Staphylococcal biofilms

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Over the last decades, biofilms have gained more and more recognition as the common mode of growth microorganisms adapt in nature (1). Furthermore, many types of human infection have been found to progress with the involvement of biofilms or originate from biofilm-associated primary infections (2). Second only to the Gram-negative *Pseudomonas aeruginosa*, staphylococci have been in the focus of biofilm researchers. As common colonizers of the human skin, staphylococci are the most frequent sources of biofilm infections on surgically implanted indwelling medical devices. These include serious infections, including endocarditis and prosthetic joint infections (PJIs), and may lead to life-threatening conditions, such as sepsis (3–5).

For the longest time, biofilm research even in pathogenic bacteria has been characterized predominantly by in-vitro investigation, from which – despite the obvious limitations - researchers attempted to extrapolate to biofilm-associated infection. More recently, biofilm research has begun to integrate in-vivo infection models to obtain a better understanding of the mechanisms governing biofilm-associated infection. Infection models for *P. aeruginosa* - the biofilm-forming pathogen most intensely studied by in-vitro research - are extremely challenging to establish and perform (6). Thus, due to the possibility to perform biofilm-associated infection models that reflect human biofilm infection, staphylococcal biofilm research has gained considerably in relative importance. As an important further prerequisite to perform mechanistic biofilm research, methods to genetically manipulate staphylococci are continuously being improved.

In this chapter, the role of staphylococcal biofilms in the commensal state and during infection, our current knowledge of the molecular basis of staphylococcal biofilm formation and its regulation, and the role of biofilm formation in vivo as an important means of immune evasion will be discussed. Finally, potential strategies to develop therapeutics to treat biofilm-associated infections by staphylococci will be presented. There will be a focus on *Staphylococcus aureus* and *Staphylococcus epidermidis*, as we know most about biofilms in these two species.

STAPHYLOCOCCAL BIOFILMS IN THEIR NATURAL HABITAT AND DURING INFECTION

Many different definitions have been used for biofilms. Most define biofilm as a microbial community of cells that is embedded in a matrix of extracellular polymeric substances that they have produced. Some researchers restrict the term "biofilm" to aggregates attached to a surface, which can be biotic or abiotic. For others, free-floating bacterial aggregates also

represent biofilms. Cells in a biofilm characteristically show a phenotype, with respect to metabolism, gene transcription, and protein production, that is different from that during planktonic growth. This has been shown for both *S. aureus* and *S. epidermidis* (7, 8).

Microbial aggregates on infected catheters or other indwelling medical devices obtained from patients resemble biofilms grown in in-vitro microtiter plates or biofilm reactors and there is no doubt that these fit the definition of a biofilm (9). Macro- or microscopic evaluation from wound infections also indicates that those infections, which often contain staphylococci among other pathogenic microbes, can be described as biofilm-associated (10). The situation becomes less clear when looking at the commensal colonization of the skin, nares or intestine, or disease-associated colonization, such as during atopic dermatitis. Some researchers would argue that the physiological situation of bacterial aggregates in those habitats may resemble that commonly found in biofilms, while others have raised doubt (11–13). Currently preventing a more detailed analysis of those potential "colonizing" biofilms is the lack of animal models that closely resemble human colonization. Therefore, in the following, only biofilms on devices and wound infections will be discussed.

Medical devices particularly prone to infection include contact lenses, peritoneal dialysis, urinary and central venous catheters (CVCs), endotracheal tubes, mechanical heart valves, pacemakers, and prosthetic joints (14). Staphylococci generally are the most frequent causes of infections on indwelling medical devices, with *S. aureus* and *S. epidermidis* representing the leading species (15). This is mostly due to their predominance in causing intravascular and prosthetic device-associated infections. However, on some specific devices, such as urinary catheters, other bacteria may dominate (16). Most commonly, indwelling medical devices are contaminated with the biofilm-forming bacteria during surgery. The source can be the skin or other colonized body sites of the patient or the health care personnel (14). In that regard, it is important that only ~ 20% of individuals are colonized by *S. aureus* – in contrast to many coagulase-negative staphylococci (CNS) including *S. epidermidis*, which are ubiquitous skin colonizers in humans (17, 18). Thus, the *S. aureus* carrier state of the patient is a risk factor for device infection, as it is for other types of *S. aureus* infection (19). Alternatively, already implanted devices can be infected via hematogenous seeding, although this is to be considered less frequent (20). Not surprisingly, immune- or otherwise compromised patients, such as AIDS patients, patients receiving immunosuppressive therapy, or premature newborns, are at increased risk of developing biofilm-associated infections on indwelling devices (14). These populations are also at increased risk regarding serious complications that may arise from infected devices. The most notable among those is blood infection (bacteremia), which may result in life-threatening sepsis. Central line (CVC)-associated bloodstream infections (CLABSIs) are among the most frequent and serious complications of medical device infection and cause thousands of deaths annually. In a 2004 study including 24,179 cases of nosocomial bloodstream infections (most of which stem from contaminated devices) in 49 US hospitals over a 7-year period from March 1995 through September 2002, CNS were responsible for 31%, and *S. aureus* for 20% of cases, far ahead any other microorganism (21).

Chronic wounds, such as chronic skin ulcers in diabetes patients, and acute burn wounds, are highly susceptible for infection by many pathogenic microorganisms. According to clinical

observation, the infecting organisms, which are usually more than one, present as a biofilm (22). The species most often found in wound infections is *S. aureus* (23, 24). From a microbiological point of view, the multispecies biofilms in wounds are much more difficult to understand than infections of indwelling medical devices, which usually present as a monospecies infections. Several studies claim that bacterial interactions exist in wounds between *S. aureus* and, for example *P. aeruginosa*, but there is no commonly accepted view of their exact nature and role (25).

THE MOLECULAR BASIS OF BIOFILM FORMATION IN STAPHYLOCOCCI

Attachment phase

Biofilm development has been extensively studied in vitro using many different setups and microorganisms. Commonly, it is described as a process of 3 main stages: attachment, proliferation/maturation, and detachment (dispersal) (26) (Fig. 1).

