Biofilm Growth and Illustrations of its Role in Mineral Formation

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ABSTRACT

Biofilm formation is a prominent feature of bacterial growth in nature. While biofilms have been described in a number of environments, little is known about their physiology. The high cell density and limited access to nutrients within biofilms would suggest quorum sensing gene activity, influenced by *lux*I or *lux*R homologues in many bacteria, and slow growth genes, regulated by *rpo*S in *Escherichia coli*, would impact biofilm physiology. Acylated homoserine lactones (acyl HSLs) are signaling molecules used in quorum sensing gene activity. Using an acyl HSL-responsive reporter strain of *Agrobacterium tumefaciens*, we have shown naturally occurring biofilms to produce these signaling molecules. In chemostat experiments, deletion of *rpo*S reduced *Escherichia coli* biofilm populations, yet did not affect planktonic populations. Naturally occurring biofilms are comprised of mixed populations. In chemostat cultures, we found that species composition and growth rate affects the ability of aquifer bacteria to form biofilms. The anionic character of biofilms enables them to interact with metal cations and to form minerals. We show one example of biofilm-mediated mineral formation on plant leaves, which can enhance the leaf fossilization process.

Numerous studies have shown that microorganisms in their natural environments are associated with surfaces. Biofilm formation commences with microbial adhesion to a surface [17[. Growth of adherent organisms results in the formation of microbial clusters, referred to as microcolonies. Continued microbial colonization and growth of adherent organisms will cause a surface to be covered by a surface community referred to as a biofilm [7]. Biofilms are a predominant mode of microbial growth in nature. They are associated with processes including leaf decomposition, fiber digestion in the digestive tract, colonization of marine surfaces by barnacles, and the formation or weathering of rocks. In contrast to planktonic bacteria, one significant characteristic of biofilm bacteria is their heightened resistance to antimicrobial agents, including disinfectants and antibiotics, making them difficult to eradicate.

Within biofilms, cells aggregate tightly together into microcolonies (Fig. 1) surrounded by water channels with few cells. The high cell density within microcolonies and biofilms suggests that cell-cell interactions would be fostered, particularly those mediated by signal

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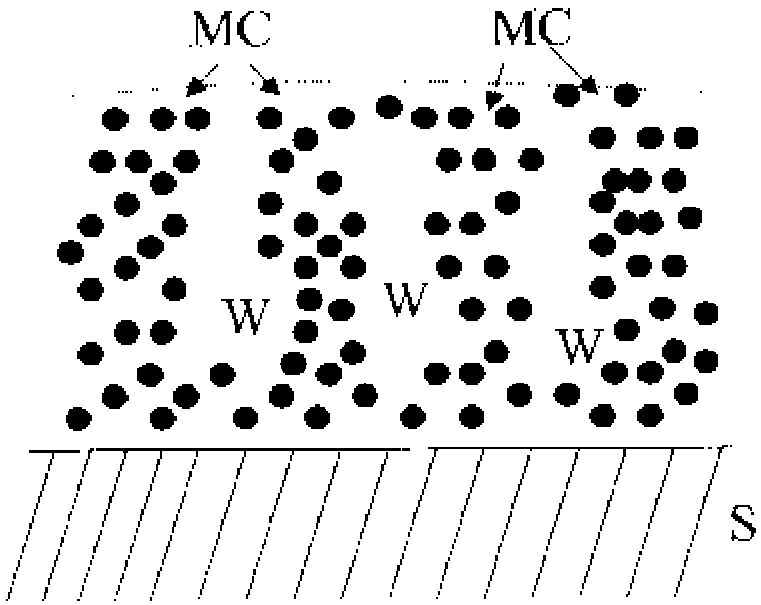

Figure 1. Biofilms are communities of microorganisms growing on a surface (S). Cell distribution within biofilms is quite heterogeneous [15]. Often individual cells are arranged in microcolonies (MC) which are surrounded by regions containing few cells, often referred to as water channels (W). A dominant feature of biofilms is the polysaccharide matrix, which extends from the colonized surface to the edge of the biofilm (dotted line). This matrix is formed from the capsule exopolymers of individual microorganisms.

molecules, such as quorum sensing acylated homoserine lactones (acyl HSLs). In addition, one would expect many chemical gradients to exist within microcolonies. In an aerobic environment, nutrients and O_2 would be present in highest concentrations at the periphery of a microcolony, while depleted in its interior. As well, a microcolony interior would contain the highest concentrations of metabolites, which may influence pH and Eh. The dominant processes influencing microbial growth in biofilms, particularly in the interior of microcolonies, would be expected to be cell-to-cell signaling, slow-growth, and possibly starvation survival. Additionally, anaerobic respiration or fermentation, as well as tolerance to changes in pH, may also be important. Other factors will be important in situations including those where biofilms contain autotrophs or anaerobes, or when they are associated with living tissues.

