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REVIEW

Staphylococcal Biofilm Development: Structure, Regulation, and Treatment Strategies

Katrin Schilcher,[a] Alexander R. Horswill[a,b]

[a]Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA
[b]Department of Veterans Affairs Eastern Colorado Health Care System, Denver, Colorado, USA

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SUMMARY In many natural and clinical settings, bacteria are associated with some type of biotic or abiotic surface that enables them to form biofilms, a multicellular lifestyle with bacteria embedded in an extracellular matrix. *Staphylococcus aureus* and *Staphylococcus epidermidis*, the most frequent causes of biofilm-associated infections on indwelling medical devices, can switch between an existence as single free-floating cells and multicellular biofilms. During biofilm formation, cells first attach to a surface and then multiply to form microcolonies. They subsequently produce the extracellular matrix, a hallmark of biofilm formation, which consists of polysaccharides, proteins, and extracellular DNA. After biofilm maturation into three-dimensional structures, the biofilm community undergoes a disassembly process that leads to the dissemination of staphylococcal cells. As biofilms are dynamic and complex biological systems, staphylococci have evolved a vast network of regulatory mechanisms to modify and fine-tune biofilm development upon changes in environmental conditions. Thus, biofilm formation is used as a strategy for survival and persistence in the human host and can serve as a reservoir for spreading to new infection sites. More-

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Address correspondence to Alexander R. Horswill, alexander.horswill@cuanschutz.edu.

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over, staphylococcal biofilms provide enhanced resilience toward antibiotics and the immune response and impose remarkable therapeutic challenges in clinics worldwide. This review provides an overview and an updated perspective on staphylococcal biofilms, describing the characteristic features of biofilm formation, the structural and functional properties of the biofilm matrix, and the most important mechanisms involved in the regulation of staphylococcal biofilm formation. Finally, we highlight promising strategies and technologies, including multitargeted or combinational therapies, to eradicate staphylococcal biofilms.

KEYWORDS *Staphylococcus aureus*, *Staphylococcus epidermidis*, biofilms, extracellular matrix, microbial communities, quorum sensing

INTRODUCTION

The term bacterial biofilm was coined in 1978 by Costerton and colleagues, who described it as a structured microbial community that is attached to a surface and encased by an extracellular matrix (1). However, cell aggregates that form in the absence of any surface (2), as well as pellicles, floating biofilms that take shape at the air-liquid interface (3), are also often considered to be types of biofilms. Since the introduction of the biofilm model over 40 years ago, it has become clear that the majority of bacteria have the inherent capacity to grow in these self-generated ecosystems. Peters and colleagues demonstrated with early electron microscopy studies that staphylococcal cells adhere to central venous catheters, where they are embedded in a "slimy material," the biofilm matrix (4, 5). The role of the self-produced biofilm matrix or extracellular polymeric substance (EPS), consisting mainly of polysaccharides, proteins, and extracellular DNA (eDNA), is to protect the cells from deleterious environmental factors, including antibiotics and the host immune system (6–9). Members of the *Staphylococcus* genus, including *Staphylococcus aureus* and *Staphylococcus epidermidis*, the most prominent member of the coagulase-negative staphylococci (CoNS), are opportunistic pathogens and both produce robust biofilms on abiotic and biotic surfaces. While nasal and skin colonization with these pathogens widely occurs in humans without any symptoms (10, 11), the switch between single free-floating cells, referred to as "planktonic state," and a multicellular biofilm is a pivotal step for staphylococci to cause different types of infections, including infective endocarditis, osteomyelitis, and prosthetic joint infections (PJIs) (12–17). Whereas *S. aureus* has many mechanisms and virulence factors to evade the immune system, the pathogenic potential of *S. epidermidis* mainly depends on biofilm formation (18). Since implants, such as prosthetics, catheters, and other devices, provide an ideal surface for bacterial adhesion, *S. epidermidis* is remarkably well adapted to cause implant-associated infections (19). Approximately 30% to 40% of nosocomial bloodstream infections are caused by CoNS, such as *S. epidermidis*, and the majority of these bloodstream infections are the direct result of an intravascular catheter infection (ICI), a fact that strongly connects the biofilm-forming capacity of *S. epidermidis* to bloodstream infections. Besides that, CoNS are among the most frequent causes of prosthetic valve endocarditis (PVE) (15% to 40% of all PVE cases), cardiac pacemaker infections (incidence rates of 0.13% to 19.9%), and surgical site infections (20).

Since the biofilm lifestyle plays a central role in staphylococcal biology, the process of biofilm formation and disassembly is tightly controlled by several regulatory systems that integrate the physiological state of the cell, environmental cues, and the dynamics within the staphylococcal community. In this context, the best-studied regulatory system of staphylococcal biofilm formation is the accessory gene regulator (Agr) quorum sensing (QS) system, a cell-to-cell communication system that coordinates cellular behavior dependent on cell density (21–23). The Agr QS system most prominently regulates the production of proteases and phenol-soluble modulins (PSMs), which are main contributors of biofilm maturation and disassembly in *S. aureus* as well as *S. epidermidis* (24–26). Our understanding of staphylococcal biofilms has progressed tremendously as a consequence of advances in biochemical tools and new imaging

technologies. Three-dimensional biofilm structure analysis *in vitro* revealed a high degree of complexity and spatial organization within staphylococcal biofilms. In addition, studies have shown that the staphylococcal EPS is highly variable in its composition, depending on nutrient availability, the host environment, and mechanical shear forces (27).

Although in the past researchers have extensively studied the molecular basis of staphylococcal biofilm formation *in vitro*, our knowledge about staphylococcal biofilm formation under *in vivo* conditions is still limited. In contrast to *in vitro* biofilm formation, *in vivo* staphylococci are subject to innate host defenses such as neutrophils, macrophages, and antimicrobial peptides (AMPs) (28, 29). During infection, staphylococcal biofilms confer protection against the host immune system as well as antibiotic treatment. Whereas biofilm recalcitrance against the immune response was long attributed to the biofilm microenvironment acting as a physical barrier for host immune cells (30), it has now become clear that biofilms shield bacterial cells from detection by the immune system by masking pathogen-associated molecular patterns (PAMPs) (13). Similarly, biofilms were originally postulated to prevent the diffusion of antibiotics, rendering the cells in the biofilm resistant to antibiotic treatment. However, new evidence suggests that cells residing inside biofilms have low metabolic activity, which increases their tolerance against antibiotics that primarily target metabolically active cells (31). Indeed, biofilm-associated cells with low metabolic activity share a high physiological similarity to persister cells and small-colony variants (SCV) (32, 33). The antibiotic tolerance of persister cells has been linked to low intracellular levels of ATP in *S. aureus* (34) as well as the Gram-negative bacterium *Escherichia coli* (35). Cells within a biofilm encounter low oxygen and nutrient availability, resulting in low metabolic cell activity and a drop in intracellular ATP levels, which presumably contribute to elevated antibiotic tolerance of the biofilm (32). Therefore, antibiofilm strategies that interfere with biofilm cells independent of their cellular activity, such as AMPs, surface modifications to prevent bacterial adhesion, nanoparticles with antimicrobial activity, and new technologies for physical biofilm removal, are considered to be highly attractive approaches (36).

This review seeks to give a comprehensive overview of staphylococcal biofilm formation, its regulation, and the resulting consequences for bacterial infections. The identification and development of antibiofilm agents has become a major research objective over the last decades, and strategies to interfere with biofilm formation and its regulation or to eradicate preexisting biofilms are discussed at the end of this review.

MOLECULAR MECHANISMS OF STAPHYLOCOCCAL BIOFILM FORMATION

Staphylococcal biofilm development is a complex process that can be divided into three main phases: (i) initial attachment, (ii) production of extracellular matrix and cell proliferation, and (iii) biofilm structuring and cell detachment. In the first phase, *S. aureus* attaches to a biotic or abiotic surface using a range of different factors and mechanisms, including sortase-attached surface adhesins (37, 38), wall teichoic acids (WTAs) (39, 40), changes in bacterial cell surface hydrophobicity (41), and eDNA production (42, 43). The attached bacteria then proliferate and build microcolonies on the surface. In the next stage, those microcolonies develop into distinct structures that establish the biofilm. During proliferation and biofilm maturation, the cells are held together by adhesive factors within the matrix. Remodeling of the biofilm occurs through disruptive factors, like nucleases and surfactants (PSMs), which are thought to be essential for the development of the three-dimensional structure of the mature staphylococcal biofilm with its distinctive towers and channels. Cell detachment during biofilm disassembly is then mainly caused by protease-driven degradation of the biofilm matrix and disruption of the biofilm by PSMs (24, 44, 45).

A further refined model of biofilm development has been postulated recently. This model expands the traditional biofilm model by two distinct new phases, referred to as the multiplication and the exodus phases, which take place after the initial attachment and before the maturation phase (46, 47). During the multiplication phase, the cells

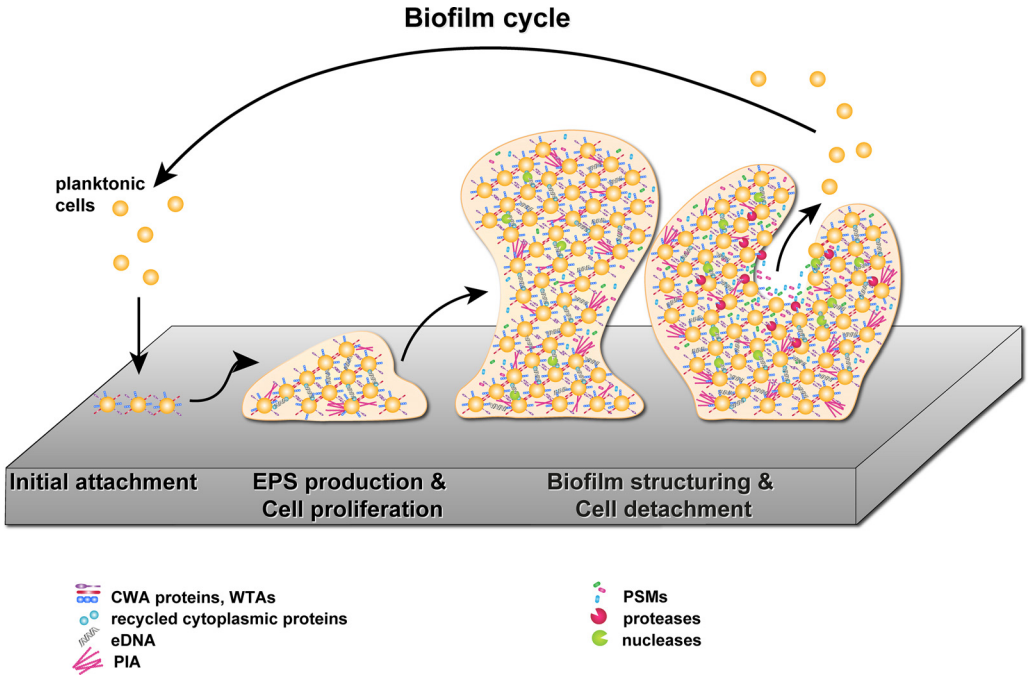

FIG 1 Staphylococcal biofilm cycle. During the first step of biofilm formation, planktonic staphylococcal cells attach to a surface with CWA proteins and WTAs (initial attachment). The production of PIA, eDNA, and adhesins to establish intercellular interactions is upregulated and leads to the formation of microcolonies on the surface (EPS production and cell proliferation). Cells within the growing biofilm express nucleases and PSMs, which are responsible for the formation of channels and towers in the matured biofilm. In the last step, the biofilm is dispersed by the action of PSMs and proteases, allowing the bacterial cells to detach from the biofilm to colonize new niches (biofilm structuring and cell detachment). The depiction of matrix components was inspired by Fig. 5 from the work of Hobley and colleagues (9).

start to grow and divide and embed themselves in a matrix consisting of proteins and eDNA. The exodus phase is then triggered by the expression and secretion of the major nuclease Nuc1, which leads to degradation of the eDNA contained in the matrix, allowing the release of a subpopulation of cells from the biofilm. Cells remaining in the biofilm then undergo rapid growth, which leads to the formation of the characteristic tower-like structure of staphylococcal biofilms (46). Since the five-phase biofilm model has been postulated only recently and the current literature is almost exclusively based on the traditional three-phase biofilm model, this review was structured according to the traditional model. For a comprehensive overview of the five-phase biofilm model, we refer the reader to the review of Moormeier and Bayles (47). An overview of the above-described *S. aureus* biofilm formation steps is provided in the following subsections and is depicted in Fig. 1.

