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## The cytotoxic *Staphylococcus aureus* PSM $\alpha$ 3 reveals a cross- $\alpha$ amyloid-like fibril

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### Abstract

Amyloids are ordered protein aggregates, found in all kingdoms of life, and are involved in aggregation diseases as well as in physiological activities. In microbes, functional amyloids are often key virulence determinants, yet the structural basis for their activity remains elusive. We determined the fibril structure and function of the highly toxic, 22-residue phenol-soluble modulins  $\alpha$ 3 (PSM $\alpha$ 3) peptide secreted by *Staphylococcus aureus*. PSM $\alpha$ 3 formed elongated fibrils that shared the morphological and tinctorial characteristics of canonical cross- $\beta$  eukaryotic amyloids. However, the crystal structure of full-length PSM $\alpha$ 3, solved de novo at 1.45 angstrom resolution, revealed a distinctive “cross- $\alpha$ ” amyloid-like architecture, in which amphipathic  $\alpha$ -helices stacked perpendicular to the fibril axis into tight self-associating sheets. The cross- $\alpha$  fibrillation of PSM $\alpha$ 3 facilitated cytotoxicity, suggesting that this assembly mode underlies function in *Staphylococcus aureus*.

### One Sentence Summary

Fibrillation-dependent cytotoxicity of PSM $\alpha$ 3 functional amyloid is encoded by a cross- $\alpha$  architecture.

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Amyloids are structured protein aggregates that encompass a variety of structures, ranging from small soluble oligomers to plaques of insoluble fibrils. Amyloids are most notorious for their involvement in human neurodegenerative diseases (e.g., Alzheimer’s and Parkinson’s diseases) (1). Insights into amyloid structures were long challenged by their polymorphic and partially disordered nature (2, 3), but advances in x-ray and electron micro-crystallography [e.g., (3–6)], cryo-electron microscopy [e.g., (7, 8)] and solid-state nuclear magnetic resonance (NMR) spectroscopy [e.g., (2, 9–12)] have substantially furthered the understanding of eukaryotic disease-associated amyloid properties and notable stability.

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Eukaryotic amyloids share a common structural feature, namely, the cross- $\beta$  spine, in which individual  $\beta$ -strands run perpendicular to the fibril axis (13).

In contrast to disease-associated amyloids, functional amyloids, evident mostly in microbes, participate in diverse activities that benefit the producing organism (14–16). Thus far, structural knowledge of microbial amyloids has been lacking, as have been the possible differences between functional and disease-associated amyloids (17, 18). Functional amyloids were recently suggested to play a role in the pathogenicity of *Staphylococcus aureus*, a prominent cause of aggressive infections and an emerging public-health concern (19, 20). These amyloids are formed by several members of a family of secreted virulent peptides called phenol-soluble modulins (PSMs). PSMs stimulate inflammatory responses, lyse human cells, and contribute to biofilm structuring (20, 21). High expression of PSM $\alpha$ s is linked to the virulence potential of methicillin-resistant *S. aureus* (MRSA) (22). Amyloid fibrillation of some PSMs promote biofilm stability (20), yet the role of the amyloid state in other PSM activities is unclear.

The 22-residue peptide PSM $\alpha$ 3 is the most cytotoxic and lytic member of the PSM family (21, 23). PSM $\alpha$ 3 forms amphipathic helices (21, 23), as shown by solution NMR (24). Yet the helix alone is not sufficient to achieve biological activities (21). We found that PSM $\alpha$ 3 formed elongated and un-branched fibrils (Fig. 1A), which bound the amyloid-indicator dye Thioflavin T, generating high levels of fluorescence emission and a characteristic amyloid-fibrillation curve (Fig. 1B and fig. S1). Whereas previously characterized amyloid proteins convert into  $\beta$ -pleated structures during fibril formation (1), we found that PSM $\alpha$ 3 maintained its  $\alpha$ -helical conformation, both in solution and in the fibrils (fig. S2 and table S1). The x-ray diffraction pattern of PSM $\alpha$ 3 indicated that the fibrils were indeed built from the stacking of  $\alpha$ -helices (fig. S3 and supplementary methods).

