Biology and Regulation of Staphylococcal Biofilm

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Despite continuing progress in medical and surgical procedures, staphylococci remain the major Gram-positive bacterial pathogens that cause a wide spectrum of diseases, especially in patients requiring the utilization of indwelling catheters and prosthetic devices implanted temporarily or for prolonged periods of time. Within the genus, if *Staphylococcus aureus* and *S. epidermidis* are prevalent species responsible for infections, several coagulase-negative species which are normal components of the microflora also constitute opportunistic pathogens that are able to infect patients. In such a clinical context, staphylococci producing biofilms show an increased resistance to antimicrobials and host immune defenses. Although the biochemical composition of the biofilm matrix has been extensively studied, the regulation of biofilm formation and the factors contributing to its stability and release are currently still being discovered.

staphylococci biofilm gene expression regulation resistance

1. Biofilm Formation and Clinical Significance

Most bacterial human diseases involve biofilm-producing pathogens. Bacteria growing in surface-associated communities, which are described as biofilms, are physiologically distinct from free-swimming, planktonic-state organisms. Biofilms can be defined as sessile microbial communities that are embedded in a self-produced extracellular matrix [1][2] of polysaccharidic or proteinaceous nature associated with DNA, yielding to so-called “hydrated surface-associated communities” [3]. While biofilms were first described in aquatic environments, biofilm formation is increasingly recognized as an important parameter in the pathogenesis of many bacterial infections. Among these infections are diseases that involve the formation of a biofilm on the biomaterials frequently used in modern medicine (e.g., catheters and polymeric or metallic implants) and hard mineral surfaces (e.g., teeth and bones) [4][5][6]. The hallmark characteristic of a biofilm is the development of a three-dimensional structure of bacteria that is stabilized within an exopolysaccharide glycocalyx [7]. The formation of bacterial biofilms is an elaborate process composed of four consecutive phases: attachment, accumulation, maturation, and spontaneous dispersal [8]. This complex and structured architecture protects the bacteria from hostile environments such as the human body [9]; this is not the case for free-floating organisms [10]. In addition, a biofilm’s mode of growth provides altered susceptibility to some antimicrobials [11][12][13] and affects bacterial killing by professional phagocytes [14][15]. The 3D structural organization of bacterial biofilms contains bacteria with different phenotypes [16][17] and various growth rates and metabolic activity, yielding a limited efficiency of the antibiotics that target cell-wall biosynthesis while the reduced oxidative metabolism limits the access of aminoglycosides to their target [18].

Biomaterials implanted for a prolonged period of time, such as durable catheters and orthopedic implants, are frequent sources of sepsis and infections, mainly due to slime-producing and biofilm-forming bacteria. Thus, a question arose with respect to the development of materials that demonstrate a reduced incidence of biofilm formation. It was rapidly noticed that bacterial adhesion (attachment) is the first step in biofilm formation, and various methods have been developed to assess adherence and biofilm formation on a given polymer surface. A brief overview was presented in the review by Götz and Peters [19]. Here, the authors showed that coagulase-negative staphylococci and *S. aureus* can bind to almost any implanted material composed of plastic, stainless steel, or titanium. Bacterial adhesion is not dependent on surface type, whether smooth or textured; or on the polymeric composition of the implant material, whether silicone or polyurethane; nor is it dependent on the presence or absence of slime. Bacterial adhesion to biomaterials is a general process that is most likely due to many surface components. With the help of a green fluorescent protein (gfp) reporter plasmid in *S. aureus* [20], adhesion and biofilm formation were investigated on various surfaces. Glass slides were coated with three different materials used as medical devices: titanium, cobalt, and Teflon [21]. As shown, *S. aureus* adhered and formed a biofilm even on titanium, the most frequently used material for hip prosthesis. It also adhered and formed a film on cobalt surfaces, while the adherence to Teflon was less pronounced.

Biofilm formation appears to be genetically programmed and finely regulated [22][23][24][25], allowing bacteria to control their microenvironment [2][17][26][27] and to actively detach from the biofilm matrix to generate metastatic infectious foci [28]. Genetic analyses were used to reveal the diversity of genetic factors contributing to biofilm formation, and it appears clearly that multiple pathways are involved in building bacterial biofilm [29][30][31][32]. These factors, especially during the early stages of biofilm formation, can be functionally replaced or compensated for by others, depending on environmental and growth conditions [25][27][33][34].

