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Staphylococcus aureus Biofilm: A Complex Developmental Organism

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Summary

Chronic biofilm-associated infections caused by *Staphylococcus aureus* often lead to significant increases in morbidity and mortality, particularly when associated with indwelling medical devices. This has triggered a great deal of research attempting to understand the molecular mechanisms that control *S. aureus* biofilm formation and the basis for the recalcitrance of these multicellular structures to antibiotic therapy. The purpose of this review is to summarize our current understanding of *S. aureus* biofilm development, focusing on the description of a newly-defined, five-stage model of biofilm development and the mechanisms required for each stage. Importantly, this model includes an alternate view of the processes involved in microcolony formation in *S. aureus* and suggests that these structures originate as a result of stochastically regulated metabolic heterogeneity and proliferation within a maturing biofilm population, rather than a subtractive process involving the release of cell clusters from a thick, unstructured biofilm. Importantly, it is proposed that this new model of biofilm development involves the genetically programmed generation of metabolically distinct subpopulations of cells, resulting in an overall population that is better able to adapt to rapidly changing environmental conditions.

Graphical Abstract

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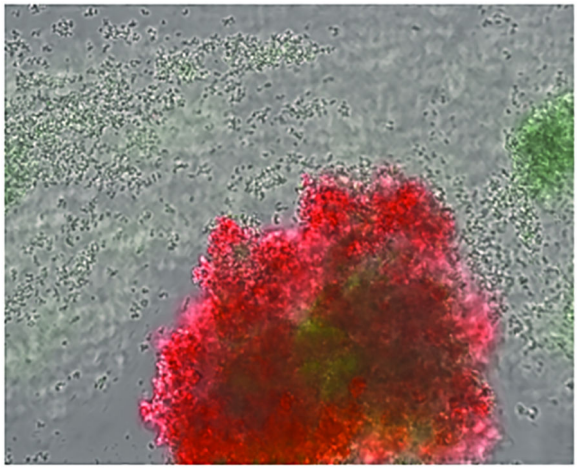

Novel technological advances that combine microfluidic flow-cell systems with time-lapse microscopy have greatly enhanced the visualization of biofilm development. Using this technology, our laboratory has revealed a more detailed view of the morphological stages and differential gene expression that occurs during *Staphylococcus aureus* biofilm development. Here, we review the complex molecular mechanisms that are required for each developmental stage, and describe a new model for the formation of structure during biofilm maturation.

Introduction

In contrast to microbiology laboratory conditions where bacteria are often grown planktonically in nutrient-rich conditions, bacteria found in the environment almost exclusively grow in nutrient-deficient conditions where they form multicellular aggregations called biofilms (Costerton et al., 1987, Hall-Stoodley et al., 2004). In order to form biofilms, bacteria generate a self-produced extracellular matrix (ECM) composed of proteins, carbohydrates, and/or extracellular DNA (eDNA) (Flemming & Wingender, 2010), which encases the cells within a sticky matrix that facilitates survival in hostile or extreme environments. In recent years, bacterial biofilms produced by human pathogens have become particularly important to study due to their increased recalcitrance to not only the host immune system (Otto, 2006), but also to antibiotics (Costerton et al., 1999, Donlan & Costerton, 2002).

The biofilm-producing pathogen, *Staphylococcus aureus*, has become notorious for causing chronic infections due to its ability to resist therapeutic treatment by forming biofilms on indwelling medical devices, including implanted artificial heart valves, catheters and joint prosthetics (McConoughey et al., 2014, Ribeiro et al., 2012). Indeed, biofilm-related infections are associated with increased morbidity and mortality, with infected medical devices often requiring surgical removal and increased durations of hospitalization. As a result, the prevalence of these and other staphylococcal diseases has led to a significant increase in expenses associated with *S. aureus* infections over the past decade, with estimated annual costs near $450 million (Parvizi et al., 2010, Song et al., 2010). Consequently, a better understanding of the development of staphylococcal biofilms at the molecular level is imperative to generate new treatment strategies for biofilm-associated

infections and to reduce the significant burdens caused by this pathogen. Here, we discuss recent advances in our understanding of the different stages of *S. aureus* biofilm development that have resulted from the use of state-of-the-art, time-lapse microscopic technology, and where possible, describe the molecular components that modulate each stage of this complex process.

Redefining the Stages of *S. aureus* Biofilm Development

While the molecular constituents involved in bacterial biofilm development vary amongst bacterial species, a basic model that is widely recognized consists of three sequential stages: 1) attachment, 2) accumulation/maturation, and 3) detachment/dispersal (Kostakioti et al., 2013, O'Toole et al., 2000, Hall-Stoodley et al., 2004). During the attachment stage, planktonic cells adhere to biotic or abiotic surfaces and proliferate into sticky aggregations called microcolonies (also known as towers or mushroom-like structures). As these microcolonies develop, bacterial cells produce an ECM that serves as a scaffold essential for establishing this three-dimensional architecture. Upon reaching a specific cell density, a mechanism is triggered to initiate ECM degradation that releases cells embedded within the biofilm to disperse and reinitiate biofilm development at distal sites.

