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REVIEW

Molecular Mechanisms of Biofilm Formation on Orthopaedic Implants: Review of the Literature

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ABSTRACT

Orthopaedic implant-associated infections (OIAIs) is one of the most catastrophic complications following joint arthroplasty or fracture fixation. Given the increasing number of orthopaedic implants which are used annually, periprosthetic infections emerge as a global problem. Their diagnosis and consequent therapeutic management remain challenging for clinicians. Biofilm formation is a complex and only partially understood process that has not been extensively studied. Understanding the underlying mechanisms involved in biofilm formation is crucial in the amelioration of both diagnosis and therapeutic management of OIAIs. We performed a literature review of the molecular mechanisms of biofilm formation and discussed the four most common and thoroughly researched microbes of biofilm-related OIAIs.

Keywords: *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus*, implant-associated infections, biofilm.

INTRODUCTION AND BACKGROUND

Implants have become an integral part of orthopedic procedures, both in joint arthroplasty and fracture fixation surgeries. Due to the large volume of orthopedic operations which are performed annually, the incidence of orthopaedic implant-associated infections (OIAI) is expected to rise. Postoperative infection rate ranges between 0.3–5% (1, 2). Microbes can colonize the surface of implants and to organize into communities that constitute the biofilm. This biofilm layer serves as a protectant for bacterial colonies on the implant, making bacteria more resistant and difficult to eradicate when using standard antibiotic treatment. Additionally, higher doses of antibiotics, with concentrations of up to 1000-1500 times higher than those needed to combat planktonic infections being required (3).

Biofilm-related infections are a considerable healthcare burden which account for approxi-

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mately 65% of all healthcare bacterial infections (4), and their definite treatment – comprising explantation of the infected hardware, surgical debridement and appropriate antibiotic therapy – has become very complicated (5).

In its natural form, a biofilm consists of a variety of microbes that can grow near each other (6). Research has focused on monobacterial biofilms growing under laboratory conditions that can easily be controlled and reproduced. Nonetheless, in reality, biofilms are constituted by multiple bacteria and in a milieu that is far more complex than that which can be reproduced in an artificial experiment.

In the initial step, infecting organisms are inserted either directly during implantation of the prosthesis or indirectly during an episode of bacteraemia. The subsequent complicated mechanisms involved in the formation of a functional mature biofilm are still under investigation. The best studied molecular biofilm formation mechanisms are those of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Bacillus subtilis*.

Formation of biofilms on medical implants can be divided into four stages: 1) primary attachment; 2) accumulative phase; 3) biofilm maturation; and 4) biofilm knock down and bacteria spreading ready for a new cycle.

We performed a literature review of the molecular mechanisms of biofilm formation of the four aforementioned bacteria which are known to be the most commonly encountered in orthopaedic implant biofilms.

Staphylococcus aureus and *Staphylococcus epidermidis*

1. Biofilm production and extraxellular matrix

Primary attachment is the first stage of biofilm formation, which is characterized by bacteria adhesion to the biomaterial that they will finally colonize. The foremost important interaction for *S. aureus* and *S. epidermidis* adhesion is that between biomaterial and autolysin AtlA or AtlE, respectively (7). Hydrophobicity of the staphylococcal cell membrane as well as that of the surface itself is also of primary importance for this first step of material surface colonization (8).

During the second stage of accumulation, bacteria aggregate to form a multidimensional, multicellular layer through microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and intercellular mechanisms of adhesion (9). During the accumulative phase, cell to cell adhesion is mediated through polysaccharide intercellular adhesin (PIA) (10), which is produced by expression of four genes in icaADBC locus. These genes are in close proximity to each other and are usually co-expressed (11).

Apart from PIA production, there is also an alternative mechanism of biofilm formation which is ica-independent. *Staphylococcus* produces a variety of adhesion proteins located in its cell membrane, which are able to interact with other surface proteins of neighbor cells. Thus, cells are tightly held within the inner layers of a biofilm (10).

In addition, the extracellular matrix of biofilms also hosts adhesive proteins that can interfere with biofilm formation. Among those proteins, biofilm associated protein (BAP) is the most important one, while accumulation associated protein (Aap) for *S. epidermidis* and extracellular matrix binding protein (Embp) for both *S. aureus* and *S. epidermidis* also play an important role (12-14).

