The Staphylococcal Biofilm: Adhesins, Regulation, and Host Response

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ABSTRACT The staphylococci comprise a diverse genus of Gram-positive, nonmotile commensal organisms that inhabit the skin and mucous membranes of humans and other mammals. In general, staphylococci are benign members of the natural flora, but many species have the capacity to be opportunistic pathogens, mainly infecting individuals who have medical device implants or are otherwise immunocompromised. Staphylococcus aureus and Staphylococcus epidermidis are major sources of hospital-acquired infections and are the most common causes of surgical site infections and medical device-associated bloodstream infections. The ability of staphylococci to form biofilms in vivo makes them highly resistant to chemotherapeutics and leads to chronic diseases. These biofilm infections include osteomyelitis, endocarditis, medical device infections, and persistence in the cystic fibrosis lung. Here, we provide a comprehensive analysis of our current understanding of staphylococcal biofilm formation, with an emphasis on adhesins and regulation, while also addressing how staphylococcal biofilms interact with the immune system. On the whole, this review will provide a thorough picture of biofilm formation of the staphylococcus genus and how this mode of growth impacts the host.

INTRODUCTION

The Staphylococcus genus includes a diverse group of commensals that colonize mammals on the skin or mucous membranes. Some of the best-known members of this genus, such as Staphylococcus aureus and Staphylococcus epidermidis, are also opportunistic pathogens and are responsible for a tremendous burden on the health care system (1, 2). One of the reasons staphylococci are problematic is their well-known ability to attach to surfaces and develop into recalcitrant community structures, often referred to as “biofilms.” Generally, biofilms are defined as communities of cells encased within an exopolymeric matrix and attached to a surface, and they are recognized as being resistant to antimicrobial therapy and host defenses (3).

The biofilm state was initially observed in studies of marine environments, in which adherent communities of bacteria were found in natural as well as industrial aquatic environments (4). Biofilm development was subsequently found to be important in many types of infections and is now a widely accepted bacterial mode of growth. According to the NIH, as many as 80% of human infections are biofilm-based (5). Biofilm infections present a clinical challenge, because they are highly resistant to antimicrobial therapies and often occur in areas of the body that are not easily accessible for treatment (6, 7). Staphylococci, in particular, represent a large portion of biofilm-based infections. S. aureus and the coagulase-negative staphylococci are the number 1 and number 3 most common etiological agents...
of hospital-acquired infections in the United States, respectively, including infections of medical devices and surgical wounds (8). Biofilm infections clearly are a significant burden on the health care system today, and the in vivo biofilm state is an important area of study.

Definition of a Biofilm
The growth of staphylococci in a biofilm has been linked to many types of infections, but one of the ongoing challenges in the field is the lack of a consensus description of the biofilm state. There is no universal agreement on what constitutes a “staphylococcal biofilm” in terms of morphology, depth, surface coverage, regulatory state, antibiotic resistance level, or whether surface attachment is even necessary. In the field, a biofilm is defined mostly by subjective observations, as well as high antibiotic resistance relative to planktonic bacteria. There have been attempts to identify biomarkers of staphylococcal biofilm formation to provide a better definition. In one promising study, Secor et al. determined that the nonribosomally generated peptide aureusimine (phevalin) was produced in higher levels by biofilm-grown S. aureus (9), suggesting that this natural product could be a biofilm biomarker. While this finding is encouraging, there is not yet enough follow-up work on aureusimine or other potential biomarkers to reach a consensus.

The other ongoing challenge in defining biofilms is the enormity of growth states that have been linked to this term. Staphylococcal biofilm growth has been linked to foreign bodies (10), endocarditis (11), osteomyelitis (12), skin infection (13), colonization (14), cystic fibrosis (15), urinary tract infection (UTI) (16), and abscess communities (17). Under such a large umbrella of different growth conditions in the host, each requiring a unique suite of bacterial factors and regulatory machinery, it is impossible to obtain a universal definition of a staphylococcal biofilm that will be agreed upon in the field. Not surprisingly, the staphylococcal requirements to develop infective endocarditis or a skin abscess, such as specific toxins and superantigens (18, 19), are not the same as those needed for an indwelling catheter infection that can be caused by many types of staphylococci. Further, a much lower bacterial load (estimated at 10,000-fold lower) is needed to colonize a foreign body than to cause a skin abscess (20). The reason for this is likely the lack of vascularization at the site and, presumably, a reduced presence of innate immunity factors (10). Considering this point, it seems logical that the virulence factor profile of the invading bacterial pathogen will be different to survive these varied host environments. As one example, S. aureus strains deficient in the agr quorum-sensing system are unable to properly initiate infective endocarditis or osteomyelitis (21, 22), while the same regulatory system is not essential to initiate a staphylococcal foreign body infection (23); in fact, the agr system seems to inhibit colonization of the foreign body (24). Thus, it is increasingly important to consider the context of infection when comparing and contrasting results with other studies.

The Biofilm Life Cycle
The biofilm life cycle is thought to consist of at least three stages (see Fig. 1): initial attachment to an abiotic or biotic surface, maturation of the biofilm, and dispersal. Some consider “microcolony formation” to be an intermediate step between attachment and maturation, but the precise differences between a microcolony and a mature biofilm are not clearly defined. Attachment involves bacterial adhesins that can stick to the surface, while maturation is mediated by cell-cell adhesion, although some adhesins possess both properties. Dispersal or disassembly is mediated by enzymes that degrade the biofilm matrix (25–27). These enzymes may be produced by the bacteria itself or be present in the environment.

A recent paper provided new insights into the stages of early biofilm development using a microtiter flow-based biofilm system (28). This study found that in an S. aureus biofilm, attachment and early accumulation were followed by dispersal of a portion of the cells, leaving behind small foci of biofilm. These foci then matured into a biofilm with tower structures. Interestingly, the early dispersal phase, termed “exodus,” was independent of the agr system but required the sae system and was specifically modulated by the sae-regulated nuclease. These findings provide novel insight into S. aureus biofilm development and the independent roles of staphylococcal regulatory systems in the biofilm life cycle.

Biofilm Matrix
The staphylococcal biofilm matrix has been a topic of interest in a number of reports, and various findings have demonstrated its heterogeneity and variability (29). The biofilm matrix contains extracellular DNA (eDNA), both from lysed bacteria and potentially from host neutrophil cell death, and is susceptible to dispersal by DNAases (30, 31). Proteinaceous adhesins have also been identified in the staphylococcal biofilm matrix. These
may be directly associated with bacteria in the biofilm or be free in the biofilm matrix (32). A number of cytoplasmic proteins have been identified that appear to moonlight as matrix components and undoubtedly have an important function (33). Finally, the extracellular polysaccharide intercellular adhesin (PIA) has also been identified as a major component of the staphylococcal biofilm, especially in certain strains of S. epidermidis (34). Proteinaceous and polysaccharide-based biofilms are susceptible to disassembly by proteases and polysaccharide-degrading agents (26, 27). Teichoic acids have also been implicated in the biofilm matrix (35), although their relative role in biofilm mechanisms has not received as much attention. Presumably, other cellular components are also present and awaiting further investigation.

**Coverage of this Review**

This review will cover recent advances in staphylococcal biofilm studies. We will discuss the mechanism of biofilm formation by staphylococcal adhesins and regulatory systems, as well as the interaction of biofilms with the host immune system, with a focus on S. aureus and S. epidermidis as the model pathogens of the genus. Finally, we will discuss the current knowledge of biofilm formation and virulence in other species of staphylococci.

### STAPHYLOCOCCAL ADHESINS

Staphylococci possess a number of surface-associated adhesins that mediate initial attachment of biofilm cells as well as intercellular adhesion during biofilm maturation (32, 36). The S. aureus genome encodes more than 20 adhesins (32, 36, 37), while coagulase-negative staphylococci have significantly fewer (38, 39). Staphylococcal adhesion and biofilm accumulation are mediated by covalently anchored cell wall proteins, noncovalently associated proteins, and nonprotein factors. The general properties of these adhesins are presented in this section, and their functions within biofilm development are included where information is available.