Like other bacterial species, *S. aureus* has been proposed to possess similar stages of biofilm development (Otto, 2013, Le et al., 2014). In fact, several biofilm studies suggest that *S. aureus* biofilms mature into thick layers of cells at which point detachment mechanisms are triggered and subpopulations of the biofilm are dispersed carving out microcolonies in the biomass that remains (Yarwood et al., 2004, Periasamy et al., 2012, Boles & Horswill, 2008). While these studies have provided immense insight into the molecular components that determine the biofilm architecture, new biofilm assays combining microfluidic flow-cell systems and time-lapse microscopy have revealed a more detailed view of the stages of *S. aureus* biofilm development. Indeed, the recent use of the BioFlux1000 system, a microfluidic flow-cell device integrated with a fluorescence microscope, has allowed *S. aureus* biofilm development to be evaluated in a nearly real-time manner (Benoit et al., 2010, Moormeier et al., 2013, Moormeier et al., 2014, Lehman et al., 2015, Vanhommerig et al., 2014, McCourt et al., 2014). Using this system, *S. aureus* biofilm development has been shown to proceed through a five-stage developmental process including: 1) attachment, 2) multiplication, 3) exodus, 4) maturation, and 5) dispersal (Figure 1) (Moormeier et al., 2014).

Attachment

To initiate biofilm formation on biotic materials, planktonic *S. aureus* cells first attach to a surface (Figure 1A) utilizing a variety of cell wall-anchored (CWA) proteins specific for different host matrix substrates. Part of this well-characterized group of surface attached proteins are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), several of which share a common cell wall targeting motif (LPXTG; see Navarre & Schneewind, 1994) and Marraffini et al., 2006), but have different binding specificities for host matrix components such as fibronectin, fibrinogen, collagen, and cytokeratin (Speziale et al., 2009). Numerous MSCRAMMs such as fibronectin-binding

proteins (FnBPA and FnBPB) (O'Neill et al., 2008, McCourt et al., 2014), serine-aspartate repeat family proteins (SdrC, SdrD, and SdrE) (Corrigan et al., 2009, Josefsson et al., 1998, O'Brien et al., 2002), clumping factors (ClfA and ClfB) (McDevitt et al., 1994, Ni Eidhin et al., 1998), collagen adhesin (Zong et al., 2005), Protein A (Nguyen et al., 2000), plasmin sensitive protein (Pls) (Huesca et al., 2002), SasG (Roche et al., 2003), iron-regulated surface determinants (IsdA, IsdB, IsdC, and IsdH) (Mazmanian et al., 2003, Miajlovic et al., 2010, Dryla et al., 2003), and bone sialoprotein (Bbp) (Vazquez et al., 2011) have been implicated in binding host matrix components to initiate cell adherence and/or biofilm development. Importantly, the attachment of most of these proteins to the bacterial cell wall is reliant on the membrane-associated protein, sortase A, which catalyzes the covalent attachment of these proteins to the penta-glycine cross-linker component of the peptidoglycan (Mazmanian et al., 1999). For a more comprehensive overview on the structures and functions of the different MSCRAMMs and other CWA proteins, the reader is referred to an outstanding recent review of this subject (Foster et al., 2014).

Although *S. aureus* is well equipped to bind multiple host matrix proteins that quickly coat implanted devices during biofilm-associated infections, recent findings suggest that these proteins play a minimal role when attaching directly to abiotic materials. In the absence of matrix molecules, such as under the conditions used in our Bioflux studies, *S. aureus* may attach to abiotic surfaces through electrostatic and hydrophobic interactions in static biofilm assays where differently charged polystyrene surfaces result in drastic alterations in attachment and overall biofilm development (Kennedy & O'Gara, 2004). Furthermore, the negatively charged teichoic acids have also been implicated in attachment to polystyrene and glass surfaces (Gross et al., 2001), in addition to the major autolysin, AtlA, which has been shown to aid in cell attachment to hydrophilic and hydrophobic polystyrene surfaces (Biswas et al., 2006, Houston et al., 2011).

Recently, the cell wall-associated protein-independent nature of binding to abiotic surfaces was further supported by testing mutants from the Nebraska Transposon Mutant Library (Fey et al., 2013). In these experiments, mutations affecting expression of CWA proteins previously described to have a function in biofilm development, including several of the MSCRAMMs listed above, *agrA*, *atlA*, and the cell wall anchoring enzymes, sortase A and B, were screened for their effect on the early stages of biofilm formation. Interestingly, only the *agr* and *atlA* mutants affected biofilm formation, demonstrating increased and decreased levels of attachment and multiplication, respectively (Moormeier et al., 2014). Indeed, the Agr quorum sensing circuit in *S. aureus* has been shown to play a role in biofilm adherence by regulating the phenol soluble modulin (PSM) peptides (Periasamy et al., 2012) (further discussed below). One of the PSMs, δ-toxin, was previously shown to inhibit attachment to polystyrene by preventing hydrophobic interactions between the cell and the polymer surface (Vuong et al., 2000). While AtlA binds to fibronectin (Houston et al., 2011), the demonstration that the autolytic activity of this enzyme is required for biofilm formation (Bose et al., 2012) suggests that the specific role of AtlA in early biofilm development may involve multiple functions.

Multiplication

After attaching to a surface, and in the presence of a sufficient nutrient source, the adherent *S. aureus* cells will begin to divide and accumulate. However, prior to the production of an ECM in which to embed, the newly formed daughter cells are vulnerable to detachment, especially in the presence of the shear forces associated with fluid flow. To maintain stability of this immature biofilm, *S. aureus* cells are known to produce a variety of factors that help to stabilize cell-to-cell interactions. It is this time of cell division and accumulation that we have termed the multiplication stage (Figure 1B).

