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The Staphylococcal Biofilm: Adhesins, regulation, and host response

Alexandra E. Paharik and **Alexander R. Horswill**

Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Abstract

The Staphylococci comprise a diverse genus of Gram-positive, non-motile 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. *S. aureus* and *S. epidermidis* are a major source of hospital-acquired infections and are the most common causes of surgical site infections and central line-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 implants, 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

Bacteria from the genus *Staphylococcus* include 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 *S. aureus* and *S. epidermidis*, are also opportunistic pathogens and responsible for a tremendous burden on the healthcare 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 a “biofilm”. Generally, biofilms are defined as a community of cells encased within an exopolymeric matrix and attached to a surface, and they are recognized as being more 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 observed in natural as well as industrial aquatic environments ([4]). Biofilm development was subsequently found to be important in many

Address Correspondence: Alexander R. Horswill, Ph.D., University of Iowa, Department of Microbiology, 540F EMRB, Iowa City, IA 52242, Phone: (319) 335-7783, FAX (319) 335-8228, ; Email: alex-horswill@uiowa.edu

types of infections and is now a widely accepted bacterial mode of growth. According to the NIH, as much as 80% of human infections are biofilm-based ([5]). Biofilm infections present a clinical challenge, as 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, and are a significant burden on the healthcare system. *S. aureus* and the coagulase-negative Staphylococci (CoNS) are the number one and number three most common etiological agents of hospital-acquired infections in the US, respectively, including infections of medical devices and surgical wounds ([8]). Biofilm infections clearly are a significant burden on the healthcare 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 (i.e., it has to look like a biofilm), 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 non-ribosomally generated peptide aureusimine (phevalin) was produced in higher levels by biofilm-grown *S. aureus* ([9]), suggesting this natural product could be a biofilm biomarker. While 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 ([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 in order to survive these varied host environments. As one example, *S. aureus* 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 Figure 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 growth. 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 eDNA, both from lysed bacteria and potentially from host neutrophil cell death, and is susceptible to dispersal by DNAses ([30, 31]). Proteinaceous adhesins have also been identified in the Staphylococcal biofilm matrix. These may be directly associated with bacteria in the biofilm, or 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]). Both 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 current knowledge on 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 *Staphylococcus aureus* genome encodes more than twenty adhesins ([32], [36], [37]), while coagulase-negative Staphylococci (CoNS) have significantly fewer ([38], [39]). Staphylococcal adhesion and biofilm accumulation are mediated by covalently anchored cell wall proteins, non-covalently associated proteins, and non-protein factors. The general properties of these adhesins are presented, and their functions within biofilm development are included where information is available.

Covalently Linked Cell Wall-Anchored Proteins

Staphylococcal cell wall-anchored (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 NPQT/PN/S motif ([40]). The Staphylococcal CWA proteins were recently discussed in a review by Foster *et al.*, who propose to classify them into four groups (see Figure 2) based on structural motifs ([40]). These are the MSCRAMMs (microbial surface component recognizing adhesive matrix molecules), the NEAT motif family, the three-helical bundle family, and the G5-E repeat family. All of these types of CWA proteins are involved in biofilm formation in the Staphylococci.

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-bnding 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 up-regulated in biofilm growth relative to planktonic ([45]). 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 occludes 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]).

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 *in vitro* ([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 a *sdrG* mutant, indicating its importance in the *in vivo* biofilm formation of *S. epidermidis* ([64]).

The coagulase-negative species *S. lugdunensis* also has a ClfA homolog called Fbl ([65], [66]). Fbl promotes both adherence to immobilized fibrinogen 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 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 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 Mn^{2+} ([82]). In contrast to SdrC, the structural stability of SdrD appears to require binding to divalent cations, specifically Ca^{2+} ([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 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* 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 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. *S. 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]). *S. 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 urinary tract infection, 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. Of 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 FnBPB, 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 10–11 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 inhibit mediate *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 up-regulated in iron limiting conditions ([122]). *S. aureus* IsdA and IsdC bind heme via their NEAT motifs (Figure 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 six 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 attached 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 Zn^{2+}, 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 uncategorized 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 *S. 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 MRSA epidemic in China, as 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 or 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 amidase and glucosoaminidase 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 ten-fold ([158]). This may indicate that *atlE* is more important later in the biofilm life cycle, when autolysis is induced and eDNA is released.

