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Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections?

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Summary of recent advances

It is well accepted that bacterial pathogens growing in a biofilm are recalcitrant to the action of most antibiotics and are resistant to the innate immune system. New treatment modalities are greatly warranted to effectively eradicate these infections. However, bacteria growing in a biofilm are metabolically unique in comparison to bacteria grown in a planktonic state. Unfortunately, most antibiotics have been developed to inhibit growth of bacteria growing in a planktonic mode of growth. This review focuses on the metabolism and physiology of biofilm growth with special emphasis on the staphylococci. Future treatment options should include targeting unique metabolic niches found within bacterial biofilms in addition to enzymes or compounds that inhibit biofilm accumulation molecules and/or interact with quorum sensing and intercellular bacterial communication.

Introduction

Bacterial antibiotics have been designed to target unique and essential prokaryotic targets including cell wall synthesis, DNA replication and protein synthesis. In addition, recent data has demonstrated that multiple classes of bactericidal antibiotics stimulate the production of hydroxyl radicals through Fenton chemistry thus accelerating bacterial cell death [1]. The efficacy of bacterial antibiotics has classically been tested using bacteria in the exponential phase of growth employing an *in vitro* broth system that is meant to simulate the planktonic growth of bacteria in blood or other sterile body fluids. Furthermore, clinical microbiology laboratories typically test the antibiotic susceptibility of bacterial pathogens with a microbroth dilution test utilizing planktonic bacteria. However, many different types of bacterial infections are known or presumed to be due bacteria growing in a biofilm state including biomaterial-related infections, chronic wounds (e.g. diabetic foot wounds), cystic fibrosis-related lung infections, endocarditis and otitis media. The National Institutes of Health (United States) estimates that 80% of all infections are biofilm-related [2]. It is well accepted that bacteria growing in a biofilm are more recalcitrant to the action of antibiotics than cells growing in a planktonic state and are associated with chronic inflammation and resistance to the innate immune system [3]. In many cases, it is unclear how clinical microbiology laboratories should test the antibiotic susceptibility of bacteria isolated from a biofilm-mediated infection. Several groups have proposed diagnostic criteria for the identification of biofilm-mediated infections

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that include (among others) association with a surface, recalcitrance to antibiotics, and ineffective clearance by host inflammatory cells [4-6]. The development of biofilm is thought to consist of four separate stages: 1) binding of planktonic bacteria to a foreign body or tissue; 2) liberation of extracellular material consisting of protein, polysaccharide and extracellular DNA (eDNA; originating from the bacteria due to autolysis) allowing for intercellular aggregation; 3) biofilm maturation which includes the development of towers and water filled channels and 4) biofilm dispersal. This review will focus on the available literature describing the physiological and metabolic differences between cells growing in a planktonic state in comparison to those growing in a biofilm; several promising aspects of treatment of biofilms will be discussed. Although several models of bacterial biofilm formation have been developed for *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli* and *Bacillus subtilis* [7], this review will focus primarily on staphylococcal biofilm models.

How do staphylococci grow in a biofilm?

Data from the Centers for Disease Control Nosocomial Infections Surveillance System demonstrates that *Staphylococcus epidermidis* and *S. aureus* are the most common cause of nosocomial infections in intensive care units [8]. *S. aureus* causes a wide variety of infections ranging from skin and soft tissue infections to more serious infections such as endocarditis or osteomyelitis [9]. In contrast, *S. epidermidis* infections are primarily associated with a foreign device (i.e. catheter related infections) and are typically perceived as biofilm-mediated [10, 11]. Due to the importance of both *S. aureus* and *S. epidermidis* in biofilm-mediated infections, mechanisms that function to mediate biofilm formation in these species have been well studied. It is out of the scope of this review to comprehensively describe the known literature related to biofilm formation within the staphylococci. The reader is referred to several recent reviews on this subject and Figure 1 [10-12]. However, although we are far from completely understanding biofilm formation, it is relevant to discuss two important aspects that are important for development of novel therapeutics.