Initial Attachment

Surface attachment is the first stage of staphylococcal biofilm formation, at which planktonic staphylococcal cells attach to biotic or abiotic surfaces. During infection, staphylococci mainly encounter biotic surfaces composed of host matrix including fibrinogen, fibronectin, vitronectin, and collagen. Binding to these biotic surfaces is facilitated by cell wall-anchored (CWA) proteins, of which the best-characterized group is termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). All MSCRAMMs contain a C-terminal cell wall-targeting motif (LPXTG motif) that covalently anchors the protein to the bacterial peptidoglycan by the enzyme sortase A (48). Several MSCRAMMs have been implicated to promote biofilm formation by facilitating the initial attachment to host matrix components. Among these are the fibronectin-binding proteins FnBPA and FnBPB (49–51), clumping factor A (ClfA) and clumping factor B (ClfB) (52–56), members of the serine-aspartate repeat family proteins (SdrC, SdrD, and SdrE) (57–60) and the bone sialoprotein-binding

protein (Bbp) (61, 62). Similar to *S. aureus*, *S. epidermidis* also uses MSCRAMMs to bind to surfaces covered with host matrix proteins for primary attachment. Three main MSCRAMMs, SdrG, SdrF, and SdrH, all members of the serine-aspartate repeat family, have been identified in *S. epidermidis* (63). To date, no ligand has been identified for SdrH. On the other hand, several studies have shown that SdrG binds fibrinogen and SdrF binds the host matrix components collagen and keratin (38). For a more detailed overview of MSCRAMMs of Gram-positive cocci, the reader is referred to a recent review by Foster (38).

Binding to abiotic surfaces of catheters and implants, but also to the polystyrene surfaces of microtiter plates often used for *in vitro* biofilm experiments, is facilitated through hydrophobic and electrostatic interactions. The extracellular glycopolymers WTAs have been identified as the main drivers of these interactions in *S. aureus* (39) and in *S. epidermidis* (40). Besides their aforementioned interaction with host matrix components, FnBPs and SdrC can also drive attachment of *S. aureus* to abiotic surfaces (64, 65), which was also shown for the biofilm-associated protein (Bap) (66). In addition, the major autolytic enzymes AtlA in *S. aureus* (67–69) and the homologous protein AtlE in *S. epidermidis* (70) are major contributors to primary attachment to abiotic surfaces like polystyrene or glass. Although it is not known exactly how these hydrolytic enzymes contribute to surface attachment, it is hypothesized that the enzymatic activity results in major changes in bacterial surface hydrophobicity, resulting in enhanced surface attachment (41). Besides their involvement in primary attachment, AtlA and AtlE are key enzymes for the release of eDNA (42, 69), a known component of the staphylococcal *in vitro* biofilm matrix (43, 71, 72). Interestingly, addition of DNase I at the start of biofilm cultures prevented attachment of several different *S. epidermidis* strains, indicating an important role for eDNA not only as a component of the biofilm matrix but already during initial surface attachment (42). It is important to note that in contrast to the case with *S. epidermidis*, eDNA seems not to play an important role for *S. aureus* surface attachment, as DNase I treatment during the first 8 h does not have an apparent effect on biofilm formation (46, 73). Finally, the accumulation-associated protein (Aap) of *S. epidermidis* was shown to mediate attachment to polystyrene surfaces through its N-terminal A domain (74). Impaired surface attachment of an Aap mutant strain could be complemented by expression of the homologous protein *S. aureus* surface protein G (SasG), indicating that SasG could play a similar role in *S. aureus* surface attachment (74).

Production of Extracellular Matrix and Cell Proliferation

After initial attachment of single cells to a surface, these cells start to proliferate, resulting in the formation of so-called microcolonies. As part of the early biofilm maturation process, the bacteria also start to produce EPS, which eventually forms the biofilm matrix. EPS mainly consists of polysaccharides, proteins, teichoic acids, and eDNA, and dependent on the EPS composition, staphylococcal biofilms can be divided in two broad categories: biofilms consisting of a polysaccharide matrix and biofilms with a proteinaceous matrix (75, 76). In polysaccharide-dependent biofilms, the main EPS component is poly-β(1-6)-*N*-acetylglucosamine (PNAG), also referred to as polysaccharide intercellular adhesin (PIA) (77, 78). The production of PIA is dependent on the intracellular adhesion (*ica*) operon, which was first identified in an *S. epidermidis* transposon mutant screen for mutants deficient in biofilm formation (79) and later also in *S. aureus* (80). The *ica* operon comprises four genes arranged in the following order: *icaA*, *icaD*, *icaB*, and *icaC* (80, 81). *In vitro* assays using UDP-*N*-acetylglucosamine (UDP-GlcNAc) as the substrate showed that the transmembrane glycosyltransferase IcaA as well as IcaD is essential for PIA synthesis (82). Coexpression of the putative membrane protein IcaC is required to produce longer oligomer chains (82) and was therefore proposed to be important for translocation of the growing polysaccharide chain to the cell surface. Only recently has it become clear that IcaC is a predicted member of membrane-bound acyltransferases and presumably responsible for the succinylation of PIA (83). It is important to note that for a long time PIA has been

described to be *N*-succinylated (84), but more recent evidence has supported the idea that PIA is indeed *O*-succinylated in staphylococci (85, 86). PIA is also partially deacetylated by the extracellular, cell surface-associated polysaccharide deacetylase IcaB (87), and deacetylated PIA is positively charged and localized on the bacterial surface in fibrous strands (87, 88). The transcription of the *icaADBC* operon is mainly regulated by IcaR, a repressor located upstream of the *ica* operon (89, 90). A more detailed description of *ica* regulation is given in "*ica* operon regulation" below.

Besides polysaccharide-dependent biofilms, staphylococci have also been shown to form biofilms with matrices mainly consisting of proteinaceous material. Such biofilms have been mainly observed for methicillin-resistant *S. aureus* (MRSA) isolates and *S. epidermidis* clinical isolates, while methicillin-sensitive *S. aureus* (MSSA) isolates are more often *ica* dependent (91–94). Importantly, the high prevalence of proteinaceous biofilms formed by *S. epidermidis* clinical isolates is likely connected with the absence of the *ica* operon in a high number of *S. epidermidis* isolates. Although the reported numbers vary, many studies have shown that only 20% to 60% of clinical *S. epidermidis* isolates carry the *ica* operon (95–99). In an early study, the surface protein Bap of *S. aureus* was identified as the main determinant for successful surface adherence as well as intercellular accumulation during biofilm development (66). A follow-up study by the same research group also showed that the *ica* operon, although present in the genome, did not contribute to biofilm formation of the *S. aureus* strain under investigation (100). Although the Bap protein is not widespread among *S. aureus* strains (66), a protein called Bap homologue protein (Bhp) is present in *S. epidermidis* (101). Furthermore, it has recently been shown that Bap proteins can build amyloid-like scaffolds that promote *S. aureus* biofilm formation (102). The role of several MSCRAMMs extends beyond initial attachment. More precisely, FnBPA, FnBPB, and SdrC all promote cell-cell interactions leading to increased biofilm formation (65, 103–105). One of the best-studied proteinaceous biofilm factors of *S. epidermidis* is the surface protein Aap (106). Originally thought to anchor PIA to the cell wall (106), Aap was recently shown to mediate biofilm formation after enzymatic cleavage by staphylococcal or host proteases (92, 107). More specifically, after cleavage the mature Aap protein accomplishes self-adhesion and intercellular adhesion via several B domain repeats (108, 109). Interestingly, the *S. aureus* Aap homologue SasG promotes biofilm formation by zinc-mediated dimerization and was shown to interact with Aap (110, 111).

A third type of staphylococcal biofilm, mainly dependent on eDNA, has attracted substantial interest. The initial discovery of this type of biofilm is supported by the observation that a mutant deficient in AtlA, the main autolysin in *S. aureus* responsible for the release of eDNA, showed a reduced biofilm formation phenotype in *in vitro* experiments (69), while mutation of the staphylococcal thermonuclease (Nuc) leads to increased biofilm formation (71). Finally, mature staphylococcal biofilms are sensitive to externally added DNase I, demonstrating that eDNA is a structural component of the biofilm matrix (43). Similar observations were made for biofilms of the Gram-negative bacterium *Pseudomonas aeruginosa* (112), indicating that the use of eDNA as a structural component of biofilms is conserved across different bacterial genera. There is also increasing evidence that cytoplasmic proteins associated with the outside of the cell via electrostatic interactions may be able to "moonlight" as eDNA-binding proteins in the biofilm matrix (113–115). Another recent study suggests that cell wall-associated lipoproteins contribute to *S. aureus* biofilm formation by linking bacterial cells together through noncovalent cross-links with high-molecular-weight eDNA found within the biofilm matrix (116). Additionally, investigation of the *S. aureus* biofilm proteome in a flow system demonstrated that secreted virulence factors and ribosomal proteins are trapped in the biofilm matrix and contribute to biofilm stability (115). In more detail, the biofilm environment is thought to be acidic, due to low oxygen levels and the release of fermentation products. The positive charge of alkaline virulence factors and ribosomal proteins in the acidic environment is then thought to mediate electrostatic interactions with surface components and eDNA, leading to biofilm stabilization (115). As described above, the biofilm matrix consists of different classes of molecules,

namely, polysaccharides, proteins, and eDNA. While these are the principal building blocks of the biofilm matrix, the exact composition of the biofilm matrix can vary depending on substrate availability and physical factors. For example, *S. epidermidis* strains isolated from environments with high shear stress, such as found in the lumen of a catheter, rather produce PIA-dependent biofilms than isolates from low-shear environments (117). This exemplifies that environmental conditions, including the physical properties of the milieu, can have tremendous effects on staphylococcal biofilms.