Attachment to a surface is considered the first stage of biofilm development, although in "free-floating" biofilms, this stage is not relevant. In contrast to motile organisms, which may reach a surface in an active fashion, staphylococci gain contact with a surface passively. Staphylococci may directly attach to the abiotic surface of indwelling medical devices, or other abiotic surfaces in the environment (27). The hydrophobic character is the main factor enabling attachment to the usually hydrophobic material surface. However, specific staphylococcal molecules also have been implicated in surface attachment. These include the *S. epidermidis* major autolysin AtlE and its *S. aureus* homologue Atl (28, 29) and wall teichoic acids (WTAs) in *S. aureus* (30). These two may be linked mechanistically due to the fact that WTAs impact binding of Atl to the cell surface, controlling cell separation (31). Of note, primary attachment is often measured merely as a short-term variation of the frequently used simple microtiter-based biofilm assay. Whether results represent true primary attachment or initial phases of proliferation cannot be determined clearly using that assay.

In vivo, direct interaction of staphylococcal cells with the abiotic surface is not considered relevant, as indwelling devices rapidly become covered by host matrix material, including fibronectin, fibrinogen, vitronectin, and other matrix molecules (27). Under those conditions, attachment to the device is facilitated by a series of specific staphylococcal surface proteins that interact with those human matrix proteins. The most important family of such surface-expressed staphylococcal binding proteins are the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). MSCRAMMs consist, from N- to C-terminus, of a signal peptide, a ligand-binding domain with characteristic repeat sequences, a cell wall-anchoring region, a membrane-spanning region, and a positively charged tail (32). At the C-terminus, the MSCRAMM contains a conserved LPXTG motif, which is covalently linked by the enzyme sortase to peptidoglycan (33). According to genome analysis, *S. aureus* has about 20 and *S. epidermidis* about 12 MSCRAMMs (34). Prominent members are the fibrinogen- and fibronectin proteins, which include clumping factors A and B (ClfA, ClfB), the serine/aspartate-rich (Sdr) protein family, and fibronectin-binding proteins A and B (FnBPA, FnBPB) (32). Non-covalently bound surface proteins may also mediate attachment. Among them are predominantly enzymes that have a primary

catalytic role, such as the Atl-type autolysins. Furthermore, there is a loosely defined group of non-covalently surface-attached proteins collectively called SERAMs (secretable expanded repertoire adhesive molecules), which bind to the surface by not yet characterized mechanisms and usually show broad specificity in human matrix protein binding (35). The extracellular adherence protein Eap (also called "MHC class II analogous protein Map) and the extracellular matrix and plasma binding protein Emp of *S. aureus* belong to this group. Finally, the giant 1.1 MDa Embp protein of *S. epidermidis* and its *S. aureus* homologue Ebh (extracellular matrix binding protein homologues) are non-covalently surface-bound fibronectin-binding proteins (36, 37).

Proliferation and matrix formation

During the second stage of biofilm development, the microcolonies that have formed after attachment grow by proliferation. Additionally, cells secrete polymeric molecules to form the biofilm matrix. Among all aspects of biofilm development, the polymeric molecules that form the biofilm matrix have received most attention. Biofilm matrix polymers are of divergent chemical nature and include polysaccharides, proteins, and teichoic acids. In addition to those actively secreted molecules, polymeric substances from dead cells are also believed to contribute to the biofilm matrix. The most notable among those is DNA, which after release from dying cells is known as extracellular DNA (eDNA).

In many biofilm-forming bacteria, exopolysaccharides represent the matrix components that are most specifically implicated with biofilm formation. Staphylococci produce one main biofilm exopolysaccharide, which is called polysaccharide intercellular adhesin (PIA), or, according to its chemical composition, poly-N-acetylglucosamine (PNAG) (38) (Fig. 2). In contrast to chitin, another important N-acetylglucosamine homopolymer found in nature, the N-acetylglucosaminyl moieties in PIA are β−1–6 linked. One characteristic feature that distinguishes PIA from most other staphylococcal surface molecules, and also from many other bacterial biofilm exopolysacharides, is its cationic character, which is due to the enzyme-catalyzed removal of ~ 15 – 20% of N-acetyl groups after secretion. This feature may be important for electrostatic interaction with other surface polymers, and thus the formation of the sticky extracellular biofilm matrix (38, 39). There appears to be no specific "anchor" for PIA on the cell surface, and it has been shown directly that this function is not accomplished by wall teichoic acids (40).

PIA biosynthesis is accomplished by the products of the *ica* (intercellular adhesion) gene locus, which comprises the *icaA*, *icaD*, *icaB*, and *icaC* genes (41). IcaA is the N-acteylglucosamine transferase, which together with IcaD has been shown to produce an N-acetylglucosamine oligomer (42). Chain growth is dependent on the presence of IcaC, a membrane protein, assumed to be the PIA exporter. IcaB, which is located on the bacterial surface, is the PIA deacetylase (39). Deacetylation is crucial for the retention of PIA on the surface and the various functions PIA fulfills in staphylococcal physiology, which in addition to biofilm formation comprise increased resistance to antimicrobial peptides (AMPs) and neutrophil phagocytosis (39). Upstream of the *ica* operon is the *icaR* gene, which is transcribed in the opposite direction of the *icaADBC* operon. IcaR works as a repressor of the *ica* operon (43).

Several studies have shown that biofilm formation in vitro or in vivo can be accomplished by *S. epidermidis* or *S. aureus* isolates that do not harbor the *ica* locus (44–46). Although, the extension, structuring, and robustness of PIA-negative biofilms appears to be lower than those formed by PIA, at least in *S. epidermidis* (47, 48). In *S. aureus* isolates, biofilm formation appears to be predominantly protein-dependent (49). Earlier studies reported differences between methicillin-resistant (MRSA)and –sensitive (MSSA) isolates in that regard, while a more recent study indicates that both MRSA and MSSA form almost exclusively protein-dependent biofilms (49, 50). In contrast, *S. epidermidis* more frequently relies on PIA to form biofilms, with an additional protein-mediated contribution (46). Replacing PIA in *ica*-negative isolates, or adding to its function in *ica*-positive isolates, are a series of macromolecules that include polymeric proteins and non-protein polymers. Among the many surface-located proteins implicated in staphylococcal biofilm formation, the *S. epidermidis* accumulation-associated protein (Aap) and its *S. aureus* homologue, SasG (51), stand out due their capacity to self-polymerize and form fibrils that interconnect cells (52–54) (Fig. 2). Aap is a 220-kD protein that is cleaved by the SepA protease to an active, mature form (55–57). At the N-terminus, the A domain of Aap contains repeats that may initiate biofilm formation in some strains and also bind to human corneocytes (58). It is followed by a lectin domain. These two domains are not present in mature Aap, which contains a large stretch of B repeats in front of a C-terminal proline/glycine-rich region and the cell wall-anchoring domain. The B repeats are responsible for self- and intercellular adhesion in a Zn^{2+}-dependent manner, assisted by cooperative folding (53, 59–61). Recently, the proline/glycine-rich region was shown to form an extended stalk that pushes the B repeat binding domain away from the cell surface, likely to enable better interaction with Aap from other cells, promoting biofilm formation (62). Notably, it has been shown that the homologous proteins SasG and Aap can interact, enabling interspecies biofilm connections (63). Several other surface proteins have also been implicated in intercellular adhesion, but in many of those cases it is difficult to distinguish between a surface/tissue attachment and genuine intercellular adhesion function. These proteins contain many surface binding proteins of the MSCRAMM family in addition to the biofilm-associated protein (Bap), found in *S. aureus* mastitis isolates, of which a homologue (Bhp) is present in *S. epidermidis* (64, 65). In support of a specific involvement in matrix formation, Bap has recently been reported to assemble into amyloid-like matrices to promote biofilm assembly (66), reminiscent of the function of Aap.

