Authors: Mark Smeltzer, Manjari Joshi, Mark Shirtliff, Daniel G. Meeker, Jeffrey B. Stambough, Janette M. Harro

QUESTION : What is the life cycle of biofilm and the mechanism of its maturation?

RESPONSE: A biofilm may be defined as a microbe-derived sessile community characterized by organisms that are attached to a substratum, interface or each other are embedded in a matrix of extracellular polymeric substance and exhibit an altered phenotype with respect to growth, gene expression and protein production. The biofilm infection life cycle generally follows the steps of attachment (interaction between bacteria and the implant), accumulation (interactions between bacterial cells), maturation (formation of a viable 3D structure) and dispersion/detachment (release from the biofilm). The life cycle of biofilm is variable depending on the organism involved. There are characteristics in the life cycle of biofilm formation. These include attachment, proliferation/accumulation/maturation and dispersal. Biofilm can either be found as adherent to a surface or as floating aggregates.

LEVEL OF EVIDENCE: Strong (this is a scientific review)

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

To answer this question the authors searched Pubmed and Google Scholar between January 1950 – August 2018. Search words included: biofilms, biofilm formation, bioflm life cycle, staphylococci biofilms, Gram positive organisms, pseudomonas aeruginosa biofilms, antibiotic resistance and prosthetic joint infections (PJIs). Relevant papers based on the above search words were reviewed.

Most studies found were animal studies, laboratory studies, in vivo studies and a few clinical studies. Due to time constraints, complete systematic review of the literature could not be performed.

A biofilm may be defined as a microbe-derived sessile community characterized by cells that are attached to a substratum, interface or each other are embedded in a matrix of extracellular polymeric substance and exhibit an altered phenotype with respect to growth, gene expression and protein production [1]. Biofilm thickness can vary between a single cell layer to a thick community of cells embedded within a polymeric matrix. Recent structural analyses have demonstrated that these biofilms possess a sophisticated architecture in which microcolonies can exist in discrete pillar or mushroom-shaped structures [2]. Between these structures, an intricate channel network provides access to environmental nutrients.

PJI can be initiated through hematogenous spread or by direct seeding via an overlying infection, penetrating trauma or contamination during surgical implantation of the prosthesis. Regardless of the seeding source or microbial species, the stepwise progression of the infection is dependent upon biofilm formation and maturation.

The biofilm infection life cycle generally follows the same steps of attachment (interaction between bacteria and the implant), accumulation (interactions between bacterial cells), maturation (formation of a viable 3D structure) and dispersion/detachment (release from the biofilm). This progression is mediated by the interplay of a number of microbial, host and environmental factors, and these are usually different in varying microbial species or even strains within species. A rapid stage progression can be seen with virulent, biofilm-forming pathogens in a susceptible host (e.g., a virulent *Staphylococcus aureus* (*S. aureus)* strain in a host with immunosuppression). In contrast, an infecting microbe with slow growth and low virulence (e.g., *Cutibacterium acnes* – formerly *Propionibacterium acnes*) in a healthy host capable of suppressing biofilm formation can produce an indolent infection with delayed progression.

By adopting this sessile mode of life, biofilm-embedded microbes enjoy a number of advantages over their planktonic counterparts. One advantage is the ability of the polymeric matrix to capture and concentrate a number of environmental nutrients, such as carbon, nitrogen and phosphate [3]. Another advantage to the biofilm mode of growth is it enables resistance to a number of removal strategies, such as antimicrobial and antifouling agent removal, shear stress, host phagocytic clearance and host oxygen radical and protease defenses. This inherent resistance to antimicrobial factors is mediated in part through very low metabolic levels and drastically down-regulated rates of cell division (e.g., small colony variants) of the deeply embedded microbes [4]. While low metabolic rates may explain a great deal of the antimicrobial resistance properties of biofilms, other factors may play a role as well. One such factor may be the ability of biofilms to act as a diffusion barrier to slow down the penetration of some antimicrobial agents [5]. For example, reactive oxidative species may be deactivated in the outer layers of the biofilm, faster than they can diffuse into the lower layers [6].

