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Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity*

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Abstract

Biofilm-associated infections are a significant cause of morbidity and death. Staphylococci, above all *Staphylococcus aureus* and *S. epidermidis*, are the most frequent causes of biofilm-associated infections on indwelling medical devices. Although the mechanistic basis for the agglomeration of staphylococcal cells in biofilms has been investigated in great detail, we lack understanding of the forces and molecular determinants behind the structuring of biofilms and the detachment of cellular clusters from biofilms. These processes are of key importance for the formation of vital biofilms in vivo with the capacity of bacterial dissemination to secondary sites of infection. Recent studies showed that the phenol-soluble modulins, surfactant peptides secreted by staphylococci in a quorum-sensing controlled fashion, structure biofilms in vitro and in vivo and lead to biofilm detachment with the in vivo consequence of bacterial dissemination. These findings substantiate that quorum sensing and surfactants have widespread importance for biofilm maturation processes in bacteria and establish a novel theory of the molecular determinants driving dissemination of biofilm-associated infection.

INTRODUCTION

The conditions that microbiologists use in the laboratory to grow bacteria—with optimal nutrient and oxygen availability—barely reflect what microorganisms encounter in nature. Many bacteria live under nutrient-limited conditions and under constant danger of being mechanically removed from food sources. To protect themselves from hostile environmental influences, bacteria often form surface-attached communities and are embedded in an extracellular matrix. In recent decades, these bacterial agglomerations have been described as "biofilms" (1). Biofilm formation is often observed in bacteria that colonize or infect humans or animals. For colonizing bacteria, such as those that live on our skin, the advantages of life in a biofilm-like mode are similar to those for bacteria that colonize abiotic surfaces: biofilms protect bacteria from being washed or scraped away, and they facilitate survival under conditions of dramatic environmental change (for example, in humidity or osmotic pressure). However, life on mammal skin also comes with additional problems for the bacteria: they are exposed to host defenses, which are meant to keep the colonizing bacterial flora in balance and avoid the overgrowth of harmful microorganisms. Bacteria that breach the epithelial barrier and infect the host encounter even more and stronger mechanisms of host defense. Biofilms provide significant protection from host defenses in both the colonizing and infectious states. They decrease the efficiency of antimicrobial peptides, a key part of epithelial innate host defense, and inhibit uptake and killing by phagocytes in human body fluids during infection. Furthermore, they provide resistance to antibiotics (2, 3).

Given the advantages that biofilms provide for bacterial survival in the host, it is not surprising that many bacterial infections involve biofilms (4). The most frequent biofilm-associated infections are nosocomial infections on indwelling medical devices, such as implanted catheters, artificial heart valves, or bone and joint prostheses. However, infective biofilms also occur in native valve endocarditis and wound infections. Dental plaque, present in virtually every human being, can also be regarded a biofilm infection. Many other infections are suspected to involve biofilms, such as lung infections in cystic fibrosis patients (5).

Molecular-level biofilm research has focused for a long time on *Pseudomonas aeruginosa*, the central bacterial pathogen involved with cystic fibrosis infections (6). However, there are also increasing recent efforts to understand the molecular basis of biofilm formation in staphylococci, which cause more frequent and more divergent biofilm-associated infections. Staphylococci play a role in wound infections, native valve endocarditis, and cystic fibrosis, and they are notorious for causing device-associated infections. In addition to *Staphylococcus aureus*, coagulase-negative staphylococci, most notably *S. epidermidis*, are frequently associated with such infections (7). These often result in dangerous bloodstream infections in the hospital, and staphylococci rank first among pathogens causing nosocomial bloodstream infections (8).

Despite the central role that biofilms play in human infections, most research on biofilm-forming pathogens has been performed using in vitro systems. This is probably because for a long time there were no appropriate in vivo models of *P. aeruginosa* lung infection. However, whether results from often extremely artificial in vitro systems can be applied to biofilm-associated infections is debatable. Molecular biofilm research in staphylococci has long been hampered by difficulties in genetically manipulating clinical staphylococcal strains. Recently developed genetic tools have made the genetic manipulation of staphylococci easier, and several studies have directly investigated molecular staphylococcal biofilm factors using gene-deletion mutants. Importantly, in vivo models of staphylococcal biofilm-associated infection are less problematic than those of *P. aeruginosa*. Thus, the recent combination of optimizing genetic manipulation of staphylococcal species and investigating specific molecular biofilm

