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Molecular basis of in-vivo biofilm formation by bacterial pathogens

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Summary

Bacterial biofilms are involved in a multitude of serious chronic infections. In recent years, modeling biofilm infection in vitro led to the identification of microbial determinants governing biofilm development. However, we lack information as to whether biofilm formation mechanisms identified in vitro have relevance for biofilm-associated infection. Here, we discuss the molecular basis of biofilm formation using staphylococci and *Pseudomonas aeruginosa* to illustrate key points, as their biofilm development process is well-studied. We will focus on in-vivo findings such as obtained in animal infection models, and critically evaluate in-vivo relevance of in-vitro findings. Although results on the role of quorum-sensing in biofilm formation have been conflicting, we now argue that integration of in-vitro and in-vivo studies allows a differentiated view of this mechanism as it relates to biofilm infection.

Keywords

Biofilms; *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Pseudomonas aeruginosa*; quorum-sensing

Introduction

Recognizing that laboratory conditions poorly represent microbial life in nature, William J. Costerton coined the term “biofilm” in 1978 to describe surface-attached microbial agglomerations (Costerton, et al., 1978). Since that early realization, biofilm research has grown into a recognized field of study within microbiology. Notably, biofilms play an immensely important role for human health, as they shelter bacteria from antibiotics and host defense during infection (Costerton, et al., 1999). The number of bacterial infections that involve biofilms varies depending on the reporting agency, with estimation being around 65% of all infections according to the Center for Disease Control (CDC), and 80% according to the National Institutes of Health (NIH). Sources of infection comprise commensal microbes that live on human body surfaces, such as staphylococci, or originally environmental microbes, such as *Pseudomonas aeruginosa*.

In the last three decades, multiple ways to investigate biofilms using in-vitro models have been developed (McBain, 2009). Ranging from simple attachment tests in microtiter plates

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to sophisticated "biofilm reactors", and from qualitative microscopic examination to elaborated mathematical evaluation of images taken by confocal laser-scanning microscopy (CLSM), these in-vitro models allowed detailed insight into the processes leading to the formation of biofilms. Especially the combination with molecular biology techniques produced important information relating to the genetic requirements of biofilm development. Procedures such as transposon mutagenesis and genome-wide screening led to the identification of genes involved for example in the production of biofilm matrix components and the regulatory principles governing biofilm development (Friedman and Kolter, 2004; Heilmann, et al., 1996; Simm, et al., 2004). Cloning of biofilm determinants and the production of isogenic deletion mutants then enabled further deciphering of the precise roles the identified genes have in biofilm formation.

However, biofilms are very complex communities that interact with the human body in a multitude of ways that are hard to mimic using in-vitro setups. Unfortunately, notwithstanding the great advances in our understanding of biofilm development that has arisen from in-vitro biofilm studies, the in-vivo investigation of the molecular processes occurring during biofilm-associated human disease has trailed behind.

Among the bacteria that are involved with biofilm-associated infections, the Gram-negative bacterium *P. aeruginosa* has received most attention. This organism is particularly notorious for causing severe chronic infection in cystic fibrosis (CF) patients (Hoiby, et al., 2010). However, adequate animal models of biofilm-associated infection in CF have not been available until recently. While the physiology and especially gene regulatory processes of *P. aeruginosa* are among the best studied among opportunistic bacterial pathogens, in-vivo relevance of those processes for the most part still remains to be established.

The Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequent causes of nosocomial infections on indwelling medical devices (Otto, 2008). As device-related infections are extremely frequent and commonly biofilm-associated, these bacteria can be regarded as the most important etiological agents of biofilm-associated infections. Staphylococci are difficult to manipulate on a genetic level, although recently there have been considerable advances in that field. On the other hand, device-related infections are more easily modeled in animals than CF.

Biofilm research is also being performed in many other microorganisms, such as for example *Escherichia* and *Vibrio ssp.* (Beloin, et al., 2008; Yildiz and Visick, 2009). However, given the wealth of information available in particular for *P. aeruginosa* and staphylococci, here we will focus on these organisms. First, we will outline the mechanisms of biofilm development, focusing on general principles rather than species-specific peculiarities. Furthermore, we will provide a critical assessment of whether in-vitro findings have bearing for the in-vivo situation, or in-vivo significance of biofilm mechanisms was established using animal infection models. Finally, we will evaluate possible avenues for the development of anti-biofilm drugs.

Biofilm infections

As they proceed, most bacterial infections involve biofilms, but there are some examples of biofilm-associated infections in which the contribution of biofilms is particularly characteristic and important. These include infections on indwelling medical devices, such as intravascular catheters, prosthetic vascular grafts, cardiac devices, prosthetic joints, and shunts. Coagulase-negative staphylococci, mainly *S. epidermidis*, are the most frequent causes of these types of infections (Rogers, et al., 2009). In addition to the strong biofilm-forming capacity of many *S. epidermidis* strains, the sheer abundance of *S. epidermidis* on

human skin may explain its frequent involvement in device-related biofilm infection (Otto, 2009).

Biofilm infections may also develop independent of indwelling medical devices for example in native valve endocarditis, open wounds, or dental plaque. Although not as frequent as many other biofilm-associated infections, biofilms in chronic CF patients receive much attention owing to the high morbidity associated with the disease. CF has for the longest time been in the center of "medical biofilm" research; and for that reason, much of what we know about biofilms has been first investigated in the main CF pathogen, *P. aeruginosa*. Of note, lungs of CF patients are not infected solely by *P. aeruginosa*, but in chronic CF infections this bacterium tends to outcompete other bacteria that infect CF patients in earlier stages of the disease, such as *Burkholderia cepacia* and *S. aureus* (Rajan and Saiman, 2002). This phenomenon appears to be specific for CF infections, as *P. aeruginosa* does not outcompete other biofilm bacteria for example in chronic wounds (Kirketerp-Møller, et al., 2008).

In-vivo biofilm models

Indwelling device-related infection has been modeled using a series of approaches and animals. The easiest and most frequently used model includes the placement of a piece of catheter or other plastic tubing under the skin of a mouse at the dorsum (Kadurugamuwa, et al., 2003; Rupp, et al., 1999). Often, biofilms are established on the tubing before insertion, which has the advantage of reproducibility over the alternative method of injecting bacteria into the lumen of the already inserted tubing. This model is supposed to mimic biofilms that originate from contaminated catheters quite closely; however it suffers – like many other animal models – from the fact that the used inocula greatly exceed the number of bacteria from which such an infection is supposed to start in a real-life scenario. In models of device-related endocarditis, inocula are smaller and biofilm infection may actually progress, but these models are surgically more challenging and require larger animals such as rats or rabbits (Hirano and Bayer, 1991; Xiong, et al., 2005). Tissue cage models, established in guinea pigs but also often performed in mice, use little, hollow ball-like cages, in which catheter tubing is placed and assayed for biofilm development (Zimmerli, et al., 1982).

