The importance of phenol-soluble modulins in *Staphylococcus aureus* infections

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Abstract | ***Staphylococcus aureus*** **asymptomatically colonise epithelial surfaces of part of the human population. However, when the epithelial barriers breach the bacteria can cause severe infections. The antibiotic resistance and high virulence of some *S. aureus* strains, especially community-acquired methicillin-resistant *S. aureus* (CA-MRSA), make infections life-threatening and hard to treat. Recently, it was discovered that CA-MRSA secrete phenol-soluble modulin (PSM) peptides. These leukocidins can recruit neutrophils to the site of infection and enable the bacteria to escape neutrophil phagosomes. Furthermore, PSMs are able to lyse host cells and are also suggested to kill bacterial cells of niche competitors. Finally, the PSM peptides are involved in structuring and detachment of biofilms. In this review, the effects and importance of these PSMs for the virulence of CA-MRSA are discussed.**

Staphylococcus aureus colonise human epithelial surfaces of about 30% of the non-hospitalized population in the U.S.A.[1]. Usually, this colonisation is asymptomatic, however, when the epithelial barriers breach the bacteria can spread to adjacent tissues or the bloodstream. Depending on the cell wall composition and production of virulence factors, *S. aureus* are able to cause many different infectious diseases, varying from superficial skin and wound infections to life-threatening disseminated infections. *S. aureus* infections are characterised by tissue destruction, the production of large quantities of pus and abscess formation. The severe inflammatory response and multi-organ dysfunction due to tissue destruction can even be fatal[2].

The occurrence of *S. aureus* infections, especially infections caused by methicillin-resistant *S. aureus* (MRSA), is rapidly increasing[3]. *S. aureus* is currently a leading cause for bacterial infections worldwide. Whereas hospital-acquired (HA-)MRSA infections are limited to people with risk factors and underlying susceptibilities, anyone can be at risk for community-acquired (CA-)MRSA infections. The combination of antibiotic resistance and high virulence, make the CA-MRSA infections life-threatening and hard to treat[4].

The outcome of an infection is highly dependent on the ability of the bacteria to evade the human immune system. *S. aureus* strains have developed many mechanisms to evade recognition and subsequent elimination[5]. An important finding was that CA-MRSA strains have the ability to destroy neutrophils by the production of leukocytic toxins[6]. These leukocidins, such as Panton-Valentine leukocidin (PVL) and α-toxin, are likely a major factor contributing to the enhanced virulence of CA-MRSA[7,8]. Previously, a lot of CA-MRSA research has focussed on PVL. However, it seems that PVL has a limited contribution to the pathogenesis of CA-MRSA[9]. Therefore, the debate about which factor is key in CA-MRSA infections is still ongoing[10].

In 2007, Wang *et al*. characterized a new type of leukocidins in CA-MRSA, namely phenol-soluble modulins (PSMs). *In vitro* experiments have shown that CA-MRSA produce higher concentrations of PSMs than HA-MRSA, suggesting that the PSM peptides contribute to the high virulence of CA-MRSA[11]. Over the last few years, a lot of knowledge has been gained about PSMs. In this review, the effects and importance of PSMs in *S. aureus* infections are discussed.

Phenol-soluble modulins: characteristics

The success of *S. aureus* infections depends mostly on the evasion of the defence mechanisms of the host. The bacteria have various mechanisms to subvert the innate immune system. For instance, *S. aureus* produce different molecules which can interfere with the complement system, provide resistance to killing by antimicrobial peptides or a respiratory burst, degrade immunoglobulins or cloak opsonins. In addition, *S. aureus* also produce leukocidins: a group of bacterial toxins which are able to kill leukocytes by disruption of the plasma membrane[12].

PSMs are small peptides which belong to the leukocidin family. The high degree of amphiphacity and the strong α-helical structure may allow the PSMs to penetrate cell membranes. *S. aureus* bacteria secrete the PSMs by a hitherto unknown mechanism. The peptides have a formylated N-terminus methionine, which is characteristic for bacterial biosynthesis. The PSM peptides are divided in two categories: α-type and β-type PSMs (Table 1). The α-type PSMs differ from the β-type PSMs in length. The α-type PSMs are shorter, about 20 amino acids long, than the β-type PSMs, which are about 40 amino acids in length[11]. The peptides do not only differ in length, but also in function. Throughout this review, the effects of the PSM peptides are discussed.

	Length:	Name:	Encoded on:	Gene:
α-type PSMs	~20 amino acids	PSMα1	core genome	*psmα*
		PSMα2	core genome	*psmα*
		PSMα3	core genome	*psmα*
		PSMα4	core genome	*psmα*
		δ-toxin	core genome	*hld*
		PSM-mec	mobile genetic element	SSC*mec*
β-type PSMs	~40 amino acids	PSMβ1	core genome	*psm*β
		PSMβ2	core genome	*psm*β

Table 1: Classification of the α-type and the β-type phenol-soluble modulin peptides.