Biofilm Structuring and Cell Detachment

Staphylococcal biofilms do not grow uniformly but rather consist of tower-like structures that are interspersed by fluid-filled channels that are developed during a process often referred to as biofilm maturation. The three-dimensional structure of biofilms is shaped by the activities of different enzymes and molecules that can degrade or interrupt the biofilm matrix (24, 118). As discussed before, the main components of the staphylococcal biofilm matrix are polysaccharides, proteins, and eDNA; accordingly, staphylococci have a vast array of exoenzymes that can target specific components of the biofilm matrix.

Protein biofilms are most susceptible to protease-mediated biofilm dispersal, and *S. aureus* produces and secretes 10 known proteases from different protease families: the metalloprotease aureolysin (Aur) (119), the two cysteine proteases SspB and ScpA (120, 121), and seven serine proteases known as SspA (also referred to as V8 protease) and SplA to -F (120, 122). The initial identification of staphylococcal proteases as biofilm-degrading agents was based on several studies investigating regulator mutants that were unable to form biofilms (103, 123, 124), a phenotype that could be correlated with elevated protease activity in these mutant strains (125–129). The importance of the individual proteases for biofilm degradation and dispersal is highly strain specific, and to date, the SspA, SspB, ScpA, and Aur proteases have been demonstrated to degrade *S. aureus* biofilms (103, 127, 129–131). The targets for two of the proteases have been identified. SspA degrades FnBPs and Bap (103, 127, 130), whereas aureolysin can degrade Bap as well as ClfB (127, 131). Although the proteins targeted by the staphopains SspB and ScpA have not been identified yet, their levels are decreased under biofilm-forming conditions, underlining their potential to inhibit biofilm formation (129). For *S. epidermidis*, three secreted proteases have been identified: the metalloprotease SepA (107, 132), the serine protease Esp, which was shown to degrade *S. aureus* biofilms (133–135), and the cysteine protease EcpA (136), all of which have low substrate specificity (137).

Another major component of the staphylococcal biofilm matrix, eDNA, is subject to degradation by nucleases. Two thermostable nucleases (Nuc1 and Nuc2), encoded by different open reading frames, are expressed by *S. aureus* (138). Transcription of *nuc1* is at its maximum during the postexponential growth phase, whereas transcription of *nuc2* is high during the early exponential phase and declines afterwards (139). Mutation of the *nuc1* gene leads to increased staphylococcal biofilm formation, whereas overexpression of *nuc1* leads to a reduction in biofilm formation (43, 71, 140). In accordance with the strong biofilm-degrading activity of Nuc1, its expression is highly repressed under *in vitro* biofilm growth conditions (141). It is interesting that eDNA needs to have a minimum size of 11 kDa in order to facilitate biofilm integrity (142), and the lack of nuclease activity is the driving factor for the preservation of high-molecular-weight eDNA (43, 71). In contrast to the well-established roles of Nuc1 during biofilm maturation and infection (141, 143), the role of Nuc2, a membrane-bound nuclease with an extracellular catalytic domain (144), has not been fully characterized yet.

Another group of molecules has emerged and been shown to participate in shaping staphylococcal biofilm structure. These molecules, so-called PSMs, are grouped in α-type (PSMα and PSMγ, which is also known as δ-toxin) and β-type (PSMβ) peptides and can disperse biofilms, most likely by disrupting noncovalent interactions between matrix components due to their pronounced amphipathic nature with potential

TABLE 1 Major staphylococcal biofilm regulators

Regulator	Functions	Involvement in biofilm development	References
Agr	Cell-cell communication system (quorum sensing), TCS with AIPs as signaling molecules; stimulation of protease production and PSMs; repression of adhesins	Important for initial attachment and biofilm disassembly	21, 22, 45, 145, 175, 176, 178, 181, 182
SaeRS	TCS; stimulation of adhesins and nuclease	Important for biofilm maturation; regulates the "exodus" event in the five-phase biofilm model	46, 141, 217
SarA	SarA family cytoplasmic regulator; repression of protease production and stimulation of PIA; regulation of adhesins	Important for biofilm formation mainly through repression of proteases; positive regulator of biofilm formation	125, 226, 237, 242
Rot	SarA family cytoplasmic regulator; main effector of the Agr QS; stimulation of adhesins and repression of proteases	Important for biofilm formation mainly through repression of proteases; positive regulator of biofilm formation.	170, 179, 245, 249
MgrA	SarA family cytoplasmic regulator; main effector of the ArlRS TCS; repression of adhesins	Important for biofilm formation mainly through repression of adhesins; negative regulator of biofilm formation	250–252
SigB	Alternative sigma factor for stress response; stimulation of adhesins and repression of proteases and nuclease; strain-dependent regulation of PIA	Important for initial attachment and biofilm disassembly	71, 240, 255, 261, 263, 264, 269
CodY	Cytoplasmic regulator for metabolic response; strain-dependent regulation of PIA; repression of proteases	Important for PIA-dependent biofilm formation	276–279, 283–286
LytSR	TCS regulating cell lysis; stimulation of LrgAB	Important for eDNA-dependent biofilm formation	315–318

surfactant-like properties (45, 118, 145). Indeed, external addition of PSMs to growing staphylococcal biofilms inhibits biofilm formation (45, 146). Periasamy and colleagues further determined that local differences of PSM expression in *S. aureus* biofilms are responsible for the formation of the characteristic fluid channels that are embedded in the biofilm matrix (45). In addition, PSM activity is also an important contributor to biofilm detachment and subsequent dissemination of staphylococcal cells *in vivo* (45). Whereas biofilm disassembly driven by soluble PSMs is well accepted, the biological role of polymerized PSMs in staphylococcal biofilms is contradictory. It was shown that PSMs of *S. aureus* not only can exist in their soluble form but also aggregate into amyloid-like fibrils that are able to stabilize the biofilm structure (147, 148). Zheng et al. confirmed that PSMs can form amyloid-like fibrils but proposed an alternative model in which PSMs, not in their amyloid form, protect eDNA from DNase-mediated degradation, which can then lead to enhanced *S. aureus* biofilm stability (149). PSMs are also important effectors for shaping the structure of *S. epidermidis* biofilms (150, 151). The surfactant-like β-type PSMs, first discovered in *S. epidermidis* (152), not only are involved in biofilm maturation and dispersal *in vitro* but also perform this function during catheter colonization *in vivo* (151). In addition, amyloid formation of *S. epidermidis* PSMs has not been observed yet, and this is in accordance with a recent study confirming a role of PSMs in biofilm structuring but not in its stabilization (26).

REGULATION OF STAPHYLOCOCCAL BIOFILM FORMATION

Biofilm formation is a social group behavior and every step, from the initial attachment to the dissemination of the mature biofilm, is tightly regulated (153). One of the main regulators of biofilm development is the QS system. In *S. aureus*, it is responsible for the switch from biofilm formation to its disassembly (22, 154, 155). Aside from QS, a vast number of environmental cues (e.g., pH, nutrient availability and fluid flow) as well as regulatory systems (e.g., Sae, SarA, Rot) are involved in the regulation of biofilm formation (23, 156, 157). The next section and Table 1 provide a comprehensive overview of the most important regulatory processes known to contribute to the formation, structuring, and disassembly of *S. aureus* biofilms. This review is mainly focused on protein-based regulatory systems. Nevertheless, there have been considerable advances in our knowledge of regulatory RNAs in staphylococci, and we refer the interested reader to two recent reviews (158, 159).

Quorum Sensing-Mediated Regulation of *S. aureus* Biofilm Development

QS enables bacteria to coordinate their behavior in a population density-dependent manner. This process involves the production of signaling molecules and their subsequent accumulation in the extracellular space surrounding the bacteria. When a certain threshold in concentration is reached, these signals are sensed by the bacteria and translated into alterations in their transcriptional profile and behavior. Therefore, QS allows bacteria to synchronize their behavior on a population-wide scale, enabling them to act like a multicellular organism. While the basic principles of QS are conserved among a wide variety of bacteria, the signaling molecules used for QS can differ greatly. The two best-studied systems are the acyl-homoserine lactone (AHL) QS system of Gram-negative bacteria and the (cyclic) peptide QS system of Gram-positive bacteria (160–162). *S. aureus* uses a QS system with autoinducing peptides (AIPs) as signaling molecules to regulate the expression of several virulence factors as well as the biosynthesis of AIPs as a function of cell population density (21, 22). The staphylococcal QS system, also known as the Agr system, is encoded by a 3.5-kb locus on the chromosome. The locus consists of the two transcriptional units, RNAII and RNAIII, driven by the two promoters P2 and P3, respectively. The RNAII transcript harbors the *agrBDCA* genes, which encode proteins required for AIP biosynthesis, transport, signal perception, and subsequent regulation of target genes (163, 164). The signaling cascade starts with the transcription and translation of AgrD, the propeptide precursor of AIP. AgrD is secreted and posttranslationally modified into the final peptide by AgrB, an integral membrane endopeptidase, and additionally processed via the type I signal peptidase SpsB (165, 166). When the AIP concentration in the environment reaches a certain threshold, known as a quorum, AIPs bind to the membrane-bound histidine kinase AgrC, which leads to autophosphorylation and initiation of the signal transduction cascade (21). AgrC then phosphorylates the response regulator AgrA, which, in turn, induces the expression of RNAIII from the P3 promoter (163, 167). RNAIII is the primary effector of the QS system that controls the upregulation of genes encoding secreted proteins, such as toxins and exoenzymes, and it also downregulates several genes encoding surface-associated adhesins (163, 168). Increased levels of RNAIII prevent the translation of the repressor of toxins (Rot) (169, 170), which is one of the main effectors of QS regulation. Although the majority of QS-regulated genes are regulated via RNAIII, some genes are directly regulated by AgrA. Most prominent among these is the direct regulation of RNAII from the P2 promoter, leading to elevated AIP production and a positive-feedback loop (171, 172). Additionally, the *psm*α and *psm*β transcripts in *S. aureus* and *S. epidermidis* are directly regulated by AgrA (26, 145, 173) as well as the *hld* gene for δ-toxin, which is encoded within RNAIII (163). The *psm*α, *psm*β, and *hld* transcripts encode peptides that belong to the family of PSMs that were already discussed above.

