the recognition of staphylococcal invaders by innate host defense, with one of the most important consequences being the stimulation of neutrophil chemotaxis.

Amyloids are aggregates of peptides or proteins that stick together to form fibrils. In humans, they are most infamous to cause neurodegenerative disorders and other diseases, a process known as amyloidosis (Dobson, 2003; Jucker and Walker, 2013). In bacteria, amyloids are now being recognized to have beneficial functions that contribute to normal bacterial physiology, which may include pathogenesis (Romero and Kolter, 2014). For example, they form curli in *Escherichia coli* and other Enterobacteriaceae (Evans and Chapman, 2014). Furthermore, most biofilms produce extracellular material that is in an amyloid form (Taglialegna et al., 2016). Finally, several human receptors appear to be stimulated strongly by amyloid ligands (Ye and Sun, 2015), including FPR2 (Tiffany et al., 2001).

In our laboratory, we had observed for many years that PSMs have a strong tendency to aggregate, which makes working with them often extremely troublesome. More recently, reports have been published describing that PSM produce amyloids. The Boles group published that several *S. aureus* PSMs produce amyloids and that they contribute to *S. aureus* biofilm stability (Schwartz et al., 2012). Recently, Tayeb-Fligelman et al. reported that PSMα3, an extremely cytolytic PSM (Cheung et al., 2012; Wang et al., 2007), produces a novel amyloid structure, which the authors claimed is linked to PSM function, most notably cytolysis (Tayeb-Fligelman et al., 2017).

Here, we analyzed all *S. aureus* PSMs for their propensity to form amyloid structures using transmission electron microscopy (TEM) as well as with an amyloid-staining dye. In addition, we critically revisited the claims that PSM amyloid formation is associated with PSM function. In particular, we analyzed whether there is a correlation between amyloid formation and PSM function using an alanine screen peptide bank of PSMα3. Finally, we propose a model explaining the observed biofilm stability-mediating properties of PSMs by attachment of PSMs to extracellular DNA, thus without the necessity to assume amyloid formation.

## 2. Materials and methods

### 2.1. Bacterial strains

Strain LAC (pulsed-field type USA300) and isogenic deletion mutants produced in this strain were used in the present study. In strain LAC Δ*psmα*Δ*psmβ*Δ*hld,* the *psmα* and *psmβ* operons are deleted in their entirety and translation of the *hld* (δ-toxin) gene is abolished by mutation of the start codon (not to interfere with the regulatory function of RNAIII, in which the gene is embedded).

### 2.2. Peptides

All PSM peptides were synthesized by commercial vendors at > 95% purity in their N-formylated forms and quality-checked by the Protein Chemistry Section, Research Technologies Branch, NIAID.

### 2.3. Transmission electron microscopy (TEM)

For analysis of amyloid formation by TEM, cells were prepared as published by Schwartz et al. (Schwartz et al., 2012). Cells were grown in TSB with 0.5% glucose for 48 h in a flow cell culture system as previously described (Periasamy et al., 2012). Cells were harvested from the glass surface of the flow cells and treated with fixative (2% paraformaldehyde in 0.1 M sodium phosphate buffer), after which tubes were sealed. For negative staining by TEM, five µl of sample was adsorbed on glow-discharged 200 mesh formvar-coated copper grids. Following a quick wash with water, the grids were stained with 2% uranyl acetate. The grids were imaged using a Hamamatsu camera (Advanced Microscopy Techniques) on a Hitachi 7500 TEM instrument at 80 kV.

### 2.4. Thioflavin T (ThT) assay for amyloid formation

*S. aureus* PSM solutions were prepared by diluting 1:100 with water from a 10 mg/ml stock in DMSO. PSM samples were incubated with filtered ThT at a final concentration of 0.2 mM.

PSMα3 and derivatives in powder form were treated with trifuoroacetic acid/hexafluoroisopropanol (1:1), dried in a chemical hood for 2 days, followed by further drying in a rotary vacuum concentrator for 2 h. Then, peptides were dissolved in 10 mM sodium phosphate buffer (pH 8.0) containing 150 mM NaCl. After sonication for 10 min, undissolved material was removed by centrifugation at 10,000 rpm for 5 min in a table top microcentrifuge. Alternatively, PSMs were diluted from 10 mg/ml stocks in DMSO in the same buffer and treated accordingly.

Thioflavin T (ThT) was purchased from AnaSpec (SensoLyte Thioflavin T β-Amyloid (1–42) Aggregation Kit). In each reaction, 200 µM peptide was mixed with 50 µM ThT in 10 mM sodium phosphate buffer (pH 8.0) and 150 mM NaCl. The fluorescence of ThT was scanned from 450 nm to 700 nm with excitation at 438 nm at the indicated times using a Tecan Safire multimode microtiter plate reader every 5 min at 37 °C.

### 2.5. DNase digestion experiment

For testing whether PSMs protect DNA from digestion by DNase, ~300 ng each of two different PCR fragments of staphylococcal DNA were incubated for 5 min with a mixture of all seven *S. aureus* PSMs, after which DNase was added and samples were incubated for 15 min. The final concentrations of PSMs in the incubation mixture were 0.1 mg/ml for each PSM, and the final concentration of DNase was 0.5 Kunitz units/ml. Then, samples were loaded on an 0.8% agarose gel, which was run for 45 min at 180 V and stained using ethidium bromide.

## 3. Results and discussion

### 3.1. Analysis of PSM amyloid fibril formation by electron microscopy and amyloid-specific dyes

In 2012, Schwartz et al. published the first study showing amyloid formation by PSMs (Schwartz et al., 2012). These authors used a mixture of all *S. aureus* PSMs at 0.1 mg/ml each or PSMα1 alone, and showed fibril formation using TEM and increased absorption of the amyloid-specific dye thioflavin T (ThT) compared to controls. For the ThT experiments, they used a 1:100 dilution from stock solutions made at 10 mg/ml in dimethylsulfoxide (DMSO), which we also routinely do in our laboratory to avoid the aggregation of PSMs that is frequently observed in concentrated aqueous stocks. For consistency, we used this method for both the ThT and TEM experiments.

We observed ThT absorption over control, indicative of amyloid formation, for the mix of all PSMs at 0.1 mg/ml, similar to the result reported by Schwartz et al. (Schwartz et al., 2012) (Fig. 1A). However, for the single PSMs, there was ThT absorption over control only for PSMα1 among the α-type PSMs (PSMα1-4, δ-toxin) (Fig. 1B) − the only single PSM for which this was also reported by Schwartz et al. (Schwartz et al., 2012). Interestingly, we found the most pronounced