### Covalently Linked Cell Wall–Anchored (CWA) Proteins

Staphylococcal CWA proteins are secreted by the Sec system and share a C-terminal cell wall anchoring motif, hydrophobic domain, and positively charged domain (40). In the majority of CWA proteins, cell wall anchoring is mediated by sortase A, which cleaves the LPXTG cell wall anchoring motif at the threonine-glycine junction and catalyzes the covalent linkage of the CWA protein to peptidoglycan (41). Some Isd proteins in the NEAT (near iron transporter) family are instead anchored by sortase B at the NPQTS/PNS motif (40). The staphylococcal CWA proteins were recently
ClfA has been shown to inhibit 87, 37, 56, 114 44, and both are upregulated in bio 80, 138, 84, 97, 53. In a murine 57, 88, 133, 103, the ClfA IgG-like fold 85, 91, 133, 103. The ClfA IgG-like fold 45, 122, 65, 93. Table 1 and 52, 80, 138, 103.

MscrAMMs were originally defined as a broad category of proteins that are cell surface–associated and able to interact with the host extracellular matrix (42). The recent definition proposed by Foster et al. limits the term MscrAMM to adhesins that contain at least two IgG-like folds and employ a ligand-binding mechanism called “dock, lock, and latch” (40). The staphylococcal MscrAMMs are the Clf-Sdr family proteins, including Bbp (bone sialoprotein-binding protein), the FnBPs (fibronectin-binding proteins), and CNA (collagen adhesion). Exposure of S. aureus to human plasma in vitro enhances both MscrAMM expression and biofilm formation, suggesting the importance of their role in in vivo biofilm infections (43).

The Clf-Sdr family consists of clumping factor A (ClfA), clumping factor B (ClfB), and the Sdr proteins. In addition to the IgG-like folds, their structure contains a serine-aspartate repeat domain called the SD region (40). ClfA and ClfB are fibrinogen-binding proteins in S. aureus (40, 44), and both are upregulated in biofilm growth relative to planktonic growth (43). Rot and agr affect bacterial binding to fibrinogen by regulating clfB but not clfA (46) (see regulation section).

ClfA is present on the cell wall throughout the growth cycle (47) and promotes bacterial clumping in solution with fibrinogen as well as bacterial attachment to immobilized fibrinogen (48, 49). The ClfA IgG-like fold domains N2 and N3 bind at the C-terminal region of the γ-chain of fibrinogen, a region that also contains platelet binding sites (50, 51). ClfA has been shown to inhibit fibrinogen binding to platelets and fibrinogen-dependent platelet aggregation, indicating that its binding site includes the platelet binding site (50, 52, 53). In a murine model of S. aureus septicemia, mice lacking the ClfA-binding motif of fibrinogen had better survival, suggesting that ClfA-fibrinogen interactions in the blood contribute to virulence (54). A clfA mutant in S. aureus had a decreased ability to cause vegetations in a rat endocarditis model (55) and reduced bacterial load in a murine abscess model (56).

### Table 1 Categorized cell wall-anchored adhesins

<table>
<thead>
<tr>
<th>Family</th>
<th>Subfamily</th>
<th>Adhesin</th>
<th>Species</th>
<th>Ligand(s)</th>
<th>References</th>
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<tbody>
<tr>
<td>MscrAMMs</td>
<td>Clf-Sdr</td>
<td>ClfA</td>
<td>Staphylococcus aureus</td>
<td>Fibrinogen γ-chain</td>
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<td>SdrG/Fbe</td>
<td>Staphylococcus epidermidis</td>
<td>Fibrinogen β-chain</td>
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<td></td>
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<td>Fbl</td>
<td>Staphylococcus lugdunensis</td>
<td>Fibrinogen γ-chain</td>
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<td></td>
<td></td>
<td>ClfB</td>
<td>S. aureus</td>
<td>Fibrinogen α-chain, cytokeratin 10, cytokeratin 8, loricrin</td>
<td>45–48, 56, 70–79</td>
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<td></td>
<td></td>
<td>SdrC</td>
<td>S. aureus</td>
<td>β-neurexins, self-association</td>
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<td>SdrD</td>
<td>S. aureus</td>
<td>Nasal epithelial cells</td>
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<td>S. aureus</td>
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<td>S. epidermidis</td>
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<td>S. epidermidis</td>
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<td>Staphylococcus capitis</td>
<td>Type VI collagen</td>
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<td>SdrI</td>
<td>Staphylococcus saprophyticus</td>
<td>Collagen, fibronectin</td>
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<td>S. aureus</td>
<td>Fibrinogen, elastin, fibronectin</td>
<td>103–113</td>
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<td></td>
<td>FnBPB</td>
<td>S. aureus</td>
<td>Fibrinogen, elastin, fibronectin</td>
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<td>CNA</td>
<td>S. aureus</td>
<td>Collagen</td>
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<td>NEAT motif</td>
<td>IsdA</td>
<td>S. aureus</td>
<td>Heme, fibrinogen, fibronectin</td>
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<td>S. aureus</td>
<td>Fc region of IgG</td>
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<td>G5-E</td>
<td>SasG</td>
<td>S. aureus</td>
<td>Nasal epithelial cells, self-association</td>
<td>133, 137, 138</td>
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<td>Aap</td>
<td>S. epidermidis</td>
<td>Corneocytes, self-association</td>
<td>133, 136, 138–140</td>
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The *S. epidermidis* MSCRAMM SdrG (also called Fbe) is homologous to *S. aureus* ClfA, although it binds to the β-chain of fibrinogen rather than the γ-chain \(^{(57, 58)}\). SdrG is the archetypal example of the dock, lock, and latch mechanism of binding using its IgG-like folds. The dock, lock, and latch model was proposed based on the crystal structure of the SdrG-fibrinogen interaction \(^{(59, 60)}\). SdrG mediates adherence to fibrinogen-coated surfaces \(^{(61, 62)}\) and is required for fibrinogen-dependent platelet aggregation \(^{(63)}\). In a rat model of central venous catheter infection, wild type *S. epidermidis* was more likely to cause infection and formed a more robust biofilm on the catheter in vivo than an sdrG mutant, indicating its importance in the in vivo biofilm formation of *S. epidermidis* \(^{(64)}\).

The coagulase-negative species *Staphylococcus lugdunensis* also has a ClfA homolog called Fbl \(^{(65, 66)}\). Fbl promotes both adherence to fibrinogen-coated surfaces and cell clumping in fibrinogen-rich solution \(^{(66)}\). Fbl and ClfA have similar binding affinities to fibrinogen, and both interact with the C-terminus of the γ-chain \(^{(67)}\). Fbl also is used as a species-specific detection method for *S. lugdunensis* \(^{(68, 69)}\).

Like ClfA, the *S. aureus* ClfB IgG-like fold region binds to fibrinogen; however, it interacts with the α-chain of fibrinogen rather than the γ-chain \(^{(48, 70)}\). ClfB promotes adherence to immobilized fibrinogen as well as *S. aureus* clumping in fibrinogen-rich solution \(^{(48, 70)}\). In contrast to ClfA, ClfB becomes depleted from the cell wall beginning in the late exponential phase, suggesting that it is susceptible to proteolytic degradation \(^{(48)}\). Further studies revealed that the metalloprotease aureolysin cleaves ClfB at two sites, resulting in the loss of fibrinogen binding \(^{(71, 72)}\). ClfB promotes biofilm formation in vitro, and aureolysin treatment disrupts ClfB-mediated biofilms, suggesting that aureolysin might facilitate biofilm dispersal by processing ClfB \(^{(73)}\). In vivo, ClfB is required for full virulence in a rat endocarditis model of infection, although the phenotype of the clfB mutant in this model was slight \(^{(74)}\). A clfB mutant also had decreased bacterial load in a murine abscess model \(^{(56)}\).
In addition to fibrinogen, ClfB also binds to the human epithelial proteins cytokeratin 10, cytokeratin 8, and loricrin. ClfB bound to the C-terminal tail region of purified cytokeratin 10, as well as cytokeratin 10 that was natively expressed in desquamated nasal epithelial cells (70, 75, 76). The ClfB IgG-like folds bind cytokeratin 10 by the dock, lock, and latch mechanism (70). Similarly, ClfB bound immobilized cytokeratin 8 and endogenous cytokeratin 8 from lysates of the HaCaT keratinocyte cell line (77). ClfB also binds loricrin, the primary protein in the cornified envelope of the stratum corneum, which is present in the anterior nares. In a murine model of nasal colonization, clfB mutant S. aureus was defective for colonization compared to wild type. Colonization by both wild type and clfB mutant S. aureus was also decreased in loricrin-deficient mice, suggesting that loricrin is a critical ligand for S. aureus nasal colonization (78). In an experimental model of human nasal colonization, clfB mutant S. aureus was eliminated significantly more quickly from the nares than its wild type parent, and ClfB was required for long-term colonization (79). These results demonstrate ClfB’s versatility in ligand binding and its importance for S. aureus colonization.