Staphylococci produce several extracellular proteins that could facilitate biofilm accumulation by promoting intercellular binding shortly after initial attachment. Some of these proteins CWA proteins categorized as MSCRAMMs (see above), like the FnBPs, ClfB, and SdrC proteins, play dual roles in both attachment and accumulation (Speziale et al., 2014). Other CWA proteins such as the *Staphylococcus epidermidis* accumulation-associated protein (Aap) (Conlon et al., 2014, Schaeffer et al., 2015) and the *S. aureus* homolog, SasG (Geoghegan et al., 2010), have also been implicated in attachment and early accumulation. In addition, CWA proteins like Protein A (Merino et al., 2009), SasC (Schroeder et al., 2009), and Bap (Cucarella et al., 2001), have all shown a propensity to aid in biofilm accumulation. While these proteins appear to have a role in the multiplication stage of biofilm development, their function during this stage was not apparent in flow-cell experiments in the absence of matrix components (Moormeier et al., 2014). Similarly, polysaccharide intracellular adhesin (PIA) has been shown to function as an ECM component during early *S. aureus* biofilm formation (Cramton et al., 1999, Cramton et al., 2001, O'Gara, 2007), however, the production of this matrix molecule appears to be strain- or condition-specific (Fitzpatrick et al., 2005, Toledo-Arana et al., 2005, Brooks & Jefferson, 2014), O'Neill et al., 2007, Rohde et al., 2007, Boles et al., 2010). Indeed, *icaA* mutant (gene encoding a N-glycosyltransferase that is essential for PIA production) derivatives of UAMS-1 and USA300 JE2 strains demonstrated normal accumulation during the multiplication stage (Moormeier et al., 2014).

Although recent results indicate that cell wall-associated proteins are not involved during the multiplication stage, protease addition during this stage was shown to abrogate biofilm formation (Moormeier et al., 2014), indicating that the accumulation of cells involves a proteinaceous component. Interestingly, this is consistent with recent findings demonstrating that *S. aureus* biofilms utilize cytoplasmic proteins as matrix components (Foulston et al., 2014). In these studies, enolase and GAPDH, which are not typically recognized as biofilm-related proteins, were shown to "moonlight" as biofilm matrix components by attaching to the surface of cells in response to the decreasing pH of the biofilm environment (Foulston et al., 2014). Although the mechanisms used by cytoplasmic proteins devoid of a signal peptide can be transported to the extracellular milieu have not been described, the authors speculate that the release of these proteins is mediated by "regulated autolysis" similar to that described for the release of eDNA during biofilm development (Sadykov & Bayles, 2012, Bayles, 2014), which may establish an early ECM during the multiplication stage (Figure 2). Indeed, this may involve the binding of enolase and GAPDH to eDNA under low pH conditions as proposed by Dengler et al. (Dengler et al., 2015). Likewise, other extracellular

proteins such as PSMs (Schwartz et al., 2016), beta-toxin (Hlb) (Huseby et al., 2010), and the immunodominant surface antigen B (IsaB) (Mackey-Lawrence et al., 2009) have been shown to bind eDNA and potentially function to stabilize the ECM. In addition, results also suggest that cytoplasmic nucleoid-associated proteins (NAPs), typically used for chromosomal structuring, may serve as an ECM component protein by binding eDNA (Goodman et al., 2011). Given these data and the results that demonstrate early-stage biofilms are protease-sensitive (Moormeier et al., 2014), cytoplasmic proteins that bind to eDNA may be important during the multiplication stage of biofilm formation before the matrix components have had a chance to accumulate.

Exodus

One of the observations of biofilm development made using time-lapse microscopy was a distinct and coordinated release of the cells approximately six hours after the initiation of the multiplication stage. This, so-called, “exodus” stage of biofilm development is an early dispersal event that coincides with microcolony formation and results in the restructuring of the biofilm (Figure 1C). Importantly, exodus is mediated by nuclease-dependent degradation of eDNA and is independent of the Agr-dispersal mechanism that occurs after microcolony development (discussed below). Degradation of eDNA within the biofilm matrix by a self-produced, secreted nuclease has repeatedly been shown to reduce the total biomass of *S. aureus* biofilms (Mann et al., 2009, Kiedrowski et al., 2011, Moormeier et al., 2014, Kiedrowski et al., 2014, Beenken et al., 2012). However, it was not until recent studies using time-lapse microscopy that it was determined that the Nuc-mediated eDNA degradation occurs very early during biofilm development and mediates the exodus event (Figure 1C). This is a tightly regulated phase of biofilm development where only a subpopulation of cells within the biofilm expresses *nuc* resulting in the secretion of nuclease that mediates the detachment of the majority of the accumulated biofilm population (Moormeier et al., 2014). Additionally, the subpopulation of *nuc*-expressing cells was absent in a *sae* mutant (Moormeier et al., 2014), consistent with a previous study demonstrating the Sae-dependent control of *nuc* expression (Olson et al., 2013). Interestingly, another Sae-regulated gene, *coa* (whose product, coagulase, converts fibrinogen to fibrin), known to aid in biofilm formation when grown in the presence of host matrix proteins (Zapotoczna et al., 2015) suggests the potential for coordinated expression of biofilm effectors during biofilm development. Although several external stimuli have been shown to induce Sae-mediated signal transduction, including the presence of antimicrobial peptides (Flack et al., 2014), the signaling events at play during biofilm growth (which lack the presence of antimicrobial peptides) remains to be elucidated.

