



Published in final edited form as:

*FEMS Microbiol Rev.* 2014 July ; 38(4): 698–719. doi:10.1111/1574-6976.12057.

## Phenol-soluble modulins – critical determinants of staphylococcal virulence

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### Abstract

Phenol-soluble modulins (PSMs) are a recently discovered family of amphipathic, alpha-helical peptides that have multiple roles in staphylococcal pathogenesis and contribute to a large extent to the pathogenic success of virulent staphylococci, such as *Staphylococcus aureus*. PSMs may cause lysis of many human cell types including leukocytes and erythrocytes, stimulate inflammatory responses, and contribute to biofilm development. PSMs appear to have an original role in the commensal lifestyle of staphylococci, where they facilitate growth and spreading on epithelial surfaces. Aggressive, cytolytic PSMs seem to have evolved from that original role and are mainly expressed in highly virulent *S. aureus*. Here we will review the biochemistry, genetics and role of PSMs in the commensal and pathogenic lifestyles of staphylococci, discuss how diversification of PSMs defines the aggressiveness of staphylococcal species, and evaluate potential avenues to target PSMs for drug development against staphylococcal infections.

### Keywords

*Staphylococcus aureus*; *Staphylococcus epidermidis*; phenol-soluble modulin; toxin; virulence; biofilm

### Introduction

*Staphylococcus aureus* is a dangerous pathogen that is a frequent cause of hospital- and community-associated infections on a global scale (Lowy, 1998, Deleo *et al.*, 2010). Many *S. aureus* hospital-associated infections present as infections of the respiratory tract, while skin and soft tissue infections dominate among community-associated infections (Lowy, 1998, Deleo *et al.*, 2010). However, *S. aureus* can also cause a series of other diseases, including endocarditis or osteomyelitis. Often, *S. aureus* infection can proceed to septicemia and become life-threatening. Furthermore, *S. aureus* is a frequent cause of biofilm-associated infections, in particular those developing on indwelling medical devices (Otto, 2008). Finally, *S. aureus* can cause food poisoning.

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*S. aureus* strains may produce a diverse and large repertoire of virulence factors, many of which are encoded on mobile genetic elements (MGE)s (Novick *et al.*, 2001). MGE-encoded virulence factors can be transferred from strain to strain by horizontal gene transfer and are usually present only in a minority of strains. Some virulence factors appear to have been acquired from coagulase-negative staphylococci (CoNS) such as *S. epidermidis* (Otto, 2013). However, some *S. aureus* virulence factors are encoded on the core genome, such as alpha-toxin and phenol-soluble modulins (PSMs), which are thus produced by virtually all strains.

Toxins, secreted molecules that directly harm the human or animal host, are arguably the most important and dangerous virulence determinants of *S. aureus*. In particular, *S. aureus* produces a series of cytolytic toxins, which are proteins that cause lysis of host cells such as red and white blood cells. Those that lyse white blood cells (leukocytes) are called leukocidins. Leukocidins of *S. aureus* belong to the beta-barrel forming family, comprising alpha-toxin and the bicomponent leukocidins (Szmigielski *et al.*, 1999). Of note, these toxins have recently been shown to exert their cytolytic activities dependent on recognition by specific receptors on the surface of the host cell (Wilke & Bubeck Wardenburg, 2010, Alonzo *et al.*, 2011, Spaan *et al.*, 2013). PSMs, the subject of the present review, are a family of peptides with multiple functions in pathogenesis. They gained recognition mostly due to their pronounced cytolytic activity toward leukocytes (Wang *et al.*, 2007), which is why they can be regarded as a new class of staphylococcal leukocidins.

Antibiotic resistance is frequent among *S. aureus* and severely complicates treatment of *S. aureus* infections (Lowy, 2003). Penicillin resistance is common among *S. aureus*; and resistance to methicillin as the antibiotic of first choice against penicillin-resistant *S. aureus* is of great concern. Many countries report methicillin resistance rates among hospital isolates that exceed 50%. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains, first reported in the late 1990s, represent an additional severe threat to public health systems (Deleo *et al.*, 2010). Antibiotic resistance is also common among many other staphylococcal species, which are in general less aggressive than *S. aureus*, but are often involved with chronic infections. *S. epidermidis* in particular is the most frequent cause of infections on indwelling medical devices (Otto, 2009).

Staphylococci are colonizers of the human or animal skin and mucous surfaces. *S. epidermidis* is the most frequent skin colonizer in humans (Otto, 2009), while *S. aureus* colonizes the nares and ano-rectal areas of about one third of the population (Rimland & Roberson, 1986, Wertheim *et al.*, 2005). Importantly, staphylococcal infections are believed to originate mainly from colonizing bacteria (von Eiff *et al.*, 2001), making colonization a risk factor for infection. However, we know much less about the physiology of staphylococci during their commensal state as compared to our knowledge of the molecular underpinnings of staphylococcal infectivity. Of note, it has been argued that the physiological status of staphylococcal commensal life may resemble that seen in biofilms (Sugimoto *et al.*, 2013), which are bacterial agglomerations embedded in an extracellular matrix commonly associated with device-related infections (Otto, 2008).

In this review article, we will present the current knowledge on PSMs, a recently discovered family of short, amphipathic peptides produced by *S. aureus* and other staphylococci, which have been recognized as premier determinants of staphylococcal virulence and physiology.

## PSMs – history, definition, and structure

The term “phenol-soluble modulins” (PSMs) was coined by Seymour Klebanoff in 1999, when he and his coworkers discovered immune-modulatory peptides of *S. epidermidis* that partitioned into the phenol phase during hot phenol extraction (Mehlin *et al.*, 1999). He described a pro-inflammatory “complex” of three peptides, PSM $\alpha$ , PSM $\beta$ , and PSM $\gamma$ , with PSM $\gamma$  being equivalent to the previously described  $\delta$ -toxin of *S. epidermidis*. The Klebanoff group reported a series of pro-inflammatory activities of the PSM “complex” (Mehlin *et al.*, 1999, Liles *et al.*, 2001), many of which we believe today were due to impurities, as pure (synthetic) peptides and isogenic deletion mutants of *psm* genes were not used in those early studies. Furthermore, PSMs have now been found to signal through a specific receptor (FPR2, see below) (Kretschmer *et al.*, 2010), which is different from the TLR2/TLR6 receptor reported to be activated by the PSM “complex” back then.

PSMs can be classified according to their length. PSMs of the  $\alpha$ -type are ~ 20–25 amino acids in length, and those of the  $\beta$ -type are about double that size (43–45 amino acids). The PSMs of the  $\beta$ -type commonly have a negative net charge, while most, but not all,  $\alpha$ -type PSMs have a neutral or positive net charge. All PSMs form an amphipathic  $\alpha$ -helix, with the  $\alpha$ -helical part stretching over the entire length of  $\alpha$ -type PSMs and forming the C-terminal part of the  $\beta$ -type PSMs, as predicted by primary and secondary structure bioinformatics analysis (Fig. 1). The structure of *S. aureus*  $\delta$ -toxin, which belongs to the  $\alpha$ -type, was determined by nuclear magnetic resonance (NMR) analysis (Tappin *et al.*, 1988), confirming amphipathy and  $\alpha$ -helicity. These features explain why PSMs are phenol-soluble. PSMs are nowadays more easily identified by their behavior during reversed-phase high-pressure liquid chromatography (RP-HPLC) rather than by their partitioning into the phenol phase. Namely, they elute at exceptionally high concentration of organic solvent, much later than most other proteins or peptides (Wang *et al.*, 2007). This feature is also likely due to the pronounced amphipathy of PSMs and their tendency to aggregate. Finally, it is important to stress that PSMs usually contain an N-terminal N-formyl methionine, indicative of the fact that they are secreted without a signal peptide (Wang *et al.*, 2007). Accordingly, *psm* genes only encode the amino acids found in the secreted PSM peptides. Some portions of secreted PSMs may lack the N-terminal N-formyl group, which is due to the activity of the cytoplasmic enzyme N-deformylase (Somerville *et al.*, 2003). Interestingly, this appears to happen at a much more pronounced extent in *S. aureus* than *S. epidermidis* (Cheung *et al.*, 2010).

## Purification and measurement of PSMs

As performed in the early studies by the Klebanoff group, a crude PSM preparation can be obtained as precipitate by hot phenol extraction followed by dialysis against distilled water (Mehlin *et al.*, 1999). Precipitation with trichloroacetic acid (TCA) or extraction with 1-butanol can be used as alternatives to enrich PSMs from the culture supernatant (Otto *et al.*,

2004, Joo *et al.*, 2011). Crude PSM extract is further purified by RP-HPLC using a gradient of organic solvent such as acetonitrile or 1-propanol. Some PSMs (e.g., PSM $\alpha$  and PSM $\beta$  of *S. epidermidis*), which elute together at the first RPLC step, need one or more additional RP-HPLC steps for purification (Mehlin *et al.*, 1999, Yao *et al.*, 2005, Joo & Otto, 2014). As a special case of PSM purification, fibrous PSM complexes can be obtained from *S. aureus* biofilms cultured in drip bioreactors with a defined media, namely peptone-NaCl-glucose (Schwartz *et al.*, 2012).

The amount of PSMs can be measured in complex bacterial culture supernatant by RP-HPLC with a proper column and eluent system (Fig. 2) (Joo & Otto, 2014). Due to the exceptionally late retention time of PSMs during RP-HPLC, PSMs are separated quite easily from other proteins in the culture supernatant. However, the fact that many PSMs do not separate from each other under those conditions necessitates coupling with mass spectrometry (MS) to quantify single PSMs accurately. The concentration of each PSM is determined by integration of the extracted ion chromatogram of the two major m/z ratios among multiply-charged ions of each PSM (Otto *et al.*, 2004, Joo & Otto, 2014). For absolute quantification, purified or synthetic PSMs should be used to calibrate (Yao *et al.*, 2005). As a useful tool for qualitative detection of PSMs, Nizet and colleagues have used an imaging mass spectrometry technique with MALDI-TOF mass spectrometry to discover PSM $\alpha$  derivatives (Gonzalez *et al.*, 2012).

## Occurrence of PSMs in staphylococci

PSMs occur in many staphylococcal strains, with the PSM profile apparently being specific for and conserved within a certain species (Vuong *et al.*, 2004, Wang *et al.*, 2007, Queck *et al.*, 2009, Rautenberg *et al.*, 2011). Although this would represent a possibly valid tool to distinguish staphylococcal species, PSM profiling has not yet been used for that purpose. It has been noted that PSM production is more frequent among pathogenic than non-pathogenic species (Rautenberg *et al.*, 2011), but whether there is a clear correlation between pathogenicity and PSM production on the species level needs further verification.

For some PSMs, such as the  $\delta$ -toxins and PSM $\beta$  peptides of *S. aureus*, *S. epidermidis* and many other staphylococci, amino acid sequences are fairly conserved between species. Some previously described peptides, such as the gonococcal growth inhibitor peptides from *S. haemolyticus* (Beaudet *et al.*, 1982, Watson *et al.*, 1988) or the slush peptides from *S. lugdunensis* (Donvito *et al.*, 1997), are now recognized to belong to the  $\beta$ -type PSMs. Genes encoding  $\delta$ -toxin and  $\beta$ -type PSMs can be found by similarity searches in many recently sequenced staphylococcal genomes (Tab. 1). In contrast, most  $\alpha$ -type PSMs, such as the PSM $\alpha$  peptides of *S. aureus*, are too short to be routinely annotated in genome sequences and in general too short to yield meaningful results in similarity searches. Performing tblastn similarity searches with all *S. aureus* and *S. epidermidis*  $\alpha$ -type PSMs, we detected some genes in staphylococci other than *S. aureus* and *S. epidermidis* that are similar to PSM $\epsilon$  of *S. epidermidis* and were not previously described (Tab. 1). However, these searches did not yield any significant results for other  $\alpha$ -type PSMs; and for the reasons outlined above, they are not likely to detect all  $\alpha$ -type *psm* genes in a genome. For most staphylococcal species,

we therefore do not have a complete list of PSM peptides, although we know from RP-HPLC/MS analyses that they produce PSMs (Rautenberg *et al.*, 2011).