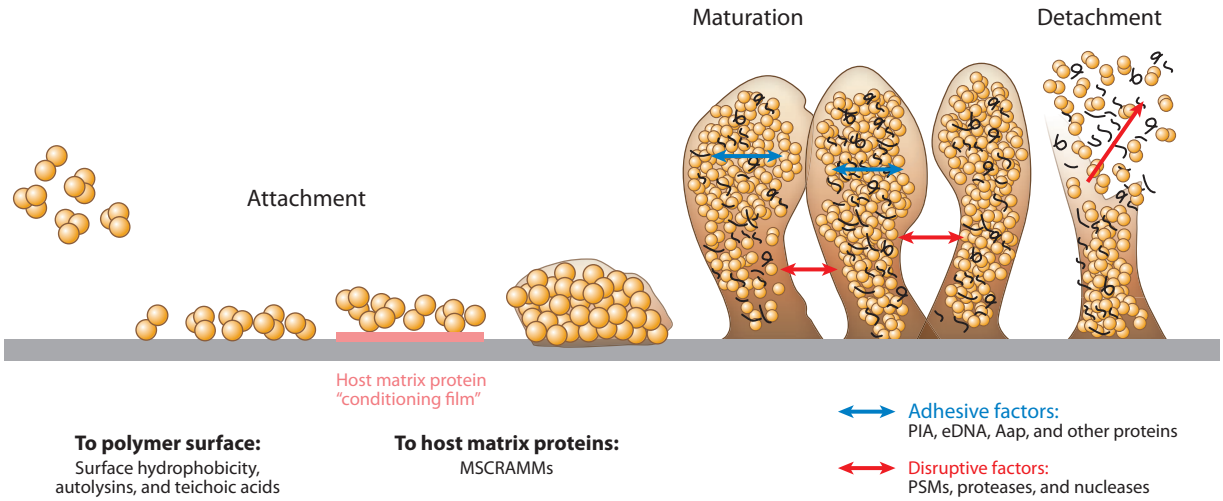

Figure 1

Phases of biofilm development. Biofilm development includes initial attachment, maturation, and final detachment. Attachment may occur directly to a surface (such as the polymeric surface of an indwelling medical device) or to a "conditioning film" formed by host matrix molecules. Then, biofilm maturation proceeds via the agglomeration of cells, which is dependent on adhesive molecules. Formation of the characteristic channel-containing biofilm structure is dependent on disruptive factors, which also ultimately facilitate the last phase of biofilm development, detachment. Molecular determinants shown or suspected to be involved in the respective biofilm development phases in staphylococci are noted on the bottom. Abbreviations: Aap, accumulation-associated protein; eDNA, extracellular DNA; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; PIA, polysaccharide intercellular adhesin; PSMs, phenol-soluble modulins.

determinants in vivo has given us important information on the molecular basis of biofilm development and biofilm-associated infection in these important human pathogens. In the following, I present an overview of molecular factors involved in staphylococcal biofilms in vitro and in vivo, with a focus on the most recent findings regarding biofilm maturation and detachment.

BIOFILM ATTACHMENT

The development of a bacterial biofilm can be divided into three phases, which involve specific molecular factors: attachment, maturation, and detachment (9) (**Figure 1**). Initial attachment can occur to abiotic or biotic surfaces. Attachment to an abiotic surface, such as the plastic surface of an indwelling medical device, is dependent on the physicochemical characteristics of the device and bacterial surfaces. This type of attachment is thus driven mostly by hydrophobic or electrostatic interactions. However, the involvement of specific bacterial surface molecules in this process, such as the surface protein autolysin or teichoic acids, has been described in staphylococci (10, 11). It is believed that those commonly abundant determinants alter the physicochemical properties of the bacterial surface rather than mediate attachment via specific, receptor-mediated interactions.

Attachment to a biotic surface such as human tissue is governed by entirely different, much more specific interactions. Staphylococci express a large variety of surface-anchored proteins that bind to host matrix proteins, collectively called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (12). These interactions are of vital importance for biofilm-associated infections on indwelling medical devices, as such devices become covered by host matrix proteins soon after insertion.

BIOFILM MATURATION

Adhesive Forces

PIA: polysaccharide intercellular adhesin

eDNA: extracellular DNA

Biofilm maturation comprises adhesive processes that link bacteria together during proliferation and disruptive processes that form channels in the biofilm structure (9). The latter are necessary for nutrients to reach cells in deeper biofilm layers. In addition to biofilm structuring, disruptive processes also ultimately cause the detachment of cell clusters from a biofilm, which controls biofilm expansion and has important consequences for in vivo biofilm infection, as it may lead to systemic dissemination. Much research has been performed on the genetics and physiology of adhesive biofilm factors.

In staphylococci, arguably the most important adhesive biofilm molecule is an exopolysaccharide named polysaccharide intercellular adhesin (PIA), also termed poly-N-acetyl glucosamine (PNAG) after its chemical composition (13). PIA is synthesized, exported, and modified by the products of the *ica* gene locus, comprising the *icaA*, *icaD*, *icaB*, and *icaC* genes (14). IcaA and IcaB form a membrane-located N-acetyl glucosamine transferase that adds N-acetyl glucosamine residues from activated N-acetyl glucosamine to the growing oligo-N-acetyl glucosamine chain (15). IcaC is assumed to represent the PIA exporter because the formation of longer chains requires the presence of this protein, which, according to sequence analysis, forms a membrane-spanning transporter (15). The surface-located IcaB de-N-acetylates some of the N-acetyl glucosamine residues (16). De-acetylation is important, as it introduces positive charges that are crucial for the PIA polymer to adhere to the bacterial surface.

The importance of PIA for in vitro and in vivo biofilm formation has been demonstrated in numerous studies (14, 17–19). It was long considered indispensable for staphylococcal biofilm formation, but more recent studies identified staphylococcal strains that are capable of in vitro and in vivo biofilm formation but do not have *ica* genes and thus do not produce PIA (20, 21). In these strains, specific proteins substitute for PIA in cell–cell adhesion. The growing list of such biofilm adhesive proteins comprises accumulation-associated protein (Aap) (22–24), extracellular matrix binding protein (Embp) (25), protein A (26), fibrinogen-binding proteins (FnbpA and FnbpB) (27), *S. aureus* surface protein G (SasG) (28), and others. The mechanisms by which these proteins contribute to cell–cell adhesion are under intense current investigation and may include the formation of large fibrils, such as in the case of Aap (29, 30).

