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Prevention and treatment of *Staphylococcus aureus* biofilms

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Abstract

S. aureus colonizes both artificial and tissue surfaces in humans causing chronic persistent infections that are difficult to cure. It is a notorious pathogen due to its antibiotic recalcitrance and phenotypic adaptability, both of which are facilitated by its ability to develop biofilms. *S. aureus* biofilms challenge conventional anti-infective approaches, most notably antibiotic therapy. Therefore there is an unmet need to develop and include parallel approaches that target *S. aureus* biofilm infections. This review discusses two broad anti-infective strategies: (1) preventative approaches (anti-biofilm surface coatings, the inclusion of biofilm-specific vaccine antigens); and (2) approaches aimed at eradicating established *S. aureus* biofilms, particularly those associated with implant infections. Advances in understanding the distinct nature of *S. aureus* biofilm development and pathogenesis have led to growing optimism in *S. aureus* biofilm targeted anti-infective strategies. Further research is needed however, to see the successful administration and validation of these approaches to the diverse types of infections caused by *S. aureus* biofilms from multiple clinical strains.

Keywords

Staphylococcus aureus; biofilm infections; persistence; antibiotic tolerance; anti-infective strategies; device related infections; prosthetic implants

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Staphylococcus aureus is a Gram-positive bacterium that typically colonizes the anterior naso-pharynx and the surface of skin.[1,2] This bacterium is found in 30–50% of healthy individuals in the United States, and one in a hundred of these individuals is colonized with methicillin-resistant *S. aureus* (MRSA). This antibiotic-resistant pathogen is, therefore, easily transmitted by direct contact, predisposing a large population of individuals to infection. Nosocomial infections are often associated with *S. aureus*, commonly transmitted either by direct contact with colonized healthcare workers or as a result of invasive medical procedures including surgeries and the introduction of medical implants.[3–5] Treating vulnerable patient populations and the ability of the bacterium to acquire multiple drug resistance further complicates effective treatment of nosocomial infections. In addition to MRSA, glycopeptide intermediate *S. aureus* and vancomycin-resistant *S. aureus* (VRSA) have emerged.[6,7] *S. aureus*-associated infections lead to an increase in hospital stays as well as hospital-associated mortality, likely due to infection with antibiotic-resistant *S. aureus*, resulting in a substantial economic burden on the medical industry, with total values of *S. aureus* infection-related hospital costs estimated at $450 million in the past decade.[8,9]

Biofilms are aggregated structured communities of bacteria encased in a matrix (often referred to as extracellular polymeric substances (EPS)), which is composed of protein, DNA and polysaccharide. During growth in biofilms, bacteria may evade host defenses and become tolerant to concentrations of antimicrobials that eliminate free-floating, single-cell (planktonic) bacteria, making biofilm infections particularly difficult to eradicate.[10,11] Additionally, a lack of biofilm-specific biomarkers makes noninvasive detection and diagnosis of these infections challenging. An important focus of biofilm research, therefore, is the identification of biofilm-specific diagnostic markers and the development of noninvasive diagnostic methods.[12,13]

The past decade has brought increased recognition that *S. aureus* biofilms are a major cause for concern in multiple infections including implant-associated infections and chronic wounds, osteomyelitis, cystic fibrosis lung infection and endocarditis.[14] As a result, research on *S. aureus* biofilm development has contributed to a better understanding of the complexity of *S. aureus* pathogenesis and significant progress in the development of therapies against biofilm infections. Although a number of these hold promise, no single effective treatment is currently available to patients suffering from *S. aureus* biofilm infections.[15–17] As summarized in Figure 1, this review describes currently used anti-infective approaches to *S. aureus* biofilm infections and provides an overview of developments in novel, effective antibiofilm therapeutic strategies. Lastly, it is important to note that there is considerable diversity in *S. aureus* strains, which must also be factored into the development of these approaches.[18,19]

S. aureus biofilm infections

Device-associated infections

An area of primary concern with *S. aureus* biofilm infections is the rapid increase in the use of medical implants and prostheses and the concomitant rise in device-related infections.[17,20] *S. aureus* is commonly associated with artificial surfaces including prosthetic

orthopedic implants, heart valves, pacemakers and vascular catheters.[17,21] These infections are facilitated by direct contact with infected individuals or carriers [22,23] or by the introduction of bacteria from the skin surface due to surgical incision. The surface of an implant is rich in proteins such as fibronectin present at the surgical wound site. These proteins are recognized by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), providing a niche for bacteria to form a biofilm.[23] For orthopedic devices, biofilms may be present on the hardware itself, bone cement and/or the surrounding fibrous tissue.[24] Clumps of detached biofilm bacteria from the surface are often also found in the joint fluid. If not cleared by host immune responses or antibiotic therapy, these bacteria can disperse from the biofilm and enter circulation, resulting in bacteremia.[17,25]

Since many clinical isolates of *S. aureus* are either methicillin or multiply drug resistant, treatment of biofilm infections is amplified by the increased tolerance of these bacteria to the few antibiotics to which MRSA remains susceptible.[11] Thus, the presence of device-associated infections may require revision surgery for replacement, [26] resulting in longer hospital stays, increased risk of a secondary infection, and potentially, removal of the implant if the infection is unabated, augmenting the burden on the patient as well as health-care systems. Increasing average lifespan also correlates with a rise in the population of elderly individuals requiring such prostheses, making MRSA or multiply drug-resistant *S. aureus* a particular concern.[17,27] Novel approaches to develop materials and coatings that can prevent attachment and subsequent *S. aureus* biofilm development have, therefore, become an important aspect of biofilm research, some of which are outlined in this review (see Table 1).

Tissue-associated infections

Chronic wounds

Chronic wounds include surgical site-associated wounds, traumatic wounds, diabetic foot ulcers, pressure ulcers and venous foot ulcers. Their chronicity is defined by the inability to successfully complete the reparative process that allows for wound healing and a return to normal functional and anatomical integrity within a span of 3 months. These wounds are commonly characterized by arrest in the inflammatory phase of healing and are often associated with bacterial infections. Bacteria in chronic wounds are frequently present as biofilms.[42,43] Given that *S. aureus* is often found as a commensal on the surface of skin and in the anterior nares, it is not surprising that it is the most common pathogen associated with wound infections (93.5% of ulcers) [44,45] in both Europe and the United States. Also, 20–50% of wound infections are due to MRSA, making them a clear problem in both inpatient care and wound clinics. The currently accepted regimen to prevent persistent infections in wounds includes a combination of physical and antimicrobial therapies. Debridement or removal of necrotic tissue and bacteria from the wound bed not only reduces the burden of biofilm infection but also exposes healthy tissue to functional immune cells that are important for clearance of bacteria from the site of infection.[46–48] However, the ability of *S. aureus* biofilms to re-establish in the wound necessitates continued sharp debridement coupled with multiple antiseptic and antibiotic treatments.[46] Although the

development of antimicrobial wound dressings has greatly facilitated the treatment of *S. aureus* infections in acute wounds, it should be noted that these target the removal of susceptible (largely planktonic) bacteria.[49] There is, therefore, an urgent need to develop antibiofilm-specific wound dressings as well as other biofilm-targeted strategies for the treatment of such infections.[50]