Phenol-soluble modulin gene expression

The genes of many toxins are located on mobile genetic elements (MGEs). Due to the mobility of these gene segments, the production of these toxins is usually restricted to a small number of strains[13]. Remarkably, only one PSM peptide, PSM-mec, is encoded on an MGE. The PSM-mec peptide is encoded by the staphylococcal cassette chromosome *mec* (SSC*mec*), which also carries the gene responsible for methicillin resistance. This indicates that there is a molecular connection between virulence and antibiotic resistance, which both have a large impact on the outcome of *S. aureus* infections[14].

Most *psm* genes are encoded on the bacterial core genome. Therefore, it is not surprising that the *psm* genes are present in all the *S. aureus* strains whose genome has been sequenced. There are seven genes which encode the PSM peptides on the *S. aureus* genome. Firstly, there are four genes on the *psmα* operon which encode the α-type PSMs: PSMα1-4. Secondly, the *psm*β operon encodes two PSM peptides of the β-type: PSMβ1 and PSMβ2. Lastly, the *hld* gene encodes δ-toxin, which is similar to the α-type PSMs (Table 1)[11].

Although *psm* genes are present in virtually all the *S. aureus* strains, there is great difference in the expression of those genes[11]. These differences in PSM expression, could explain why one *S. aureus* strain is more virulent than the other. Furthermore, since PSMs have both cytolytic and proinflammatory effects (see *Phenol-soluble modulin activity*), the production must be tightly regulated to times when the immune cells can be efficiently inactivated. The expression of PSM peptides is, like many other *S. aureus* virulence factors, strictly controlled by the accessory gene regulator (*agr*) quorum-sensing system[11,15,16].

The *agr* quorum-sensing system consists of two transcriptional units, RNAII and RNAIII, which are transcribed in opposite directions (Fig. 1). RNAII encodes four proteins: AgrABCD. AgrB is a transmembrane protein, which processes the AgrD precursor into an octapeptide. This autoinducing peptide (AIP) is secreted and further modified by AgrB. AIP is regarded as an autocrine pheromone peptide because it signals the state of the cell density to other members of a bacterial population. The sequence of this peptide varies between *S. aureus* strains. The pheromone peptide of one strain can inhibit the *agr* quorum-sensing system of another strain[17-20].

AIP binds to the extracellular part of the histidine kinase AgrC. AgrA is a response regulator and forms together with AgrC a classical bacterial two-component regulatory system. Thus, AgrC can modulate the activity of AgrA. Active AgrA binds the P2 and P3 promoter regions on the *agr* locus to increase transcription of RNAII, creating a positive feedback loop, and RNAIII, respectively[17,21]. The enhanced transcription of RNAIII leads to an increase in the transcription, and in some cases even the translation, of several virulence factors[19,20]. However, RNAIII has no considerable impact on the expression of PSM peptides. Strikingly, the *psmα* and *psm*β genes are virtually the only genes which are upregulated by the *agr* system independent of RNAIII. Indeed, RNAIII even caused downregulation of *psmα* and *psm*β transcription, which is opposite to the overall strong effect of *agr* on PSM production. Clearly, the RNAIII-independent upregulation overrules the RNAIII-dependent downregulation of *psm* expression (Fig. 1) [16,19].

Both the core genome encoded PSMs as well as PSM-mec are directly regulated by AgrA rather than by RNAIII[16,22]. In an active state, AgrA can bind directly to the promoter regions of PSMs, thereby increasing the PSM production. Thus, the PSM expression is controlled by the *agr* system, depending only on the RNAII

