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Staphylococcus Biofilms

Janet Jan-Roblero, Sandra Rodríguez-Martínez,
Mario E. Cancino-Díaz and Juan C. Cancino-Díaz

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Abstract

The majority of staphylococci produce biofilm on medical devices, which is the main mechanism to infect humans. Staphylococcal biofilms attach to abiotic or biotic surfaces, forming aggregates and protecting themselves against the immune system and the antimicrobial compounds of the host. Few studies on biofilm formation mechanism in *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CNS) have been performed; however, there is a great interest in studying and controlling biofilm formation of this genus. This chapter exhibits the state of the art on biofilm formation in *S. epidermidis* and other staphylococcal species. The main goal of this chapter is to recognize the importance of biofilm formation in *Staphylococcus*. The participating molecules in staphylococcal biofilm formation are described. Currently, biofilm producer strains of *Staphylococcus* and mainly CNS have been frequently isolated at hospitals, causing significant economic losses. This chapter includes promising solutions in order to prevent medical device-associated infections, as the development of medical devices possessing anti-biofilm materials or surfaces that act against the adhesion or viability of the microorganisms.

Keywords: Biofilm, *Staphylococcus epidermidis*, *Staphylococcus aureus*, medical devices, anti-biofilm

Introduction

I. *Staphylococcus* biofilms

During the last years, the study of biofilms has become relevant due to their significance on many microbiology areas. In the health field, biofilms have been of great relevance because many pathogenic and non-pathogenic bacteria can produce biofilm as a part of its virulence

mechanism and protection against the host. A biofilm is considered a complex microbial community (or communities) attached to a defined surface and embedded within a cell matrix. Regarding the surface, biofilms may be formed on a wide variety of chemical or biological surfaces. Regarding bacteria of the *Staphylococcus* genus, biofilm is the main virulence mechanism of the coagulase-negative staphylococci (CNS) species. Biofilm formation in staphylococci is carried out in at least three stages: i) bacterial attachment to a defined surface, a process termed primary attachment; ii) assembly of these originating bacteria into a small cluster, also known as microcolony or cellular accumulation; and iii) biofilm growth and disassembly (also known as detachment or dispersal) mediated by a mechanical process or by active metabolites produced by the biofilm-embedded bacteria.

I.1. Medical and epidemiological relevance of staphylococci biofilms

Staphylococci are commensal bacteria inhabiting the human skin and mucus. However, they have been identified as infection-causing agents associated to biofilms. Animal models of biofilm-associated infections using staphylococci have allowed to determine the importance of their biofilms as a virulence mechanism. Therefore, staphylococci, particularly *Staphylococcus epidermidis*, are currently the most studied microorganisms regarding their biofilm formation capacity. Nosocomial Infections Surveillance System recognizes that *Staphylococcus aureus* and CNS (e.g., *S. epidermidis* and most of the remaining staphylococci species) are the most frequent nosocomial pathogens isolated from patients at the intensive care unit. Epidemiological data show that CNS are the third most common infective agent causing native valve infective endocarditis (NVIE), and they occupy the first place in prosthetic valve infective endocarditis (PVIE), demonstrating their importance for these two clinical entities.

Regarding *S. epidermidis*, it is an inhabitant of the human skin microbiota. *S. epidermidis* is an opportunist pathogen that causes disease only in patients subjected to predisposing factors. This includes patients with particular features such as premature newborns, inborn immunological impairments, or concomitant medical conditions, for example, human immunodeficiency virus (HIV) infection, immunosuppression after solid organ or bone marrow transplants, and chemotherapy-related neutropenia. Epidemiological data point out *S. epidermidis* as the most commonly isolated microorganism from foreign materials-related infections such as infected prosthetic joints, central venous catheters (CVC), cerebrospinal fluid shunts, intracardiac devices, artificial heart valves, and vascular grafts. Regarding prosthetic joints infections, *S. epidermidis* is the main infective agent of prosthetic joint implants. In UK, CNS and *S. epidermidis* are isolated from a 36% of total hip and 49% of total knee arthroplasty infections [1]. In an additional study on infected total hip and knee arthroplasties, it is pointed out that nearly 70% of the CNS isolates were identified as *S. epidermidis* [2].