Biofilm-associated wound infection is difficult to mimic, because the skin of commonly used test animal species is inherently different from human skin. Pig skin infection appears to come as close as possible to human skin infection (Roche, et al., 2012), but pigs are not available as test animals for most researchers. Similarly, dental plaque formation is extremely difficult to simulate.

Because *P. aeruginosa* has been the key subject of biofilm study, biofilm researchers have tried for a long time to establish an animal model of CF biofilm-associated lung infection. Earlier models have been criticized as not being representative of chronic infection for two main reasons (Hoffmann, 2007): First, the commonly used *P. aeruginosa* strain, PAO1, is a non-mucoid isolate causing acute types of infection that are not representative of the clinical situation in chronic infection. Second, the bacteria were embedded in an artificial biofilm made for example of agar to prevent mechanical clearing. However, recently a model was established that uses a clinical isolate without the need for artificial embedding (Hoffmann, et al., 2005).

Establishing a biofilm infection: attachment

Biofilm formation is commonly considered to occur in three main stages: (i) attachment to a surface, (ii) proliferation and formation of the characteristic, mature biofilm structure, and

finally (iii) detachment, which is also often called dispersal (O'Toole, et al., 2000). Recent research provided molecular insight in all three stages (Fig. 1).

Many studies investigated the attachment of biofilm-forming bacteria to abiotic surfaces. In general, the bacterial characteristics that determine the degree of attachment to such surfaces are of physico-chemical nature, most notably hydrophobicity, which is determined by the overall composition of the bacterial surface. Nevertheless, there are reports on specific determinants of attachment. However, it needs to be stressed that the techniques used to investigate attachment determinants are often simple and similar to those employed for the investigation of biofilm formation overall. Consequently, there is a significant overlap between factors reported to be involved with the attachment and proliferative stages of biofilm development. For example, in *S. aureus* and *S. epidermidis*, teichoic acids and the surface protein autolysin were linked to abiotic surface attachment (Gross, et al., 2001; Heilmann, et al., 1997), but it can be assumed that their impact is indirect, via alteration of surface hydrophobicity. The *S. epidermidis* autolysin AtlE has a demonstrated role in device-related infection (Rupp, et al., 2001), but it is difficult to judge whether the detected effect was due to attachment or the primary role of AtlE in cell growth and division. In *P. aeruginosa*, flagella (O'Toole and Kolter, 1998), pili (Deziel, et al., 2001), fimbria (Vallet, et al., 2001), extracellular DNA (eDNA) (Whitchurch, et al., 2002), and the Psl exopolysaccharide (Ma, et al., 2009) were attributed functions in surface attachment. Several of these factors work by facilitating transport of the *P. aeruginosa* to surfaces where it can form a biofilm, reflecting that *P. aeruginosa* is motile in contrast to staphylococci. However, it is certainly debatable whether the requirement for motility to reach a surface represents an attachment phenomenon in the stricter sense. Psl and eDNA also were reported to impact biofilm formation in later stages; a specific role in attachment of those factors is thus questionable for the reasons outlined above.

Biofilm attachment to abiotic surfaces may play an important role when biofilms persist in the hospital setting on medical instruments, doorknobs, etc. as fomites of infection. However, attachment to an abiotic surface is very likely only of minor importance for biofilm formation in vivo. Attachment to tissue or medical devices in the human body is mainly governed by the interaction of bacteria with human matrix proteins, which effectively cover devices soon after insertion. In staphylococci, the major underlying molecules are a class of surface-attached bacterial proteins coined MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Clarke and Foster, 2006; Foster and Hook, 1998). Many MSCRAMMS have a demonstrated role for staphylococcal infection or colonization in vivo, such as fibronectin- (McElroy, et al., 2002) and fibrinogen-binding proteins (Josefsson, et al., 2001), or the *S. aureus* surface protein SasX, which was linked to an ongoing outbreak of methicillin-resistant *S. aureus* (Li, et al., 2012). Furthermore, MSCRAMMs were shown in multiple in-vitro assays to promote adhesion to human matrix proteins such as fibrinogen (Pei, et al., 1999), fibronectin (Maxe, et al., 1986), and others. In the case of *P. aeruginosa*, indications for a role in attachment of specific determinants during infection are exclusively derived from in-vitro experiments, often using only abiotic surfaces. Therefore, confirmation that specific *P. aeruginosa* attachment factors play a role during infection has to await detailed in-vivo investigation.

Formation of a biofilm: matrix formation

After attachment to tissue or matrix-covered devices is accomplished, infectious bacterial biofilms grow by proliferation and production of an extracellular matrix. The function of the matrix is to provide adhesion between bacterial cells, thereby enabling the formation of a multilayered biofilm. In-vitro evidence indicates that the biofilm matrix consists of a multitude of components of different chemical nature, including exopolysaccharides,

proteins, eDNA, and other polymers. These components may also facilitate the formation of bacterial agglomerations that do not necessarily constitute a biofilm, and provide protection from antibiotics and host defenses independently of biofilm formation (Mai, et al., 1993; Vuong, et al., 2004).

S. aureus and *S. epidermidis* produce an exopolysacharide named polysaccharide intercellular adhesin (PIA), also called poly-N-acetyl glucosamine (PNAG) (Cramton, et al., 1999; Mack, et al., 1996; Maira-Litran, et al., 2002) (Fig. 2). PIA/PNAG represents probably the most important component of the extracellular matrix in staphylococci, although there is evidence that in-vitro and in-vivo staphylococcal biofilms can form without PIA/PNAG (Rohde, et al., 2007). In these cases, other matrix components substitute for the missing exopolysaccharide. An important feature of the PIA/PNAG molecule is its partial de-acetylation, which produces positively charged residues that likely have an important role in interacting with other, negatively charged matrix components, resulting in a tightly connected matrix network (Vuong, et al., 2004). The importance of PIA/PNAG in biofilm-associated infection was demonstrated in several animal infection models (Begun, et al., 2007; Kropec, et al., 2005; Rupp, et al., 1999; Rupp, et al., 1999; Vuong, et al., 2004). Importantly, PIA/PNAG is also found in other biofilm-forming bacteria, including many staphylococci and even Gram-negative bacteria (Kaplan, et al., 2004; Wang, et al., 2004).