Other polymers have also been implicated in staphylococcal biofilm formation. Teichoic acids are a characteristic component of Gram-positive cell surfaces. They were shown to contribute to *S. aureus* and *S. epidermidis* biofilm formation (11, 31). Most likely, they interact with other surface polymers via electrostatic interaction, thereby contributing to the complex network that forms the staphylococcal cell surface. DNA released from lysed bacteria, called extracellular DNA (eDNA), also forms part of that network (32). As in the case of teichoic acids, the negative charge of DNA may play a crucial role in interacting with other surface structures.

Notably, knowledge about the structural components of the extracellular biofilm matrix is crucial to understand the possible roles of biofilm structuring and detachment factors discussed in the next section, because enzymes that degrade these structures may have a function in biofilm maturation.

Disruptive Forces and Detachment

In contrast to extensive efforts undertaken for at least a decade to delineate biofilm adhesive factors, the investigation of biofilm disruptive processes has only recently been intensified. Interestingly, research on the regulatory principles behind biofilm maturation preceded the only very recently identified molecular determinants of biofilm disruptive processes. The Agr (accessory gene regulator) quorum-sensing system (see sidebar Quorum Sensing and Agr)

has been suspected to govern biofilm maturation since 2000, when Vuong et al. showed increased biofilm formation by isogenic *S. aureus agr* mutants (33). This finding, assumed to be a consequence of decreased expression of Agr-regulated biofilm detachment molecules, was later confirmed in *S. aureus* and *S. epidermidis* both in vitro and in vivo (34–36). More recently, waves of biofilm growth and detachment were linked to Agr by measuring *agr* expression in growing biofilms using fluorescent reporter constructs (37) and exogenous addition of the extracellular Agr-inducing signal (38). From these observations, a model was developed, in which *agr* expression in mostly outer layers of a biofilm leads to detachment, which is followed by regrowth (39). More defined recent data showed that *agr* is expressed not only on the surface but also in deeper layers. Most likely, expression of *agr* in different biofilm layers is required for the efficient formation of channels (34). However, we do not understand yet which signals or mechanisms lead to the nonuniform expression of *agr* that is necessary for biofilms to obtain their characteristic structure.

Ever since the Agr quorum-sensing system was identified as a primary regulator of biofilm maturation, there has been increased interest in defining the Agr-regulated effectors that directly promote the maturation processes (for an overview of biofilm maturation and detachment factors, see **Table 1**). Before I discuss the effectors of biofilm maturation, it should be noted that Agr does not control expression of most of the important biofilm adhesive molecules, such as PIA (33). This contrasts with many molecules involved in attachment, such as many surface proteins that are downregulated by Agr and thus expressed only during the initial attachment phase (40). Generally, the mere cessation of the production of biofilm adhesive molecules does not appear to contribute significantly to biofilm maturation.

One possible mechanism of biofilm maturation is the production of enzymes that degrade biofilm matrix components. As PIA has a central function in staphylococcal biofilm formation, it was assumed that a PIA-degrading enzyme (PIAse) may contribute to biofilm maturation. However, such an enzyme has never been found in staphylococci. Some other microorganisms also produce PIA, and an enzyme that serves as a PIAse, dispersin B, was described in the periodontal pathogen *Aggregatibacter actinomycetemcomitans*, in which it appears to facilitate detachment (41). The lack of a gene homologous to that encoding dispersin B in staphylococcal genomes underlines that staphylococci very likely do not have a PIAse.

It is now well accepted that specific proteins contribute to biofilm formation, at least in the subset of strains that do not produce PIA or in which biofilm formation appears to be largely independent of PIA (20). Proteases are thus candidates for biofilm maturation enzymes in such strains. Additionally, they may function as accessory facilitators of biofilm maturation in staphylococcal strains that form PIA-dependent biofilms. The first hint that proteases may facilitate detachment came from an experiment in which a broad-specificity protease often used in the laboratory, protease K, was added to biofilms of a strain in which

QUORUM SENSING AND AGR

Quorum sensing is a regulatory mechanism in microorganisms that controls gene expression in a cell-density-dependent manner. In staphylococci, the quorum-sensing system is called Agr (accessory gene regulator). It upregulates the expression of toxins and degradative exoenzymes, such as proteases, and downregulates the expression of many surface adhesion proteins upon entry in the stationary growth phase. The Agr system uses a peptide pheromone (autoinducing peptide, AIP), which, when a threshold concentration is reached at a certain cell density, binds to a membrane-located histidine kinase (AgrC). AgrC in turn activates a response regulator protein, AgrA. AgrA binds to two promoters, activating transcription of the Agr operon itself (auto-feedback) and that of RNAIII, the intracellular effector of the system. In addition, AgrA can directly activate expression of selected operons such as those encoding PSMs. Mutants in Agr have strongly decreased virulence in models of acute staphylococcal infection.