Non-Proteinaceous 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]).

The polysaccharide intercellular adhesion (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 in ([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, as their role in biofilm development more relates 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 extracellular DNA (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 up-regulated under low iron conditions ([176]). The *eap* mutant biofilm phenotype is dependent on these iron-limiting 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 like fibrinogen ([174]) and it is also SaeRS and iron regulated ([176, 182]). Similar to Eap, Emp is required for biofilm formation on 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 down-regulation 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 *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 presented here will be on the biofilm-like infections. The majority of these studies have been performed with *S. aureus*, but some studies on *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 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 PSMα and PSMβ transcripts ([190]), and also autoinduces the quorum sensing machinery. RNAIII is the main effector of the system and directly regulates production of virulence factors, and also regulates the translation of the repressor of toxins (Rot) ([191]). In the Staphylococcal strains where global changes in *agr*-dependent gene expression have been assessed ([192], [193]), the general dogma is that induction of the *agr* system leads to up-regulation of secreted enzymes and toxins, while simultaneously down-regulating 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, like 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]), 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 *in vitro* ([24], [199–201]). As the biofilm develops, small populations experience *agr* re-activation 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 Figure 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 phenol-soluble modulins (PSMs) ([23]). These peptides have surfactant activity that is anti-biofilm 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 *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 *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 in order 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, like 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 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 period ([215]). The inoculum dose can be varied, as 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 the ability to colonize the catheter and develop a biofilm ([216]). 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 actually 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], [217]). 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 ([216]).

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 the ability to establish osteomyelitis ([22]). *S. aureus* is also the leading cause of infective endocarditis ([218]), 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 the ability to establish infection ([21]). Interestingly, the *agr* system showed progressive activation within the endocarditis vegetations ([219]), 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 ([220_223]).

It is generally accepted that low levels of *agr* expression are observed in chronic infections ([224_226]), 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 RNAIII levels are extremely high. The more informative experiment is tracking *agr* function over time within the infection, which was performed with infective endocarditis ([219]), 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, like *Pseudomonas aeruginosa*, in cystic fibrosis are known to be in biofilm state, but there is limited information on *S. aureus*, although link to biofilms has been suggested ([15]). What is clear is that *agr* negative mutants do frequently appear during biofilm growth *in vitro* ([227]) and during biofilm infections ([226, 228]). In part, this could be due to the increased fitness cost of expressing the *agr* system in presence of antibiotic pressure ([229]). When *S. aureus* reenters a normal state, the maintenance requirement of the *agr* system is restored ([230]).

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 possible treatment for *S. aureus* infections ([231_233]), 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 a second quorum-sensing molecule that is present in both Gram-positive and Gram-negative organisms, and is thought to be a bacterial interspecies signaling molecule ([234, 235]). 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 ([236]). 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. Similar findings were reported in *S. aureus* by Yu *et al.*, 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 ([237]). An *S. aureus* AI-2 mutant also displayed increased survival in human blood and macrophages ([238]). 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 ([237]).

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 ([239]). 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 ([240], [241]) and increased nuclease activity ([242]). 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 ([243], [244]).

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*, SigB cascade was reported to be highly transcriptionally active, although a *sigB* mutant had no difference in disease outcome in the mice ([245]). However, an *S. epidermidis sigB* mutant did have a defect in colonization in a rat model of foreign body infection ([246]). Expression of *S. aureus* SigB is also activated by human pulmonary surfactant and after internalization by human bronchial epithelial cells ([247], [248]).