*S. aureus* and *S. epidermidis* are the only species for which peptide purification approaches led to a complete list of produced PSMs and PSM-encoding genes (Mehlin *et al.*, 1999, Vuong *et al.*, 2004, Yao *et al.*, 2005, Wang *et al.*, 2007) (Fig. 3). *S. aureus* produces four PSM $\alpha$  peptides, encoded in the *psm $\alpha$*  locus, two PSM $\beta$  peptides, encoded in the *psm $\beta$*  locus, and the  $\delta$ -toxin, which is encoded within RNIII, the effector molecule of the Agr system (Wang *et al.*, 2007). *S. epidermidis* produces the  $\alpha$ -type PSMs PSM $\alpha$ , PSM $\delta$ , PSM $\epsilon$ , and the  $\delta$ -toxin (Mehlin *et al.*, 1999, Vuong *et al.*, 2004, Yao *et al.*, 2005). PSM $\alpha$  and PSM $\delta$  are somewhat similar to the *S. aureus* PSM $\alpha$  peptides and encoded together in a locus that resembles the *S. aureus* *psm $\alpha$*  locus, indicating that they are evolutionarily related. Furthermore, *S. epidermidis* produces two PSM $\beta$  peptides. The *S. epidermidis* *psm $\beta$*  locus contains another gene, *psm $\beta$ 3*, but we have never found a secreted peptide corresponding to that gene (Vuong *et al.*, 2004, Queck *et al.*, 2009). In some strains including the genome-sequence strains RP62A and ATCC12228, the *psm $\beta$ 1* gene is duplicated, leading to enhanced production of PSM $\beta$ 1 (Wang *et al.*, 2011).

Notably, all known *psm* genes are encoded on the core genome or on MGEs that are present in virtually all strains of the species. As a noticeable exception, the PSM-mec peptide is encoded in staphylococcal cassette chromosome *mec* (SCC*mec*) types II, III and VIII, representing a rare example of combined presence of antibiotic resistance and virulence factors on a staphylococcal MGE (Queck *et al.*, 2009, Chatterjee *et al.*, 2011). In addition to the MRSA strains harboring these SCC*mec* elements, PSM-mec is frequently produced by methicillin-resistant *S. epidermidis* (Queck *et al.*, 2009); and *psm-mec* genes are also found in other methicillin-resistant staphylococci (Monecke *et al.*, 2012). The presence of a *psm $\beta$*  homologue on a plasmid of *S. simulans* ssp. *staphylolyticus* is a further exception to the common encoding of *psm* genes on the genome. Of note, to clearly distinguish PSM peptides carrying the same name, a PSM peptide should always be described together with the species, such as in “*S. epidermidis* PSM $\beta$ 1”.

Interestingly, the PSM production pattern reflects the clearly distinct “lifestyles” of *S. aureus* and *S. epidermidis*. Despite the capacity of *S. epidermidis* to produce strongly aggressive and cytolytic PSMs such as PSM $\delta$  (Cheung *et al.*, 2010), the organism shifts the production of PSMs to less aggressive forms, such as the PSM $\beta$  peptides (Otto, 2009, Wang *et al.*, 2011). In contrast, many virulent *S. aureus* strains produce high amounts of the strongly cytolytic PSM $\alpha$  peptides and only low amounts of PSM $\beta$  peptides (Wang *et al.*, 2007).

## Regulation of PSMs

Similar to several other major staphylococcal exotoxins, PSMs are positively regulated by the global regulatory quorum sensing system, accessory gene regulator (Agr) (Wang *et al.*, 2007, Queck *et al.*, 2008) (Fig. 4). Notably, Agr thus connects the response to cell density (quorum-sensing) with virulence (Novick, 2003). The *agr* locus is comprised of a divergent promoter system that transcribes two important mRNAs. RNIII is a regulatory RNA that

controls the transcription of several major exotoxins and surface proteins, and also contains the gene coding for the PSM  $\delta$ -toxin, named *hld* for hemolysin delta. RNAPII codes for two genes belonging to a classical two-component signal transduction system, a histidine kinase (*agrC*), and a response regulator (*agrA*), and two genes that code for an auto-inducing peptide (AIP) precursor (*agrD*) and an AIP maturation and export protein (*agrB*). AgrA becomes activated when AgrC detects a critical concentration of secreted AIP. In turn, activated AgrA induces the transcription of *rnaiii* and its own operon (*rnaii*), constituting a positive feedback loop. A functional Agr regulatory system is essential for *S. aureus* virulence as demonstrated by severe attenuation of pathogenicity in isogenic *agr* mutants (Abdelnour *et al.*, 1993, Cheung *et al.*, 1994, Gillaspay *et al.*, 1995, Cheung *et al.*, 2011).

In contrast to the regulation of virtually all Agr target genes via RNAPIII, *psm* genes are regulated by direct binding of AgrA to their promoters (Queck *et al.*, 2008). This represents an exceptionally direct control by the Agr system, indicating that cell density-dependent regulation of PSMs preceded a later connection to further virulence determinants. The fact that the PSM  $\delta$ -toxin is encoded within *rnaiii*, which thus probably evolved around the  $\delta$ -toxin gene *hld*, further supports this notion. Although the regulation of PSM transcription is dominated by AgrA (Queck *et al.*, 2008, Chatterjee *et al.*, 2011), additional levels of PSM regulation exist. For example, the biofilm mode of growth appears to lead to enhanced relative production of  $\beta$ -type PSMs over  $\alpha$ -type PSMs, as shown in *S. epidermidis* (Wang *et al.*, 2011), but how differential production of  $\alpha$ - versus  $\beta$ -type PSMs is achieved mechanistically is not known. Recent studies show that the PSMs, in addition to other toxins, are degraded by aureolysin, a protease which is regulated by several global regulators, which include Agr, staphylococcal accessory regulator (SarA), and SaeRS (Zielinska *et al.*, 2011, Cassat *et al.*, 2013, Rasigade *et al.*, 2013). Accordingly, SarA impacts PSM production by a general impact on protease activity (Zielinska *et al.*, 2011).

The *psm-mec* gene is embedded in a gene encoding a regulatory RNA (Kaito *et al.*, 2011), in a way very similar to the connection of *hld* with RNAPIII (Fig. 5). The *psm-mec* regulatory RNA decreases *agrA* activity (Kaito *et al.*, 2013), and thus has an indirect effect on PSM production. However, the effect of the *psm-mec* regulatory RNA on PSM production is quite low in extent, reaching a maximal factor of  $\sim 3$ , and is only seen in some strains (Kaito *et al.*, 2013). Lastly, Agr also regulates transcription of *psm-mec* itself in an RNAPIII-independent manner (Chatterjee *et al.*, 2011).

*S. aureus* can survive within a variety of host cells to hide from the immune system and disseminate to other sites in the host (Gresham *et al.*, 2000, Kubica *et al.*, 2008, Giese *et al.*, 2011, Thwaites & Gant, 2011, Cassat *et al.*, 2013). Importantly, intracellular environments were found to activate the expression of PSMs (Geiger *et al.*, 2012, Surewaard *et al.*, 2012). When phagocytosed by polymorphonuclear leukocytes (PMNs, or neutrophils), *S. aureus* quickly produces high levels of the intracellular signaling molecule, (p)ppGpp, which activates the production of PSMs by an unknown mechanism (Geiger *et al.*, 2012). Unlike the Agr quorum sensing system, which requires time for the accumulation of enough AIP for activation of AgrA (Carnes *et al.*, 2010, Pang *et al.*, 2010), activation of the stringent response is independent of AgrA. It is likely that both the stringent response (Geiger *et al.*, 2012) and Agr (Surewaard *et al.*, 2012) are induced by *S. aureus* to ensure that PSMs are

produced immediately and continually in intracellular environments, maximizing the capacity of *S. aureus* to trigger phagosomal escape and subsequent cell lysis.

Finally, it was reported that PSM transcription is up-regulated, in addition to other important virulence factors, in the presence of calf serum (Oogai *et al.*, 2011). It is tempting to speculate that *S. aureus* may up-regulate PSM production as a means to compensate for the observed sequestration of PSMs by lipoproteins in human serum (Surewaard *et al.*, 2012). Taken together, while the Agr global regulatory system is by far the main effector of PSM production, other factors, which include the regulatory *psm-mec* mRNA, proteolytic degradation, and various responses to the intracellular and extracellular environments, all play important roles affecting PSM concentrations in vitro and in vivo.

## Export of PSMs

The fact that PSMs carry an N-terminal N-formyl methionine indicates that they are secreted without a signal peptide using a non-canonical protein export system. Recently, the PSM export system was identified to be an ABC transporter, which was named phenol soluble modulins transporter (Pmt) (Chatterjee *et al.*, 2013) (Fig. 6). The *pmt* locus encodes four genes, *pmtA-D*, of which *pmtA* and *pmtC* code for ATPases, whereas *pmtB* and *pmtD* encode membrane-associated proteins. In *S. aureus*, Pmt was shown to be able to export all types of PSMs into the surrounding milieu. Pmt is present in all *Staphylococcus* species and absent from non-staphylococcal bacterial genomes, suggesting that Pmt is specific for staphylococci, as is PSM production. Notably, Pmt is essential for bacterial survival, because in its absence PSMs accumulate in the cytoplasm, causing acute growth deficiency and cellular defects. As expected, Pmt is required for mediating PSM-associated virulence phenotypes. Due to its key impact on virulence and additional essentiality for bacterial survival, and because it is highly conserved among the staphylococci, Pmt appears to be an exceptionally promising target for interference with staphylococcal pathogenesis (Chatterjee & Otto, 2013).

## PSMs in the natural habitat of staphylococci

PSMs have been attributed at least two main functions that are related to their detergent-like features and may facilitate the life of staphylococci in their natural habitat, that is, on the epithelial surfaces of humans and other mammals. First, the observed PSM-dependent spreading of *S. aureus* on agar plates most likely translates to PSMs having a key function in the spreading of staphylococci on epithelial surfaces (Tsompanidou *et al.*, 2013). Second, PSMs may assist in the emulsification of nutrients in those environments, although this remains to be demonstrated. Of note, PSMs are produced at the high levels that these functions presumably require. In fact, ~ 60% of the secreted protein mass in *S. aureus* in-vitro culture was shown to be PSMs (Chatterjee *et al.*, 2013). Finally, PSMs are responsible for biofilm structuring (Wang *et al.*, 2011, Periasamy *et al.*, 2012), which is discussed further below. As biofilm-like bacterial agglomerations are believed to exist on epithelial surfaces, this PSM function likely also contributes to the physiology of staphylococci in their commensal state. However, in general we still lack detailed information on the role of PSMs in the non-infectious lifestyle of staphylococci.