To understand the atomic basis of these  $\alpha$ -helical fibrils, we solved the micro-crystallographic fibril structure of full-length PSM $\alpha$ 3 at 1.45 Å resolution (Fig. 1 and table S2), using de novo phasing methods (25). The structure revealed amphipathic  $\alpha$ -helices positioned perpendicular to the fibril axis, which stacked into sheets that ran parallel to the fibril axis and mated through the hydrophobic faces of the helix (Fig. 1, D and E, and figs. S4 and S5). This “cross- $\alpha$ ” amyloid-like fibril is has not been observed previously in structures of eukaryotic amyloids solved to date. The structural characteristics of PSM $\alpha$ 3 fibrils were nevertheless reminiscent of those displayed by cross- $\beta$  fibrils, which also feature in-register stacking of a structural element into sheets, that mate through a dry interface (Fig. 2 and fig. S6). The chemical properties governing cross- $\alpha$  fibril stability, i.e., buried surface area and shape complementarity between sheets, resembled those of cross- $\beta$  structures (figs. S4 to S7 and table S3). These structural characteristics suggested that the binding of the amyloid-indicator dye Thioflavin T to PSM $\alpha$ 3 fibrils (Fig 1B and figs. S1 and S8) probably occurs via cavities running parallel to the fibril axis. These cavities bear the characteristics of repeating structures that exist mainly in  $\beta$ -rich amyloid fibrils, but also within some  $\alpha$ -helical rich environments (26, 27). Thioflavin T binding to these cavities is often mediated by aromatic side chains (26), which were indeed abundant in the PSM $\alpha$ 3 sequence (Fig. 1C). Overall, PSM $\alpha$ 3 fibrils not only shared the morphological and tinctorial properties of amyloid fibrils, but also exhibited a cross- $\alpha$  architecture reminiscent of cross- $\beta$  amyloids,

notwithstanding the fundamental difference that the fibrils were formed of  $\alpha$ -helices rather than  $\beta$ -strands (Fig. 2).

To explore whether fibrillation plays a role in PSM $\alpha$ 3 cytotoxicity, we performed mutagenesis analysis to identify PSM $\alpha$ 3 mutants that do not fibrillate, and discovered F3A and the K9P/F11P double mutant (A, Ala; F, Phe; K, Lys; P, Pro) (figs. S8 and S9). The two mutants displayed much lower T-cell cytotoxicity compared to wild-type PSM $\alpha$ 3 (Fig. 3A). In contrast, the G16A mutant (G, Gly), which forms fibrils recognized by Thioflavin T, thus serving as control, was highly cytotoxic (Fig. 3A and figs. S8 and S9). Whereas the K9P/F11P double mutant was mostly unstructured in solution, both G16A and F3A mutants maintained  $\alpha$ -helical conformation (fig. S8), reinforcing the notion that helical conformation alone is not sufficient for cytotoxicity. Furthermore, the addition of a biocompatible surfactant maintained  $\alpha$ -helicity, but diminished fibrillation and abrogated PSM $\alpha$ 3 toxicity (Fig. 3B and figs. S8 and S10). The same pattern of fibrillation-dependent cytotoxicity was recorded also against human embryonic kidney 293 (HEK293) cells (fig. S11), suggesting that the lytic activity of PSM $\alpha$ 3 fibrils is not cell-specific. It is possible that this cytotoxicity stems from self-assembly of helices that form large “carpets” of amphipathic sheets (fig. S6) on the membrane surface, triggering its deformation (28). The exact conformation that contributes to amyloid toxicity is still under debate. In some human disease-associated amyloids, the toxic entity has been attributed to a prefibrillar conformation, whereas the mature  $\beta$ -rich fibrils detoxify the amyloid (29). Several eukaryotic amyloid proteins contain  $\alpha$ -helices in their monomeric or prefibrillar intermediate states [e.g., (30)], or even in the fibril state [e.g., (27)], suggesting a link to the cytotoxicity induced by the fibrillation of PSM $\alpha$ 3 into purely helical species.