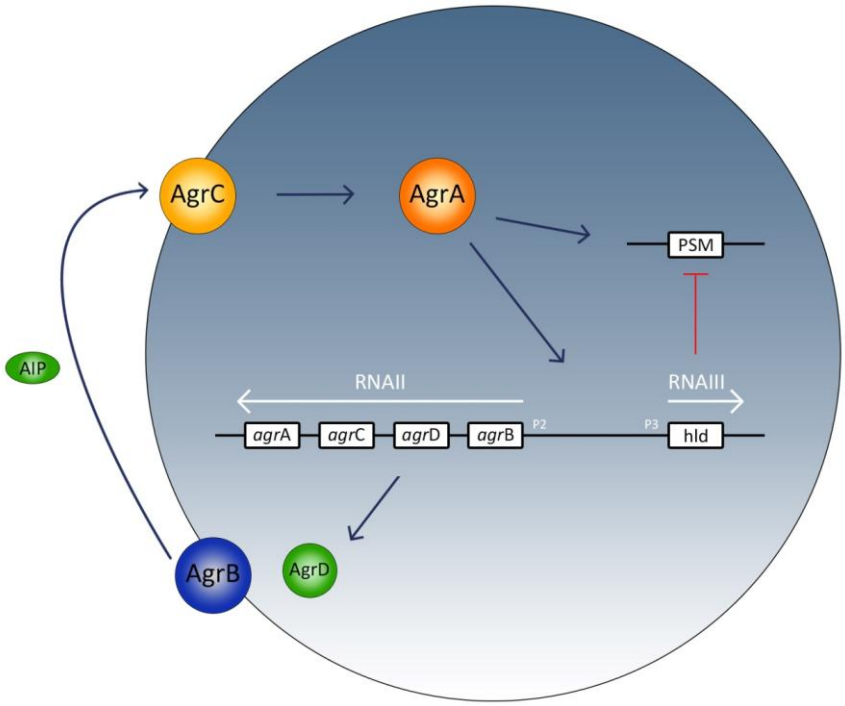

Figure 1: Control of* psm *transcription by the* agr *quorum-sensing system in S. aureus. In the right environmental setting, the P2 and P3 operons in the genome of *S. aureus* are activated, which results in the RNAII and RNAIII transcripts, respectively. RNAII encodes four proteins: AgrABCD. AgrB processes AgrD into an autoinducing peptide (AIP). Subsequently, AIP is secreted and modified by AgrB. In turn, AIP binds to the extracellular part of the histidine kinase AgrC, which is part of a two-component system. AgrC activates AgrA, which stimulates the transcription of RNAII, creating a positive feedback loop, and RNAIII. RNAIII increases the transcription of secreted virulence factors. However, RNAIII inhibits rather than upregulates the *psm* transcription. PSM production is regulated by direct binding of AgrA to the promoter of the *psm* genes. The stimulatory signal of AgrA clearly overrules the inhibition by RNAIII.

transcript[11,16].

Apart from its regulatory role in the *agr* quorum-sensing system, the RNAIII transcript also encodes δ-toxin. It is hypothesised that the *agr* quorum-sensing system regulated PSM expression before it was involved in the regulation of a wide variety of virulence factors. It is likely that the control of additional virulence factors occurred when the RNAIII encoding region developed around the gene encoding δ-toxin[16]. When the *agr* system is activated, there is a vast increase in the production of RNAIII. This leaves the question how expression of δ-toxin is regulated. Since RNAIII is a part of the *agr* quorum-sensing system, there is likely another regulatory mechanism involved.

The expression of δ-toxin might depend on the alarmones ppGpp and pppGpp. The enzyme RSH synthesises the alarmones under stringent conditions, such as amino acid deprivation. It was shown that the alarmones can lead to an increase in *psmα1-4* and *psmβ1-2* transcription. Although the transcription depends on the *agr* quorum-sensing system, AgrA expression did not change during the stringent response[23]. The exact molecular mechanism behind the upregulation of *psm* transcription remains to be elucidated. However, the upregulation of PSMs under stringent conditions shows that there are various regulatory mechanisms involved in PSM expression.

Phenol-soluble modulin activity

After production of the PSMs, the peptides are secreted from the bacterial cell. The PSM peptides have several effects, namely cytolytic, pro-inflammatory and antimicrobial effects.

Cytolytic | The severe infections caused by CA-MRSA are characterised by massive influx and subsequent lysis of neutrophils, which causes severe damage to the surrounding tissues. Furthermore, it was reported that PSMs in the blood can lyse erythrocytes, which contributes to the pathogenesis[24]. However, the clinical relevance of erythrocyte lysis can be debated because PSMs in the blood are neutralised by serum lipoproteins[25]. The observed cell lysis is the result of disruption of the plasma membrane caused by micromolar concentrations of the α-type PSMs or PSM-mec. The cytolytic effects are probably due to the α-helical structure and a high degree of amphiphacity of the peptides. This would allow the PSMs to incorporate in the plasma membrane, thereby forming pores which is lethal to the cell[11,14].

Proinflammatory | Whereas only some PSM subtypes have cytolytic effects, all PSMs have proinflammatory effects[11,14,26]. When the PSMs are present in nanomolar concentrations, the peptides act as chemoattractants for neutrophils. Surprisingly, formyl peptide receptor 1 (FPR1), which recognises formylated bacterial peptides and causes chemotactic migration, did not respond to

the PSM peptides. Instead, formyl peptide receptor 2 (FPR2) is activated by all eight different core genome encoded PSMs[27,28]. The formyl groups on the PSM peptides are not essential but have a considerable impact on FPR2 activation[27]. FPR2 is mainly involved in diseases such as Alzheimer's disease because it responds to a variety of amyloidogenic peptides[29,30]. This would suggest that PSMs also have amyloid properties. Indeed, in *S. aureus* biofilms there are amyloid-like fibers present which were shown to consist of PSMs. However, these fibers are very different from the monomeric PSM peptides which are secreted by *S. aureus*[31]. This raises the question whether FPR2 is designed to respond to bacterial infections and accidentally got involved in the pathogenesis of diseases like Alzheimer's or if the receptor originally has a different function and incidentally also recognises PSMs.

When FPR2 is activated by PSMα peptides, neutrophils respond by secreting reactive oxygen species (ROS). Like many other microbial toxins, the biological activity of the PSM peptides can be inhibited by myeloidperoxidase (MPO) and ROS[26]. The exact mechanism behind this inhibition of PSMs is still unknown. Besides the mechanism, also the purpose of this inhibitory feedback loop is not yet understood. The bacteria could use the MPO/ROS system to control the PSMs, since the gene expression is controlled via the positive feedback loop of the *agr* quorum-sensing system. The PSM inhibition by neutrophils shows that there is a very complex interaction between the immune system and the bacteria.