Non-protein extracellular polymers implicated in biofilm formation include teichoic acids and eDNA. Teichoic acids occur in two forms: wall teichoic acid (WTA), which is covalently linked to the cell wall, and lipoteichoic acid (LTA), which is surface-anchored via a lipid moiety that intercalates in the cytoplasmic membrane lipid bilayer (67). A specific contribution of the teichoic acid surface polymers to the biofilm matrix is likely, but has only been shown for WTA of *S. epidermidis* (68). Teichoic acids devoid of D-alanylation, which alters their net charge, show diminished biofilm formation in *S. aureus* (30). Teichoic acids are also implicated in receptor-mediated adhesion to the nasal epithelium (69). Finally, eDNA contributes to biofilm formation in vitro (70). Likely due the highly polymeric and anionic character of DNA, it is extremely "sticky" and thus, eDNA is prone to interact with

many other surface molecules, contributing to the formation of the extracellular biofilm matrix network.

Structuring and detachment

Biofilms do not grow as undifferentiated "bricks", but have a characteristic three-dimensional structure, which contains towers, often described as "mushroom"-like, as well as fluid-filled channels between those towers (26). The second (maturation) stage of biofilm development comprises disruptive forces to create those structures in addition to the adhesive mechanisms described above. These are mediated by enzymes that degrade biofilm polymers, such as nucleases and proteases, and surfactant-like molecules, like the staphylococcal phenol-soluble modulins (PSMs), which disrupt non-covalent interactions (71, 72). The same forces can also ultimately cause biofilm detachment, which facilitates systemic dissemination and subsequent complications during device-associated infection. Both biofilm structuring and detachment/dispersal effects have been described for the PSMs, while research on degradative enzymes has been mostly limited to detachment/dispersal.

PSMs are a family of alpha-helical peptides with pronounced amphipathy, thus exhibiting characteristics that give them surfactant-like properties (73). Members of the PSM family are produced in *S. aureus*, *S. epidermidis*, and most likely virtually all staphylococci (73, 74). PSMs can be grouped in α-type peptides (~20–25 amino acids in length), which in *S. aureus* comprise the PSMα and δ-toxin peptides, and the longer β-type PSMs (~44–45 amino acids) (73). Confocal-laser scanning microscopy, a method often used to visualize biofilm structure, revealed that the characteristic channel-containing biofilm structure is not present in *psm* deletion mutants, which grow in an undifferentiated and extended fashion (48, 75). In contrast to biofilm polymer-degrading enzymes, PSMs structure PIA -dependent and –independent biofilms (48). PSMs work on growing biofilms; when biofilms are formed, external addition of PSMs has only limited biofilm-dispersing activity. Somewhat contrastingly, PSMs have also been described to form amyloid structures that prevent the enzymatic degradation of pre-formed biofilms (76). A double mutant in the *psm*α and *psm*β loci was impaired in preventing degradation, while the δ-toxin was not specifically addressed in that study. While there appears to be agreement on the in-vitro capacity of PSMs to form amyloid-like structures, the role of PSM amyloids in biofilms remains controversial (77).

Among biofilm-degrading enzymes, proteases are the most important (72), while enzymes degrading PIA are not produced by staphylococci. *S. aureus* and *S. epidermidis* produce a series of exoproteases. *S. aureus* secretes ten proteases, including seven serine proteases (SspA and SplA-F), two cysteine proteases (SspB and ScpA), and one metalloprotease (Aur) (78). *S. epidermidis* produces at least three major proteases, a homologue of the staphopain cysteine protease SspB, a metalloprotease called SepA, and a homologue of the *S. aureus* SspA (V8) serine protease (17). Mutations in specific *S. aureus* protease genes, or addition of protease inhibitors, impair biofilm formation or lead to biofilm degradation, respectively, in biofilms that are protein-dependent (49, 79). While specific matrix protein targets of several of the *S. aureus* proteases have been identified (80, 81), they overall show relatively low target sequence specificity (82). The *S. epidermidis* protease SspA (Esp) has been

shown to be able to degrade protein-dependent *S. aureus* biofilms (83). As biofilm formation in *S. epidermidis* is often strongly PIA-dependent, research on the role of *S. epidermidis* proteases in biofilm development has been limited.

The biofilm matrix component eDNA is subject to degradation by nucleases, which originally has been shown using human DNase I (70), which is abundant in human serum. Both *S. aureus* and *S. epidermidis* secrete nuclease activity. *S. aureus* is known to produce two secreted nucleases, Nuc1 and Nuc2 (84). Expression of Nuc1 reduces biofilms and a *nuc1* mutation in strain USA300 shows increased in-vitro biofilm formation (85), as would be expected from abolished eDNA degradation. However, isogenic *nuc1* or *nuc2*, or combined *nuc12* mutants in a different strain (UAMS-1) did not exhibit enhanced in-vitro or, notably, in-vivo biofilm formation (86).

REGULATION OF STAPHYLOCOCCAL BIOFILM FORMATION

Biofilms have been recognized as the common form of microbial growth. It is thus not surprising that many regulators that adapt staphylococcal gene expression to changing environmental conditions have an impact on biofilm formation. Here, there will be a focus on regulatory effects on the main biofilm-forming factors and on the biofilm effects of the major global regulators, the quorum-sensing system Agr, the Sar family of regulators, and the alternative sigma factor, SigB.