Although the basic principle of QS is the same in all bacteria, QS can have profoundly different impacts on biofilm formation in various bacterial species. *P. aeruginosa*, for example, uses its QS system in the early stages of biofilm development, where it initiates the differentiation of individual cells into a complex multicellular biofilm (174). The Agr QS system of *S. aureus* has the opposite effect on biofilm formation. Surface-associated proteins, such as FnBPs and SdrC, are important for cell attachment and subsequently the initiation of biofilm formation but are repressed by the Agr QS system (175, 176). Therefore, initiation of *S. aureus* biofilm formation requires conditions in which QS activity is low. Studies using fluorescent Agr reporter constructs have shown that the Agr QS system is not uniformly expressed in *S. aureus* biofilms, but only local patches of *S. aureus* cells show QS activity. Activation of the QS system in these local patches is followed by the detachment of these cells, creating voids in the biofilm that are filled by regrowing cells in these areas. Activation of QS in these cells at a later time point creates a periodic wave pattern of QS induction and cell detachment that is characteristic for *S. aureus* biofilms (177). One of the major forces driving the switch between biofilm formation and detachment is the degradation of the proteinaceous biofilm components by the Agr-regulated proteases

(126, 127, 129, 178). Their upregulation by the Agr QS system is presumably a consequence of the negative regulation of Rot via the RNAIII effector (170, 179), whereas the core genome-encoded PSMs (PSMα, PSMβ, and δ-toxin) are directly regulated via AgrA (26, 145, 173). Therefore, activation of the Agr QS system leads to upregulation of PSMs and matrix-degrading enzymes, which are responsible for biofilm structuring and detachment of staphylococcal cells from the mature biofilm (45, 178, 180, 181). In agreement with these findings, inactivation of the Agr QS system enhances biofilm formation on certain surfaces, presumably by obstructing or delaying the dissemination process of the biofilm (182). Although the Agr QS system is an important regulator of *S. aureus* toxin production (183), the contribution of Agr to infections is also closely tied to its impact on biofilm development. The interconnections of Agr, biofilms, and infections are discussed below.

Impact of Agr on biofilm-associated infections. Biofilm formation is a hallmark of chronic infections, and as outlined above, the Agr QS system is necessary for efficient *S. aureus* dissemination from a biofilm infection and subsequent spreading into neighboring tissues, a process that heavily relies on PSMs, nucleases, and proteases (24, 45, 184). Interestingly, *agr* mutants are defective in establishing osteomyelitis as well as endocarditis (185, 186). However, the beneficial role of the Agr QS system in staphylococcal infections is not always clear-cut. This point is emphasized by the fact that staphylococci adapt to the lifestyle of chronic infections by acquiring mutations in the *agr* operon. Naturally occurring *agr* mutants have been isolated from cystic fibrosis patients, persistent bacteremia, and biofilm-associated infections of indwelling medical devices (187–190). Similar results were obtained with four *S. epidermidis* isolates from indwelling catheter-associated infections, which all exhibited downregulation of RNAIII accompanied by enhanced initial attachment, eDNA release, and formation of thicker microcolonies (191). In addition, low Agr activity and low PSM production promote bacterial aggregation and biofilm formation in synovial fluid (192). An explanation for these observations might be the fact that activation of QS systems is assumed to display a high metabolic burden for the cell, leading to frequent spontaneous mutations within the QS system (193). The loss of the Agr QS system is tightly linked to a phenotype with elevated biofilm formation (146, 182, 190). While this can confer a fitness advantage in chronic infections (194, 195), it is also a potential maladaptation in the context of bacterial dissemination (196).

Modulators of Agr signaling and biofilm formation. Bacterial cells constantly sense environmental cues and change their transcription profiles accordingly to maintain viability under diverse conditions. These environmental cues can be physicochemical parameters such as pH, temperature, and shear forces from fluid flow. Several of these parameters and nutrient availability can influence biofilm formation via the Agr QS system. Among the earliest environmental cues that has been shown to affect the Agr system are acidic metabolites derived from glucose catabolism (197). A subsequent study by Weinrick and colleagues showed that a decrease in pH in the absence of glucose was sufficient to inhibit *agr* transcription (198). In addition, glucose depletion of existing biofilms was shown to activate the Agr QS system, resulting in biofilm dispersal (178). In contrast to *S. aureus*, the addition of excess glucose is not necessary to promote biofilm formation in *S. epidermidis*, although it is not known whether this is related to Agr regulation (199). Another interesting link between glucose and biofilm formation was presented by Seidl and colleagues (200). The catabolite control protein A (CcpA), which regulates gene expression in response to different carbon sources, was shown to be important for biofilm growth in the presence of glucose. Moreover, the formed biofilm was susceptible to DNase, proteinase K, and sodium metaperiodate, showing that the biofilm matrix most likely consisted of proteins, eDNA, and PIA. In agreement with this observation, CcpA stimulated the upregulation of *cidA*, which encodes a holin involved in eDNA release, and *icaA*, a gene essential for PIA production (200). It is important to mention that an earlier study already established that *ccpA* deletion results in downregulation of RNAIII (201). Further studies will be needed to address this discrepancy. Since we have already established above that the Agr QS

system is a negative regulator of biofilm formation, these described observations are a plausible explanation why the majority of biofilm studies with *S. aureus* are carried out using glucose-supplemented media (71, 177, 202).

Metals are essential micronutrients for bacteria, and many enzymes involved in vital bacterial processes rely on metals as cofactors. Within the host, *S. aureus* must compete for iron that is bound to iron-binding proteins such as hemoglobin, ferritin, lactoferrin, and transferrin (203, 204). In *S. aureus*, intracellular iron concentrations are regulated by the ferric uptake regulator (Fur), which is also able to interact with other metals such as cobalt, copper, manganese, and nickel (205). Biofilm formation in *S. aureus* is induced under iron-limiting conditions (206), and the increase of biofilm formation was demonstrated to be regulated by Fur, Agr, and the *S. aureus* exoprotein expression (Sae) regulator through the production of the two secreted proteins, the extracellular adhesion protein (Eap) and the extracellular matrix protein-binding protein (Emp) (207). Furthermore, Fur is required for elevated expression of Agr, Rot, and the Sae regulator under iron-limited conditions (208). These three regulatory systems play critical roles in *S. aureus* biofilm formation and are described in further detail elsewhere in this section. In contrast to the aforementioned studies, *S. aureus* strain SA113 showed reduced biofilm formation in the presence of an iron chelator, and the effect could be reversed by addition of iron to the growth medium (209). These contrasting results show that biofilm formation of *S. aureus* in response to various iron concentrations is likely strain dependent. For *S. epidermidis*, it was shown that iron excess as well as iron deficiency leads to impaired biomass accumulation and changes in the biofilm structure, ultimately leading to stunted biofilm formation (210).

The effect of physicochemical factors on staphylococcal biofilm formation have become a major research topic over the last couple of years. When colonizing indwelling medical devices such as catheters, staphylococci are subjected to mechanical shear forces from fluid flow, the local geometry, and surface chemistry of the medical device. Fluid flow can change the ability of bacterial cells to form biofilms (211, 212) by removing the QS signal molecules from the site of the producing bacteria (212, 213). Nonetheless, these signal molecules might then activate QS in cells located further downstream of the flow. This can lead to a relatively higher biofilm formation rate in regions located at sites on the upstream end of the flow than at sites further downstream. Even in environments with fluid flow, certain topographic regions, like small crevices, show a limited flow rate that allows the accumulation of QS molecules and subsequent activation of the *S. aureus* QS system (212). Another important factor regarding QS under flow conditions is biofilm thickness. Fluid flow is effective in removing QS signals only at the biofilm-liquid interface, while AIPs produced at the bottom of the biofilm are less affected by fluid flow. Therefore, AIPs can accumulate deep within the biofilm and activate the *S. aureus* QS system, subsequently leading to biofilm dissemination (212). Even though the interconnection of fluid flow, QS, and biofilm formation has not been directly studied for *S. epidermidis*, among a collection of over 100 clinical isolates, significantly more strains from high-shear environments either carried the *ica* operon or had other genetic determinants to increase biofilm formation (117). Altogether, these findings suggest that staphylococcal biofilm formation is increased in areas with high fluid flow due to the removal of the QS signal molecules in the case of *S. aureus* (212) and due to the presence of biofilm-enhancing determinants in *S. epidermidis* (117).

Regulatory Network of Staphylococcal Biofilm Formation

Several other regulatory systems are important during the biofilm development process, and some of these systems are interconnected with the Agr QS system. This network allows *S. aureus* to fine-tune its response to changing environmental conditions and modulate biofilm development. A simplified sketch of the Agr QS system and its regulatory network with the most important global regulators is depicted in Fig. 2 and described in the following section.

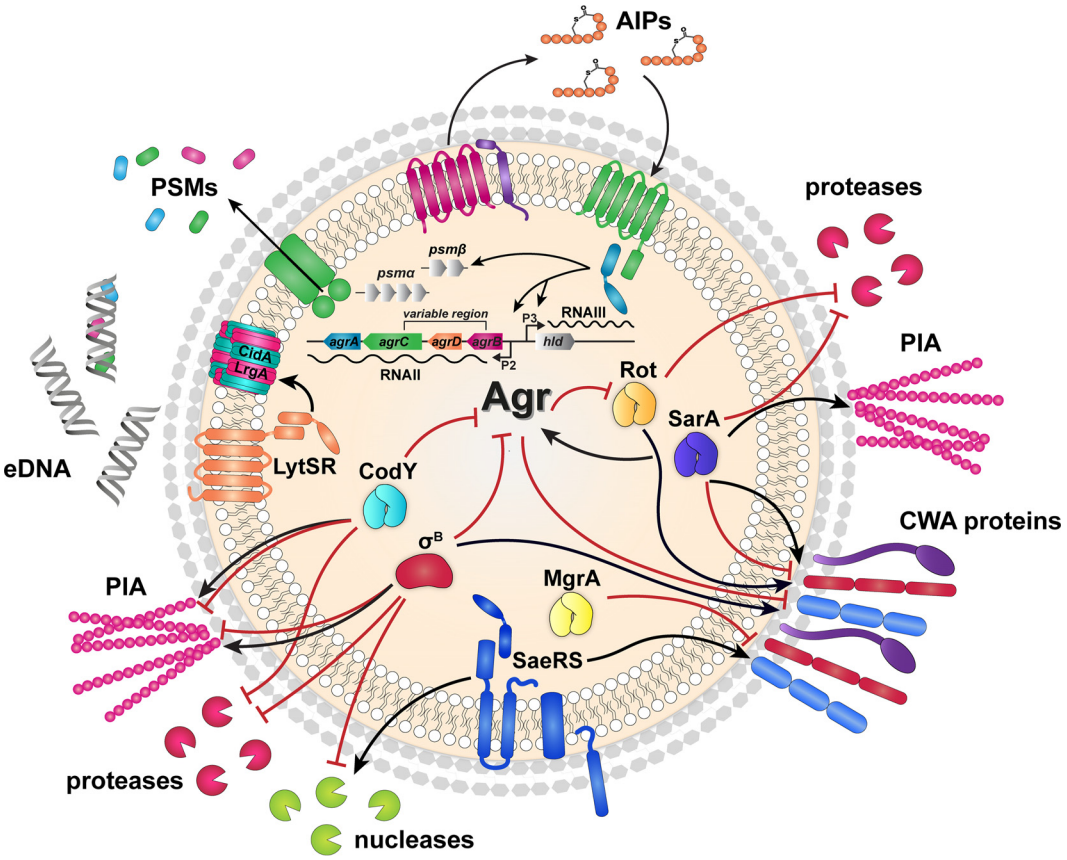

FIG 2 Regulatory network of staphylococcal biofilm formation. Shown is a simplified overview of the Agr quorum sensing system, its regulatory interaction with the most important biofilm regulators (LytSR, SigB, CodY, SaeRS, MgrA, SarA, and Rot) and their influence on the molecular determinants of biofilm formation. Black arrows indicate stimulation, and red blunted arrows indicate repression.