Cystic fibrosis

One in every 2500 children is born with mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR) protein, making CF one of the most common lethal genetic disorders associated with the Caucasian population. The most frequently occurring mutation is a deletion in a phenylalanine (ΔF508) of the CFTR protein required for efficient ion transport across the cell membrane. The resulting imbalance of ion transport and osmotic regulation leads to dehydration of cells lining the internal organs that significantly impacts airway cells.[51,52] The lungs of patients with CF are compromised in the ability to produce airway surface liquid necessary for efficient mucociliary clearance of pathogens from the airways. Patients with CF are, therefore, susceptible to chronic infection by multiple opportunistic pathogens, [53,54] but the major colonizers are *Pseudomonas aeruginosa* and *S. aureus*.[52]

Studies indicate that CF lung infections are associated with the presence of biofilms and long-term bacterial persistence in the host.[55] Although *P. aeruginosa* is the cause of most complications in adults, children (0–17 years) are prevalently colonized with *S. aureus*. [55,56] These infections are often caused by MRSA and correlate with worsened lung function, measured by forced expiratory volume. Persisting MRSA (>2 years), as is the case for chronic biofilm-associated infections, increases the risk of death in children with CF.[56] Although the impact of *S. aureus* biofilm infections in CF is evident, the lack of a detailed understanding about the specific mechanisms of pathogenesis of *S. aureus* biofilms during CF is currently impeding the progress of treatments specific to chronic *S. aureus* infections in CF.

Endocarditis

Infective endocarditis (IE) is an infection of the endocardium or of prosthetic surfaces in the heart, usually caused by bacteria, and *S. aureus* is a major cause of IE (40–50% in the United States).[57,58] Patients with mechanically injured (from previous valvular disease) or inflamed heart valves are particularly susceptible to *S. aureus* biofilm-associated endocarditis. Injured valves provide a surface for attachment and biofilm formation as well as intracellular infection of inflamed endothelium, which induces further tissue destruction, higher turbulence and deposition of clotting factors.[58] *S. aureus* has multiple surface binding determinants to components of the coagulation cascade, making it well adapted to attachment to the growing vegetation. Biofilm *S. aureus* (vegetation particles) can then replicate on damaged valvular endothelium and disseminate (embolization) to cause systemic disease, [58] resulting in complications such as congestive heart failure, sepsis, persistent bacteremia and intra-cardiac abscess formation, which contribute to a higher rate of in-hospital mortality in these patients.[57]

Surgical procedures involving implantation of prosthetic cardiovascular devices further increase the risk of such infections, [58] and *S. aureus* endocarditis is increasing with the use of cardiovascular implants such as prosthetic heart valves, grafts, hemodialysis catheters and pacemakers. For example, there has been a concurrent increase in cases of *S. aureus* endocarditis in patients who have undergone previous surgical implantation of cardiac prostheses since *S. aureus* is easily disseminated with direct contact during these procedures. Problematically, cases of IE are usually identified using the modified Duke criteria, [59] which, although proven effective for the diagnosis of IE, permit diagnosis only after the infection has become systemic. No protocols exist for the identification of infections in the early stages of vegetation growth since these patients are usually asymptomatic. The ability of biofilms to evade host responses may also contribute to the difficulty in diagnosing IE. [60]

Current therapeutic approaches of *S. aureus* biofilm infections and their drawbacks

It is generally agreed that treating biofilm-associated infections as early as possible results in the best treatment outcomes. [12,13] However, the ability of biofilms to persist for long periods (months to years) without detection or effective eradication by the host immune system often makes early diagnosis and treatment untenable. Physical removal of the source of infection (catheters or surgical removal of orthopedic hardware, nonadsorbable sutures and necrotic tissue) and antibiotic treatment are the only methods currently used for treating these infections. Unfortunately, these methods are not always successful in eradicating the infection.[17,42]

Antibiotic treatment

A significant problem generally with *S. aureus* infections is the rapid development of antibiotic resistance. In *S. aureus* biofilm infections, this may be compounded by an increase in antibiotic minimum inhibitory concentrations (MICs) compared with planktonic isogenic bacteria indicating antibiotic tolerance.[11] In addition, exposure of increased numbers of *S. aureus* in a biofilm to antibiotic selection pressure is also associated with the potential development of antibiotic resistance.[61] Vancomycin is the most commonly administered drug for *S. aureus* biofilm-associated infections.[62] However, clinicians are cautious about the administration of this drug owing to the propensity of *S. aureus* to develop resistance.[6] Evidence for this as a cause for concern is the recent development of vancomycin-intermediate *S. aureus* and VRSA strains.[6] Furthermore, increased tolerance of biofilms to vancomycin (planktonic MIC ~ 2 μg/ml, biofilm MIC ~ 20 μg/ml) requires the use of a combination of other drugs (such as rifampin and linezolid).[11,63,64] However, the combination of vancomycin with rifampin has shown conflicting results, and multiple studies indicate that although this combination might be effective against MSSA it may not hold promise for use in treating MRSA biofilm infections.[63,65,66] Similarly, the use of vancomycin with other drugs including oxacillin, linezolid and tigecycline is open to debate. [14] Nevertheless, rifampin remains the only antibiotic that shows a high degree of efficacy against biofilm-associated staphylococci, and when used in combination with other

antibiotics, it represents the best current treatment along with surgical debridement with retention for treating prosthetic joint infections.[66]

Daptomycin, a cyclic lipopeptide molecule, is a novel antibiotic that has been used for vancomycin-unresponsive *S. aureus* infections. Daptomycin disrupts the cytoplasmic membrane of bacteria, resulting in rapid depolarization and cessation of DNA, RNA and protein synthesis. Daptomycin was found to be the most effective of five drugs tested (linezolid, clindamycin, vancomycin, tigecycline) in clearing *S. aureus* from an existing biofilm.[67] However, a small population of biofilm bacteria remained tolerant to daptomycin, and this drug has shown ambiguous results when used in combination with other antibiotics.[65,68] Treatment of biofilm-associated infection with antibiotic use may also result in the development of dormant “persister” populations of cells that can withstand antibiotic treatment. Since the mechanism of daptomcyin does not require cells to be growing in order to cause membrane damage, it is likely that if used in combination with appropriate antimicrobials it may provide increased efficacy. Moreover, recent work using acyldepsipeptide compounds that activate endogenous proteases to specifically target persister cells and cause lysis provides an innovative approach to this problem.[69,70]