In 1998, Davies et al. reported that a quorum-sensing regulator in *P. aeruginosa* positively impacted biofilm formation (87), a result soon generalized regarding the relationship of quorum-sensing control and biofilm formation in bacteria. Soon after, Vuong et al. reported increased biofilm formation in isogenic mutants of the Agr quorum-sensing system in *S. aureus* (88), in clear contrast to the generalized *P. aeruginosa* finding. Ever since, mechanistic investigation has shed more light on the impact quorum-sensing systems have on biofilm formation in several different bacteria. As a common theme, quorum-sensing systems appear to frequently control biofilm structuring processes, which may result in more or less extended biofilms depending on the specific conditions (71). Agr in staphylococci controls two main biofilm structuring and dispersal factors described above, PSMs and proteases, with the control of *psm* genes being exceptionally direct and tight (8, 89, 90). As a result, biofilms of *agr* mutants grow in an unstructured and extended fashion, as shown for both *S. aureus* and *S. epidermidis* (75, 91), and applying the extracellular autoinducing peptide (AIP) signal of the Agr quorum-sensing system results in biofilm dispersal (79). However, it remains poorly understood which mechanisms lead to the spatial and temporal heterogeneity of Agr expression that is observed in biofilms (75) and which are a theoretical prerequisite for the Agr-mediated biofilm structuring effect. Finally, the autoinducer-2 quorum-sensing system, which remains less well investigated in staphylococci, represses biofilm formation in an *ica*-dependent manner (92, 93). Both quorum-sensing systems thus affect biofilm formation negatively, but using different mechanisms.

The prototype of the Sar paralogue family of regulators, SarA, impacts biofilm formation via regulation of Agr, as well as in an Agr-independent manner (94–97). The strong regulatory impact of SarA on protease production appears to play a preeminent role in this effect (98),

and this, historically, was how the biofilm effect of *S. aureus* proteases was discovered. Furthermore, SarA positively controls *ica* transcription in both *S. aureus* and *S. epidermidis* (95, 97), while its paralogue SarZ appears to have opposite effects in the two species – positive in *S. epidermidis* and negative in *S. aureus* (99, 100). Generally, which environmental signals stimulate Sar expression is not known, and therefore the biological context of biofilm regulation by Sar paralogues remains poorly defined.

Sigma factors are transcription initiation factors that provide promoter recognition specificity to RNA polymerase (101). The "alternative" sigma factor SigB is expressed during stationary growth phase and environmental stress and is involved in controlling manifold aspects of staphylococcal physiology and virulence. SigB regulation of biofilm formation in *S. epidermidis* biofilms was reported to occur by an impact on *ica* transcription via SigB dependence of the *icaR* promoter (102), and in *S. aureus* possibly via SarA-dependent protease regulation (103, 104). However, other studies found no impact of SigB on biofilm formation in *S. aureus* and *S. epidermidis* (97, 105).

IN VIVO ROLES OF STAPHYLOCOCCAL BIOFILM FACTORS

The recognition that microbes grow in biofilms in nature as well as during many types of infection has revolutionized microbiology in the second half of the previous century. However, in comparison to the wealth of in-vitro biofilm studies, biofilm microbiology has not yet widely included in-vivo experimentation to understand mechanisms of biofilm-associated infection and determine whether general principles established in-vitro bear in-vivo relevance. In part, this is due to the problem that animal models for biofilm-associated lung infection, by most biofilm researchers' favorite model organism, *P. aeruginosa*, are extremely challenging. The situation is somewhat better for staphylococcal biofilm-associated infection, as catheter infections can quite easily be mimicked by placing a plastic device under the skin of test animals, such as mice or rabbits. Furthermore, more sophisticated models of biofilm-associated staphylococcal endocarditis are available.

Staphylococcal biofilm infection has originally been associated mainly with *S. epidermidis*. Biochemical characterization of PIA and genetic approaches to find biofilm-related factors using transposon mutagenesis of *S. epidermidis* then resulted in the identification of the *ica* and *atlE* loci as important for matrix formation or initial adherence, respectively, during in-vitro biofilm formation (29, 41). For that reason, these were the factors first assayed in animal models of biofilm-associated infection. An isogenic transposon mutant in the *ica* genes (*S. epidermidis* M10) was tested for the impact of PIA production on infection outcome in a mouse model of device-associated infection, using a subcutaneously implanted catheter, and a rat CVC-associated infection model (106, 107). In the mouse subcutaneous catheter model, the wild-type produced significantly more biofilm on the catheter and a more pronounced abscess (106). In the rat model, more animals challenged with the wild-type strain developed CVC-associated infection than did those challenged with the *ica*-negative M10 strain (107). The impact of PIA and AtlE were also assayed in a different strain background in the rat CVC-associated model. Both PIA and AtlE had a significant impact on biofilm formation on the device as well as on associated bacteremia (108). Interestingly, later it was also shown that the deacetylation of PIA by IcaB is crucial for the

development of biofilm-associated infection using a mouse subcutaneous catheter model (39). As for *S. aureus*, there is no direct evidence from animal infection models suggesting a key role of PIA in biofilm-associated infection. Furthermore, one study showed that the *S. aureus* homologue of AtlE, the major autolysin Atl, does not impact biofilm-associated infection (109). These results reflect in-vitro findings indicating that biofilm formation in *S. aureus* is mainly dependent on other factors.

Biofilm formation in *S. aureus* is believed to depend on surface proteins in a majority of strains. However, only for very few surface proteins, has an impact on biofilm-associated infection been directly demonstrated using biofilm-associated infection models and deletion mutants. These include *S. aureus* Bap and protein A (Spa), for which a significant impact on pathogenesis in mouse catheter implant infection models was found (64, 110). For some other surface proteins, there is some circumstantial evidence for roles in in-vivo biofilm formation that is derived from using antibodies. For example, human IgG recognizing ClfA was protective in a rabbit endocarditis model (111). As for *S. epidermidis*, the difficulty in producing isogenic deletion mutants has hampered research along those lines, but circumstantial evidence obtained by heterologous expression has been obtained indicating a role of the surface protein SdrF, which is produced by a subset of *S. epidermidis* strains (112), in the initiation of experimental device driveline-related infection in mice (113). In general, the paucity of experimental in-vivo evidence underlining the importance of surface proteins in biofilm-associated infection may be due to their pronounced functional redundancy. More recently, an ex-vivo approach was taken to investigate biofilm formation during PJI. Dastgheyb et al. used synovial fluid obtained during surgery from uninfected human joints. Using the Nebraska transposon bank, which contains a copy of every non-essential gene in *S. aureus*, they identified ClfA, ClfB, FnBPA and FnBPB, as well as characteristically low Agr activity and PSM concentration in synovial fluid as driving forces of the extensive formation of antibiotic-resistant biofilms seen in PJI (114–116).

