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REVIEW PAPER

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Biofilms, bacterial signaling, and their ties to marine biology

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Abstract Much of what is know about quorum sensing has come from the study of marine biology. The original description of the phenomenon was based on the study of marine bacteria and the luminescent pathway. More recently, aquatic organisms have been found to inhibit bacterial fouling of surfaces by blocking signaling pathways in the bacteria. These signaling effects have, over the last 5 years, been linked to biofilms. However, this correlation is not as straight forward as originally believed. Here, a brief overview of quorum sensing, and background on biofilms is provided, followed by a discussion of more recent work looking at the effects that environment may have on signal expression.

Keywords Biofilm · Bacterial communication · Biofilm control · Marine biotechnology

Introduction

The current understanding of bacterial signaling, and its relationship to biofilm formation owes much to the study of marine biotechnology. Bacterial signaling was first discovered during examination of the light organelles of deep-sea organisms. The relationship between bacterial signaling and biofilm formation was further tied together by research showing that the macroalga *Delisea pulchra* uses furanones to inhibit signaling, and subsequently to reduce surface colonization by bacteria [15]. This work has done much to further our knowledge of bacterial signaling and biofilm formation, as well as enhancing biotechnology with implications for science, industry, bioremediation, and human health.

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Bacterial signaling

Bacterial signaling/communication, also termed "quorum sensing", has generated much excitement over the last decade. Nealson and Hastings discovered quorum sense in the 1970s while studying light production by deep-sea marine organisms [31]. The organelles of flashlight fish were examined and shown to owe their light production to a species of bacteria, *Vibrio fischeri*, that colonizes the light-producing organs. Since these bacteria are virtually ubiquitous throughout the ocean, their ability to limit light production to the organelles of their host symbiont was of particular interest. The study reveled that the bacteria produce a chemical, termed an "autoinducer", which causes the bacterium to alter its genetic expression. Only when sufficient levels of autoinducer are present does *V. fischeri* turn on the luciferase genes (*lux*) that control light production [30].

Further analyses elucidated a two-gene regulatory system that, when induced, transcribes the *lux* genes. The *luxI* gene produces a diffusible signaling molecule (the autoinducer) was found to be an acyl-homoserine lactone molecule (*N*-3-oxohexoyl-L-homoserine lactone) [17]. Signal "reception" requires that the signaling agent reach a threshold level, above which receptor protein is induced. Under normal conditions *Vibrio* produce a relatively low level of homoserine lactones. Therefore, the only way to elevate the concentrations to the levels necessary for induction is either by chemical buildup in a confined environment or by obtaining cellular densities sufficient for producing the needed concentrations [20]. This type of signaling has been dubbed "quorum sensing" due to the quorum of bacteria needed to achieve sufficient concentration of signaling molecule to tip the equilibrium to the coordinated complex. Once induced, the luxR protein becomes an activated promoter transcribing the various *lux* genes, which in the case of *Vibrio*, includes the luciferase gene and therefore light production. Additionally, *luxI* is also up-regulated and

the production of luxI protein is increased, in a feedback manner, further increasing signaling [20].

Biofilm formation

Biofilms are a major concern in industrial processes due to their detrimental effects on many industrial and medical systems, and their resistance to treatment, making them a reservoir for bacterial contamination. Biofilms are involved in microbially induced corrosion, causing millions of dollars in damage to oil pipes, water tanks and many other industrial systems [4, 6, 28], and play a major role in the fouling of water pipes, heat exchangers, and filtration devices [4, 38]. Biofilm bacteria additionally degrade industrial products such as emulsifiers and natural oils used for industrial coatings as well as spoiling processed foods and pharmaceuticals [47]. In these systems biofilms act as a safe haven from disinfectants and remediation techniques making the removal of bacteria a challenge for many industries [8, 9]. Biofilm research is currently coming to the forefront of the medical industry as research begins to elucidate the role of biofilms in nosocomial, implant and chronic infections [8]. The impact that biofilms have on society is vast and currently quite costly.