2. Molecular Control of *S. aureus* Biofilm Development and the Role of *ica*

The formation of mature, three-dimensional biofilms is a complex process composed of different phases: attachment, accumulation, maturation, and dispersal [35][36]. While the initial binding to abiotic (protein-free) surfaces in vitro is mostly based on hydrophobic interactions, primary attachment during infection occurs via the binding of specific bacterial surface receptors that recognize host matrix proteins [37]. This group of cell-wall-anchored proteins, named MSCRAMMs (for microbial surface components recognizing adhesive matrix molecules) [38][39], presents a conserved structure containing 4–5 domains with the binding domain exposed to the extracellular medium. The accumulation phase appears to be related to the production of polysaccharide adhesins that allow interactions between bacterial cells [23][40]. Thus, the primary determinant of the accumulation phase of staphylococcal biofilm formation relates on the production of the polysaccharide intercellular adhesin (PIA), a process that is dependent on the expression of genes of the *icaADBC* operon [41][42]. Biochemical studies have demonstrated that the PIA consists of polymeric *N*-acetylglucosamine in which the cells are embedded and protected against humoral and cellular host immune defense and against antibiotic treatments [14][43][44]. PIAs act as an intercellular adhesin, allowing for the integration of bacterial DNA [45] and constituting a stable, organized

structure. They appear to play a role in the formation of multiple bacterial clusters that are involved in biofilm maturation and include the accumulation-associated protein [23][46] and other proteins, such as clumping factor A (ClfA) [23], the staphylococcal surface protein (SSP1), and the biofilm-associated protein (Bap) [23].

With the increasing number of sequenced genomes due to progress in high-throughput sequencing capacity, an *ica* locus has been identified in several staphylococci species: *S. caprae*, *S. roterodami*, *S. carnosus*, *S. saprophyticus*, *S. cohnii*, *S. capitis*, *S. sciuri*, *S. hominis*, and *S. simulans.* It appears to serve the same function as in *S. aureus*. Note that if *S. aureus* remains a potent human pathogen, most of these species represent potential human opportunistic pathogens.

The *ica* operon was first identified in *S. epidermidis* [29][47] and has been studied most extensively in that species. The *ica* operon is subject to environmental regulation [48]. For example, anaerobic growth was found to induce expression of the *ica* operon and PIA production in both *S. epidermidis* and *S. aureus* [27]. Expression of the *icaADBC* operon appears tightly controlled in *S. aureus*, evidenced by the fact that it is expressed at very low levels under in vitro growth conditions [49]. Beenken et al. found that the mutation of *ica* and the resultant inability to produce PIA had little impact on in vitro biofilm formation or the colonization of an abiotic surface [25]. A group also compared an *S. aureus* strain and its corresponding *ica* mutant in a tissue cage model of infection and demonstrated that the *ica* mutant retained the capacity to colonize at a similar level to the wild-type strain [50], a result which was confirmed by others [51]. Taken together, the expression of *ica* plays a major role in biofilm formation but is not essential in the colonization of a surface. Interestingly Rachid et al. [52] showed that the expression of *ica* is at least partially controlled by the stress response transcription factor, σ^B [53]. Studies performed with *S. aureus* have demonstrated that the regulation of *ica* expression and the ability to form a biofilm involve regulatory elements other than σ^B and IcaR [54]. Among these additional regulatory loci, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*) represent important partners. Note that the interaction of SarA with *agr* results in the promotion of biofilm formation. It was also shown that a mutation of *sarA* resulted in a reduced capacity to form a biofilm, a phenomenon which is independent of the *icaADBC* operon but involves various regulatory pathways, including *sar*, *tcaR*, and sRNA [55][56][57]. Factors that influence staphylococcal biofilm formation have been reviewed by Goetz and Otto [23][58].

The range of environmental factors altering biofilm formation appears to be indicative of the highly diverse habitats in which staphylococci are able to form biofilms. For example, the presence of oleic acid induces *S. aureus* biofilm formation. This probably results from an ionic interaction of the positively charged PIA with the negatively charged oleic acid. The effect is even more pronounced under oxygen-limited conditions [59][60][61], a fact consistent with the observation that anaerobiosis is an important stimulus for *ica* expression [27][62]. A mature biofilm reveals an architecture that ensures the provision of nutrients and oxygen to all cells in the biofilm [3]. As they grow, bacteria begin to arrange in a three-dimensional structure composed of an array of pillars and mushroom-shaped structures. These structures are connected by convoluted channels that deliver nutrients and contribute to the elimination of waste. The maturation of biofilms has been studied by imaging and transcription profiling studies [10][25][63]. A primary discovery that emerged from microarray experiments is that persistence within a mature biofilm requires an adaptive response that limits the deleterious effects of pH reduction associated with anaerobic

metabolism [25]. The cell envelope is a very active compartment as the expression of genes that encode binding proteins, proteins involved in the synthesis of murein and glucosaminoglycan, PIA, and other enzymes involved in the cell-envelope metabolism appears to be significantly upregulated. Thus, a biofilm is a dynamic structure that evolves with environmental conditions, such as physical shear forces, and as a result of the processes that are sensed and regulated by the bacteria. Once cell clusters reach a sufficient size, groups of cells either detach (dispersal phase) or die. Thus, it is the cycle of cell growth, detachment, and regrowth that underlies the observed patterns of organized gene expression [33][64].