Redundancy of accumulation molecules in staphylococcal biofilm

The most well studied staphylococcal accumulation molecule is polysaccharide intercellular adhesin (PIA or poly-N-acetylglucosamine [PNAG]) [13]. PIA is synthesized by enzymes encoded by the *icaADBC* operon [13]. In *S. epidermidis*, PIA is essential for the development of large (50 μ m) three-dimensional towers, resistance to fluid shear stress, and resistance to biocides and neutrophil-dependent killing [10,14,15]. The contribution of PIA to virulence of *S. epidermidis* has been demonstrated in two relevant animal models of infection [16,17]. However, several studies have demonstrated that not all strains of *S. epidermidis* encode the *icaADBC* operon [18,19] and, although most isolates of *S. aureus* encode *icaADBC*, the expression level within a biofilm is very low and seems to be dispensable for biofilm formation [20-22]. Therefore, as predicted, other accumulation factors have been described including eDNA, Aap, Bap [Bhp], Protein A, FnBpA/FnBpB, SasG, and Embp [23-30]. Recent studies by Rohde and colleagues found that 27% of clinically relevant *S. epidermidis* isolates produced PIA-independent biofilms further demonstrating their clinical relevance [31]. An evolving hypothesis suggests that staphylococci may change the composition of the extracellular matrix depending upon the extracellular niche. Supporting this hypothesis, studies from Vergara-Irigaray and colleagues have recently shown that *S. aureus* can alternate between an FnBp and PIA/PNAG biofilm depending on the environmental conditions [32]. What is not known is whether the metabolism differs between different proteinaceous biofilms and those that are primarily encased in PIA/PNAG. Further work is clearly needed to determine if unique proteins are expressed within these different biofilms and whether the antibiotic susceptibility or virulence differs in relevant animal models of infections.

Heterogeneity and metabolism of staphylococcal biofilm

As previously mentioned, bacteria growing within a biofilm are metabolically unique from those cells growing in exponential phase (e.g. planktonic cells). Lopez and colleagues termed the biofilm mode of growth “natural stationary phase [7].” Due to a measured nutritional and oxygen gradient, bacteria within a biofilm exist in different metabolic zones [33]. The current paradigm suggests that bacteria at the surface of the biofilm are the most aerobic and metabolize preferred carbon sources whereas those bacteria at the bottom of the biofilm are either inert or reside in an anaerobic niche and utilize secondary carbon sources (amino acids or peptides) [33]. Rani and colleagues recently demonstrated that *S. epidermidis* growing in a biofilm existed in four metabolic states: aerobic, fermentative growth, dead and dormant [34]. The detection of significant dormant areas brings forward the hypothesis that persister cells may function to mediate innate antibiotic resistance within a staphylococcal biofilm [35]. As defined by Lewis, persister cells are those that enter into a dormant, multidrug-tolerant state; it is hypothesized that persister cells can repopulate a biofilm once the patient is not longer administered antibiotics [35]. Although it is unclear where persister cells may be found within the biofilm (dormant area at bottom of biofilm?), it has been documented that they can be isolated within a staphylococcal biofilm and presumably have a highly relevant role in the development of innate antibiotic resistance [35,36]. Singh and colleagues recently isolated persister cells from a *S. aureus* biofilm following treatment with oxacillin, cefotaxime, amikacin, ciprofloxacin and vancomycin; however, all persister cells reverted to back to a susceptible phenotype upon subsequent time kill curve analysis [36]. Although persister cells have been linked to toxin/antitoxin systems in *E. coli*, it is unclear whether these same mechanisms are functioning in other species biofilms as evidence suggests that persistence is a redundant system [35,37].

The transcriptional response of staphylococcal biofilm growth in comparison to exponentially growing cells has demonstrated that, on average, biofilms are growing under microaerobic/anaerobic conditions as genes such as lactate dehydrogenase, formate dehydrogenase and nitrate reductase are upregulated during biofilm growth [20,38,39]. In addition, genes that encode amino acid transporters or amino acid catabolism (e.g. arginine deiminase) are also up-regulated within a biofilm in comparison to planktonic cells. Direct measurement of *S. aureus* biofilm flow cell effluent confirmed glucose is catabolized to pyruvate and then catabolized primarily to lactic acid via lactate dehydrogenase [40]. In contrast, very little lactic acid is produced by the staphylococci under aerobic growth conditions [41]. A model is clearly emerging whereby biofilm is a heterogenic mixture of cells utilizing different carbons sources (for instance glucose vs. amino acids/peptides) and responding to different levels of oxygen tension. Therefore, the development of appropriate anti-biofilm approaches must take into account at least the following: 1) unique accumulation molecules (e.g. proteinaceous vs. polysaccharide) and the differences that may reside within these biofilms (unknown at this time); 2) the development of persister cells; and 3) the metabolic heterogeneity that is inherent to growth in a biofilm.