Biofilms have been defined as "bacteria that attach to surfaces aggregate[d] in a hydrated polymeric matrix of their own synthesis" [8]. However surface-related does not mean static; biofilms are very dynamic systems. Their formation is a progressive process in which colonizing bacteria move to or are transported to a surface, attach, and, through a series of steps, produce a biofilm. Initial attachment can be as passive as the hydrophobic attraction of a non-motile organism to the surface or as involved as the active use of organelles to facilitate attachment [24, 32, 49, 50]. Bacteria have developed multiple strategies for adsorption and attachment to surfaces. Many bacteria are attracted to surfaces by the amphiphilic (having both hydrophobic and hydrophilic regions) nature of their outer surface, which although soluble in water, is attracted to a variety of materials, making a number of hydrophobic materials, such as synthetic polymers, readily available for bacterial colonization [34]. One adsorption technique employed by bacteria for surface attachment is the use of membrane-bound proteins with high affinity for certain surface materials. Examples include aporusticyanin protein to bind *Thiobacillus ferroxidans* to pyrite, or fibronectin- and collagen-binding proteins to adhere *Staphylococcus aureus* to eukaryotic cells [2, 19]. One limitation of this type of attachment system is the necessity for the bacteria to come into close proximity to the surface before the protein can attach. Another bacterial adsorption technique takes advantage of the sticky nature of the much longer extracellular polysaccharides, produced by some planktonic bacteria, to aid in the initial adherence [1]. Although much less specific than the proteins, polysaccharides have a length advantage, allowing the bacteria to overcome the repulsive electrostatic surface forces. Additionally, the bacterial-excreted polymers often have multiple chemical functional groups, allowing covalent bonding, hydrogen bonding, hydrophobic interactions and static electric attraction which can aid in adsorption to multiple surface chemistries [7, 29].

Motile bacteria have developed additional attachment techniques. Flagellar bacteria (specifically *Pseudomonas*) have attractive moieties positioned at the distal ends of their flagella [32]. As with polysaccharides, this allows the cell to attach to surfaces without entering the ionic double layer, therefore reducing the energy needed for adsorption. An even more active attachment strategy is the use of type IV pili, which the bacteria will "shoot" out from the cell wall at a surface. The pili, like the previously mentioned flagella, have attractive moieties at their ends which adhere/attach to the surface. The pilus is then drawn back into the cell, pulling the cell toward the surface [32, 43, 50].

The passive formation of conditioning layers further aids in bacterial surface attachment. The natural adsorption of dissolved amphiphilic molecules to surfaces, termed a conditioning layer, occurs very rapid commonly within 30 minutes of surface immersion [5, 6, 34]. The conditioning layer provides a much wider variety of chemical groups for the attraction and adsorption of bacterial cells, and reduces the repulsive effects at the surface. Furthermore, it has been hypothesized that, since the conditioning layer concentrates many organic molecules, these molecules can provide a nutrient source for surface-associated bacteria [5].

Biofilm-forming bacteria have the ability to respond to surface adsorption, for example, by up-regulating the production of extracellular polysaccharides within as little as 45 min of attachment [10]. Nonetheless, biofilm formation does not occur immediately; while surface recognition is rapid, mature biofilms can take any where from 12 h to weeks to form [6]. Biofilm development takes place in stages, and has been compared to the development of a higher (multicellular) organism. At the surface, many of the motile bacteria continue to move by twitching or swarming. Twitching allows the bacteria to move across the surface and aggregate into microcolonies, accelerating the biofilm formation process [32], whereas gliding or swarming motility appears to allow the bacteria to quickly colonize open surface space [43]. However, once biofilm formation is initiated the bacteria are bound to the surface and movement is virtually stopped. The cells produce exopolysaccharides, long amphiphilic molecules with a high degree of flexibility. These molecules can act as tether lines providing additional mechanical adhesive (surface-related) and cohesive (between cells) stability [29].

Even with little movement biofilms are still very dynamic systems, growing from single cells and microcolonies to cover the surface, in some cases obtaining thickness of greater than a centimeter, by gaining cells in one of two ways. The first, as discussed above, is the use of twitching motility to form cellular aggregates from

individual cells [46]. The second is by growth, or cellular division, during which the cells continue to divide thereby expanding the biofilm away from the surface [6]. As the biofilms expand and grow, the increase in cell numbers is accompanied by the need for increasing amount of nutrients. In most systems, this means that cells closest to the solid surface (i.e. furthest from solution) quickly become limited for nutrients. The consumption of nutrients by cells in the biofilm, coupled with diffusional limitations, sets up a system in which the cells closest to the surrounding environment receive the majority of the nutrients. Biofilm bacteria have evolved three ways, that we know of, to deal with this. First, the cells at the center or bottom of the biofilm colonies (i.e. furthest from the nutrients) take on a stationary phase-like behavior, reducing the levels of nutrients necessary for survival. Second, biofilms have been shown to maintain flow channels within their structure and increase both the surface area of the biofilm and convectional transport by forming channel-like openings. Computational modeling of the biofilm shows that channeling provides significant increases in nutrient levels throughout the biofilm [37]. Third, biofilm bacteria slough and detach (the regulation of which may be, in part, nutrient dependent), releasing small biofilm aggregates and individual cells that can more readily use dissolved nutrients [36].