3. Biosynthesis of PIA/dPNAG and Its Regulation

In 1987, Gordon Christensen published a paper on the phenotypic variation of *S. epidermidis* slime production in vitro and in vivo [65]. Today, the “slime” they described was the exopolysaccharide PIA (polysaccharide intercellular adhesin), whose chemical structure was first described in *S. epidermidis* in 1996 [66]. Later, PIA was also referred to as ß-(1,6)-N-acetylglucosamine (PNAG) [67]. The more chemical-sounding name PNAG is not really a correct description of the glucosamine polymer as it ignores the fact that N-deacetylation takes place at certain intervals, which is essential for biofilm formation. PIA represents a linear homoglycan of at least 130 beta-1,6-linked 2-deoxy-2-amino-D-glucopyranosyl residues which are from 80 to 85% N-acetylated. The rest are non-N-acetylated and positively charged. Since a correct chemical description was cumbersome, the name PIA was chosen in the initial description of the structure [66]. PIA is a polymer of partially de-*N*-acetylated ß-1,6-linked *N*-acetylglucosamine (dPNAG).

4. Roles of Biofilm in the Tolerance to Multiple Drugs

In a biofilm, the bacterial cells are attached to a surface where, depending on the nutrient content of the environment, they multiply more or less actively and form a multilayered structure. The maturation to a three-dimensional biofilm is also called the accumulation phase. Such biofilms are formed in humid or marine environments in water pipes, on ship hulls, and other on stainless steel surfaces where they cause biofouling [68], which causes enormous costs [69][70]. Typically, such a biofilm consists of a heterogeneous spectrum of micro- and macro-organisms whose cells are embedded in a self-produced matrix and whose metabolic products lead to the corrosion of the metal [71]. In particular, the production of extracellular polymeric substances (EPSs) by microorganisms facilitates adhesion to material surfaces such as metals. These complex biofilm structures are highly resistant to extreme stress conditions, and only aggressive bactericidal detergents or harsh physical treatments such as sonication exhibit antifouling properties [72].

There are similarities and differences between biofouling and biofilm-associated infections. They have in common that microorganisms primarily bind to surfaces and change these surfaces by their binding so that further microorganisms can bind and thus form a robust biofilm, whereby EPSs make an important contribution to the compactness of the biofilm. While biofouling is a mixture of various microorganisms, biofilm-associated infection is usually due to a single bacterial species. The National Institutes of Health (NIH) evaluated that biofilm-producing

bacteria are involved in 65% of all microbial infections and are responsible for 80% of chronic infections. The annual incidence of biofilm-related infections in the United States represents roughly 2 million cases, causing 268,000 estimated deaths, and is accompanied by USD 18 billion in direct costs for the therapy of these infections [2][73]. The bacterial species frequently involved in such infections are *S. epidermidis*, *S. aureus*, *Enterococcus*, *Bacillus*, and *Candida* spp. The origin of these microorganisms may be from the skin or from other indwelling devices such as central venous catheters or dental work [74].

With biofilm-associated infection, the largest problem is that many therapeutic approaches fail because a high proportion of the bacterial cells in a biofilm matrix are “phenotypically” insensitive to most antibiotics. Researchers deliberately speak here not of resistance, since the latter implies certain resistance genes in the classical sense. In 1994, after penicillin was marketed, it was observed that staphylococci can enter a physiological state called persistence (or multidrug tolerance) in which lethal antibiotics failed to kill them [75]. Multiple factors appear to contribute to the global insensitivity of biofilm bacteria [13][76]:

- Enhanced antimicrobial resistance is a general phenomenon of biofilms and is the result of numerous specific factors which depend on the species involved, the environment of the biofilm, and the antimicrobial agent used;
- The implant material on which a biofilm is formed is not or is only scarcely perfused, preventing antibiotic diffusion at a sufficiently high concentration;
- The penetration and diffusion of antibiotics into a thick biofilm is hampered;
- The growth rate of bacterial cells in a biofilm is reduced (most antibiotics are efficient against actively growing bacteria);
- The physiology of cells in a biofilm differs from that of planktonic cells.