I.2. Experimental models to study biofilm formation

The clinical relevance of biofilm formation on foreign materials has been demonstrated using cell culture models, a *Caenorhabditis elegans* infection model, and animal models of device infections, for example, CVC or prosthetic device infection models. The first study on the importance of biofilm formation in vivo using animal models and genetically distinct *S.*

epidermidis isolates with both positive- and negative-biofilm phenotypes failed to show evidence that could demonstrate that biofilm-forming isolates are more virulent in comparing with those possessing a biofilm-negative phenotype. Nevertheless, compelling results were obtained on subsequent studies using genetically defined strains and comparing the wild-type strain with its respective isogenic mutant strain. Using a mouse model of subcutaneous catheter infection and a rat model of venous catheter infection, the polysaccharide intercellular adhesion (PIA)-producing *S. epidermidis* 1457 strain was more virulent than its isogenic counterpart, the biofilm-negative 1457-M10 strain [3]. In a different model of CVC infection, the *icaRADBC*-expressing *S. epidermidis* strain and its *icaRADBC*-negative isogenic mutant displayed the same result [4]. An infection model of *Caenorhabditis elegans* was used in order to study the biofilm-positive phenotype of the *S. epidermidis* 9142 strain, in comparison to the *icaA* mutant, resulting in a higher virulence of the wild type than the mutant [5]. Recently, using a catheter infection model, *icaADBC* inactivation apparently had no effect on colonization, whereas *aap* inactivation completely abolished *S. epidermidis* ability to establish the infection [6]. One explanation for the null pathogenicity of the mutant strain regarding biofilm formation is the lack of protection against the innate immune system. Experiments conducted with cell culture showed that the biofilm-positive 1457 strain was less susceptible to antimicrobial peptides (AMPs) and to phagocytosis performed by polymorphonuclear granulocytes (PMNs) compared to the biofilm-negative 1457-M10 isogenic strain [7]. Physiological status is also important, when *S. epidermidis* 1457 grown on biofilm conditions was less susceptible to phagocytes than it was grown on planktonic conditions [8].

PIA-dependent biofilm formation also interferes with the host's complement activation. Biofilm-positive wild-type bacteria pre-opsonized with normal human serum are more resistant to complement-mediated elimination than the corresponding biofilm-negative isogenic bacteria [8]. It has been also shown that *S. epidermidis* biofilm formation interferes with the phagocytosis process and macrophage activation. This biofilm-forming phenotype may contribute to the chronic persistence of *S. epidermidis* in inflammatory conditions.

Conversely, *S. epidermidis* produces a set of pro-inflammatory peptides termed phenol-soluble modulins (PSMs), which are produced in a tightly regulated manner by the accessory gene regulator (*agr*) system. It has been demonstrated that PSM δ is able to lyse neutrophils, supporting the concept that these peptides are relevant for *S. epidermidis* pathogenesis. However, PSM δ is expressed at low levels by the biofilm-producing *S. epidermidis* 1457 strain, grown either in biofilm conditions or in planktonic conditions [9].

II. Mechanisms and molecules participating in staphylococci biofilm formation

In this chapter, we will divide the study of the biofilm formation process in three phases. During primary attachment, bacteria adhere to the biotic or abiotic surface in order to colonize it, whereas on the accumulation phase, bacteria build a tridimensional multi-cell and multi-layer array. Then, staphylococci are able to disassemble biofilm structure in order to release those cells capable to colonize other sites on the surface. *S. epidermidis* and *S. aureus* biofilm models have been the most studied among staphylococci and the overall biofilm formation process is very similar. In this chapter, we will address *S. epidermidis* biofilm as the base model.

II.1. Participating molecules on the biofilm primary attachment phase

An essential step performed during the primary attachment stage is the tight binding of bacteria to the foreign material (medical device). This bacterial tight binding leads to a successful establishment of a medical device-associated infection. Regarding *S. epidermidis*, it has been found that cell wall proteins are the main elements of such interactions and this is similar for *S. aureus*. Genetic evidence has allowed establishing that bacterial binding to unmodified polystyrene (non-biotic surface) is conveyed by the *S. epidermidis* AtlE autolysin protein [10]. AtlE is a 115 KDa protein that belongs to the bacterial peptidoglycan (PGN)-hydrolases group that plays an important role in bacterial cell wall degradation. The protein is composed by an N-terminal signal peptide, a propeptide, a catalytic domain possessing N-acetylmuramyl-L-alanine amidase activity, three repeated sequences (R1-3), and one C-terminal catalytic domain, possessing N-acetylglucosaminidase activity. In addition to its role during cell wall turnover, AtlE is also important for unmodified polystyrene binding. This function was demonstrated by the *S. epidermidis* O-47 strain harboring a mutation caused by the *atlE::Tn917* transposon, which has an impaired ability to adhere to the polystyrene surface [10]. The binding mechanism of AtlE to the polystyrene surface is unclear; however, it is thought that the first event is AtlE recruitment on the bacterial cell wall through the R1-3 domain and those domains possessing enzyme activity. Based on AtlE expression and functional activation studies, it has been suggested that this protein leads to significant changes in cell wall hydrophobicity contributing to the primary attachment process [11]. Another assigned function for AtlE is its autolysin activity in order to cleave the cells wall and thus releasing extracellular DNA (eDNA), which is a common component in staphylococci biofilm [12]. *S. aureus* autolysin also shares this function at this biofilm formation phase.