Apart from PIA and proteins, the extracellular component of biofilms also includes DNA, which has only recently gained researchers' attention as a major contributor to both biofilm structural solidity and possible refractoriness to antibiotics (15). Actually, in a biofilm of *S. epidermidis*, using PCR amplification from extracellular DNA (eDNA) proved that eDNA was identical to bacterial DNA, thus strengthening the possibility that the latter resulted from degradation of a bacterial subpopulation (16). Cell lysis is mediated by cid operon in *S. aureus* (17), while the major autolysin of *S. epidermidis* (AtlE) plays the perspective role (16). As mentioned before, AtlE is involved in the first stage of biofilm synthesis. Since it is not a transmembrane protein, its role probably results indirectly from eDNA release rather than directly by adhesion to other proteins (16). *S. aureus* eDNA interactions and mechanisms of biofilm production are still investigated but seem to resemble those of *S. epidermidis*.

2. Quorum sensing in biofilm formation

Biofilm production is controlled by a complex network of signal molecules, reflecting its multifactorial nature. Cell to cell signaling, called quo-

rum sensing, is the mechanism through which the complex interaction of gene expression and bacterial population is harmonized. Bacterial population density is in direct connection to signal molecules produced by cells, which by binding to specific quorum sensing receptors inhibit genes responsible for various functions under strict population density conditions (18). Both in *S. aureus* and *S. epidermidis* the biofilm production depends on environmental conditions (e.g., culture media) and bacterial density. Bacterial communities produce and discharge signal molecules, called autoinducers, which begin a cataract of cellular responses (18). In *Staphylococcus*, quorum sensing is coded by accessory gene regulator (agr) locus. Agr gene is activated during the shift from the exponential to stationary growth face. It comprises four genes (agrA, agrC, agrD, and agrB) which are co-transcribed. Each of the aforementioned genes is responsible for the secretion of a signaling molecule named autoinducing peptide (AIP) through encoding different components of a complex system. In *S. aureus*, four agr allelic genes exist and are implicated in "bacterial interference", a phenomenon resulting in inhibition of agr activity between different groups of allele carriers.

RNAIII, the effector molecule of agr system, is produced only when the latter is activated through P3 promoter. RNAIII regulates a variety of important membrane and extracellular proteins (19). Agr is considered a virulence regulator since many such factors such as toxic shock toxin and enterotoxins are under its strict management (20). In *S. aureus*, agr quorum sensing system seems to decrease biofilm synthesis through increasing biofilm disconnection (21).

Apart from agr system, the transcriptional members of the staphylococcal-specific SarA family encoded by the relevant gene (SarA) are also key regulators implicated in biofilm formation. Their production highly relies on the growth phase. SarA up-regulates biofilm formation by enhancing ica transcription through interference with the ica promoter region. MgrA – another member of SarA family – interferes with agr system activation and control of cell lysis. Even though icaADBC genes of *S. aureus* and *S. epidermidis* share common sequences, biofilm synthesis seems to be mediated by different pathways. In *S. epidermidis*, the stress sigma factor σB suppresses transcription of icaR (a negative regulator of icaADBC), activates one of the three promoters needed for sarA expression and minimally activates icaADBC. In *S. aureus*, the aforementioned mechanisms are all activated, but in addition to those, σB system employs a pivotal function on ica-independent biofilm synthesis through inhibition of RNAIII and decrease in extracellular proteins.

Pseudomonas aeruginosa

1. Biofilm formation and structural components

As mentioned before, bacterial attachment to a surface is the first step for biofilm formation. As far as *P. aeruginosa* is concerned, the gene in charge to put this non-reversible attachment through is SadB (22). Important factors involved in biofilm formation include extracellular matrix production (apparently the most important one), bacteria mobility, cell to cell signaling and interactions between bacteria populations.

Bacterial motility is not uniform, with a subpopulation being stationary upon attachment and another one being mobile on the surface attached using flagella or type IV pili. The stationary subpopulation is the one that forms the initial colonies of the biofilm by growing on specific positions (23, 24).

The extracellular matrix (ECM) mainly consists of polysaccharides, proteins and eDNA (25), with the exact proportion of each depending on growth conditions, biofilm age and *Pseudomonas* strain (26). It is the factor that plays the pivotal role in biofilm formation mediated through cell to cell interactions, cell attachment and resistance to antimicrobial treatment. The three polysaccharides synthesized by *Pseudomonas* are (1) Pel, which is encoded by pel gene cluster and plays an important role in membrane formation, contributing to biofilm synthesis (27); (2) Psl, encoded by psl gene and engaged in cell to cell as well as cell to surface attachment (28, 29); and 3) alginate, usually found in lung strains of patients suffering from cystic fibrosis (30).