Quorum sensing refers to the ability of some bacteria to regulate certain aspects of their physiology as a function of population density. Many bacteria release and respond to diffusible cell density signals for this purpose. In gram-negative species, cell density signals are often acyl HSLs. While a role for acyl HSL signaling in biofilms has been postulated for several years, only recently have studies begun to provide evidence for quorum sensing in biofilms. In 1997 we detected acyl HSL production from naturally occurring freshwater stream biofilms [23], and more recently from biofilms on in-dwelling urinary catheters [29], using an *Agrobacterium tumefaciens* detector strain harboring an acyl HSL-responsive reporter gene fusion [11]. This strain responds to a wide range of acyl HSL derivatives and provides sensitive detection of acyl HSLs. Davies et al. [9] recently showed that *las*I mutants of *P. aeruginosa*, unable to synthesize the acyl HSL N-3-oxo-dodecanoyl-HSL, formed abnormal biofilms that are sensitive to detergent. In experiments with nitrifying biofilms, Batchelor [1] showed that exogenously added N-3-oxohexanoyl-

HSL could enhance starvation recovery. Givskov [13] demonstrated that the furanones, acyl HSL analogues prevented biofilm formation on the growing tips of the marine macroalga, *Delisea pulchra*. The concentration of these furanone analogues on *D. pulchra* was not toxic to the marine bacteria studied – it just prevented their biofilm formation.

Slow growth has also been associated with biofilm bacteria. Brown et al. [3] showed that the characteristic antibiotic resistance of sessile biofilm bacteria was equivalent to that shown by chemostat-grown planktonic bacteria at low growth rate. More recently Møller et al. [24] correlated acridine orange staining intensity of ribosomes in chemostat cultures with growth rate. Using this approach, they showed growth rates to be fastest at the periphery of microcolonies and slowest in the microcolony interior.

Several genes are activated by slow growth. In *Escherichia coli*, at least 30 genes are regulated by *rpo*S [19], which codes an alternate sigma factor expressed during slow growth [26]. As bacteria within biofilms experience slow-growth [24], we examined the impact of an *rpo*S deletion on *E. coli* biofilms. When *E. coli* was grown using a modified Robbins device (MRD) [25], coupled to a chemostat [32], loss of *rpo*S had minimal effect on planktonic populations. In contrast, biofilm populations were reduced by almost 50-60%. Flow cell experiments also showed the biofilm structure to be affected by the deletion of *rpo*S [Adams and McLean, unpublished). This study provides the first direct evidence of the importance of slow growth in biofilm physiology.

In their natural environment, biofilms almost always contain mixed populations. To study the impact of community composition on biofilm formation, Whiteley et al. [unpublished] obtained 20 isolates from a karst aquifer and studied their ability to form a biofilm using a chemostat coupled to a MRD [32[. As determined by dilution plating, the

