

PSEUDOMONAS AERUGINOSA BIOFILM STRUCTURE, BEHAVIOR AND
HYDRODYNAMICS

by

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November 29, 2004

I dedicate this work to my beloved husband Justin P. Gage

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ABSTRACT

Biofilm formation by bacterial pathogens is an important factor in the progression and treatment of many infectious diseases. Biofilm structural development is a dynamic process dependent on many cellular and environmental parameters including Quorum Sensing (QS) and hydrodynamics. Since QS is dependent on a threshold autoinducer concentration, it was hypothesized that the flow dynamics in the bulk fluid surrounding the biofilm would play an important role in expression of QS and the genes that are under its control. In order to investigate the relative contribution of hydrodynamics and QS on biofilm development, biofilms were grown from wild type *Pseudomonas aeruginosa* PAO1 and the cell signaling *lasI* mutant PAO1-JP1 under laminar and turbulent flows. When morphology of the biofilms were quantified using Image Structure Analyzer (ISA) software, a multivariate analysis demonstrated that both QS and hydrodynamics influenced biofilm structure, suggesting that QS was not required for biofilm development but affected structural heterogeneity in biofilms. GFP reporter based gene expression analysis of QS regulated *lasB* (coding for elastase) expression during biofilm development in laminar flow further supported these results.

Detachment has been recognized as another factor that may define structural morphology of biofilms. Under flow conditions hollow biofilm clusters were formed as a result of active detachment process, termed as “seeding dispersal”. A differentiation of a “seeding” microcolony into an interior motile, swarming, phenotype and a non-motile surrounding, “wall phenotype” formed as a prelude to the dispersal process in which the interior cells swarmed out of the microcolony from local break out points and spread over the wall of the flow cell. A critical microcolony diameter of approximately 100 μm was required for differentiation suggesting that regulation was related to cell density and mass transfer conditions. It was found that rhamnolipid (*rhlA*) biosurfactant was not required and QS system (PAO1-JP2) was shown to be important in this process, possibly by sensing nutrient limitation within the biofilm microcolonies.

These results strengthen a current view of multi-cellularity and coordinated behavior in prokaryotes as well as a dynamic network of overlapping pathways and cellular mechanisms that act on biofilm development in a complex interrelated manner.

CHAPTER 1

BIOFILM STRUCTURE, BEHAVIOR AND HYDRODYNAMICS

Introduction

During the past decade our appreciation for biofilm structural complexity and its significance in natural, as well as man made settings, have been augmented by the concurrent development of sophisticated optics based imaging instrumentation and molecular visualization techniques. Fundamental discoveries concerning biofilm structure made by Scanning Confocal Laser Microscopy (SCLM) images of *in situ* biofilms revealed sessile bacteria growing in matrix-enclosed microcolonies interspersed between open water channels (Lawrence, 1991; De Beer *et al.*, 1994). This complex architecture is now known to facilitate efficient nutrient uptake by allowing the flow to permeate into the biofilm from the bulk liquid via the channels (Stoodley *et al.*, 1994), thereby delivering nutrients and other essentials to deeply embedded parts of the biofilm community. Intriguingly, visual characteristics of biofilms growing in diverse environments are strikingly similar, suggesting convergent biofilm survival strategies conferred in part by structural specialization. For example, biofilms growing in fast moving water tend to form filamentous streamers whether in acid mine drainage runoff (Edwards *et al.*, 2000), photosynthetic algal or bacterial mats in thermal hot springs (Reysenbach and Cady, 2001) or periphyton in rivers. In quiescent water biofilms tend to form isotropic mushroom or mound like structures such as those seen in stromatolites. Similar structures can be formed in the laboratory by a diverse range of microorganisms (Figure 1.1).