Treatment guidelines are typically specific to the type of infection and for *S. aureus*, dependent upon antibiotic susceptibility.[71] These represent clinical practice guidelines based on randomized controlled clinical trials comparing existing and novel treatments to determine optimal antibiotic regimens and are often debated within the clinical community; [71] however, not all include biofilm infections.[72] In the first published guideline for biofilm infections, the recommendation for *S. aureus* is the use of frequent, appropriate, empiric antibiotic therapy, especially for institutions with recurring MRSA infections.[12] Since most antibiotics show limited efficacy against biofilm-associated infections, novel strategies such as anti-infective surfaces or using combinatorial treatment approaches offer the most potential.[66] Although antibiotic resistance continues to be a major problem, recent studies have shown that changes in the method of delivery and timing of use may reinstate antibiotic efficacy. For example, localized delivery (method) of vancomycin and/or tobramycin using calcium sulfate beads improved their efficacy against planktonic bacteria (~10^6 colony-forming unit/ml reduction) and prevented (timing) formation of biofilms associated with orthopedic infections.[11] Similarly, the use of silver nanoparticles for delivery of drugs into biofilms has recently been found to increase their effectiveness.[73,74] Coadministration of drug efflux pump inhibitors, together with their drug substrates to increase intracellular concentrations of the compound, leading to bacterial killing, is also being evaluated.[75]

Physical removal

Another method currently employed for *S. aureus* biofilm infections is surgical removal of the focus of infection. As mentioned previously, this may be in the form of debridement for wounds or a combination of irrigation and debridement for orthopedic implants. One of the major causes of concern with implant infections is the early development of biofilms, with *S. aureus* commonly isolated as the cause.[76–78] Retention of implants, however, may result in failure to resolve these infections, requiring revision surgery with antibiotic

treatment. Irrigation and/or pulsed lavage are other physical removal techniques that have been used extensively. Unfortunately, strategies that apply only physical means have met with limited success. For example, pulse lavage irrigation was ineffective in eliminating *S. aureus* biofilms from coupons modeled to mimic total knee arthroplasty implants (cobalt chrome, polyethylene, polymethylmethacrylate).[79] These and other studies demonstrated that all currently utilized physical removal methods may fail to effectively clear *S. aureus* biofilms from the infection site, and further studies are necessary to improve these techniques.[77,80,81]

New promising approaches for prevention and treatment of *S. aureus* biofilm infections

The therapeutic challenge of biofilms distinct from planktonic infections has resulted in a concomitant change in the rationale behind *S. aureus* anti-infective treatments toward the development of biofilm-specific strategies. The approaches currently being investigated for targeting biofilms fall into two broad categories as outlined below and each will be discussed in turn.

- **Prevention**
- Preventing attachment of bacteria to surfaces
  - Antibacterial coatings
  - Antiadhesion surfaces
- Vaccines
- **Treatment or disruption of established biofilms**
  - Matrix degrading enzymes
  - Dispersal triggering agents
  - Small-molecule inhibitors
  - *Targeting* S. aureus *regulatory networks*

Prevention

Preventing attachment of bacteria to surfaces—*S. aureus* biofilm development is associated with four broad phases, namely attachment or adherence, proliferation (micro-colony formation), maturation and dispersal (Figure 1). However, the mechanism of the first phase may depend on whether *S. aureus* attaches to an abiotic or biotic surface. Whereas attachment to abiotic surfaces such as glass, metals (Co-Cr, 316SS, titanium, etc.) and plastics (polyester, silicone, polyethylene, etc.) can be nonspecific, *S. aureus* adherence to biotic surfaces depends on bacterial MSCRAMM (the largest class of surface proteins anchored to cell wall peptidoglycan) recognition of host proteins.[82] Thus, abiotic attachment is facilitated by Van der Waal's forces, electrostatic and steric interactions, [83] in addition to a 'conditioning film' formed by host matrix proteins such as fibrinogen, fibronectin and collagen, which may be more important in adhesion than the surface

material.[24,66] These early stages, therefore, may play an important role in selection of isolates that colonize devices since infected cardiac implants have been shown to have higher affinity for fibronectin.[82] The problem of *S. aureus* biofilms and device-associated infections has, therefore, led to the development of anti-infective approaches designed to prevent initial colonization. This includes coatings that prevent the attachment of bacteria (antiadhesion) to and/or growth on (antibacterial) artificial surfaces (see Table 1) in addition to vaccine approaches.

Antibacterial coatings: Approaches currently being evaluated involve the development of bacteriostatic and bactericidal coatings in addition to engineering surfaces to prevent attachment of bacteria.[84,85] For example, silver nanoparticle-coated catheters are being evaluated for use in preventing *S. aureus* attachment. Although *in vitro* studies have shown potential, there are concerns about the cytotoxicity of silver to host tissue due to accelerated thrombin formation and platelet activation, putting patients at higher risk for thrombosis. Nevertheless, these surfaces are now being tested for better host compatibility, making this approach promising for clinical use in the coming decade.[36,86,87] Similarly, titanium, stainless steel and other commonly used implant materials are being coated with antibiotics such as vancomycin to prevent growth of *S. aureus* on these surfaces.[88–90] A concern with this approach, however, is the potential for selection of antibiotic-resistant subpopulations of *S. aureus* in spite of antibiotic combination therapy. Table 1 summarizes some of the coating strategies in development against *S. aureus* biofilm infections.

Antiadhesion surfaces: Another approach to reduce bacterial adhesion to abiotic surfaces is the development of materials that retard adhesion used in combination with the administration of antibiotics or antimicrobials. This dual strategy aims to prevent planktonic bacteria from easily attaching to the implant surface while allowing killing of this antibiotic-susceptible population. The first strategy can be accomplished either by changing the surface physical properties (such as hydrophobicity/hydrophilicity, texture, charge and roughness) such that bacteria are no longer able to easily attach. A second strategy that facilitates the attachment of host cells to the implant has best been described by Gristina *et al.*as a “race for the surface” whereby the risk of developing infections on a surface can potentially be lowered by allowing host cells to competitively occupy it before bacteria are able to do so.[91] Used in conjunction with agents that directly target bacterial host attachment proteins (such as fibronectin binding proteins (FnBPs), collagen binding proteins CnA) [92] and other proteins involved in biofilm formation (biofilm-associated protein (Bap)), [93] this may result in a viable option for preventing *S. aureus* biofilm infections. Although promising, these methods are chiefly applicable to infections on artificial or abiotic surfaces such as implants (prostheses) and foreign devices (such as catheters, stents) and may not be readily applicable to bacterial adherence on host tissue (although nanoparticles may offer anti-infective potential). However, in the case of orthopedic and dental implants, any surface roughness or chemical modifications designed to reduce bacterial attachment need to be assessed for their influence on successful osseointegration to prevent loosening.[94] Finally, important considerations in the development of antiadhesive surfaces include preserving the primary function of the implant (mechanical stability, surface articulation or maintenance of

patency – as in catheters, shunts) and ensuring that the material and/or coating is not cytotoxic to host cells.[87,89,95,96]