The last advantage of the biofilm mode of growth is the potential for dispersion via detachment. As mentioned, micro-colonies can exist in discrete, mushroom-shaped structures. These microcolonies may detach under the direction of mechanical fluid shear or through a genetically programmed response that mediates the detachment process [7]. Under the direction of fluid flow, this microcolony travels to other regions of the host to attach and promote biofilm formation on virgin areas. Therefore, this advantage allows a persistent bacterial source population that is resistant to antimicrobial agents and host immune clearance, while at the same time enabling continuous shedding to promote bacterial spread.

S. aureus Biofilm Formation

Although many bacterial pathogens are capable of forming biofilms in a range of clinical contexts, *S. aureus* is the main etiological agent associated with PJI.

The initial phase of biofilm formation is characterized by the attachment of planktonic cells to a surface. In a planktonic mode of growth, *S. aureus* up-regulates the expression of key mediators for immunoavoidance (e.g., Protein A) and the attachment to biotic surfaces. These mediators are a variety of proteins anchored in the

cell wall, the largest group of which are termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [8]. Binding of MSCRAMMs to host components such as fibronectin, fibrinogen, collagen and cytokeratin are an important first step in the attachment of *S. aureus* to initiate biofilm formation [9]. Attachment to abiotic surfaces is also determined by properties and physicochemical characteristics of the abiotic surface as well as the bacterial surface, with hydrophobic and electrostatic interactions playing a major role [10].

However, it is worth noting that many abiotic surfaces, as is the case with many implanted medical devices, are rapidly coated in host matrix components upon implantation. Therefore, surfaces that have been engineered to be "biofilm-resistant" have failed in vivo since *S. aureus* mediates attachment to these conditioned surfaces [11]. The presence of a devitalized surface coated with host extracellular matrix proteins decreases the infectious dose required to cause infection to less than 100 viable *S. aureus* cells, thereby increasing the ability of *S. aureus* to cause biofilm infections by over 75,000 fold [12].

Following this initial attachment, bacteria proliferate and produce an extracellular matrix (ECM), often referred to as slime or glycocalyx, comprised of proteins (both host derived and bacterial), carbohydrates and extracellular DNA (eDNA). These serve as a scaffold for maturation and 3D structuring of the biofilm [11]. Ultimately, through coordinated degradation of ECM via proteases, nucleases, delta hemolysin and other factors (e.g., phenol soluble modulins), bacterial cells are released from the biofilm with the potential to seed secondary sites of infection [13]. Below is a brief discussion of the factors and mechanisms responsible for these stages of the *S. aureus* biofilm life cycle.

The next phase of biofilm formation entails the proliferation and accumulation of attached bacterial cells. During this early phase, intercellular attachment plays a key role in stabilizing the early biofilm before a significant amount of ECM can be produced to protect the attached cells from disruptive forces such as shear force [11]. One key contributor to intercellular adhesion is the polysaccharide intercellular adhesin (PIA), first studied in *Staphylococcus epidermidis* [14]. The MSCRAMMs (discussed above) and certain cytoplasmic proteins shown to bind to eDNA are also know to contribute [15–17]. Together, these factors not only play a role in early intercellular adhesion but also constitute major components of the ECM produced by biofilm-associated cells.

Recent studies utilizing technology allowing for nearly real-time evaluation of biofilm progression have suggested the addition of a stage of biofilm development following proliferation/accumulation referred to as an "exodus" phase [18]. This exodus phase is characterized by an early dispersal event with a reduction in total biomass from a biofilm. This is reportedly achieved through the coordinated bacterial expression of secreted nucleases by a subpopulation of bacterial cells resulting in degradation of eDNA and subsequent bacterial release [18]. The purpose of this phase and its necessity for the overall progression of the biofilm life cycle remain to be determined. However, given the timing of these observations within the overall progression of biofilm formation, it has been suggested that a dynamic shift occurs in which early events are largely protein-mediated and subsequent events are mediated by both protein and eDNA [11]. Although some literature would suggest that certain biofilms tend to be exclusively dependent upon PIA, protein or eDNA, these studies propose a more dynamic model of development with temporal and spatial changes in ECM components [11].