Proteins that are part of ECM include type IV pili, Cup, CdrA, LecB, and Fap. Type IV pili, apart from playing a role in bacterial motility as mentioned before, also contributes to adhesion to biotic and abiotic surfaces as well as binding with eDNA, thus directly linking *Pseudomonas* to eDNA matrix (31-34). CupA, B and C fimbria all

take part in bacterial adherence and subsequent biofilm synthesis (35). CdrA and B prompts autoaggregation through psl (36). LecB and Fap also play important roles in biofilm formation through mechanisms that are still under investigation (37-39).

Extracellular DNA is one of the most important components in biofilm formation (40, 41). It represents only a small percentage of biofilm components in the initial phase of biofilm formation, but the amount of eDNA augments during biofilm maturation (40). This probably occurs through lysis of a bacterial population mediated by PQS quorum sensing system (further discussed) (42). Studies have shown that DNase, if included in biofilms, did not inhibit bacterial growth but prevented young biofilm formation (40, 42). Conversely, mature biofilms cannot be degraded but become more sensitive to treatment.

2. Quorum sensing in *P. aeruginosa* biofilm

In *P. aeruginosa* quorum sensing, signal molecules are primarily responsible for virulence factor expression (43). Three systems (Las, Rhl and Pqs) are implicated, each of which having its specific signal molecule: 3-oxo-C12-HSL, C4-HSL and PQS, respectively (44). The Las and the Rhl systems work in concordance, with Las directly affecting the expression Rhl system (45), while Pqs works between Las and Rhl (46). As mentioned before, PQS quorum sensing of initial bacterial colonies contributes to eDNA production and thus, to biofilm production. Quorum sensing regulates multiple other factors apart from eDNA production, including rhamnolipid as well as Pel polysaccharide production (47).

Moreover, genes encoding CupA and CupB fimbriae and LecA and LecB lectins are regulated by quorum sensing (48). Even though quorum sensing is important and needed in the growth of microcolonies under specific conditions, it is not required for biofilm formation. Nonetheless, its critical role in biofilm resistance to host immune responses or antimicrobials has been proven. *P. aeruginosa* bacteria characteristically produce various products which may not be beneficial at all for themselves but only for their environment and the rest of the surrounding bacteria (49). These "public good" compounds include iron siderophore pyoverdine, biosurfactant rhamnolipid (responsible for mobile populations' movement) and extracellular matrix material needed for cell to cell connection. *P. aeruginosa*, when grown in flow chambers, creates biofilms with mushroom shaped colonies (23). Immobile subpopulations create the stalk, while mobile ones create the cup (24). PQS is necessary for that mushroom structure formation. Actually, it is a subpopulation of stalk forming bacteria (named pilA) that expresses pqs genes. This pilA subpopulation produces eDNA through PQS quorum sensing, while mobile populations are attached to eDNA through type IV pili, beginning cup formation (50).

Bacillus subtilis

1. Extracellular matrix of *B. subtilis* biofilms

B. subtilis is a motile Gram-positive bacterium which forms firm, robust and complex biofilms using several mechanisms. The primary action of the organism is to change from a mobile, planktonic state to an immobile one, called sessile, *via* inhibition of flagellar genes (51). These immobile forms begin to connect to each other through cell to cell molecules, with simultaneously producing extracellular matrix (ECM), which is indispensable for biofilm formation.

As mentioned before for other organisms, ECM contains polysaccharides, proteins and nucleic acid (52). Genes responsible for ECM production are part of the epsA-O gene operon (53). Recently, two important proteins that help sustain the biofilm whole and undivided, namely TasA and TapA, have been brought to light. They are encoded by the three gene-operon tapA-sipW-tasA. TapA is required for anchoring TasA into the cell wall (54). An additional protein, called BslA, was also proven to be a contributor to the final stages of biofilm formation through hydrophobins, proteins that construct a hydrophobic layer at the top of the biofilm, which is protecting it from water (55).

2. Signaling in *B. subtilis* biofilm formation

B. subtilis displays great variability of cell phenotypes. Although carrying the same genetic code, the bacterial cells differentiate in distinct phenotypic populations so as to serve different and multiple purposes (56).

The three pivotal regulators, without any of which the complex architecture of the bacterial colonies cannot form, are DegU, ComA and

Spo0A (57). Their action in biofilm rigidity is mediated through activation of specific genes. As mentioned before, all cells in the planktonic state are mobile. This is due to the expression of genes, only activated when the three above regulators are unphosphorylated (58).