The Sae two-component system. The *S. aureus* exprotein expression (Sae) two-component system (TCS) consists of the SaeS sensor histidine kinase and the SaeR response regulator (214, 215). SaeS recognizes host signals such as human α-defensins and regulates the expression of various exoproteins important for pathogenesis and biofilm formation, among them α-hemolysin (Hla), the MSCRAMMs FnbA and FnbB, and the extracellular thermonuclease Nuc (141, 216–220). Fibronectin-binding proteins are known to be important for biofilm formation at the level of intercellular accumulation, allowing cells to bind together via homophilic interactions between surface proteins (103, 104), while Nuc was shown to cleave eDNA, a structural component of *S. aureus* biofilms (43, 71). An early biofilm dispersal event, termed exodus, that is part of the five-phase biofilm model, takes place before tower formation in *S. aureus* biofilm maturation and is independent of Agr QS (46). With a set of elegant experiments, it was shown that expression of Nuc preceded the exodus event, which likely led to degradation of eDNA in the biofilm matrix (46). The exodus event could directly be linked to the SaeRS system, as neither an *sae* nor a *nuc* mutant exhibited the exodus phenotype. Corroborating these results, Moormeier and colleagues showed that *nuc* transcription was regulated by the SaeRS system in their biofilm model (46). SaeS shows polymorphism across *S. aureus* strains (221). For example, a point mutation in SaeS ($SaeS^P$) present in *S. aureus* Newman leads to hyperactivity of the SaeRS TCS, which was linked to the inability of this strain to form robust biofilms (221, 222). In a recent study, it was also determined that several Sae-regulated virulence genes in *S. aureus* are stochastically expressed in a subpopulation of cells in the nascent biofilm with an expression pattern matching the one of the thermonuclease Nuc (223). In *S. epidermidis*, mutation

of *saeRS* leads to enhanced biofilm formation (224) concomitant with higher Aap expression and eDNA release (225).

The SarA protein family. The staphylococcal accessory regulator SarA is a general transcription factor that binds to AT-rich sequences and activates or represses target gene expression (226, 227). SarA is transcribed on three different overlapping transcripts, initiated by three distinct upstream promoters (P1 to P3) (228). The SarA regulator directly modulates the expression of certain virulence factors and upregulates *agr* expression by bending the *agr* promoter region to enhance AgrA-dimer interaction (226, 229, 230). SarA also induces the expression of *hla*, *fnbA*, and *fnbB* but inhibits the transcription of protein A (*spa*) (216, 226, 231–234). The most important role of SarA regarding biofilm formation is the repression of extracellular proteases. A *sarA* mutant produces high levels of each protease (128, 235), has a decreased capacity to bind fibronectin (236), and is not able to form a static or flow cell biofilm (123). The observed absence of biofilm formation in *sarA* mutants could be restored only by concomitant inhibition of all three classes of proteinases (serine, cysteine, and metalloprotease) (125) or by simultaneous mutation of the four extracellular proteases Aur, ScpA, SspA, and SspB (237). SarA itself is subject to positive regulation by the alternative sigma factor SigB (σ^B) and consequently also by the σ^B regulator RsbU (238–241). These two regulators are discussed in more detail in the section below.

Although *sarA* mutation drastically decreases biofilm formation in most *S. aureus* strains, some exceptions exist. As described above, *S. aureus* strain Newman, for example, has a point mutation in SaeS which results in the constitutive activation of the SaeRS regulatory system and represses some protease production. Therefore, mutation of *sarA* in strain Newman is counterbalanced by the protease repression via the constitutively active SaeRS system, enabling this strain to form biofilms, even when *sarA* is mutated. These observations show that SaeRS and SarA synergistically repress protease production and drive biofilm formation in *S. aureus* (235). In *S. epidermidis*, SarA-mediated regulation of biofilm formation is highly strain dependent. It positively regulates the expression of the *ica* operon, and *sarA* mutations lead to biofilm-negative phenotypes in certain *S. epidermidis* strains (242). However, mutation of *sarA* in a PIA-negative *S. epidermidis* strain induced biofilm formation by overexpression of extracellular matrix-binding protein (Embp) and eDNA production via the upregulation of the metalloprotease SepA, which, in turn, processes the autolysin AtlE (243).

Besides SarA itself, the SarA regulator family also includes SarS, SarT, SarR, SarU, SarX, SarZ, MgrA, and Rot. The latter is known to promote the expression of surface proteins and immunomodulators and represses the production of extracellular toxins and enzymes (244–246). The regulatory activity of Rot is prevented by downregulation of *rot* translation via the QS effector RNAIII (169, 247), which, for example, leads to the activation of staphylococcal superantigen-like proteins (Ssl) in *agr*-negative *S. aureus* strains (248). Mutation of *rot* showed a significant decrease in biofilm formation in many clinical isolates (170). Similar to the observations made for SarA (described above), Rot was also shown to repress protease transcription, and the deletion of protease-encoding genes, as well as treatment with protease inhibitors, restored biofilm formation in a *rot* mutant. In contrast to the above-described *sarA* mutant, the general cysteine protease inhibitor E-64 or staphostatin SspC, a specific inhibitor of staphopain B, is necessary to restore biofilm formation (170). Further evidence suggests that Rot can potentially regulate all four protease loci, but the degree of regulation is highly medium dependent (170, 179, 245, 249). In summary, SarA and Rot, two members of the SarA regulator family, are important regulators of biofilm formation primarily through the repression of protease production. Another important member of the SarA protein family is the global regulator MgrA, which acts as the main effector of the ArlRS TCS (250, 251). MgrA acts as a repressor of eight cell wall-anchored proteins, and mutation of *mgrA* leads to loss of bacterial clumping but also to an increase in biofilm formation. The latter was attributed to the derepression of SasG, a surface protein that promotes intercellular interactions (250, 252) and was implicated in abiotic surface attachment (74).

The alternative sigma factor SigB. The survival and successful adaptation of staphylococci under extreme environmental conditions are possible through the regulation of stress response genes via alternative sigma factors. Sigma factors direct the binding of the catalytic core of the RNA polymerase to specific promoter regions to induce gene transcription. The known *S. aureus* sigma factors are σ^A, σ^B, σ^H, and σ^S, with σ^A being the housekeeping sigma factor for transcription; σ^S seems to be important during starvation and exposure to elevated temperatures, and the alternative sigma factor σ^B acts as a transcription factor in response to different environmental stresses (240, 253–256). The recently identified sigma factor σ^H was indicated to play a role in staphylococcal natural competence regulation (257), but according to a more recent study, it does not regulate all components of the natural competence machinery and therefore can only induce low-level competence for natural genetic transformation (258). σ^B (*sigB*) is expressed from and regulated by the *rsbUVW-sigB* operon. The anti-sigma factor RsbW renders σ^B inactive when the cells are not under environmental stress. RsbU, which dephosphorylates RsbV (anti-anti-σ^B factor), is a positive regulator of σ^B and essential for the activation of σ^B (259). Donegan and Cheung showed that full σ^B activity is also dependent on the upstream *mazE* promoter (P_{mazE}) of the MazEF toxin-antitoxin (TA) system, due to its transcriptional linkage with the downstream *sigB* operon (260). P_{mazE} itself is positively regulated by SarA and downregulated by σ^B (260).

Among the >250 genes regulated by σ^B, genes for several adhesins for early stages of biofilm formation are upregulated (e.g., FnbA and ClfA) and various genes for exoproteins, known to be important for biofilm dispersal (e.g., nuclease and proteases), are repressed by σ^B (241, 255, 261–264). σ^B reduces the RNAIII levels of the Agr system, and it can also influence SarA expression depending on the strain background and environmental conditions (238, 265). Furthermore, σ^B was shown to downregulate the SaeRS TCS in certain strains (266).

It was also shown that σ^B promotes microcolony (267) and biofilm (268) formation. An *S. aureus* mucosal isolate was shown to form a biofilm in an *in vitro* static biofilm model induced by high-salt conditions. A mutation in the σ^B-encoding gene abolished the induction of biofilm formation by osmotic stress, showing that σ^B is crucial for stress-induced biofilm formation (268). In addition, *sigB* mutation has been demonstrated to influence PIA-dependent biofilm formation. It is important to mention that the available literature on PIA-dependent biofilm formation and its regulation by σ^B is contradictory (268–271). This phenomenon can most likely be explained by *S. aureus* strain-specific properties and the use of different media and biofilm systems across these studies. For example, mutation of *sigB* in the methicillin-sensitive mucosal isolate *S. aureus* MA12 correlated with reduced transcription of the *ica* operon (268). In contrast, the biofilm-negative phenotype in a *sigB* deletion mutant in *S. aureus* SH1000 and the USA300 strain LAC, a community-associated methicillin-resistant *S. aureus* (CA-MRSA) isolate, did not correlate with changes in the *ica*-dependent PIA levels but instead was caused by enhanced Agr activity and consequent increased protease activity (126). This might be explained by the fact that both strains have the capacity to form PIA-independent biofilms (178, 241, 272). In a recent study, Valle and colleagues evaluated the contribution of σ^B to biofilm formation in several genetically unrelated *S. aureus* strains. In this study, *sigB* mutants produced higher levels of PIA-dependent biofilms, which correlated with increased accumulation of the IcaC protein, indicating that σ^B acts as a repressor of PIA biosynthesis (271). Besides the aforementioned σ^B-dependent biofilm regulation via the *ica* operon and proteases, σ^B also negatively regulates the expression of the secreted thermonuclease Nuc. Cell-free supernatants of an USA300 *sigB* mutant were able to inhibit biofilm formation of different *S. aureus* strains. Subsequent fractionation and mass spectrometry analysis showed Nuc to be the active component in the supernatant responsible for biofilm inhibition. Deletion of the *nuc* gene in several *S. aureus* strains, including the USA300 lineage, led to enhanced biofilm formation, and mutation of *nuc* in a *sigB* background partially repaired the biofilm-negative phenotype of the *sigB* mutation (71). In *S. epidermidis*, mutation of *rsbU* as well as σ^B leads to decreased biofilm formation. This phenotype is explained by the

upregulation of IcaR and consequent downregulation of the *ica* operon in the aforementioned mutants (273–275). In summary, the alternative sigma factor σ^B exerts regulatory control over several genes involved in the formation of different types of biofilms, including protein-, eDNA-, and PIA-containing biofilms.