Teichoic acids are characteristic major components of the cell surface in Gram-positive bacteria (Glaser, 1973). Teichoic acids are negatively charged and have been shown to contribute to biofilm formation in staphylococci. Most likely, they interact with other surface polymers and function as a scaffold for protein attachment (Gross, et al., 2001; Sadovskaya, et al., 2005).

P. aeruginosa produces three exopolysaccharides, the glucose-rich Pel polysaccharide (Friedman and Kolter, 2004), the mannose-rich Psl polysaccharide (Friedman and Kolter, 2004), and alginate (Evans and Linker, 1973; Govan and Deretic, 1996; Hoiby, 1974). Alginate is an acylated polysaccharide composed of guluronic and mannuronic acid monomers (Fig. 2). There is a wealth of studies providing detailed information on the genetic regulation of the production of these polysaccharides, indicating, for example, that production is regulated in a way opposite to factors that are involved in acute infection (Goodman, et al., 2009). Importantly, alginate is overproduced during the establishment of a chronic CF infection, resulting in what is called a “mucoid” phenotype (Evans and Linker, 1973). The role of these biofilm polysaccharides in CF has not yet been addressed using defined genetic mutants and animal infection models, despite early discovery of the genes involved in alginate biosynthesis (Deretic, et al., 1987; Goldberg and Ohman, 1984). However, it has been shown that alginate biosynthesis contributes to virulence in acute forms of *P. aeruginosa* infection (Goldberg, et al., 1995).

Proteins may have an accessory, or for example in the absence of staphylococcal PIA, a primary role in the formation of the biofilm matrix (Rohde, et al., 2007). In *S. epidermidis*, a protein called accumulation-associated protein (Aap) contributes to establishment of intercellular connections by forming fibrils on the cell surface (Banner, et al., 2007; Hussain, et al., 1997; Rohde, et al., 2005). In *S. aureus* and *S. epidermidis*, additional surface proteins, such as protein A, *S. aureus* surface proteins (Sas) C and G, extracellular matrix binding protein (Embp), biofilm-associated protein (Bap) and the fibronectin-binding proteins FnbpA and B, were implicated in matrix formation (Christner, et al., 2010; Corrigan, et al., 2007; Cucarella, et al., 2001; Merino, et al., 2009; O’Neill, et al., 2008; Schroeder, et al., 2009). Recent data indicate that in-vitro biofilm formation of *S. aureus* as a species, in particular among methicillin-resistant strains, may rely more on eDNA and proteins, whereas PIA/PNAG may play a more important role in *S. epidermidis* and methicillin-

susceptible *S. aureus* (Izano, et al., 2008; O'Neill, et al., 2007; Pozzi, et al., 2012). An in-vivo role of several staphylococcal proteinaceous biofilm factors was established in animal infection models (Cucarella, et al., 2001; Shinji, et al., 2011). However, in many cases it is unclear whether the observed effects are due to a contribution to biofilm development, tissue attachment, or biofilm-independent immune evasion mechanisms. In *P. aeruginosa*, recent findings indicate that a protein called CdrA is involved in exopolysaccharide cross-linking (Borlee, et al., 2010); but there is no in-vivo evidence yet for a role of proteinaceous matrix components in *P. aeruginosa* CF infection.

In recent years, it was found that eDNA, which is released from dying cells, is a component of the extracellular biofilm matrix (Whitchurch, et al., 2002). DNA is a polyanionic molecule believed to interact with other matrix polymers of opposite charge, thereby contributing to the matrix network in a way similar to other polymers with distinct charge properties. In many biofilm-forming organisms eDNA has an analogous effect (Rice, et al., 2007; Thomas, et al., 2008; Whitchurch, et al., 2002). Whether eDNA has a role in biofilm-associated infection is difficult to assess. Arguing against an in-vivo role of bacterial eDNA is the presence of the potent DNase I in human serum, which at least in vitro has been shown to degrade bacterial biofilms that contain eDNA as a key matrix constituent (Kaplan, et al., 2012; Whitchurch, et al., 2002).

Formation of a structured biofilm and dispersal mechanism: quorum sensing and surfactants

When grown in vitro, mature biofilms, have a characteristic "mushroom"-like structure, which contains channels that are believed to be essential for providing nutrients to cells in deeper biofilm layers (O'Toole, et al., 2000). This indicates that in addition to the well-studied adhesive matrix components that mediate aggregation, biofilm maturation requires cell-cell-disruptive factors. In the outmost layers of a biofilm or upon strong expression, such disruptive factors also cause cell detachment or dispersal.

Quorum-sensing (QS), a phenomenon where increased cell density triggers changes in gene expression, has received much attention as a regulator of biofilm formation and maturation. In staphylococci, QS is established by the accessory gene regulator (Agr) system, which produces a secreted, post-translationally modified peptide that interacts with a two-component system in an auto feedback loop, ultimately resulting in a considerable shift in gene expression patterns during early stationary growth phase (Ji, et al., 1995; Recsei, et al., 1986) (Fig. 3). In general, Agr up-regulates toxins and other acute virulence factors and down-regulates surface proteins such as MSCRAMMs. *P. aeruginosa* QS is more complicated and contains three systems interconnected in hierarchical order, which together govern the expression of hundreds of genes (Schuster, et al., 2003) (Fig. 4). The Las system senses 3-oxo-C12-homoserine lactone; the Rhl system senses C4-homoserine lactone; and the Pqs system senses a specific quinolone referred to as *Pseudomonas* quinolone signal (PQS; (Juhas, et al., 2005).