Treatment approaches for Staphylococcal biofilm

In most cases, the best treatment for a staphylococcal foreign body mediated biofilm infection is to remove the offending device [42]. However, in implantable infected prostheses (e.g. knee or hip), pacemaker leads or other cardiac implantable devices where removal is difficult, appropriate treatment options are greatly warranted. Unfortunately, due to the lack of randomized clinical trials, standard clinical practice regarding treatment of biomaterial-related infections has not been fully developed. The most logical approach to the prevention of staphylococcal biofilm formation is to inhibit initial binding of the bacterium to the biomaterial. This typically involves the impregnation of antimicrobial compounds into the biomaterial; one of the most commonly used catheter impregnations in clinical practice is chlorhexidine/silver

sulfadiazine that has decreased microbial colonization in clinical trials [43]. However, although many antiseptic/antibiotic/antimicrobial peptide biofilm impregnation approaches are currently being investigated [44], alternative strategies are clearly needed to treat a developed or developing biofilm.

Antibacterial agents

Only a few clinical studies have been performed to test the clinical efficacy of antibacterials against staphylococcal biofilm infections. Although rifampin is never used as a single agent due to rapid development of resistance (point mutation in *rpoB*), several clinical studies have shown its efficacy in combination with other antimicrobials to treat biofilm infections including tigecycline [45,46], linezolid [47-49] quinupristin/dalfopristin [46,48,50] and ciprofloxacin [51,52]. A recent study by Olson et al. utilized a guinea pig tissue cage model to replicate a *S. epidermidis* biomaterial-based infection; this model was used to assess the efficacy of daptomycin, vancomycin or both in combination with rifampin [53]. It was determined that treatment with daptomycin or vancomycin alone was not statistically different than the no-treatment control. However, the combination of rifampin to either vancomycin or daptomycin sterilized 5/6 tissue cages. In each case, the one tissue cage that was not sterile contained rifampin-resistant isolates. Surprisingly, addition of rifampin alone in the tissue cage model sterilized 5/6 cages (the one tissue cage contained rifampin-resistant mutants) suggesting that the function of both vancomycin and daptomycin in the model was to suppress the subpopulation of rifampin resistant mutants. These data demonstrating the efficacy of rifampin against staphylococcal biofilms may suggest that targeting RNA polymerase is a highly effective strategy against the metabolic diversity of cells found in a biofilm. Additional screens should be performed to determine if targeting RNA metabolism is an effective strategy at controlling biofilms. A similar antibacterial strategy has been employed in a *P. aeruginosa* biofilm model [54]. Pamp and colleagues found that metabolically active *P. aeruginosa* cells (found on the surface) were resistant to colistin due to PmrA-PmrB two component regulatory system mediated lipopolysaccharide modification or in *mesAB-oprM*-mediated efflux. However, non-metabolically active bacteria found in the core of the biofilm were susceptible to the action of colistin. Conversely, the metabolically active cells found on the surface were susceptible to ciprofloxacin whereas the non-metabolically active cells were resistant. As expected, the combination of ciprofloxacin and colistin were able to sterilize the biofilms. Therefore, investigation of the unique metabolism and heterogeneity of bacterial biofilms may lead to novel treatment regimens; i.e. treatment of each metabolic niche with unique compounds.

