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Sociomicrobiology: the connections between quorum sensing and biofilms

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In the past decade, significant debate has surrounded the relative contributions of genetic determinants versus environmental conditions to certain types of human behavior. While this debate goes on, it is with a certain degree of irony that microbiologists studying aspects of bacterial community behavior face the same questions. Information regarding two social phenomena exhibited by bacteria, quorum sensing and biofilm development, is reviewed here. These two topics have been inextricably linked, possibly because biofilms and quorum sensing represent two areas in which microbiologists focus on social aspects of bacteria. We will examine what is known about this linkage and discuss areas that might be developed. In addition, we believe that these two aspects of bacterial behavior represent a small part of the social repertoire of bacteria. Bacteria exhibit many social activities and they represent a model for dissecting social behavior at the genetic level. Therefore, we introduce the term 'sociomicrobiology'.

Introduction

In general, biofilm cells encounter much higher local cell-densities than free-floating, planktonic cell populations. An obvious consequence of this is the elevated levels of metabolic by-products, secondary metabolites and other secreted or excreted microbial factors that biofilm cells encounter. Of particular interest are intercellular signaling or quorum-sensing molecules. Because biofilms generally consist of aggregates of cells, one could argue that they represent an environmentally relevant context for quorum sensing. For some species, there is evidence that quorum sensing is important for the construction and/or dissolution of biofilm communities. In this review, we will begin with a discussion of what is known about the role quorum sensing plays in biofilm development in different systems. We will then focus specifically on *Pseudomonas aeruginosa* as a model system and, finally, consider quorum sensing in the context of a biofilm. Because this review focuses on the intersection of two fields of microbiology that involve social activity of bacteria, we introduce the term sociomicrobiology, meaning 'investi-investigations of any group-behaviors of microbes'. This is particularly fitting because we discuss controversial areas where the relative contributions of genetic and environmental influences that govern biofilm formation are not clear. This is reminiscent of discussions that continue in the general areas of sociobiology and sociology.

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Common molecular schemes used for quorum sensing in bacteria

Quorum sensing is a term used to describe intercellular signaling in bacteria. Although several quorum-sensing systems are known, perhaps the two most thoroughly described systems are the acyl-homoserine lactone (acyl-HSL) systems of many Gram-negative species and the peptide-based signaling systems of many Gram-positive species [1–3]. We will also briefly discuss the widespread AI-2 system that is found in several Gram-positive and Gram-negative species [1]. Before discussing these systems in the context of biofilms, we will review these three basic signaling mechanisms. For acyl-HSL quorum sensing, a single enzyme is required for synthesis of the signal from cellular metabolites [4–6]. Generally, these synthases belong to the LuxI family (named for the signal synthase of the *Vibrio fischeri lux* system). The signal is an acylated homoserine lactone that can diffuse across the cell membrane. The homoserine lactone ring is conserved in all signals identified to date; however, depending upon the system, the acyl side chain can vary in length and degree and type of substitution. As signal levels build, due to either an increase in local cell density or in areas of restricted local diffusion, the signal interacts with a cytoplasmic DNA-binding receptor protein belonging to the LuxR family [7,8]. The LuxR homolog–signal complex then modulates expression of quorum-sensing-regulated genes.

Peptide-based signaling usually involves the production of small linear or cyclic peptides that are translated as a larger pro-peptide inside the cell; these are then further processed during secretion [9]. In contrast to acyl-HSL-based signaling, peptide signals are not detected inside the cell. In some cases a membrane-bound sensor protein belonging to the two-component signal transduction family interacts with the peptide. Peptide-bound sensor then activates an associated response regulator, which modulates expression of quorum-sensing-regulated genes.

The AI-2 quorum-sensing system was first described in *Vibrio harveyi* and has been implicated in interspecies communication [10]. The extracellular signaling molecule of the *V. harveyi* AI-2 system is a furanosyl borate diester [11]. Synthesis of this molecule is directed by the *luxS* gene product [12]. Many species have been shown to

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contain *luxS* homologs, although the signaling mechanism has not been completely defined or verified in most of these systems.