The interaction between *S. epidermidis* and an artificial unmodified surface (polystyrene) is mediated by non-specific interactions without the participation of a receptor-specific ligand. On surfaces coated with the host's extracellular matrix (ECM), both *S. epidermidis* and *S. aureus* express cell surface proteins leading to a specific interaction with the components of this ECM of the host. Proteins exhibiting ECM-binding activity are important in order to initiate the infection of medical devices because once foreign materials are introduced inside the body, they are covered by ECM materials (e.g., fibronectin; fibrinogen; vitronectin; collagen). It has been described that *S. epidermidis* AtlE can adhere to vitronectin-covered surfaces, whereas the GehD lipase is involved in interactions with collagen [13]. In addition to these proteins, both *S. epidermidis* and *S. aureus* express proteins possessing a specific function for their interaction with ECM. These belong to the serine-aspartate repeat (Sdr) protein group and they are members of the microbial surface components family that recognize adhesive matrix molecules (MSCRAMM) [14]. In *S. epidermidis*, three Sdr proteins referred to as SdrF, SdrG, and SdrH have been identified [14]. SdrG (also known as Fbe) is a protein containing the LPXTG motif that is covalently bound to the bacterial cell wall surface and specifically recognizes fibrinogen and thus *S. epidermidis* cells expressing SdrG adhere to fibrinogen-covered surfaces [15]. The gene coding for SdrG/Fbe is found on *S. epidermidis* clinical isolates. The SdrG protein contains four distinct regions: an N-terminal export motif sequence, the A region containing the fibrinogen-binding activity, the B region with unknown function, and the R

region containing the serine-aspartate repeat sequence. SdrG specifically binds to a 14-amino acid-long peptide sequence on the N-terminal of fibrinogen's beta chain. SdrF, display a similar organization to SdrG and it specifically binds to collagen I [16]. So far, a specific function has not been assigned to the A region of SdrF. However, it has been demonstrated that its B region is sufficient to interact with collagen I and apparently, this binding occurs through the alpha1 and alpha2 chains of type I collagen [16]. Using a *Lactococcus lactis* heterologous expression system and a murine infection model, it has been established that SdrF may contribute to cardiac assist device driveline infections [17]. SdrF also participates in binding to unmodified Dacron surfaces covering drivelines. Anti-SdrF antibodies inhibited 50% of *S. epidermidis* 9491 binding to collagen using an in vivo model [17], indicating that additional collagen-binding factors may participate.

eDNA function during *S. epidermidis* and *S. aureus* biofilm formation has been established as another crucial component for cell attachment to a surface. Some studies confirm that eDNA is a structural component of the biofilm's matrix in both species. Independent studies have demonstrated that eDNA is released through increased cell lysis. In *S. epidermidis*, autolysis is carried out mainly by the autolysin activity of AtlE. A role for eDNA in *S. epidermidis* 1457 was evidenced during primary attachment through the addition of DNase I that results in inhibition of bacterial binding to a glass surface. In spite of the fact that eDNA participates in the primary attachment phase, a function during intercellular attachment phase (accumulation phase) has been ascribed [18]. During the surface colonization phase by *S. aureus* under flow conditions, eDNA is crucial during the transition between primary attachment and accumulation phases [19]. This points out that eDNA plays an important role in early stages of staphylococci biofilm formation.

II.2. Participating molecules during the biofilm accumulation phase

The main component during the accumulation phase is the expression of molecules possessing intercellular (cell-cell) adhesion properties leading to cell aggregation and to subsequent biofilm development having a multi-cell and multi-layer tridimensional structure. Based on the early electron microscopy studies, it has been shown that *S. epidermidis* are embedded within an amorphous matrix. Afterwards, the studies were focused on the biochemical analysis of the matrix components. These efforts resulted in the discovery of the PIA polymer, a component participating in great proportion on intercellular adhesion.

The PIA structure was first described in biofilm-forming *S. epidermidis* 1457 and RP62A strains. Through biochemical analysis, the existence of the structurally related polysaccharide I (>80%) and polysaccharide II (<20%) was determined and separated based on their different ionic properties. Using chemical analysis and NMR spectrometry, it has been demonstrated that polysaccharide I is a lineal homoglycan consisting of beta-1,6-linked 2-amino-2-deoxy-D-glucopyranosyl residues. Approximately 80–85% of them are N-acetylated (GlcNAc) and the rest are not N-acetylated, this polymer has an overall negative charge. PIA's polysaccharide II has a low proportion of N-acetylated 2-amino-2-deoxy-D-glucopyranosyl residues and it is modified with succinate residues linked by ester bonds, which confers it with anionic features [20]. The synthesis of an actively functional PIA molecule requires the expression of