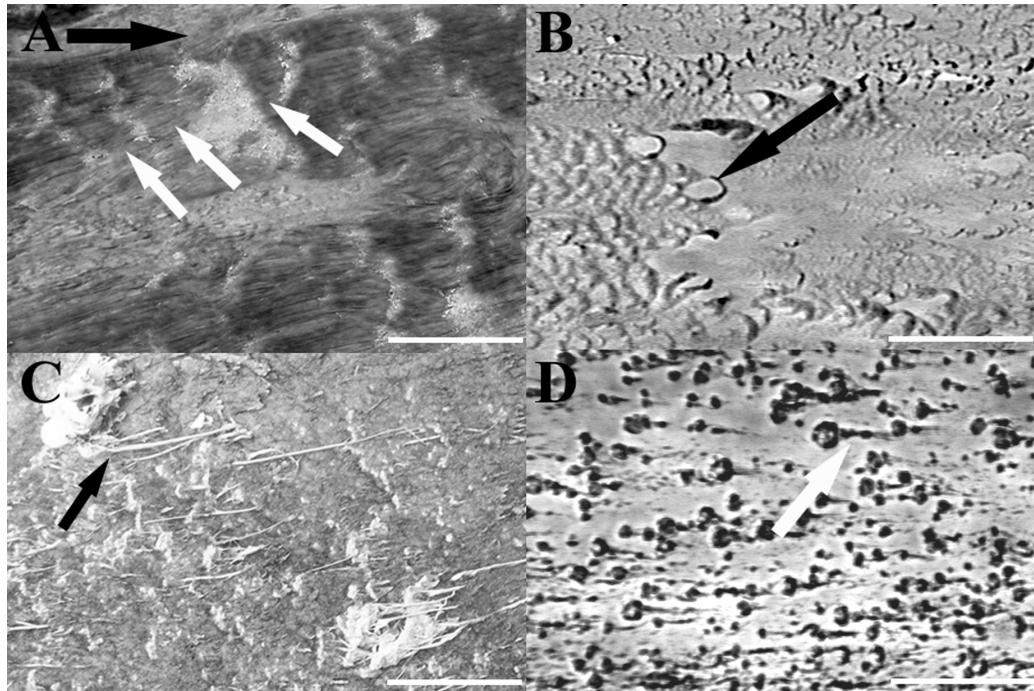


Figure 1.1. Structural phenotypes of both natural and laboratory biofilms are strikingly similar. A, a microbial biofilm growing in a hot spring in Black Sands geyser basin, Yellowstone National Park USA, forming ripple structures (individual ripples are indicated by white arrows) much similar to the ones formed by the mixed species biofilms in the laboratory, B (an individual ripple is indicated by a black arrow). C, Microbial biofilm with streamers in Canary Spring, Yellowstone National Park USA and similar streamers formed by a laboratory grown *Pseudomonas aeruginosa* biofilm, D (a streamer on panel C and D are indicated by a black and white arrow respectively). The fluid flow direction in all panels is the same and indicated by a black arrow on panel A. The scale bars on panel A and C are approximately 10 cm and 50 μm on B and D.

In mammalian biological systems it is clear that crucial physiological parameters, such as blood glucose and body temperature, are precisely regulated by a network of mechanisms, which allows the individual cells in the tissues to exist in precisely controlled homeostatic environments. Similarly, the homeostatic environment and collective integration of microbes into structured biofilm communities may have allowed the development of complex interactions and networks of regulations between the individual cells. In turn providing them advantages beyond mere survival – a further development of complex multi-cellular like system. Perhaps it is no wonder that attached

biofilms and microbial mats appeared early in the fossil record and have endured ca. 3.3 billions years to exist in modern environments (Rasmussen, 2000; Westall, *et al.*, 2001). The fundamental importance of biofilm formation by microbes is possibly revealed by the numerous redundancy and overlap of pathways involved in biofilm development as revealed by gene knock out studies which identify numerous genes as being “essential” or “required” for biofilm formation (Caiazza and O’Toole, 2003; Cucarella *et al.*, 2001; Cramton *et al.*, 1999; Hamon and Lazazzera, 2001; Froliger and Fives-Taylor, 2001; Gavín *et al.*, 2002; Whitchurch *et al.*, 2002). However, on closer inspection, in many of these studies biofilm formation is not totally prevented by the knocking out a specific gene but instead is either retarded or reduced.