Only when cells transform into their immobile, sessile form it is that the three regulators are activated to urge cell differentiation (59). For example, Spo0A activation presents in two levels, a high one and a low one, with the former inducing ECM production and the latter driving a subpopulation of *B. subtilis* to form spores (60). Cannibalism is an additional interesting ability of the Spo0A cells, which uses dead cells as a source of feed and an indirect way to incorporate new DNA into their genomes, thus expanding both genetic variability in the colony as well as colony survival capacity (61). ComA activated cells, a procedure that is controlled via quorum sensing, can differentiate in two distinct cell types which can switch to another serving multiple purposes as needed. DegU activation is closely related to the expression of BslA, the water repellent protein that protects biofilms (55, 62). Specifically, it leads a subpopulation of cells to differentiate into "miners" which promote nutrition production into the colony (63). This subpopulation of cells is preferentially at the top of the biofilm, near the hydrophobins known proteins of the BslA family. To further study the specific locations of the various subpopulations, it has been proven that mobile planktonic cells preferably sit in the bottom of the colony, serving in its expansion, while ECM producer cells sit in the center of the colony (59). Spores and DegU cells are on the top, facilitating dispersal and protecting the colony from water respectively.

Extensive research has explored which were the triggering events driving cell differentiation in *B. subtilis* colonies, a crucial step in its biofilm formation and conservation. These events seem to be both endogenous and exogenous, mainly leading to phosphorylation of the three major regulators: DegU, ComA and Spo0A.

Spo0A activation is usually mediated by phosphorylation of five different kinases (KinA-E), including complex mechanisms and pathways, with SinR-SlrR complex and AbrB protein being in the center of them (64-66). Now, activation of those kinases is dependent on different cues, ranging from potassium for KinC to glycerol (67), mamganese and L-malic acid for KinD (68), while KinA and KinB sense low oxygen or high iron (69). DegU-P can either act as a gene inhibitor or activator, depending on its phosphorylation status (70, 71). External cues driving its activation implicate osmolarity changes or specific peptides (72).

ComA activation starts with ComX binding on ComP sensor kinase, which then mediates ComA phosphorylation. ComA activation pathway finally produces competent cells as well as capable of producing spores (73).

All the aforementioned cataracts are inhibited by the Rap family of phosphatases through dephosphorylation. RapGH dephosphorylates DegU-P, RapABEJ dephosphorylates Spo0A-P and RapCFGHK dephosphorylates ComA-P. This way, an additional communication network contributes to the complex differentiating mechanisms of *B. subtilis* cells (74).

Finally, it is noteworthy to mention that, since soil is the natural host of *B. subtilis*, its biofilms are preferentially found on plant roots contributing to plant protection against various pathogens. Actually, the organism and its host interact to further drive the maturation of colonies, unrevealing the importance of biofilms for plants (75).

3. Biofilm dispersal

Bacteria have unique mechanisms for detachment from biofilms and migration to other surfaces (76, 77). These mechanisms, including rupture of biofilm matrix or release of extracellular proteins leading to a decrease in bacterial aggregation, contribute not only to formation of new biofilms to other surfaces but also to re-organization of the existing biofilm. External stimuli may trigger bacterial migration – e.g., carbon or oxygen deficiency or other environment conditions – within a biofilm (78-80). This procedure, called biofilm dispersal, is of paramount importance in medicine, specifically orthopedics, as it may justify persistence of disease even after implant removal.

As far as biofilm dispersal of the organism is concerned, molecular mechanisms are not as clear. Bacterial dispersal is mediated through many pathways such as environmental cues or gene products, all of which finally interact with intracellular levels of c-di-GMP (81). It is considered that high intracellular c-di-GMP levels upregulate matrix production and biofilm forma-

tion, while the opposite down-regulate matrix production and lead to the dispersal of cells from the biofilms. In Pseudomonas, the gene bdlA encodes the BdlA protein, a chemotaxis regulator which affects the intracellular c-di-GMP levels.

D-amino acids are also able to obstruct biofilm formation. They have been proved to be implicated in biofilm degradation and dispersal, but the involved mechanisms as well as their exact effect – driving or inhibiting growth under certain circumstances – remain hazy. They act by blocking surface attachment in *S. aureus* and affect TapA functionality in *B. subtilis*. ❑

CONCLUSIONS

Biofilm formation is the main reason why subacute and chronic implant-associated infections are difficult to eradicate with antibiotic therapy. Implant-related infections are associated with high morbidity risk and financial costs. Early implant failure or fracture non-union is an alert sign of implant associated infection. Proper detection and identification of the causative agent is of paramount importance for a reliable diagnosis and development of a successful therapeutic plan, which includes implant removal for the time being. Although existing research has led to a better deciphering of the ways bacteria use to form and maintain biofilm, further studies should be conducted to investigate and clarify the exact mechanisms governing biofilm disassembly for the development of new therapeutic strategies without implant removal. ❑

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