all four *icaADBC* genes [21] constituting the *ica* operon. The *icaR* gene confines the repression of the *ica* operon expression. The synthesis process has been studied in detail using *S. carnosus* recombinant strains expressing different combinations of the *icaADBC* genes and using UDP-GlcNAc as the sugar donor [21]. The IcaA protein belongs to the glycosyltransferase family 2. It is an integral membrane protein consisting of 412 amino acids and four transmembrane domains. This protein performs the synthesis of beta-1,6-linked GlcNAc oligosaccharide composed by up to 20 GlcNAc units. The IcaD protein is required for IcaA full activity in vitro. IcaD is a membrane protein of 101 amino acids possessing two putative membrane space domains and it is thought that it may be a chaperone guiding IcaA protein folding and its membrane insertion and it also may act as a link between the IcaA and IcaC proteins [21]. Essential to PIA synthesis is the presence of the IcaB, an integral membrane protein of 355 amino acids possessing 10 predicted transmembrane domains, which may be involved in the externalization and elongation of the nascent polysaccharide [21]. IcaB is a member of the polysaccharide deacetylase family that includes chitin deacetylases or the chitooligosaccharide deacetylase NodB of *Rhizobium melioli*. IcaB in its mature form is a secreted protein consisting of 259 amino acids with a predicted signal sequence, which is responsible for PIA N-deacetylation and it is crucial for PIA activity during biofilm formation and for *S. epidermidis* virulence [22]. A strain harboring an *icaB* deletion mutation, in which the *icaB* gene has been eliminated, produces a weakly retained PIA at the cell's surface, as it does not contain N-deacetylated GlcNAc [22].

Conversely, the first observation made through biochemical analysis on biofilm matrix extracts indicated the presence of oligosaccharide, proteins, and nucleic acids. The specific proteins that comprise a biofilm have been identified and characterized; one of them is the biofilm-associated protein Bap [23]. The Bap is rarely found in invasive *S. epidermidis* biofilms from human infections [23] and it similarly occurs for *S. aureus*. Another protein found in biofilms is SesC, which has been proposed as playing an important role during biofilm formation [24]. SesC is a surface protein of 68 KDa containing the *S. epidermidis* LPXTG motif and it is related to the *S. aureus* clumping factor A (ClfA). SesC protein is strongly expressed in biofilm conditions in contrast to planktonic conditions by the *S. epidermidis* 1457 strain [24]. An anti-SesC antibody inhibited biofilm formation in vitro in several *S. epidermidis* isolates. All 105 *S. epidermidis* isolates collected from nose swabs of infection possessed the *sesC* gene in their genomes [24]. Actively or passively immunized animals using SesC as antigen displayed a decreased biofilm formation using the in vivo CVC infection model [25]. Nevertheless, a specific role of SesC during the intercellular adhesion of the accumulation phase of the biofilm remains to be demonstrated.

II.3. Multifunction proteins during the biofilm accumulation phase

Protein factors contributing to the accumulation phase of staphylococci biofilm have features of multifunctional proteins. In *S. aureus*, it has been found that fibronectin-binding proteins (FnBPs) (FnBPA and FnBPB), ClfA protein and *S. aureus* surface protein (SasG), may be considered multifunctional proteins as they do not have an exclusive role in either of the biofilm phases: primary attachment or accumulation [26]. FnBPs are constituted by an N-

terminal end, the A domain, and within the C-terminal, the LPXTG-anchoring domain separated by tandem repeats, which are involved in binding to fibronectin. Both, FnBPA and FnBPB are involved in the biofilm's accumulation phase in isolates from hospitals and under flow conditions. This concept of multifunctional proteins with important roles during some of the biofilm formation and surface colonization phases is also applied to *S. epidermidis* with its respective proteins participating in the accumulation phase: the accumulation-associated protein (Aap) and the extracellular matrix-binding protein (Embp).

The Embp protein and its *S. aureus* orthologue, designated as Ebh, were simultaneously pinpointed during identification studies of protein factors [27]. In a clinically relevant *S. epidermidis* isolates collection, the *embp* gene was detected in 90% of all strains [2]. Furthermore, studies conducted in vivo indicated the presence of anti-Embp antibodies in patients with prosthetic joint infections by *S. epidermidis*, suggesting that the Embp protein is expressed and it has a role during the infection. Surprisingly, when using a bacterial cell model in flow conditions, it was observed that addition of anti-Embp antibodies to the system inhibited biofilm formation by the *S. epidermidis* 1457 strain [28]. This result leads to propose the Embp protein as a potential candidate for preventive strategies against biofilm formation. Experimental evidences suggest that Embp has a defined role during the primary attachment phase. This proposal is supported by the fact that Embp overexpression had no effect on bacterial attachment on an unmodified polystyrene surface, although it had a negative effect on bacterial binding to fibronectin-covered surfaces [29]. Additionally, it was observed that the Embp-fibronectin interaction is necessary for the biofilm's accumulation phase on plastic surfaces [29].