Table 1 Biofilm maturation and detachment determinants in staphylococci

Determinant	Species	Proposed function	In vitro relevance (references)	In vivo relevance[a] (references)
PSMs				
PSMα peptides	*S. aureus*	Disruption of noncovalent molecular interactions	(34)	(34)
PSMβ peptides	*S. aureus*	Disruption of noncovalent molecular interactions	(34)	(34)
δ-toxin	*S. aureus*	Disruption of noncovalent molecular interactions	(34)	(34)
PSMβ peptides	*S. epidermidis*	Disruption of noncovalent molecular interactions	(66)	(66)
Protease				
Aureolysin	*S. aureus*	Digestion of protein-based biofilm matrix by proteolytic activity	(38)	N.D.[b]
Nucleases				
Nuc1	*S. aureus*	Digestion of DNA-based biofilm matrix by nucleolytic activity	(46, 48)	(48) Not relevant
Nuc2	*S. aureus*	Digestion of DNA-based biofilm matrix by nucleolytic activity	(46, 48)	(48) Not relevant
Regulators				
CidA	*S. aureus*	Control of autolytic activity, which leads to eDNA release	(45, 49)	N.D.
LrgAB	*S. aureus*	Inhibitor of CidA-mediated lysis	(45, 49)	N.D.
Agr	*S. aureus* *S. epidermidis*	Control of PSMs and proteases	(33, 34) (35, 36)	(34) (36)
SarA	*S. aureus*	Control of proteases	(43)	N.D.

[a]As determined in animal models of biofilm-associated infection.
[b]Not determined.

biofilm formation is PIA-independent, and a reduction in biofilm mass was observed (38). In the same study, results from the investigation of several protease gene-deletion mutants gave inconsistent results. When Agr-mediated detachment was stimulated using the Agr autoinducing peptide (AIP), mutants in the secreted protease aureolysin showed increased biofilm formation, but mutants in the serine proteases V8 and SplABCDEF showed decreased biofilm formation. Thus, only the aureolysin mutant displayed the expected phenotype, whereas the other two mutants showed effects contradicting the model of protease-mediated quorum-sensing-dependent biofilm detachment. However, strangely, the V8 and SplABCDEF mutants had increased overall proteolytic activities, possibly explaining the unexpected outcome of the biofilm experiments (38).

Several other observations support a potential role of proteases in promoting staphylococcal biofilm detachment. First, in a transposon mutagenesis screen, several clones were isolated with increased biofilm formation that had mutations in protease genes (42). Second, exposure to protease inhibitors such as α-macroglobulin and phenylmethylsulfonyl fluoride (PMSF) led to thicker biofilms (42). Clearly, however, the results regarding the role of proteases in staphylococcal biofilm maturation are still quite preliminary and in part inconsistent. Furthermore, it needs to be stressed again that this mechanism was investigated only in the subset of staphylococcal strains that show PIA-independent biofilm formation. Moreover, the SarA global regulatory system appears to have a much stronger impact on extracellular protease production than Agr and a concomitant

impact on biofilm formation in the investigated protein-dependent biofilms (43). However, there is no clear evidence for a role of SarA in biofilm maturation processes or in promoting waves of biofilm detachment and regrowth, as there is for Agr. Finally, as discussed further below, there is no evidence for in vivo relevance of protease-mediated biofilm detachment.

Extracellular DNA is being recognized as a biofilm matrix constituent in a large number of bacteria. Enzymes degrading DNA, i.e., nucleases, can have biofilm matrix-degrading capacities and thus an impact on biofilm maturation. For example, human serum DNaseI degrades staphylococcal biofilms (44). Staphylococcal thermonuclease (*nuc2*) was shown to be involved in biofilm development, as a *nuc2* mutant formed a thicker biofilm than did the wild-type strain (45, 46). *S. aureus* encodes two nucleases (47), and the other nuclease gene, *nuc1*, showed a similar effect on biofilms, despite its DNA-degrading activity being much less pronounced (48). Release of DNA as a consequence of cell lysis is under the control of genes that govern what has been called "bacterial programmed cell death," such as *cidA* and *lrgAB* (45, 49). The matrices of biofilms in advanced stages with a large proportion of dead cells and accumulated eDNA may thus be degraded by secreted staphylococcal nucleases. However, staphylococcal nucleases are not regulated by Agr (46). Thus, it is hard to imagine how nucleases would contribute to the reported quorum-sensing-dependent structuring of biofilms in earlier stages. Finally, staphylococcal nucleases do not appear to have in vivo relevance for biofilm development (see below).