S. aureus Sdr proteins SdrC, SdrD, and SdrE are encoded in a single locus and have striking similarity in sequence and structural arrangement with the Clf proteins (80). SdrC can bind a host ligand as well as self-associate to promote biofilm formation. A study using a phage display peptide library found that SdrC binds to human β-neurexins, which are expressed on neuronal cells (81). Although the effect of this has not been tested in vivo, S. aureus endocarditis and sepsis are associated with polyneuropathy, meaning that SdrC interactions with neurexins could contribute to S. aureus pathogenesis. A later study showed that SdrC self-associates at the N2 domain and promotes biofilm formation, a process that is inhibited by Mn2+ (82). In contrast to SdrC, the structural stability of SdrD appears to require binding to divalent cations, specifically Ca2+ (83). Like SdrC, SdrD mediates adherence to human desquamated nasal epithelial cells (84). SdrE in S. aureus (80) induces platelet aggregation (85). SdrE also inhibits complement activation by two mechanisms. It binds the complement regulatory protein factor H, which inhibits activation of the alternative complement pathway (86). SdrE also inhibits classical complement pathway-mediated opsonization and phagocytosis by binding to the classical complement regulator C4b-binding protein (87).

SdrF in S. epidermidis binds type I collagen, mediated by its B domain repeats (88). Interestingly, SdrF was also found to bind with high affinity to Dacron, the polymeric surface of drivelines that are used in ventricular assist devices (VAD) for end-stage congestive heart failure. Since S. epidermidis is a common etiological agent of medical device infections, including VAD infections, this finding is relevant to its pathogenesis. Anti-SdrF antibodies decreased infection in a murine model of S. epidermidis VAD driveline infection, suggesting possible therapeutic interventions for these infections (89). SdrF also was found to bind other plastic materials based on ionic interactions (90). These results suggest that inhibition of SdrF binding to prosthetic devices may be a promising avenue for treatment of S. epidermidis infections.

S. epidermidis also has SdrG and SdrH, whose sequences are similar to typical SD proteins but are not present in cell wall preparations of S. epidermidis, indicating that they may be improperly sorted to the cell wall. However, antisera from patients following infection with S. epidermidis were reactive to the A domains of SdrG and SdrH, suggesting that they are expressed during infection (91).

Other coagulase-negative staphylococci possess Sdr family proteins that have been characterized in limited detail. Staphylococcus capitis SdrX has an SD repeat region, although the N-terminal domain is not strongly similar to S. aureus Sdr proteins. SdrX was reported to bind type VI collagen and mediate bacterial adherence to a type VI collagen-coated surface (92). Staphylococcus saprophyticus has the Clf-Sdr family protein SdrI, which binds collagen (93) and fibronectin (94), the latter of which is a unique property among the Sdr proteins. SdrI was also found to contribute to the hydrophobicity of the S. saprophyticus surface, a property that is known to enable bacterial adherence to host epithelia (95). In a murine model of UTI, SdrI was critical for persistence but not initial colonization (96).

Bbp (bone sialoprotein-binding protein) is an Sdr-family protein in S. aureus that is considered to be an allelic variant of SdrE (87). S. aureus isolates from osteomyelitis infections were observed to bind bone sialoprotein by an unknown adhesin (97), which was eventually identified to be Bbp (98, 99). Like SdrE, Bbp binds the classical complement regulator C4b-binding protein, inhibiting classical-pathway-mediated opsonization and phagocytosis (87). Bbp appears to be associated with invasive osteomyelitis infections in particular. In 60 patients with deep S. aureus infections following orthopedic surgery, 95% of the isolates were positive for the bbp gene (100), while of 53 S. aureus isolates from bloodstream infections, 47% were positive for bbp (101).
The presence of antibodies to Bbp was also shown to be effective in distinguishing osteomyelitis from soft tissue infections in patients with diabetic foot ulcers (102).

FnBPs

*S. aureus* has two fibronectin-binding proteins, FnBPA and FnBPP, which are encoded by *fnbA* and *fnbB*, respectively (103). Like the Clf-Sdr family proteins, the FnBPs contain an N-terminal region that forms IgG-like folds; these domains bind fibrinogen and elastin (104, 105). In place of the SD repeat region, the FnBPs have a region of 10 to 11 tandem repeats that recognize fibronectin (106). FnBP binding to fibronectin induces bacterial invasion into epithelial cells, endothelial cells, and keratinocytes (107–109). The FnBPs have been found to affect biofilm formation and virulence. In *S. aureus*, a double knockout of *fnbA* and *fnbB* lost the ability to bind fibronectin and to form biofilms on microtiter plates and under shear flow conditions. Complementation of either *fnbA* or *fnbB* alone on a plasmid restored these phenotypes, as well as the ability of *S. aureus* to agglutinate (110). The FnBPs are thought to promote biofilm formation by a self-association mechanism that is distinct from ligand binding, making them multifunctional in the *S. aureus* biofilm life cycle (111, 112). The FnBPs also were shown to enhance virulence in an experimental model of endocarditis (113).

The collagen-binding adhesin CNA was initially reported to be necessary and sufficient for *S. aureus* binding to the collagen-rich substrate cartilage (114). CNA consists of an A domain with several collagen-binding sites and a domain containing B repeats (114, 115). Crystal structure characterization of the CNA-collagen interaction suggested a “collagen hug” model, a variation of the dock, lock, and latch ligand-binding scheme (115). CNA blocks activation of the classical complement pathway (116) and contributes to virulence of *S. aureus* keratitis (117), osteomyelitis (118), septic arthritis (119), and endocarditis (120).

*S. epidermidis* also has a collagen-binding protein, the GehD lipase. GehD is not LPXTG-anchored, although it is cell wall–associated, and its structure does not resemble CNA of *S. aureus*. However, purified GehD binds to immobilized collagen and mediates *S. epidermidis* binding to collagen and therefore may contribute to colonization or pathogenesis (121).

The NEAT motif family consists of the Isd (iron–regulated surface determinant) proteins. These CWA proteins bind heme or hemoglobin, facilitating its transport into the bacterial cell, and they are upregulated in iron limiting conditions (122). *S. aureus* IsdA and IsdC bind heme via their NEAT motifs (Fig. 2) (123). These proteins also play a role in survival against host immune defense. *S. aureus* IsdA is the most abundant CWA protein in iron starvation conditions and also decreases surface hydrophobicity, which makes *S. aureus* more resistant to bactericidal fatty acids and peptides in human skin (124). IsdA also is able to bind human fibrinogen and fibronectin (125). In a murine model of systemic infection, *S. aureus* *isdB* expression varied among organs to which bacteria localized in the host, and *IsdB* was required for colonization of the heart (126). This suggests that *isd* expression depends upon iron availability in each host niche of infection. In *S. lugdunensis*, IsdC was found to induce biofilm formation under iron-limiting conditions and to induce attachment to polystyrene as well as self-associate to promote intercellular adhesion (127).

The sole three-helical bundle cell wall–anchored protein is staphylococcal protein A (SpA), which is present in all strains of *S. aureus* and whose sequence variation is used for strain typing (128). SpA binds the conserved Fc region of immunoglobulin IgG, which allows immune evasion (129, 130) and has also been found in the biofilm matrix in vitro (131). SpA is also released from the cell wall, and released SpA has been shown to promote bacterial survival in human blood, suggesting that free SpA contributes to disruption of the host immune response (37, 132). Presumably, free SpA could also provide adhesion in the biofilm matrix.