The Aap protein is covalently bound to the cell wall and consists of an A domain and a B domain. The A domain has 584 amino acids and includes an export signal at its N-terminal, 16 amino acid repeats and a globular region of 212 amino acids with alpha-helical and beta-sheet contents. This 212-amino acid-long region is highly conserved between Aap and its *S. aureus* orthologue, SasG. Through bioinformatics analysis, it has been shown that this domain possess lectin-type activity [6]. The B domain consists in a variable number of repeats of 128 amino acids, the G5 subdomains [2]. The number of G5 subdomains in the B domain is different among the *S. epidermidis* strains, for example, the RP62A reference strain possesses 13 G5 subdomains, whereas the *S. epidermidis* 1457 strain has only seven [6]. This fact has been also observed in clinic isolates with clonal genotypes subsequently recovered from ongoing infections on devices in patients [2]. This observation leads to the hypothesis stating that the number of G5 subdomains of the Aap B domain may represent a mechanism contributing to the immune evasion of *S. epidermidis* mediated by the modification of the major epitopes on the cell's surface [2]. Aap is detected on the bacterial cell wall and its retention mechanism is through anchoring of its C-terminal by a covalent bond with the cell wall PGN [30]. A more detailed analysis by confocal microscopy showed that Aap is strictly localized at the bacterial cell surface, whereas minimal amounts of Aap are released within the biofilm matrix [30]. This result is supported by electron microscopy observations in which Aap appears as elongated fibers of 120 nm projecting outwards from the cell wall and in form of tufts [31].

The importance of Aap for *S. epidermidis* biofilm formation was recognized during studies in which the expression of the B domain does not modify the primary adherence properties, although it is very important to cellular aggregation, indicating that Aap is a protein that participates in the intercellular adhesion [32]. Similarly, the importance of the B domain for intercellular adhesion was also described for SasG in *S. aureus* [33]. Another fact that evidences the properties of Aap in intercellular adhesion is the ability of the B domain to undergo homodimerization in the presence of Zn [34]. The proposed mechanism of intercellular adhesion through Aap is that the protein must undergo proteolytic processing in order to remove the A domain [32–33]. Thus, Aap proteolytic processing does not normally occur under in vitro growth conditions [32].

Although the intercellular adhesion property of Aap was recognized, currently there is evidence supporting its significant role in the primary attachment phase as well. The binding of *S. epidermidis* NCTC 11047 strain expressing Aap to squamous epithelial cells was partially inhibited by the addition of the recombinant A domain of Aap [35]. In a clinical isolate, the binding of *S. epidermidis* CSF41498 strain expressing a non-processed Aap (thus containing the A domain) to polystyrene was completely impaired by the addition of an anti-A domain antiserum, whereas an anti-B domain antiserum did not affect its adhesion ability [36]. Thus, a new bifunctional role of Aap during the biofilm formation is suggested: its participation on the primary attachment phase through its A domain and also its participation on the accumulation phase through its B domain [32, 35–36].

II.4. Molecular mechanisms for mature biofilm disassembly

A primary biofilm disassembly mechanism used by *S. aureus* and *S. epidermidis* is the production of extracellular enzymes or surfactants that degrade or solubilize the adhesive components of the biofilm matrix. Because this matrix cover bacterial cells within the biofilm colony, its degradation results in cell detachment from the colony and its release toward the environment. The products of the matrix-degrading genes, which are implicated on the dispersion of the staphylococcal biofilm, include proteases, DNases, and surfactants.

The *agr* system is a putative regulator controlling the production of the enzymes degrading the biofilm matrix. The *agr* is controlled by a cyclic autoinducing peptide (AIP) that is synthesized and secreted within the environment. When the AIP concentration reaches a critical threshold level, it activates a two-component signal transduction cascade leading to the production of secretory virulence factors [37]. The extracellular proteins induced by the *agr* system are multiple proteases and pore-forming toxins termed PSMs. *S. aureus* does not form a biofilm when the *agr* system is highly active and its reactivation within the mature biofilm results in its disassembly [38]. Furthermore, the *agr* system of *S. aureus* is more active in cells detached from the biofilm [39]; the same phenomenon was observed in *S. epidermidis* [9], contributing evidence showing that induction of the *agr* system results in biofilm disassembly.

Extracellular protease production has been implicated on the disassembly of the mature biofilm. In *S. aureus*, the mutation of the protease-coding genes results in a significant increase of biofilm formation under flow conditions and the disassembly decrease when the *agr* system

is inactivated [38]. Additionally, protease inhibitors have a promoter role during biofilm formation in *S. aureus* under conditions that normally accelerate its disassembly [38]. Similarly, mutations leading to extracellular protease overexpression, such as those on the *sarA* and *sigB* genes, enhance a planktonic growth in *S. aureus* [40]. This leads to a concept of an inverse correlation between protease expression and biofilm formation.