The phenomenon of the general antibiotic insensitivity of bacterial cells in a biofilm is characterized by the fact that biofilm-associated cells are insensitive, whereas “the same” cells in suspension are sensitive [77]. This suggests that insensitivity is not related to classical antibiotic resistance gene but to an altered physiological state in the biofilm mode of growth. Kim Lewis called the small fraction of essentially invulnerable cells in a biofilm “persisters” that exhibit multidrug tolerance (MDT) [78]. In *Escherichia coli*, the toxin–antitoxin (TA) modules RelE-RelB and HipB-HipA (high-persistence) seam to play a role in the persister phenotype. The overproduction of RelE or HipA causes an increase in the persister population. HipA inhibits translation by the phosphorylation of EF-Tu [79], stimulates the RelA-dependent synthesis of (p)ppGpp [80], and phosphorylates glutamyl-tRNA synthetase (GltX), which becomes inactivated by phosphorylation by HipA [81]. RelE cleaves mRNA at the ribosomal A site with high codon specificity [82]. The overexpression of RelE or HipA leads to a slowdown translation and thus the growth of *E. coli*, which presumably protects the cells from lethal factors such as antibiotics. It is known from ß-lactam antibiotics that they act mainly on dividing cells and are less effective on non-growing cells.

In staphylococci, the generation of persister cells is less clear than in *E. coli*. There are four different families of TA systems described, but their physiological roles are elusive [83]. The chromosomal *mazEF* system encodes the RNase toxin MazF and the antitoxin MazE [84]. MazF specifically targets UACAU sequences of *spa* (staphylococcal protein A) and *rsbW* (anti-sigmaB factor) in *S. aureus* mRNA in vivo, whereas translational reporter fusions indicated that the protein levels of the encoded products were unaffected. Despite a comparable growth rate to the wild-type, an *S. aureus mazEF* deletion mutant was more susceptible to β-lactam antibiotics, suggesting that the genes involved in antibiotic stress response or cell wall metabolism are controlled by this TA system [84].

Long before *E. coli*, a connection between reduced growth and increased antibiotic tolerance was described in staphylococci in the form of “small colony variants” (SCVs) [85]. From patients with persistent and relapsing infections, *S. aureus* SCVs were isolated which were auxotrophs for menadione, hemin, and/or a CO_2 supplementation. All these SCVs were resistant to aminoglycosides. The phenotype of such respiratory deficient mutants was further analyzed in a stable *hemB* mutant of *S. aureus* [86]. Such a *hemB* mutant showed the typical SCV phenotype, such as slow growth and a resistance to aminoglycosides; it also showed decreased pigmentation, low coagulase activity, reduced hemolytic activity, and a high persistence in endothelial cells. Respiratory mutants, both those that are naturally occurring or genetically constructed, demonstrate the importance of the metabolism in virulence and drug tolerance [87]. In *S. aureus*, there are many global regulators that impact virulence factor expression in SCVs [88].

5. Staphylococcal Biofilm in the Clinical Situation

At the end of the 1990s in the United States, experts estimated that biofilms were associated with 65% of nosocomial infections and that the annual cost of treatment of these biofilm-associated infections was higher than USD 1 billion [2][89]. *S. aureus* and other staphylococci are frequently found on implanted materials such as catheters, hip prosthesis, or surgical materials [5][90][91][92]. A recent study identified methicillin-resistant coagulase negative staphylococci as a major cause of biofilm-associated infections and possibly responsible for critical clinical situations. This interesting study relied on the analysis of numerous samples originating from hospital environments and from various hospital wards. The authors identified different staphylococcal species that produce bacterial biofilms: *Staphylococcus haemolyticus*, *S. epidermidis*, *S. hominis*, and *S. warneri*. The authors isolated approximately 300 MR-CoNS among the 558 samples from community and hospital environments. *S. haemolyticus* and *S. epidermidis* were the predominant species, representing roughly 73% of the CoNS identified. Significant biofilm production was detected in 91% of isolates, suggesting that the absence of production is marginal in clinical and environmental CoNS [93]. The staphylococci isolates that were derived from hospital wards were more associated with biofilm production than the community-derived isolates. Distinguished from the isolates identified in hospital wards, environmental strains were devoid of *icaAD* and *bap* genes and thus produced mainly proteinaceous biofilms.

Recent studies documented biofilms as community phenomena by assessing the interaction between bacteria and surface-associated-biofilm-producing organisms. Toledo-Silva reported nicely that numerous non-*aureus* species of staphylococci were able to interact with biofilm-producing *S. aureus*. The authors isolated *S. chromogenes*, *S.*

epidermidis, and *S. simulans* from bovine milk samples and showed that *S. chromogenes* (devoid of *ica*) stimulates the biofilm formation of *S. aureus* and alters the dispersion of *S. aureus*-formed biofilm. The study highlighted possible interactions between CoNS and *S. aureus* in the biofilm communities, most likely through interactions between the respective *agr* quorum systems [94]. Further research is needed to study bacterial biofilms as community phenomena.

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