The transcriptional repressor CodY. Virulence factor production and biofilm formation can also be regulated by nutritional availability and the metabolic potential of the cell. Gram-positive bacteria can adapt to the stationary growth phase and starvation by regulating their gene expression via CodY, a GTP-sensing pleiotropic transcription repressor (276). More than 100 genes in *S. aureus* are directly or indirectly regulated by CodY (277, 278). Nutrient availability is sensed by CodY through the amount of the effector molecules, branched-chain amino acids (BCAAs; isoleucine, valine, and leucine), and GTP. Under sufficient nutrient conditions, CodY can interact with its effector molecules, leading to a conformational change of CodY, which promotes enhanced affinity of CodY to its DNA binding sites (276). CodY is thought to inhibit transcription by blocking RNA polymerase from binding to the promoter or acting as a roadblock for mRNA transcription (278). Under nutrient-limited conditions, the intracellular concentration of GTP and BCAAs decreases, CodY loses its DNA binding affinity, and CodY-repressed genes are activated. In *S. aureus*, CodY-regulated genes are involved in the primary metabolism, transport, and virulence (277, 278), and notably, CodY strongly represses the *agr* locus (279). *In vitro* binding assays confirmed interaction of CodY not with the P2 and P3 promoter regions of the Agr system but rather with a region within the *agrC* gene (278). A recent study confirmed the elevated expression of the *agr* locus and RNAIII in a *codY* mutant, as well as *in vitro* binding of CodY within the *agrC* gene. Nevertheless, the same study showed that this had no effect on *agrA* expression. In addition, Roux and colleagues observed *in vitro* binding of CodY to the P2 and P3 promoters, although with low affinity (280). Since RNAIII positively regulates transcription and translation of toxins and hemolysins (234, 281), CodY-dependent derepression of RNAIII in a *codY* mutant leads to higher hemolytic activity in culture supernatants (279). CodY was recently shown to repress *rsaD*, a small regulatory RNA (sRNA) that contributes to cell death regulation during weak acid stress (282), potentially leading to eDNA release and biofilm formation.

PIA-dependent biofilm formation is also regulated by CodY, and different biofilm phenotypes were shown in *codY* mutant strains. A transposon insertion in the *codY* gene in the clinical isolate S30 yielded reduced biofilm formation and PIA production (283). In contrast, a *codY* allelic replacement mutation in the two *S. aureus* clinical isolates SA564 and UAMS-1 showed an increase in biofilm formation, most likely resulting from the higher transcription of *icaA* and elevated PIA synthesis during *in vitro* growth (279). CodY-mediated regulation of the *icaADBC* locus is independent of IcaR (279), SarA, and RNAIII (278). In addition to the regulation of PIA-dependent biofilms, CodY represses two important modulators of biofilm formation and maturation, namely, extracellular proteases and *nuc*, the latter of which is indirectly regulated via the repression of the Sae TCS (284, 285). Most recent work from the Brinsmade lab investigated the contribution of CodY to biofilm formation in the clinical isolate SA564, a robust biofilm producer. Based on their results, they suggest that elevated PIA synthesis in cells with low CodY activity is the main contributor to biofilm formation. Moreover, in their model, PIA and eDNA can associate and have a synergistic contribution to biofilm formation rather than being mutually exclusive (286). Taken together, these findings show that the nutritional status of the cell has great influence on biofilm formation and is integrated into the regulatory network of *S. aureus* via CodY.

***ica* operon regulation.** PIA synthesis and regulation of the *ica* operon are governed by a complex regulatory network, the details of which are not completely understood. The *icaADBC* operon is negatively regulated by IcaR and TcaR (90). IcaR is a DNA-binding protein (287) located upstream of the *icaADBC* operon and acts as a repressor of *icaA* transcription in *S. epidermidis* (89) and *S. aureus* (90). A recent report showed that the 3′ untranslated region (3′ UTR) of *icaR* can base-pair with the Shine-Dalgarno

sequence at the 5′ UTR of *icaR*, which interferes with translation, generates a double-stranded RNase III substrate, and consequently leads to reduced IcaR-mediated biofilm repression (288). Synthesis of IcaR is upregulated by the stress response regulator Spx (289) and indirectly downregulated by the AraC/XylS-type regulator Rbf via SarX (290, 291). TcaR, a transcriptional regulator of the teicoplanin-associated locus (292), was shown to act together with IcaR as a repressor of the *ica* operon in *S. aureus* and *S. epidermidis* (90, 293). In contrast, transcription of *icaA* is induced by the TCS SrrAB (staphylococcal respiratory response regulator) under anaerobic growth conditions (294, 295). Besides that, other environmental stresses, including sodium chloride (296, 297) and subinhibitory concentrations of certain protein synthesis inhibitors, such as tetracycline, were shown to upregulate the *ica* operon and induce PIA synthesis in staphylococci (298). In addition, *ica* operon expression was also shown to be activated by SarA (270) and partially by σ^B (268, 297). Going further, transcription of the *ica* locus is regulated by a specific 5-bp motif in the promoter region, and loss of this binding site causes a PIA-overproducing and biofilm-positive phenotype (287). Besides the described regulatory systems, PIA synthesis can be altered by phase variation via the insertion of the IS*256* element into the *ica* operon of *S. epidermidis* and *S. aureus* (299, 300) or through mutation by slipped-strand mispairing in a tandem repeat in *icaC* (301).

An additional layer of regulatory elements are noncoding RNAs, of which several have been shown to participate in *ica* regulation in different staphylococcal species. One such noncoding RNA is *icaZ*, present only in *ica*-positive *S. epidermidis* strains and not in *S. aureus*. This regulatory molecule is a novel long noncoding RNA that was shown to downregulate *icaR* mRNA translation by targeting the 5′ UTR of *icaR* (302). Two additional RNA regulator molecules have been identified very recently. In contrast to *icaZ*, both are small noncoding RNAs, referred to as RsaI and RsaE. In *S. aureus*, RsaI is repressed by CcpA in the presence of high glucose concentrations and derepresses biofilm formation by binding to the 3′ UTR of *icaR* (303). RsaE has an interesting bifunctional role in *S. epidermidis* biofilm development, even though the effect is highly strain specific. The RNA molecule exists in a full-length 100-nucleotide form and a shorter processed form. Processed RsaE interacts with the 5′ UTR of *icaR* and leads to increased PIA production. The unprocessed form, on the other hand, interacts with the antiholin-encoding *lrgA* mRNA, leading to bacterial lysis and eDNA release, and potentially enhances biofilm matrix production (304). Given its unarguable importance for staphylococcal biofilm formation, it is not surprising that the *ica* operon and subsequent PIA biosynthesis are subject to regulation and control by a multitude of different regulatory systems that allow the bacterium to tightly control production of this polysaccharide.

The LytSR two-component system. Cell death and lysis of *S. aureus* are regulated by the LytSR TCS (305) via a bacteriophage-like holin/antiholin system (306–308). The holin is encoded by *cidA* and facilitates cell lysis through increased extracellular murein hydrolase activity (306, 307). Confirming the role of CidA, a *cidA* mutant strain exhibited decreased lysis resulting in lower amounts of eDNA and impaired biofilm formation (202). The antiholin counteracts the activity of CidA and is encoded by *lrgA* (309). Mutation of the *lrgAB* operon results in the formation of more adherent biofilms with a higher eDNA content, demonstrating its role as a CidA antagonist (43). As the Cid/Lrg system controls such vital processes as lysis and cell death, its expression has to be tightly controlled. Several regulatory systems exert control over the Cid/Lrg system, including the LysR-type transcriptional regulator CidR (310), the alternative sigma factor σ^B (311), and, as already mentioned in the beginning of this section, the TCS LytSR (305). The *lrgAB* operon is also regulated by SarA and the Agr QS system (312), as well as by Rot (313). In addition, the presence of certain metabolites can alter the transcription of the Cid/Lrg system (314). The best-studied regulation of the Cid/Lrg system is exerted by the LytSR TCS, which positively regulates the *lrgAB* operon. LytSR is induced by the dissipation of the membrane potential ($\Delta\Psi$), and upregulation of *lrgAB* by alterations in $\Delta\Psi$ is subsequently abolished in a *lytS* mutant. Furthermore, mutation of *lytS* decreases *lrgAB* transcription and leads to a thicker and more adherent biofilm with

more eDNA in its matrix (315). These phenotypes clearly underscore the role of LytSR in regulation of the Cid/Lrg system and biofilm formation. Interestingly, expression of *lrgAB* is specifically upregulated in tower structures of *S. aureus* biofilms (316), and a small fraction of towers formed by a *lytS* mutant still exhibited strong *lrgAB* expression (317). This suggests that a LytS-independent pathway for LytR activation exists in these tower structures (317). The LytSR TCS is also present in *S. epidermidis* strains, and one study showed that mutation of *lytSR* in *S. epidermidis* strain 1457 leads to elevated biofilm formation (318), indicating that LytSR has an as-yet-unexplored role in *S. epidermidis* biofilm formation.

The LuxS/AI-2 system. The LuxS/autoinducer-2 (AI-2) system, originally identified in *Vibrio harveyi* (319, 320), is thought to be an interspecies communication system due to its wide distribution in many bacterial species. LuxS synthesizes AI-2 (321), a furanosyl borate diester (322) that acts as the extracellular signaling molecule of the system. Sequence analysis showed that a functional LuxS/AI-2 system is conserved in different *S. aureus* strains (323). However, several studies have investigated the role of AI-2 in staphylococcal biofilm formation, with disparate results. Two early studies, one for *S. aureus* and one for *S. epidermidis*, identified LuxS as a negative regulator of biofilm formation, and external addition of AI-2 to a *luxS* mutant strain restored the wild-type phenotype (324, 325). These results were attributed to the activation of *icaR* transcription by AI-2, resulting in the downregulation of *icaA* (324). It is also important to note that an *S. epidermidis luxS* mutant was highly impaired in the production of PSMs, which are important for biofilm maturation and dispersal (326). The role of LuxS as a negative regulator of biofilm formation in *S. aureus* was corroborated in a recent study that showed increased PIA production in a LuxS-negative strain, and this phenotype was linked to increased expression of the positive biofilm regulator Rbf (327). In contrast to the aforementioned studies, Xue and colleagues observed downregulation of *icaR* and subsequent upregulation of the *ica* operon by AI-2 in *S. epidermidis* (328). In addition, upregulation of the *ica* operon was observed when the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) was added in micromolar concentrations (328). Yu and colleagues, on the other hand, observed changes in *S. aureus* biofilm formation only when DPD was added in the low nanomolar range; when this signal was provided at higher concentrations, no effect on biofilm formation was observed (324). This shows that despite the discussed role of AI-2 as a universal signaling molecule, the effect of AI-2 on each species might be very different and even concentration dependent.