A survey of quorum-sensing-related biofilm phenotypes

Many groups have examined whether quorum sensing controls biofilm formation. In some cases, quorum sensing does not appear to be involved in biofilm formation. However, quorum sensing has been shown to influence biofilm development for several species.

Attachment

Attachment of a bacterium to a surface, or substratum, is the initial step in the formation of a biofilm [13,14]. The nature of the attachment surface in addition to several microbial factors have been shown to affect adherence [15].

In general, the cyclic-peptide-dependent accessory gene regulator (agr) quorum-sensing system in *Staphylococcus aureus* represses several surface adhesins that mediate contact with the host matrix [16]. These include fibrinogen- and fibronectin-binding proteins. Under certain conditions, *agr* mutants adhere more efficiently than wild-type strains to both biological and abiotic surfaces [17–19].

The gastrointestinal pathogen *Helicobacter pylori* has a *luxS* homolog that has been implicated in attachment. A *luxS* mutant was shown to adhere approximately twice as well as the wild-type strain [20]. Conversely, LuxS of the pathogen *Salmonella enterica* serovar Typhimurium was shown to be required for biofilm formation on human gallstones [21].

Maturation

The maturation of a biofilm community occurs downstream of adherence. The architecture of mature biofilms can vary from flat, homogenous biofilms, to highly structured biofilms, characterized by void spaces and towers of cells encased in an extracellular matrix. The architecture of a biofilm affects the distribution of chemical gradients and potentially the antimicrobial tolerance profiles of bacteria in the biofilm, although the latter remains to be tested experimentally [13]. Several factors have been shown to influence biofilm architecture, including motility, extracellular polymeric substance matrix (EPS) production and rhamnolipid production [22–24].

Acyl-HSL-based quorum sensing has been shown to influence biofilm maturation for the Gram-negative bacterium *Serratia liquefaciens* [25]. Quorum sensing regulates swarming motility in *S. liquefaciens* [26]. Wild-type *S. liquefaciens* biofilms are heterogenous, consisting of cell aggregates and long filaments of cells. A mutation in the acyl-HSL synthase gene, *swrI*, resulted in thin biofilms that lacked aggregates and filaments. Two quorum-sensing-regulated genes, *bsmA* and *bsmB*, were also implicated in biofilm development. The BsmA and BsmB proteins showed no homology to proteins of known function. The *cepI/R* quorum-sensing system of *Burkholderia cepacia* H111 has also been shown to control biofilm maturation. Huber *et al.* [27] showed that strains with mutations in either *cepI* or *cepR* formed biofilms that were arrested at the microcolony stage of growth, whereas the wild-type strain formed more robust biofilms that covered the attachment surface.

The *ahyR/I* acyl-HSL quorum-sensing system of *Aeromonas hydrophila* has also been shown to be required for biofilm maturation [28]. A strain harboring an *ahyI* mutation formed biofilms that were structurally less-differentiated than the wild-type strain. Interestingly, the *ahyI* mutant also showed a gradual reduction in biofilm-associated viable counts, leading the authors to suggest that the *ahyI* mutant biofilm cells were more susceptible to biofilm-related stress. This phenotype could be partially overcome by exogenous addition of butyryl-HSL, the cognate acyl-HSL of the system. For all three of the systems mentioned, the functional consequence of this altered architecture is unclear.

The LuxS-type quorum-sensing system in *Streptococcus mutans* is also involved in biofilm development. A mutation in *luxS* resulted in mature biofilms with less overall biomass [29,30]. The architecture of the mature biofilm was different for the mutant strain. A *luxS* mutant biofilm grown on hydroxyapatite disks was loose and rough in appearance compared with the wild-type strain, which formed biofilms that were smooth and confluent. A two-component regulatory system, *smu486* and *smu487*, was also identified as potentially being involved in the quorum-sensing-dependent biofilm phenotype [30].