In-vivo information on biofilm structuring factors is available for the PSMβ peptides of *S. epidermidis*, which have been shown to drive dispersal and systemic dissemination from an implanted device in a mouse model (48). Mutants in the *psmα*, *psmβ*, or *hld* (encoding δ-toxin, now classified as a PSM) loci of *S. aureus* show the same effect, with that of a triple *psmαβhld* mutant being comparable to that of an *agr* mutant (75). However, at least in the case of α-type PSMs (PSMα peptides, δ-toxin), these outcomes may be influenced also by the cytolysis-mediated role of PSMs in immune evasion (117). Which role proteases play in biofilm-associated infection remains largely undefined. With proteases presumably having multiple roles in infection, their specific role in in-vivo biofilms is difficult to define. In a recent study using the USA300 strain LAC, a deletion mutant in all ten protease genes did not reveal a significantly changed bacterial load on the device in experimental implanted device infection in mice (118). Similarly, *S. aureus* *nuc1* and *nuc2* mutants do not have a significant in-vivo effect during biofilm-associated infection (86), casting some doubt also on the role of eDNA in in-vivo biofilms.

STAPHYLOCOCCAL BIOFILM FORMATION AS AN IMMUNE EVASION AND ANTIBIOTIC RESISTANCE MECHANISM

In-vivo biofilm formation is subject to several factors that are specifically due to the in-vivo environment, such as, most notably, interaction with innate host defenses, in addition to interaction with antibiotics during antibiotic therapy. Innate host defenses consist primarily of phagocytes, such as neutrophils and macrophages, and antimicrobial peptides (AMPs), which are a part of the intracellular killing mechanisms phagocytes employ (119). AMPs are also produced in the skin, where they are secreted to control skin-colonizing or –infecting bacteria (120). Secreted AMPs may thus also form part of the host's defenses on subcutaneously placed catheters (Fig. 3).

Biofilm formation per se has long been known to provide resistance to attacks by phagocytes, which are impaired in penetrating through the biofilm matrix (121, 122). Similarly, the biofilm matrix may be impermeable to certain AMPs and antibiotics. On the other hand, some can readily penetrate, which is likely dependent on the AMP's or antibiotic's chemical features (123). It has been estimated that biofilms provide 10 – 1000 times increased tolerance to antibiotic effects (124). One antibiotic that has been shown to be exceptionally active against biofilms and is often proposed to treat biofilm infections is rifampicin (rifampin) (125). However, resistance to rifampicin can develop quickly during treatment (126). Interestingly, recent in-vivo research indicates that specific other antibiotics, such as ceftaroline, are more active against *S. aureus* biofilms than those commonly used in the clinical setting (127). Decreased anti-biofilm efficacy of antibiotics whose penetration is not decreased is due the specific physiology of cells in a biofilm. For example, the reduced proliferative and metabolic activity of biofilm cells makes them more tolerant to antibiotics that target those processes, such as cell wall-targeting antibiotics (e.g., beta-lactams) or antibiotics that target DNA or protein synthesis (128). Finally, biofilms have been shown to contain more so-called "persisters", dormant cells which are indifferent to antibiotics, as compared to the majority of the bacterial population, an effect that enables this subset of cells to withstand antibiotic concentrations far above the MIC (129).

In addition to those general effects, staphylococcal biofilms show specific alterations in physiology, as compared to planktonic cells, that dampen inflammation and the innate immune response. First, Agr as a regulator of many toxins, which have pro-inflammatory effects, shows limited expression in biofilms (75, 91). However, whether this effect has in-vivo consequences remains to be shown directly. In support of that notion, many *S. aureus* and *S. epidermidis* isolates obtained from chronic and device-associated infections are functionally Agr-negative (91, 130). It appears as if phenotypically or genotypically Agr-negative isolates have selective advantages during chronic infection, of which the mechanistic underpinnings still need to be elucidated.

Second, *S. aureus* biofilms have been shown to reduce inflammatory effects, such as phagocyte influx. Specifically, *S. aureus* biofilms in murine biofilm-associated infection appear to skew the immune response away from a microbicidal phenotype by circumventing locus specific pathogen recognition pathways (131) – a finding in line with reduced Agr activity. Furthermore, during experimental PJI, *S. aureus* biofilms stimulate the influx of

myeloid-derived suppressor cells (MDSCs), resulting in reduced phagocyte influx (132), and human PJIs are associated with MDSCs (133). Finally, cyclic di-AMP, released by cell lysis, has been shown to stimulate a type I interferon-mediated anti-inflammatory effect in biofilms (134). However, it is not clear whether these immune evasion mechanisms are specific to a biofilm infection as opposed to other *S. aureus* infection types, or, in the case of cyclic di-AMP release, occur in vivo. Somewhat in contrast, it has also been shown that in a long-lasting murine biofilm infection, downregulated responses in regulatory T-cells (Tregs) during later stages of the infection provide for an unrestrained inflammatory response (135).

Some prominent biofilm matrix molecules have been described to promote immune evasion mechanisms independently of their role in matrix formation. PIA in its de-acetylated form provides resistance to neutrophil phagocytosis and killing by AMPs (39). Possibly, this activity is responsible for the effects PIA has shown in non-biofilm associated experimental infection (136). The *S. aureus* Bap protein, in addition to promoting adhesion, prevents *S. aureus* internalization by epithelial cells (137). This contrasts with fibronectin-binding proteins, which have long been known to facilitate such internalization (138). Which role internalization and potential intercellular persistence in non-professional phagocytic cells has in biofilm-associated infection still needs to be investigated in more depth. There is evidence indicating that the formation of small-colony variants (SCVs), cells with severely reduced metabolism, contributes considerably to such persistence (139).

THERAPEUTIC STRATEGIES FOR STAPHYLOCOCCAL BIOFILM-ASSOCIATED INFECTIONS

Biofilm infections remain extremely resistant to antibiotic therapy. Consequently, there are currently no alternatives, and excision and replacement of an infected device are often the only remedy. In the following, some strategies to develop alternative biofilm therapeutics will be presented. Most of them are still in the pre-clinical stage.