Biofilm channels are phenomena produced by various biofilm-forming species of bacteria. This type of morphology, although not present in all biofilms, increases convective transport within the biofilm [12, 13]. How these channels are maintained is not completely understood. Certainly sloughing and detachment play a role in removing cells from the surface; however, regulation of growth and nutrient limitation may play an additional role. There is speculation that channel maintenance may even be regulated by signaling pathways.

Biofilm resistance

One characteristic used to distinguish biofilms from their planktonic counterparts is the resistance of biofilm bacteria to most treatments, including disinfectants, antimicrobials, and antibiotics. Biofilm bacteria have been found to remain viable at MICs up to 1,000 times higher than those of their planktonic counterparts [44]. Biofilm resistance can be attributed to three basic differences between biofilm and planktonic organisms: diffusional limitations, local environment, and phenotype.

Biofilm bacteria are embedded in a matrix material consisting of 99% water, which allows rapid diffusion of small particles. However, the gel-like nature of the biofilm can reduce and prevent the diffusion of larger particles. The matrix confers both structural and adhesion/cohesion properties, binding the cells to the surface and each other. The adhesive nature of the extracellular polymeric substances (EPS) will cause a number of moieties to adsorb to the matrix, further slowing diffusion. The rates of diffusion are also limited in the case of a reactive chemical, i.e. reaction-diffusion. The best examples of this are oxidizers such as hypochlorite (bleach), which readily reacts with organic molecules. As bleach diffuses into the biofilm it reacts with the outermost cells and matrix material, lowering the concentration and therefore leaving little hypochlorite to diffuse farther. These reactions greatly reduce the diffusion rates and is why bleach and other reactive disinfectants are not as effective on biofilms as on planktonic organisms [23, 39].

Biofilms have been shown to alter the local environment to enhance their survival, changing such properties as pH and the dissolved oxygen concentration [28]. These changes can reduce the effectiveness of some treatments. For example, biofilms are known to vary the local pH, and some oral biofilms have regions of pH less than 4.9 [26]. Most antibiotics are only effective at a narrow pH range, making many of them ineffective in the low pH regions of natural biofilms [35]. A second example is the use of dismutase and catalase by biofilms to reduce the damage caused by reactive oxygen groups. Biofilm bacteria produce catalytic enzymes capable of reacting with hydrogen peroxide [18]. If hydrogen peroxide or the superoxidative burst of a neutrophil were used on a planktonic bacterium, the reactive oxygen molecules would quickly degrade the cell. By contrast, the biofilm is much less sensitive to this threat [3] having more than 14 times the resistance to this type of treatment compared to planktonics, despite the fact that biofilm bacteria produce one-fifth the amount of catalase. This is mostly likely due to reaction-diffusion by the reactive oxygen groups penetrating the biofilm. In this case, the oxygen groups are degraded by both the biomass and the catalase, reducing their penetration into the biofilm giving the biofilm time to up-regulate catalase production [25].

Finally, a dramatic phenotypic change occurs as the bacteria go from a planktonic to a biofilm state. The bacteria physiologically shift to the stationary-phase-like behavior, and like most stationary-phase planktonic cells become resistant to a range of disinfectants. However, there are additional changes in protein expression by biofilm cells that differ from those of stationary-phase planktonic cells, making biofilms a completely different mode of bacterial life. These include a decrease in a number of antibiotic targets, as well as increases of efflux pumps and other resistance mechanisms [16, 21].

Biofilm physiology

As mentioned, bacteria dramatically change their phenotype in converting from the planktonic form to biofilms. These changes are most evident when looking at the differences in protein expression. Sauer reported a 70% difference when comparing protein expression of *Pseudomonas aeruginosa* planktonics and biofilms [40].

This difference is as great as that seen between various species of bacteria, or, in other words, biofilms are as different from planktonics of their own species as, e.g., *P. aeruginosa* is to *Pseudomonas putida* [40]. Other researchers have shown that, although not as dramatic, there are also changes at the RNA level, with at least 73 additional genes transcribed in the biofilm form [48]. This knowledge opens the possibility of finding new biofilm-specific control strategies, by targeting biofilm-specific genes and proteins.