Aggregation and dissolution or dispersal

Aggregation in liquid culture has been correlated with a propensity to form biofilm communities. Liquid-culture aggregates probably have many of the same characteristics as a biofilm community, including cells held together by an extracellular matrix and steep chemical gradients within the aggregate. Cell aggregates, or flocs, are observed in both industrial (e.g. wastewater treatment plants) and natural (e.g. marine snow) settings.

There is growing appreciation within the biofilm field that individual cells of a variety of bacterial species are capable of actively leaving a biofilm. Presumably, this dispersal process could serve to enable bacteria to colonize new surfaces and reinitiate the biofilm developmental process [31,32]. Using quorum sensing to regulate this process makes sense. In crowded conditions, where resources are becoming limited, quorum sensing would be an ideal way to mediate exodus from a biofilm. As described below, there is some evidence to support the notion that for certain bacterial species quorum sensing controls dispersal.

Acyl-HSL based quorum sensing in the phototroph, *Rhodobacter sphaeroides*, has been shown to control cellular aggregation [33]. Mutations in the acyl-HSL synthase of the *R. sphaeroides* quorum-sensing system, called *cer* (for community escape response), resulted in hyperaggregation of cells in liquid culture. The ecological role of quorum sensing in this organism remains unclear.

The enteric pathogen, *Yersinia pseudotuberculosis*, shows a similar phenotype for quorum-sensing mutants in its acyl-HSL *ypsI/R* quorum-sensing system [34]. Mutations in the regulator *ypsR* cause the organism to

aggregate in liquid culture. Expression of a 42 kDa surface protein with homology to flagellin was found to be activated in the *ypsR* background and might mediate aggregation. Mutations in both the regulator and the acyl-HSL synthase resulted in increased swimming motility.

The plant pathogen, *Xanthomonas campestris*, has a novel quorum-sensing system that has been implicated in biofilm dispersal [35]. The signal for this system, DSF (for diffusible signal factor) has not yet been identified, however, the gene responsible for its production (*rpfF*) and a two-component system that senses the signal (encoded by *rpfC* and *rpfG*) have been identified [36,37]. Mutations in this system result in aggregates in liquid culture and increased biofilm formation. The secreted polysaccharide, known as xanthan, mediates intercellular aggregation in this organism. *X. campestris* produces an extracellular mannosidase, responsible for cleaving xanthan, which is regulated by the DSF/*rpf* quorum-sensing system and contributes to dissolution of aggregates [38].

Similar to *X. campestris*, the enteric pathogen *Vibrio cholerae* uses quorum sensing to regulate production of the secreted exopolysaccharide encoded by the *vps* operon. This exopolysaccahride mediates intercellular aggregation and adherence to surfaces. A homolog of a repressor involved in *luxS*-based signaling, designated HapR, represses expression of *vps* exopolysaccharide biosynthesis, and a *hapR* mutation results in exopolysaccharide overproduction and a smaller, wrinkled colony, or 'rugose' phenotype when grown on solid medium [39–42]. The current model is that attaining a quorum leads to reduction in *vps* exopolysaccharide synthesis. In addition to stimulating biofilm formation and aggregation, it has been suggested that *vps* overproduction might enable *V. cholerae* to survive navigation across the acid pH barrier of the stomach and ultimately promote infection.

An interesting point when considering these data is the criteria that are used to determine the role that quorum sensing plays in biofilm formation. A common theme in the studies discussed above is that mutants are constructed in key quorum-sensing regulators and then biofilm phenotypes are evaluated. Perhaps its not surprising that quorum sensing has been found to be important for biofilm formation under these conditions. There is growing evidence that quorum sensing constitutes a global regulatory system in many different species. Generating a mutation in a global regulator would produce pleiotropic phenotypes, and anything that affects motility, surface appendage expression or the chemistry of the cell surface might translate to a biofilm phenotype. Perhaps the best way to evaluate the role of quorum sensing is to monitor the signaling process *in situ* in a developing biofilm of a wild-type strain and determine if the onset of quorum sensing corresponds to any discernible transition in development, such as changes in structure or an increase in antimicrobial tolerance. Although this type of analysis would not be trivial, a more accurate understanding of quorum sensing in biofilm development might be achieved.