In the context of evolution and adaptation it is appealing to consider that biofilms provided the homeostasis, in the face of fluctuating and harsh conditions of the primitive earth, which allowed the development of complex interactions between individual prokaryotes. This homeostasis may have even possibly allowed the development of more complex cellular functions such as motility, which initially may have allowed cells to move within biofilms and later to spread out to colonize new surfaces (Stoodley *et al.*, 2002a). Interestingly, biofilm formation is also found in prokaryotes from the most deeply rooted branches of the phylogenetic tree in both the *Archaea* and *Bacteria* kingdoms, the *Korarchaeota* and *Aquificales* respectively (Jahnke *et al.*, 2001; Reysenbach *et al.*, 2000). Taken together these data suggest that biofilm formation is an ancient and integral component of modern day prokaryotic life. To control them against their renowned resilience we may be fighting over 3 billion years of evolutionary adaptation for survival on surfaces in hostile environments.

Historical Aspects of Biofilm Structure

Some of the earliest evidences of biofilm documentation can be traced back to Van Leeuwenhoek's crude description of microorganisms on tooth surfaces, and in 1940 observation by Heukelekian and Heller (1940) that growth and activity for some marine bacteria were substantially enhanced by surface incorporation, and three years later by Zobell (1943) that the concentration of marine bacteria on surfaces was dramatically higher than in the surrounding bulk media. By using a specific polysaccharide-stain Jones *et al.*, (1969) demonstrated that the matrix material surrounding the sessile cells was what would later be a hallmark of biofilms, a polymer of various sugars. The tenacity and resistance to conventional disinfectants, now known to be common characteristics of biofilms were later discovered by Characklis (1973). In general the concept of biofilm structure before the advent of sophisticated optical and molecular tools was overly simplified and biofilms were perceived to be "slabs" of matrix material with randomly embedded cells. However, four-dimensional images of biofilm using SCLM revealed an organized complex architecture suggesting a potential link to function and behavior. As our knowledge of biofilms progressed Costerton *et al.* (1987, 1994, 1999 and 2001) has extended the concept of prokaryotic life in biofilms to a wide variety of microbial ecosystems ranging from the natural to the man made, including man himself, all of which has opened a new arena for considering the role of biofilms in industry and medicine. In light of these discoveries, microbial biofilms are now recognized as being widely distributed occupying every niche on the planet and have now become one of the most hotly pursued research areas in microbiology.

Factors Relating Hydrodynamics and Structure

Our current understanding recognizes biofilm structural development as a dynamic multifactorial entity which is constantly fluctuating both in time and space (Hall-Stoodley and Stoodley, 2002). Contrary to its terminology, the biofilm has a rich repertoire of structures which can range anywhere from patchy monolayers, to thin or thick flat biomasses to more organized mushrooms, ripples and filamentous streamers (Stoodley *et al.*, 1999c) (Figure 1.2).

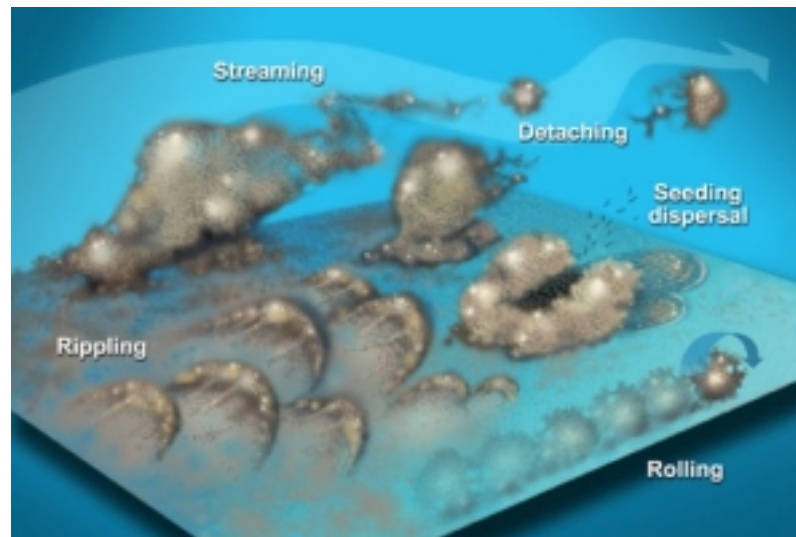


Figure 1.2. Dynamic behavior and associated structural phenotypes of bacterial biofilms in flowing fluids. Schematic by P. Dirckx, MSU-CBE, 2003.