The second messenger cyclic-di-AMP. In bacteria, signaling nucleotides such as cyclic AMP (cAMP) and guanosine tetraphosphate (ppGpp) are involved in the regulation of carbon metabolism and the stringent response but have also been linked to the expression of virulence genes and biofilm formation (329). Two additional signaling nucleotides, cyclic-di-GMP and cyclic-di-AMP, have gained much attention in the scientific community. The second messenger c-di-GMP is predominantly found in proteobacteria, in which it was shown to regulate a multitude of different processes, including motility (330), cellulose production (331), and biofilm formation (332). In contrast, c-di-AMP signaling is mainly associated with Gram-positive bacteria, especially from the phylum *Firmicutes* (333). Production of c-di-AMP by *S. aureus* was first reported in 2011 by Corrigan and colleagues and is involved in the regulation of cell size, as well as membrane and cell wall stress (334). *S. aureus* synthesizes c-di-AMP from two molecules of ATP by the diadenylyl cyclase (DAC) enzyme DacA and degrades it with the phosphodiesterase (PDE) GdpP (334). Disruption of *dacA* in *S. aureus* was not possible in a screen for essential genes, indicating that c-di-AMP formation is an essential process for *S. aureus* (335). Intracellular c-di-AMP levels are sensed at least by the four receptor proteins KtrA, KdpD, PstA, and CpaA (336). Deletion of *gdpP* in *S. aureus* SEJ1 led to a 3-fold increase in biofilm formation (334), indicating that high c-di-AMP levels foster biofilm formation in this strain. It is not clear if this is a general phenotype of *S. aureus*, because neither *S. aureus* USA300 LAC nor its corresponding *gdpP* mutant was able to form a biofilm under the conditions used by Corrigan and

colleagues (334). In another recent study, several homogenous oxacillin-resistant (HoR) *S. aureus* isolates with either single nucleotide polymorphisms (SNPs) or deletions in *gdpP* showed a decrease in *icaADBC* and *agr* expression and elevated expression of penicillin binding protein 2 (PBP2). These regulatory changes were accompanied by impaired polysaccharide-type biofilm development and a change to a more proteinaceous biofilm (272). Besides its influence on the polysaccharide and protein components of the biofilm matrix, c-di-AMP is also involved in eDNA release; more specifically, mutation of *gdpP* results in reduced levels of eDNA. In accordance with this observation, growth of *S. aureus* under biofilm-inducing conditions results in a GdpP-dependent drop in c-di-AMP levels (337). As different studies have shown different biofilm phenotypes for *gdpP* mutants, the influence of c-di-AMP on biofilm formation is likely to be strain dependent. Furthermore, *dacA* expression is elevated in tower structures of flow cell biofilms as well as in a static biofilm model, compared to planktonic growth. Surprisingly, between 30% and 50% of total c-di-AMP produced in biofilm cultures was found to be extracellular due to autolysis of *S. aureus*, and the extracellular c-di-AMP levels were sufficient to significantly elevate interferon beta (IFN-β) protein expression in an orthopedic implant biofilm infection model (338). In summary, these findings indicate that c-di-AMP levels might play a role in the biofilm matrix composition of staphylococci and could have an important impact on host defenses during chronic biofilm infections.

THE IMPORTANCE OF STAPHYLOCOCCAL BIOFILMS DURING INFECTION

Among the many types of infection that can be caused by staphylococcal species, biofilm-associated ones are the most challenging to treat owing to the increased resistance of staphylococci to antibiotic treatments and the host immune system (16). In adults, *S. aureus* is the major pathogen associated with osteomyelitis (339, 340). After initial biofilm formation, the host immune system attacks the invading bacteria, resulting in host tissue damage that further promotes biofilm development (16). Another common but serious problem is the formation of biofilms on indwelling medical devices (e.g., catheters) and implants (341–343). Usually, the indwelling device or implant readily becomes coated by host matrix, which eventually allows initial attachment and subsequent biofilm formation by *S. aureus* and other staphylococci (344). The major source of device-related infections is cross-contamination during surgery, and in this regard, colonization of the skin or other body sites of the host or health personnel is a major risk factor for implant-associated infections (343, 345, 346). Another type of infection that is very often associated with staphylococcal biofilms is a chronic wound. Staphylococci are among the pathogens most frequently isolated from chronic wounds (347), where they often reside as cellular aggregates, engulfed in a self-produced extracellular matrix (348). In that case, the wound healing process can be substantially prolonged due to secreted factors as well as the strong immune response to the bacteria present in the wound (349–351). According to recent studies, *S. aureus* has also become the leading cause of infective endocarditis (IE) worldwide (352–354). Whereas the role of biofilms in IE is obvious when IE is associated with an implanted cardiac device, involvement of biofilm formation in native valve IE is still under debate (355). Nevertheless, it has been shown that there is a correlation of *in vitro* biofilm formation and the ability to cause persistent bacteremia (356, 357), indirectly associating staphylococcal biofilm formation with IE.

Immune Evasion Mechanism

Staphylococcal biofilms can circumvent immune-mediated clearance by several mechanisms. First, it was proposed that the deficiency of phagocytes to kill staphylococci within a biofilm is mainly due to the physical diffusion barrier of a biofilm where immune cells are barely able to penetrate through the matrix (30). However, this is not supported by *in vitro* data, showing that human leukocytes are able to adhere and penetrate the biofilm matrix, even under conditions mimicking physiological shear (358). To evade the host immune system, *S. aureus* rather relies on directly attacking host immune cells with secreted factors

(e.g., nucleases, PSMs, and hemolysins) and by skewing host immunity toward anti-inflammatory responses that favor staphylococcal persistence (13, 359). It was shown in an *in vivo* model that *S. aureus* biofilms are able to circumvent bacterial recognition pathways, such as Toll-like receptor (TLR) 2 and TLR9 signaling (360). Recent data by Bhattacharya and colleagues show that cells in an *S. aureus* biofilm secrete high concentrations of the leukocidins Panton-Valentin leukocidin (PVL) and γ-hemolysin and that either of these can induce NETosis to avoid clearance by neutrophils (361). NETosis, also referred to as neutrophil extracellular trap (NET) formation, is a mechanism employed by neutrophils that involves the controlled release of chromatin, modified histones, DNA, and granular proteins, resulting in the formation of web-like structures that can entrap and kill planktonic cells but are not able to clear cells within a biofilm (361, 362). In addition, modification of PIA by deacetylation of its poly-*N*-acetylglucosamine molecule is important for biofilm formation but was also shown to mediate resistance to neutrophil phagocytosis and AMPs (87). The immune response against *S. epidermidis* biofilms is generally less pronounced than for planktonic cells. This has been attributed to reduced activation and production of the transcription factor NF-κB and the cytokine interleukin-1β by macrophages (363), reduced antibody-mediated phagocytosis (364), and reduced deposition of the complement protein C3b and immunoglobulin G on the bacterial surface (365). These phenotypes have mainly been linked to the presence of PIA in the biofilm matrix (366), but the exact molecular mechanisms responsible for immune evasion of *S. epidermidis* biofilms are still to be determined.

Antibiotic Resistance

In general, it has been postulated that cells within a biofilm are 10 to 1,000 times more tolerant to antibiotics and AMPs than are planktonic cells (367). Although some antibiotics and AMPs show a partial decrease in their ability to penetrate the biofilm matrix (368–371), the high tolerance of biofilms to therapeutic agents is mainly attributed to the altered cell metabolism within a biofilm (372). Staphylococcal biofilms often show a high degree of cell heterogeneity with distinct specialized subpopulations of cells in the community. Cell heterogeneity can exist in staphylococcal biofilms due to the development of persister cells or different QS expression patterns or, for instance, by the acquisition of antibiotic resistance genes through horizontal gene transfer (373, 374). Indeed, high bacterial cell density within a biofilm community has been suggested to promote the transfer of resistance genes between bacterial cells (375). Besides the genetic diversity in biofilms, these communities are often a reservoir for antibiotic persister cells, which represent a subpopulation of cells that can survive antibiotic treatment without becoming resistant (376). In general, cells within a biofilm often show reduced proliferative and metabolic activity, leading to an increased tolerance to antibiotics targeting the bacterial cell wall, as well as DNA or protein synthesis inhibitors (31–33). Mapping of spatial patterns of DNA replication, protein biosynthesis, and oxygen concentrations in *S. aureus* and *S. epidermidis* biofilms allowed the distinction of four subpopulations: aerobically growing, fermentative growing, dormant, and dead cells (377).

The antibiotic rifampin has a high diffusion rate within biofilms, host cells, and bone tissue as well as bactericidal activity against *S. aureus* and *S. epidermidis* in the stationary phase of growth. Although rifampin is highly effective against staphylococcal biofilms and is often proposed for the treatment of staphylococcal PJIs, there is also a high risk of emergence of rifampin resistance during treatment, and therefore, it is administered only in combination with other antimicrobial agents (378–381). It is also generally assumed that bacterial cells within a biofilm are likely to encounter subinhibitory concentrations of antibiotics, which were shown to potentially stimulate biofilm production and alter the composition of the biofilm matrix (382–384).

ANTIBIOFILM STRATEGIES

The above-mentioned recalcitrance of staphylococcal cells within a biofilm makes it very difficult to effectively eradicate a biofilm once formed. Thus, implant-associated staphylococcal biofilm infections normally require surgical debridement and prolonged

antimicrobial therapy (385). Besides these conventional therapeutic antibiofilm strategies, a broad range of substances with different mechanisms to prevent or eradicate staphylococcal biofilms have been identified. Those molecules either interfere with the Agr QS system (e.g., savirin and apicidin), degrade the biofilm matrix (e.g., recombinant DNase I and Dispersin B), or have bactericidal activity against bacterial cells within biofilms (e.g., lysostaphin and AMPs). Another highly promising strategy is to modify biomaterial surfaces such as catheters and implants to prevent staphylococcal adhesion and subsequent biofilm formation. In that regard, implants coated with surfactants, antibiotics, or antimicrobial peptides alone or in combination have shown promising effects (386–388). Another exciting new strategy to combat staphylococcal biofilms is the application of nanoparticles as delivery systems for conventional antibiotics or in the form of metal nanoparticles with antimicrobial activity. Poly(lactide-co-glycolide) (PLGA) nanoparticles, for example, have been successfully used as delivery systems for the antibiotics levofloxacin and ciprofloxacin to inhibit *S. aureus* biofilm formation (389, 390). Metal nanoparticles have been used in combination with near-infrared light irradiation for photothermal ablation of staphylococcal biofilms (391), and liposomes were shown to be promising delivery vehicles for antibiotics such as levofloxacin (392, 393) and vancomycin, with the latter having activity against mature biofilms (394).

Quorum-Sensing Modulators

Interference with bacterial communication, also called quorum quenching, has developed into a popular and productive research field over the last decades (395, 396). The idea is to identify molecules that specifically target the QS system, leading to attenuation of exoenzyme and toxin production while having no inhibitory effect on bacterial growth in order to limit resistance development. For example, a high-throughput screen was used to identify savirin (*S. aureus* virulence inhibitor), a small-molecule inhibitor of the Agr QS system that enhances host defense and *S. aureus* clearance in a murine skin infection model (397). QS inhibitory activity was also reported for natural products extracted from the leaves of the European chestnut (*Castanea sativa*) (398) and, recently, the fungus-derived apicidin (399). Parlet et al. showed that apicidin successfully prevents *agr* activation and efficiently attenuates MRSA virulence in apicidin-treated mice (399). Another promising strategy is the development of AIP mimetics to inhibit the AgrC receptor. This strategy is based on the fact that there are four Agr classes in *S. aureus*; each synthesizes a unique AIP molecule (AIP-I to AIP-IV) (395, 400), and except for AIP-I and AIP-IV, they show cross-inhibition, also known as Agr interference (22, 164, 401). A series of AIP mimetics based on the structure of AIP-III exhibited AgrC inhibition in all four Agr classes (402, 403). An additional study revealed the structural features important in AgrC-AIP interaction (404) that will eventually stimulate the development of new QS inhibitors. One consequence of Agr inhibition is the development of a thicker biofilm *in vitro*. This likely stems from the inhibition of Agr-regulated PSMs and protease biosynthesis, which are important for biofilm maturation but also foster dispersal of cells from the biofilm (45, 123, 126, 178). Therefore, QS inhibitors could be associated with an increase in *S. aureus* biofilm formation, raising concerns about their impact on chronic infections. Nonetheless, as already noted above, *agr* mutants have a defect in establishing infections like osteomyelitis and endocarditis (185, 186), and thus, QS inhibition might impact the biofilm structure in the infection but also lead to an overall less virulent phenotype. A possible approach to destroy mature *S. aureus* biofilms *in vivo* is the activation of the Agr QS system by the addition of exogenous AIPs, which was shown to lead to complete disassembly of mature biofilms and restoration of antibiotic susceptibility (178, 181).