Pseudomonas aeruginosa as a case study

In 1998, a report in Science described the role of the *P. aeruginosa las* quorum sensing in biofilm formation [43]. In this first study on the connection between quorum sensing and biofilm formation, *lasI* mutants deficient in the synthesis of 3-oxododecanoyl-HSL formed biofilms that were flat, densely packed, and homogenous relative to the highly structured, heterogeneous biofilms of the wild-type parent PAO1. In contrast to the wild-type strain, the biofilms formed by the mutants were also dispersed by addition of the detergent sodium-dodecyl sulphate (SDS). This study suggested that two important aspects of social behavior, quorum sensing and biofilm formation, are connected in *P. aeruginosa*.

Several subsequent studies on quorum sensing and biofilm development in *P. aeruginosa* have now been published [44–47]. It quickly became apparent that the influence of quorum sensing on biofilm structure was dependent upon experimental conditions. Heydorn, *et al.* [44] reported that a *lasI* mutant of PAO1 formed flat homogenous biofilms that were indistinguishable from the wild-type under their experimental conditions. The flat biofilms were similar in appearance to the quorum-sensing mutant biofilms reported in the 1998 study, leading to some confusion in the field. There is not necessarily any inconsistency between these two reports, however, the difference is in the architecture of the wild-type *P. aeruginosa* biofilms. Interestingly, Hentzer *et al.* [45] showed that the furanone compounds (structurally similar to acyl-HSL signals) produced by the red algae, *Delisea pulchra*, interfered with *P. aeruginosa* biofilm formation by disrupting signaling. To heighten the confusion, Stoodley, *et al.* [46] initially reported that under high shear conditions biofilms formed by a quorum-sensing mutant were indistinguishable from the parent, however, discernable structural differences were found following a more refined analysis [47].

If one accepts that mutations in quorum sensing affect the ability of *P. aeruginosa* to form highly structured biofilms, the question of 'Why?' still remains. A simple explanation could be, as some have suggested, that quorum-sensing mutants form fragile biofilms and appear flat because developing structures continually break away [45]. All of these reports point to a basic limitation in methodology for analyzing the influence of quorum sensing on biofilm biology [43–46]. We always depend on gross structural analysis of the biofilm by microscopy. Is it more important that we can observe differences under some conditions, or is it more important that we don't observe differences under some conditions? We believe it is important that differences are observed under certain conditions and that this is providing a glimpse into the biology of a biofilm. It is less significant that this is conditional because our only basis of comparison is the visual appearance of the biofilm. There are probably many significant aspects of biofilm biology not related to observable architecture. However, there are other points of view and at this juncture any point of view is nothing more than an article of faith.

In addition, we are not sure what *P. aeruginosa* quorum-sensing-regulated functions are important for

biofilm formation. This might be partly due to the fact that quorum sensing in *P. aeruginosa* is global and, depending upon the study and experimental conditions, has been shown to regulate the expression of 170–400 genes [48–50]. However, some quorum-sensing-regulated functions have been shown to affect biofilm development. Davey and O'Toole [24] showed that rhamnolipid production was required for maintenance of architecture in structured biofilms. A *rhlA* mutant formed biofilms that were flat and unstructured compared with the parent strain. This is a similar result to the original report [43], although in this case a *rhlI* mutant formed biofilms similar in architecture to the parent. This was puzzling because the *rhl* quorum-sensing system of *P. aeruginosa* controls rhamnolipid production. However, Schuster, *et al.* and others [48–50] showed that the rhamnolipid gene expression also responds to the *las* signaling system. There might be sufficient activation of rhamnolipid synthesis in a *rhlI* mutant via the *las* system to enable the development of structured biofilms. Because the *las* system is required to activate the *rhl* system (among many other genes), a *lasI* mutant produces negligible amounts of rhamnolipid. This might highlight the effect culturing conditions can have on the influence of quorum sensing on biofilm development.