Although the underlying mechanisms that shape biofilm architectural development are yet to be completely characterized, many different external and internal parameters, both biotic and abiotic, have been shown to play a role in the process. Given the redundancy and overlap of regulatory pathways involved in the biofilm maturation (Caiazza and O'Toole, 2003; Cucarella *et al.*, 2001), it is difficult to assign any one

particular parameter to any resultant property in the biofilm structure. In addition, biofilms are known for their complex heterogeneous patterns which can vary even over small distances of less than a millimeter, making comparison of isogenic variants to the wild type (WT) parent difficult. These properties of the biofilm in fact pose one of the biggest challenge in the biofilm structural studies and are, perhaps, one of the main causes for discrepancies in scientific reports (O'Toole and Kolter, 1998; De Kievit *et al.*, 2001; Heydorn *et al.*, 2002; Nivens *et al.*, 2001; Davies *et al.*, 1998; Purevdorj *et al.*, 2002; Stoodley *et al.*, 1999b). Nevertheless, in this chapter we attempt to cover the major discoveries associated with the biofilm architecture and discuss some instances where they in turn may be affected by environmental hydrodynamics.

Understanding the significance of hydrodynamics in the biofilm life cycle is crucial if we consider the habitats in which the most abundant biofilms tend to accumulate. These biofilms are usually found in hydrated systems growing under a wide range of shear stress from the almost stagnant such as in unmixed lakes and the depths of seabeds to high shear turbulent flow in the rivers. For instance filamentous biofilms of both archeal and bacterial origins are known to proliferate in fast-flowing environment of hot springs (Reysenbach and Cady, 2001) and acid mine drainage runoff (Edwards *et al.*, 2000), suggesting the presence of different survival adaptations in these high flow and shear environments. In the evolutionary perspective microbial survival in these non-permissive streams may select for the formation of surface associated communities over free swimming planktonic cells, which would be easily swept by the flow to potentially hostile environments downstream. The viscoelastic nature of pure and mixed culture (including *Desulfovibrio spp.*, *Pseudomonas aeruginosa*, *Streptococcus mutans*,

Staphylococcus aureus, tap water, pond water, and hot spring cyanobacterial and algal biofilms (Stoodley, P. unpublished data) may be an important adaptive strategy for survival in flowing environments, by allowing the biofilm to absorb a certain amount of fluid energy elastically, but under sustained elevated shear permit flow along surfaces while remaining attached (Körger *et al.*, 2001; Towler *et al.*, 2003; Klapper *et al.*, 2002; Winston *et al.*, 2003; Stoodley *et al.*, 1999d). Even when shear does induce detachment *in vitro* studies suggest that only localized parts of the biofilm detach, the homeostatic environment provided by the remaining attached “mother colony” allows survival for rapid regrowth. In addition to viscoelastic properties, the ability to coaggregate facilitates microbial proliferation in high shear environments (Handley *et al.*, 2001; Rickard *et al.*, 2002). Interestingly, coaggregation is observed primarily during the stationary growth phase in intergeneric, intrageneric and intraspecies manner via lectin-saccharide interaction (Rickard *et al.*, 2000; Rickard *et al.*, 2002). Finally, there is evidence of biofilm structural modulation in response to varying shear conditions, perhaps another example of biofilm structural flexibility to better suit the fluctuating environment. For instance, in laminar or low shear, flow the biofilm microcolonies often assemble into amorphous aggregates, roughly hemispherical or cylindrical in shape. Whereas in turbulent or high flow, the circular symmetry tends to diminish and filamentous streamers form instead (Stoodley *et al.*, 1999a). While these *in vitro* studies directly demonstrate the importance of hydrodynamic shear, we will now discuss some of the other influences of hydrodynamics on biofilm structural development, such as mass transfer of nutrients and signaling molecules.