Recent reports provided important insight into the QS-controlled factors that structure biofilms and cause detachment. In both *P. aeruginosa* and staphylococci, these are surfactants believed to function via the disruption of non-covalent interactions between biofilm cells and matrix molecules. The surfactant molecules responsible for biofilm maturation in staphylococci are phenol-soluble modulins (PSMs), amphipathic, alpha-helical peptides that are controlled by the Agr QS system in an exceptionally direct manner (Periasamy, et al., 2012; Queck, et al., 2008; Wang, et al., 2011). In *P. aeruginosa*, the QS-controlled surfactants are amphipathic glycolipids, the rhamnolipids (Boles, et al., 2005; Davey, et al., 2003). Rhamnolipid synthesis is induced in the center of a mushroom cap,

which is consistent with it being subject to cell density control (Lequette and Greenberg, 2005). As *P. aeruginosa* is a motile bacterium, dispersal may commence with the up-regulation of motility; and recent evidence suggests that indeed both rhamnolipid and type IV pili are involved in *P. aeruginosa* biofilm dispersal (Pamp and Tolker-Nielsen, 2007). Notably, while the general principle of biofilm maturation and dispersal appears thus to be conserved among phylogenetically distinct bacteria, the chemical nature of the surfactants is different, indicating convergent evolution. Interestingly, both PSMs and rhamnolipid have additional functions in the killing of neutrophils, a key mechanism of immune evasion especially in the case of *S. aureus* (Jensen, et al., 2007; Wang, et al., 2007).

During infection, detachment is of utmost importance as it may lead to the dissemination of a biofilm-associated infection. The role of Agr and PSM surfactants for dissemination has recently been demonstrated in animal models of *S. aureus* and *S. epidermidis* catheter-related infection, underscoring the importance of surfactant-mediated QS control of biofilm-associated infection (Periasamy, et al., 2012; Wang, et al., 2011).

Especially in staphylococci, biofilm maturation was also proposed to occur by enzymatic degradation of biofilm matrix components, most notably by proteases and nucleases (Boles and Horswill, 2008; Kiedrowski, et al., 2011). However, only some of these enzymes are under QS control. Importantly, QS does not regulate production of PIA/PNAG (Vuong, et al., 2003) and PIA/PNAG-degrading enzymes that were found in other bacteria (Kaplan, et al., 2004), are apparently absent from staphylococci. Furthermore, there is no evidence for in-vivo relevance of enzyme-based detachment. In fact, in a recent report, nuclease was found not to contribute to in-vivo biofilm dispersal in *S. aureus* (Beenken, et al., 2012).

In *P. aeruginosa*, QS appears to regulate Pel exopolysaccharide synthesis, although there are conflicting reports as to how production is affected (Sakuragi and Kolter, 2007; Ueda and Wood, 2009). Finally, it should be mentioned that D-amino acids were shown to trigger biofilm dispersal in the non-pathogenic *Bacillus subtilis* (Kolodkin-Gal, et al., 2010) and recently in *S. aureus* (Hochbaum, et al., 2011). However, the underlying regulated determinants are not known.

Biofilm formation as a lifestyle change

The first report about the role of QS in bacterial biofilm formation described the *P. aeruginosa* Las system as important for the formation of structured, extended biofilms, as a *lasI* mutant formed an undifferentiated, flat biofilm compared to the wild-type strain (Davies, et al., 1998). Subsequent research in staphylococci underscored the function of QS in biofilm structuring; but *agr* mutants formed a thicker rather than flatter biofilm compared to the wild-type strains (Vuong, et al., 2003; Vuong, et al., 2000). Also in *P. aeruginosa*, further investigation of the relationship between QS and biofilm development produced results that could not be aligned with the initial, simple model of direct, positive control of biofilm formation by QS (Kirisits and Parsek, 2006).

Similarly, the role of QS in biofilm-associated infection has remained a complicated issue. In *P. aeruginosa*, evidence for a significant role of QS in biofilm-associated cystic fibrosis infection is based on the detection of QS signals in the sputum of cystic fibrosis patients (Singh, et al., 2000). In *S. epidermidis*, a contrasting role of QS for catheter attachment and infiltration of surrounding tissue was shown (Vuong, et al., 2004). Furthermore, while QS was reported to have a positive role in many infections, clinical isolates in both staphylococci and *P. aeruginosa* that were obtained from chronic, biofilm-associated infections were often demonstrated to be QS mutants, directly arguing against a positive role of QS on biofilm development (Bjarnsholt, et al., 2010; Traber, et al., 2008; Vuong, et al., 2004).

A model that unifies these conflicting results and offers a possible way to rationalize them is based on distinguishing two types of virulence, acute and chronic, and recognizing that QS is important for the expression of acute virulence, and the formation of a differentiated biofilm with the capacity for dissemination, while chronic, biofilm-associated infection develops with a down-regulation and/or mutation of the QS system(s) (Fig. 5). Reflecting the contrasting bacterial approaches to infection in cases of high or low QS activity, QS has been described as a lifestyle determinant of biofilm-forming pathogenic bacteria. Accordingly, determinants of acute and chronic virulence are regulated by QS in an opposite fashion. In staphylococci, toxins and degradative exoenzymes as characteristic mediators of acute virulence are up-regulated by QS, whereas non-aggressive colonization and biofilm factors such as MSCRAMMS are down-regulated. Similarly, in *P. aeruginosa*, QS up-regulates proteases while it down-regulates the biofilm exopolysaccharide alginate. Recent findings provide further support of this model. In *S. aureus*, QS mutants are found in elevated numbers in chronic infection, but these mutants have lost the ability to infect other individuals, for which active QS is crucial (Shopsin, et al., 2010). In *P. aeruginosa*, it has been shown that QS mutants occur in increasing numbers in late stages of CF infection, but rhamnolipid production for example is maintained in earlier stages (Bjarnsholt, et al., 2010). Finally, cyclic diguanylate (c-di-GMP), a recently identified regulatory molecule governing biofilm formation among many other mechanisms, has been attributed a key role as regulator of “lifestyle” changes in many biofilm-forming bacteria (Gomelsky and Hoff, 2011; Hickman, et al., 2005). However, there is yet no in-vivo evidence for the role of c-di-GMP during biofilm infection. Despite new insights, an important question remains unanswered: does the entire population in chronic biofilms consist of QS mutants or are some non-mutant cells “reserved” to potentially regain the ability for dissemination under changing environmental conditions? Commonly, clinical microbiology laboratories only culture one representative isolate from an infection, but to answer this question, it will be crucial to analyze a much higher number of isolates.

Biofilm resistance to antimicrobial agents and mechanisms of host defense

Biofilms have strongly increased capacity to resist antibiotic treatment and attacks by human host defenses (Costerton, et al., 1999). However, the mechanisms underlying this phenomenon are poorly understood. Resistance to antibiotics was explained by a series of different mechanisms (Mah and O’Toole, 2001). First, the biofilm matrix may represent a diffusion barrier for antibiotics. This may be the case for some, but certainly not all antibiotics, and appears to be dependent on their physico-chemical characteristics. Second, biofilm cells are in a different physiological status compared to actively growing, planktonic cells, which minimizes sensitivity to antibiotics that target active cell processes. Third, expression of specific protective molecules may be higher in the biofilm mode of growth, and antibiotics may even directly promote the expression of protective mechanisms. All these mechanisms certainly contribute to biofilm resistance to antibiotics in vitro, but it is unknown which of them matter in vivo.