Bioactive Molecules Interfering with Staphylococcal Biofilms

In general, there are two classes of bioactive molecules that can potentially be used to treat biofilm-associated infections. The first class are biofilm dispersal agents, which can degrade the biofilm matrix, and the second class are biofilm eradication agents, which are capable of killing the bacteria embedded in a biofilm, including metabolically

inactive cells. Among the most promising classes of molecules to fight biofilm infections are biofilm-degrading enzymes. The usability of such enzymes is highly dependent on the composition of the biofilm matrix. For PIA-dependent biofilms, the PIAse Dispersin B was shown to be an effective agent (387, 405, 406), while eDNA-based biofilms can be dispersed by DNase I (43, 181) and protein-based biofilms are susceptible to proteinase K and different proteases secreted by staphylococci themselves, such as staphylococcal serine proteases, staphopains, and the *S. epidermidis* serine protease Esp (24, 133, 181). Importantly, the dispersal process of biofilms leads to the spreading of bacterial cells, which can then lead to a reinfection of the host (407). Therefore, biofilm dispersing agents need to be coadministered with antibiotics in order to effectively combat biofilms and prevent subsequent bacterial regrowth (387, 408). In addition, administration of proteases as biofilm dispersing agents could lead to degradation of nontarget proteins or peptides, resulting in host tissue damage and consequently reducing the therapeutic potential of proteases. For example, the human antimicrobial peptide LL-37 is cleaved by staphopain B (409) and aureolysin (410) and essentially all staphylococcal proteases have known host targets (411), including immune cells, immunoglobulins, and components of the complement system (412–415).

The second class of bioactive molecules to fight staphylococcal biofilms are the biofilm-eradicating molecules. Probably the most prominent member of this class of molecules is lysostaphin, a lysin produced by *Staphylococcus simulans* biovar *staphylolyticus*. It was originally discovered and proposed for the treatment of *S. aureus* infections over half a century ago (416, 417) and has recently garnered attention for its ability to combat staphylococcal biofilms, in combination with antibiotics, in *in vitro* studies and in a mouse catheter model (418–421). A very diverse group of biofilm-eradicating agents are natural and engineered AMPs, which were shown to have a plethora of different modes of action, ranging from pore formation and inhibition of DNA and protein synthesis to the disruption of the biofilm matrix (422, 423). An interesting AMP is the human cathelicidin peptide LL-37, because it not only shows activity against planktonic cells (424) but also exhibits strong antibiofilm activity against preformed *S. aureus* biofilms (425). Although AMPs are very promising candidates for antibiofilm therapy, cases of resistance development have been observed, including proteolytic degradation, alteration of the cell wall, and active secretion (426). Additionally, staphylococci use regulatory systems to increase their resistance to AMPs, one of which is the *S. aureus* TCS GraRS (427). A thoroughly studied class of AMPs are bacteriocins, with some of them showing increased activity against staphylococcal biofilms alone or in synergistic interactions with antibiotics (428–430). For a comprehensive review about different groups of bacteriocins with antibiofilm activity, the reader is referred to the work of Mathur et al. (431) and Newstead et al. (432).

Bacteriophage-Based Antibiofilm Strategies

An alternative strategy to fight biofilm-associated infections is the use of bacteriophages, viruses that specifically infect bacterial cells (433). Historically, bacteriophage therapy against bacterial infections had already been used over 100 years ago, but with the discovery of antibiotics, this form of treatment lost its popularity. However, the rise of multidrug-resistant bacteria has sparked new interest in the potential use of bacteriophages and their products to combat bacterial infections (434). Bacteriophages produce endolysins, which are able to enzymatically degrade the bacterial peptidoglycan, resulting in cell lysis and biofilm clearance (435, 436). There are three possible approaches to eradicate biofilms with bacteriophages: single-phage application, mixed-phage application with two or more phages, and phage-antibiotic combinations. An example that demonstrates the ability of a single phage to disperse staphylococcal biofilms was the recent isolation of phages UPMK_1 and UPMK_2. When applied separately, both phages have promising antibiofilm activity against pregrown *S. aureus* biofilms (437). The successful application of a bacteriophage mixture as an antibiofilm agent was demonstrated by Alvez and colleagues (438). They reported the isolation of a novel bacteriophage, DRA88, which in combination with bacteriophage K was

demonstrated to strongly reduce the biofilm mass of *S. aureus*, including MRSA clones, and CoNS. In addition, combinatorial therapy with phages and antibiotics has shown promising results to eradicate biofilms of drug-resistant bacteria without the emergence of new antibiotic resistance (439–441). Another interesting approach is the use of phage-derived enzymes capable of dispersing bacterial biofilms. Recently, LysCSA13, an endolysin encoded by the bacteriophage CSA13, was shown to decrease staphylococcal biofilm mass up to 90% on several surfaces, including polystyrene, glass, and stainless steel (442, 443). Similar results were reported for the bacteriophage lysins LysGH15 and CF-301, and the latter also showed promising activity against bacterial mixed-species biofilms formed by *S. aureus* and *S. epidermidis* (444, 445). The two major advantages of phage therapy to treat biofilm-associated infections are the capability of phages to infect and kill biofilm embedded bacteria (433, 446) and their relatively narrow host range, which prevents the undesirable killing of bacterial commensals during treatment (447, 448). Nevertheless, a recent study determined that the amount of biofilm mass, strain susceptibility, initial phage titer, phage entrapment in the extracellular matrix, and phage inactivation are all factors contributing to successful biofilm penetration and need to be considered during the design of an effective phage-based therapy (446).

CONCLUDING REMARKS

Staphylococci, most prominently *S. aureus* and *S. epidermidis*, exist as multicellular communities or so-called biofilms in their natural habitats. When growing in a biofilm, the bacteria are encased in an extracellular matrix and display an altered metabolic state compared to that of planktonic cells (449), which leads to increased resistance to host defenses and antibiotic treatment (33). In order to coordinate the formation of biofilms, staphylococci rely on an extensive network of regulatory systems enabling the bacteria to sense and respond to an abundance of biotic and abiotic factors. The staphylococcal QS system is one of the best-studied regulatory systems and allows the bacteria to use the biofilm as a protected reservoir that serves as an initiating point for colonization of new body sites. As outlined in this review, the staphylococcal QS system is subject to regulation by many environmental factors and host components, in addition to physicochemical factors, like mechanical shear stress from fluid flow. Most interestingly, a recent study has shown that QS follows a heterogeneous activation pattern under flow conditions (212). Under stronger flow conditions, QS is essentially turned off, but the presence of areas of no or limited fluid flow, like small crevices or inside specific biofilm regions, allows the accumulation of AIP signals and subsequent activation of the staphylococcal QS system. An additional example of bimodal QS was recently published by García-Betancur and colleagues. They showed that *S. aureus* populations consist of two distinct subpopulations, one with QS turned on and one having it turned off. A high concentration of environmental magnesium triggers the downregulation of the QS system and most likely promotes *S. aureus* biofilm formation (450). These examples show that even though the connection between QS and biofilm formation has been studied extensively, there are still many aspects to investigate. For example, it is still not clear how different infection sites in the host can influence biofilm formation, either through the staphylococcal QS system or through other regulatory systems, and which environmental cues contribute to staphylococcal biofilm-associated infections.

In addition, multispecies biofilms more closely reflect the complex bacterial communities that are usually found in the host instead of single-species biofilms. For example, *S. aureus* and *P. aeruginosa* are frequently coisolated from chronic wound infections and cystic fibrosis lungs (347, 451–453), which represent types of infections associated with biofilm formation. Not surprisingly, the interaction between staphylococci and other microbial species has profound effects on antimicrobial resistance and the spatial organization within the biofilm. Several studies have shown that during mixed-species biofilm growth, although being present in the biofilm together, the participating species are spatially segregated and inhabit different niches within the

biofilm (454, 455). Regarding alterations of antimicrobial resistance during mixed-species biofilm formation, *S. aureus* biofilms, when grown in the presence of cell-free *P. aeruginosa* supernatant, show increased sensitivity to cell wall-targeting antibiotics but also gain resistance to vancomycin (456, 457). In contrast to these observations, when *S. aureus* is grown with *P. aeruginosa* in a multispecies biofilm, *S. aureus* shows higher sensitivity to several classes of antibiotics (458). The ability of *S. aureus* to form interspecies biofilms is not limited to bacterial species but was also observed with the fungus *Candida albicans*. In these mixed-species biofilms, *S. aureus* forms microcolonies on top of the *C. albicans* biofilm and becomes more resistant to vancomycin (459–462). Considering that infections of the cystic fibrosis lung are always of a polymicrobial nature, it is reasonable to assume that *S. aureus* can also form mixed-species biofilms with several other bacterial and fungal species which have not been investigated yet.

Therefore, it will be crucial for future development of antibiofilm agents to incorporate mixed-species biofilm studies and to have a stronger focus on *ex vivo* and *in vivo* biofilm systems in order to understand which aspects of the already available knowledge on *in vitro* biofilms can be directly transferred to *in vivo* situations.

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Katrin Schilcher is a postdoctoral fellow at the University of Colorado Anschutz Medical Campus. She obtained her B.Sc. and M.Sc. in molecular biology and molecular microbiology from the University of Graz, Austria. She then moved to Switzerland and received her Ph.D. from the University of Zurich in the laboratory of Dr. Annelies Zinkernagel, where she studied antibiotic-mediated modulation of *Staphylococcus aureus* biofilm formation and host-pathogen interaction. After completion of her Ph.D., Katrin joined the laboratory of Dr. Alexander Horswill to investigate the role of linear peptides in staphylococcal cell-cell signaling and host-pathogen interactions. She unraveled a new biosynthetic pathway for linear peptide processing and export from *Staphylococcus aureus* and is currently continuing her work on linear peptide signaling. Her research has been supported by an Early Postdoc Mobility fellowship from the Swiss National Science Foundation and a Postdoctoral Fellowship from the American Heart Association.

Alexander R. Horswill obtained his Ph.D. in 2001 from the University of Wisconsin-Madison studying *Salmonella* short-chain fatty acid metabolism under the direction of Professor Jorge Escalante-Semerena. From 2001 to 2005, Dr. Horswill was a Damon-Runyon postdoctoral fellow in the laboratory of Dr. Stephen Benkovic in the Department of Chemistry at Penn State University. In 2005, Dr. Horswill joined the Department of Microbiology at the University of Iowa as an Assistant Professor. He started a research program focused on mechanisms of *Staphylococcus aureus* quorum sensing, biofilm development, and host-pathogen interactions. In 2017, Dr. Horswill relocated his research group to the University of Colorado Anschutz Medical Campus, and currently he is a Full Professor in the Department of Immunology & Microbiology.