**G5-E Repeat Family: Aap/SasG**

G5-E repeats are found in cell wall–anchored adhesins in Gram-positive organisms, and are so named because of the five conserved glycine residues in each repeat. G5 domains consist of 78 residues and form 6 beta strands, with E domain spacers that are of similar sequence to G5, but only 50 residues (133). The *S. aureus* G5-E repeat protein SasG and its *S. epidermidis* homolog Aap have similar structures and are thought to function similarly in adhesion and biofilm formation. SasG and Aap each have an N-terminal A domain that mediates attachment to abiotic and host surfaces via unknown ligands. In *S. epidermidis* Aap, the A domain alone promotes attachment to polystyrene (134, 135) as well as to human corneocytes (136). *S. aureus* SasG promotes attachment to human desquamated nasal epithelial cells via its A domain (137). Multiple studies have shown that the G5-E repeats of SasG and Aap are able to dimerize by binding to Zn²⁺, forming a “twisted rope” structure (133, 138). This property is thought to enable intercellular adhesion when adjacent SasG or Aap proteins...
dimerize via their G5-E domains. In S. epidermidis, Aap has been shown to induce biofilm formation following proteolytic removal of its A domain by exogenous proteases, although the S. epidermidis proteases that may process Aap are not identified (139). Aap has also been shown to be required for full in vitro biofilm formation in S. epidermidis, as well as for virulence in a rat catheter model of infection (134). Recently, a small 18-kDa scaffolding protein, called small basic protein, or Sbp, was found in the S. epidermidis biofilm matrix and affects both PIA-dependent and Aap-dependent biofilm formation. In Aap-mediated biofilm formation, Sbp was found to interact with the B domain of Aap in the biofilm matrix, suggesting its role as a structural component of the biofilm (140).

Uncategorized CWA Proteins

The remaining un categorized cell wall–anchored proteins are Bap and several Sas proteins, including SasA/SraP. Bap is an S. aureus cell wall–anchored protein that was identified in a transposon screen for mutants defective in biofilm formation (141). It has a unique structure consisting of three major domains, each with sequence homology to different cell wall–anchored proteins in other bacterial genera. Bap was found to mediate attachment to an abiotic surface as well as intercellular adhesion, making it a potent enhancer of biofilm formation. In a murine model of catheter infection, the bap mutant had decreased bacterial load. Bap is also present in several coagulase-negative staphylococci, and a mutant of the bap homolog in S. epidermidis had decreased biofilm formation relative to wild type (142). A later study demonstrated that Bap promoted adherence, but inhibited invasion, of epithelial cells in vitro by binding the Gp96 receptor (143). The authors propose that this property of Bap enhances virulence of biofilm-based infections by resisting bacterial engulfment into host cells, although this has yet to be directly tested.

S. aureus SasA/SraP (serine-rich adhesin for platelets) is a member of the serine-rich repeat family of cell wall–bound proteins found in several Gram-positive pathogens, primarily oral streptococci (144, 145). Homologs of SraP have also been identified in the coagulase-negative staphylococcal species S. epidermidis and Staphylococcus haemolyticus (146, 147). SraP is a sortase-anchored, cell wall–bound adhesin that binds platelets, and it also possesses a ligand-binding domain that is thought to promote intercellular adhesion and biofilm formation. A sraP mutant was reported to have decreased biofilm formation, and SraP bound to S. aureus whole cell lysates, suggesting that SraP may self-associate or bind other targets on neighboring S. aureus cells to promote biofilm development (148). The ligand-binding domain was recently structurally characterized and found to contain a lectin-like module that binds N-acetylneuraminic acid (149), an abundant sugar on host glycosylated proteins. The S. haemolyticus serine-rich repeat protein UafB mediates binding to fibronectin, fibrinogen, and human uroepithelial cells (150).

SasX is another cell wall–anchored adhesin that has been shown to play an important role in virulence. SasX was linked to the spread of a methicillin-resistant S. aureus (MRSA) epidemic in China, because its prevalence in MRSA clones increased via horizontal transfer, suggesting its importance in the pathogenic success of MRSA. SasX was also shown to be crucial for a murine model of nasal colonization, murine MRSA skin infection, and bacterial aggregation in vitro (151). SasX has recently been shown to be a promising vaccine candidate for S. aureus infection. Active or passive immunization to SasX decreased S. aureus virulence in murine models of skin infection, lung infection, and nasal colonization (152).

There are also several S. aureus Sas proteins that are poorly characterized. SasC is an LPXTG-anchored protein that contains a FIVAR domain and a domain of unknown function consisting of repeats. SasC, specifically its FIVAR domain, induced cell aggregation, binding to polystyrene, and biofilm formation (153). Genome sequence analysis of S. aureus revealed the putative cell wall–anchored adhesins SasB, SasD, SasF, SasJ, SasK, and SasL, but their structure and function have not been studied further (154).

Surface-Associated Proteinaceous Adhesins

The autolysins AtlA and AtlE are found in S. aureus and S. epidermidis, respectively. Atl and AtlE share a similar amino acid sequence and structure, with bacteriolytic amidas e and glucosaminidase domains (155). They are known to be involved in cell wall turnover, cell division, and cell lysis (156, 157). Autolysins have two properties that could promote biofilm formation: the ability to attach to extracellular matrix materials and to augment the biofilm matrix with eDNA by inducing cell lysis. Adhesin activity was first identified in a transposon mutant of S. epidermidis atlE that had decreased ability to adhere to polystyrene and vitronectin (155). In an S. epidermidis biofilm grown on medical device biomaterials in vitro, atlE expression decreased during the first 12 hours of biofilm growth relative to planktonic culture, but by 48 hours, expression was upregulated 10-fold (158). This may indicate that atlE is more
important later in the biofilm life cycle, when autolysis is induced and eDNA is released.

**Nonproteinaceous Surface-Associated Adhesins**

Wall teichoic acids and lipoteichoic acids have been shown to play a role in adhesion, colonization of host cells, and biofilm formation. Wall teichoic acids are covalently linked to the peptidoglycan and consist of alternating phosphate and ribitol, while lipoteichoic acids attach to the outer leaflet of the cell membrane and have alternating phosphate and glycerol (36). Teichoic acids are highly charged, a property that was found to be critical for *S. aureus* colonization of abiotic surfaces. A mutant lacking d-alanine in its wall teichoic acid lost the ability to form a biofilm *in vitro*. This was due to its greater net negative charge, which decreased its adherence to plastic surfaces (159). In *S. epidermidis*, wall teichoic acids induced adherence to immobilized fibronectin (160). Teichoic acids have been identified in the biofilm matrices of *S. epidermidis* and *S. aureus* (35, 161, 162).

PIA is a secreted polysaccharide that is synthesized by the ica operon and has been thoroughly studied in the context of biofilm formation, immune evasion, and pathogenesis. Several reports state that PIA is required for *S. epidermidis* biofilm formation and virulence (163–165), and it is considered to be the most important intercellular adhesin of the staphylococci (34). The role of PIA in staphylococcal biofilm formation has been reviewed by O’Gara (166) and by Rohde et al. (167), and the regulation of the ica locus in the staphylococci is reviewed in reference 168.

**SECRETED PROTEINS IN THE BIOFILM MATRIX**

A number of secreted staphylococcal proteins have been implicated in biofilm formation, the most prominent being AtlA/AtlE (discussed above as surface associated), proteases, nucleases, and phenol-soluble modulins (PSMs). The exo-enzymes and PSMs will be discussed in more detail in the regulation section below, because their role in biofilm development relates more to dispersal than accumulation. Other secreted proteins that have been linked to biofilm formation are covered here.

Alpha-toxin (Hla) is a potent cytolysin secreted by *S. aureus* that also affects inflammation and contributes to pathogenesis by multiple mechanisms. Many studies have shown that alpha-toxin mutants are attenuated in virulence (169). However, the contribution of this toxin to biofilm formation is less clear. In a study of *in vitro* biofilm formation on polystyrene, an *S. aureus hla* mutant had dramatically reduced biofilm in a standard microtiter plate assay and under flow conditions. Its initial attachment to the surface was also decreased, indicating that an inability to bind to the surface contributes to decreased mature biofilm (170), although the exact mechanism of this phenotype remains unclear.

*S. aureus* beta-toxin (Hlb) is a secreted sphingomyelinase that has hemolytic and lymphocytic activities (171, 172). Beta-toxin also has a sphingomyelinase-independent “biofilm ligase” activity, which refers to its ability to cross-link strands of DNA in the biofilm matrix. Mutants in *hlb* were deficient in *in vitro* attachment, flow-cell biofilm formation, and vegetation formation in a rabbit endocarditis model (173). Hlb is the first staphylococcal protein identified that binds eDNA in the biofilm matrix, providing more evidence of the importance of this matrix component.