Extracellular Polymeric Substances (EPS) (Slime) Matrix

One of the key factors in the biofilm structure is the Extracellular Polymeric Substance or Extracellular Polysaccharides (EPS) which form the biofilm matrix. EPS provides the slimy matrix in which cells are localized, and also acts as a protective layer against potentially harmful agents as well as a carbon and energy source at times of nutrient deprivation (Liu and Fang, 2002). Additionally, EPS has been shown to be crucial for flocculation (Frølund *et al.*, 1996) and for microstructure of methanogenic granular sludges (Schmidt and Ahring, 1999). The chemistry of the EPS is complex and although carbohydrate is often the predominant constituent (Cescutti *et al.*, 1999; Sutherland and Kennedy, 1996) a wide variety of organic substances such as proteins (Fang and Jia, 1996; Veiga *et al.*, 1997), humic acids (Frølund *et al.*, 1995), and deoxyribonucleic acids (Tsuneda *et al.*, 2001; Zhang *et al.*, 1999; Whitchurch *et al.*, 2002). However, it is not yet clear if the extracellular proteins or nucleic acids play an active structural role or are, more passively, the entrapped contents of lysed cells. Traditionally EPS was generally thought to carry a net negative charge (Donlan, 2002), however, more recent *in situ* studies reveal that the bacterial EPS composition is heterogeneous both chemically and spatially. For instance Wolfaardt *et al.*, (1994) noticed that hydrophobic, fucose-rich EPS regions contained both positive and negative charges, suggesting existence of the specific functional regions within complex biofilm communities (Moller *et al.*, 1997). Interestingly, Stoodley *et al.*, (2001) have demonstrated that the EPS chemistry can be altered by cation cross-linking, and in the process the mechanical strength of the biofilm was substantially increased. The strength and structure of the biofilms can also be regulated by varying the chain length of EPS

polymers and by polymer modification. In *P. aeruginosa* O- acetylation of the alginate EPS (Nivens *et al.*, 2001) or over expression of alginate itself (Henzter *et al.*, 2001) produced structurally more differentiated biofilms compared to the parent strain.

It also appears that biofilms can adjust the mechanical properties of their EPS in response to hydrodynamic shear. Biofilms grown in high shear conditions had a stronger EPS matrix and subsequently more strongly adhered cells than those grown under lower shear (Stoodley *et al.*, 2001). The suggested mechanism behind the observation is that when the biofilm stretches in response to higher shear the individual polymer strands may become physically aligned allowing additional electrostatic as well as hydrogen bonding to occur between the closely pulled neighboring polymers (Flemming *et al.*, 2000). Although the EPS chemistry and its physical arrangement in the biofilm matrix may explain differences in biofilm strength, it is also important to keep in mind the physiological responses such as increased EPS production as suggested by Applegate and Bryers (1991) or by regulation of metabolic pathways in response to shear (Lui and Tay, 2001).

Nutrient Source and Growth in Relation to Hydrodynamics

The clonal growth of attached cells is one of the mechanisms by which the biofilm can initiate structural development (Heydorn *et al.*, 2000). In the process of cell division, the resulting daughter cells can pile up to form initial microclusters, then with localized growth and proliferation it can subsequently differentiate into more complex architectural phenotypes (Stoodley *et al.*, 1999a; Stoodley *et al.*, 1998; Lawrence *et al.*, 1991; Nivens *et al.*, 2001; Henzter *et al.*, 2001). While many environmental conditions,