Surface-anchored proteins play an obvious role in adherence to and invasion of host cells and tissues, as well as in biofilm formation, and therefore, these proteins represent critical factors that facilitate *S. aureus* colonization and survival during infection. Surface proteins that play a specific role in biofilm formation include Bap, clumping factors (ClfB), FnBPs, surface proteins SasC, SasG and protein A. ClfB, FnBPs and protein A are widely distributed.[82,84] Bap, SasG (homologous to *Staphylococcus epidermidis* accumulation-associated protein) and SasC, however, are not present in all isolates. Furthermore, the role of a number of these proteins in biofilm development is not well characterized. In an alternative approach to target these proteins, a recent study utilized an array of chemical compounds to inhibit the activity of *S. aureus* transpeptidase sortase A, responsible for anchoring surface proteins to the cell wall.[97] Therefore, given further study, surface proteins represent potential novel therapeutic targets to disrupt adhesion or adherence and mitigate biofilm formation.

Vaccines—Research in the development of vaccines that prevent *S. aureus* infections has grown considerably with the rise in antibiotic resistance. Most vaccine candidates, however, fail to take into account the biofilm mode of growth, which may have contributed to their ineffectiveness.[98–100] None have succeeded in passing phase III clinical trials, and therefore, no vaccine is currently in clinical use. For example, a bivalent vaccine developed against the capsular polysaccharides (CP 5/8) with non-toxic *P. aeruginosa* exotoxin A used as a carrier protein showed potential efficacy against planktonic *S. aureus* infections.[101] However, given the presence of strains that lack a capsule (including the prevalent USA300 strain [102]), this vaccine would not be expected to be effective in multiple strains including many associated with biofilm infections.[103] Although both animal models and human trials showed the development of high titers of antibodies lasting for up to 10 months, this vaccine did not proceed to phase III clinical trials due to these drawbacks. Other studies have investigated the use of these polysaccharides with limited success (see Table 2).[104] Similarly, the *S. aureus* iron surface determinant B (IsdB), which is highly conserved and shown to be protective in animal models, was investigated as a vaccine target in a large randomized, placebo-controlled phase II study in patients receiving elective cardiothoracic surgery.[105] This trial not only resulted in failure to achieve the required end points, but was stopped because the vaccine did not reduce the rate of postoperative *S. aureus* infection compared with the placebo and was associated with higher mortality in the vaccinated patients who developed infections with *S. aureus*.

Few vaccine approaches have specifically targeted biofilms. Nevertheless, vaccines against the polysaccharide intercellular adhesin (PIA) component of the biofilm matrix have been developed and tested.[119,120] PIA or poly-*N*-acetyl-β-(1,6)-glucosamine (PNAG) was one of the first molecules identified in biofilm accumulation and continues to be a vaccine candidate since antibodies to deacetylated PNAG epitopes appear to function as better opsonins for humoral protection.[121] However, the number of *S. aureus* strains capable of PIA-*independent* biofilm formation (particularly in clinical isolates) remains to be epidemiologically determined, [122] and since polysaccharides are generally poor

immunogens, it will be important to investigate conjugate vaccines with appropriate protein/peptide carriers. These important drawbacks may be responsible for the limited success of PIA vaccines to date.[109,119] Similarly, studies using CnA (skin, wound infections), FnBPs (heart valves, endocarditis) and ClfB (bone, osteomyelitis) as vaccine targets have demonstrated that, especially concerning biofilm infections, localization and the type of infection need to be considered.[12]

A central consideration in developing vaccines against *S. aureus* biofilms is the significant change in gene and protein expression compared with planktonic bacteria.[123–125] Thus, including biofilm-specific antigens in multivalent vaccines has been investigated and may be particularly important since immune responses elicited by planktonic or biofilm *S. aureus* may be different. For example, planktonic *S. aureus* and the virulence factors enterotoxin A/B, alpha toxin, have been shown to elicit a T_H1-type pro-inflammatory response using *in vitro* models.[126,127] In contrast, implant infection models have shown *S. aureus* biofilms to be resistant to a pro-inflammatory (T_H1) response, but protected by a T_H2-type defense.[128] More recently, a better understanding of T_H17 cells has begun to shape the understanding of *S. aureus* immunity.[99] Similarly, effective antigen presentation is an important avenue for research.[25] A loss in the ability of macrophages and other phagocytic cells to engulf *S. aureus* in biofilms has been shown to be a mechanism of *S. aureus* immune evasion.[129] Furthermore, macrophages have also been shown to have pro-inflammatory and anti-inflammatory phenotypes.[60] These studies raise the possibility of potentiating an anti-*S. aureus* immune response to overcome *S. aureus* actively biasing the host toward an anti-inflammatory and profibrotic response, which may select for biofilm formation and persistence of bacteria in the host.[60] Further understanding the interplay between innate and adaptive immune mechanisms, therefore, represents another crucial area of research.

Another vaccine approach that exploited differences in surface and extracellular protein expression between biofilm and planktonic bacteria led to a multivalent vaccine comprising *both* planktonic and biofilm-specific polypeptides that showed efficacy in combination with vancomycin in a rabbit model of osteomyelitis.[130] This strategy was innovative in developing a more broadly effective vaccine that included biofilm-associated infection since it targeted bacteria in *all stages* to effect eradication. Based on this rationale, a pentavalent vaccine designed for either biofilm or planktonic infection demonstrated 100% *S. aureus* clearance in a murine tibial osteomyelitis model.[131] Further studies are required to determine the suitability of this vaccine for human trials. Although controversy exists regarding *S. aureus* vaccines, studies suggest that taking both planktonic and biofilm growth into account as well as the specific infection environment and multiple strains will be important for significant progress to be made in this field.[99,100,119] A summary of the most promising vaccine approaches subjected to clinical trials is included in Table 2, and the reader is referred to specific references for further information.