## PSMs as cytolytins

Several PSMs have an extraordinary ability to lyse many eukaryotic cell types, such as bone cells (osteoblasts) (Cassat *et al.*, 2013, Rasigade *et al.*, 2013), endothelial and epithelial cells (Giese *et al.*, 2011), monocytes (Wang *et al.*, 2007), erythrocytes (Cheung & Otto, 2011), and most importantly, PMNs (Wang *et al.*, 2007, Cheung *et al.*, 2010, Forsman *et al.*, 2012). The wide variety of cell types that are subject to PSM-mediated lysis is in accordance with the notion that PSM cytolytic activity is due to membrane perturbation in a receptor-independent fashion (Kretschmer *et al.*, 2010) (Fig. 7). However, not all PSMs show this pronounced cytolysis of eukaryotic cells. For instance, the PSM  $\beta$ -type peptides are generally much less cytolytic than the  $\alpha$ -type peptides. Whether this is due to their commonly negative net charge is not known. Notably, *S. aureus* PSM $\alpha$ 3 and *S. epidermidis* PSM $\delta$  are by far the most potent cytolytic PSMs described to date, with pronounced cytolytic potencies observed in the low micromolar range (Wang *et al.*, 2007, Cheung *et al.*, 2010). Interestingly, the cytolytic activities of the PSMs are enhanced in the presence of *S. aureus*  $\beta$ -toxin (Cheung & Otto, 2011) and PVL (Hongo *et al.*, 2009) in addition to antimicrobial peptides (Cogen *et al.*, 2010), signifying that the PSMs may not always exert their cytolytic effects independently.

Much of our understanding about the lytic nature of the PSMs stems from extensive research performed with *S. aureus*  $\delta$ -toxin, which forms small transient pores in artificial lipid vesicles (Pokorny *et al.*, 2002). Phosphatidylcholine (POPC), which is a major phospholipid constituent of eukaryotic membranes (Devaux & Morris, 2004), is generally used to study membrane perturbation. A more detailed study of the interaction of the PSMs of *S. aureus* and *S. epidermidis* with POPC vesicles revealed that POPC-lytic activities of PSMs did not correlate with those previously described for leukocytes and erythrocytes (Duong *et al.*, 2012). In particular the PSM $\beta$  peptides showed strongly lytic behavior toward POPC phospholipid vesicles, implying that the pore-forming activities of PSMs are more complex than originally thought and possibly dependent on the membrane lipid composition. Some PSMs can lyse bacterial protoplasts (Kreger & Bernheimer, 1971) and even whole bacteria, such as *Streptococcus pyogenes* and *Legionella pneumophilla* (Verdon *et al.*, 2008, Cogen *et al.*, 2010, Joo *et al.*, 2011). This is discussed further below.

A hallmark of a typical subcutaneous *S. aureus* infection is the rapid infiltration of PMNs. PMNs are important host innate immune cells that have a key role in controlling local infections by taking up bacteria by phagocytosis (Amulic *et al.*, 2012). Phagocytosed bacteria, compartmentalized into phagosomes, are exposed to a battery of antimicrobial agents. However, virulent *S. aureus* strains such as CA-MRSA strains have developed strategies to evade killing by cells of the host innate immune system (Voyich *et al.*, 2005, Kobayashi *et al.*, 2010, Rigby & DeLeo, 2012). These strategies include the ability to survive within the PMNs, which provides protection from detection by the immune system and also represents a means of transportation to disseminate to other sites of the body (Rogers, 1956, Gresham *et al.*, 2000, Thwaites & Gant, 2011). Several recent studies have shown that PSM $\alpha$  peptides are responsible for the lysis of neutrophils after ingestion (Geiger *et al.*, 2012, Chatterjee *et al.*, 2013, Surewaard *et al.*, 2013) (Fig. 7). Furthermore, expression levels of PSMs correlate with cytotoxicity within PMNs (Rasigade *et al.*, 2013).



Among the classical bicomponent *S. aureus* leukocidins, only LukAB (also known as LukGH) (Ventura *et al.*, 2010, Dumont *et al.*, 2011) contributes to PMN lysis after phagocytosis (Ventura *et al.*, 2010, DuMont *et al.*, 2013). PSM $\alpha$  expression is also important for the phagosomal escape or survival of *S. aureus* within osteoblasts (Rasigade *et al.*, 2013), professional phagocytes other than neutrophils, and non-professional phagocytes such as endothelial cells (Grosz *et al.*, 2013), indicating a general crucial role in intracellular survival of *S. aureus*.

## Recognition of PSMs by the host

The PSMs are potent pro-inflammatory agents that mediate their activities primarily through the interaction with human formyl peptide receptor 2 (FPR2) (Kretschmer *et al.*, 2010) (Fig. 8). FPR2 belongs to a family of G protein-coupled receptors that aid cells of the innate immune system to recognize pathogen-associated molecular patterns (PAMPs) produced by bacteria. In humans, three FPR paralogs exist; FPR1, FPR2/ALX (previously known as FPR-like 1), and FPR3. FPR1 and FPR2 are expressed on monocytes, macrophages, PMNs, immature dendritic cells and some other cell types, while FPR3 is only present on monocytes and macrophages (Migeotte *et al.*, 2006).

The FPRs detect N-formyl peptides, such as the N-formylated PSMs. However, they were shown to also recognize non-formylated forms of PSMs (Kretschmer *et al.*, 2011), which are secreted under certain conditions (Somerville *et al.*, 2003). At nanomolar concentrations, the PSMs activate many FPR2 receptor-dependent effector functions, which include the induction of chemotaxis, exocytosis and superoxide generation, pro-inflammatory cytokine production, up-regulation of CD11b expression, and the induction of intracellular calcium flux (Fu *et al.*, 2006, Wang *et al.*, 2007, Queck *et al.*, 2009, Kretschmer *et al.*, 2010, Chaffin *et al.*, 2012, Forsman *et al.*, 2012). These pro-inflammatory activities are essential for an effective immune response to invading pathogens. The activation of FPR2-dependent pro-inflammatory activities by non-formylated PSMs from *S. aureus* and *S. epidermidis* is generally less pronounced than that by PSMs with an N-formyl methionine residue (Kretschmer *et al.*, 2011).

The specificity of the PSMs for FPR2 was discovered with the help of two small protein inhibitors (the chemotaxis-inhibitory protein of *S. aureus* (CHIPS) (de Haas *et al.*, 2004) and FPR2/ALX-inhibitory protein (FLIPr) (Prat *et al.*, 2006)). These inhibitors, secreted by *S. aureus*, block the ability of FPR1 and FPR2 to recognize formylated peptides, respectively. Based on the specificity of FPR2 for the detection of PSMs, it is thought that the innate immune system may discriminate between virulent *S. aureus* strains, such as CA-MRSA, and commensal staphylococci, which generally have a reduced PSM output (Kretschmer *et al.*, 2010). Notably, FPR2-specific inhibition by FLIPr dramatically reduces chemotaxis and IL-8 release in neutrophils as stimulated by culture filtrate of the CA-MRSA strain USA300 (Kretschmer *et al.*, 2010), emphasizing the importance of PSM recognition in the interaction of the innate immune system with *S. aureus*. Evidently, PSMs and FLIPr have an opposite impact on FPR2 activation. Most likely, production of FLIPr and PSMs is controlled by the bacteria in a way that allows establishment of an infection without immune recognition. This is achieved by the strict control of PSM production by the Agr quorum-sensing system

(Queck *et al.*, 2008), delaying PSM production until the bacterial density is high. Then, strong PSM production likely outcompetes FLIPr to promote inflammation via FPR2. However, the in-vivo kinetics of FLIPr and PSM production still need to be investigated. Similarly, it is yet unknown whether PSM-dependent induction of inflammatory responses is part of the bacterial pathogenesis program or PSMs represent pathogen-associated molecular patterns that the immune system recognizes to launch a defensive response.

While the PMNs are critical for innate immune defense, macrophages and dendritic cells (DCs) play an essential role in linking innate and adaptive immunity by serving as antigen presenting cells (APCs). In particular, APCs process and present antigens to T-lymphocytes, which then initiate the stimulation of an adaptive immune response. Interestingly, a recent study demonstrated that PSMs modulate murine DC function; PSM-stimulated DCs increased in vitro priming of regulatory T cells, which led to a suppression of Th1 immune responses (Schreiner *et al.*, 2013). Murine macrophages stimulated with isogenic *psma* and *hld* CA-MRSA mutants showed decreased production of IL-6, IL-12 and TNF $\alpha$  cytokines as compared to stimulation with the corresponding wild-type strain (Spentzas *et al.*, 2011) confirming that both PSM $\alpha$  peptides and  $\delta$ -toxin are important mediators of inflammatory cytokine production. Taken together, the PSMs appear to be capable of manipulating the host immune system in various ways to promote survival of *S. aureus* during infection.

## PSMs in different infection types

### PSMs in skin and soft-tissue infections and sepsis

In humans, skin and soft tissue infections represent ~ 90% of all clinical manifestations of community-associated *S. aureus* disease (Deleo *et al.*, 2010). However, *S. aureus* can cause severe invasive disease resulting in bacteremia (sepsis). Even under antibiotic therapy, invasive *S. aureus* disease is associated with high mortality (Klevens *et al.*, 2007). Notably, on average, greater levels of PSMs are detected in the supernatants of CA-MRSA strains as compared to HA-MRSA strains, suggesting a correlation with the virulence potential of those strains (Wang *et al.*, 2007, Li *et al.*, 2010). Accordingly, isogenic *psma* CA-MRSA mutants were severely attenuated in their ability to cause subcutaneous abscesses in the skin of mice compared to the wild-type strain (Wang *et al.*, 2007) (Fig. 9a). Furthermore, mice infected with isogenic *psma* CA-MRSA mutants were significantly delayed in reaching moribund status compared to mice infected with the wild-type strain in a bacteremia model of infection (Wang *et al.*, 2007), confirming a link between high expression of PSM $\alpha$  peptides and the virulence potential of CA-MRSA. In contrast, *psm* $\beta$  deletion mutants had none and *hld* mutants only a slight impact on disease progression in these mouse models. Similarly, in a rabbit model of skin infection, a *psma* deletion mutant was strongly impaired in forming abscesses, to an extent similar to that caused by an *agr* deletion mutant and clearly exceeding that of a *lukSF* (PVL) deletion mutant, which did not show a significantly different ability to form abscesses compared to the wild-type strain in that model (Kobayashi *et al.*, 2011) (Fig. 9b).

As for the impact of the *psm-mec* locus on virulence, remarkable differences were reported in murine models of skin and bacteremia infections (Queck *et al.*, 2009, Kaito *et al.*, 2013). In *S. aureus* strains that produce relatively high levels of PSM-mec, a significant attenuation

in virulence was observed in isogenic *S. aureus psm-mec* mutants, suggesting that the PSM-mec peptide contributes to virulence similarly to the  $\alpha$ -class PSMs (Queck *et al.*, 2009). In contrast, deletion of the *psm-mec* locus caused an increase in virulence in some HA-MRSA isolates (Kaito *et al.*, 2013) as a result of the negative effect of the *psm-mec* RNA on Agr, but not in others (Chatterjee *et al.*, 2011, Kaito *et al.*, 2013). The impact that the *psm-mec* locus has on virulence thus is highly strain-dependent and appears to result from a combination of the PSM-mec peptide, which has a positive effect, and the *psm-mec* RNA, which has a negative effect on virulence.

### PSMs in biofilm-associated infection

Biofilms are surface-attached agglomerations with a distinct physiology and pronounced capacity to withstand attacks by host defenses and antibiotics (Costerton *et al.*, 1999). Biofilm-associated infections often develop on indwelling medical devices; staphylococci are the most frequent pathogens involved with such infections (Otto, 2008). The factors that determine aggregation of cells in a biofilm, such as the production of an extracellular matrix, are fairly well understood. This includes staphylococci, in which the matrix consists of proteins, extracellular DNA, teichoic acids, and a specific exopolysaccharide called polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG), according to its chemical composition. In contrast, the factors that contribute to the structuring of biofilms and the detachment of cells from biofilms, a process vital for the systemic dissemination of a biofilm infection, have remained poorly understood until recently (Otto, 2013).