In addition to exposing a new stage in biofilm development, the studies conducted by Moormeier *et al.* (Moormeier et al., 2014) reveal a drastic shift in ECM as biofilm integrity progresses from a reliance on protein components only (attachment and multiplication stages, Figure 1A and 1B) to a dependence on both DNA and proteins (exodus stage, Figure 1C). Thus, the concept of exclusively PIA-based, protein-based, and eDNA-based biofilms should be replaced with a more dynamic model of biofilm development where the composition of the ECM changes both temporally and spatially as the biofilm develops. This model is similar to that described by Ma et al. (Ma et al., 2009) where significant changes in

the matrix components were observed during *P. aeruginosa* biofilm development. Hence, studies aimed at further dissecting the molecular events mediating both Sae and Agr signaling are warranted.

What is the biological role of the exodus stage? Although the answer to this question is not known, it may be significant that the more complex developmental bacterium, *Myxococcus xanthus*, also exhibits a "restructuring" event as part of its developmental cycle. Indeed, prior to fruiting body formation, the *M. xanthus* population is reduced approximately 80% through a process that may involve the function of a toxin-antitoxin system (Wireman & Dworkin, 1977, Nariya & Inouye, 2008). Thus, it is possible that the reduction in the cellular population during early biofilm development (either through cell death or exodus) is a prerequisite to the formation of secondary structure (see below). Indeed, in the absence of exodus, such as in a *S. aureus nuc* mutant, microcolony formation is not observed (Moormeier et al., 2014). Clearly, additional studies are required to provide a more complete understanding of the role exodus plays in biofilm development, as well as the mechanisms controlling how *S. aureus* modulates its biofilm ECM as the biofilm transitions from one stage to the next.

Maturation

A key aspect of biofilm maturation for any bacterial species is the formation of microcolony structures that provide increased surface area for nutrient exchange and waste removal, as well as to promote the dissemination of the biofilm cells to distal sites (Hall-Stoodley et al., 2004, Stewart & Franklin, 2008). Like other bacterial species, there have been numerous studies reporting on the formation of microcolony-like structures during *S. aureus* biofilm development (Mann et al., 2009, Moormeier et al., 2013, Moormeier et al., 2014, Thomas et al., 2014, Thurlow et al., 2011, Yarwood et al., 2004, Periasamy et al., 2012), however, the mechanism that promotes their formation is still being elucidated.

One model described previously (Periasamy et al., 2012) posits the formation of biofilm microcolony structures as a subtractive process, carving out channels from a thick mat of biofilm cells as a result of the PSM-mediated dispersal. However, observations of biofilm development using time-lapse microscopy clearly reveal the formation of microcolonies from distinct foci of cells that remain in the basal layer shortly after the commencement of exodus (Figure 3A). In a separate experiment, in which biofilm formation was monitored at a lower magnification, microcolony emergence from a basal layer of cells was also observed (Figure 3B). Thus, in contrast to the previous model, we envision an additive process where rapidly growing microcolonies emerge from a basal layer of slower growing cells (Moormeier et al., 2013). Interestingly, these studies also showed the emergence of different microcolony types that grow at different rates and with different gene expression patterns and physical properties. For example, using fluorescent reporters fused to the promoters of the *cidABC* and *lrgAB* cell death-associated operons, two different microcolony types were clearly delineated. The first was a rapid growing microcolony that exhibited constitutive *lrgAB* expression but delayed *cidABC* expression, presumably in response to the hypoxic nature of the microcolony as it increases in size (Figure 3A). The second microcolony type appeared to grow at a slower rate and expressed *cidABC* constitutively, with no observable

lrgAB expression. In addition, the rapidly growing microcolonies were found to stain positive with propidium iodide (a DNA intercalating dye used to detect dead cells and/or eDNA), unlike the slower growing microcolonies (Moormeier et al., 2013). Interestingly, the two microcolony types also exhibited what appeared to be differences in dispersal rates (Moormeier *et al.*, 2013), a difference that is readily observed in macroscopic imaging (Figure 3B), where dispersal can be seen as "streaking" of biofilm growth emerging from some, but not all, microcolonies formed.

Given the differential expression of the *cidABC* and *lrgAB* operons within the two recently described microcolony types, it is conceivable that *S. aureus* biofilms undergo some level of metabolic diversification, where select cells within the developing biofilm are "programmed" to differentiate into different microcolony types that exhibit distinct metabolic activities. Consistent with this is the observation that microcolony formation during *P. aeruginosa* biofilm development is linked to pyruvate metabolism. For example, inactivation of genes involved in pyruvate utilization, as well as the depletion of pyruvate from the growth medium, were found to abrogate microcolony development (Petrova et al., 2012), suggesting that pyruvate metabolism is a distinct feature of microcolony physiology. Similarly, disruption of the *S. aureus ackA* and *pta* genes involved in the conversion of pyruvate to acetate both resulted in a dramatic shift in the types of biofilm microcolonies that were formed (manuscript in preparation). Likewise, growth of the biofilm under anaerobic conditions, which prevent respiratory activity (in the absence of a terminal electron acceptor), resulted in a similar shift in the types of microcolonies that were formed. Collectively, the results of these studies suggest the existence of a mechanism underlying the formation of structure during biofilm development that involves the metabolic differentiation of cells and the emergence of microcolonies from a basal layer that remain after the exodus stage. Importantly, this does not exclude the possibility that further modification of the biofilm structure is also mediated by the function of PSMs as envisioned by Periasamy *et al.* (Periasamy et al., 2012).