S. aureus secretes a potent DNase, also known as thermonuclease or micrococcal nuclease, which has been implicated on cell detachment from the biofilm. *S. aureus* mature biofilm is disintegrated by the exogenous addition of DNase I or restriction enzymes [41]. It has been shown that a nuclease-mutant *S. aureus* strain exhibited an increase of biofilm formation under flow conditions regarding to the wild-type strain [41]. These findings suggest that nucleases may function as endogenous mediators for biofilm disassembly in this species.

PSMs are peptides possessing surfactant features, which are produced by both *S. aureus* and *S. epidermidis*, and they are capable of contributing for mature biofilm disassembly. PSMs are regulated by the *agr* system and their amphiphilic alpha-helix structure confers it with a surfactant-type property. PSMs promote both disassembly of the mature biofilm of *S. epidermidis* in vitro and dissemination from colonized catheters on a mouse model of device-related infection [9]. Additionally, antibodies against PSMs inhibit bacterial dispersal from the implanted catheter, indicating that the disassembly manipulation strategy may prevent the spreading of the infection.

III. Regulation of biofilm formation in staphylococci

Biofilms are a lifestyle adopted by a wide variety of microorganisms that requires the consumption of an enormous amount of energy. Thus, it is expected that biofilm growth may be controlled by more regulatory mechanisms regarding planktonic growth. Some of the factors that impact on biofilm formation are mentioned in the following sections.

III.1. Regulation of the factors participating on the primary attachment phase

The *agr* is a quorum-sensing system in staphylococci that regulates the expression of adhesion molecules, thus it participates in the primary attachment phase. These adhesion molecules, such as the MSCRAMMs, display an increased expression when cell density is low, a situation favoring the onset of the infection by staphylococci. Once the colonization has concluded, the increased activity of the *agr* quorum-sensing system represses the expression of unnecessary colonization factors. Among these, the MSCRAMMs are included, which are negatively regulated by *agr* in *S. aureus* [42]. In *S. epidermidis*, the knowledge regarding colonization factors and their regulation is more limited. Results obtained by transcriptional profiling and the assessment of MSCRAMM expression [43], suggest that some of the latter do not follow the classic notion of regulation mediated by the *agr* system.

III.2. Regulation of PIA synthesis

The regulation of PIA expression is probably the best-studied system among those regulation systems involved in staphylococci biofilm formation. Anaerobiosis significantly increas-

es PIA expression [44]. This constitutes an important finding for biofilm physiology, as the oxygen concentration would restrict biofilm formation at the oxygen-loaded arterial bloodstream. In an already established biofilm, PIA expression would be higher at the most deep biofilm sections because oxygen concentration significantly decreases. Conversely, it has been found that sub-inhibitory concentrations of specific antibiotics increase the transcription of the *ica* operon in *S. epidermidis* [45].

Some overall regulators of *S. aureus* or *S. epidermidis* participate in the *ica* operon transcription regulation or PIA expression, such as the SarA DNA-binding protein and the alternative sigma factor SigB that increase the expression of the *ica* operon, whereas the luxS quorum-sensing system represses the expression of this operon [46]. Contrastingly, the *agr* system does not regulate PIA. The exact mechanism explaining the influence of SarA and SigB on the *ica* operon transcription is complex. Briefly, the SigB regulator represses the *icaR* gene transcription, as its protein product, IcaR, in turn, represses the transcription of the *icaADBC* operon [47]. Besides, SarA regulates positively the *icaA* gene, independently from IcaR [48].

III.3. Regulation of the PSMs expression

It has been discussed that the *agr* quorum-sensing system represses the expression of surface proteins after the primary attachment. The major *agr* control relies on the expression of the PSMs. The expression of the *agr* system within a biofilm is limited to its periphery, in which the *agr* regulator controls cell detachment from the biofilm by regulating the increased expression of the PSM effector molecules [49]. The staphylococcal PSM δ is a major effector molecule for cell detachment from the biofilm and it is tightly controlled by the *agr* system of *S. aureus* [50]. In *S. epidermidis*, the PSM β is the most important.

III.4. Biofilm regulation against host's defenses and antibiotics

One of the advantages possessed by bacteria in the biofilm state is high resistance toward antibiotics and the host innate defense, such as AMPs and the phagocytosis performed by neutrophils. However, the molecular basis of this phenomenon has been recently investigated. Two of the main mechanisms contributing to biofilm resistance are: (1) keeping antibacterial substances from reaching their target, for example, by limited diffusion or repulsion and (2) biofilm's specific physiology that limits the efficiency of antibiotics, mainly those targeting active cells, and it may include specific subpopulations of resistant cells ("persistent").