*S. epidermidis* in particular, often regarded as an opportunistic pathogen, is the leading cause of chronic catheter-related infections in hospital settings (Otto, 2009, Rogers *et al.*, 2009). *S. epidermidis* has a strong aptitude to form biofilms on inert surfaces (Kogan *et al.*, 2006, Rohde *et al.*, 2007). PSM $\beta$  peptides are strongly produced in *S. epidermidis*, and in a relatively higher proportion compared to other PSMs during biofilm as compared to planktonic growth (Wang *et al.*, 2011). PSM $\beta$  peptides were shown to structure *S. epidermidis* biofilms, leading to the characteristic biofilm structure with channels deemed important for nutrient delivery to all biofilm layers (Wang *et al.*, 2011) (Fig. 10). Furthermore, these peptides were found to be crucial for the detachment of biofilm clusters in vitro and dissemination from biofilms on indwelling devices in vivo, as demonstrated in a mouse model of indwelling device-associated infection (Wang *et al.*, 2011). In *S. aureus*, all PSM peptides were shown later to contribute to these phenotypes in vitro and in vivo (Periasamy *et al.*, 2012). The extent to which other PSMs of *S. epidermidis* contribute to biofilm structuring and detachment has not yet been analyzed using gene deletion mutants. The finding that the PSM surfactant peptides are responsible for biofilm structuring and detachment are in line with similar findings in other bacteria (Branda *et al.*, 2001, Boles *et al.*, 2005, Angelini *et al.*, 2009), indicating a common mechanism involved in these phenotypes that is dependent on surfactant molecules. However, the chemical nature of the surfactants underlying biofilms structuring is different in different bacteria. Notably, the experiments on biofilm dissemination in *S. epidermidis* and *S. aureus* biofilm-associated infection were the first to show the impact of biofilm-structuring surfactants on dissemination from in-vivo biofilms.

A recent report demonstrated that PSMs can polymerize to form fibrillar, amyloid structures that help stabilize *S. aureus* biofilms in vitro (Schwartz *et al.*, 2012). Interestingly, the amyloid structures isolated from *S. aureus* cultures consist of only deformed PSMs. However, further research is needed to investigate whether these structures exist in vivo and how they fit into the pathogenic lifestyle of *S. aureus* and *S. epidermidis* during biofilm infection.

### PSMs in bone infection (osteomyelitis)

*S. aureus* is the leading cause of bone infections (osteomyelitis) in adults and infants (Arnold *et al.*, 2006, Vander Have *et al.*, 2009). CA-MRSA-related osteomyelitis in infants is often associated with increased interval and severity compared to HA-MRSA-related osteomyelitis (Martinez-Aguilar *et al.*, 2004, Arnold *et al.*, 2006, Bocchini *et al.*, 2006). Intracellular residence within osteoblasts appears to be essential for the chronic presence and dissemination of *S. aureus* (Tuchscher *et al.*, 2010, Tuchscher *et al.*, 2011). Recently, the global *S. aureus* regulators Agr, SarA, and SaeRS were shown to regulate the expression of aureolysin, a protease responsible for degrading important *S. aureus* exoproteins (Zielinska *et al.*, 2011, Gonzalez *et al.*, 2012, Cassat *et al.*, 2013, Rasigade *et al.*, 2013). Although there is conflicting evidence supporting the roles SaeRS and SarA in aureolysin-mediated degradation of exoproteins (Cassat *et al.*, 2013, Rasigade *et al.*, 2013), it is clear that PSM $\alpha$  peptides are crucial for cytotoxicity toward murine and human osteoblasts in vitro (Cassat *et al.*, 2013, Rasigade *et al.*, 2013) and that they are subject to aureolysin-mediated degradation during bone infection (Cassat *et al.*, 2013). Furthermore, PSM $\alpha$  peptides were essential for bone remodeling and destruction in a mouse model of osteomyelitis infection as visualized by high-resolution microcomputed tomography (Cassat *et al.*, 2013). Taken together, these results implicate the PSM $\alpha$  peptides as important virulence determinants in the pathogenesis of osteomyelitis.

### Delta-toxin and atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease that commonly affects infants (Williams & Flohr, 2006). Mast cells are believed to be key contributors to the pathology of AD and other allergic diseases through the release of inflammatory mediators from intracellular granules activated by antigen cross-linked to Fc3RI receptor-bound IgE (Galli *et al.*, 2011, Galli & Tsai, 2012). Although more than 90% of AD patients are tested positive for *S. aureus* (Rudikoff & Lebwohl, 1998), the link between *S. aureus* and AD pathogenesis has remained unclear. However, a recent study implicates a link between *S. aureus* colonization and allergic skin disease and correlates these observations with the potent mast cell-degranulating activity of  $\delta$ -toxin (Nakamura *et al.*, 2013). Notably, other *S. aureus* PSMs lacked such activity. Importantly, non-cytolytic concentrations of both formylated and non-formylated forms of  $\delta$ -toxin are equally potent in stimulating mast cell degranulation, these activities are not dependent on FPR2, and the intracellular signaling pathways stimulated by  $\delta$ -toxin leading to mast cell degranulation are different from those induced by antigen cross-linked to IgE. The ability of  $\delta$ -toxin to induce mast cell degranulation was also confirmed in vivo using in murine models of inflammatory skin disease. Furthermore, skin colonization with an isogenic *S. aureus* *hld* mutant promoted less IgE and IL-4 production and inflammatory skin disease when compared to mice colonized with the wild-type  $\delta$ -toxin

expressing *S. aureus* strain. Moreover, enhancement of IgE production and skin inflammation by  $\delta$ -toxin was abolished in mast cell-deficient mice and restored by adoptive transfer of mast cells (Nakamura *et al.*, 2013).

Commensal staphylococci such as *S. epidermidis*, which are not linked to AD, also produce  $\delta$ -toxin peptides that are highly similar to that produced by *S. aureus* (Scheifele *et al.*, 1987). *S. epidermidis*  $\delta$ -toxin showed some, but reduced activity compared to *S. aureus*  $\delta$ -toxin in causing mast cell degranulation (Nakamura *et al.*, 2013). Whether  $\delta$ -toxin producing *S. epidermidis* isolates contribute to allergic skin disease requires further investigation.

## Relationship of PSMs with other *S. aureus* toxins

Clearly, the PSMs are not the only toxins that contribute to skin and soft tissue and many of the other infections mentioned above. For instance,  $\alpha$ -toxin (Hla), an important pore-forming and pro-inflammatory (Thelestam *et al.*, 1973, Bhakdi & Tranum-Jensen, 1991, Walev *et al.*, 1993, Ezechuk *et al.*, 1996, Valeva *et al.*, 1997, Hocke *et al.*, 2006, Liang & Ji, 2006, Bartlett *et al.*, 2008) toxin, contributes to abscess formation in mice (Inoshima *et al.*, 2011) and rabbits (Kobayashi *et al.*, 2011). Furthermore, bi-component pore-forming leukotoxins are increasingly recognized as important virulence determinants involved in *S. aureus* pathogenesis. These leukotoxins include LukED, LukAB (LukGH) and PVL (Diep *et al.*, 2010, Alonzo *et al.*, 2011, Dumont *et al.*, 2011). They are cytolytic towards cells of myeloid origin of different animal species. Notably, cytolysis triggered by those toxins has recently been found to be receptor-dependent (Alonzo *et al.*, 2013, DuMont *et al.*, 2013, Spaan *et al.*, 2013), as has  $\alpha$ -toxin-dependent cell lysis and pro-inflammatory activities (Wilke & Bubeck Wardenburg, 2010, Inoshima *et al.*, 2011). Studies using isogenic *S. aureus* deletion mutants showed that LukED and LukAB contribute to the virulence of *S. aureus* in murine sepsis (Alonzo *et al.*, 2011) and renal abscess models (Dumont *et al.*, 2011), respectively. The contribution of PVL to the pathogenesis especially of CA-MRSA-induced disease is controversial (Otto, 2010, Otto, 2011).

While PSMs play a key role as virulence determinants during many types of *S. aureus* infection, the pathogenesis of *S. aureus* disease is multi-factorial. The PSMs may work in concert with other toxins, possibly in a synergistic fashion as reported for PVL and PSMs (Hongo *et al.*, 2009), to exert maximal pathogenesis as in the case of skin and soft tissue infections. Notably, unlike  $\alpha$ -toxin, LukAB, LukED and PVL, the cytolytic activity of the PSMs very likely does not require a receptor (Kretschmer *et al.*, 2010). Thus, the non-specific lytic nature of the PSMs may provide a distinct advantage over the aforementioned toxins, inasmuch as they may attack a wide variety of cell types, and offer a possible explanation as to why the PSMs contribute to pathogenicity in a variety of *S. aureus* diseases.

## PSMs as antimicrobials

Although periodically, antimicrobial activities of  $\delta$ -toxin analogs and other PSM-like peptides of staphylococci have been reported (Beaudet *et al.*, 1982, Dhople & Nagaraj, 1995, Dhople & Nagaraj, 2005), more intensive studies on such activities of PSMs have only been performed more recently (Tab. 2). In these studies, PSM $\delta$  and  $\delta$ -toxin of *S.*

*epidermidis* were found to be active predominantly against *Streptococcus pyogenes* (Cogen *et al.*, 2010). Furthermore, *S. epidermidis*  $\delta$ -toxin recruited host antimicrobial peptides (AMPs), such as LL-37, CRAMP, and hBDs, to reduce survival of *S. pyogenes* in a synergistic fashion (Cogen *et al.*, 2010). In another study, most intact PSMs exhibited very low or no antimicrobial activity on *S. pyogenes*, but proteolytically processed PSM $\alpha$ 1 and PSM $\alpha$ 2 showed significantly increased activities (Joo *et al.*, 2011). The N-terminal two amino acids, formyl-methionine and glycine, of these PSMs are missing in the antimicrobially active derivatives. Whether these processed PSMs contribute to bacterial interference in vivo is unclear. Also, which proteases are involved in the processing is not known, but recent findings on additional proteolytically processed derivatives of PSM $\alpha$ 1 and PSM $\alpha$ 4 implicates aureolysin. Overall, the biological significance of the antimicrobial activities found in some PSMs is unclear. The facts that these activities are rare and the antimicrobially active processed PSM $\alpha$  peptide derivatives are only produced in low amounts support the notion that they are a side effect of the general membrane-damaging effect of PSMs. The features that most PSMs exhibit suggest that they evolved predominantly to harm eukaryotic rather than prokaryotic membranes. Nevertheless, that staphylococci are protected from the antimicrobial effects of PSMs by the Pmt export system (Chatterjee *et al.*, 2013) indicates that they may at least theoretically contribute to bacterial interference with other genera, which remains to be clearly demonstrated in real-life bacterial communities.