Swarming motility is another process controlled by quorum sensing. Mutants in both the *las* and *rhl* systems are deficient in swarming motility [51]. Although the link between swarming motility and biofilm development isn't firmly established in *P. aeruginosa*, surface-associated motility is a key aspect of biofilm development in a variety of species. Finally, quorum sensing is known to regulate iron acquisition systems, such as biosynthesis of the siderophore pyoverdin [52]. Iron limitation has been previously shown to inhibit biofilm development [53].

To revisit earlier discussion: a key question is 'What does the biofilm's conditional dependence on quorum sensing mean?' One possibility is that quorum sensing is simply insignificant. The fact that only a few laboratory culturing conditions have been identified to date for which quorum sensing appears to be important for biofilms suggests that these conditions are contrived and result in experimental artifacts that are not relevant to biofilm formation in environmentally and clinically relevant environments. After all, numerous species appearing to lack quorum-sensing systems have been shown to form biofilms with significant architecture. Another possibility might reflect our limitations in being able to experimentally characterize biofilms and identify (unknown) quorum-sensing effects. With the exception of observing how a biofilm looks by microscopy and determining its antimicrobial tolerance, we are limited in how we can characterize a biofilm. Therefore, although wild-type and quorum-sensing mutant biofilms might look similar under certain conditions, their functional characteristics and capabilities as a community can be significantly different. We know that quorum sensing regulates several secreted factors that affect virulence and presumably can affect local extracellular chemistry. We also know that quorum sensing is required for virulence in several animal model systems [54,55]. Therefore, at least in the context of virulence, one would predict the functional capabilities of wild-type biofilms and those formed by quorum-sensing mutants to be very different, although they might appear similar under the microscope. Maybe the real issues that should be addressed relate to the role quorum sensing plays in determining the functional capabilities of a biofilm. Again our view is that contrived laboratory conditions reveal important connections that are otherwise difficult to ascertain.

How might quorum sensing function in a biofilm?

To date, all of the quorum-sensing mechanisms that have been described in detail have been studied in the context of planktonic cultures. This is understandable because it simplifies the signaling process. In shaken liquid culture, all bacteria are presumed to be physiologically similar, are producing signal molecules at the same rate, and are exposed to the same concentration of signal molecule. Any degree of heterogeneity in the population can be dismissed because the researcher is measuring an average of the population. In the context of a biofilm, this is not the case and signal diffusion might be very complicated. Heterogeneity in the population that arises from mutation cannot be dismissed and might give rise to clonally derived pockets within the biofilm. In this section we will pose some important questions that need to be answered to understand how quorum sensing might work in a biofilm community.

Are signal molecules freely diffusible in a biofilm?

Acyl-HSLs can vary in hydrophobicity, depending upon the length of the acyl side chain. Apparently, long chain acyl-HSLs partition the hydrophobic environment of the cell membrane. Biofilm cells are usually encased in an extracellular matrix, consisting of a mixture of secreted proteins, polysaccharides, nucleic acids and dead cells. Acyl-HSLs are assumed to diffuse freely through this matrix, although, depending upon the relative hydrophobicity of matrix components it could serve as a sink, sequestering signal molecules. The argument for chemical sequestration by the biofilm matrix can be made for other quorum-sensing systems, regardless of the type of signal molecule. For species possessing multiple acyl-HSL signals, such as *P. aeruginosa*, this issue might be central to understanding signaling patterns in biofilms. *P. aeruginosa* has two main acyl-HSL signals, with one (butyryl-HSL) being more hydrophilic than the other (3-oxododecanoyl-HSL). Therefore differences in the relative diffusion of the two signals could ultimately dictate signaling patterns within the biofilm. Unfortunately, thorough studies have not been conducted to address this possibility.

Another point to consider regarding acyl-HSL-based quorum sensing is the recent identification of environmental species capable of degrading acyl-HSLs and using them as energy or carbon sources. Degradation can occur through the action of either lactonases that break the homoserine lactone ring open or acylases that cleave off the acyl side chain. Gram-positive bacteria, such as *Bacillus* spp., have been shown to degrade acyl-HSL molecules. This has also been shown for Gram-negative

species; first reported for *Variovorax paradoxicus* [56–59]. In the context of environmental biofilms this poses an intriguing possibility. In a mixed species biofilm, signal-consuming organisms might influence signal gradients. This would be particularly true for signals that diffuse out of cell aggregates of signal producers. The signal consumers could 'insulate' other bacteria in the biofilm from signaling molecules by surrounding them and consuming the signals they produce (J. Leadbetter, personal communication). Indeed, environmental communities have been shown to be poised for degradation of acyl-HSLs.