Overall these studies highlight several current issues in *S. aureus* vaccine design, some of which also apply to other anti-infective approaches. First, immunogens will need to be present on multiple clinical isolates. Second, despite intense research on *S. aureus* pathogenesis, it is unclear whether induction of humoral or cell-mediated immunity is desirable for protection and this may be particularly an issue for biofilm-associated *S.*

aureus.[60,109,129] A successful vaccine will likely contain more than one antigen coupled with an adjuvant that stimulates both humoral and cell-mediated immunity, particularly since *S. aureus* has the ability to both invade cells and to develop biofilms.[15,109,120,130] The disparate strategies that *S. aureus* uses to breach host defenses suggest that until there is a better understanding of immune effectors in *S. aureus* pathogenesis fundamental questions will remain regarding *S. aureus* vaccines. Lastly, another issue in the study of vaccine and anti-infective approaches involves animal models that recapitulate clinical infections (particularly those associated with biofilms, which are often indolent and chronic).[12,98] While expense as well as research requirements for rapid results and a high infection rate favor the use of short-term acute infection models, recent studies using a vaccine against the *S. aureus* PVL toxin demonstrated that both murine and monkey models did not translate to human infection.[98,132,133] Therefore, although there has been progress with models for prosthetic joint and catheter infections, [134] fundamental questions have arisen about how human immunity to *S. aureus* differs from animal models [98,99] and research is needed to develop models that better characterize biofilm infections.

Thus, targeted preventative approaches appear to be limited at the present time without a better understanding of conserved antigens across numerous clinical strains, the antigenic complexity of virulence factors and capsular antigens, as well as including both biofilm and nonbiofilm *S. aureus* to serve as successful *S. aureus* immunogens. Finally, until there is a better understanding of *S. aureus* pathogenesis, including the diverse conditions for colonizing different host sites of infection and the complexity of regulation of virulence factors, vaccine approaches require further research.

Treatment or disruption of established biofilms

Treatment of biofilm-associated *S. aureus* infection is confounded by the ability of biofilms to evade effective resolution by the host immune system. Coupled with the fact that these infections may not be diagnosed until already formed, an important anti-infective strategy is the development of treatments that target established biofilms. An area of research aimed at mitigating biofilm infections, therefore, involves understanding the mechanisms of biofilm development *and* detachment as part of the dynamic biofilm life cycle (Figure 1), where bacteria return to a planktonic phenotype vulnerable to both antibiotic treatment and host immune responses in order to disperse and colonize new sites. Several different mechanisms have been shown to be involved in the dispersal/disassembly phase of *S. aureus* biofilms. [135] These mechanisms include matrix degrading agents (DNase, proteases, dispersin B, phenol soluble modulins (PSMs)), uncharacterized inducers of dispersal such as D-amino acids and *cis*-2-decanoic acid, as well as activators of dispersal due to environmental cues (such as nutritional or pH stress) or the induction of regulatory networks.

Matrix degrading enzymes—The biofilm extracellular matrix consists primarily of protein, extracellular DNA (eDNA) and polysaccharide providing a physical barrier that protects bacteria in a biofilm from host immune defenses as well as antibiotics. For example, the matrix can impede the penetration of antimicrobial compounds into the biofilm, thus slowing their effective killing function.[136–138] Binding to “decoy proteins” that immobilize antibiotics in the matrix where they are unable to prevent cell wall biosynthesis

or the production of enzymes capable of degrading antibiotics such as β-lactamases are both mechanisms that contribute to antibiotic tolerance. [131] Moreover, retardation of antibiotic penetration may also contribute to the development of genetic resistance. For these reasons, approaches that disrupt the matrix are currently being investigated. These include the exogenous addition of enzymes to disrupt the polysaccharide (dispersin B) or the extracellular DNA (DNase/thermonuclease) components of the EPS.[14] Dispersin B, an enzyme produced by the periodontal pathogen, *Actinobacillus actinomycetemcomitans,* disrupts polysaccharide components of staphylococcal biofilms. However, some studies suggest that the susceptibility of *S. aureus* to dispersin B differs between strains depending on the chemical composition of the matrix, with a number of clinically relevant resistant strains capable of forming polysaccharide-independent biofilms.[122,139]

Similarly, DNase I is effective in targeting *S. aureus* biofilms and disrupting the matrix by breaking down the extracellular DNA released as a result of autolysis of a subpopulation of cells. [140,141] DNase I may be more effective in disrupting early biofilms, reflecting an important role for eDNA in biofilm attachment.[142–144] Trypsin and proteinase K have also been used to disrupt protein components of the biofilm matrix.[14,145,146] Protease inhibitors have also been shown to promote biofilm formation under conditions that otherwise induce dispersal and mutational defects in *sarA* and *sigB*, which upregulate extracellular proteases, restrict *S. aureus* biofilm development.[135] While these methods have met with less enthusiasm due to the possibility of protein-induced inflammatory responses in the host, a combination of these approaches may be viable for use in device-related *S. aureus* infections by employing an approach similar to “antibiotic lock” solutions (treating with a high concentration of antibiotic in the catheter lumen).[147,148]

Dispersal-triggering agents—Although methods that disrupt the biofilm matrix and address the problem of penetration of other compounds into the biofilm are promising, the development of antimicrobial-resistant sub-populations remains a primary concern, especially since development of subpopulations occurs at a higher rate in biofilm cultures compared with planktonic cultures.[149] Therefore, antibiotic-independent approaches that target *S. aureus* biofilms represent a critical parallel strategy. The dispersal of single cells from biofilms has been shown to result in increased susceptibility to antibiotics.[150,151] However, if detached as clumps *S. aureus* can remain tolerant to antibiotics, so the manner of dispersion is also an important consideration.[152] Researchers are, therefore, also evaluating *S. aureus*-specific factors that initiate biofilm dispersal/disassembly. Nonspecific planktonically expressed dispersal agents including surfactants such as the PSMs have been shown to be effective in disrupting most *S. aureus* biofilms.[153–155] A drawback of these molecules, however, is that they are intrinsically inflammatory and may cause undesired effects in the host. The discovery of PSMs [156] and the role of these peptides in biofilm pathogenesis indicate that further study is needed, particularly in light of reports suggesting they may lyse phagocytes.[157] Recent work, however, demonstrated that PSMs are soluble under planktonic conditions but form insoluble amyloid fibers during growth as biofilms. Insolubility was concurrent with a loss of surfactant properties and the ability of PSMs to cause phagocyte cell lysis, which may affect the use of these molecules as dispersal agents. [158,159]

Another small molecule, *cis*-2-decenoic acid, a fatty acid messenger produced by *P. aeruginosa*, caused an increase in the number of bacteria released by *S. aureus* biofilms, suggesting its use as a dispersal agent.[160] The mechanism by which dispersal occurred, however, is not fully understood, and further studies are required to validate these findings. The use of nanoparticles to penetrate biofilms and deliver antiadhesive and/or antiaggregative small-molecule inhibitors, or dispersal agents to the biofilm surface, is an area of intense research interest and also represents a promising approach.[28,97,161]