### Structure-function analysis of PSMs and role of cytolytic versus pro-inflammatory capacity during infection

Recently, an alanine substitution peptide library of PSM $\alpha$ 3 was used to investigate which structural determinants are responsible for the diverse biological activities of PSMs (Cheung *et al.*, 2013) (Fig. 11). PSM $\alpha$ 3 was chosen because it is the most cytolytic PSM and significantly contributes to all phenotypes associated with PSMs, including hemolysis, biofilm structuring and pro-inflammatory activities (Wang *et al.*, 2007, Kretschmer *et al.*, 2010, Cheung & Otto, 2011, Periasamy *et al.*, 2012). The results obtained with the alanine substitution peptide library of PSM $\alpha$ 3 identified several important variants that have unique phenotypes. Namely, PSM $\alpha$ 3 L7A and K17A showed reductions in cytolytic but retained pro-inflammatory activities, whereas PSM $\alpha$ 3 N21A and N22A showed the reverse phenotype. PSM $\alpha$ 3 K6A and PSM $\alpha$ 3 K12A showed marked reductions in both cytolytic and pro-inflammatory activities. To investigate whether the in-vitro pro-inflammatory phenotypes of those derivatives were also reflected in vivo, a murine peritonitis model was used to measure the numbers of infiltrating leukocytes after exposure to select PSM $\alpha$ 3 alanine variants. Interestingly, only PSM $\alpha$ 3 L7A and K17A induced levels of leukocyte influx similar to those induced by wild-type PSM $\alpha$ 3. *S. aureus* constructs expressing these PSM $\alpha$ 3 derivatives from the genome will be valuable to investigate the biological role of PSM-receptor interaction in *S. aureus* disease in vivo. Furthermore, the identified non-toxic PSM $\alpha$ 3 derivatives may serve to develop a toxoid vaccine.

Changes at the hydrophobic side of PSM $\alpha$ 3, especially the exchange of large side chains such as phenylalanine, led to a reduction in biofilm-structuring activities and sometimes to

antimicrobial activity toward *S. pyogenes* not observed in native PSM $\alpha$ 3 (Cheung *et al.*, 2013). The latter suggests that large hydrophobic side chains in PSMs prevent the lysis of bacterial versus that of eukaryotic cells. Accordingly, PSM $\alpha$ 3, which has only strong capacity to lyse eukaryotic cells, has five phenylalanine residues, whereas PSM $\delta$  of *S. epidermidis*, which lyses both eukaryotic and prokaryotic cells, has only one.

## Diversification of PSMs and PSM production

Based on recent findings, it has become evident that PSMs have undergone divergent evolution to develop different functions. For example, many  $\alpha$ -type, but not  $\beta$ -type, PSMs are cytolytic and only the  $\delta$ -toxin leads to mast cell degranulation (Wang *et al.*, 2007, Cheung *et al.*, 2010, Nakamura *et al.*, 2013). The PSM $\beta$  peptides do not appear to have a distinct role, unless this role has not yet been identified. However, their lack of cytolytic function may be beneficial for commensal bacteria such as *S. epidermidis* in their effort to use PSM peptides in a “non-aggressive” fashion primarily to structure biofilms and facilitate life on epithelial surfaces, without simultaneously harming host cells.

While the capacity to produce strongly cytolytic PSMs is not limited to *S. aureus*, as we know from the cytolytic PSM $\delta$  of *S. epidermidis* (Cheung *et al.*, 2010), only *S. aureus* appears to produce high amounts of cytolytic, “aggressive” PSMs to lyse host cells and thereby evade elimination by host defenses. Additionally, *S. aureus* uses increased production of PSMs to augment virulence, as seen with highly virulent *S. aureus* strains such as CA-MRSA as compared to HA-MRSA (Wang *et al.*, 2007, Li *et al.*, 2009). Whether *S. epidermidis* or other staphylococcal species up-regulate production of “aggressive” PSMs under conditions of systemic invasion or specific disease conditions is not known.

## Targeting PSMs for anti-staphylococcal drug development

Historically, inactivated bacterial toxins proved to be successful candidates for active immunization to protect against bacterial disease, of which, the diphtheria, tetanus, acellular pertussis (DTaP) vaccine is a classic example (Adkins *et al.*, 2012). However, unlike in *S. aureus*, the toxins produced by *Corynebacterium diphtheria*, *Clostridium tetani*, and *Bordetella pertussis* are, for the most part, the only and primary toxins that are responsible for disease development. In contrast, *S. aureus* produces a plethora of toxins that have different contributions to different *S. aureus* diseases. As an additional complication, the toxins produced by *S. aureus* are not uniformly expressed among all isolates, making the identification of a toxin target for immunotherapy against *S. aureus* disease challenging (Proctor, 2012). With the exception of  $\alpha$ -toxin (Kennedy *et al.*, 2010) and PVL (Brown *et al.*, 2009), few other *S. aureus* toxins have been thoroughly investigated as candidates for active vaccination against a variety of pathogenic staphylococci (Spellberg & Daum, 2011).

Targeting the PSMs for anti-staphylococcal therapy and drug development would be advantageous for several reasons: the PSMs form a major constituent of the *S. aureus* exoprotein repertoire; they have an integral role in different facets of *S. aureus* disease; they show little variation in amino acid sequences between *S. aureus* strains; and the genome-encoded *psm* genes are present in all sequenced *S. aureus* strains (Wang *et al.*, 2007). Therefore, neutralizing the cytolytic and pro-inflammatory activity of all PSMs would

reduce their potency against host cells and possibly their overall contribution towards *S. aureus* disease progression.

Although protective humoral immune responses to *S. aureus* infection are generally not observed (Spellberg & Daum, 2011), a recent serological study showed a correlation between increased antibody levels to *S. aureus* exotoxins ( $\alpha$ -toxin, PVL, *S. aureus* enterotoxin C1,  $\delta$ -toxin, and PSM $\alpha$ 3) and a lower incidence of sepsis in hospitalized adults with invasive *S. aureus* infections (Adhikari *et al.*, 2012). These data indicate that the PSMs are immunogenic, since naturally circulating anti-PSM antibodies are detected in human adults (Adhikari *et al.*, 2012). Furthermore, this observation correlates with studies that show robust anti-PSM antibody responses in mice and rabbits after active immunization with PSMs (Nolte & Kapral, 1981, Wang *et al.*, 2011, Spaulding *et al.*, 2012). In addition, rabbits immunized with a combination of PSMs show weak protection in a bacteremia infection model (Spaulding *et al.*, 2012). The protective effect may be improved with the discovery of non-toxic PSM $\alpha$ 3 derivatives (Cheung 2013), as high concentrations of PSMs are known to be cytotoxic in vitro (Wang *et al.*, 2007).

The benefits of passive administration of anti-PSM antibodies were demonstrated in a study, in which antibodies against the *S. epidermidis* PSM $\beta$  peptides effectively blocked the dissemination of *S. epidermidis* from bacteria-coated catheters in mice (Wang *et al.*, 2011). However, passive immunization with anti-PSM antibodies may not be generally beneficial in biofilm-related *S. aureus* and *S. epidermidis* infections, because – while they may inhibit dissemination - they may also lead to more compact and thicker biofilms (Wang *et al.*, 2011). Accordingly, Agr-dysfunctional, and therefore PSM-negative, *S. epidermidis* and *S. aureus* strains are frequently isolated from biofilm-associated infections (Vuong *et al.*, 2004, Traber *et al.*, 2008). Thus, the benefits of passive vaccination with anti-PSM antibodies in the case of biofilm-associated staphylococcal disease need to be thoroughly investigated further. In contrast, passive immunization with antibodies directed against cytolytic PSMs may be more adequate in acute forms of staphylococcal disease. Problems with this approach are the following. First, PSMs are produced at high levels; likely requiring a high concentration of antibodies, at least if the primary mode of function is sequestration rather than promotion of opsonophagocytosis, whose efficacy in the case of *S. aureus* vaccines is debatable (DeLeo & Otto, 2008). Second, PSMs may exert much of their contributions to pathogenesis in the intracellular environment (Surewaard *et al.*, 2012), which may be difficult to reach for antibodies in sufficient amounts. Third, the variety of PSMs produced by *S. aureus* would require an antibody cocktail, further complicating such an approach.

Keeping these difficulties in mind, developing drugs or mAbs against Pmt, the dedicated PSM ABC transporter (Chatterjee *et al.*, 2013), has several key advantages over targeting single PSMs or other *S. aureus* toxins (Chatterjee & Otto, 2013). Inhibiting PSM transport would reduce the overall pathogenicity of *S. aureus* in vivo because of the interference of the secretion of all PSMs. Furthermore, because the fact that the *pmt* genes are found genome-encoded among all genome-sequenced staphylococci, it is probable that other pathogenic staphylococci may also be targeted by a potential drug inhibiting Pmt. Additionally, deletion of the *pmt* genes is harmful to *S. aureus* viability (Chatterjee *et al.*,



2013) suggesting that blocking Pmt may have a direct bactericidal in addition to a virulence-diminishing effect.

Lastly, while lysis of immune cells very likely is of key importance for the strong impact of  $\alpha$ -type PSMs on staphylococcal virulence, the biological role of PSM pro-inflammatory capacity and receptor-mediated recognition of PSMs by the human innate immune system is not understood. If indeed FPR2-dependent activation turns out to be crucial for the pathogenesis of staphylococcal disease, FPR antagonistic molecules, such as CHIPS and FLIPr, may be evaluated as potential drugs against staphylococcal infections.

In conclusion, the PSMs, FPR2-receptor mediated signaling, and especially Pmt are all possible targets for anti-staphylococcal drug development. However, as attractive as these single targets may be, successful protection against *S. aureus* may depend on disarming multiple targets that are involved in *S. aureus* virulence (Cheung & Otto, 2012).

## Conclusion/open questions

PSMs are a fairly recently discovered group of peptides. Naturally, therefore, many aspects of PSM biology and biochemistry remain to be analyzed. First, our knowledge on *psm* genes and PSM peptides is restricted to *S. aureus*, *S. epidermidis*, and the *psm* $\beta$ -like genes that can be found by similarity searches in other staphylococci. A comprehensive analysis of PSMs of most staphylococcal species is still missing. Second, how the physico-chemical features of PSMs determine their function in surface spreading, emulsification, biofilm formation, and interaction with membranes during cytolysis remains to be more clearly analyzed. Most of this unknown and the only information we have on PSM structure is derived from experiments on *S. aureus*  $\delta$ -toxin. One important question to be answered is the role of PSM aggregation, in particular in the reported form of ordered amyloid fibers, during biofilm formation and in-vivo biofilm infection. Third, the structural determinants of FPR2 activation by PSMs are not understood, as is the role of PSM-receptor interaction in pathogenesis, especially in comparison with the non-receptor-mediated cytolytic function of PSMs. Regarding cytolysis, further details of the interaction of PSMs with different cell types, including professional and non-professional phagocytes before and after ingestion, and the role of these interactions during infection and in possible synergy with other toxins need to be elucidated in more detail. Finally, PSMs may be used as vaccine targets, while small molecule inhibitors that target PSM export could induce killing bacteria during infection. However, while this newly recognized family of virulence determinants is clearly a promising therapeutic target, drug development efforts are still in their infancy.

## Acknowledgments

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH).