*S. aureus* secretes multiple proteins that have been called secreted expanded repertoire adhesive molecules (SERAM) (174). Two of the SERAMs, the extracellular adherence protein (Eap; also called the MHC class II analog protein or Map) and the extracellular matrix-binding protein (Emp) have a demonstrated connection to biofilm formation (175, 176). Eap is a secreted adhesin that enhances *S. aureus* adherence to the extracellular matrix, and it has been shown to bind fibrinogen, fibronectin, vitronectin, and thrombospondin-1 with varying affinities (177–180). Eap can also self-associate to induce aggregation of *S. aureus* (177). Due to its ability to bind several matrix proteins, Eap is required for biofilm formation in the presence of serum (175). Regulation of the *eap* gene is dependent on the SaeRS two-component system (181, 182), and the gene is upregulated under low-iron conditions (176). The *eap* mutant biofilm phenotype is dependent on these iron-limited conditions (176). The Eap protein has a number of known immunomodulating properties that have been summarized elsewhere (174). Less is known about Emp, but this protein can also bind matrix proteins such as fibrinogen (174), and it is also SaeRS and iron regulated (176, 182). Similar to Eap, Emp is required for biofilm formation in iron limiting conditions (176).

**REGULATION OF BIOFILM FORMATION**

Global changes in gene regulation occur throughout the course of the staphylococcal biofilm life cycle. Microarray studies have shown that the biofilm lifestyle requires a gene expression profile to allow tolerance of the
low pH within a biofilm, as well as a metabolic quiescence that includes downregulation of transcription, translation, and aerobic processes \( (183, 184) \). Several global regulators, such as the \( agr \) quorum sensing system, sigma factor B, and SarA, have strong connections to staphylococcal biofilm formation \emph{in vitro} and during infection and will be summarized further below. These regulators have also been examined in more acute pathogenesis mechanisms, and the focus here will be on the biofilm-like infections. The majority of these studies have been performed with \( S. aureus \), but some studies of \( S. epidermidis \) are also included. It should be noted that a number of other regulators have also been linked to biofilm formation, including MgrA \( (185) \) and ArlRS \( (186) \), but these are beyond the scope of this review.

**agr Quorum-Sensing System**

The \( agr \) (accessory gene regulator) system is a peptide quorum-sensing system present in all the staphylococci and is a dominant regulator of pathogenesis and biofilm development in \( S. aureus \). Its molecular characteristics and importance in pathogenesis have been thoroughly studied and reviewed in detail \( (187, 188) \). The \( agr \) system functions by sensing extracellular levels of an autoinducing peptide (AIP) that is produced by staphylococci during growth. The chemical nature of this AIP signal is variable depending on the species and can even have multiple types within a species. Briefly, the AIP is released outside the cell, where it accumulates, and at a particular concentration (usually in the low nM range), the AIP binds to the surface-exposed AgrC histidine kinase, activating a two-component response. This results in phosphorylation of the response regulator AgrA, which in turn induces expression of the primary output of the system, the regulatory transcript RNAIII \( (189) \). In parallel, AgrA activates transcription of the PSMa and PSMβ transcripts \( (190) \) and autoinduces the quorum-sensing machinery. RNAIII is the main effector of the system and directly regulates production of virulence factors; it also regulates the translation of the repressor of toxins (Rot) \( (191) \). In the staphylococcal strains in which global changes in \( agr \)-dependent gene expression have been assessed \( (192, 193) \), the general dogma is that induction of the \( agr \) system leads to upregulation of secreted enzymes and toxins while simultaneously downregulating adhesins.

Understanding and interpreting the literature on the \( agr \) system is a challenging task, in part due to the depth of the literature but also due to the complexities of the system. Focusing on \( S. aureus \), one of the most overlooked issues is that the dynamic range of the \( agr \) system is tremendously variable across strains \( (187) \), meaning some strains barely produce RNAIII, whereas others, such as the USA300 strains, produce very high levels \( (194) \). In recent years, the molecular nature of this variability has begun to be examined \( (195) \), providing preliminary explanations for why some \( S. aureus \) strains have muted \( agr \) function, resulting in reduced RNAIII levels. The challenge becomes interpreting the results of \( agr \) mutant studies, where in a USA300 strain the mutation has a dramatic impact on many phenotypes \( (196–198) \), but in others, such as some clonal complex 30 strains \( (195) \), it has little impact. Further complicating this issue, some older lab strains, such as 8325-4, have known mutations that lead to dysregulation of \( agr \) function \( (199) \). Thus, care must be taken in interpreting results of studies, especially in animal studies of infections, and unfortunately, the \( agr \) function of strains used for some of these studies is not known.

Several studies have investigated the role of quorum sensing in the biofilm life cycle. The current model is that biofilm initiation and maturation require low \( agr \) expression, while subsequent \( agr \) activation within the biofilm induces dispersion to the planktonic state. Indeed, multiple studies of \( S. aureus \) and \( S. epidermidis \) have shown that isogenic \( agr \) mutants display increased biofilm formation \emph{in vitro} \( (24, 199–201) \). As the biofilm develops, small populations experience \( agr \) reactivation and disperse from the biofilm \( (27, 187, 202) \). An established \( S. aureus \) biofilm can be fully dispersed by the addition of AIP to induce \( agr \) activation \( (179, 203) \), and the dispersion process is mediated by \( agr \)-regulated proteases, most prominently the staphopain enzymes \( (204) \). The \( agr \) regulation of the proteases is via Rot, whose transcriptional repression of the proteases is relieved when \( agr \) is induced \( (205, 206) \). A model for this regulatory pathway is shown in Fig. 3. Currently, the major missing piece of this model is the specific biofilm matrix proteins that are targeted by the staphopain enzymes, and this is a topic of ongoing investigation. The other prominent \( agr \)-regulated factors linked to biofilm dispersion are the PSMs \( (23) \). These peptides have surfactant activity that is antibiofilm in nature. On the whole, these results support the model that under \( agr \)-repressive conditions or with \( agr \) null mutants, \( S. aureus \) cells have increased biofilm capacity \emph{in vitro} due to the absence of secreted dispersal factors. However, in a biofilm infection, the importance of the \( agr \) system for initiation depends on the type of infection (see below), presumably because in some tissue sites \( S. aureus \) must secrete \( agr \)-regulated immunomodulating factors to survive. Once the biofilm has been established, both \emph{in vitro}
and in vivo studies indicate that activation of the agr system can lead to dispersal of the cells and dissemination to new sites.

Environmental conditions are a critical factor in controlling agr function. S. aureus can metabolize many sugars (207), and the low pH generated from excretion of short-chain fatty acids can repress agr activity (208). For development of an in vitro S. aureus biofilm, excess sugar (e.g., glucose) must be provided to trigger the pH decline and promote biofilm formation (179). The expression of agr-regulated factors, such as the staphopains, must be repressed for S. aureus cells to attach and initiate biofilm development. For other staphylococcal species, this low pH requirement is less clear. In S. epidermidis, the addition of excess glucose was not essential for promoting biofilm formation (134), but whether this is related to agr function is not known. The control of agr function is also beginning to be appreciated in the host environment. Proteins found in human serum are known to repress S. aureus agr activity (209), including apolipoprotein B (210, 211). Hemoglobin is another abundant host protein with known agr-inhibitory properties (212). Environmental contaminants that accumulate in the body, such as triclosan, have also been linked to enhanced biofilm formation (213). Altered environmental conditions can impact biofilm structure as well, as recently demonstrated with the conversion of PSMs into protease-resistant amyloid fibers (214).

The role of agr during infection is complex and has been the focus of many studies. The contribution of agr to acute pathogenesis has been reviewed previously (187), and we will focus on chronic infections with biofilm-like properties. One of the most common models of biofilm infection is a catheter placed subcutaneously in the flank of a mouse. Staphylococci are inoculated into the lumen of the catheter and allowed to develop into a biofilm over an extended time (215). The inoculum dose can be varied, because it is known that foreign bodies greatly reduce the bacterial load required to colonize (10). Using this
model, *S. aureus* agr mutants have no defect in their ability to colonize the catheter and develop a biofilm (C. Ibberson, C. Parlet, and A.R. Horswill, unpublished observations). However, these mutants are less able to disseminate into other tissues (23), consistent with the concept that the *agr* system is a dispersing mechanism. Using a similar rabbit model of indwelling medical device infection, an *S. epidermidis* agr mutant had an increased ability to colonize the device (24). For both *S. aureus* and *S. epidermidis*, it has been demonstrated that PSMs are important for dispersing from a foreign-body biofilm (23, 216). It seems likely that the many exo-enzymes secreted by these pathogens are also important for dissemination through the host, and our preliminary studies confirm this hypothesis (C. Ibberson, C. Parlet, and A.R. Horswill, unpublished observations).