Spontaneous *sigB* mutants arise *in vitro* in biofilm formation and in chronic Staphylococcal infections ([249], [250]). 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 ([250]). 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 ([251]). 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 ([252], [253]). Early studies showed that a *sar* mutant had decreased levels of the *agr* transcripts RNAII and RNAIII ([254–256]). 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 ([253]). 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 ([257]). SarA represses transcription of the collagen adhesion *cna* even in the absence of *agr*, and directly binds to the *cna* promoter region ([258], [259]). 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 ([260]). Secreted proteases and nuclease are also up-regulated in *sarA* mutants ([193], [261], [262]). 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], [263–265]). 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 ([264]). However, a later study showed that while a *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 in fact 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 ([264]). A later report found that treatment with a cocktail of protease inhibitors for all the secreted proteases resulted in increased biofilm formation of a *S. aureus sarA* mutant in the clinical isolate UAMS-1 ([266]). 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 ([267]). 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 increase biofilm formation ([268]). 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 ([269]). 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 ([270]), which was shown by Christner *et al*. to induce processing of the autolysin AtlE, leading to increased lysis and eDNA release ([269]). 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 ([271–273]). 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, PMNs (polymorphonuclear leukocytes) and macrophages are the first responders to Staphylococcal infection ([17, 274–276]). Although one study reported minimal PMN influx in a murine model of catheter-associated *S. aureus* biofilm growth ([273]), others have demonstrated that activated PMNs are prevalent at the site of infection in human patients with orthopedic device-associated Staphylococcal biofilm infections ([277, 278]). Multiple studies have reported that human PMNs in *in vitro* co-culture with *S. aureus* localize to the biofilm and can phagocytose bacteria ([279, 280]). 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 ([281]). These effects were observed with or without opsonization, which suggest 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 NETs (Neutrophil Extracellular Traps), although there is evidence that these effects are dampened relative to planktonic bacteria ([282]). *S. epidermidis* is more resistant to *in vitro* killing by human PMNs when grown in a biofilm than grown planktonically ([283]). The *S. epidermidis* extracellular polysaccharide PIA is thought to play a role in evading PMN killing, as it has been shown that an *S. epidermidis ica* mutant exhibits increased susceptibility to phagocytosis and killing by human PMNs *in vitro* ([284]).

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 ([285], [286]). 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 co-culture than its wild type counterpart, suggesting that biofilm cells expressing *agr* could kill PMNs and therefore evade phagocytosis and killing by PMNs ([279]). 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]).

Neutrophil Extracellular Traps (NETs) were first described by Brinkmann *et al.*, who showed that activated PMNs produce thread-like projections composed of DNA and granule components ([287]). 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 ([288]). *S. aureus* extracellular nuclease has been shown to degrade NETs, thereby allowing the bacteria to resist NET-mediated killing ([289], [290]). Since the secreted nuclease is unique not found in all Staphylococci, speces that lack it may be more susceptible to NETs, although an additional surface-attached nuclease is conserved among the Staphylococci ([291]).

The interactions between Staphylococcal biofilms and host macrophages have also been investigated. These studies have led to a model (see Figure 4) in which Staphylococci promote an anti-inflammatory, pro-fibrotic environment via alternative macrophage activation ([271], [273]). Macrophages can undergo at least two varieties of activation, including classical and alternative ([292]). 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 destruct intracellular pathogens ([293]) and promotes collagen formation, extracellular matrix proliferation, and wound healing ([294]). Planktonic Staphylococci have been shown to induce classical activation of macrophages and are readily phagocytosed ([295]). However, in a *S. aureus* biofilm co-culture with macrophages, Scherr *et al.* observed little macrophage phagocytosis of *S. aureus* cells, and few macrophages in close proximity to the biofilm ([279]). A co-culture 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 ([295]). Another group has also reported that *clfA* expression prevents macrophage phagocytosis by a mechanism that is independent of binding to fibrinogen ([296]). 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 co-culture 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 down regulation was observed following co-culture 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 ([279]). Another report demonstrated that following co-culture with phagocytic PMNs, *S. aureus* experienced widespread changes in regulation. Stress response proteins such as catalase were up-regulated, 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. Of note, the Sae system, which positively regulates several secreted toxins and other virulence-associated proteins ([196, 297–299]), was up-regulated several fold after exposure to PMNs ([300]). The Sae system was later shown to differentially regulate its various targets in response to specific neutrophil stimuli ([301]), and *sae* mutants have decreased survival in *in vitro* PMN phagocytosis assays ([299]). 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.

Several studies have characterized the *S. aureus* proteins that are targeted by the host antibody response following infection or colonization ([302–304]). This group of antigens is referred to as the immune proteome, and 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 ([305, 306]). The genus *Staphylococcus* comprises 47 species: 8 of which are coagulase-positive or coagulase-variable, 38 of which are coagulase-negative, and one, *S. schleiferi*, that 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 ([307, 308]). One example is *S. pseudintermedius,* a commensal of dogs that is implicated in canine opportunistic infection ([309]). Biofinformatic 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* ([310, 311]).