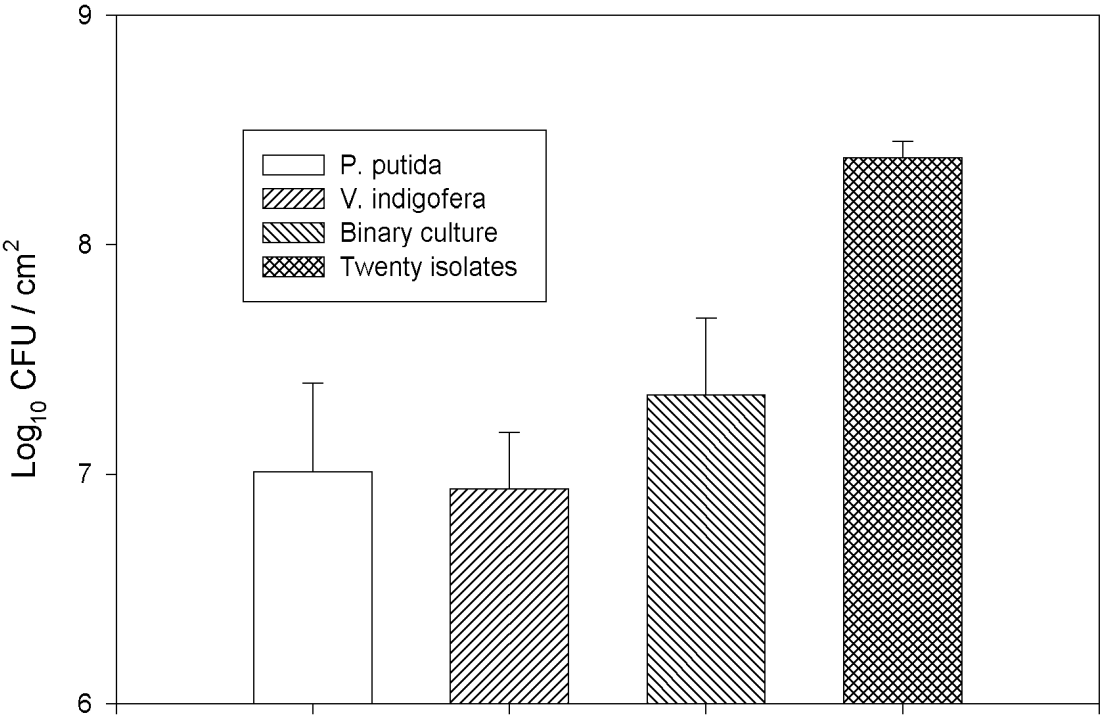

Figure 2. An illustration of the effect of community composition on 48-h chemostat-grown biofilms [32] of bacteria isolated from a karst aquifer (M Whiteley, JR Ott, EA Weaver, and RJC McLean [unpublished]. Error bars represent one standard deviation.

greatest biofilm cell density occurred when all twenty isolates were cultured together (Fig. 2). Monoculture biofilms exhibited the least cell density. Another interesting aspect of this study is that biofilm cell density was higher at the lower growth rate. This observation would suggest that biofilm formation is a response to starvation.

One consequence of biofilm formation is that the surface chemistry of a substratum is altered upon bacterial colonization. Bacteria can colonize a number of diverse substrata of varying hydrophilic or hydrophobic character [8, 18, 27, 31, 33]. Often adhesion is facilitated by the presence of a layer of molecules adsorbed to the substratum referred to as a conditioning film [4, 28]. Once colonized, the extracellular cell surface polymers (EPS) and metabolic activities of the bacteria within a biofilm determine the surface chemistry of