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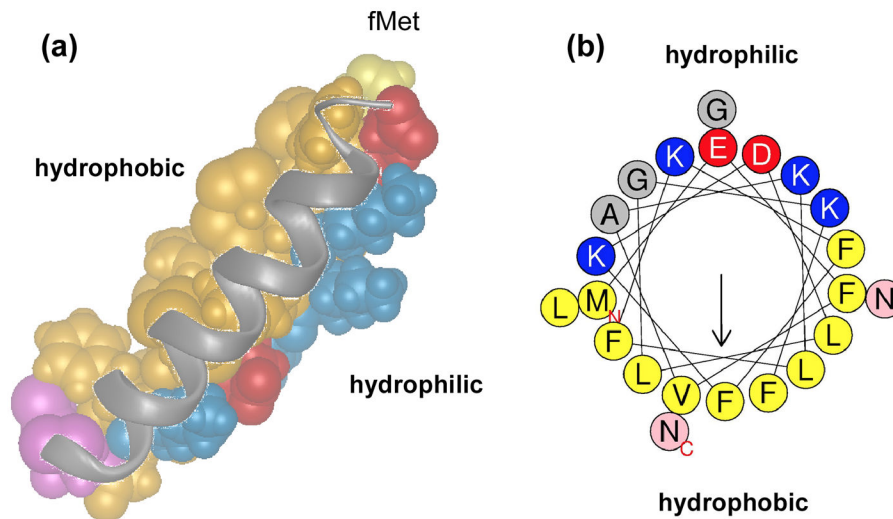
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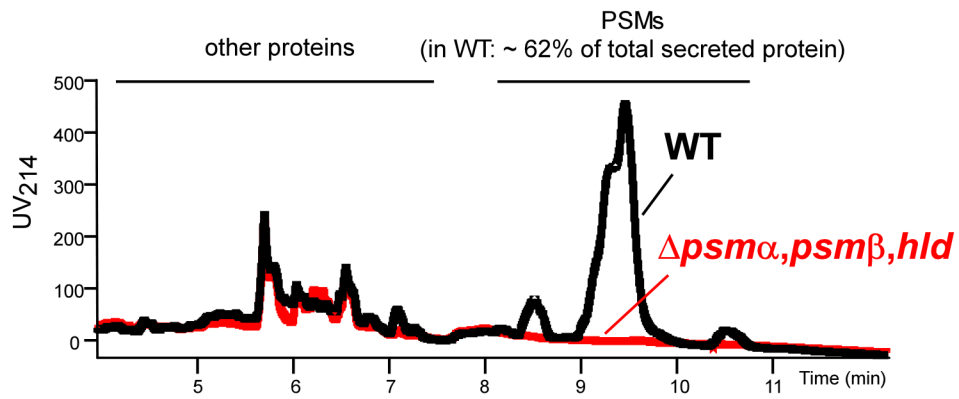


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**Fig. 1. Structure of PSMs**

Shown is a structural representation of PSM $\alpha$ 3 modeled after NMR data available for  $\delta$ -toxin (a) and an  $\alpha$ -helical wheel representation of the same peptide (b) as examples. Hydrophobic amino acids are in yellow/brown, hydrophilic amino acids in blue (positive charge) or red (negative charge). The two C-terminal asparagine residues are in pink. Note that in the  $\alpha$ -helix formed by PSM $\alpha$ 3 hydrophilic and hydrophobic residues occupy opposite locations, making the  $\alpha$ -helix distinctly amphipathic.



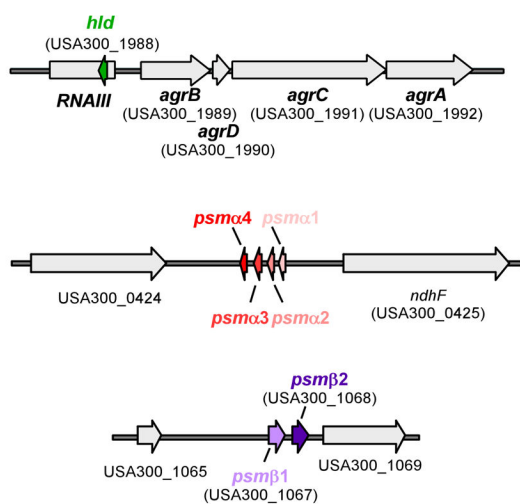
**Fig. 2. Analysis of PSMs by RPLC/MS**

Shown is RPLC chromatography routinely used for RPLC/MS analysis of PSMs. Culture supernatant of an *S. aureus* strain (USA300) was directly applied to a  $2.3 \times 30$  mm C8 reversed phase column and a brief gradient from 10 to 50% followed by an extended gradient from 50 to 90% water/0.1% trifluoroacetic acid (TFA) to acetonitrile/0.1% TFA as described in Wang et al. (Wang *et al.*, 2007) was run. Note that PSMs are separated from other proteins, but not all PSMs completely separate from other PSMs, necessitating coupling to MS for measurement of single PSM quantities. WT, wild-type USA300 (LAC); *psm* $\alpha$ , *psm* $\beta$ , *hld*, isogenic mutant not expressing any PSM.

**S. aureus**

<b>δ-toxin</b>	<b>fMAQDIISTIGDLVKWIIDTVNKFTKK</b>	<b>0</b>
<b>PSMα1</b>	<b>fMGIIAGIIKVIKSLIEQFTGK</b>	<b>+1</b>
<b>PSMα2</b>	<b>fMGIIAGIIKFIKGLIEKFTGK</b>	<b>+2</b>
<b>PSMα3</b>	<b>fMEFVAKLFKFFKDLLGKFLGNN</b>	<b>+1</b>
<b>PSMα4</b>	<b>fMAIVGTIIKIKAIIDIFAK</b>	<b>+2</b>
<b>PSMβ1</b>	<b>fMEGLFNAIKDVTVAAINNDGAKLGT</b>	<b>-2</b>
<b>PSMβ2</b>	<b>fMTGLAEAIANTVQAAQQHDSVKLGT</b>	<b>-1</b>

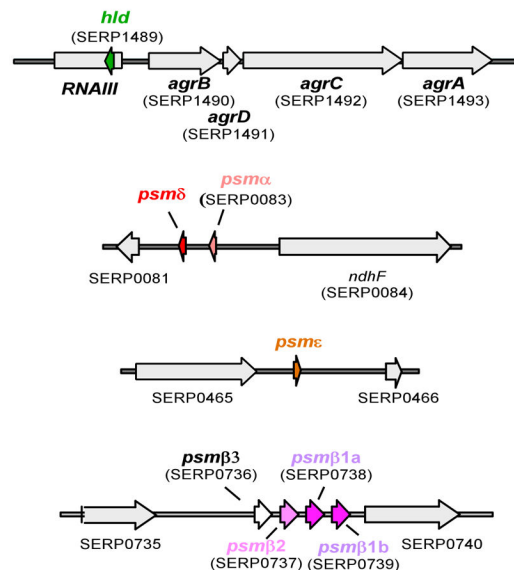
amphipathic α-helical domain



**S. epidermidis**

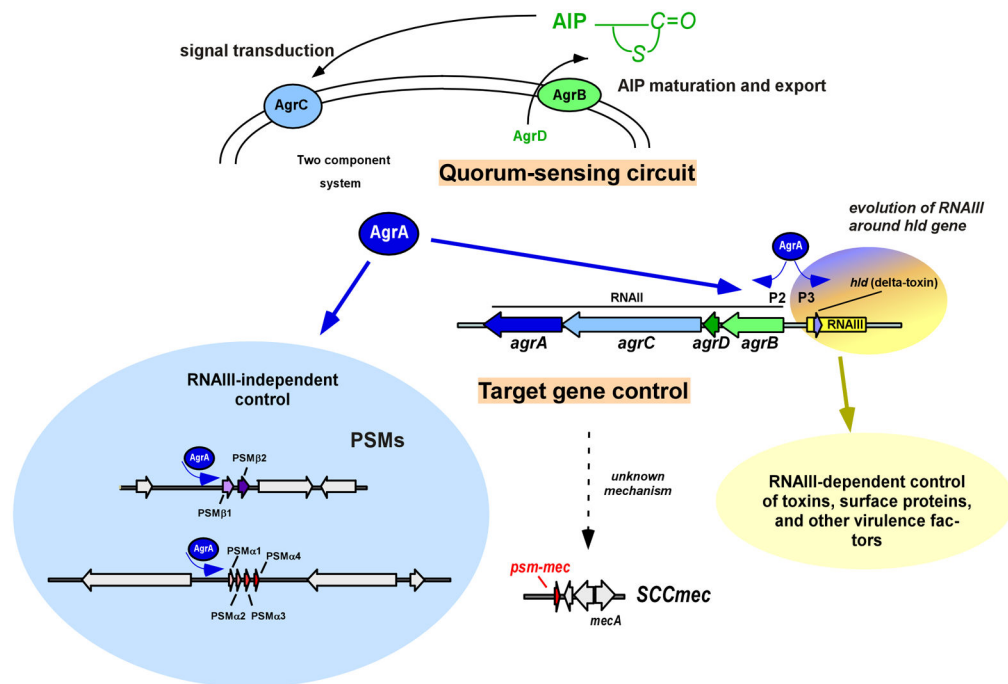
<b>δ-toxin</b>	<b>fMAADIISTIGDLVKWIIDTVNKFKK</b>	<b>25 0</b>
<b>PSMα</b>	<b>fMADVIAKIVEIVKGLIDQFTQK</b>	<b>22 -1</b>
<b>PSMδ</b>	<b>fMSIVSTIIEVVKTIVDIVKKFKK</b>	<b>23 +2</b>
<b>PSMε</b>	<b>fMFIINLVKKVISFIKGLFGNNENE</b>	<b>24 0</b>
<b>PSMβ1</b>	<b>fMSKLAEAIANTVKAADQDWTKLGT</b>	<b>44 -2</b>
<b>PSMβ2</b>	<b>fMEQLFAIRSVVDAGINQDWSQLASGI</b>	<b>44 -4</b>

amphipathic α-helical domain



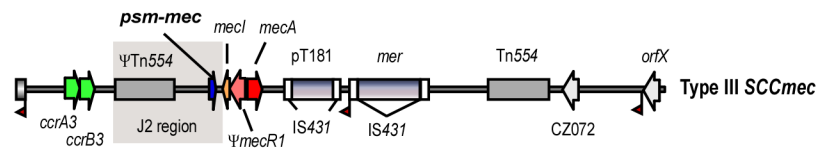
**Fig. 3. PSMs in S. aureus and S. epidermidis**

Amino acid sequences are shown at the top. Numbers at the right show the net charge of the peptides at pH 7.0, rounded to whole numbers, and considering N-formylation. Genes are shown at the bottom. Gene numbering is according to strains *S. aureus* USA300 FPR3757 (Diep *et al.*, 2006) and *S. epidermidis* RP62A (Gill *et al.*, 2005), respectively.



**Fig. 4. Regulation of PSMs by Agr**

The Agr quorum-sensing circuit, as described in the text, is shown at the top. Direct regulation of the *psmA* and *psm $\beta$*  operons by AgrA is shown left (blue shadowing) and RNAIII-dependent Agr control of other target genes at the right (yellow shadowing). The likely evolution of the connection of quorum-sensing, RNAIII-dependent and –independent control is apparent in the encoding of the PSM  $\delta$ -toxin (*hld*) by RNAIII. Agr also controls the *psm-mec* gene in an RNAIII-independent manner, but binding of AgrA to the *psm-mec* promoter has not yet been demonstrated.