as well as the inherent nature of organism itself would define the resulting biofilm growth and structure, the type of energy source and its availability, namely its transport into biofilm cells are one of the major factors to consider. In general nutrients are dissolved in the liquid flow and must diffuse first through the mass transfer boundary layer (external mass transfer) and then through the biofilm matrix (internal mass transfer) in order to reach the bacterial cells (Characklis and Marshall, 1990). The thickness of the boundary layer is inversely correlated to the flow pattern over the biofilm surface, meaning that at higher shear condition, the thickness of the boundary layer decreases and results in increased rate of nutrient diffusion into the biofilms. This was shown by increased biofilm density observed in high shear compared to low shear environment (Peyton, 1996, Gantzer *et al.*, 1991; Characklis and Marshall, 1990). Numerous studies utilizing SCLM have demonstrated the effect of nutrient composition on biofilm structure (Massol-Deya *et al.*, 1995; Wolfaardt *et al.*, 1994; Grotenhuis, 1991; Caldwell, 1992; Moller *et al.*, 1997). De Kievit *et al.*, (2001) have showed that in a static condition, *P. aeruginosa* biofilms formed a thick, multilayer biofilm in M9 medium yet a sparse monolayer in the FAB medium. However, when the biofilms were subjected to the flow the biofilms supplied with different media were virtually indistinguishable. Intriguingly, global regulator of carbon metabolism, CsrA, was shown to have a profound effect on the biofilm formation in *Escherichia coli* (Jackson *et al.*, 2002) as well as in *P. aeruginosa* by regulating in part the expression of twitching motility phenotype, which is in turn required for biofilm structural development (O'Toole *et al.*, 2000). In addition, in response to high shear the biofilms have been shown to regulate their metabolic pathways (Liu and Tay, 2001) and display enhanced proton translocation, resulting in robustly

adhered biomass. These studies demonstrate intricate interplay between the nutrient composition and the hydrodynamics in defining the biofilm growth and subsequent architecture as well as the importance of consideration of these parameters in the experimental set-up upon the relative contribution of factors on structure.

Detachment and Dispersal Mechanisms

The detachment process is an important component of the biofilm life cycle and plays a fundamental role in dissemination and contamination and ultimately the long term survival in either natural (Nickel *et al.*, 1994) or man made settings of industrial and medical importance (Zottola *et al.*, 1994; Walker *et al.*, 1995; Piriou *et al.*, 1997). Despite the importance of detachment in biofilm development, very little is known about the biological, chemical, and physical mechanisms underlying the detachment process (Stewart *et al.*, 2000). However, apart from recognized internal mechanisms like enzymatic dissolution of the matrix (Allison *et al.*, 1998), physical forces such as hydrodynamics or shear is known to cause biofilm detachment via either erosion of single cells or sloughing of large aggregates of biomass (Bryers, 1988, Stoodley 2002b).

Currently biofilm structure is thought of primarily in terms of growth and accumulation via polymer production however, it is becoming clear that detachment process may also play an important if not equal role in the morphological characteristics and structure of mature biofilms (Van Loosdrecht *et al.*, 1995, 1997; Stewart, 1993; Picioreanu, 2001). Reports of the “hollowing” out of microcolonies by cells actively leaving the interiors are being noted by a number of laboratories (Sauer *et al.*, 2002) and the remaining “hollow mounds” have been described in the literature (Tolker-Nielsen *et*

al., 2000) in *P. putida*. However, the phenomenon may be more widespread, Kaplan *et al.* (2003) reported that “non-motile” *Actinobacillus actinomycetemcomitans* microcolonies grown statically on agar swarmed out of the colony and spread over the surface, but the actual “swarming process was not observed. Hollow microcolonies have also been seen in *Staphylococcus epidermidis* on agar plates (Stewart, P., personal communication). However, in this case transmission electron micrographs (TEM) suggest that the hollowing may have occurred through lysis of cells within the colony. In ongoing experiments in our lab we have observed the differentiation of *P. aeruginosa* biofilm cluster into distinct phenotypes of nonmotile cells forming rigid walls surrounding the hollow structure with free swimming cells in the center of the cluster (Figure 1.3).

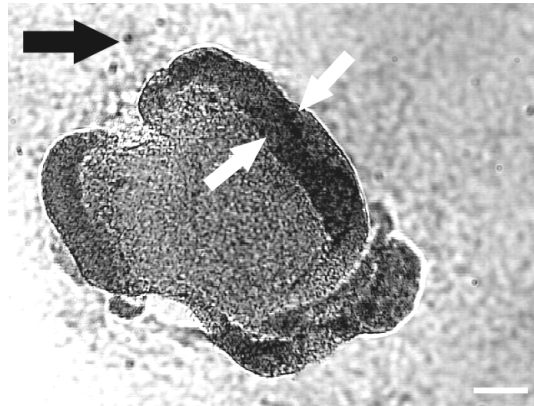


Figure 1.3. A structurally specialized *P. aeruginosa* biofilm cluster. The cluster is composed of an outer layer of non-motile cells forming a "wall" (indicated by white arrows). In the interior of the cluster cells swim about rapidly before flowing out leaving the cluster empty. The scale bar was 20 μm and the flow direction is indicated by the black arrow.