A hallmark of chronic infections is the incapacity of the acquired immune system to clear the infection. In the case of biofilms, this is believed to be mostly due to the shielding of recognizable epitopes by lowly immunogenic matrix components. Whether mechanisms of innate host defense may efficiently attack bacteria in established biofilms is poorly understood. The most important innate host defense mechanism is the elimination of bacteria by professional phagocytes. Activation of these immune cells depends on the recognition of pathogen-associated molecular patterns, but these may also be hidden by matrix components that do not themselves trigger phagocyte activation efficiently. Whether phagocytes are prevented from infiltrating into a biofilm is controversial (Leid, et al., 2002). However, it is certainly imaginable that the biofilm matrix provides at least some protection

from phagocyte intrusion, as was postulated early (Krieg, et al., 1988; Vaudaux, et al., 1985).

Anti-biofilm therapy

Finding a cure for biofilm infection is one of the most difficult and challenging tasks in anti-bacterial drug development. Clearly, there has not been much success yet. This is due to the fact that there are significant problems associated with all approaches that were undertaken or conceived to develop anti-biofilm therapeutics.

Biofilm formation on indwelling medical devices may theoretically be prevented by surface alteration to prevent bacterial attachment or by inclusion of anti-bacterial therapeutics in the device that prevent early stages of biofilm formation. There has been some limited success using these approaches (Rodrigues, 2011), but the fact that biofilms develop on human matrix proteins rather than directly on the device surface poses a significant problem for this strategy.

Another approach consists in targeting bacterial biofilm determinants. However, it is now clear that different bacteria use chemically different molecules to establish biofilms. Thus, there is a great problem with marketability, even if such an approach would succeed, because only specific bacteria could be targeted. Additionally, one would have to consider that other microbes might take the place of the targeted biofilm formers when they are eliminated.

Are there conserved mechanisms of biofilm formation that may represent the basis for a more broadly applicable biofilm therapeutic? The most frequently proposed such mechanism is QS. However, QS systems are only conserved in Gram-negative, less in Gram-positive bacteria. Moreover, the more recent differentiated view of the role of QS in infection argues strongly against this approach (Otto, 2004). QS inhibitors, such as the frequently discussed acyl homoserine lactone system-targeting furanones (Hentzer, et al., 2002) or inhibitors of the staphylococcal Agr system (Wright, et al., 2005), may have potential to reduce toxicity in acute infection, but they appear counterproductive for chronic biofilm-associated infection. In addition, compounds such as QS inhibitors, which target virulence expression instead of killing the bacteria, were originally believed to have a low potential of triggering the development of resistance. However, resistance to furanone-based QS inhibitors has been recently reported (Maeda, et al., 2012), suggesting the need for a thorough re-evaluation of this drug development strategy.

Targeting biofilm matrix components directly has also been proposed. For example, the PIA/PNAG exopolysaccharide is being evaluated as a vaccine target (Maira-Litran, et al., 2004). There is also an enzyme, dispersin B, that specifically degrades PIA/PNAG (Kaplan, et al., 2003). Use of a degradative enzyme may work well in vitro but it is hard to imagine that it works during infection. Finally, it has been proposed to develop antisera against antigens that are expressed strongly in biofilms (Harro, et al., 2010). However, the problem is less that humans would not be able to produce opsonic antibodies, rather that the bacteria may be inaccessible for professional phagocytes.

Altogether, unfortunately, an efficient anti-biofilm therapeutic is currently not in sight. This situation calls for intensified molecular research in the biofilm field, most importantly with a more pronounced focus on in-vivo relevance. In addition, prophylactic prevention of biofilm formation in hospital settings, such as by increased hygiene measures, should be emphasized.

Conclusions and future outlook

In the last decades, many biofilm components and mechanistic details of biofilm formation and regulation have been discovered using in-vitro research. However, it also became clear that different experimental setups often lead to strongly varying results; and overall, it is difficult to draw conclusions from in-vitro biofilm research on in-vivo biofilm-associated infection. Even important new concepts in the biofilm field, such as the contribution of eDNA to biofilm formation, have not yet been confirmed to have in-vivo relevance. To that end, there should be a stronger focus on (i) the development and use of appropriate animal models of biofilm-associated infection and (ii) evaluating how clinical samples from biofilm infections can be obtained and analyzed to give more detailed information on in-vivo biofilms. For example, recent advances in genome-wide transcriptomic profiling of such samples will allow more detailed insight into the physiological processes in in-vivo biofilms. Furthermore, especially in the *Pseudomonas* field, biofilm research has frequently used strains that are not characteristic for biofilm, but rather acute infection. The use of such strains should be discontinued in biofilm research except for the analysis of general molecular mechanisms. Altogether, biofilm researchers have to re-evaluate thoroughly which in-vitro and in-vivo experimental approaches give a valid representation of biofilm-associated infection, especially given the urgent need to develop anti-biofilm therapeutics.