The maturation phase of the biofilm life cycle entails the 3D structuring of biofilms into classic architectural structures (towers and mushroom-like structures) and the development of microcolonies displaying some degree of phenotypic diversity [10,11]. This complex structuring is coordinated through the balance of adhesive and disruptive factors [10]. Adhesive factors include the ECM components discussed above such as PIA, proteins and eDNA. Disruptive factors include enzymes that degrade these components such as proteases and nucleases, as well as the surfactant-like molecules, phenol-soluble modulins (PSMs). These disruptive factors allow for the remodeling and maturation of biofilm structures. For example, studies have demonstrated that channels are created throughout a biofilm via the surfactant-like activity of PSMs, allowing nutrients to reach deeper layers of the biofilm [19]. Therefore, these studies describe biofilm maturation as a subtractive process. Alternatively, some studies suggest an additive process of maturation from observations of microcolonies emerging from slower growing basal layers of biofilms [20]. It is likely that both additive and subtractive processes contribute to the complex structuring observed during biofilm maturation.

The final step of the biofilm life cycle involves the dispersal of cells with the ability to travel to distal sites to disseminate infection. The mechanism by which *S. aureus* regulates this step is largely mediated by the accessory gene regulator (*agr*) quorum-sensing system [19,21]. The *agr* system responds to cell density through the accumulation of signal molecules, allowing for dispersal to occur once a threshold density is reached [22]. The *agr*-regulated factors that have been proposed to mediate dispersal include secreted proteases and resultant degradation of protein components of ECM [23]. Dispersal has also been proposed to be mediated by the *agr*-mediated production of PSMs, which act by disrupting molecular interactions within biofilms [19].

In addition to these staphylococcal factors responsible for PJI development, the complicit nature of the host towards biofilm formation also plays a role. In an early *S. aureus* biofilm infection, the intense inflammatory response is produced by the host. *S. aureus* is readily able to resist clearance from the host through a large number of virulence factors that specifically attack the host and promote immunoavoidance. The expression of *S. aureus* virulence factors, timed by the quorum sensing system, promotes the host to release T_H1 cytokines, including interleukin (IL)-12, interfenn gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-17 resulting in a shift of the adaptive immune system to an ineffective T_H17 and T_H1 cell-mediated immune response. This type of response is incapable of clearing a biofilm infection, thereby enabling *S. aureus* to form a fully mature biofilm and a persistent infection. The other branch of adaptive immunity, the T_H2 antibody-mediated response, is readily effective at clearing the infection in the early phase of biofilm formation before it progresses to a fully mature phenotype. However, this antibody-mediated response is shut down both by the host cytokines associated with the initial response to *S. aureus*, most notably IFN-γ, and by the *S. aureus* production of superantigens, capsule and other toxins. Additionally, *S. aureus* produces a number of highly immunogenic decoy antigens (e.g., lipase) that augments the ability of *S. aureus* to cause disease and reduces antibody production against more vital antigens [24]. By the time the antibody-mediated immune system recovers and mounts an effective response against the biofilm infection, the fully mature biofilm is able to resist clearance. Even if cleared through surgical intervention and infection resolution, this host immune response manipulation and variable antigen expression allows *S. aureus* to re-infect patients throughout their lifetime.

Once in this fully mature phase, the infection can remain quiescent for years or even decades, or more typically, will show remarkable signs of chronic inflammation [25]. This host response is often due to the metastasis of metabolically active and virulent planktonic subpopulations that have dispersed/detached from the localized biofilm aggregate. Antibiotic therapy is effective against these

active populations allowing for temporary suppression of clinical signs and symptoms of the underlying biofilm disease. However, upon antibiotic treatment cessation, exacerbation of the disease will necessarily result.