Do all cells in a biofilm produce signal molecules at the same rate?

Regardless of the quorum-sensing system being studied, the substrates for signal production are derived from general metabolites, the composition of which reflects the overall physiologic state of the bacterium. In the case of acyl-HSLs, the substrates for signal synthesis are *S*-adenosyl methionine (SAM) and acylated acyl carrier protein (acyl-ACP) [4,6]. The availability, and in the case of acyl-ACP, the composition of the substrate pool will depend upon the metabolic state of the cell [60]. Cells buried in the interior of a biofilm show decreased metabolic activity. This in turn could affect SAM and acyl-ACP levels. Therefore, one might predict that the levels of acyl-HSL synthesis would differ in the interior of the biofilm compared with the metabolically active exterior. In turn, this would affect signal gradients in the system. The same argument can be made regarding the amino acid pools available for peptide signal synthesis.

Would differences in metabolic activity have an impact on signal synthesis rates? The positive autoregulation of signal synthases observed in many quorum-sensing systems complicates this question. If a subpopulation of the biofilm community were induced for quorum sensing, this would result in a corresponding subpopulation of cells that would have higher levels of signal synthase activity. Therefore knowing where in a biofilm population quorum sensing is first induced and how rapidly the rest of the population is induced following the initial onset of quorum sensing would be vital for predicting rates of signal synthesis throughout a biofilm population.

Where is quorum sensing first induced in a biofilm?

This is a question that has been previously addressed experimentally. De Kievit *et al.* [61] used *lasI* and *rhlI* transcriptional fusions to the gene coding for the green fluorescent protein (GFP) to monitor quorum-sensing-regulated gene expression in *P. aeruginosa* biofilms. They found that gene expression occurred primarily at the substratum, within the depths of the biofilm. Yarwood *et al.* [62] took a similar approach using an RNAIII-GFP fusion in *S. aureus* to monitor expression of a gene controlled by the *agr* quorum-sensing system [62]. GFP expression was observed in pockets throughout the biofilm accompanied by a rapid loss of fluorescence. Detachment of the quorum-sensing-induced cells from the biofilm was suggested to be the explanation for this observation. Both studies represent initial attempts to monitor quorum-sensing-regulated gene expression in a biofilm. Further studies on the onset of quorum sensing and how biofilm architecture and physical forces, such as flow, affect this are in order.

There are a many key questions that remain to be answered. *P. aeruginosa* has been shown to form biofilms that vary from flat and homogenous to highly differentiated with towers and mushrooms of cells separated by