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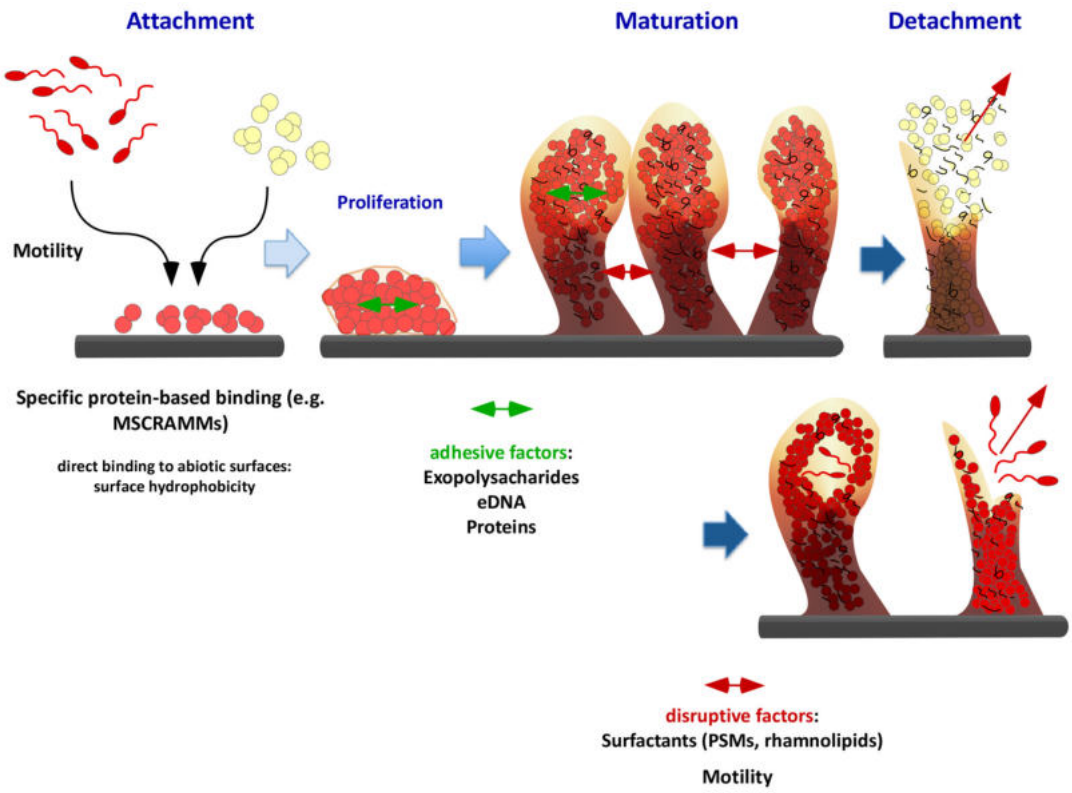

Fig. 1. Phases of in-vivo biofilm development

Biofilms develop via initial attachment, which depends on transport of the bacteria to a surface, which is passive in the case of non-motile bacteria such as staphylococci (yellow), and active in the case of motile bacteria such as *P. aeruginosa* (red). Attachment itself is governed by specific protein-protein interactions of bacterial surface with human matrix proteins. Attachment to an abiotic surface such as a catheter depends on bacterial surface hydrophobicity, but this mechanism is believed to have minor importance in vivo. Subsequent steps do not differ in principle between motile and non-motile bacteria. They involve proliferation, embedding in an extracellular matrix, and maturation. The latter depends on cell-cell disruptive factors, recently identified to be primarily surfactants. Strong production of surfactants, which are controlled by QS, leads to biofilm detachment (dispersal). In the case of motile bacteria, up-regulation of motility, starting in the center of biofilm “mushroom caps” assists dispersal.

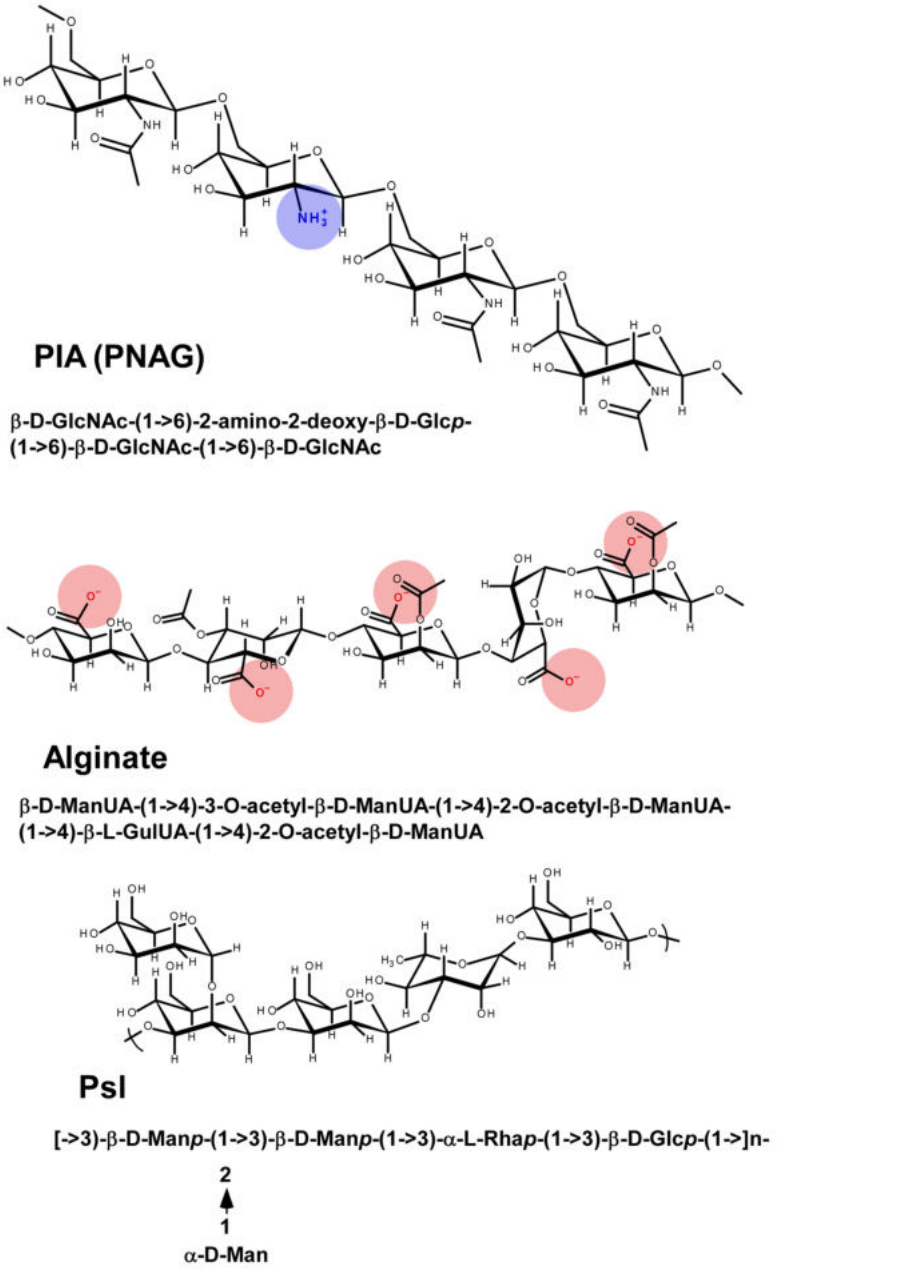

Fig. 2. Biofilm exopolysaccharides in *P. aeruginosa* and staphylococci

The major biofilm exopolysaccharide of staphylococci (and some other bacteria) is PIA (or PNAG), a homopolymer of beta-1,6-linked N-acetyl-glucosamine residues, of which about a quarter become de-acetylated after export. De-acetylation creates free amino groups, which at neutral or acid pH give the molecule a cationic character (shown in blue). Major exopolysaccharides of *P. aeruginosa* are the mannuronic acid/guluronic acid-based, negatively charged alginate (negative charges, red) and the mannose-rich neutral Psl. GlcNAc, N-acetyl-glucosamine; ManUA, mannuronic acid; GulUA, guluronic acid; Man*p*, mannopyranose; Rha*p*, rhamnopyranose; Glc*p*, glucopryranose.