Biofilms Formed by Other Microbial Species

In addition to *S. aureus*, a number of other microbial species are able to form infectious biofilms in PJIs [26]. These include other facultative anaerobic, gram-positive, non-motile bacterial species, including coagulase negative staphylococci and *Streptococcus* and *Enterococcus* species. The stages of biofilm formation are similar, and these microbes use a number of homologs to the biofilm-associated virulence factors already described for *S. aureus*. Species other than these gram-positive microbes contribute towards PJI, particularly the facultative anaerobic gram-negative bacilli, including *Escherichia coli* and *Pseudomonas aeruginosa* and anaerobes to a lesser extent.

Gram-negative bacterial biofilms, especially *P. aeruginosa,* have long been studied in the biofilm research field due to their ubiquitous nature in the environment and disease, and their preponderance in chronic wounds and cystic fibrosis lung infections. Although the stages progress through early attachment, mature attachment, accumulation, maturation and dispersion/detachment, the mechanisms by which these steps are accomplished show important differences to gram-positive pathogens.

The motility provided through flagella allows *P. aeruginosa* to facilitate close association with surfaces, such as those in indwelling medical devices. The microbial cells will then proceed to irreversible attachment. Additionally, Type IV pili provide for differential virulence factor production associated with shear stress as well as allow subpopulations to migrate on the surface through twitching motility. As the biofilm accumulates, the formation of complex multicellular structures occurs that demonstrate heterogeneity of nutrients, pH and oxygenation. During maturation, the development of membrane blebs, nanofilaments, eDNA structural support and electrical coupling of the embedded bacterial cells also occurs. As the population swells, the homoserine lactone quorum sensing system induces the production of the surfactant and anti-leukocyte pseudomonal rhamnolipids to prevent clearance and add to the burgeoning inflammatory response. The microbes can then either disperse as single-celled planktonic populations or detach from the biofilm in large conglomerated flocs that allow for metastasis of the infection while enjoying the protective environment of the biofilm matrix.

Clinical Relevance: Treatment and Resolution

During the early acute stage of infection and inflammation, the biofilm is in an early accumulation phase. During this phase, the growing biofilm demonstrates higher susceptibility to antimicrobial therapy than the fully mature, quiescent and metabolically inactive biofilm phenotype. This increased susceptibility to antimicrobial therapy during the acute phase of PJIs translated into efficacious treatment without surgical intervention [28]. When effective combination antimicrobial therapy was used alone to treat PJIs with clinical signs of less than one month in duration, over 83% of patients were successfully treated without surgical intervention. However, once symptoms lasted for greater than six months, successful treatment of antibiotic therapy fell to just over 30%. Therefore, the potential for effective therapy of PJIs without surgical intervention may be a possibility if the infection is diagnosed early and targeted antibiotic therapy is quickly initiated with emphasis on adding Rifampin/Rifampicin when a *Staphylococcus* spp is the etiological agent. After this early therapeutic window, proper surgical debridement along with combination antibiotic therapy is necessary for optimal infection resolution.

Clinical Relevance: Diagnosis

Rapid, effective and sensitive discovery and identification and antibiotic sensitivity determination of the pathogenic bacterial species must be accomplished in order to effectively combat PJIs. Once identified, effective therapeutic counter-measures and treatment can be applied. Currently, pathogen identification requires microbial culture followed by diagnostic analyses that normally require additional rounds of replication in culture or purification of specific bacterial/fungal products. At best, microbial identification may require days to weeks, depending on the growth rate of a specific pathogen. These limitations of bacteria are dramatically exacerbated in diagnosing and speciation of the etiological agent in PJIs. Culture from tissue samples can be effective during the early stages of infection when the biofilm is in an accumulation phase and planktonic populations are present. However, all too often, patients have received antimicrobial therapy prior to proper tissue sampling, thereby eliminating the easily detected planktonic populations, leaving behind only small microbial aggregates that are often missed during biopsy. Also, as the biofilm matures, the host immune response walls off the infectious nidus to form these same hard-to-detect biofilm aggregates.

In conclusion, understanding the progression of biofilm life cycles and the mechanisms that pathogens use to regulate this progression is essential for the development of therapeutic approaches aimed at preventing, disrupting and eradicating biofilm-associated infections.

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