In this work, we have demonstrated, at atomic resolution, that cross- $\alpha$  fibrillation of PSM $\alpha$ 3 into amyloid-like fibrils is required for cytotoxicity and suggest a key role for cross- $\alpha$  fibrils in *S. aureus* pathogenicity. PSM $\alpha$ 3 is thus a functional amyloid, displaying architecture and properties similar to those of eukaryotic cross- $\beta$  fibrils, but differs in its secondary structure elements. Among the large variety of super-helical assemblies found in nature,  $\alpha$ -helices that stack perpendicular to the fibril axis are rare; the few examples include de novo-designed amphiphilic peptides (28, 31, 32) and ultra-stable proteins of multiple tandem copies of a helix-loop-helix unit (33) that bear no sequence relationship to PSM $\alpha$ 3. We thus conclude that the cross- $\alpha$  architecture is robust and compatible with divergent sequences. It remains to be seen whether PSM $\alpha$ 3 is a unique example of a natural cross- $\alpha$  fibril. The crystal structure of the PSM $\alpha$ 3 should contribute to research on protein aggregation, biomaterial design, and antibacterial therapeutics.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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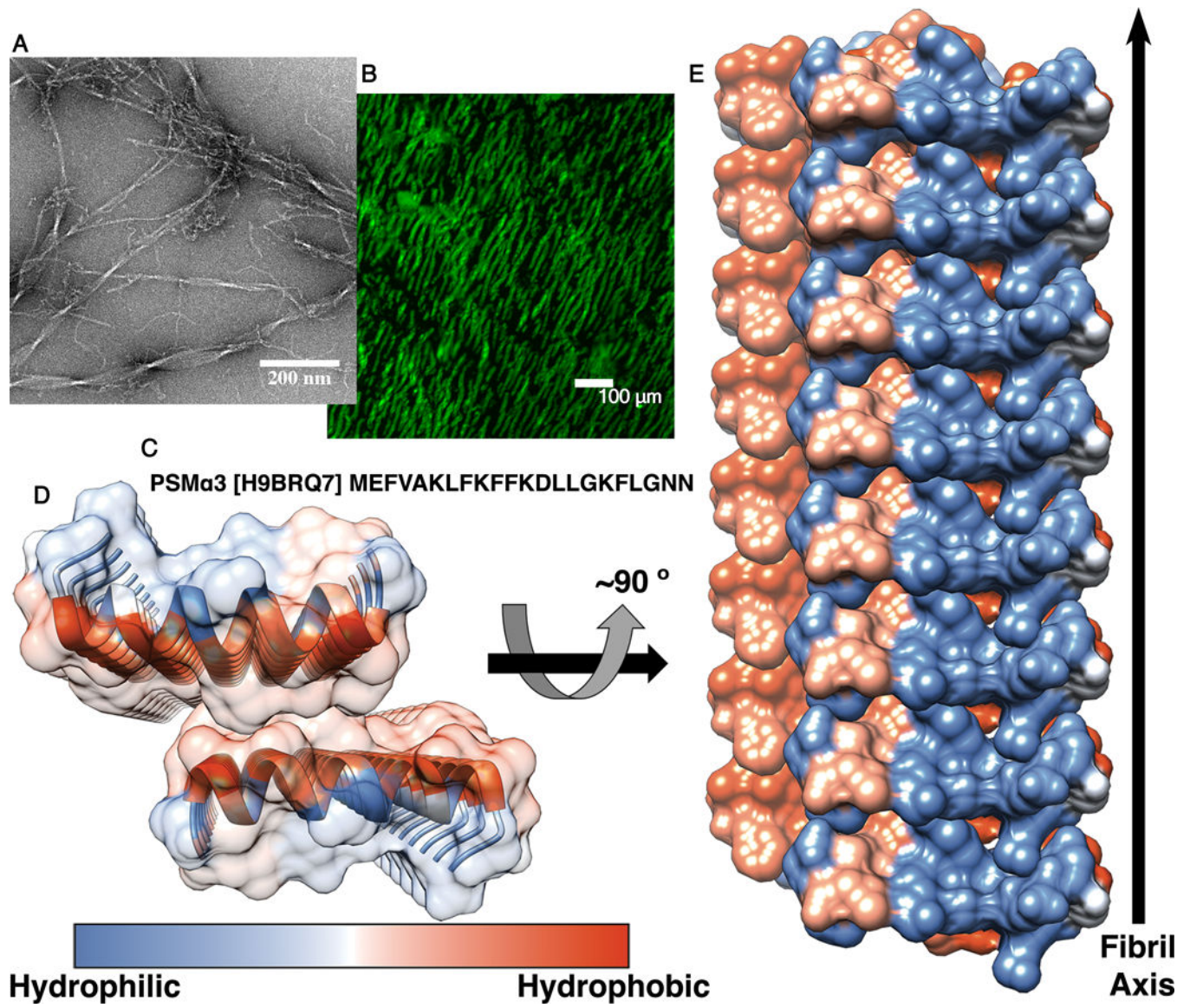
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**Fig. 1. The cross- $\alpha$  amyloid-like fibril of the full-length PSM $\alpha$ 3.**