The observation that metabolically distinct microcolony types emerge during the maturation stage suggests another possible role for this stage – to provide diversity in preparation for the inevitable onslaught of unanticipated environmental stresses. Indeed, diversity is the name of the game in most healthy biological systems. For example, a diversified forest is better able to withstand the damaging effects of drought and disease if it is comprised of a diverse array of different tree species (Haas et al., 2011). Although the *S. aureus* biofilms under study in our laboratory (and likely associated with many implant-related infections) are genetically identical, they are still able to diversify through the coordinated expression of genes that control the metabolic status of the individual cells. The presence of metabolically diverse subpopulations may not only lessen (or eliminate) the time required to adapt to nutrient and oxygen stress, it may also provide an important metabolic context to resist antibacterial factors (e.g. via drug tolerance and/or promoting persister cell formation) present within the environment. Of course, if the stress becomes too great, the biofilm has a mechanism that promotes the dispersal of biofilm cells that enhances the chances that these cells encounter a more habitable environment.

Dispersal

Most *S. aureus* biofilm studies have focused on trying to understand the constituents that enable biofilm attachment and accumulation. However in recent years, there have been several studies examining the factors that contribute to the control of the biofilm dispersal. Dispersal of *S. aureus* biofilms has largely been shown to be under the control of Agr quorum sensing (Vuong et al., 2000, Yarwood et al., 2004, Periasamy et al., 2012) (Figure 1E), and like other quorum sensing systems, the Agr system is dependent on cell density and the accumulation of signal molecules called autoinducers. In *S. aureus*, an octapeptide pheromone called auto-inducing peptide (AIP) (Tong et al.) accumulates in the culture medium, and upon reaching a threshold concentration, binds to and activates the histidine kinase, AgrC. Once activated, AgrC phosphorylates the response regulator, AgrA, which then initiates transcription from the P3 promoter of the *agr* operon, producing a regulatory RNA molecule (RNAIII) that regulates expression of several virulence factors and biofilm-associated genes (Novick & Geisinger, 2008, Abdelnour et al., 1993, Dunman et al., 2001).

The first studies examining the contribution of the Agr system in *S. aureus* biofilm development demonstrated that *agr*-deficient strains formed more robust biofilms when compared to their wild-type counterparts (Vuong et al., 2000). Yet, it was not until flow-cell studies evaluating the function of Agr quorum sensing during *S. aureus* biofilm development that demonstrated P3 promoter expression in a subpopulation of cells located primarily within microcolonies that appeared to oscillate in waves over time coinciding with detached cells of the biofilm (Yarwood et al., 2004). Since then, this has been further corroborated by a study demonstrating increased P3 expression within microcolonies under low flow rates due to accumulation of AIP (Kim et al., 2016), as well as similar results using the BioFlux1000 system (Figure 4).

While the initial report provided support that Agr activity has a function in dispersal of biofilms, the Agr-regulated factors that mediate dispersal were not identified. However, two subsequent studies provided evidence of contrasting modes of Agr-mediated dispersal mechanisms. In one study, a direct correlation was demonstrated between P3 activation and dispersal of intact biofilms, which they propose was due to increased protease activity and subsequent degradation of the protein-based ECM (Boles & Horswill, 2008). While this provides a link to protease activity, Agr is not the only known regulator of the secreted proteases. Indeed, several other *S. aureus* transcriptional regulators, such as SarA, SigB, SaeRS, and Rot, and the newly defined *msaABCR* operon, have all been shown to mediate protease activity and biofilm maturation (Tsang et al., 2008, Lauderdale et al., 2009, Mootz et al., 2013, Mrak et al., 2012, Mootz et al., 2015, Sahukhal et al., 2015).

In a different study, Agr-dependent dispersal was proposed to involve the production of the phenol soluble modulin (PSM) peptides. These short amphipathic, α-helical peptides have been shown to be under the regulatory control of the Agr system through direct binding of AgrA to the *psm* operon promoters, and have been implicated in dispersing staphylococcal biofilms (Wang et al., 2007, Periasamy et al., 2012). Indeed, *S. aureus* isogenic mutants defective in the production of PSMα, PSMβ, or δ-toxin resulted in thicker biofilms (Periasamy et al., 2012). Like the Yarwood et al. study (Yarwood et al., 2004), it was also

shown that the induction of Agr and the *psm* operon promoters correlated with waves of dispersal during late stages of biofilm development (Periasamy et al., 2012).