The coagulase-negative Staphylococci (CoNS) are the third most common cause of human healthcare-associated infections ([8]) and the number one cause of bovine intramammary

infections ([312–314]). They have been reviewed thoroughly in ([44]) and ([1]). Within the CoNS, *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, 315–317]). 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, 318]). Other CoNS 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 ([319, 320]). *S. haemolyticus* is preferentially isolated from axillae and pubic areas ([318, 321]), and *S. saprophyticus* from the gastrointestinal tract, rectum, and urogenital tract, typically in younger individuals ([1, 322, 323]).

Of the coagulase-negative Staphylococci, *Staphylococcus 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%–7.8% of infectious endocarditis cases in non-drug users ([324]), with morality rates ranging from 38%–42% ([324, 325]). These high mortality rates are similar to those of *S. aureus* endocarditis, and are suggestive of aggressive infection. It is also implicated in infections of medical devices, such as catheters and prosthetic joints ([326–328]). 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 ([329]).

For mechanisms of pathogenesis, *S. lugdunensis* possesses several virulence factors, including surface adhesins, that have been reviewed in ([330]). A mutant in the cell wall-anchoring enzyme Sortase A was attenuated in an experimental endocarditis model, confirming that like in *S. aureus*, adhesins contribute to *S. lugdunensis*-mediated endocarditis ([331]). *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 ([332]). Biofilm formation may be a significant factor in infection, as *S. lugdunensis* clinical isolates from prosthetic implant infections have been shown to be strong biofilm formers *in vitro* ([333]). In addition to its biofilm-forming activity, *S. lugdunensis* has several putative cytolysins, including the SLUSH (*Staphylococcus lugdunensis* synergistic hemolytic) peptides, which are similar to *S. aureus* delta-toxin Hld, and another Hld-like protein ([330, 334]). 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 ([335]) and postoperative endophthalmitis ([336]). 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 ([337]). 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 ([338]).

Since *S. saprophyticus* colonizes the rectum and urogenital tract, it is unsurprising that it is a common cause of urinary tract infections (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 ([339], [340]). *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 ([341]). 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* ([342]).

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 CoNS 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 ([343], [344]). *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 ([345]). Other studies have demonstrated that the appearance of heterogeneous populations can develop within a *S. aureus* biofilm ([227]), and further studies have shown these sub populations can interact to promote vancomycin-intermediate resistance phenotypes ([346]). 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 among 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 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 reign in the enthusiasm by trying to keep certain growth states separate and uniquely defined, such as colonization ([347]), 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 ([348]), polysaccharide aggregates ([349]), and fibrinogen-based clumping ([350]), 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 much 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 in order 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 different ways, and these conditions need to be considered when modeling biofilm development *in vitro* or *in vivo*. We need to properly understand all these different complexities to best position therapeutic development for treating biofilm infections.

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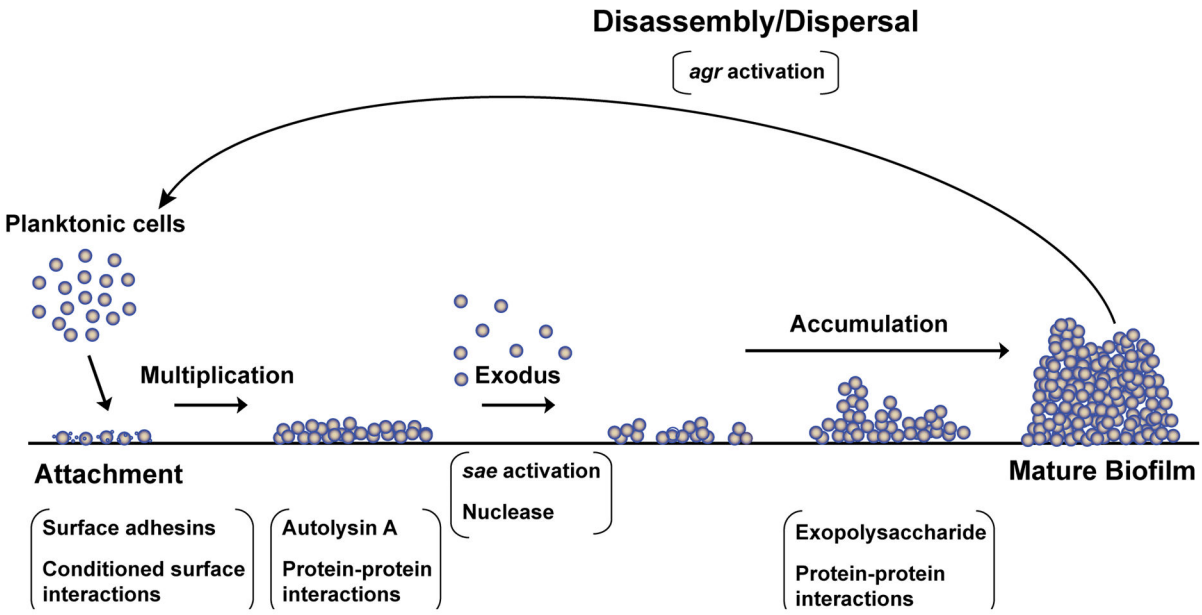