Small-molecule inhibitors—A number of small-molecule inhibitors with activity against *S. aureus* biofilms have recently been identified.[14,28,162] Most of these studies have been completed *in vitro,* and further investigation is needed to examine their effects *in vivo*. However, an inhibitor of the *S. aureus* RnpA protein essential for efficient mRNA turnover in the cell ameliorated systemic infection in a mouse model and further showed antimicrobial properties against *S. aureus* biofilms in an *in vitro* catheter model.[163] Similarly, preliminary studies indicate that the D-isomers of the amino acids proline, tyrosine and phenylalanine may inhibit *S. aureus* biofilm development. Notably these molecules were ineffective in preventing initial attachment of bacteria to surfaces, but inhibited the development of mature biofilms from nascent aggregates or microcolonies.[164–166] D-isomers appeared to be effective in targeting the protein, but not polysaccharide components of the matrix. Since *S. aureus* forms protein-independent biofilms, this may be a limitation of this approach. Similarly, a *Bacillus subtilis*-derived molecule, norspermidine, was shown to have protein-dependent anti-*S. aureus* biofilm activity.[164,165,167] However, studies by other groups failed to replicate these results and recently the authors retracted these results based on the fact that they were unable to reproduce the original findings.[167] Indeed, a better understanding of the mechanism, not only of these molecules, but numerous other effectors and therapeutic strategies on *S. aureus* biofilms, is needed.

Targeting *S. aureus* regulatory networks—Biofilm bacteria may use quorum sensing (QS) as a mechanism to modulate the expression of virulence factors, adhesins and extracellular components, once a critical mass or “quorum” of bacterial cells has been reached. Modulation of QS systems is an important strategy in controlling biofilm infections in bacteria such as *P. aeruginosa* based on the rationale that QS signals that trigger the switch from planktonic to the biofilm mode of growth can be inhibited, thereby preventing biofilm development or inducing biofilm dispersal.[168] Targeting QS, therefore, represents a novel anti-infective strategy for combined therapeutic approaches to treat biofilm-associated infections. However, in *S. aureus*, the accessory gene regulator (*agr*) QS system is pleiotropic in its regulation of downstream effectors and crucially, since *agr* is repressed in biofilms, appears to be distinct from other bacterial systems in the regulation of biofilm development. Therefore, activation of the *agr* QS network (achieved by the release of an auto-inducing peptide (AIP), a cyclic thiolactone-containing molecule that increases with cell density) results in the dispersal of *S. aureus* from the biofilm.[145,162] Increased concentration of AIP leads to activation of the *agr* regulatory network that controls the expression of multiple virulence factors such as PSM and extracellular proteases, via RNAIII, the main effector for downstream virulence gene expression. The complexity of the

agr regulon and the discovery to date of numerous *agr* associated transcription factors, two-component signaling systems and downstream effectors is beyond the scope of this review, so the reader is referred to several excellent recent reviews on *S. aureus* QS and gene regulation.[113,169,170] Therefore, while developing agents that inhibit *agr* expression would theoretically be a useful strategy for blocking virulence determinants that play an important role in acute *S. aureus* infections, because *agr* activation induces the dispersal of bacteria from a mature biofilm,[162] *agr* inhibitors would be expected to promote biofilm development. Researchers have, therefore, attempted to target factors downstream of the *agr* locus.

RNAIII peptide inhibitor (RIP) was investigated as a means to disrupt the numerous genes activated by this effector and found to prevent the formation of *S. aureus* biofilms on the surface of central venous catheters and reduce the minimum biofilm inhibitory concentration (MBC) for ciprofloxacin, vancomycin and imipenem when combined with RIP.[171,172] Studies with RIP initially suggested another *agr*-linked QS in *S. aureus*; however, these studies are controversial since other groups have not been able to reproduce them. There is also a lack of understanding regarding the mechanism by which RIP inhibits biofilm formation without affecting the *agr* QS circuit. Without validation, these studies are directly contradictory to the role of *agr* QS in biofilm dispersal.[113] Nevertheless, a nonpeptide RIP analogue, discovered by screening a small compound natural product library, has been reported. The analogue from witch hazel, hamamelitannin, was active against device-associated infections in a rat model and may show promise.[173]

Although the understanding of QS regulatory systems has increased significantly in the past few years, links between these key networks await further elucidation. Notably, another regulatory locus, staphylococcal accessory regulator (*sar*) plays an opposing role with *agr* in *S. aureus* biofilm formation, with inhibition of *sar* or overexpression of *agr* both showing potential as strategies for inhibiting biofilms.[174–176] This study, however, indicated variable effectiveness among *S. aureus* clinical isolates, and other studies suggest that *sarA* is able to facilitate the expression of *agr*.[161,175,176] Therefore, important underlying molecular mechanisms require further study before this approach can be successful as an anti-infective strategy for *S. aureus* biofilms.

Concerns have also been raised about inhibiting *agr* since this QS system is present in many commensal staphylococci including *S. epidermidis*, which is also responsible for a substantial percentage of biofilm infections on medical devices. Although the *agr* system is indisputably a major regulator, the existence of numerous other regulatory elements in *S. aureus* requires further research to better understand the role of *agr* in the balance between biofilm accumulation and secreted virulence factor expression in staphylococci and how *agr* modulators would affect this balance.[23,175] Nevertheless, the extensive regulation by the *agr* system, as well as its interconnections with other global regulators such as *Sar, Rot* and *CodY,* suggests that targeting regulatory systems is a promising avenue for development of anti-infective therapies against *S. aureus* biofilm infections.[176–178]