The ability of the PSM peptides to recruit neutrophils to the site of infection would indicate that these immune cells are somehow beneficial for the bacteria. It is hypothesised that the α-type PSMs are so effective in destroying neutrophils, that the bacteria actively recruit neutrophils to cause more damage to the immune system[27]. Furthermore, it has been shown that *S. aureus* are able to survive after phagocytosis by neutrophils[32-34]. If the bacteria escape neutrophils after the cells have relocated, the bacteria can spread without being detected by the immune system[35]. This is supported by the fact that the *S. aureus* infections in neutropenic patients are less severe[36].

Even though recruitment of neutrophils to the site of infection could be beneficial to the bacteria, *S. aureus* also secrete inhibitors of the FPR2 receptor: FPR2-inhibitory protein (FLIPr) and FLIPr-like[37,38]. It appears that during certain stages of colonisation and infection it is preferable for the bacteria to remain undetected by the immune system. For CA-MRSA it has been shown that the FLIPr gene is expressed under different conditions than the PSM genes. Whereas the PSM genes are tightly controlled by the *agr* quorum-sensing system, the FLIPr gene is expressed upon contact with the contents of neutrophil granules[16,39]. During the early stages of infection, when it is beneficial for the bacteria to remain unrecognised by the immune system, the PSM expression is largely turned off. FLIPr and FLIPr-like may help to inhibit any residual activity of the PSMs[27].

Antimicrobial | Recently, it was reported that *S. aureus* PSMs do not only target host immune cells, the peptides can also target other microbes. The antimicrobial activity is caused by derivatives of the PSM peptides, suggesting that the peptides gain antimicrobial properties in environments where the peptides can be proteolytically processed[40,41]. Strikingly, *S. aureus* PSMs have virtually no activity against *S. epidermidis* or different *S. aureus* strains. In contrast, *Streptococcus pyogenes*, niche competitors of *S. aureus*, were highly sensitive to the peptides. Joo *et al*. suggest that the PSMs do not provide a competitive advantage within the species or genus, but that other bacteria suffer from the PSM peptide production. Therefore, it is suggested that the antimicrobial properties of PSMs contribute to the exceptional capacity of CA-MRSA to colonise and spread in populations[40].

However, there is no evidence that, in addition to *S. pyogenes*, there other bacteria which also become the victim of the PSM derivates. It is very striking that the antimicrobial activity seems to be species specific, since *S. epidermidis* and various *S. aureus* strains are not affected by the PSMs[40]. Neutrophil lysis by PSMs does not depend on a receptor[27], thus where does the specificity of PSM derivatives in selecting their targets come from? Bacteria within the *Staphylococcus* genus might have developed mechanisms to counteract the antimicrobial effects of the PSMs, which could result in specific antimicrobial activity against niche competitors. The effect of *S. aureus* PSMs should be tested with various bacteria from different genera. This should provide more insight in the working mechanisms of the antimicrobial activity of PSM peptides.

Phenol-soluble modulins after phagocytosis

As discussed in the previous section, the ability of PSMs to recruit neutrophils may indicate that the immune cells are somehow beneficial for the bacteria. Although *S. aureus* are not considered to be intracellular pathogens, the bacteria are able to survive in several cell types. Cells incapable of phagocytosis, including endothelial cells, epithelial cells and fibroblasts, can be invaded by a bacterial surface-exposed fibronectin-binding protein which recognises host integrins[42]. Furthermore, *S. aureus* are able to survive, but not replicate, in the phagosomes of neutrophils and macrophages[32-34].

Mainly neutrophils play an essential role in *S. aureus* infections. As immune cells, neutrophils have a protective role, however, excessive numbers of

neutrophils correlate with an increased bacterial burden[35,43]. Thwaites and Gant proposed that *S. aureus* survival in neutrophils allows bacterial persistence in the bloodstream. Furthermore, if bacteria are phagocytosed and the neutrophils travel throughout the body, the immune cells may protect the bacteria from immune recognition and facilitate dissemination[35].

After *S. aureus* are taken up by the professional phagocytes, the bacteria reside in a phagosome. Here, the bacteria are exposed to antimicrobial components, including reactive oxygen species (ROS) and granular contents, such as lysozyme[1,44]. In order to survive after phagocytosis, *S. aureus* have developed mechanisms to evade these host defence mechanisms. The bacteria can interfere with the release of the granular contents and evade the effects of the antimicrobial substances[6]. But how would the bacteria escape from the phagosome to cause further infections?

Recently, it was shown that production of AIP in a confined space is enough to activate the *agr* quorum-sensing system[45]. This would indicate that when *S. aureus* are trapped in a phagosome, the concentration of AIP could cross the activation threshold and stimulate the production of various virulence factors, including PSMs. Furthermore, the finding that PSMs are inhibited in a blood environment supports the idea that PSMs play an important role in the phagosome. It was shown that neutrophil lysis and FPR2 activation induced by *S. aureus* PSMs are inhibited by human blood serum. Several serum lipoproteins are able to bind and neutralise the PSM peptides in the blood[25]. Therefore, PSMs can only exert their function in surroundings which lack serum lipoproteins, such as tissues and phagosomes. Thus, it is likely that PSMs are produced intraphagosomally.