Limited antibiotic diffusion provided by the biofilm is mainly due to the nature of the biofilm matrix. However, this limited diffusion is the resistance mechanism toward some antibiotics, such as ciprofloxacin in *P. aeruginosa* [51], whereas some others (e.g., rifampicin and vancomycin) are able to cleave the exopolysaccharide envelope of *S. epidermidis* [52]. Interestingly, PIA has the ability to protect the cells within the biofilm from both cationic or anionic AMPs, as it possess an overall positive or negative charge, and thus PIA interacts or repels molecules depending on its charge. Similarly, the poly-gamma-glutamic acid (PGA) exopolymer, of *S. epidermidis* and a number of CNS species, contributes to the resistance toward AMPs of either charge.

Phagocytosis, mainly performed by neutrophils, is a major mechanism by which the innate immune system eliminates microorganisms invading the human body. Staphylococci in a biofilm are not readily subjected to phagocytosis by neutrophils. The responsible elements for this constraint are the PIA exopolysaccharide and the PGA exopolymer, and therefore they contribute to biofilm resistance toward the host's innate defense mechanisms.

IV. Therapeutic strategies against biofilm formation in medical devices

Medical devices are widely used for diagnostic and therapeutic treatment in most medical specialties. Infection risk is a frequent complication linked to the permanent use of medical devices such as orthopedic or heart prostheses, vascular catheters, urinary catheters, and endotracheal tubes. A promising solution in order to prevent medical device-associated infections is to develop devices possessing materials or surfaces that act against microorganism adhesion or their viability. The first strategy was the use of biocides in coatings. A number of clinical assays have been conducted producing conflicting results. Some authors suggest that the extended use of biocide on the coating may lead to an increase of microbial resistance toward the microbiocide agent. The other strategy consists in the development of materials impeding bacterial adhesion.

IV.1. Biological strategies for biofilm treatment

The chemical diversity of the biofilm matrix, including protein material, eDNA, and polysaccharides, is susceptible to degradation by a wide variety of exogenously added enzymes. Some research groups have observed that proteinase K and trypsin may disperse the mature biofilm of *S. aureus* and *S. epidermidis* [53]. Bovine DNase addition has also been successful for dispersing the mature biofilm of *S. aureus* [54]. Similarly, the enzymes able to degrade PNAG cleave biofilms containing this polysaccharide as their primary component. An enzyme called dispersin B (DspB) inhibits biofilm formation and promotes its disassembly in several *S. epidermidis* and *S. aureus* strains having PNAG as the main component of their respective biofilm matrices [54]. Finally, the treatment with lysostaphin was effective in a catheter mouse model of *S. aureus* biofilm [55], suggesting that it may be a general therapy against staphylococcal biofilm infections.

A current topic is the development of an antimicrobial coating interfering with quorum-sensing mechanisms. This has been observed for halogenated furanones synthesized by the red algae *Delisea pulchra* possessing anti-adhesive properties against a wide range of bacteria [56].

IV.2. Anti-adhesive chemical strategies

IV.2.1. Hydrophobicity and surface charge

Bacterial adhesion depends on hydrophobicity of the cell and material constituting the surface. The self-autoassembled monolayers (SAMs) can modulate the exposure of their different residues on a surface and they are used in bacterial adhesion studies as models of surfaces

with chemically controlled properties. SAMs with hydrophilic residues (OH, NH₂) tend to decrease bacterial adhesion when compared to those with hydrophobic surfaces containing methyl groups (CH₃) [57]. Some hydrophilic linings, such as hydrogels or medical devices with chemically modified surfaces have been developed in order to restrict biofilm development. Some clinical studies have reported that urinary catheters lined with heparin can reduce *Proteus mirabilis* biofilm [58]. However, it has been observed that heparin may stimulate biofilm formation by *S. aureus* [59]. This demonstrates that initial adhesion is not always sufficient to avoid biofilm development. Treatment with plasma can also create hydrophilic residues at the surface of medical devices producing antimicrobial activity.

IV.2.2. Steric barriers

The chemical modifications of the surface may also consist on grafting long-chain polymers in order to form brush-type structures on it. The density of the chains provides a steric barrier that repels bacterial adhesion. The most widely studied polymers are derivatives of polyethylene oxides. In fact, residues of SAMs with ethylene glycol (4EG and 3EG) have lower bacterial adhesion in comparison to hydrophilic surfaces [57]. Polymers with ester residues (CHO₂-) or cyclic hydrocarbons (C₄H-, C₆H-) exhibit less bacterial attachment strength than materials containing ethylglycol or hydroxyl group fragments [60].

IV.2.3. Anti-adhesive strategies based on topographic modifications of the surface

In the theories of bacterial adhesion, the appearance of the surface of the material was not considered. The relief of a surface depends on the scale, that is, for bacterial adhesion, the submicron scale is used. The reliefs are divided into: i) areas with irregular or random traits defined as rough; ii) areas with organized features, often made by an engineering process, defined by the term surface topography.