Biofilm dispersing enzymes

Two specific biofilm dispersing enzymes, DNase I and dispersin B, have recently gained attention as potential anti-biofilm agents [55]. Two separate studies have shown that eDNA generated by bacterial lysis is important for initial adherence and subsequent accumulation of staphylococcal biofilm [30,56]. Addition of DNase I to staphylococcal biofilm was found to inhibit initial biofilm development and subsequent maturation; in addition, a *nuc* (staphylococcal nuclease) mutant exhibited thicker biofilms [57]. Pulmozyme is a recombinant form of DNase I that is used to treat patients with cystic fibrosis [58]; addition of this enzyme disrupts pre-formed biofilms due to digestion of eDNA. Dispersin B is a hexosaminidase produced by *Aggregatibacter actinomycetemcomitans* that hydrolyzes PIA/PNAG; a polysaccharide synthesized by the *icaADBC* operon in *S. aureus* and *S. epidermidis* [59]. Dispersin B has excellent activity against PIA/PNAG producing *S. epidermidis*, however, is not presumed to have significant activity against *S. aureus* biofilms (due to lack of PIA/PNAG synthesis) [60]. In general, *S. epidermidis* biofilms are Dispersin B susceptible and DNase I resistant whereas *S. aureus* biofilms are DNase I susceptible and Dispersin B resistant [60]. As discussed previously, a significant drawback to the use of Dispersin B therapeutically is

that not all clinically relevant staphylococcal biofilm infections produce significant amounts of PIA/PNAG.

Quorum sensing inhibition

A significant amount of interest has been applied to the discovery of inhibitors of intercellular communication via quorum sensing pathways [61]. Since the loss of quorum sensing is not detrimental to growth, it is believed that the development of resistance to the inhibitors would not be rapid. A significant amount of investigation has developed several quorum sensing antagonists against development of *P. aeruginosa* biofilms and virulence [61], however, the clinical relevance of inhibition of the major quorum sensing system in the staphylococci may depend on the type of biofilm accumulation molecule that is utilized (i.e. PIA/PNAG vs. proteinaceous). The major and most well studied quorum sensing system in staphylococci is the accessory gene regulator *agr* [62]. Briefly, the *agr* system is comprised of four genes *agrB*, *agrD*, *agrC*, and *agrA*. ArgBD synthesizes and secretes autoinducing cyclic thiolactone peptides (AIPs) that are recognized by ArgC (membrane bound sensor); ArgC phosphorylates AgrA which activates RNAIII. RNAIII is an RNA regulatory molecule which downregulates surface adhesins and upregulates secreted toxins, proteases, etc. Construction of an *agr* allelic replacement mutation in a PIA/PNAG positive isolate of *S. epidermidis* resulted in a biofilm that was much thicker than wild type [63]. This increased thickness is most likely due to the lack of δ -toxin and phenol soluble modulins which act as surfactants releasing bacteria at the surface of the biofilm [11,64]. *agr* mutants of *S. aureus* also exhibit thicker biofilms, however, recent investigation from Boles and Horswill demonstrated that synthesized AIP disrupts a PIA/PNAG-independent biofilm [65]. Further work is needed to identify the target that AIP is activating to release the biofilm from the biomaterial; in addition, it is unknown how synthesized AIP may function against PIA/PNAG-dependent biofilms.

Conclusions

Similar to strategies utilized for vaccines against staphylococcal disease [66], treatment modalities against bacterial biofilm should evolve into a multi-tiered approach. Combination therapy may involve antimicrobials that target specific metabolic niches or metabolically active in contrast to non-metabolically active bacteria. In addition, further therapies may involve compounds that are able to disperse pre-formed biofilms from the surface of the biomaterial either through digestion of accumulation molecules (e.g. DNA, protein, polysaccharide) or through inhibition or stimulation of bacterial intercellular communication systems. Further investigation of biofilm biology is warranted to identify these metabolic interactions.

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Figure 1.

Four stages of staphylococcal biofilm formation. A) Initial adherence to the foreign body utilizing MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules), other adhesins (AtlE, Aae) and extracellular DNA (eDNA). B) Accumulation phase is mediated by multiple molecules including polysaccharide intercellular adhesin (PIA)/ Poly N-acetyl glucosamine (PNAG) (illustrated by blue extracellular material), proteins (green) such as biofilm associated protein (Bap), accumulation associated protein (Aap) or fibronectin binding protein (FnBp) and eDNA (illustrated by double helix). C) Maturation is defined as the synthesis of towers containing, in part, eDNA from lysed bacteria and the generation of multiple metabolic niches that include bacteria growing under aerobic conditions (purple cocci), microaerobic/anaerobic conditions (red cocci), dormant (black cocci) and dead cells (yellow cocci). D) Dispersion, which is mediated by *agr*, and includes synthesis of proteases, nucleases, and phenol soluble modulins.