The limitation of amino acids in a phagosome induces a stringent response in *S. aureus* bacteria, leading to increased *psm* transcription. This seems to be important for *S. aureus* survival. However, the exact effect of intraphagosomal PSM production is still unknown. There are three postulated consequences: (I) PSMs lead to the destruction of the phagosome, which allows the bacteria to escape. The bacteria remain in the cytosol to hide from the immune system. (II) The bacteria escape from the phagosome and lyse the neutrophil from the inside. (III) The PSMs induce necrosis of the phagocytes[23].

In literature there are some contradictory reports on which PSMs could be responsible for phagosomal escape. It was shown that in the phagosome of a neutrophil the PSMα promoter can be activated. This would suggest that PSMs may form pores in the phagosomal vesicle, which facilitates bacterial escape and destroys the neutrophil from the inside[25]. However, Giese *et al.* found that α-type PSMs, which are responsible for pore formation, are not essential for phagosomal escape. In contrast, the β-type PSMs and δ-toxin seem to mediate the escape from the phagosomes. This is in concert with the finding that phagosomal escape was not related to host cell death. However, it is also very striking because α-type PSMs are structurally similar to δ-toxin[46]. These contradictory findings indicate that there is still a lot to discover about the role of PSMs in the intracellular survival and escape of *S. aureus*.

Original role of phenol-soluble modulins in biofilms?

Besides intracellular hiding, *S. aureus* bacteria are also able to evade the immune system by the formation of biofilms. Biofilms are multicellular aggregates embedded in a matrix which consists of polysaccharides, extracellular DNA, structural and enzymatic proteins, and other environmental factors. The formation of biofilms has a large impact on the bacterial resistance to antibiotics and the immune system. There are two main mechanisms which contribute to the biofilm resistance: (I) the matrix capsule of the biofilm prevents the antibacterial component to reach its target, and (II) the physiology of the biofilm reduces the efficacy of antibiotics. *S. aureus* is a leading cause for biofilm-associated infections. Usually, there is only one *S. aureus* type/strain present in a biofilm. This can be explained by the specificity of the AIP of the *agr* quorum-sensing or possibly by the antimicrobial properties of the PSMs[20,40,47].

There are three steps in the biofilms life-cycle: attachment, maturation and detachment (Fig. 2). *S. aureus* attaches to human matrix proteins, including fibrinogen and fibronectin. In addition, *S. aureus* have the extraordinary ability to bind to plastic surfaces, such as indwelling medical devices[47]. This increases the infection risk for patients who are already susceptible.

After attachment, there is maturation of the biofilm. This stage is characterized by intracellular aggregation and biofilm structuring[47]. At particular sites in the biofilm, under the strict control of the *agr* quorum-sensing system, the bacterial cells start to produce PSMs[48-51]. The PSM peptides are involved in the structuring of the biofilm by influencing expansion, thickness and surface smoothness[50]. Periasamy *et al*. suggest that, despite the lower concentrations, β-type PSMs have the most pronounced impact on biofilm structuring. They hypothesise that by producing the non-cytolytic PSMβ peptides, the bacteria are able to structure the biofilms without causing a strong activation of the immune system. However, all the PSM types are expressed during biofilm formation. Furthermore, the total volume of the biofilm is hardly affected by expression of PSMβ peptides only[50]. Thus, even if PSMβ peptides are the major contributors to biofilm structuring, the other PSM types, except PSM-mec[14], are probably also required.