As an alternative to enzymatic degradation of biofilm matrix molecules, biofilm maturation may be accomplished by surfactants. This theory involves the disruption of noncovalent rather than covalent interactions by a mechanism that is probably much less target specific. There are important examples of surfactant molecules in other bacteria that promote biofilm development: surfactin in the soil bacterium *Bacillus subtilis* (50, 51) and rhamnolipid in the opportunistic pathogen *P. aeruginosa* (52, 53). Staphylococci have recently been shown to produce a family of amphipathic, α-helical peptides with surfactant properties, the phenol-soluble modulins (PSMs) (54, 55). Although PSMs are present in virtually all staphylococcal species, the PSM pattern and the amino acid sequences of PSMs vary greatly from species to species (56). All PSMs have been identified in *S. aureus* and *S. epidermidis* (**Figure 2**) (54, 55, 57–59). The *psm* genes form part of the core genome and are thus expressed by all strains of a species, albeit in varying degrees. The only exception is the PSM-mec peptide, which is encoded in the staphylococcal chromosome *mec* mobile genetic element carrying methicillin-resistance genes (59). PSMs have a variety of functions, which include proinflammatory capacities, such as the promotion of neutrophil chemotaxis, activation, and cytokine release (55, 60). PSM proinflammatory activities are receptor-mediated (60). Furthermore, several PSMs, most notably the PSMα peptides of *S. aureus* and the PSMδ peptide of *S. epidermidis*, efficiently lyse important human immune and other cell types such as neutrophils, monocytes, and erythrocytes (55, 61). Likely as a consequence of both proinflammatory and cytolytic capacities, deletion mutants in the *psmα* operon of *S. aureus* have strongly decreased virulence in mouse and rabbit sepsis and skin infection models (55, 62). Some PSM peptides also have antibacterial capacities and thus a potential role in bacterial interference (63, 64). Notably, expression of all PSMs is under exceptionally strict control by the Agr system, inasmuch as it is accomplished by direct binding of the AgrA response regulator protein to *psm* operon promoters (55, 57, 65). This control is more direct than that of many other Agr-regulated virulence determinants, which are controlled via a regulatory RNA. Most likely, control of PSMs by Agr preceded that of other factors during evolution.

PSM: phenol-soluble modulin

Staphylococcal δ-toxin, a long-known peptide toxin now classified as a member of the PSM family, was implicated already in 2000 as a potential mediator of Agr's impact on biofilm

Figure 2

Phenol-soluble modulins (PSMs) present in *Staphylococcus aureus* (Sa) and *Staphylococcus epidermidis* (Se). PSMs are a family of core genome–encoded peptides with surfactant properties secreted by staphylococci. PSM-mec, which is encoded on a mobile genetic element, is found in some methicillin-resistant strains of *S. aureus* and *S. epidermidis*. PSMs usually do not share significant amino acid sequence similarity, but all contain an amphipathic α-helix important for surfactant properties.

development (33). Discovery of the PSM family then prompted a more detailed investigation of the contribution of those peptides to biofilm maturation. The role of PSM peptides in biofilm maturation was first analyzed in *S. epidermidis* (66). That study focused on the PSMβ peptides, as these are produced at high amounts in *S. epidermidis*, especially in a biofilm mode of growth (58, 66). Furthermore, they lack the cytolytic properties of many of the α-type PSM peptides—suggesting they may have a role in other aspects of staphylococcal physiology, such as biofilm development (55, 61). An isogenic mutant of the *psmβ* operon in *S. epidermidis* developed a more compact and extended biofilm compared to the wild-type strain, very similar to that seen in an *agr* mutant of the same strain (66). These findings indicated that many of the Agr-dependent biofilm maturation processes in *S. epidermidis* are due to expression of PSMβ peptides. Notably, the PSM-based mechanism of biofilm maturation does not depend on the type of staphylococcal biofilm (PIA-dependent or -independent). This was confirmed using *S. epidermidis* strains with PIA-dependent and -independent biofilm formation.

Soon afterward, all of the PSM peptides produced by *S. aureus* underwent analysis regarding their contribution to biofilm maturation using isogenic gene-deletion mutants in the *psmα* and *psmβ* operons of the clinically important community-associated methicillin-resistant *S. aureus* USA300 and USA400 strains (67), as well as mutants in which production of δ-toxin was abolished (34). This analysis also included mutants in which all *psm* loci were deleted. The absence of one class of PSM peptides (PSMα, PSMβ) or of δ-toxin had a significant impact on biofilm volume, thickness, roughness, and channel formation, indicating that the presence of all PSM peptides is necessary for fully functioning biofilm development (34). Importantly, in that study inducible expression of PSM peptides led to biofilm detachment under flow conditions. Furthermore, the absence of the characteristic Agr-dependent waves of biofilm detachment and regrowth first described by Yarwood et al. (37) was attributed to PSM peptides using confocal

laser scanning microscopy. Thus, this analysis underscored the key importance of PSM peptides in staphylococcal biofilm maturation and detachment.

ROLE AND MOLECULAR BASIS OF IN VIVO BIOFILM DETACHMENT

The importance of biofilm detachment for infection resides in the dissemination of infection from the surface of an indwelling medical device to other sites via the lymph and bloodstream. Detached biofilm bacteria may establish secondary biofilm infections elsewhere, possibly with increased severity, such as for example endocarditis. Additionally, detached bacteria may cause acute, nonbiofilm infections, such as sepsis.

For biofilm-forming pathogens, the interpretation of results from in vitro models is problematic. Although in vitro models may closely reflect environmental biofilms, such as those forming in wastewater tubing, biofilm-associated infection is difficult to model adequately in vitro. Some adjustments may be made, such as coating microtiter assay plates with human matrix molecules or adding human serum, to get closer to the in vivo situation during infection. However, ultimately only animal models will allow conclusions as to whether mechanisms discovered in vitro bear relevance for pathogenic biofilm-forming bacteria.