Antimicrobial peptides

With most antibiotics not being efficacious against biofilm cells due to their mode of action that only targets active cell processes, one strategy focuses on developing bactericidal antibacterial compounds. In the center of this approach are AMPs, most of which work by forming pores in the bacterial membrane (140). However, AMPs are subject to a series of resistance mechanisms that are based on the barrier function of the biofilm matrix in addition to more specific mechanisms, such as efflux pumps and AMP repulsion. Furthermore, staphylococci have learned to sense the presence of AMPs during their co-evolution with humans and react with efficient countermeasures (141, 142).

Material alterations

Many attempts have been made to deal with the adhesion of bacteria to medical devices by altering the surface so as to lower adhesive features, or by coating the device surface with antibacterial compounds (143). The latter mostly comprise antibacterial metals, such as silver or copper, in addition to antibiotics or AMPs. Such optimized devices, mainly catheters, are in clinical use and have had some success; however, biofilm formation still

happens and it appears impossible to completely inhibit adhesion by altering device surface composition. Unfortunately, once adhesion is accomplished, altered device surfaces do not impact further biofilm growth. One reason for the limited success of altering device surfaces is the abovementioned fact that they are prone to being covered by human matrix proteins, to which bacteria attach independently of the device surface.

Vaccines

So far, there is no working vaccine for *S. aureus*, even for non-biofilm associated infections; and many have argued that development of an *S. aureus* vaccine is inherently difficult (144). It has been proposed to design vaccine approaches for biofilm infections depending on which antigens are present in the biofilm mode of growth (145), but no such attempts have been actively pursued. There appears to be some pre-clinical success with anti-PIA antibodies, as shown in an *S. aureus* periprosthetic osteomyelitis rat model (146). As for *S. epidermidis*, there has been success using immunization with the surface protein SesC in a subcutaneous foreign body in a rat model (147). However, vaccination against *S. epidermidis* as a beneficial part of the skin microbiota is generally debatable. Furthermore, the many positive results from anti-staphylococcal vaccine experiments in animals have so far never resulted in a vaccine that works in humans.

Bacteriophage and lysins

Bacteriophage therapy is controversial for several reasons, but it has the advantage of having a bactericidal mechanism, and thus not being subject to the efficacy-lowering bacterial physiology of biofilms or persister cells (148). Then again, cell surface-located bacteriophage receptor molecules may not be accessible in the biofilm matrix. However, in several cases bacteriophages have proven efficacious against in-vitro staphylococcal biofilms. For example, bacteriophages ϕIPLA-RODI and ϕIPLA-C1C reduced biofilms of *S. aureus* and *S. epidermidis* in vitro (149). Bacteriophage K is also often proposed as an anti-staphylococcal biofilm agent (150, 151). Furthermore, bacteriophage lysins may represent a means to enzymatically disrupt biofilms, such as bacteriophage lysin CF-301 (152, 153). In-vivo efficacy of bacteriophages or bacteriophage lysins against *Staphylococcus* biofilm-associated infection still needs to be demonstrated.

Biofilm-degrading enzymes

The idea to enzymatically disrupt staphylococci in biofilms for treatment is not limited to bacteriophage-derived lysins. Lysostaphin, a lysin produced by *S. staphylolyticus*, is used in the laboratory to digest staphylococcal cells and has been investigated intensely as an anti-staphylococcal agent (154). It has also been shown to work against in-vitro *S. aureus* biofilms, an effect that could be enhanced when used together with antibiotics (155–157). Notably, lysostaphin eradicated *S. aureus* in a catheter-associated infection model in mice when applied through the catheters (158). *S. epidermidis* shows increased resistance to lysostaphin due to the different composition of the pentapeptide bridge in peptidoglycan, the target of lysostaphin (159). Another enzyme-based potential therapeutic strategy consists of applying matrix-degrading enzymes. Dispersin B is a PIAse produced by *Actinobacillus actinomycetemcomitans* (160). It has been shown to degrade staphylococcal biofilms in vitro that are dependent on PIA, thus, predominantly *S. epidermidis* biofilms (161, 162). Other

matrix-degrading enzymes, such as proteases, have also been proposed to treat biofilm infections (79, 155), a strategy obviously also dependent on the mode of biofilm matrix formation. In-vivo efficacy of biofilm matrix-degrading enzymes still needs to be shown.

Quorum-sensing blockers

Finally, quorum-sensing blockers are often proposed for biofilm infections; however, due to the biofilm-structuring and –dispersing effect of the Agr quorum-sensing system, this approach appears counterproductive in staphylococci. Nevertheless, one needs to consider that severe and life-threatening conditions, such as bloodstream infections, often only arise after dispersal from a biofilm. For those, quorum-sensing blockers or other approaches targeting aggressive virulence factors, such as toxins, may be invaluable. Anti-toxin antibodies show great promise in that regard (163).

CONCLUDING REMARKS

Biofilm formation in staphylococci remains a serious clinical problem due to the enormous and continued difficulties in treating staphylococcal biofilm-associated infections. Therefore, mechanisms of staphylococcal biofilm formation have received much attention over the last decades; and many important results, for example on staphylococcal biofilm genetics and regulation, have been achieved using in-vitro setups of biofilm development. However, it has become clear that biofilm-related in-vitro observations are often not transferable to the in-vivo level, with the in-vivo situation differing in substantial aspects, such as the interaction with host matrix proteins and immune defenses. With the interest in staphylococcal biofilm formation clearly stemming from the involvement of staphylococci in infections, there should be a more pronounced focus on the in-depth evaluation of biofilm formation in an in-vivo context in the future. This is equally imperative in pre-clinical research on potential anti-biofilm therapeutics, which currently often lacks this crucial aspect. Such approaches should contain more animal models, which still can be optimized to better reflect human biofilm-associated infection, and ex-vivo research using human cells, particularly immune cells. The increasing recent interest in the genetics and immunology underlying the asymptomatic colonization of human skin and mucous surfaces, which can be seen as proceeding in a biofilm-like fashion, will also be of great value to gain a comprehensive understanding of staphylococcal biofilm physiology in the commensal and infectious states.