From our studies we have observed that these structures have resulted from release of individual or eruption of aggregates of cells from interior portion of the cluster back to the bulk media surrounding the biomass. This seeding dispersal process may

provide the detached cells the chance to explore more favorable conditions elsewhere. Although the mechanisms of seeding dispersal as well as role of hydrodynamics in this process are largely unknown, it appears to possess its own distinct genetic regulatory pathway different from those involved in the developmental process (O'Toole, 2000) and could be one of the major dispersal mechanisms in the biofilm life cycle.

The Role of Motility in Biofilm Formation and Structure

So far 80% of all known bacteria are motile (Aizawa, 1996) and able to move over the surfaces via twitching motility mediated by type IV pili and by flagella based swarming motility (Costerton *et al.*, 1999; Harshey *et al.*, 1994). In the biofilm developmental process, type IV pili-mediated twitching and flagellar based motility was shown to play an important role in biofilm initiation by surface aggregation in *P. aeruginosa* (O'Toole and Kolter, 1998) as well as in *Aeromonas spp* biofilms (Gavin *et al.*, 2000). Motility has been shown to influence the adhesion of bacteria to various surfaces in the flowing systems (Korber *et al.*, 1994; Mueller *et al.*, 1996) as well as in the static systems (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Watnick and Kolter, 1999). However, the consideration of hydrodynamics is also important as revealed by De Kievit *et al.* studies (2001) where type IV pili were important during static biofilm growth as well as in very low-flow or intermittently flowing environments but not in a constantly flowing, high shear system. The authors suggest that the cells subjected to the shear force, have limited surface movement via type IV twitching motility and initial microcolony formation result primarily from cellular division rather than a combination of cell clustering and division. Similarly flagellar motility has been

shown to be required for the initial attachment and development of a biofilm in number of different microorganisms (O'Toole and Kolter, 1998). Although there are some evidences suggesting that motility enhanced and strengthened adhesion at high flow compared to low flow conditions (McLaine and Ford, 2002), the dependence of the flagellar motility on the fluid velocity is yet to be fully characterized.

In summary there are a myriad of factors which influence biofilm development and structure. However interaction between hydrodynamics and each of these parameters are poorly characterized. Although in this work we discussed the influence of hydrodynamics and shear as the major factors that are involved in the biofilm architecture, it is important to keep in mind that all of the identified, and yet to be discovered factors, are involved in a dynamic interrelated manner and there is no single global regulating pathway so far known to control this process.

Concluding Remarks

In this chapter we have discussed the roles of hydrodynamics and shear on the biofilm structural maturation as well as how they may affect the major factors that are involved in the process. In vitro studies performed in the lab as well as the observations of biofilms growing in the natural environment demonstrate the importance of hydrodynamics and shear in the biofilm structural development. Moreover, many survival strategies acquired by biofilms to suit wide range of environmental shear, now assist the successful colonization of wide variety of medical and industrial settings including human beings themselves, which can exhibit shear pattern no different than one seen in nature. For instance many surfaces of medical and industrial devices such as water

distribution pipelines, transepidermal devices, catheter and dialysis machines as well as many parts of the human body such as teeth, lungs, circulatory system, lymph, tear ducts, ear wax etc all experience both normal as well as mechanical shear and are suitable surfaces for unwanted biofilm accumulation. Although our understanding of biofilm structure and its development has significantly advanced during the past two decades we are still facing the fundamental question of how to relate specific structures to function and behavior in different environments. The answer will assist in our battle to control biofilms against a period of over 3 billion years during which prokaryotes have adapted survival strategies to remain on surfaces in flowing fluids.