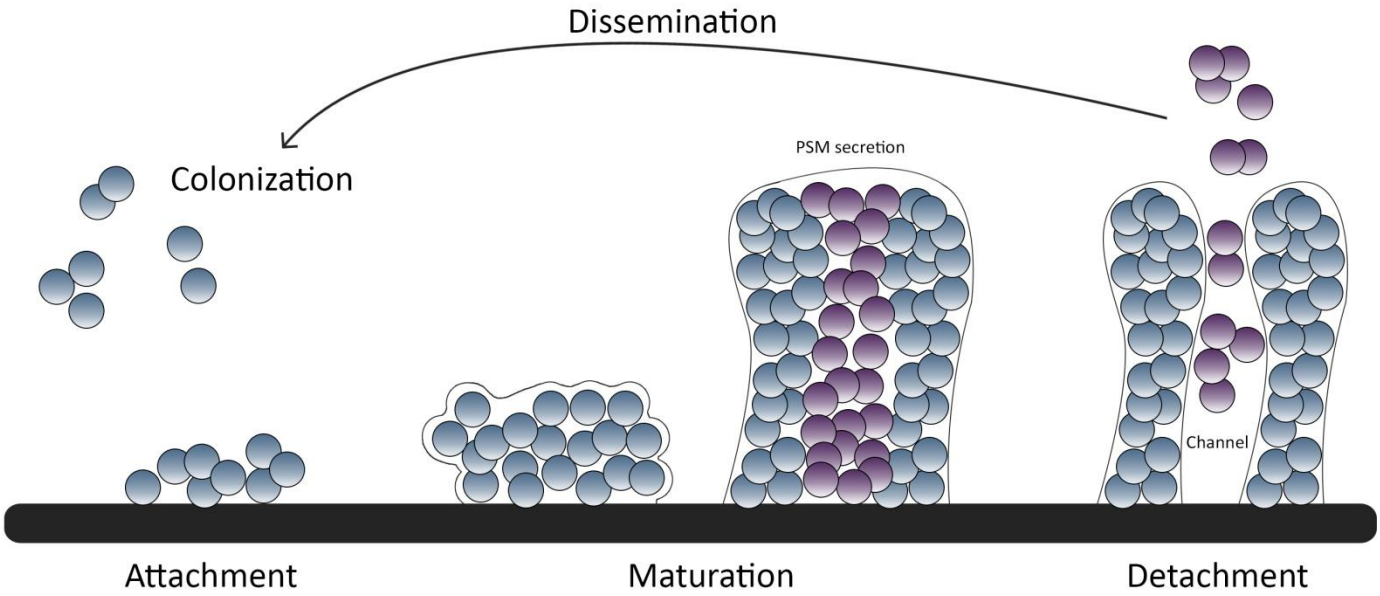

Figure 2: Phenol-soluble modulins involved in structuring and detachment of* S. aureus *biofilms. The formation of biofilms proceeds in three steps. First, there is attachment of the bacteria to a biotic or an abiotic surface. Subsequently, there is proliferation and maturation of the biofilm. Part of the bacterial cells start to secrete PSMs within the biofilm (purple cells). PSMs influence the thickness, surface smoothness and expansion of the biofilm. Furthermore, the surfactant-like features of the PSMs enable the peptides to disrupt cellular interactions. This loosens the biofilm agglomerations, resulting in the detachment of entire biofilm clusters. The detached bacterial cells can disseminate and form new colonies throughout the body. What is left of the original biofilm, are "tower" shaped structures with channels in between. The channels are essential to transport nutrients to the bacterial cells in the deeper layers of the biofilm.

In addition to their role in biofilm maturation, PSM peptides also play a role in the final stage of the biofilm life-cycle: detachment. The surfactant-like features of PSMs enable the peptides to disrupt cellular interactions within the biofilms, thereby loosening the sticky biofilm agglomerations[49,50,52]. If there is strong PSM production at a certain location, entire clusters of the biofilm may detach. This leaves the specific three-dimensional structure in the shape of a "mushroom" or "tower", which is characteristic for biofilms. The detachment of biofilm clusters provides the opportunity for the bacterial cells to spread and to colonise other sites. Furthermore, the detached cells leave a gap, which forms a channel to deliver nutrients to the bacterial cells in the deeper layers of the biofilm (Fig. 2)[47,48,50,53].

Under specific growth conditions, *S. aureus* produce fibers which contribute to the biofilm stability. These fibers possess some of the characteristics which are attributed to amyloid proteins, including relative SDS insolubility, binding to amyloid-specific dyes, a β-sheet structure and the ability to form highly stable polymerized aggregates. Biofilms which contain these amyloid-like fibers are more resistant to both matrix degrading enzymes and mechanical stress[31].

Schwartz *et al*. showed that the amyloid-like fibers in the *S. aureus* biofilms consist of PSMs. Contrary to the soluble PSMs, aggregation of PSM peptides into amyloid-like fibers contributes to the stability of the biofilm rather than causing disassembly. It is likely that the activity of the PSM peptides is altered when they aggregate into extracellular fibers. Furthermore, there is a change in the structure of the peptides; soluble PSMs have an α-helical structure, however, after aggregation, the peptides adopt a structure with more β-sheets[31]. It is still unclear which mechanism drives the ability of PSMs to switch from a monomeric to a fibril state. Schwartz *et al*. suggest that an interplay of several environmental cues, e.g. pH and osmolarity, determines the commitment of PSM peptides to the fibrillation pathway. They offer two hypotheses for a mechanism which results in the aggregation of PSMs to fibers: (I) the formation of PSM fibrils *in vivo* is synchronized by a nucleator protein which catalyses the formation of fibrils. (II) Fibril formation is related to deformylation of the N-terminus methionine. Although PSMs are secreted with a formylated methionine at the N-terminus the fibers contain primarily deformylated PSM peptides, indicating that deformylation might be linked to fibril formation[31].

Although PSM peptides seem to play a dual role, contributing both to the detachment and the stability, several studies have now shown that PSMs play an important role in the formation of biofilms[31,47,50]. Therefore, Periasamy *et al.* have hypothesised that the role of PSMs in the formation of biofilms is the "original function" of the peptides. This hypothesis is supported by the fact that virtually all staphylococci, which are all excellent colonisers of epithelial surfaces, produce PSMs. Furthermore, the PSMs are encoded in the bacterial core genome, which indicates the importance

of the peptides[53]. Because all the detaching cells produce PSMs, it would be very beneficial for these peptides to develop a function in immune evasion. This enhances the chances of dissemination and further colonisation.

Are phenol-soluble modulins essential for virulence?