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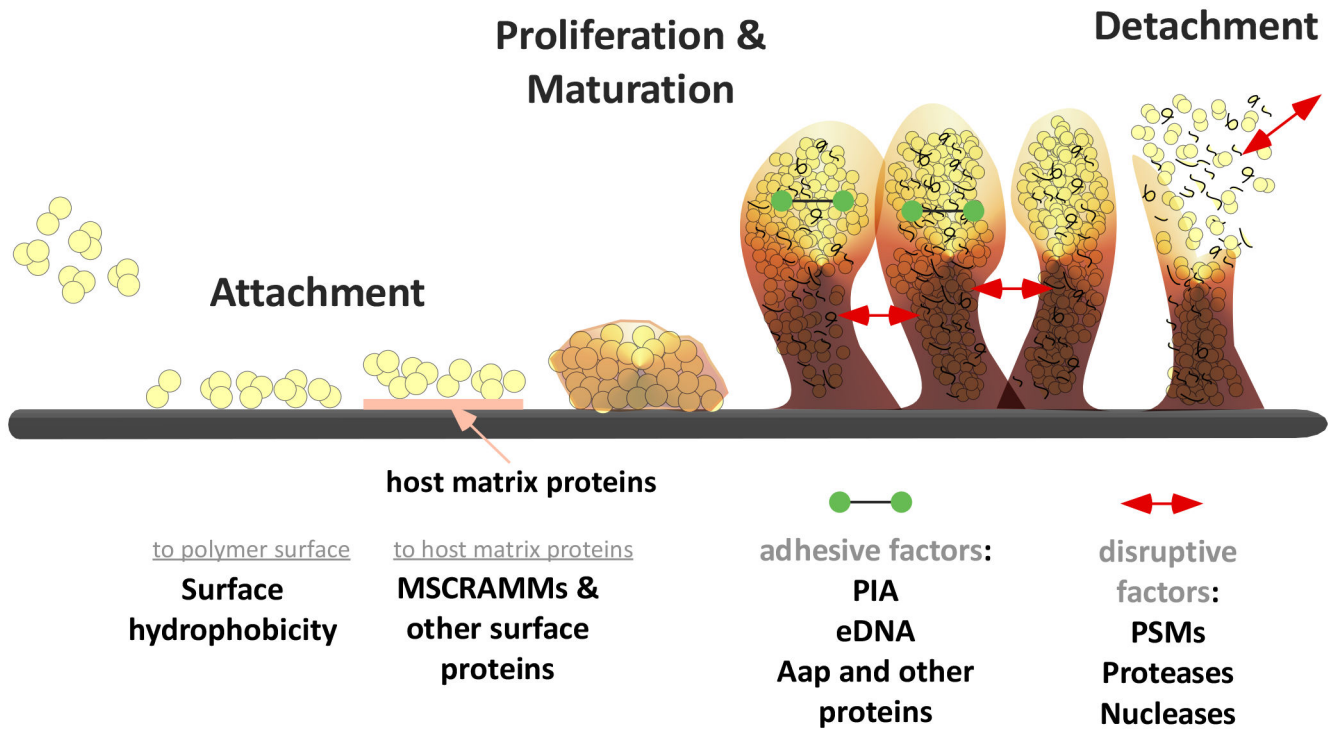
**Proliferation &
Maturation**

Detachment

Attachment

host matrix proteins

to polymer surface

**Surface
hydrophobicity**

to host matrix proteins

**MSCRAMMs &
other surface
proteins**

adhesive factors:

**PIA
eDNA
Aap and other
proteins**

disruptive
factors:

**PSMs
Proteases
Nucleases**

Fig. 1. Staphylococcal biofilm development.
Attachment of cells to a surface (in the case of surface-attached biofilms) occurs via
hydrophobic interactions to an abiotic surface, or via surface proteins that bind in a specific
fashion to host matrix proteins covering an indwelling medical device. Growth of the biofilm
in the proliferation/maturation stage is accompanied by the production of cell-cell-adhesive
matrix components (such as, PIA, eDNA, and proteins) as well as disruptive factors (such as,
PSMs and degradative secreted enzymes). Those disruptive factors can also cause
detachment, a process of great importance for the initiation of complications of biofilm–
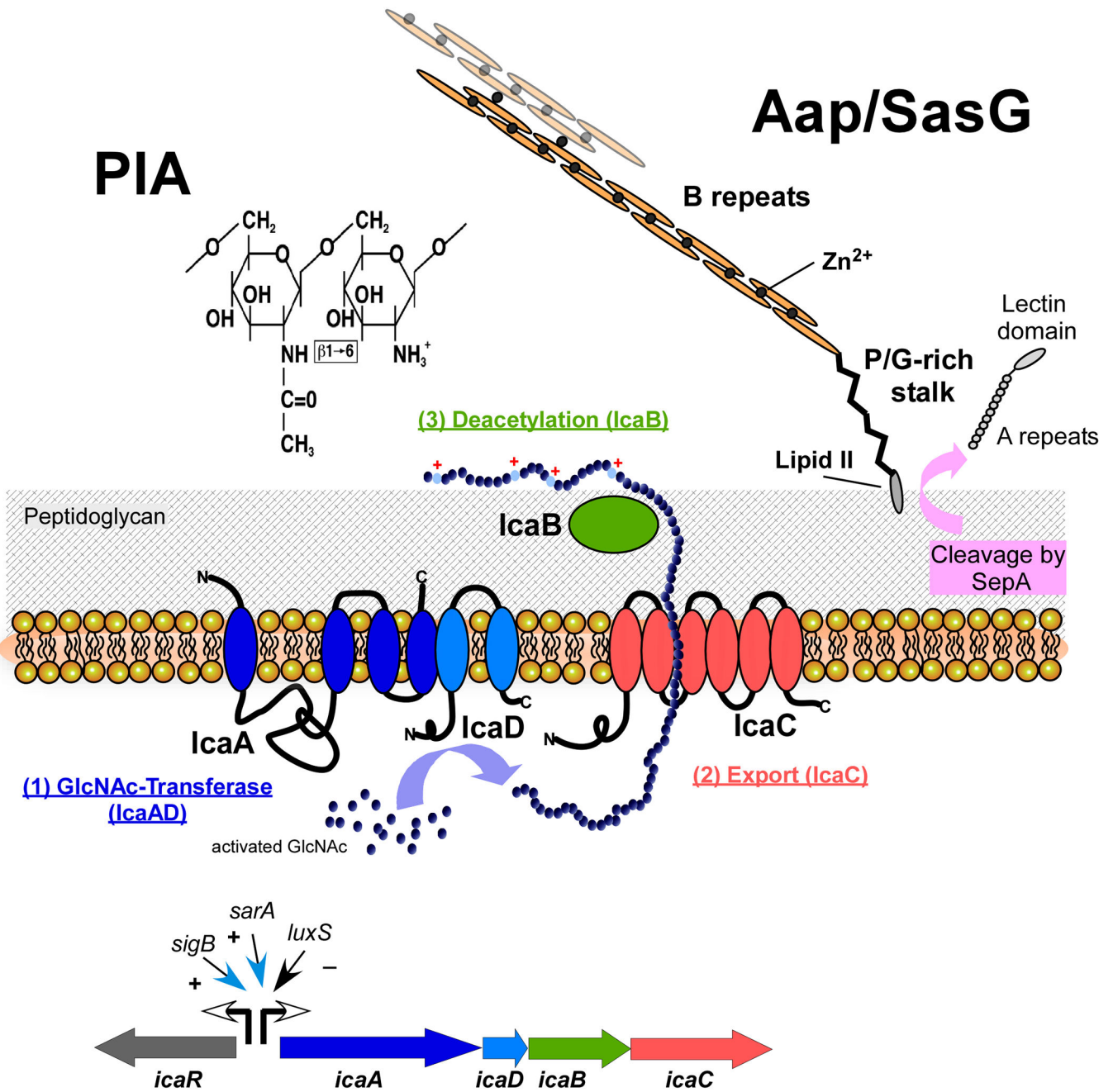associated infection, such as bacteremia.
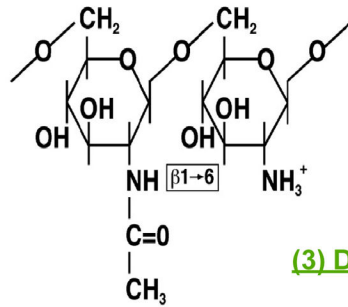
Fig. 2. Predominant staphylococcal biofilm matrix components.
The exopolysaccharide PIA (polysaccharide intercellular adhesin) (left), produced by many
S. aureus and *S. epidermidis* isolates, is a β1–6-linked homopolymer of N-
acetylglucosamine. It is synthesized in the cell by the combined activity of the membrane
enzymes IcaA and IcaD, and likely exported by IcaC. The extracellular surface-bound
enzyme IcaB removes a certain percentage (~ 15 – 20%) of N-acetyl moieties, which gives
the otherwise neutral PIA molecule a positive net charge, anchoring PIA to the negatively
charged cell surface. In addition to the *ica* biosynthetic genes *icaADBC*, the PIA