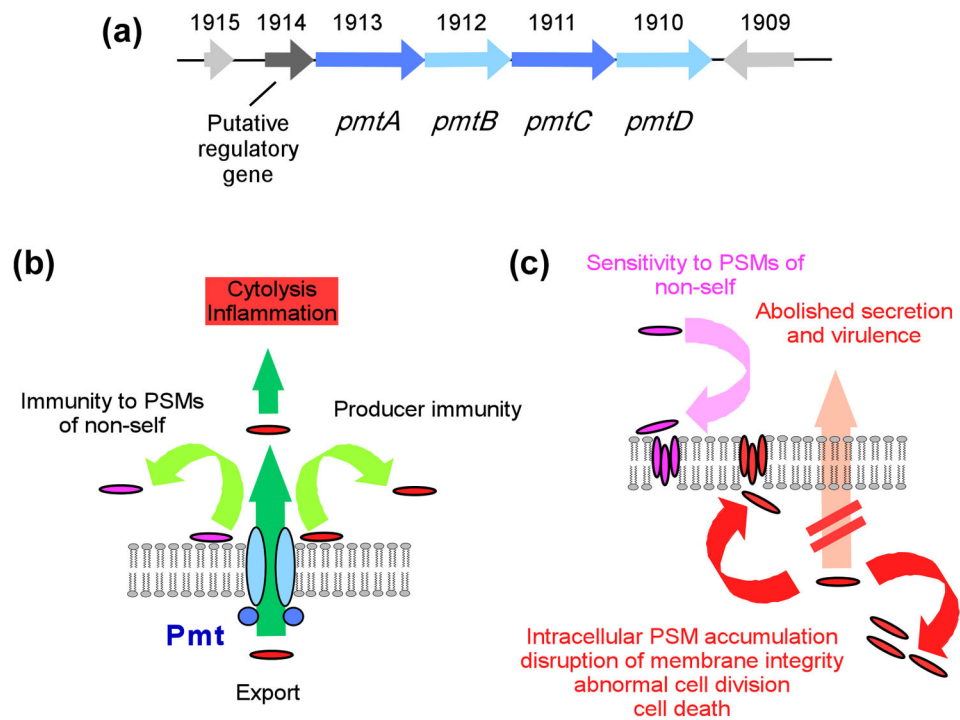


PSM-mec **fMDF**TGVITSIIDLIK**TCIQAFG** -2

in SCCmec II, III, VIII. or similar in:  
*S. aureus*, *S. epidermidis*, *S. fleuretti*, *S. hominis*, *S. pseudintermedius*,  
*S. saprophyticus*, *S. simulans*, *S. vitulinus*

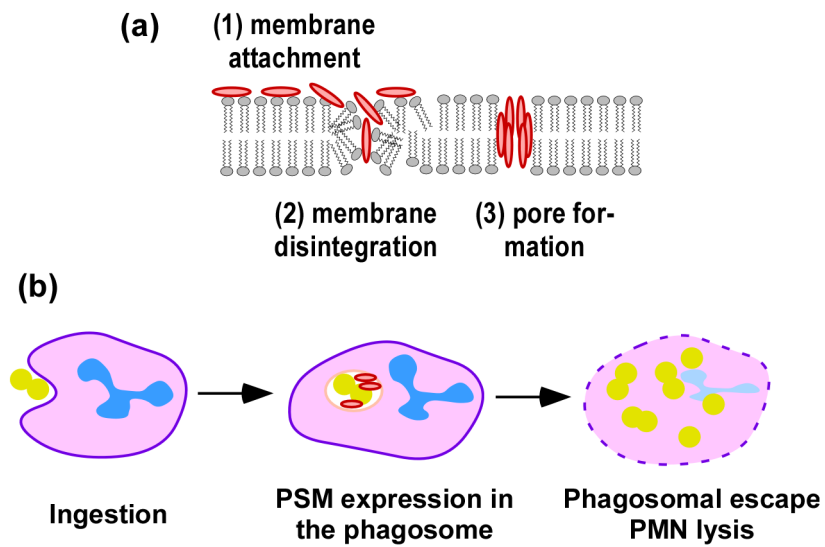
**Fig. 5. PSM-mec**

The PSM-mec peptide and the *psm-mec* RNA surrounding the PSM-mec-encoding gene are encoded on SCCmec elements of types II, II, and VIII, of which SCCmec type III is shown here as example.



**Fig. 6. PSM export**

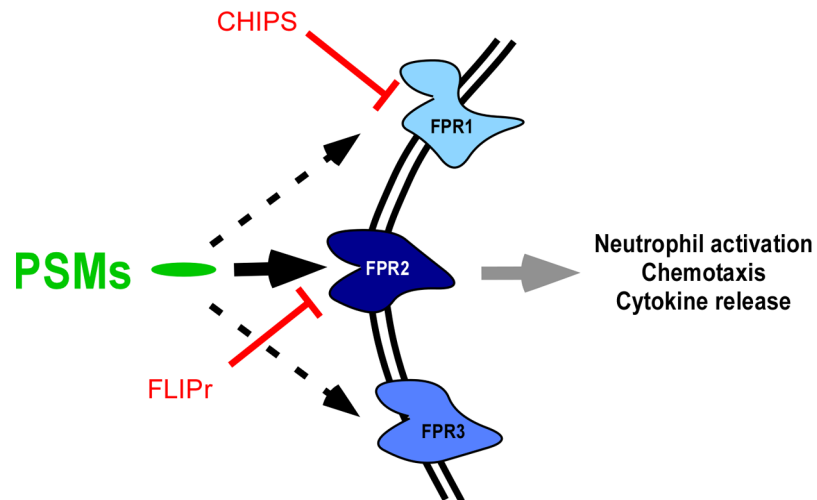
The genetic locus encoding the four Pmt ABC transporter components is shown at the top (a). Numbers refer to gene numbers in the USA300 FPR3757 genome (Diep *et al.*, 2006). (b) Pmt functions. (c) Consequences of Pmt absence/blocking.



**Fig. 7. PSM-mediated cytolysis**

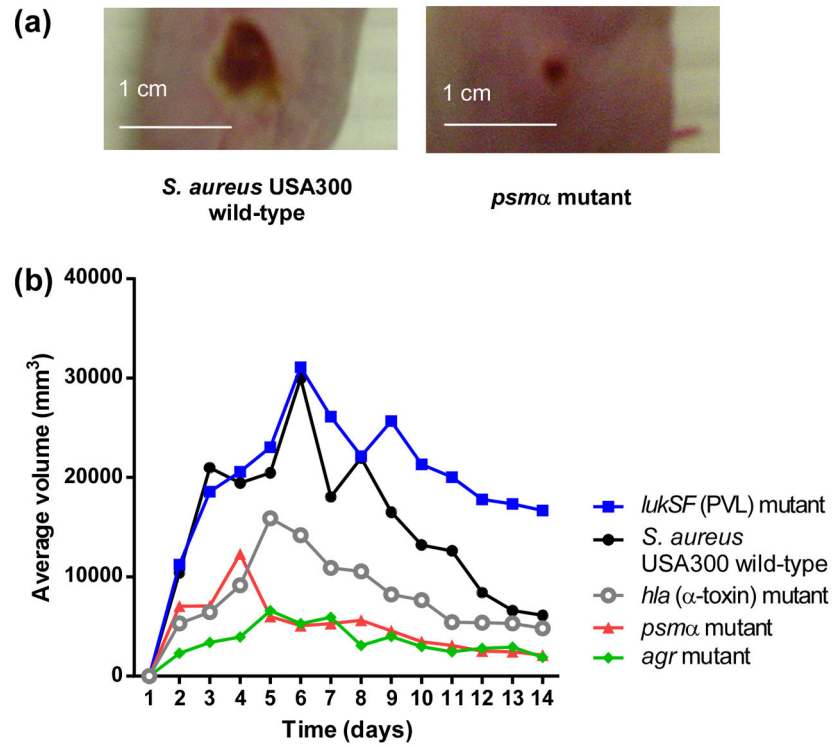
Likely mechanism of receptor-independent membrane attachment and disintegration, and subsequent pore formation by PSMs. This model is based on experimental evidence obtained with *S. aureus*  $\delta$ -toxin, which forms receptor-independent short-lived pores in artificial membranes (Verdon *et al.*, 2009). (b) Mechanism of intracellular neutrophil killing by PSM $\alpha$  peptides of *S. aureus* (Surewaard *et al.*, 2013).





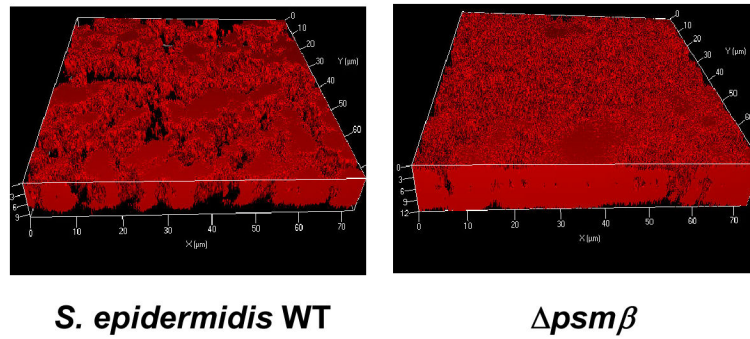
**Fig. 8. PSM receptor interaction**

In the nanomolar range, PSMs bind to and activate FPR receptors, with by far the strongest activation occurring with FPR2. Pro-inflammatory, FPR2-mediated activities of PSMs include neutrophil activation, chemotaxis, and release of specific cytokines such as IL-8. N-formylated and N-deformylated PSMs activate FPR2 receptors, with activation by formylated PSMs being somewhat stronger. The *S. aureus* virulence factors CHIPS and FLIPr block recognition by FPR1 and FPR2, respectively.



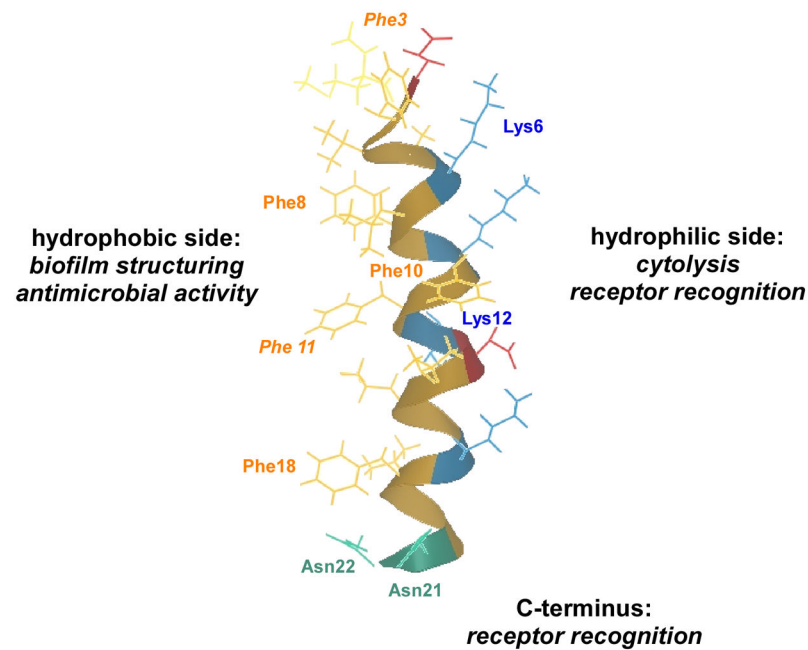
**Fig. 9. PSM $\alpha$  peptides and skin infection**

Representative pictures of abscesses formed by the USA300 CA-MRSA strain and its isogenic *psmA* deletion mutant in mice (Wang *et al.*, 2007). (b) Comparative analysis of virulence determinants in a rabbit skin infection model (Kobayashi *et al.*, 2011). The abscess volume is shown on the y-axis. Significantly smaller abscesses compared to the wild-type strain were found with the *hla*, *psmA*, and *agr*, but not *lukSF* mutants.



**Fig. 10. PSMs and biofilm structuring**

Confocal laser scanning microscopy pictures of biofilms formed by an *S. epidermidis* wild-type strain (strain 1457) and an isogenic deletion mutant of the *psm* $\beta$  operon (Wang *et al.*, 2011). Note the thicker biofilm formed by the mutant and the absence of channels in the biofilm.