For chronic infections involving host tissue, the function of the *agr* system has also been assessed. *S. aureus* is one of the leading causes of osteomyelitis, a chronic infection of the bone that has known biofilm-like properties (12). *S. aureus* agr mutants are attenuated in their ability to establish osteomyelitis (22). *S. aureus* is also the leading cause of infective endocarditis (217), another chronic infection, where bacteria attach to the heart valve and develop into a vegetation composed of matrix proteins, platelets, and immune cells. Similar to osteomyelitis, *S. aureus* agr mutants show a defect in their ability to establish infection (21). Interestingly, the *agr* system showed progressive activation within the endocarditis vegetations (218), which is in contrast to what is observed in biofilms under *in vitro* conditions (179). As an added complication of endocarditis, septic emboli can dislodge and enter the bloodstream, and these emboli have biofilm characteristics that make them more resistant to antibiotics than planktonic bacteria and are potentially life-threatening for the patient (219–222).

It is generally accepted that low levels of *agr* expression are observed in chronic infections (223–225), suggesting that the loss or decreased activity of *agr* is an adaptation to allow persistence in the host environment. However, this is somewhat misleading, since the comparison for these claims is usually *in vitro* broth culture, in which *agr* function and RNAlI levels are extremely high. The more informative experiment is tracking *agr* function over time within the infection, which was performed with infective endocarditis (218) but has not been attempted more systemically in other infection types. Some of the studies on low *agr* expression are based on *S. aureus* isolates from the cystic fibrosis lung. Growth of bacterial pathogens, such as *Pseudomonas aeruginosa*, in cystic fibrosis are known to be in a biofilm state, but there is limited information on *S. aureus*, although a link to biofilms has been suggested (15). What is clear is that *agr*-negative mutants do frequently appear during biofilm growth *in vitro* (226) and during biofilm infections (223, 227). In part, this could be due to the increased fitness cost of expressing the *agr* system in the presence of antibiotic pressure (228). When *S. aureus* reenters a normal state, the maintenance requirement of the *agr* system is restored (229).

As a global regulator, the *agr* quorum-sensing system has widespread effects on gene expression that can strongly impact the staphylococcal biofilm life cycle. However, the complexity of the system and the factors that alter its expression can result in varied effects of the *agr* system *in vivo*. When studying the role of the *agr* system during infection, the *agr* expression level of the staphylococcal strain must be considered, as well as the infection niche and the relevant host factors that may modify the importance of *agr*. Since inhibition of *agr* has begun to be investigated as a possible treatment for *S. aureus* infections (230–232), further work needs to be done to clarify the types of *in vivo* conditions and infections that would benefit from this.

**AI-2**

AI-2, or autoinducer-2, is another quorum-sensing molecule that is present in both Gram-positive and Gram-negative organisms, and it is thought to be a bacterial interspecies signaling molecule (233, 234). AI-2 has been shown to inhibit biofilm formation and virulence in both *S. aureus* and *S. epidermidis*. In *S. epidermidis*, a mutant that does not produce AI-2 had greatly increased biofilm formation *in vitro* and increased virulence in a rat central venous catheter infection model (235). This mutant also had increased expression of the secreted polysaccharide synthesis operon *ica* and increased polysaccharide production, which is thought to be the mechanism for its biofilm phenotype. Yu et al. reported similar findings, in which an AI-2 mutant had increased biofilm formation *in vitro* and in a murine catheter infection model. AI-2 was also shown to positively regulate expression of the *ica* operon repressor *icaR*, leading to increased production of extracellular polysaccharide in the AI-2 mutant (236). An *S. aureus* AI-2 mutant also displayed increased survival in human blood and macrophages (237). Yu et al. also investigated the relationship between AI-2 and *agr* regulation in *S. epidermidis* biofilm formation. The two were found to have an additive effect, in which a double mutant had higher biofilm formation than either single mutant (236).
SigB
Sigma B (SigB) is an alternative sigma factor of RNA polymerase that is activated in stress response and leads to global changes in promoter specificity and, thus, gene expression (238). Strains lacking SigB have tremendous changes in regulatory profiles that alter biofilm formation and virulence. *S. aureus* mutants in the global regulator *sigB* are unable to form a biofilm *in vitro* (199). This phenotype is mediated both by increased protease activity (239, 240) and increased nuclease activity (241). Part of the reason for increased protease activity has been linked to hyper-activation of the *agr* system in SigB-defective strains (199). In *S. epidermidis*, a *sigB* mutant also was reported to have decreased biofilm formation and increased expression of *icaR*, which represses production of the extracellular polysaccharide PIA (242, 243).

SigB also plays an important role in the response of staphylococci to the *in vivo* environment. In a murine intravenous infection model with *S. aureus*, the SigB cascade was reported to be highly transcriptionally active, although a *sigB* mutant had no difference in disease outcome in the mice (244). However, an *S. epidermidis* *sigB* mutant did have a defect in colonization in a rat model of foreign body infection (245). Expression of *S. aureus* SigB is also activated by human pulmonary surfactant and after internalization by human bronchial epithelial cells (246, 247).

Spontaneous *sigB* mutants arise *in vitro* in biofilm formation and in chronic staphylococcal infections (248, 249). These mutants are small colony variants, which have elevated extracellular protease activity and decreased biofilm formation. They also have increased intracellular persistence and are thought to contribute to virulence of *S. aureus* infections (249). These results suggest that biofilm populations and infecting bacteria are heterogeneous in gene sequence as well as expression level of key virulence factors.

SarA
The *sar* (staphylococcal accessory regulator) locus was discovered in a transposon mutagenesis screen for fibrinogen-binding-negative mutants (250). In addition to decreased fibrinogen binding, the *sar* mutant had an altered exoprotein profile, with increased protease, lipase, and α-hemolysin. The *sar* locus produces three transcripts from three separate promoters, all of which contain the ORF for the DNA-binding protein SarA (251, 252). Early studies showed that a *sar* mutant had decreased levels of the *agr* transcripts RNAII and RNAIII (253–255). EMSA (electrophoretic mobility shift assay) studies have shown that purified SarA directly binds to three sites within a region spanning the P2 and P3 promoters of the *agr* locus (252). SarA regulatory activity therefore occurs partially via its effects on *agr*.

SarA also directly regulates several genes that affect biofilm formation. A putative SarA binding site has been identified upstream of several SarA-regulated genes, providing a mechanism for direct regulation by DNA binding (256). SarA represses transcription of *cna* even in the absence of *agr* and directly binds to the *cna* promoter region (257, 258). A microarray revealed that in addition to positively regulating *fnbA* and *fnbB*, SarA negatively regulates *isaB* and *spa*, and all of these gene promoters contain a putative SarA binding site (193). SarA also positively regulates *bap*, a cell wall adhesin found in bovine isolates of *S. aureus*, via direct binding to its promoter (259). Secreted proteases and nuclease are upregulated in *sarA* mutants (193, 260, 261). Since the opposite phenotypes are observed in *agr* mutants, it is apparent that this effect occurs via an *agr*-independent pathway.

Multiple studies have reported that in *S. aureus*, SarA is required for biofilm formation, both *in vitro* and *in vivo* (175, 262–264). Various mechanisms for this phenotype have been proposed. The first study to report the *S. aureus sarA* mutant biofilm defect also showed that this mutant had decreased *ica* transcription and PIA production and suggested that this could partially account for the biofilm phenotype (263). However, a later study showed that while an *S. aureus sarA* mutant did not produce a biofilm, an *ica* operon knockout had no decrease in biofilm formation (183). These studies were performed *in vitro* as well as *in vivo* using a murine model of catheter-associated biofilm infection. The results indicate that *ica* regulation is not the sole factor behind the *sarA* knockout biofilm phenotype and that PIA production may not be critical under certain biofilm conditions.

Biofilm formation in *sarA* mutants is also thought to be inhibited by their increased protease and nuclease activity. Although one study found that protease inhibitor treatment did not alter the phenotype of a *sarA* mutant, only the initial attachment was tested, rather than the endpoint of biofilm growth (263). A later report found that treatment with a cocktail of protease inhibitors for all the secreted proteases resulted in increased biofilm formation of an *S. aureus sarA* mutant in the clinical isolate UAMS-1 (263). This suggests that increased protease activity contributes to the *sarA* biofilm phenotype. The same study showed that a *sarA nuc*
double mutant had improved biofilm formation relative to a single sarA mutant, demonstrating that the effect of SarA is also partially mediated by nuclease production. In a group of three S. aureus clinical isolates, sarA mutants had decreased biofilm formation regardless of their various levels of agr expression (266). This study also found that protease inhibitor treatment improved biofilm formation in sarA mutants of each clinical isolate, confirming the impact of secreted proteases on biofilm formation. On the whole, these results suggest that SarA-mediated effects on biofilm formation are mediated by secreted enzymes more than PIA and are at least partially independent of agr.