biosynthesis locus also contains a regulatory gene, *icaR*. Several global regulators impact transcription from the *icaR* and *icaADBC* promoters.

The accumulation-associated protein (Aap), which is present in *S. epidermidis* and has a homologue in *S. aureus* called SasG, is produced as a 220-kD precursor protein, from which the secreted protease SepA cleaves off the N-terminal A-repeat and lectin domains. It is anchored to the cell wall via sortase-catalyzed covalent linkage to lipid II. Mature Aap forms extended fibrils out of B repeat domains, whose polymerization is dependent on Zn^{2+} ions. Zn^{2+} is also required for the interconnection of Aap/SasG proteins from different cells, which can happen in an interspecies manner.
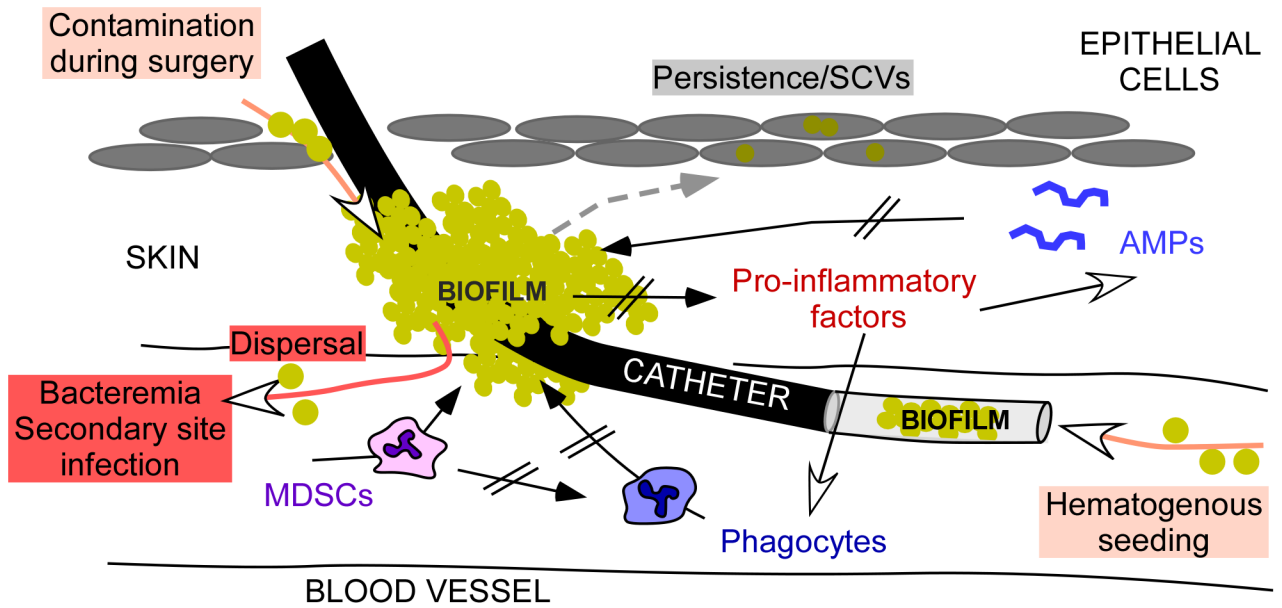
Fig. 3. Staphylococcal biofilm-associated infection on medical devices.
Mechanisms underlying staphylococcal device-associated infection are depicted in an
exemplary fashion for an intravascular catheter-associated biofilm. Many of those are still
hypothetical. Biofilm formation on a catheter may originate from bacteria introduced as a
contamination during surgery/catheter insertion; those are believed to initiate biofilms on the
catheter outside. Alternatively, biofilms in the lumen of the catheter can originate from
bacteremia and other infection sites due to hematogenous seeding from those sites.
Compared to planktonic growth, biofilms secrete less pro-inflammatory factors, which
normally cause considerable responses by innate host defenses, such as phagocyte influx and
AMP production. In addition to eliciting less such responses, the biofilm matrix provides a
shelter from AMPs and phagocyte intrusion. *S. aureus* biofilms also attract myeloid-derived
suppressor cells (MDSCs), which add to decreasing inflammatory responses, particularly
phagocyte influx. Finally, internalization of bacteria for example by epithelial cells can
produce SCVs, prone to persist and cause recurrent infections.