As discussed in the previous sections, PSMs have proinflammatory and cytolytic effects. Furthermore, PSMs are involved in biofilm structuring and detachment, and the peptides allow phagosomal escape in the neutrophil (Fig. 3). This all indicates that PSMs are major contributors to CA-MRSA virulence. But are the peptides the crucial factor for CA-MRSA infections?

Many studies illustrate the importance of the production of PSM peptides during *S. aureus* infections. Firstly, the production of PSM peptides in CA-MRSA and HA-MRSA correlates to the severity of the infections these strains cause. Moreover, when the α-type PSMs are deleted, the CA-MRSA strains are severely attenuated in an animal infection model. Infections by these CA-MRSA deletion mutants are comparable with HA-MRSA strains[11]. Secondly, CA-MRSA strains are more capable of evading killing by human neutrophils than HA-MRSA. This effect is due to resistance to destruction and/or the production of factors which cause rapid host cell lysis, e.g. PSMs[6]. Thirdly, the biofilms do not develop normally when PSM peptides are absent[50]. Thus, the PSMs contribute to the development of an environment in which the bacteria can grow and cause severe infection. Finally, the PSMs are embedded in the core genome of the bacteria[11]. The high expression levels of the genome-encoded virulence factors rather than the acquisition of additional virulence genes on MGEs determines the level of invasiveness[54]. This is demonstrated by PSM-mec which does not significantly alter disease progression[14]. It seems that the hypervirulent phenotype of CA-MRSA is due to differential expression of molecules, such as PSMs, encoded by the core genome[9,54].

Even though PSMs have many different roles in *S. aureus* infections, it seems unlikely that an infection would depend solely on one factor. In nature there is always a balance: *S. aureus* strains would be extremely vulnerable, because if the PSMs do not work properly or if the host develops a way to neutralize this factor, it would be easy for the immune system to get rid of the bacteria. Nevertheless, many studies show that *S.*