Among the determinants of biofilm formation known in staphylococci, only some have been investigated in vivo. Most notably, despite the frequent depreciation of PIA as an important factor of staphylococcal biofilm formation (based on the discovery of a series of determinants that impact staphylococcal biofilm formation in vitro in a PIA-independent manner), PIA is still the only staphylococcal biofilm factor with substantial evidence for in vivo importance (16, 18, 19). For many surface proteins, including Aap, that are being discussed as potential contributors to staphylococcal biofilm-associated infection, evidence is limited to association analyses with origin from biofilm-associated infection. Also, many surface proteins with a suspected contribution to biofilm infection have other roles in pathogenesis, such as protein A or Fnbp, and thus it is difficult to discern a specific, biofilm-related role in infection in vivo. Similarly, there is no evidence for proteases functioning as biofilm maturation determinants in vivo, despite frequent statements about those enzymes having such a role.

In particular, the role frequently attributed to eDNA in biofilm formation of pathogenic bacteria may be limited to in vitro conditions; there are at least three reasons to doubt that eDNA contributes to biofilm formation in vivo. First, there is no direct evidence that it does, although admittedly such evidence would be hard to achieve given that it cannot be obtained by using isogenic-deletion mutants. Second, a potent DNAse in human serum, DNaseI, was shown to degrade staphylococcal biofilms in vitro (44); this makes it difficult to imagine how bacterial biofilms would grow in vivo if biofilm formation were dependent on the production of eDNA. Third, there is recent evidence that staphylococcal nucleases, which have a significant impact on in vitro biofilms, do not affect biofilm-associated infection (48). This suggests not only that nucleases do not facilitate biofilm maturation in staphylococci in vivo but also that the contribution of eDNA to staphylococcal biofilms may be largely an in vitro artifact. Rather, the in vivo task of staphylococcal nucleases during infection may be to destroy neutrophil extracellular traps (68), a recently discovered, still controversial means of innate host defense (69).

So far, PSMs are the only biofilm maturation determinants for which there is evidence of such a function in vivo. When catheter pieces coated with equal amounts of wild-type or isogenic *psmβ*-deletion mutant strains were inserted into mice, dissemination of bacteria to the lymph nodes and organs was observed almost exclusively in wild-type bacteria (66). Furthermore, anti-PSMβ antibodies prevented the spread of *S. epidermidis* from biofilms on catheters to other sites in vivo (66). In a control experiment, PSMβ peptides did not affect

the development of non-biofilm-associated infection. These findings indicated that PSMβ peptides in *S. epidermidis* promote the dissemination of biofilm-associated infection by an effect on biofilm detachment. In a subsequent mouse study performed using *S. aureus*, all PSM peptides were shown to contribute to systemic dissemination in vivo (34). However, establishment of second-site infection or delayed clearance from second sites may also be influenced by the fact that many PSMs function as cytolytic agents, reducing the efficacy of host defense mechanisms. Then again, PSMβ peptides of both *S. epidermidis* and *S. aureus* are barely cytolytic, and thus may potentially represent a PSM class specifically evolved for biofilm maturation and detachment.

QUORUM SENSING IN BIOFILM-ASSOCIATED INFECTION

The *S. aureus* PSM in vivo study also included a detailed assessment of the role of Agr in the dissemination of biofilm infection. It confirmed preliminary results that had shown an increased dissemination into catheter-surrounding tissue of wild-type *S. epidermidis* compared to an isogenic *agr*-deletion mutant strain (36). Specifically, the behavior of an *agr* mutant of *S. aureus* resembled that of a mutant in which all *psm* genes were deleted, inasmuch as dissemination to lymph nodes, the skin adjacent to the catheter, and organs was strongly decreased compared to the USA300 wild-type strain (34).

The in vitro and in vivo findings on the role of Agr in biofilm maturation and detachment also explain observations indicating an increased frequency of naturally occurring *agr* mutants that can be isolated from catheter infections (36, 70, 71). These isolates show increased capacity to develop compact, extended biofilms on catheters, but have apparently lost the ability to detach and disseminate, and may thus be interpreted as a dead end of infection. Accordingly, it has been shown that *S. aureus agr* mutants do not spread to other individuals (72). Finally, when *agr* mutants are isolated from a catheter-attached population of bacteria, it is not known whether *agr* mutants constitute the whole population or whether some wild-type bacteria are left that may outgrow again to form a structured biofilm with the capacity to disseminate.

CONCLUDING REMARKS

The Agr quorum-sensing system and the Agr-regulated PSMs have now been identified as key controllers and effectors, respectively, of biofilm structuring, detachment, and the dissemination of biofilm-associated infection in the two most important staphylococcal pathogens, *S. aureus* and *S. epidermidis*. Not only may these findings lead to the development of drugs or vaccines interfering with the dissemination of staphylococcal biofilm infection, but they also set a new paradigm for the role of quorum sensing and surfactants in bacterial biofilm infection in general. Although surfactants of varying chemical nature have been implicated in in vitro biofilm maturation processes in several bacteria, the new findings in staphylococci suggest a general role of such determinants in biofilm-associated disease caused by various bacterial pathogens, such as *P. aeruginosa*.

SUMMARY POINTS

1. Biofilm maturation is governed by adhesive and disruptive molecular determinants.
2. Adhesive biofilm determinants include exopolysaccharides, proteins, and extracellular DNA.