There are conflicting reports of the effect of sarA mutation on S. epidermidis. In two S. epidermidis clinical isolates, sarA mutation was found to drastically decrease biofilm formation (267). In the same study, S. epidermidis SarA was also found to positively regulate transcription of the ica operon and bind to the icaA promoter, indicating that SarA induces biofilm formation via PIA production. In contrast, Christner et al. showed that SarA represses biofilm formation in an S. epidermidis clinical isolate by two mechanisms (268). Mutation of sarA dramatically increased biofilm formation in the aap-negative, ica-negative S. epidermidis clinical isolate 1585 as well as in an S. epidermidis 1457 icaA mutant. The first mechanism of biofilm enhancement in the sarA mutant was increased expression of the cell wall adhesin embp (extracellular matrix binding protein). A double sarA embp mutant had decreased biofilm formation relative to the sarA mutant, indicating that the sarA phenotype was partially dependent on Embp. The second mechanism of biofilm enhancement was found to be increased protease expression in the sarA mutant, although this is contrary to previous findings in S. aureus. S. epidermidis sarA mutants have increased production of the metalloprotease SepA (269), which was shown by Christner et al. to induce processing of the autolysin AtlE, leading to increased lysis and eDNA release (268). This study shows that SarA is a positive regulator of the eDNA- and Embp-dependent biofilm.

**EVASION OF THE HOST IMMUNE SYSTEM**

Staphylococcal biofilms are noted for their resistance to host immune clearing, and there have been significant efforts to characterize the mechanisms that contribute to this resistance (270–272). Studies have investigated the effects of staphylococcal biofilms on immune cell function, the host antibody response to chronic staphylococcal infection, and the staphylococcal transcriptional response to host innate immune cells.

In the innate immune system, polymorphonuclear leukocytes (PMNs) and macrophages are the first responders to staphylococcal infection (17, 273–275). Although one study reported minimal PMN influx in a murine model of catheter-associated S. aureus biofilm growth (272), others have demonstrated that activated PMNs are prevalent at the site of infection in human patients with orthopedic device-associated staphylococcal biofilm infections (276, 277). Multiple studies have reported that human PMNs in in vitro coculture with S. aureus localize to the biofilm and can phagocytose bacteria (278, 279). In an S. epidermidis biofilm grown in vitro, PMNs were able to attach to the biofilm, release granule components from both primary and secondary granules, and phagocytose biofilm bacteria (280). These effects were observed with or without opsonization, which suggests they are mediated at least in part by bacterial components that interact with the PMNs.

PMNs can attack staphylococcal biofilms by phagocytosis, release of toxic granule components, and production of neutrophil extracellular traps (NETs), although there is evidence that these effects are dampened relative to planktonic bacteria (281). S. epidermidis is more resistant to in vitro killing by human PMNs when grown in a biofilm than grown planktonically (282). The S. epidermidis extracellular polysaccharide PIA is thought to play a role in evading PMN killing, because it has been shown that an S. epidermidis ica mutant exhibits increased susceptibility to phagocytosis and killing by human PMNs in vitro (283). Similarly, the S. aureus capsular polysaccharide inhibits opsonophagocytosis of planktonic bacteria by PMNs in vitro and is required for full virulence in a murine model of septic arthritis (284, 285). Although these studies did not directly test phagocytosis of biofilm bacteria, the results suggest that PIA and capsule in a staphylococcal biofilm may shield the bacteria from the host immune response. The agr system may also allow the staphylococcal biofilm to resist PMN killing. In S. aureus, an agr mutant biofilm was less cytotoxic to PMNs in coculture than its wild type counterpart, suggesting that biofilm cells expressing agr could kill PMNs and therefore evade phagocytosis and killing by PMNs (278). This corroborates an earlier study testing interactions of planktonic wild type and agr mutant S. aureus with PMNs, which also found that the agr mutant induced decreased PMN lysis (198).

NETs were first described by Brinkmann et al., who showed that activated PMNs produce thread-like projections composed of DNA and granule components
NETs bind to microbes in vitro and degrade bacterial extracellular virulence factors as well as kill the bacteria. NET formation is thought to occur via a regulated cell death pathway termed NETosis that is distinct from necrosis and apoptosis (287). S. aureus extracellular nuclease has been shown to degrade NETs, thereby allowing the bacteria to resist NET-mediated killing (288, 289). Since the secreted nuclease is unique and not found in all staphylococci, species that lack it may be more susceptible to NETs, although an additional surface-attached nuclease (Nuc2) is more conserved among the staphylococci (290).

The interactions between staphylococcal biofilms and host macrophages have also been investigated. These studies have led to a model (see Fig. 4) in which staphylococci promote an anti-inflammatory, profibrotic environment via alternative macrophage activation (270, 272). Macrophages can undergo at least two varieties of activation, including classical and alternative (291). Classically activated (M1) macrophages are characterized by their ability to present antigen and degrade intracellular pathogens, while alternatively activated macrophages are inefficient at both of these. Alternatively activated (M2) macrophages have high arginase (Arg–1) activity, which decreases their ability to destroy intracellular pathogens (292) and promotes collagen formation, extracellular matrix proliferation, and wound healing (293). Planktonic staphylococci have been shown to induce classical activation of macrophages and are readily phagocytosed (294). However, in an S. aureus biofilm coculture with macrophages, Scherr et al. observed little macrophage phagocytosis of S. aureus cells and few macrophages in close proximity to the biofilm (278). A coculture study of S. aureus biofilms and macrophages reported that macrophages that interacted closely with the biofilm performed little phagocytosis and exhibited gene expression patterns consistent with M2 activation. Further, the same study showed that macrophage death was induced in cells that were close to the biofilm (294). Another group has reported that clfA expression prevents macrophage phagocytosis by a mechanism that is independent of binding to fibrinogen (295). These results suggest that biofilms can promote a macrophage phenotype that favors the progression of infection and produce factors that are cytotoxic to macrophages.

Multiple studies have performed microarrays on S. aureus following coculture with innate immune cells to determine global regulatory changes that facilitate staphylococcal survival. A microarray study of S. aureus following interactions with PMNs and macrophages revealed more extensive changes in regulation after exposure to macrophages. Although various S. aureus genes were differentially regulated in response to PMNs, a striking global downregulation was observed following coculture with macrophages. In both an immature and a mature biofilm, downregulation of hundreds of genes was observed, corresponding to a global decrease in metabolic processes (278). Another report demonstrated that following coculture with phagocytic PMNs, S. aureus experienced widespread changes in regulation. Stress response proteins such as catalase were upregulated, as well as virulence factors such as hemolysins and fibrinogen-binding proteins. There was also a shift in the metabolic profile relative to broth culture, and changes in global regulators occurred. Of note, the Sae system, which positively regulates several secreted toxins and other virulence-associated proteins (196, 296–298), was upregulated several fold after exposure to PMNs (299). The Sae system was later shown to differentially regulate its various targets in response to specific neutrophil stimuli (300), and sae mutants have decreased survival in in vitro PMN phagocytosis assays (298). These results demonstrate that S. aureus has an arsenal of tools to survive PMN and innate immune assault and that the Sae system in particular is a crucial element.

FIGURE 4 Macrophage activation pathways. Biofilm growth of Staphylococci has been shown to favor the M2 phenotype in macrophages, which is characterized by increased arginase and profibrotic activity, as well as decreased antimicrobial clearance. These changes are thought to contribute to the persistence of staphylococci in biofilm infections. This figure is reproduced from reference 272.

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**Staphylococcal Biofilms**
Several studies have characterized the *S. aureus* proteins that are targeted by the host antibody response following infection or colonization (301–303). This group of antigens is referred to as the immune proteome, and it identifies proteins that are expressed in vivo and may be important to virulence. These findings also may suggest targets for the development of a vaccine for *S. aureus*, which has been an area of interest in recent years.