Five-year view

S. aureus is a life-threatening opportunistic pathogen with the ability to form robust biofilms and readily adapt to diverse environments in the human host. *S. aureus* biofilm development is a multifactorial process that can occur on both implant and tissue surfaces.[17,23] Due to increased antibiotic tolerance and the persistence of *S. aureus* in biofilms, these infections defy treatment with antibiotics alone, reflecting a trend that will continue in the next five years, no matter how carefully the use of these agents is monitored. The study of *S. aureus* biofilm development has led to discoveries of how surface-associated determinants, QS and regulatory pathways of secreted and surface anchored virulence factors differ during biofilm growth. However, the complexity of growth conditions, the high degree of genomic diversity amongst *S. aureus* strains, the complexity of biofilm pathogenesis (including the ability to evade immune effectors) and the difficulty in translating animal models to human trials have slowed breakthroughs in therapeutic approaches. This highlights the urgency for basic and translational research to develop novel anti-infective therapeutics effective against diverse *S. aureus* biofilm infections and multiple clinical strains. Anti-infective strategies will involve a decreased reliance on antibiotics as the sole treatment for *S. aureus* infections and the investigation of interventions targeting both biofilms and planktonic bacteria, some of which have already been implemented in clinical settings. Nevertheless, the antimicrobial resilience and ability to evade host immune responses observed by *S. aureus* biofilms requires further research to investigate the underlying mechanisms of pathogenesis used by *S. aureus* to persist in biofilms under multiple conditions. Recently, specific guidelines for the clinical diagnosis and treatment of biofilm infections and the development of antibiofilm therapeutics have been published.[12,179] Partnerships that bring together key clinical, academic, industry and regulatory stakeholders to discuss biofilm infections will help antibiofilm therapeutics keep pace with the projected rise in device-related biofilm infections.[179] More funding for biofilm research along with a collaborative, interdisciplinary approach will likely see novel *S. aureus* biofilm-directed therapies reaching the patient in the next five years. The recognition that *S. aureus* biofilms are a distinct clinical problem has led to significant advances in understanding *S. aureus* biofilm development and in multiple strategies for targeted therapeutic intervention specific to *S. aureus* biofilms. Although none of these constitutes a magic bullet on the immediate horizon, combinatorial and adjunctive strategies will no doubt lead to more effective treatments in the near future.

Expert commentary

One of the most challenging issues plaguing clinicians in the past decade has been the rise in infections involving multidrug and MRSA, especially multidrug-resistant MRSA.[1,7] A less well known but equally problematic issue, however, involves the ability of *S. aureus* to form structured aggregated communities known as biofilms that protect bacterial cells from host defenses and antibiotic treatment. Biofilm bacteria are phenotypically distinct and even methicillin-sensitive strains may tolerate much higher concentrations of antibiotic than isogenic planktonic bacteria, making these infections difficult to eradicate. It is now recognized that a majority of chronic staphylococcal infections are due to biofilms, particularly those associated with an indwelling medical devices.[8] However, most

therapeutic strategies are applicable only to planktonic or acute *S. aureus* infections. Therefore, there is an urgent unmet need for new therapeutic strategies that target *S. aureus* in biofilms.

The rationale for the development of biofilm-specific treatment strategies is broadly divided into three key aspects of biofilm-associated infections. The first is the distinction between planktonic and biofilm bacteria.[124,130] Biofilms are not comprised simply of free-floating bacteria that have passively attached to a surface and become covered in extracellular material. Rather, biofilm bacteria actively express extracellular proteins, virulence factors and surfactants that are distinct from their planktonic counterparts.[124] Treatments targeted to infections using only planktonic *S. aureus* (such as antibiotic susceptibility testing), therefore, are likely to be ineffective against the biofilm phenotype. Second, biofilms are intrinsically more tolerant to antibiotics in comparison to free-floating bacteria with MIC and MBC up to 1000 higher,[95] often exceeding cytotoxic thresholds or clinically achievable concentrations. Treatment of *S. aureus* biofilms with antibiotic concentrations using planktonic MICs, therefore, may not only be ineffective but may lead to the development of antibiotic-resistant subpopulations. Thus, adjunctive or parallel approaches are necessary to circumvent the potential problem of antibiotic-resistant sub-populations.[69] Third, since *S. aureus* is capable of successfully colonizing and establishing biofilms in various host environments (implant surfaces, lung, skin and bone), it is important to take this into account when designing anti-infective therapies against these infections.

References

Reference annotations

* Of interest

** Of considerable interest

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Key issues

- *S. aureus* is a potentially multiply drug-resistant pathogen frequently found as biofilms in both implant- and tissue-associated infections.
- An increasing aging population requiring prostheses and implants is vulnerable to *S. aureus* biofilm infection, including multiply drug-resistant strains.
- *S. aureus* biofilms are structured aggregates of bacteria encased in an extracellular matrix of carbohydrate, DNA and proteins, phenotypically distinct from single-cell (planktonic) bacteria.
- *S. aureus* in biofilms is more difficult to eradicate since biofilm bacteria may evade host responses and tolerate much higher concentrations of antibiotic compared with isogenic planktonic bacteria.
- *S. aureus* biofilm infections are typically characterized by chronic or recurring infections.
- In addition to the many clinical strains of *S. aureus* causing infections, phenotypic differences between planktonic and biofilm bacteria must also be taken into account in the development of effective preventive or therapeutic antibiofilm strategies.
- *S. aureus* biofilm research is further advancing our understanding of the ability of this complex opportunistic pathogen to adapt to different host niches and cause infection.
- Current strategies for preventing *S. aureus* biofilm infections include vaccines and the development of coated or modified biomaterial surfaces; however, many of these strategies are still in the research phase and most have yet to be successfully implemented.
- There is an urgent unmet need for new therapeutic strategies that target *S. aureus* biofilm growth.