Figure 1.

The biofilm life cycle. Recent studies have identified steps present in early stages of biofilm formation. After attachment, bacteria form a lawn of growth, which undergoes an exodus period that leaves several small foci of cells. The exodus phase is mediated by the SaeRS system via nuclease enzyme activity. The foci of cells then develop into a mature biofilm, containing tower structures. Final dispersal is mediated by the *agr* system via secreted enzymes and PSMs.

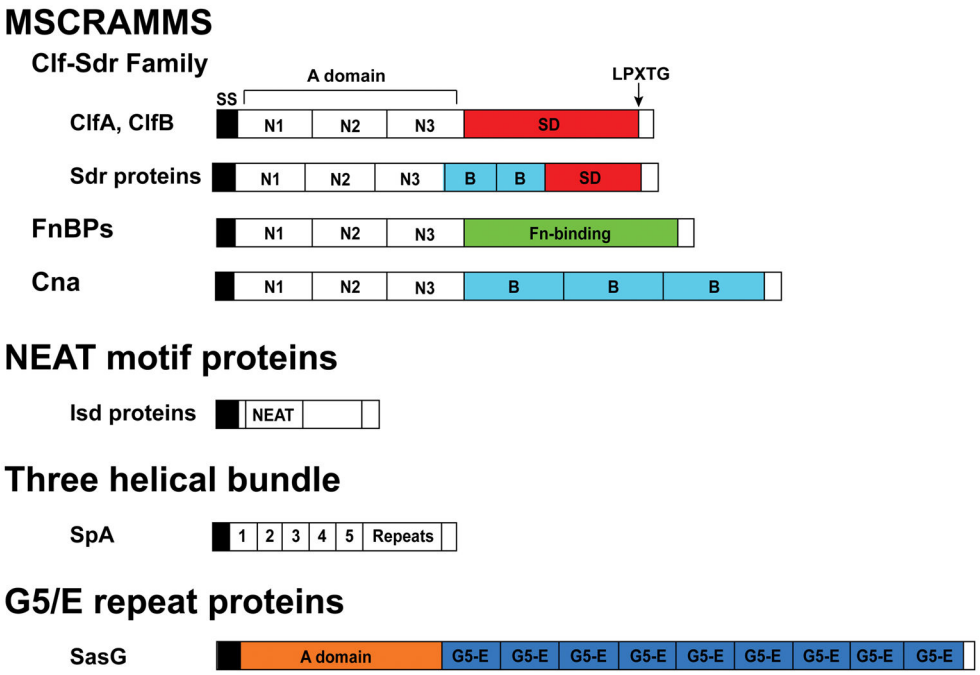

Figure 2.

Cell wall-anchored adhesins. All the cell wall-anchored adhesins contain an N-terminal signal sequence (SS) and a C-terminal portion that is cleaved by Sortase A at the LPXTG sequence. MSCRAMMs contain three IgG-like folds N1, N2, and N3, followed by specific ligand-binding domains. In the Sdr protein subfamily, a variable number of B repeats is found between the IgG-like folds and the SD repeat region. SdrC is shown, which contains two of these B repeats. Similarly, the Isd proteins contain one, two, or three NEAT motifs. IsdA is shown, which has one. In SpA, there are four or five IgG-binding domains, sometimes referred to as domains E, D, A, C, and B. There follows a region containing a variable number of tandem repeats.