Sloughing and programmed desorption

For years, fully developed biofilms were thought to be static systems, often changing little in morphology and having only minimal metabolic activity [6]. However, it is now recognized that continual bacterial signaling takes place in the biofilm, as discussed below, and that the biofilm continues to grow despite the appearance of maintaining a static condition. What causes this appearance is that cells detach from the mature biofilm at approximately the same rate that the biofilm grows [45]. Detachment comes in two forms: the conventional sloughing that has been known for years, and a ''programmed detachment'' in which the bacteria apparently dissolve their EPS matrix and either float or swim away [36, 40]. Programmed detachment could very well be a ''seeding'' mechanism in biofilm, forming planktonic bacteria that can further propagate, migrate, and colonize additional surfaces.

Bacterial signaling within biofilms

One of the more recent and exciting areas of microbiology has been that of bacterial signaling/communication (quorum sensing) [11]. A number of articles have linked biofilm formation to quorum sensing [11, 25, 33]. This is certainly reasonable since the ''quorum'' needed for the reception of such a signal is certainly present in a mature biofilm; however the specific relationships are currently being debated [27].

There are a number of different signaling systems employed by bacteria, although the overall mechanism seems to be consistent throughout the prokaryotic world. In these systems, like that of the *lux* system previously discussed, a small surfactant-like molecule (the ''signal'' or autoinducer) is produced which, depending on the bacterium, is either freely diffusible or must be transported outside the cell. These molecules are constitutively produced and exported from the cell. In most environments, they diffuse away from the cell and, having only a moderate half-life, do not build up in the environment. However, when bacterial concentrations are high enough these signaling molecules reach some threshold value and begin to effectively bind with a receptor protein. The inducer, in its activated form, is a promoter that allows transcription of a number of genes. This phenomenon is especially relevant to the high cell densities and lower diffusion rates of a biofilm.

The most extensively studied signaling systems in biofilm formation are the *las* and *rhl* systems of *P. aeruginosa*. The *las* system is apparently dominant, in that it partially regulates the *rhl* system. While several researchers have shown that signaling-deficient mutants have biofilm morphologies that differ from those of the parent strain, it is the mechanism of these differences that is argued [11, 20]. Hypotheses have included the need for signaling to initiate biofilm formation, a detachment factor expressed by signaling-regulated genes, as well as the control of channels by signaling pathways. The current understanding of the *las* and *rhl* genetic regulation pathways are best diagrammed in Shirtliff et. al., in which the work of many other researchers is compiled to more completely show the interrelationships of this regulator system [42]. The interrelationship between bacterial signaling and other genes is very complicated. As discussed, feedback regulation of signaling enhances its own expression. The *las* system is known to control elastase (*las*B), toxin A (*tox*A) and *rhl* genes (and many others including *las* A, *las* R, *las* I, *xcp* P, *wxp* R and *apr*), while the *rhl* system controls rhamnolipids expression (*rhl*I, *rhl* AB, and *rpo* S) [14, 20, 42]. Many of these genes are virulence factors, suggesting a role of signaling in infection. While signaling genes regulate a large array of genes, they are themselves also regulated by Mn, Fe, carbon (shown for glucose and suggested for other substrates), and O_2 concentration as well as stress response (RpoS) [25, 42]. This complexity has slowed the study of bacterial signaling and may explain the conflicting results published by various researchers.

Anti-biofilm possibilities

Marine research, as in the study of quorum sensing, has aided in the development of bacterial signals as biofilm control agents. Researchers at the University of New South Wales found that *Delisea pulchra*, a macroalga, was able to inhibit marine fouling on its surface [15] by excreting a variety of molecules, identified as furanone derivatives, that reduce biofilm fouling [22]. A further study of these molecules showed that they were involved in disrupting the signaling pathways of a number of bacteria. This work has been supported by additional research showing that a number of bacteria use a furanone-based signaling molecule in their quorum sensing systems [41].

Research and discussion

Biofilm fouling remains a serious problem in many industries and a frequent cause of infections in humans. The medical industry has discovered the marine environment to be an amazing resource for a variety of

chemical agents ranging from anticancer drugs to antibiotics. Work on furanones at the University of New South Wales shows this to be a very exciting compound with respect to biofilm prevention. Since the marine environment is very favorable for biofilm formation and other types of fouling, marine organisms, e.g., *Delisea pulchra* are most likely to be highly adapted to dealing with this problem. Therefore the use of a screen to examine the effectiveness of marine extracts to prevent biofilms may lead to interesting findings.