**OTHER STAPHYLOCOCCAL SPECIES**

The study of staphylococcal biofilm formation has largely focused on *S. aureus* and *S. epidermidis*. However, other staphylococcal species are also pathogenic biofilm-formers. Staphylococci are classified based on their production of the blood-clotting enzyme coagulase, a secreted protein that induces the conversion of fibrinogen to fibrin (304, 305). The genus *Staphylococcus* comprises 47 species: 8 are coagulase-positive or coagulase-variable, 38 are coagulase-negative, and 1, *Staphylococcus schleiferi*, has both a coagulase-negative and a coagulase-positive subspecies. Of the coagulase-positive staphylococci, *S. aureus* is the sole species that is primarily associated with human disease (1). However, there have been reports of other coagulase-positive staphylococci colonizing or causing disease in human hosts who have significant contact with animals (306, 307). One example is *Staphylococcus pseudintermedius*, a commensal of dogs that is implicated in canine opportunistic infection (308). Bioinformatic and proteomic analyses have been employed to study the cell wall adhesins of *S. pseudintermedius*, leading to further study of two proteins (SpsD and SpsL) to which canines have antibodies, indicating their expression in vivo (309, 310).

The coagulase-negative staphylococci are the third most common cause of human health care–associated infections (8) and the number 1 cause of bovine intramammary infections (311–313). They have been reviewed thoroughly in references 1 and 44. Within the coagulase-negative staphylococci, *S. epidermidis* is the most frequent cause of medical device–related infections and is able to infect virtually any medical implant including catheters, central lines, ventilators, prosthetic joints, and pacemakers (8, 314–316). One reason for its high rate of infection may be its prevalence in the normal skin flora and ability to colonize many surfaces of the human body, including the anterior nares, axillae, and inguinal and perineal areas (1, 317). Other coagulase-negative staphylococci species inhabit various niches of the human body. *S. lugdunensis* is found particularly in the lower extremities of the body in the perineal and groin areas, as well as in the axillae (318, 319). *S. haemolyticus* is preferentially isolated from axillae and pubic areas (317, 320), and *S. saprophyticus*, from the gastrointestinal tract, rectum, and urogenital tract, typically in younger individuals (1, 321, 322).

Of the coagulase-negative staphylococci, *S. lugdunensis* is considered to be the most similar to coagulase-positive staphylococci (1). It is a skin commensal and opportunistic pathogen, responsible for 0.8 to 7.8% of infectious endocarditis cases in non–drug users (323), with mortality rates ranging from 38 to 42% (323, 324). These high mortality rates are similar to those of *S. aureus* endocarditis and are suggestive of aggressive infection. *S. lugdunensis* is also implicated in infections of medical devices, such as catheters and prosthetic joints (325–327). Recent studies have also demonstrated that *S. lugdunensis* is a significant cause of skin and soft-tissue infections, with up to 53 per 100,000 per year (328).

For mechanisms of pathogenesis, *S. lugdunensis* possesses several virulence factors, including surface adhesins, which are reviewed in reference 329. A mutant in the cell wall–anchoring enzyme sortase A was attenuated in an experimental endocarditis model, confirming that in *S. aureus*, adhesins contribute to *S. lugdunensis*-mediated endocarditis (330). *S. lugdunensis* also has an *ica* locus for PIA synthesis, although its role in biofilm formation is not clear, since *S. lugdunensis* biofilms in *vitro* were not sensitive to degradation by either of the PIA-targeting factors Na-metaperiodate or dispersin B (331). Biofilm formation may be a significant factor in infection, because *S. lugdunensis* clinical isolates from prosthetic implant infections have been shown to be strong biofilm formers in *vitro* (332). In addition to its biofilm-forming activity, *S. lugdunensis* has several putative cytolysins, including the SLUSH (S. lugdunensis synergistic hemolytic) peptides, which are similar to *S. aureus* delta-toxin Hld, and another Hld-like protein (329, 333). However, the activity and virulence role of these SLUSH proteins is not known.

*S. haemolyticus* has been implicated in a range of opportunistic infections in humans, including prosthetic device infections (334) and postoperative endophthalmitis (335). In a study of *S. haemolyticus* isolated from bloodstream infections, 66% of the isolates formed robust biofilms in *vitro*, but the same strains were all negative for PCR of the *ica* operon for PIA synthesis (336). This suggests that *S. haemolyticus* has other means of biofilm formation. The Bap adhesin was present in several biofilm-positive *S. haemolyticus*
nosocomial infection isolates, identifying at least one PIA-independent mechanism that may contribute to *S. haemolyticus* biofilm formation and infection (337).

Since *S. saprophyticus* colonizes the rectum and urogenital tract, it is unsurprising that it is a common cause of UTIs. *S. saprophyticus* is the second most frequent cause of UTI in young, sexually active women, although it also causes UTIs in populations of all ages (338, 339). *S. saprophyticus* has several adhesins that contribute to virulence, including SdrI and the serine-rich repeat protein UafB (see adhesins section). It also secretes urease, an enzyme that hydrolyzes urea to carbon dioxide and ammonia, which raises the pH of the urinary tract and can result in calculi formation. Urease was shown to be a virulence factor in a rat model of *S. saprophyticus* UTI (340). Chemical inhibition of urease was able to prevent the pH change caused by *S. saprophyticus* growth in an artificial urine medium, although the effectiveness of this as a treatment has yet to be tested in vivo (341).

**CONCLUSIONS AND FUTURE PERSPECTIVES**

The goal of this review on staphylococcal biofilms is to summarize the latest literature on the function of adhesins, regulatory cascades, and the host response to these biofilms, with a focus on the noted pathogens *S. aureus* and *S. epidermidis*, and also coverage on other coagulase-negative staphylococci biofilms. The significant body of work available indicates that there are numerous mechanisms to assemble a mature staphylococcal biofilm, and these structures vary depending on the substratum, the adhesins particular staphylococci express, and the matrix materials available. Within a biofilm infection, the host niche clearly also has an important role in biofilm matrix composition and impacting the regulatory pathways controlling expression of staphylococcal biofilm factors.

A number of recent studies provide convincing evidence that the staphylococcal biofilm cells are equipped to thwart the host immune response, making them more resistant to the host immune system than planktonic cells. Recent findings have also shown that staphylococcal biofilms contain heterogeneous populations, with a subset of cells contributing to the development of antibiotic resistance. In several bacteria, the phenomenon of persister cells has been observed, which are a small portion of a bacterial population that remains following antibiotic treatment (342, 343). *S. aureus* persisters were first observed in a murine model of deep wound infection. Treatment with vancomycin killed 99% of *S. aureus* cells, while the remaining 1% continued to be unaffected by vancomycin even after another day of treatment, suggesting a persister population (344). Other studies have demonstrated that the appearance of heterogeneous populations can develop within an *S. aureus* biofilm (226), and further studies have shown that these subpopulations can interact to promote vancomycin-intermediate resistance phenotypes (345). Clearly, biofilms are a diverse population with varying phenotypic properties that can contribute to the progress of an infection in complex ways.

Despite all the advancements, much remains to be elucidated regarding the defined nature of the in vivo biofilm state. Although the term “biofilm” is broadly applied to various growth states ranging from benign skin colonization to endocarditis, the universal qualities of these that specifically define biofilm characteristics are not clear, making it challenging at times to compare results across studies. There have been efforts to identify universal biomarkers of a staphylococcal biofilm (9), as well as to clarify the roles of virulence determinants that are unique to certain biofilm infection types, but there is still a pressing need for more studies in this direction to standardize the field. In part, researchers themselves have created this dilemma by trying to link every staphylococcal growth state to a biofilm without considering the limitations of such a diverse umbrella. Attempts have been made to rein in the enthusiasm by trying to keep certain growth states separate and uniquely defined, such as colonization (346), but the popularity of “biofilms” keeps this terminology at the forefront of any literature on staphylococcal communities. Further, with the growing literature on other community states, such as synovial aggregates (347), polysaccharide aggregates (348), and fibrinogen-based clumping (349), there is a growing need for investigations to compare and contrast properties of these states with classical biofilm features.

The study of staphylococcal biofilm development has advanced greatly over the past decade, and we have endeavored in this review to cover many of the recent advances. In the future, biofilm studies will need to be extended to more host-relevant conditions to properly model and understand the in vivo biofilm state. Too often, in vitro studies leave out host factors that can impact biofilm maturation in many ways, and these conditions need to be considered when modeling biofilm development in vitro or in vivo. We need to properly understand all these complexities to best position therapeutic development for treating biofilm infections.
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