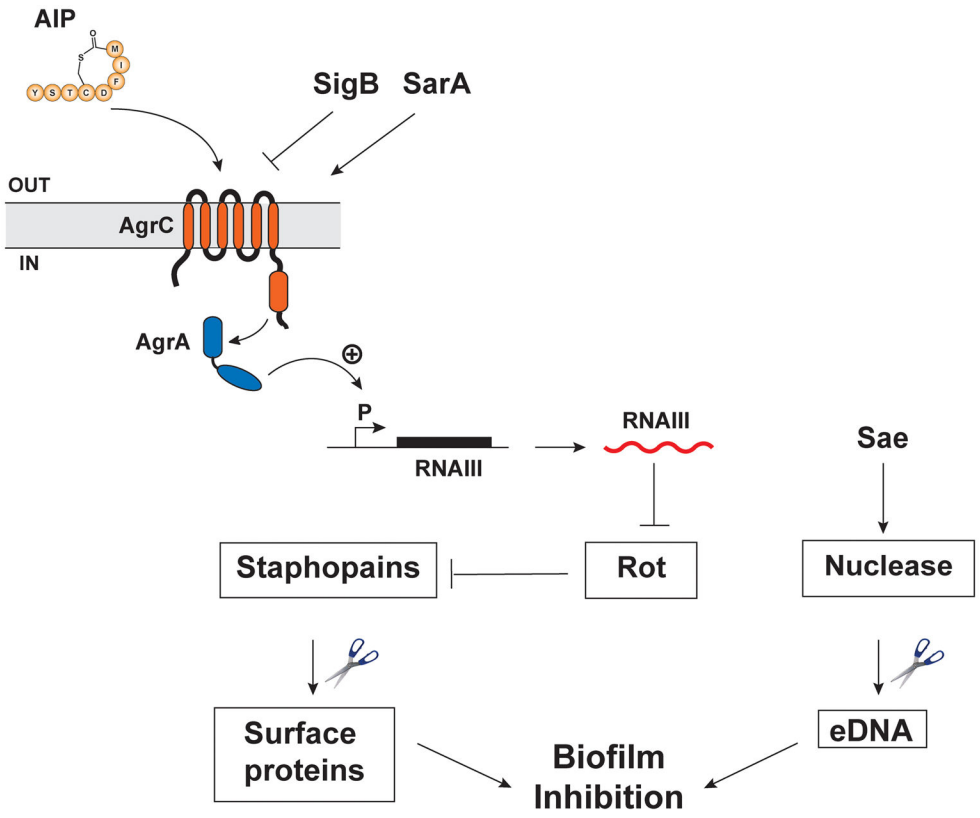

Figure 3.

Regulatory networks in biofilm formation. The *agr* quorum-sensing system induces expression of secreted Staphopain proteases by inhibiting translation of Rot (repressor of toxins), a negative regulator of the proteases. These proteases then degrade proteins on the Staphylococcal surface and in the biofilm matrix. The SaeRS system induces production of the nuclease enzyme that cleaves eDNA in the matrix. Sigma Factor B (SigB) inhibits *agr* expression, while SarA has been shown to directly enhance it.