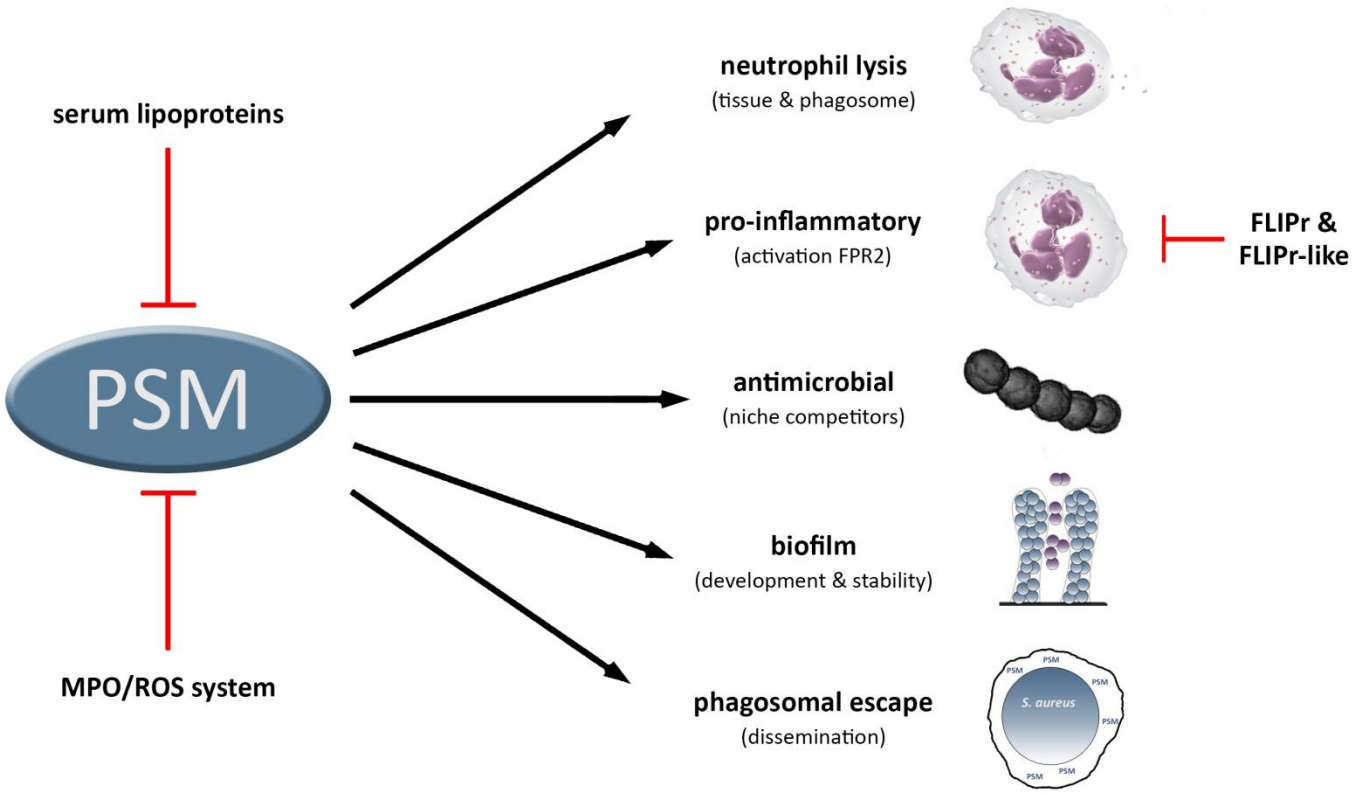

Figure 3: Overview of the effects and inhibitions of* S. aureus *phenol-soluble modulins[57]. PSMs have various effects during infection. Firstly, PSMα peptides can cause neutrophil lysis, probably by the formation of pores in the cell membrane. Secondly, PSMs can act as chemoattractants by activating FPR2 on neutrophils. Under some circumstances *S. aureus* produce FLIPr and FLIPr-like, which inhibit FPR2. Thirdly, it is suggested that PSMs have antimicrobial properties. However, the peptides are not effective on bacteria within the same species or genus. Fourthly, PSMs are involved in the maturation, detachment and stability of biofilms. Finally, PSMs allow escape from the neutrophilic phagosomes, which facilitates dissemination. The host also has mechanisms to inhibit the PSM peptides. In a blood environment, the serum lipoproteins bind and neutralize the PSMs. Furthermore, MPO and ROS, which are secreted from neutrophilic granules, can inhibit PSM peptides.

aureus produce various lytic factors, including PSMs, which can individually determine the outcome of an infection[55]. Therefore, PSMs are probably not THE crucial factor which leads to infection. However, the peptides might be key to tip the scale of the balance in favour of the bacteria.

Vaccination against phenol-soluble modulins

Many *S. aureus* strains have developed resistance against antibiotics. Therefore, there is a great need for new treatment methods, such as vaccination. Even though vaccination against *S. aureus* is thoroughly investigated, there is currently no U.S. Food and Drug Administration approved vaccine[56]. Nevertheless, the search for new treatments is still ongoing. Recently, a successful first-in-human Phase I clinical trial was published where a combined vaccine against *Candida albicans* and *S. aureus* was tested[57]. Furthermore, also antitoxins produced by bacteria themselves are investigated[58].

Despite the virulence and immune evasion capacity of CA-MRSA, not everyone is infected with these bacteria. This suggests that there are circumstances in which the immune system is capable of controlling invasive infections. However, there is no proof that lasting immunity against *S. aureus* exists, which is demonstrated by the fact that patients often have recurrent infections with the same strain. In addition, even though a vaccine might boost the immune system, biofilms might be able to protect the bacterial cells. Therefore, vaccination might not prevent *S. aureus* infections. However, a vaccine could be used to decrease the severity of the disease[55].

A vaccine could enhance the amount of (pre-existing) antibodies against the *S. aureus* bacteria itself. This would improve opsonisation and thus phagocytosis by neutrophils and other phagocytes. However, it has been shown that phagocytosis of CA-MRSA by neutrophils is very efficient[6]. Moreover, as described before, CA-MRSA have the ability to survive after phagocytosis and subsequently lyse the neutrophil. Therefore, a vaccine which enhances phagocytosis does not seem feasible[9].

In contrast, an antitoxin approach, where bacterial toxins are neutralized by antibodies, might be very efficient. Because of the importance of PSMs in CA-MRSA infections, neutralization of the peptides might decrease the severity of the disease. It has been shown that sepsis patients have significantly lower antibody levels against most toxins, including PSMα3. Furthermore, a previous *S. aureus* infection is protective against sepsis[55]. If a vaccine could create memory B-cells with antibodies against the various PSMs, the peptides can be neutralized quickly upon *S. aureus* infection. The pre-existing antibodies against the toxins may not prevent an infection, but it could tip the scale of the balance back to the host.

Concluding remarks

It is remarkable that PSMs are involved in many different processes during an infection (Fig. 3). Strikingly, in many of these processes PSM peptides seem to play a dual role. For example, the peptides are involved in the development and stability of the biofilm. Contrastingly, the PSMs also cause detachment of entire clusters from the biofilm, leading to the formation of fluid channels. Another example is the interplay between the bacteria and the immune system of the host. On one hand PSMs cause neutrophil lysis. On the other hand, the peptides are proinflammatory and attract neutrophils to the site of infection. However, MPO and ROS, which are released from the neutrophilic granules during infection, are able to inhibit the PSMs. In addition, *S. aureus* themselves can produce FLIPr and FLIPr-like, which are able to inhibit the inflammatory effects by binding to the FPR2 on neutrophils. This interplay between host and bacteria of activation and inhibition shows the immense complexity of an infection.

Although some processes are still unclear or questionable, such as endosomal escape or the effect of PSMs on microbes, there are many studies which underline the importance of PSMs in CA-MRSA infections. It is unlikely that the peptides are the only factor required for infections, but PSMs might push the virulence over a certain threshold, thereby allowing the bacteria to cause severe infections. Therefore, treatment, or at least a decrease in the severity, of *S. aureus* infections might be found in the interference with PSMs. If vaccination results in antibodies which neutralize the PSMs, the severity of the disease will most likely decrease. This is the first step towards prevention of *S. aureus* infections altogether.

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