3. Disruptive biofilm determinants are vital for the formation of a structured biofilm, detachment of cell clusters from a biofilm, and the dissemination of biofilm-associated infection.
4. In staphylococci, PSMs, proteases, and nucleases promote biofilm structuring and detachment in vitro.
5. PSMs facilitate dissemination of staphylococcal biofilm-associated infection.
6. Quorum-sensing-dependent production of surfactant molecules appears to be a general mechanism of biofilm structuring and detachment in many bacteria.

DISCLOSURE STATEMENT

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LITERATURE CITED

1. Costerton JW, Lewandowski Z, Caldwell DE, et al. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* 49:711–45
2. Hoiby N, Bjarnsholt T, Givskov M, et al. 2010. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35:322–32
3. Otto M. 2006. Bacterial evasion of antimicrobial peptides by biofilm formation. *Curr. Top. Microbiol. Immunol.* 306:251–58
4. Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–22
5. Singh PK, Schaefer AL, Parsek MR, et al. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407:762–64
6. Hoiby N, Ciofu O, Bjarnsholt T. 2010. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol.* 5:1663–74
7. Uckay I, Pittet D, Vaudaux P, et al. 2009. Foreign body infections due to *Staphylococcus epidermidis*. *Ann. Med.* 41:109–19
8. 1999. National Nosocomial Infections Surveillance (NNIS) System report, data summary from January 1990–May 1999, issued June 1999. *Am. J. Infect. Control.* 27:520–32
9. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* 54:49–79
10. Heilmann C, Hussain M, Peters G, et al. 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol. Microbiol.* 24:1013–24
11. Gross M, Cramton SE, Gotz F, et al. 2001. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect. Immun.* 69:3423–26
12. Patti JM, Allen BL, McGavin MJ, et al. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu. Rev. Microbiol.* 48:585–617
13. Mack D, Fischer W, Krokotsch A, et al. 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J. Bacteriol.* 178:175–83

14. Heilmann C, Schweitzer O, Gerke C, et al. 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol. Microbiol.* 20:1083–91
15. Gerke C, Kraft A, Sussmuth R, et al. 1998. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J. Biol. Chem.* 273:18586–93
16. Vuong C, Kocianova S, Voyich JM, et al. 2004. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J. Biol. Chem.* 279:54881–86
17. Mack D, Haeder M, Siemssen N, et al. 1996. Association of biofilm production of coagulase-negative staphylococci with expression of a specific polysaccharide intercellular adhesin. *J. Infect. Dis.* 174:881–84
18. Rupp ME, Ulphani JS, Fey PD, et al. 1999. Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect. Immun.* 67:2627–32
19. Rupp ME, Ulphani JS, Fey PD, et al. 1999. Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. *Infect. Immun.* 67:2656–59
20. Rohde H, Burandt EC, Siemssen N, et al. 2007. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* 28:1711–20
21. Kogan G, Sadovskaya I, Chaignon P, et al. 2006. Biofilms of clinical strains of *Staphylococcus* that do not contain polysaccharide intercellular adhesin. *FEMS Microbiol. Lett.* 255:11–16
22. Hussain M, Herrmann M, von Eiff C, et al. 1997. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect. Immun.* 65:519–24
23. Conrady DG, Brescia CC, Horii K, et al. 2008. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. *Proc. Natl. Acad. Sci. USA* 105:19456–61
24. Rohde H, Burdelski C, Bartscht K, et al. 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol. Microbiol.* 55:1883–95
25. Christner M, Franke GC, Schommer NN, et al. 2010. The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. *Mol. Microbiol.* 75:187–207
26. Merino N, Toledo-Arana A, Vergara-Irigaray M, et al. 2009. Protein A–mediated multicellular behavior in *Staphylococcus aureus*. *J. Bacteriol.* 191:832–43
27. O'Neill E, Pozzi C, Houston P, et al. 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *J. Bacteriol.* 190:3835–50
28. Geoghegan JA, Corrigan RM, Gruszka DT, et al. 2010. Role of surface protein SasG in biofilm formation by *Staphylococcus aureus*. *J. Bacteriol.* 192:5663–73
29. Banner MA, Cunniffe JG, Macintosh RL, et al. 2007. Localized tufts of fibrils on *Staphylococcus epidermidis* NCTC 11047 are comprised of the accumulation-associated protein. *J. Bacteriol.* 189:2793–804
30. Gruszka DT, Wojdyla JA, Bingham RJ, et al. 2012. Staphylococcal biofilm-forming protein has a contiguous rod-like structure. *Proc. Natl. Acad. Sci. USA* 109:E1011–18
31. Sadovskaya I, Vinogradov E, Flahaut S, et al. 2005. Extracellular carbohydrate-containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. *Infect. Immun.* 73:3007–17
32. Whitchurch CB, Tolker-Nielsen T, Ragas PC, et al. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295:1487
33. Vuong C, Saenz HL, Gotz F, et al. 2000. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.* 182:1688–93
34. Periasamy S, Joo HS, Duong AC, et al. 2012. How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proc. Natl. Acad. Sci. USA* 109:1281–86
35. Vuong C, Gerke C, Somerville GA, et al. 2003. Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J. Infect. Dis.* 188:706–18
36. Vuong C, Kocianova S, Yao Y, et al. 2004. Increased colonization of indwelling medical devices by quorum-sensing mutants of *Staphylococcus epidermidis* in vivo. *J. Infect. Dis.* 190:1498–505