To mediate dispersal, PSMs are thought to function as surfactants disrupting molecular interactions within the biofilm matrix (Otto, 2013, Peschel & Otto, 2013). While the surfactant-like properties of the PSMs may indeed play a major role in biofilm dispersal, there is also contrasting evidence suggesting that the aggregation of the PSMs into nonsoluble amyloid-like fibers might abrogate biofilm dispersal and contribute to the maintenance of biofilm structure (Schwartz et al., 2012). Hence, the production of PSMs may not initiate dispersal, but rather the state in which the PSMs are assembled may contribute directly to biofilm integrity. Furthermore, it has become apparent that the presence of eDNA promotes the formation of these amyloid-like structures (Schwartz et al., 2016) suggesting a necessity for the production and interplay between ECM components to allow proper biofilm development. Clearly, more studies are needed to understand the interplay between the amyloid- and surfactant-like properties of the PSMs in biofilm stabilization and dispersal.

Conclusions

The use of new microfluidics technology to visualize *S. aureus* biofilm development has provided an enhanced perspective on the specific events that occur during this ill-defined process. These events appear to be more complex than previously appreciated and involve metabolic heterogeneity and differential gene expression that may be a hallmark of biofilms produced by all bacterial species. Based on this new perspective, we defined five stages of *S. aureus* biofilm development: 1) attachment, 2) multiplication, 3) exodus, 4) maturation, and 5) dispersal (Figure 1), and discussed the molecular mechanisms that contribute to each stage. In addition, we argue that the categorization of biofilm based on matrix types (PIA, protein, and eDNA) does not provide an accurate representation of *S. aureus* biofilm; rather, we envision biofilm development as a dynamic process involving the contributions of multiple matrix components. The emergence of distinct microcolony types exhibiting differential gene expression and different ECM components suggests the existence of temporal changes in the ECM composition during *S. aureus* biofilm development, and also the presence of spatial variations in ECM composition within the same biofilm. Given the heterogeneity of the different stages of biofilm development, it is important to incorporate real-time and time-lapse assays in conjunction with endpoint and static biofilm assays to best evaluate biofilm development. Ultimately, studies of this nature will lead to a more comprehensive understanding of the complexity of biofilm development and will enhance our ability to generate new therapeutic strategies to combat infections caused by these sophisticated multicellular communities.

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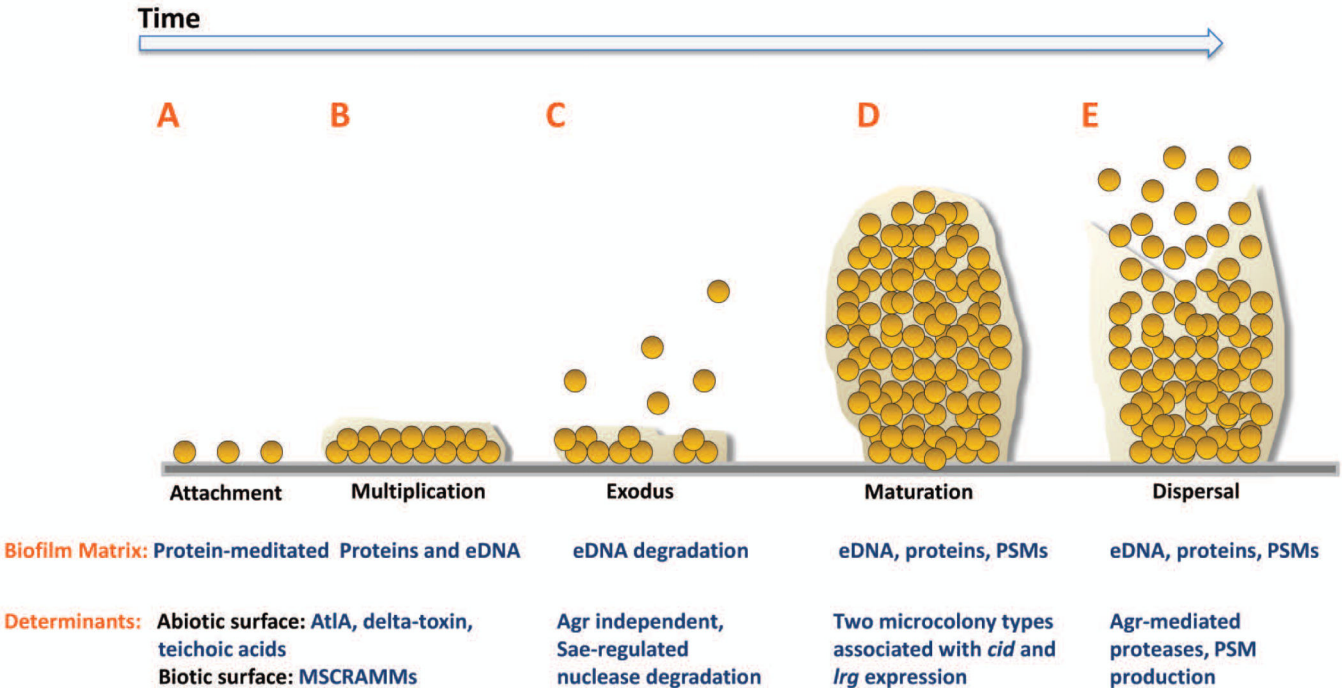

Figure 1. Model of *Staphylococcus aureus* biofilm development

S. aureus biofilm development is described in five stages: A) attachment, B) multiplication, C) exodus, D) maturation, and E) dispersal. A. *S. aureus* cells attach to abiotic or biotic surfaces via hydrophobic interactions or MSCRAMMs, respectively. B. After cells attach, the biofilm develops into a confluent 'mat' of cells composed of an eDNA and proteinaceous matrix. C. Upon reaching confluency, a period of mass exodus of cells occurs in which a subpopulation of cells is released from the biofilm via Sae-regulated nuclease-mediated eDNA degradation to allow for the formation of three-dimensional microcolonies. D. Microcolonies form from distinct foci of cells that have remained attached during the exodus stage. This stage is characterized by rapid cell division that forms robust aggregations composed of proteins including PSMs and eDNA. E. Activated Agr-mediated quorum sensing initiates biofilm matrix modulation and dispersal of cells via protease activation and/or PSM production. AtlA, autolysin A; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; eDNA, extracellular DNA; PSM, phenol soluble modulins; Agr, accessory gene regulator.