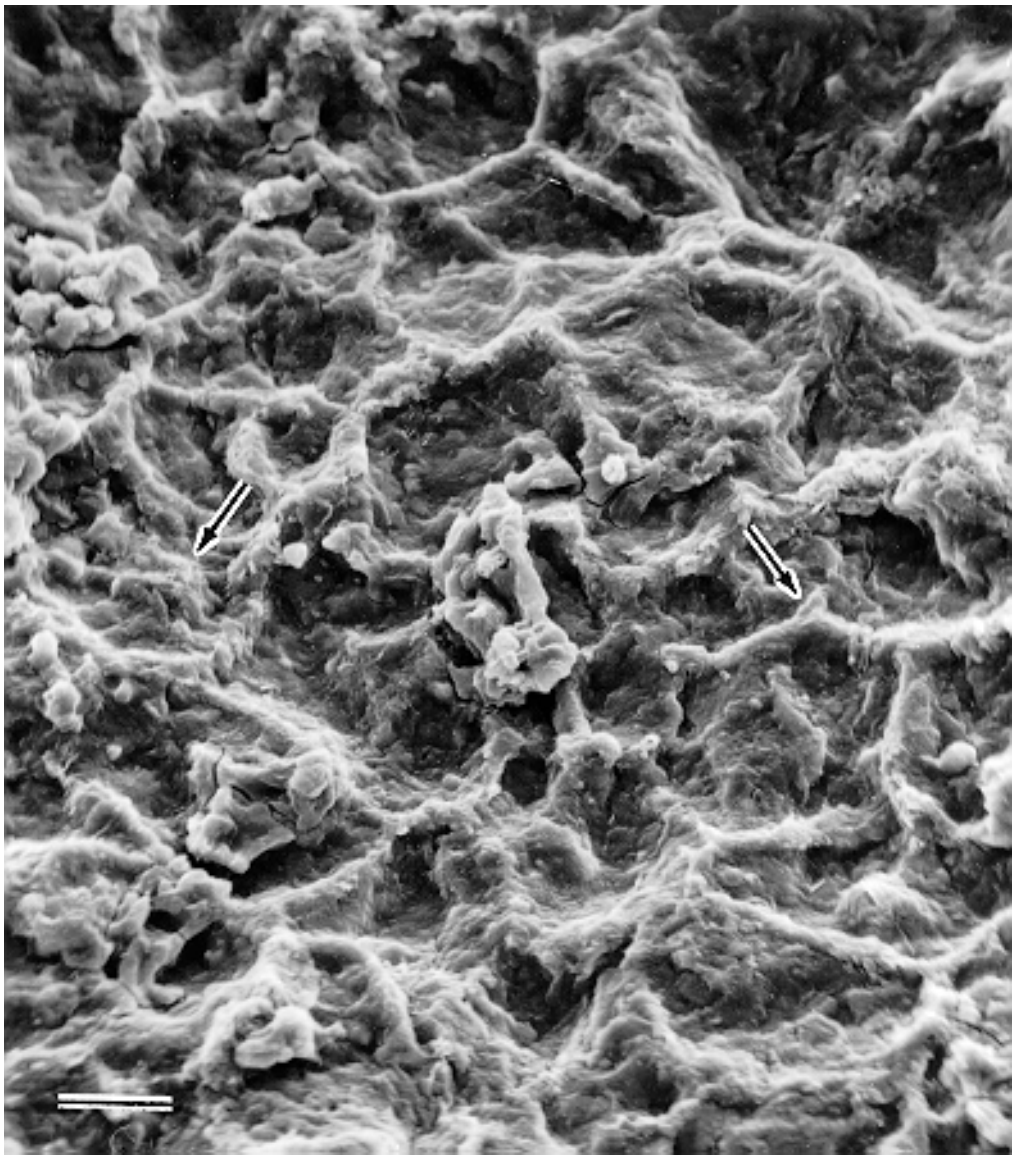

Figure 3. Morphological evidence of bacteria (arrows) from biofilms are seen in a scanning electron micrograph of *Nelumbites minimus*, leaf surface Potomac Group (Lower Cretaceous, Albian) of Maryland [10]. Bar represents 10 μm. This micrograph was kindly supplied by GR Upchurch, Jr., Department of Biology, Southwest Texas State University.

a substratum. Bacterial cell surfaces are typically anionic due to the presence of carboxylate or phosphate moieties in capsular or cell wall polymers. As a result, a colonized substratum will acquire an anionic character, regardless of its original chemistry. In aquatic environments, metal ions including Ca^{2+}, Mg^{2+} and Fe^{3+} will readily bind to and precipitate within anionic biofilms. Many examples of biofilm-mediated mineral formation are documented in the literature (for recent reviews see [2, 20 ,21]). Some examples include travertine formation by photosynthetic microorganisms in hot springs [6] stromatolite formation [5], and the association of fossilized microorganisms in cherts [30]. One unique aspect of biofilm formation was shown by Dunn et al. [10]. They showed that biofilms present on leaves in aquatic environments could nucleate mineral formation and so accelerate fossilization. Indeed scanning electron microscopy examination of leaf fossils from the Cretaceous (Fig. 3) shows evidence of biofilms, a finding that suggests a pivotal role of biofilms during the leaf fossilization process.

The chemical environment is not uniform throughout biofilms. Rather it is quite heterogeneous due to metabolic activities of component organisms and diffusion gradients due to the EPS-containing biofilm matrix. Chemical microenvironments within a biofilm can influence mineral formation or dissolution. In one instance, McLean et al. [22] illustrated how a *Proteus mirabilis* biofilm could protect the acid labile mineral, struvite, from dissolution in a pH 5.8 solution. Metal binding by EPS and respiratory activity of biofilm organisms is instrumental in microbial induced corrosion [12, 14, 16].

In summary, while the distribution of biofilms is well established, aspects of biofilm physiology and growth are not as well studied. Evidence to date supports the importance of quorum sensing and slow growth in biofilms. Other aspects of biofilm-specific physiology remain to be elucidated. In nature, biofilms are complex communities within which multiple species exist. Future studies of biofilm biology need to address interspecies interactions including those involving other prokaryotes, eukaryotes and even bacteriophage.

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