One study showed that adherence of *S. epidermidis* was similar on titanium surfaces, both rough and smooth [61]. SEM observations showed that this strain tends to adhere to grooves and depressions possessing dimensions similar to that of bacteria [62]. Regarding surface topography, it has been found that a surface constituted by titanium nanotubes is more hydrophilic than a conventional titanium surface. These properties have a biomedical application in orthopedics by decreasing bacterial adhesion [63]. Additionally, nanotubes could be filled with biocides in order to enhance its activity against biofilm. Superhydrophobic surfaces are being developed with nano or micro-features in order to create bacteria-free medical devices.

IV.2.4. The influence of nanofeature physical structure on bacterial response

Nanofeatures may adopt different shapes: nanotubes, notches, channels or grooves, holes or pillars. There are few studies regarding the relationship between nanofeatures and bacterial adhesion. Ercan et al. compared *S. aureus* and *S. epidermidis* adhesion on commercial titanium surfaces with nanotubes of 20–80 nm of diameter. A decreased bacterial adhesion was observed for larger diameters (60 and 80 nm) [64]. However, the study conducted by Yu et al. produced opposite results: staphylococci adhesion increased proportionally to nanotube

diameter (30, 70, and 120 nm) [65]. The nanofeature's array is also important for bacterial adhesion and it may form large patterns having several effects on microbial adhesion. For instance, the crest-shaped array (2 μm wide, 3 μm spacing, and different lengths) in polydimethyl siloxane elastomer was bioinspired from the shark's skin. This elastomer structure exhibited no signs of *S. aureus* biofilm formation after 14 days, unlike the smooth surface, which allowed the formation of a mature biofilm [66].

As mentioned above, the nanoscale level, size, and bacterial shape regarding nanofeature dimensions play a significant role. Bacterial features (adhesion, surface charge) are also important to the adhesion process. The surface of a nanostructure must be tested with several bacterial strains, as they exhibit different adhesion behaviors. For instance, a titanium surface nanostructured by femtosecond laser ablation and mimicking the superhydrophobic surface of the lotus leaves was not colonized after 18 hours by *P. aeruginosa*, whereas *S. aureus* adhesion was stronger when compared to the smooth surface [67]. This result suggests that some nanostructure surfaces may not be appropriate for medical applications in which the adhesion properties of the microorganisms are unknown.

Conversely, silver nanoparticles (AgNPs) are gaining interest for biomedical applications because of their features having a higher surface/mass ratio and a potent antibacterial activity. These AgNPs may be applied as monolayers at the surface of biomaterials. A study on glass surfaces modified with AgNPs was carried out and it was found that they possess a great stability in aqueous media, an extended Ag^+ release without AgNPs detachment and a strong anti-biofilm activity against *S. epidermidis* RP62A [68]. These AgNP-coated surfaces could be applied on a great variety of biomaterials. Nevertheless, it is important to conduct more studies to verify the anti-biofilm capacity with clinical isolates of different staphylococci species.

5. Conclusions

Staphylococcus biofilms is a virulence factor widely distributed in this genus, currently there are many studies about this subject; however, there are still questions to be answered about the process of biofilm formation. Some molecules involved in biofilm formation are recognized; nevertheless, the interaction between them is unknown, such as the formation of the structural network of the biofilm, the assembly and disassembly process, and the mechanisms of intrinsic and extrinsic regulation during these events. Many molecular pieces remain to be resolved, which allow us to fully understand the construction of a biofilm. Furthermore, it is evident that a strain of *Staphylococcus* can form different types of biofilm (PIA-dependent or dependent protein), suggesting that staphylococcal biofilm is dynamic and adaptable to growth conditions. In fact, biofilm dynamics can be interpreted as a mechanism of resistance to environmental variations. The use of medical devices covered with anti-biofilm materials represents an alternative strategy but it is not decisive. The picture is complicated by the biological and physical characteristics of the different types of biofilm, the high genetic diversity into the genus, and the lack of comprehensive knowledge on biofilm formation properties, which leads to a complex and complicated scenario that prevents a successful anti-

biofilm treatment. By understanding the processes of biofilm, it could control its formation and have biofilm-free medical devices.

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Author details

Janet Jan-Roblero¹, Sandra Rodríguez-Martínez², Mario E. Cancino-Díaz² and Juan C. Cancino-Díaz^{1*}

*Address all correspondence to: jccancinodiaz@hotmail.com

¹ Microbiology department, Escuela Nacional de Ciencias Biológicas-IPN, Mexico City, Mexico.

² Immunology department, Escuela Nacional de Ciencias Biológicas-IPN, Mexico City, Mexico.

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