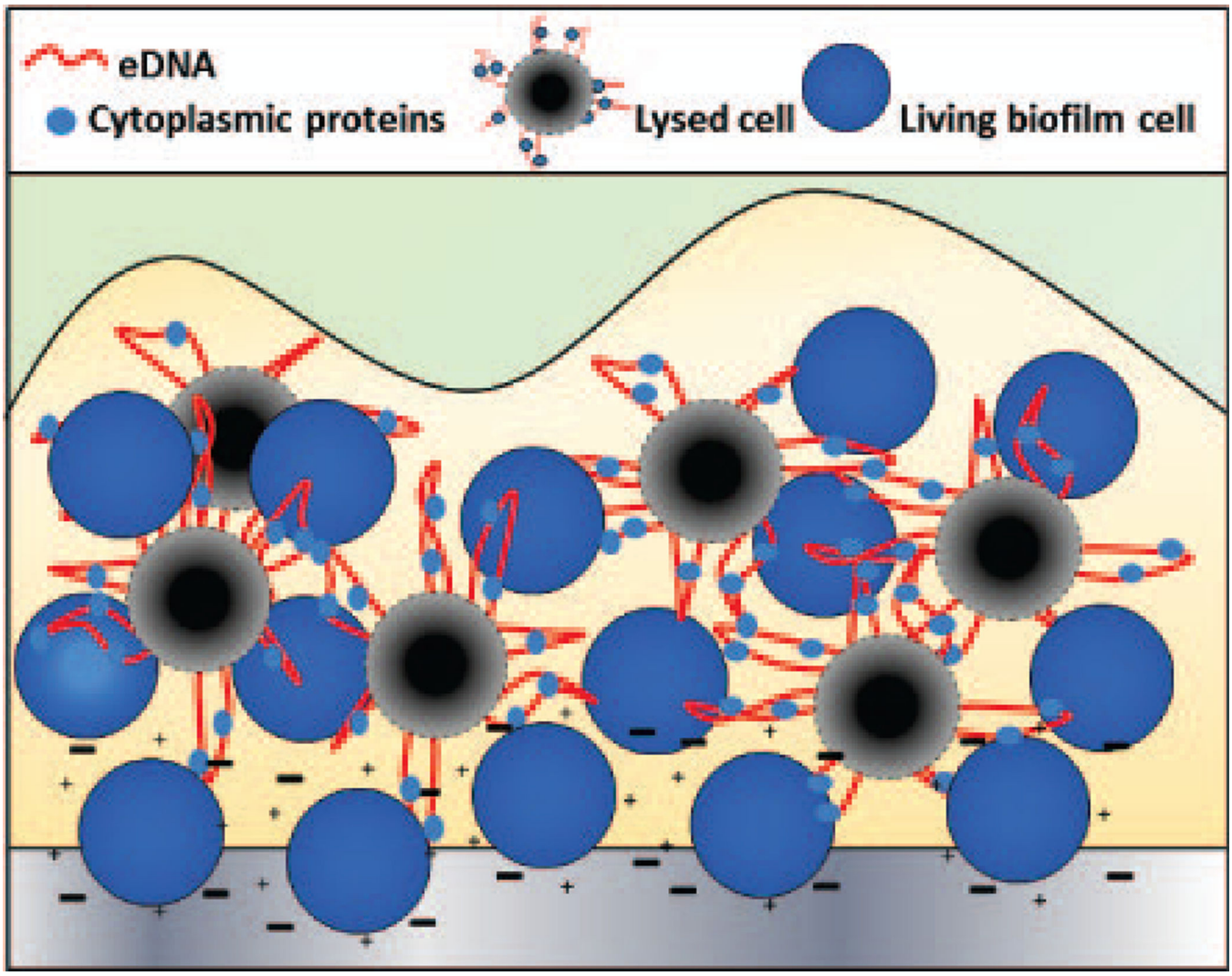

Figure 2. Model of cellular interactions during the multiplication stage of biofilm development

During the initial stages of *S. aureus* biofilm development, planktonic cells attach to a surface through electrostatic interactions (indicated by + and – symbols) involving teichoic acids, PSMs, and autolysin A. As biofilm development progresses into the multiplication stage, a subpopulation of cells dies and lyses (black circles) releasing extracellular DNA (red lines) and cytoplasmic proteins (light blue ovals) into the extracellular milieu, encasing the existing living cells (blue circles) in a mixture of cytoplasmic proteins and genomic DNA.