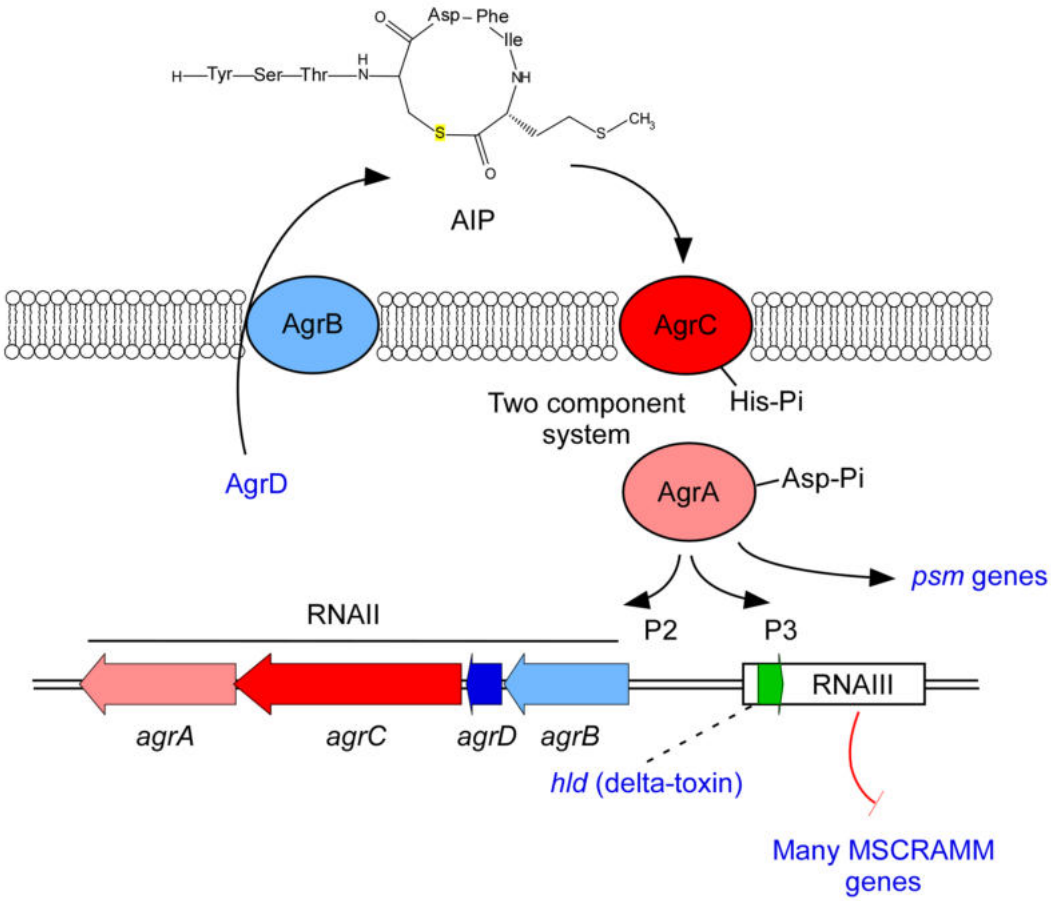

Fig. 3. Quorum-sensing in staphylococci

QS in staphylococci is exerted by the *agr* locus, which contains the *agrA*, *agrC*, *agrD*, and *agrB* genes (RNAII transcript) and RNAIII, the intracellular effector of the system, which also contains the *hld* gene for the PSM δ-toxin. AgrD is a pre-pheromone, which is exported and modified by AgrB, resulting in a characteristic thiolactone-containing autoinducing peptide (AIP). Activation of the AgrC/AgrA two-component system by AIP binding leads to transcription of RNAIII and RNAII, the latter leading to auto feedback and fast up-regulation of *agr* and *agr* target expression at a certain threshold of cell density. Agr-regulated biofilm-relevant genes are first and foremost PSMs, which are regulated by direct binding of AgrA to their promoters, rather than via RNAIII. In contrast, many MSCRAMMs are negatively regulated by RNAIII, reflecting that tissue attachment is a mechanism no longer needed during later stages of infection.