(A) An electron micrograph of PSM $\alpha$ 3 fibrils. (B) Fluorescence microscopy images of Thioflavin T stained PSM $\alpha$ 3 fibrils. (C) The sequence of *S. aureus* PSM $\alpha$ 3 (UniProt accession number is indicated in brackets). (D and E) The crystal structure of PSM $\alpha$ 3 at 1.45 Å resolution, colored according to hydrophobicity (a colored scale bar is shown). (D) A view down the fibril axis. PSM $\alpha$ 3 forms parallel  $\alpha$ -helical stacks, viewed as ribbons along with a semitransparent surface representation. Facing helical sheets are oriented head to tail. (E) A view perpendicular to the fibril axis. The helices, shown in surface representation, run horizontally. Eight layers of  $\alpha$ -helices forming the cross- $\alpha$  structure are depicted. Theoretically, fibrils can contain tens of thousands of layers. The  $\alpha$ -helical sheets interact via their hydrophobic face, creating a tight interface. The higher order packing of the crystal structure shows continuous rows of sheets that generate alternating hydrophobic and hydrophilic interfaces (fig. S6A). Single-letter abbreviations for the amino acid residues are

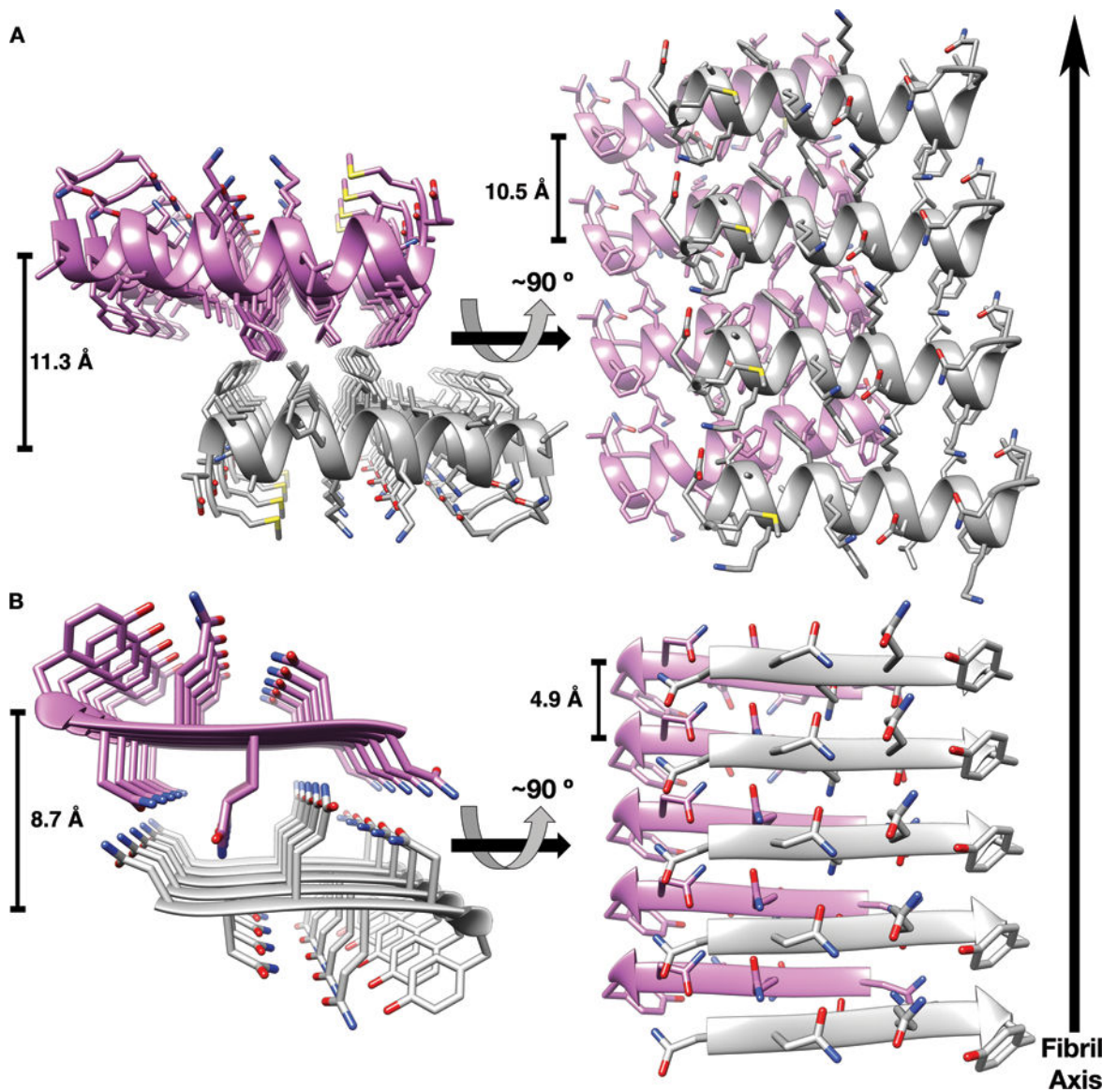
as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; K, Lys; L, Leu; M, Met; N, Asn; and V, Val.