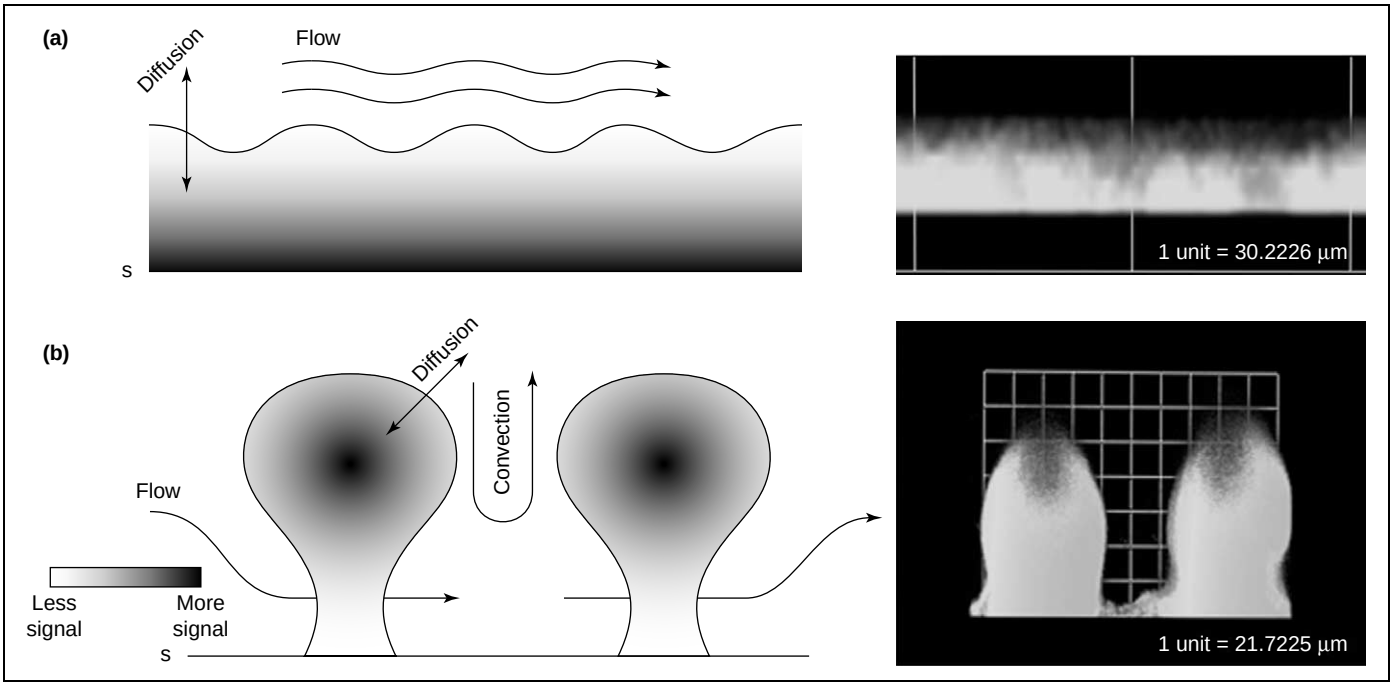

Figure 1. Hypothetical signal gradients in a biofilm system. This schematic represents a side-view of **(a)** a flat and **(b)** a structured biofilm (s indicates the surface or substratum). This diagram represents speculation regarding potential signal gradients (indicated by the gray scale), with higher signal concentrations indicated by darker coloration. Factors, such as diffusion constants for the signal, mass transfer and non-uniform signal production, within different regions of the biofilm could all affect signal gradients. The two micrographs at the right of the figure represent side-views of confocal micrographs of *P. aeruginosa* PAO1 forming a flat and a structured biofilm.

void spaces. Several environmental conditions have been shown to influence biofilm structure, including carbon source and flow rate. One important question is what influence does biofilm architecture have on the amount of biomass needed to constitute a quorum? The three-dimensional architecture of flat, homogenous biofilms would undoubtedly result in signal gradients distinct from a structured biofilm (Figure 1). A related question is what effect does mass transfer have on the onset of quorum sensing in a biofilm community? As flow rate increases in a biofilm system, signals might be removed at a greater rate and hence more biofilm biomass would be required to achieve a quorum. Of course we have been limiting our discussion to solid–liquid interface biofilms. Biofilms forming at liquid–gas or solid–air interfaces would probably have their own distinct characteristics that would influence signaling.

Summary

Investigations of the role quorum sensing plays in biofilm systems for different organisms and how quorum sensing works mechanistically in a biofilm community remain in their infancy. A clear challenge facing the field is to determine what parameters of a biofilm community influence the onset of quorum sensing and subsequent patterns of gene expression. Another key challenge is to determine the functional consequences of quorum sensing in a biofilm community. Does induction of quorum sensing influence the pathogenic potential of biofilm communities of some species, or perhaps alter the antimicrobial tolerance of the biofilm? Finally, the role of quorum sensing in mixed species systems remains to be explored. Does interspecies signaling occur frequently in mixed species systems, or do signal consuming organisms severely limit the extent of signaling that occurs? The answer to these questions will undoubtedly provide some expected results as well as surprises.

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