37. Yarwood JM, Bartels DJ, Volper EM, et al. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. *J. Bacteriol.* 186:1838–50
38. Boles BR, Horswill AR. 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog.* 4:e1000052
39. Yarwood JM, Schlievert PM. 2003. Quorum sensing in *Staphylococcus* infections. *J. Clin. Invest.* 112:1620–25
40. Recsei P, Kreiswirth B, O'Reilly M, et al. 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by agr. *Mol. Gen. Genet.* 202:58–61
41. Kaplan JB, Velliyagounder K, Ragunath C, et al. 2004. Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *J. Bacteriol.* 186:8213–20
42. Boles BR, Thoendel M, Roth AJ, et al. 2010. Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. *PLoS ONE* 5:e10146
43. Beenken KE, Mrak LN, Griffin LM, et al. 2010. Epistatic relationships between sarA and agr in *Staphylococcus aureus* biofilm formation. *PLoS ONE* 5:e10790
44. Kaplan JB, LoVetri K, Cardona ST, et al. 2012. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. *J. Antibiot.* (*Tokyo*) 65:73–77
45. Mann EE, Rice KC, Boles BR, et al. 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. *PLoS ONE* 4:e5822
46. Kiedrowski MR, Kavanaugh JS, Malone CL, et al. 2011. Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. *PLoS ONE* 6:e26714
47. Tang J, Zhou R, Shi X, et al. 2008. Two thermostable nucleases coexisted in *Staphylococcus aureus*: evidence from mutagenesis and in vitro expression. *FEMS Microbiol. Lett.* 284:176–83
48. Beenken KE, Spencer H, Griffin LM, et al. 2012. Impact of extracellular nuclease production on the biofilm phenotype of *Staphylococcus aureus* under in vitro and in vivo conditions. *Infect. Immun.* 80:1634–38
49. Rice KC, Mann EE, Endres JL, et al. 2007. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* 104:8113–18
50. Branda SS, Gonzalez-Pastor JE, Ben-Yehuda S, et al. 2001. Fruiting body formation by *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* 98:11621–26
51. Angelini TE, Roper M, Kolter R, et al. 2009. *Bacillus subtilis* spreads by surfing on waves of surfactant. *Proc. Natl. Acad. Sci. USA* 106:18109–13
52. Davey ME, Caiazza NC, O'Toole GA. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 185:1027–36
53. Boles BR, Thoendel M, Singh PK. 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol. Microbiol.* 57:1210–23
54. Mehlin C, Headley CM, Klebanoff SJ. 1999. An inflammatory polypeptide complex from *Staphylococcus epidermidis*: isolation and characterization. *J. Exp. Med.* 189:907–18
55. Wang R, Braughton KR, Kretschmer D, et al. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* 13:1510–14
56. Rautenberg M, Joo HS, Otto M, et al. 2011. Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence. *FASEB J.* 25:1254–63
57. Vuong C, Durr M, Carmody AB, et al. 2004. Regulated expression of pathogen-associated molecular pattern molecules in *Staphylococcus epidermidis*: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. *Cell Microbiol.* 6:753–59
58. Yao Y, Sturdevant DE, Otto M. 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *J. Infect. Dis.* 191:289–98
59. Queck SY, Khan BA, Wang R, et al. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. *PLoS Pathog.* 5:e1000533
60. Kretschmer D, Gleske AK, Rautenberg M, et al. 2010. Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. *Cell Host Microbe* 7:463–73

61. Cheung GY, Rigby K, Wang R, et al. 2010. *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLoS Pathog.* 6:e1001133
62. Kobayashi SD, Malachowa N, Whitney AR, et al. 2011. Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. *J. Infect. Dis.* 204:937–41
63. Cogen AL, Yamasaki K, Sanchez KM, et al. 2010. Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. *J. Invest. Dermatol.* 130:192–200
64. Joo HS, Cheung GY, Otto M. 2011. Antimicrobial activity of community-associated methicillin-resistant *Staphylococcus aureus* is caused by phenol-soluble modulin derivatives. *J. Biol. Chem.* 286:8933–40
65. Queck SY, Jameson-Lee M, Villaruz AE, et al. 2008. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol. Cell* 32:150–58
66. Wang R, Khan BA, Cheung GY, et al. 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. *J. Clin. Invest.* 121:238–48
67. Otto M. 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu. Rev. Microbiol.* 64:143–62
68. Berends ET, Horswill AR, Haste NM, et al. 2010. Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J. Innate Immun.* 2:576–86
69. Brinkmann V, Reichard U, Goosmann C, et al. 2004. Neutrophil extracellular traps kill bacteria. *Science* 303:1532–35
70. Traber KE, Lee E, Benson S, et al. 2008. agr function in clinical *Staphylococcus aureus* isolates. *Microbiology* 154:2265–74
71. Shopsin B, Drlica-Wagner A, Mathema B, et al. 2008. Prevalence of agr dysfunction among colonizing *Staphylococcus aureus* strains. *J. Infect. Dis.* 198:1171–74
72. Shopsin B, Eaton C, Wasserman GA, et al. 2010. Mutations in agr do not persist in natural populations of methicillin-resistant *Staphylococcus aureus*. *J. Infect. Dis.* 202:1593–99