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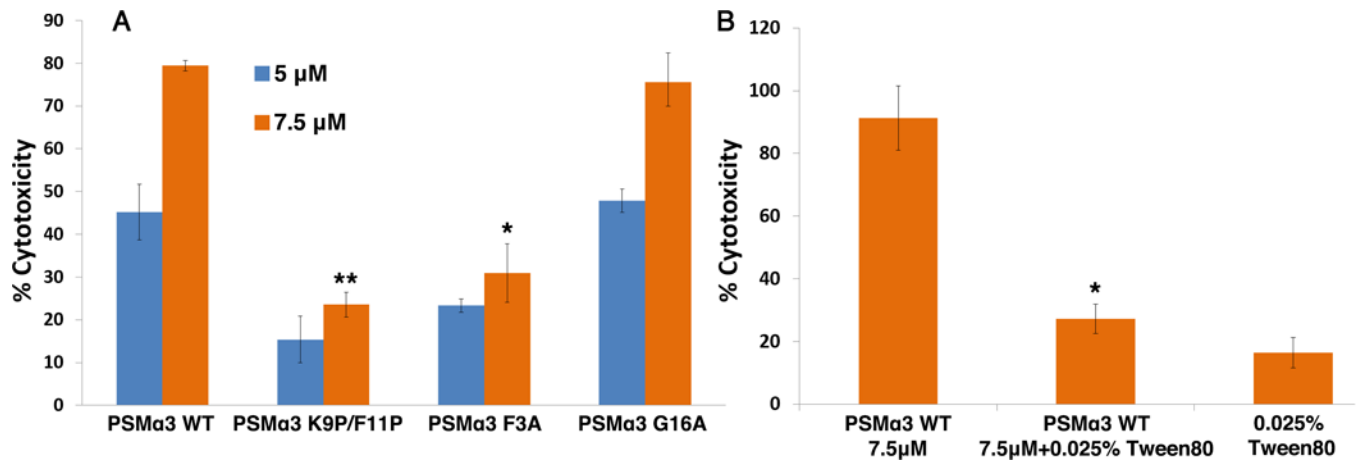
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**Fig. 2. PSM $\alpha$ 3 cross- $\alpha$  fibril is reminiscent of amyloid cross- $\beta$  structure.**

(A) The crystal structure of PSM $\alpha$ 3. Two mating  $\alpha$ -helical sheets are shown. (B) The steric zipper structure of the NNQQNY N, Asn; Q, Gln; Y, Tyr) segment from yeast prion Sup35 (4) (PDB code 1YJO) forms the cross- $\beta$  spine of amyloid-like fibrils. The two mating  $\beta$ -sheets are composed of parallel  $\beta$ -strands. In both PSM $\alpha$ 3 (A) and NNQQNY (B) structures, side-chains protruding from the two sheets intermesh to form a dry, tightly self-complementing interface. The two sheets, in purple and gray, are shown as ribbons, with side chain as sticks. Heteroatoms are colored by atom type (nitrogen in blue, oxygen in red, and sulfur in yellow). In the left panels, the view looks down the fibril axis, and in the right panels, the view is roughly perpendicular to the fibril axis. The  $\alpha$ -helices (A) and  $\beta$ -strands (B) run horizontally. Distances between mating sheets and between strands along the sheet are displayed (table S3).



**Fig. 3. PSMα3 toxicity against human T-cells is dependent on its ability to form fibrils.**

(A) PSMα3 is toxic to human T-cells in a dose dependent manner. The F3A mutant and the K9P/F11P double mutant, which do not form fibrils (figs. S8 and S9), exhibited much lower levels of cytotoxicity compared to wild-type PSMα3. G16A, a mutant that is helical and which forms fibrils that bind Thioflavin T, served as a control mutant and proved cytotoxic (figs. S8 and S9). (B) Cytotoxicity of PSMα3 was significantly reduced with the addition of Tween 80, a biocompatible surfactant that diminishes fibrillation (figs. S8 and S10). In both panels, error bars represent the SEM of three replicates. The experiment was performed at least three times on different days. \*  $P < 0.05$  and \*\*  $P < 0.001$  compared to 7.5 μM wild type PSMα3.