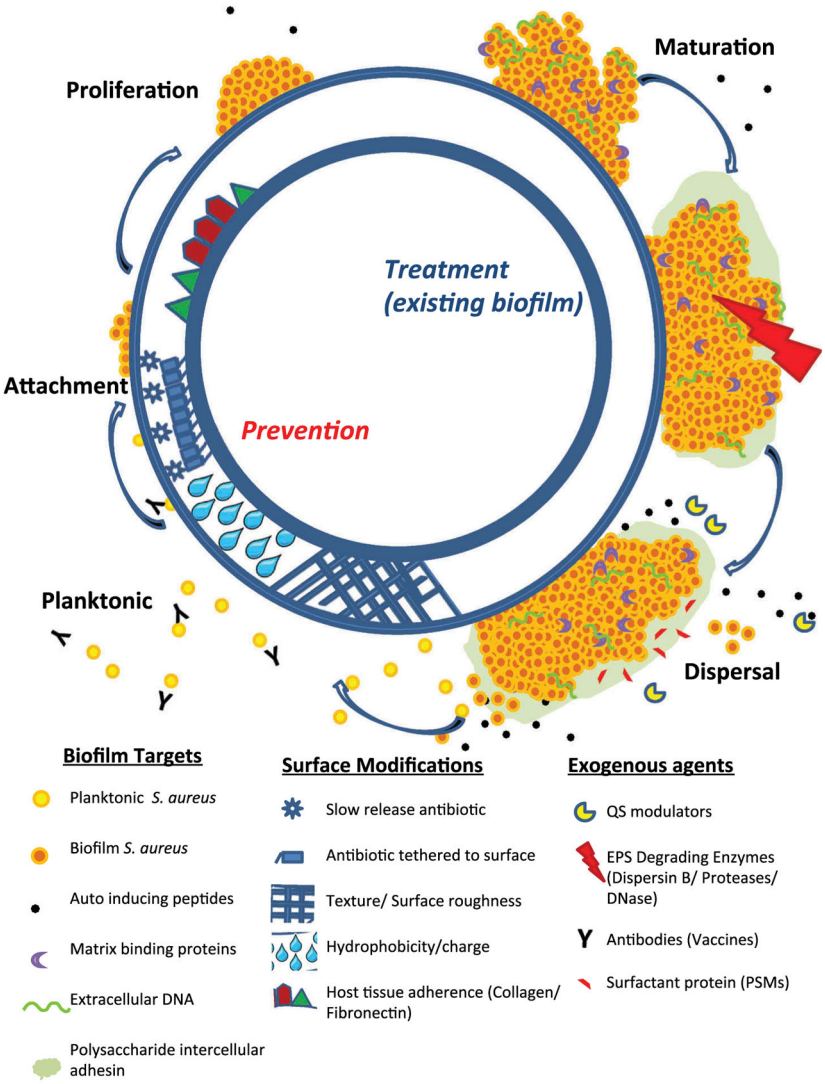

Figure 1. Strategies for prevention and treatment of *S. aureus* biofilms

A summary of the life cycle of a *S. aureus* biofilm, depicting the various stages of attachment, subsequent development, dispersal and colonization of new sites, is shown. Each of these stages represents possibilities for therapeutic disruptive intervention strategies. Broadly these strategies break down into (1) disruption at the surface (inner part of ring) through physical surface modification or surface-mediated delivery of antimicrobial/ antibiofilm agents or (2) systemic or local delivery from the surrounding tissue or body fluids (outer part of the ring). Biofilm single cells and clusters are attached to a representative surface depicted by the blue ring. Since established biofilms can exhibit all stages of the growth cycle simultaneously due to highly localized structural heterogeneity, it is likely that for many patients multiple antibiofilm strategies will be required for effective prevention or treatment of the infection. EPS: Extracellular polymeric substances; PSM: Phenol soluble modulin; QS: Quorum sensing.

Table 1

Some examples of materials incorporating antimicrobial surfaces or coatings to prevent *S. aureus* attachment and/or biofilm development on implant surfaces. Drawbacks (if known) are included (marked by closed circles).

Anti-*S. aureus* coating	**Principle and reported drawbacks**	**References**
Aryl rhodanines	Small molecule. Prevents attachment of bacteria to surfaces but is not antibacterial. Effective against multiple Gram-positive species	[28,29]
Quaternary ammonium silane	Quaternary ammonium groups have antimicrobial activity. These were tested in a singular ATCC *S. aureus* strain	[30]
	• Concerns with masking of active groups by dead bacteria, tissue	
Calcium chelators	Deprive bacteria of essential Ca^{2+}	[31]
	• Strain-specific inhibition of biofilm formation (depends on expression of clumping factor B)	
Polymer brushes	Repulsive osmotic forces, discourages bacterial adhesion to the surface	[32]
	• Few bacteria that successfully attach have been shown to form biofilms albeit at a slower rate	
Organoselenium	Catalyzes the formation of superoxides, reducing possibility of bacterial survival on surface	[33]
	• Dead bacteria and host tissue masking the compound coating, however, is a concern	
PLL-g-PEG	PLL-g-PEG reduces adsorption of host matrix proteins onto the surface, preventing bacterial attachment	[34]
Silver nanoparticles	Ag+ ions enter cells to interact with protein and DNA, disrupting cell division and respiration, leading to cell death	[35,36]
	• Showed disappointing results due to inactivation of silver by blood components	
Lysostaphin	Antimicrobial, coating on orthopedic implants (mouse model)	[37]
Chitosan	Osteoconductive, antimicrobial coating. Biocompatible with host tissue	[38–40]
	• Further studies required (strain specificity, attachment *in vivo*)	
Sharklet™	Engineered surface microtopography based on biomimicry of shark skin patterns	[41]

ATCC: American Type Culture Collection; PLL-g-PEG: Poly(L-lysine)-grafted-poly(ethylene glycol).

Table 2

Summary of some vaccine approaches to prevent infection with *S. aureus*, their stage in clinical trial investigation and drawbacks (if known).

Vaccine agent	Clinical trial progress and potential drawbacks	References
IsaA, LytM (endopeptidase), Nuc, Atl pro-peptide and four PSMα	Animal model (mouse/bacteremia) • Preclinical • Does not specifically target biofilms	[106]
V710 (Merck). Targets IsdB, a cell wall-anchored protein expressed during iron limitation	Phase I completed. Phase II/III interrupted. Many isolates tested were from wound sites • Infection environment may alter efficacy • Not analyzed in context of biofilms; however, recent studies using multiple antigenic determinants including IsdB in biofilms address this issue	[107,108]
Sanofi Pasteur SAR279356 (poly-*N*-acetyl glucosamine) extracellular matrix polysaccharide	Terminated in phase II trials Not all strains form a polysaccharide dependent matrix	[109]
SA3Ag (ClfA) CP5, CP8	Phase I ongoing All strains do not express CP5 and CP8, which might alter the efficacy	[110]
Veronate (INH-A21) pooled immunoglobulin from patients with high titer of fibrin and fibrinogen binding adhesins	Phase II studies tracking sepsis were promising • Failed phase III trials • Vaccine was active against *S. epidermidis* and *Candida albicans* • Failure possibly due to lack of specificity	[99,111]
Pagibaximab (BXYX-A110) mAb against cell wall component (LTA acid)	Phase II completed. Studies have mostly been targeted to prevention of *S. aureus*-associated sepsis in infants Strain-dependent variations of LTA would alter the response	[100,112]
AP4-24H11 mAb against AIP-4 to block quorum sensing	Mouse model of abscess formation • Preclinical Depends on strain AIP group (1–4) Further studies with mAb against all groups of AIP will allow use with multiple strains	[113,114]
MEDI 4893 (MedImmune)	Phase II • Targets a single virulence factor • Does not take strain diversity into consideration	[115,116]
PVL-targeted vaccines	Preclinical • Controversial results • Relevance of PVL debated • Vaccine would be effective in small population of PVL+ CA− strains MRSA	[117,118]

AIP: Auto-inducing peptide; Atl: Autolysin; CA: Community-acquired; ClfA: Clumping factor A; CP: Capsular polysaccharide; IsaA: Immune-dominant staphylococcal antigen A; LTA: Lipoteichoic acid; mAb: Monoclonal antibody; MRSA: Methicillin-resistant *S. aureus*; Nuc: Nuclease; PSMα: Phenol-soluble modulins α; PVL: Panton–Valentine leukocidin.