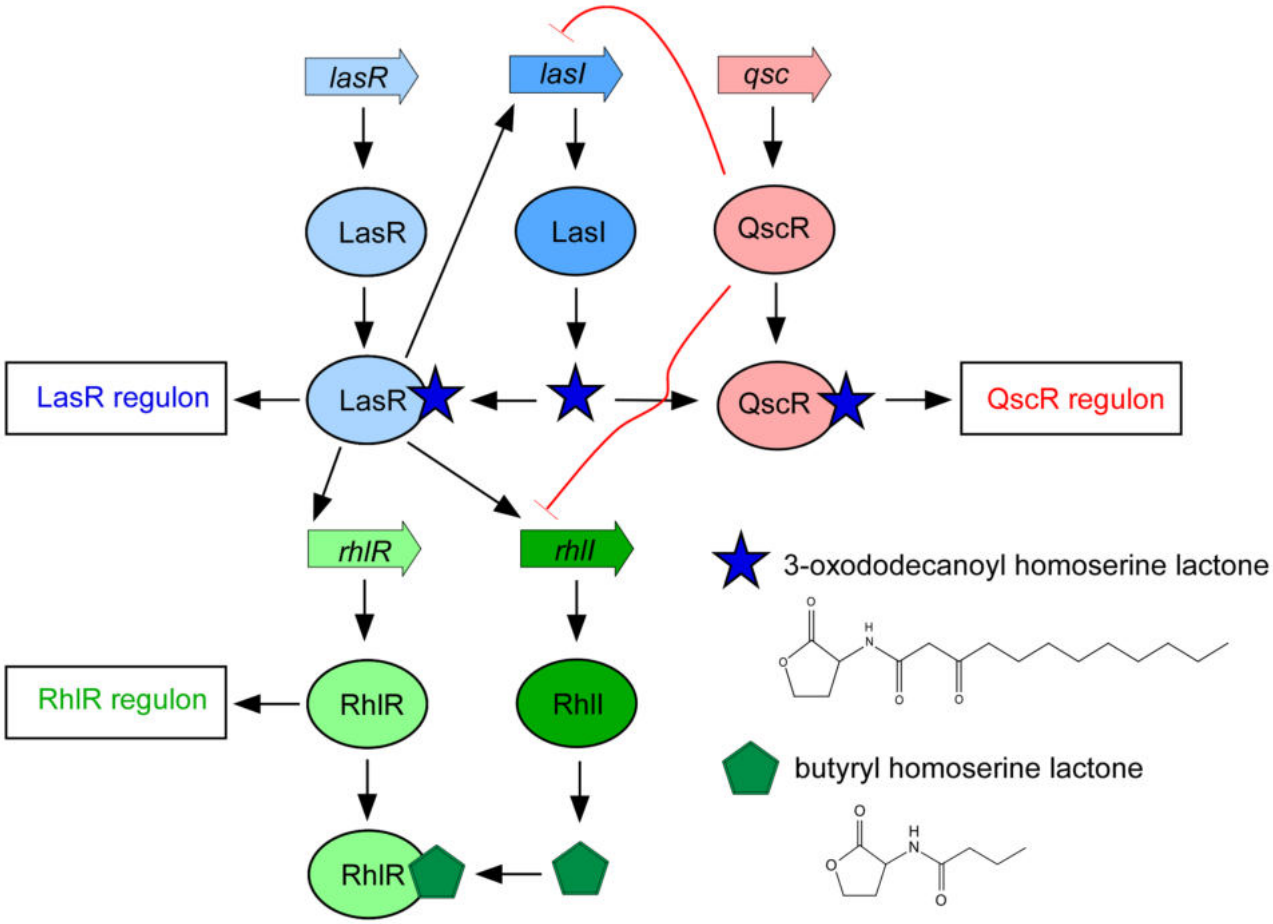

Fig. 4. Quorum-sensing in *P. aeruginosa*

P. aeruginosa uses at least 3 QS systems, which are arranged in hierarchical order. The *rhl* system is under control of the *las* system; both use an acyl homoserine lactone (AHL) signal that is produced by the LasI or RhlI AHL synthetases, respectively. Target genes are under control of the DNA-binding regulators LasR, RhlR, and QscR, defining the respective QS regulons. QS-characteristic auto feedback loops are established by the fact that the AHL synthetase genes are under control of the corresponding regulator proteins. The *qsc* system responds to, but does not produce AHLs. The QscR DNA-binding protein, in addition to controlling production of the *qsc*'s systems target genes, inhibits expression of the AHL-producing LasI and RhlI enzymes.

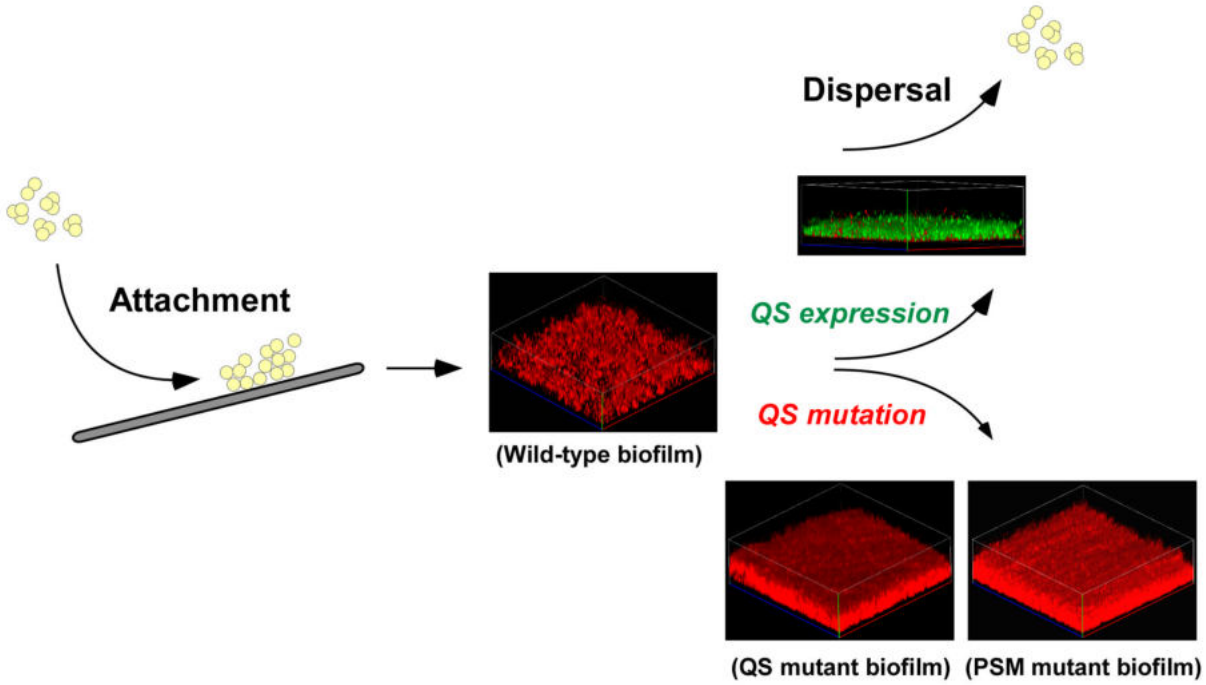

Fig. 5. Role of quorum-sensing in biofilm-associated infection

QS systems (such as the staphylococcal Agr shown here) contribute to maturation and dispersal of biofilms. Accordingly, biofilms of an Agr QS wild-type strain, as shown by CLSM in the middle, contain channels between cellular agglomerations. Active expression of the QS system (as shown on the top right in green, using an *agr* promoter *gfp* fusion construct) leads to dispersal. During prolonged chronic infection, the QS system in biofilms cells may be irreversibly inactivated by mutation, leading to excessive growth of compact biofilms, which likely have lost the capacity to disperse and disseminate. The phenotype of a surfactant mutant, in which all *psm* genes controlled by Agr have been inactivated (bottom right), has the same phenotype as the *agr* QS mutant (bottom left), underlining the importance of surfactants in QS-mediated control of biofilm maturation and detachment.