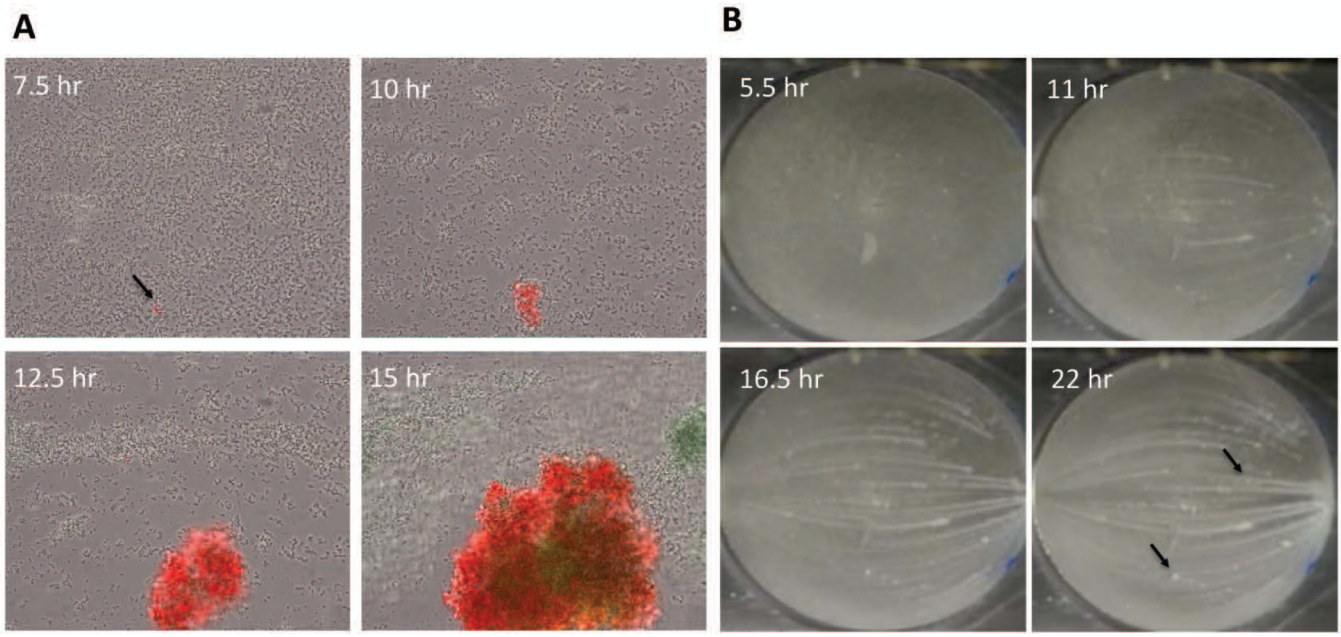

Figure 3. Microcolony initiation

A) *S. aureus* cells containing an *lrgAB::gfp* promoter fusion plasmid were inoculated into a Bioflux1000 microfluidics system and allowed to form a biofilm over a time-course experiment in which epifluorescence images were acquired at regular time points. Shown are images collected at regular intervals after the initiation of medium flow. Note the emergence of the microcolony originating from what appears to be a single (or relatively few) *lrgAB*-expressing (green) cells. (B) Macroscopic images of *S. aureus* biofilm grown in an FC flow-cell system. Shown are images collected at 5.5, 11, 16.5, and 22 hrs after the initiation of medium flow. Note the emergence of microcolonies from a basal layer of cell starting at 5.5 hrs, as well as the presence of streaking downstream of most (but not all; see arrows) of the microcolonies.

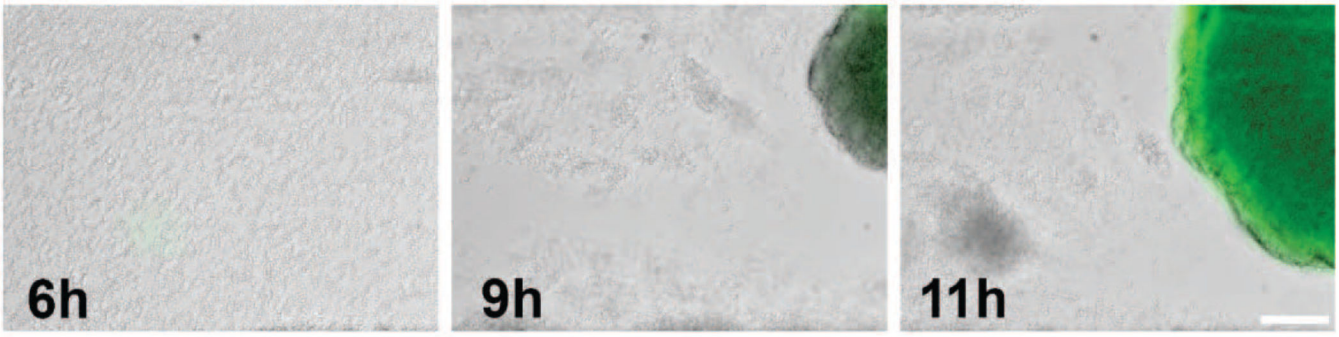

Figure 4. Agr expression in microcolonies

S. aureus cells containing a *agr-p3::gfp* promoter fusion plasmid were inoculated into a Bioflux1000 microfluidics system and allowed to form a biofilm over a time-course experiment in which epifluorescence images were acquired at 0, 6, 9, and 11 hrs after the initiation of medium flow. Note the emergence of Agr expression presumably after the AIP octapeptide reaches a threshold density required for induction of P3 expression.