A preliminary study has been undertaken at the Center for Biofilm Engineering to find such a product. A collaboration has been developed with Sequoia Sciences, and funded by the NIH, to examine novel chemistries from aquatic and terrestrial plants to screen for biofilm-inhibitory compounds. A preliminary screen of 12,160 purified extracts turned up 269 active mixtures containing potential biofilm-inhibitory agents. Compounds from these active mixtures are being further purified and examined for antifouling properties. Initial work on the active mixtures has identified novel compounds that inhibit the formation of biofilms.

A research project has also been initiated to examine the effects of environment on bacterial signaling in *P. aeruginosa*. A plasmid containing a 250-bp GFP fusion to *lasB*, under the regulation of the *las* B transcriptional start site, has been placed into *P. aeruginosa* strain PAO1. The plasmid allows for the reporting of up-regulation of the *las* system. By systematically varying the specific environmental conditions, their effects on signaling regulation can be determined. GFP expression is normalized by cell number and compared to a control to determine any change in regulation. Although this work is ongoing, certain details are coming to light. The expectation was that signaling would increase or decrease with the concentration of a given compound. However, what was more commonly observed were maxima and minima. An example is shown in Fig. 1, where fluorescence is highest at a moderate succinate concentration and decreases with both high and low concentrations. One interesting phenomenon is a transient plateau in fluorescence, in which the curve first levels and then increases to a higher steady-state fluorescence, possibility due to feedback regulation of this system (Fig. 2). This preliminary work suggests that signaling regulation is even more complex than previously thought. Bacterial signaling may provide a very useful tool for the control of bacteria; however, there are likely other factors that need to be taken into account to obtain the greatest effectiveness from such agents. An example is that ''programmed'' biofilm detachment, whether controlled by signaling or otherwise, may have genetic mechanisms to prevent the release of cells into an environment that is unfriendly to bacteria. Therefore, some of the regulatory proteins involved in this controlled detachment may include those responsing to nutrient levels, toxins and pH and able to down-regulate release into a harsh environment. This type of information is needed to understand and improve effectiveness of biofilm control agents.

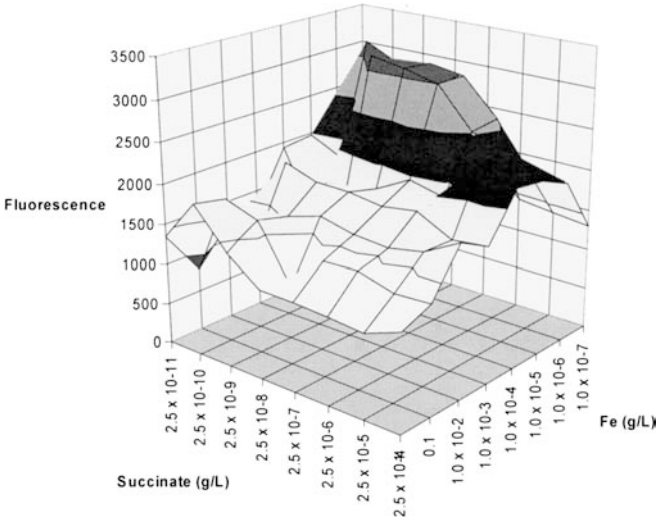

Fig. 1 A surface map of fluorescence intensity versus succinate and iron concentration for a culture of *Pseudomonas aeruginosa* carrying the LasB reporter

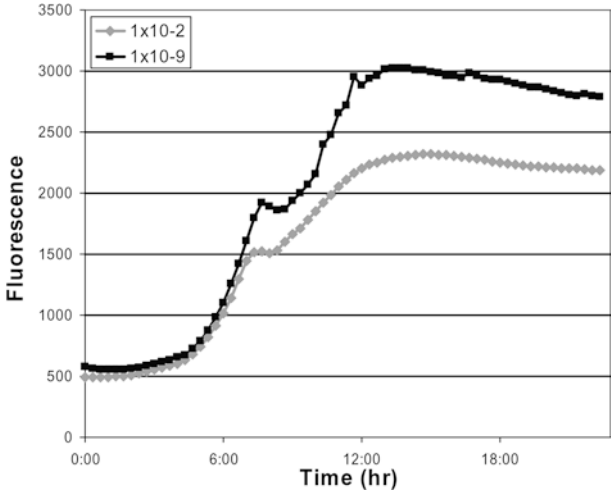

Fig. 2 A plot of fluorescence intensity versus time for a culture of *P. aeruginosa* carrying the LasB reporter and grown at two different Mn concentrations. *Diamonds* Mn concentration of 1×10^{-2} g/l, *squares* 1×10^{-9} g/l

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