**Fig. 11. PSM structure-function analysis**

An alanine exchange library of the PSM $\alpha$ 3 peptide was created and used to determine changes in PSM function caused by structural changes (Cheung, *et al.*, 2013). Overall, large hydrophobic side chains impacted biofilm-structuring capacities (such as some phenylalanine residues shown in regular font) and predominantly cationic amino acids impacted cytolytic and pro-inflammatory functions. In addition, the C-terminus exclusively affected receptor-mediated but not cytolytic activities. Finally, removal of large hydrophobic side chains such as all phenylalanine residues led to derivatives that showed antimicrobial activities, indicating that these large hydrophobic side chains are involved in preventing cytolysis of bacterial versus eukaryotic membranes in PSMs.

Table 1

PSM homologues identified by similarity searches

Species/strain	PSM name/annotated as (suggested name in brackets)	Amino acid sequence	Gene <sup>L</sup>	Protein <sup>L</sup>	Length (amino acids)	Net Charge
<i>PSMβ</i> homologues						
<i>S. aureus</i>	PSMβ1	MEGLFNAIKDTVTAAIINNDGAKLGTSTIVSIVENGVLGKLFQGF	A284_07765	AGC90870.1	44	-2
	PSMβ2	MTGLAEAIANTVQAAQQHDSVKLGTSTIVDIVANGVGLLGLKLFQGF	A284_07760	AGC90869.1	44	-2
<i>S. epidermidis</i>	PSMβ1	MSKLAEAIAINTVKAQQDDWTKLGTSTIVDIVESGVSVLGKIFQGF			44	-2
	PSMβ2	MEQLFDAIRSVVDAGINQDWSQLASGIAGIVENGISVSKLLGQ			44	-4
<i>S. warneri</i> SG1	PSMβ1 (PSMβ1)	MEGLIKAIKDTVEAGVNNNDGAKLGTSTIVSIVENGVGLSKLFGF	A284_07755	AGC90868.1	44	-2
	PSMβ1 (PSMβ2a)	MTKLAEAIAINTVQAAQGHDKGAKLGTSTIVSIVENGVSVLGKLFQGF	A284_07750	AGC90867.1	44	-1
	PSMβ1 (PSMβ3a)	MTKLAEAIAINTVKAQQHDKGAKLGTSTIVSIVENGVSVLGKLFQGF	A284_07745	AGC90866.1	44	0
	PSMβ1 (PSMβ3b)	MTKLAEAIAINTVQAAQGHDKGAKLGTSTIVSIVENGVSVLGKLFQGF	A284_07745	AGC90866.1	44	0
	antibacterial protein (PSMβ2b)	MSKLVQAISDAVQAQQNDWAKLGTSTIVSIVENGVSVLGKLFQGF	SH1745	BAE05054.1	44	-1
<i>S. pneumoniae</i> JCSJ1435	unnamed protein (PSMβ1a)	MEKIANAVKSAIEAGQNQDWTCLGTSILDIVSNGVTELSKIFQGF	SH1744	BAE05053.1	44	-2
	unnamed protein (PSMβ2a)	MSKLVQAISDAVQAQQNDWAKLGTSTIVSIVENGVSVLGKLFQGF	SH1743	BAE05052.1	44	-1
	unnamed protein (PSMβ1b)	MEKIANAVKSAIEAGQNQDWTCLGTSILDIVSNGVTELSKIFQGF	SH1742	BAE05051.1	44	-2
	unnamed protein (PSMβ2b)	MOKLAEAIAAAAQAGQDKDWGKMGTSIVGIVENGISVLGKIFQGF	SH1741	BAE05050.1	44	-1
	unnamed protein (PSMβ3)	MTELFDAIKNTIEAGINHDWVTLGTSIADIVANGINVISGFFFG	SPSE_1635	ADX76893.1	43	-5
<i>S. pseudinterme dius</i> B99	putative hemolysin-HIC (PSMβ1) <sup>2</sup>	MGLDFESITNIVTSAIQHDDWVNMGAAIATVQHGVELIAKLFGLG	SPSE_1546	ADX76805.1	45	-4
	putative hemolysin-HIC (PSMβ2) <sup>2</sup>	MGLDFESITNIVTSAIQHDDWVNMGAAIATVQHGVELIAKLFGLG	SPSINT_0962	ADV05490.1	45	-4
<i>S. pseudinterme dius</i> H10-03	hypothetical protein (PSMβ2) <sup>2</sup>	MTELFDAIKNTIEAGINHDWVTLGTSIADIVANGINVISGFFFG	SPSINT_0872	ADV05400.1	43	-5
	hypothetical protein (PSMβ1) <sup>2</sup>	MEGLFEAIKNTVQAGIADGAKIGSGVLDIISNGASLIQKALGL	SSP1598	BAE18743.1	44	-2
<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> ATCC 15305	putative antibacterial protein (PSMβ1)	MADLFNAIKETVQAGIADGAKLGTSTIVSIVENGVSVLKLFQGF	SSP1597	BAE18742.1	44	-2
	putative antibacterial protein (PSMβ2)	MTGIVEAIGNAVNVGLAHDWATMGVSIADVLAKGVDSVLGFFK	SSP0130	BAE17275.1	43	-3
	putative antibacterial peptide (PSMβ3) <sup>2</sup>	MSGIVDAISKA VQAGLDKDWATMATSIAADAIKGVDFIAGFFN	SLUSHA	CCB52870.1	43	-3
<i>S. lugdunensis</i> N920143	SlushA	MSGIIEAITKAVQAGLDKDWATMGTSIAEALAKGIDAISGLFG	SLUSHB	CCB52869.1	43	-3
	SlushB	MDGIFEAISKA VQAGLDKDWATMGTSIAEALAKGVDFIAGLFLH	SLUSHC	CCB52868.1	43	-3

Species/strain	PSM name/annotated as (suggested name in brackets)	Amino acid sequence	Gene <sup>1</sup>	Protein <sup>1</sup>	Length (amino acids)	Net Charge
<i>S. carnosus</i> TM300	phenol soluble modululin (PSMβ1)	MEGLFNAIKGTVEAAINQDGGQLAVNIVDIVQNGIQIVSKFIGA	SCA_0784	CAL27694.1	44	-3
	phenol soluble modululin (PSMβ2)	MLTKLAQAIVDTVNA.AQAQDPAPAKLGTISIVDIVQNGVGVGKLFQGF	SCA_0785	CAL27695.1	45	-1
<i>S. simulans</i> bv. <i>staphylolyticus</i> NRRL B-2628 plasmid pACK1	unnamed protein product (PSMβ-pACK1)	MSGIVDAISQA VQSGLEQDWVTMTGNTIAKALAAQGIDVISGFFG			43	-4
δ-toxin homologues						
<i>S. epidermidis</i>	δ-toxin	MAADIISTIGDLVKWIIDTVNKFKK			25	0
<i>S. aureus</i>	δ-toxin	MAQDIISTIGDLVKWIIDTVNKFTKK			26	0
<i>S. carnosus</i> TM300	(δ-toxin)	MAGDIVGTIGDFVKKLIETVVKFTNK			26	-1
<i>S. simulans</i>	(δ-toxin)	MAGDIVGTIGDFVKKLIETVVKFTQK	AJ223775.1	CAA11542.1	26	-1
<i>S. intermedium</i>	(δ-toxin)	MAGDIISTIVDFIKLIAETVKKFTK	AY860843.1	AAW55662.1	25	0
<i>S. pseudinterme dius</i> HKU10-03	(δ-toxin)	MAADIISTIVEFVKLIAETVEKFIK			25	-2
<i>S. pseudinterme dius</i> ED99	(δ-toxin)	MAADIISTIVEFVKLIAETVAKFIK			25	-1
<i>S. warneri</i> SG1	(δ-toxin 1)	MAADIISTIGDLVKLIINTVKKFQK	A284_04270	AGC90171.1	25	1
	(δ-toxin 2)	MTADIISTIGDFVKWILDVTKKFTK	A284_04275	AGC90172.1	25	0
PSMε( <i>S. epidermidis</i> ) homologues						
<i>S. epidermidis</i>	PSM ε	MFIINLVKKVISIFIKGLFGNNENE			24	0
<i>S. pseudinterme dius</i> ED99	(PSM ε)	MFIIDLKIKVIEFLKGLFGNK			21	1
<i>S. pseudinterme dius</i> HKU10-03	(PSM ε)	MFIIDLKIKVIEFLKGLFGNK			21	1
<i>S. lugdunensis</i> N920143	(PSM ε)	MFVNLIKKVFIEFIKKLFGNK			21	3
<i>S. lugdunensis</i> HKU09-01	(PSM ε)	MFVNLIKKVFIEFIKKLFGNK			21	3
<i>S. warneri</i> SG1	(PSM ε)	MQFTIDLKIKKAVDFEKLFGNK			22	1

<sup>1</sup> if annotated. The *psm* genes in *S. aureus* and *S. epidermidis* are found and annotated in many strains. See Fig. 3 for gene numbers in strains USA300 and RP62A.

<sup>2</sup> encoded separately. Other *psmβ* genes are commonly grouped in one genetic locus.

Table 2

PSMs with antimicrobial activities.

Strain	PSM type	Derivatization	Target	MBC (μM)	Note	Reference
CoNS isolate	unknown	-	<i>Neisseria gonorrhoeae</i> G-10		16 kD protein dissociated to 1.4 kD	(Beaudet, <i>et al.</i> , 1982)
<i>S. epidermidis</i>	δ-toxin	intact	<i>S. aureus</i> 113	4–8	KAM <sup>2</sup> used except for <i>S. pyogenes</i> (25% Todd-Hewitt broth, 75% PBS)	(Cogen, <i>et al.</i> , 2010)
			<i>S. pyogenes</i> , NZ131	16		
			<i>Escherichia coli</i>	8–16		
	PSMδ	intact	MRSA, Sanger 252	16–32		
			MRSA, USA300	32		
			<i>S. aureus</i> , 113	8		
			<i>S. pyogenes</i> NZ131	16		
			<i>Escherichia coli</i>	8		
<i>S. aureus</i>	δ-toxin	synthesized and modified 16 to 26 residues-derivatives	<i>Escherichia coli</i>	2.1–3.9 <sup>1</sup>	intact <i>S. aureus</i> δ-toxin is not antimicrobial	(Dhople & Nagaraj, 1995, Dhople & Nagaraj, 2005)
			<i>S. aureus</i>	3.7–22 <sup>1</sup>		
	PSMα1	N-terminal two residues are absent	<i>S. pyogenes</i>	8	THY <sup>2</sup> used	(Joo, <i>et al.</i> , 2011)
			<i>S. pyogenes</i>	11		(Gonzalez, <i>et al.</i> , 2012)
			<i>S. epidermidis</i> , ATCC35984	42		
	PSMα2	N-terminal two residues are absent	<i>S. pyogenes</i>	8	THY used	(Joo, <i>et al.</i> , 2011)
			<i>Staphylococcus epidermidis</i> 12228	16	TSB <sup>2</sup> used	
	PSMα3	Synthesized alanine exchange derivatives: F3A, V4A, F8A, F10A, F11A, L14A, F18A, L19A	<i>S. pyogenes</i> , <i>Micrococcus luteus</i>	3		(Cheung, <i>et al.</i> , 2013)
	PSMα4	N-terminal three residues are absent	<i>S. pyogenes</i>	10	aureolysin-dependent	(Gonzalez, <i>et al.</i> , 2012)

<sup>1</sup> MIC (μM)

<sup>2</sup> KAM, killing assay media (Dorschner, *et al.*, 2006); THY, Todd-Hewitt broth containing 0.2% yeast extract; TSB, tryptic soy broth

<sup>3</sup> determined by growth inhibition zones