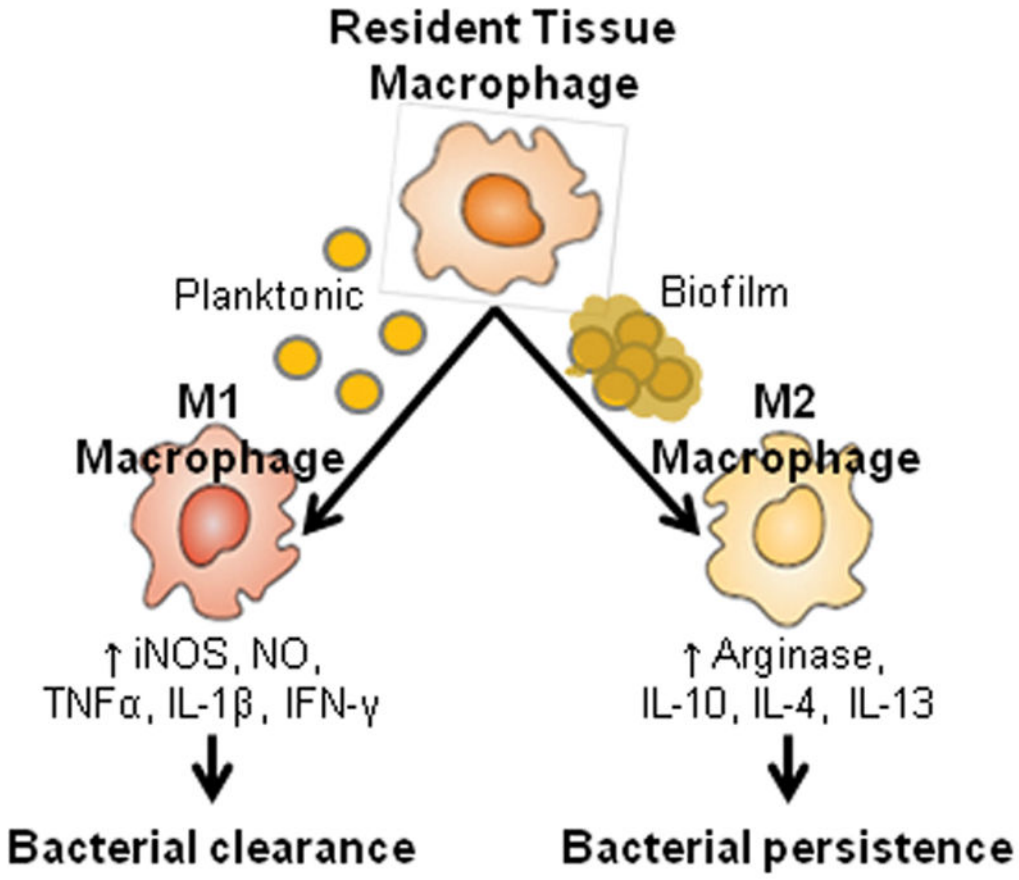

Figure 4.

Macrophage activation pathways. Biofilm growth of *Staphylococci* has been shown to favor the M2 phenotype in macrophages, which is characterized by increased arginase and pro-fibrotic activity, as well as decreased antimicrobial clearance. These changes are thought to contribute to the persistence of Staphylococci in biofilm infections. This figure is a reproduction from ([273]).

Table 1

Adhesins

Family	Subfamily	Adhesin	Species	Ligand(s)	Refs
MSCRAMMs					
	Clf-Sdr				
		ClfA	*S. aureus*	Fibrinogen γ-chain	45_56
		SdrG/Fbe	*S. epidermidis*	Fibrinogen β-chain	57_64
		Fbl	*S. lugdunensis*	Fibrinogen γ-chain	65_69
		ClfB	*S. aureus*	Fibrinogen α-chain, Cytokeratin 10, Cytokeratin 8, Loricrin	45_48, 56, 70_79
		SdrC	*S. aureus*	β-neurexins, self-association	80_82
		SdrD	*S. aureus*	Nasal epithelial cells	80, 83, 84
		SdrE	*S. aureus*	Factor H, C4b-binding protein	80, 85_87
		Bbp	*S. epidermidis*	Bone sialoprotein, C4b-binding protein	87; 97_102
		SdrF	*S. epidermidis*	Type I collagen, Dacron	88_90
		SdrG	*S. epidermidis*	Unknown	91
		SdrH	*S. epidermidis*	Unknown	91
		SdrX	*S. capitis*	Type VI collagen	92
		SdrI	*S. saprophyticus*	Collagen, Fibronectin	93_96
	FnBPs				
		FnBPA	*S. aureus*	Fibrinogen, Elastin, Fibronectin	103_113
		FnBPB	*S. aureus*	Fibrinogen, Elastin, Fibronectin	103_113
		CNA	*S. aureus*	Collagen	114_120
NEAT Motif					
		IsdA	*S. aureus*	Heme, fibrinogen, fibronectin	122_125
Three Helical Bundle					
		SpA	*S. aureus*	Fc region of IgG	37; 128_132
G5-E					

Family	Subfamily	Adhesin	Species	Ligand(s)	Refs
		SasG	*S. aureus*	Nasal epithelial cells, self-association	133, 137, 138
		Aap	*S. epidermidis*	Corneocytes, self-association	133_136, 138_140