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CHAPTER 2

THE ROLE OF CELL SIGNALLING IN BIOFILM DEVELOPMENT

Introduction

During the past 30-40 years with the advancement of sophisticated genetic and biochemical tools increasing evidence of chemical communication systems in bacterial population has been provided. Requirement for threshold of cell concentration during process of bioluminescence production in marine symbiotic bacteria *Vibrio fischeri* (Nealson and Hastings 1979), formation of fruiting body in myxobacteria (Dworkin and Kaiser 1982) and development of competence for genetic transformation in streptococci (Dawson and Sia 1931) are among the first evidence of cell-to cell signaling systems in bacterial communities. At present the term Quorum Sensing (QS) is defined as a cell-density dependent bacterial intercellular signaling mechanism that enables bacteria to coordinate the expression of certain genes to coordinate the group behavior.

Although the signaling mechanisms differ, QS systems are utilized by both gram positive and negative bacterial species (extensively reviewed by Miller and Bassler 2001). In general to sense their population density Gram negative bacteria use small chemical molecules called acylated homoserine lactones (AHLs) (reviewed by Fuqua *et al.*, 2001) and gram positives use oligo-peptides (Kleerebezem *et al.*, 1997), both collectively referred to as autoinducers. While the autoinducers are constitutively expressed, a threshold level must be achieved in the extracellular environment in order to initiate the QS system (in the closed system most often it occurs during late exponential and stationary phase). Some signal molecules are able to freely diffuse through the cell

membrane but some require active transport system (Pearson *et al.*, 1999). Inside the cell, in association with the transcriptional activators the threshold level of autoinducers is capable of inducing the expression of various genes.

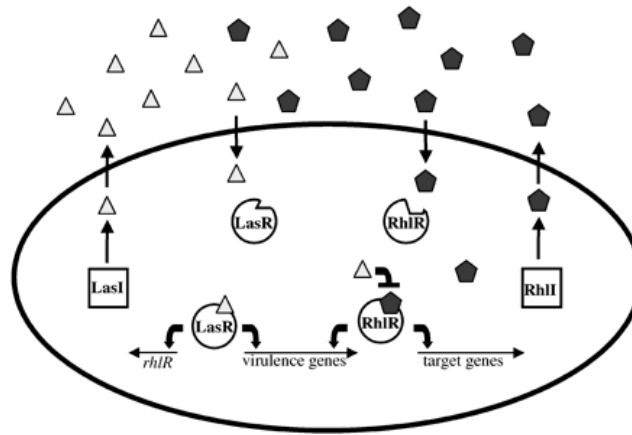


Figure 2.1. Example *P. aeruginosa* LasI/LasR-RhII/RhIR QS system. *P. aeruginosa* uses two LuxI/LuxR-like autoinducer-sensor pairs for QS regulation of a variety of genes. The LasI protein produces the homoserine lactone signaling molecule N-(3-oxododecanoyl)-homoserine lactone (triangles), and the RhII protein synthesizes N-(butyryl)-homoserine lactone (pentagons). Ongoing research continues to reveal increasingly complex regulatory pathways with many interlinked positive and negative feedback loops. After Miller and Bassler 2001.

The target genes are involved in various physiologic activities such as symbiosis, competence, conjugation, antibiotic production, motility, sporulation etc (Lee and Morrison 1999, Li *et al.*, 2001a and b Grossman 1995, Gutowski-Eckel 1994 and Ji *et al.*, 1995). Understanding of the QS systems in clinically relevant strains has become the focus of intense study during the last decade when many QS regulated genes were also found to be involved in virulence (Costerton *et al.*, 1999). The idea that the biofilms are optimum sites for expression of phenotypes regulated by QS (Williams and Stewart 1994), has led to numerous studies of QS mechanisms in the bacterial biofilms.

Interspecies QS Interactions

Since most signal molecules are conserved across many different species, particularly in gram negative bacteria, it would be reasonable to predict that some form of interspecies communication is likely in natural ecosystems where different acyl HSL-producing species inhabit a common locale. Interspecies QS system would play an important synergistic or competitive role, in the dynamics of the microbial communities. In fact, recent advances in the field indicate that cell-cell communication via QS occurs not only within but between bacterial species (Bassler *et al.*, 1997, Surette *et al.*, 1999). *In vitro* study of mixed species of biofilm demonstrated that *Burkholderia cepacia* was capable of perceiving the AHL signals produced by *P. aeruginosa*, while the latter strain did not respond to the signal molecules produced by *B. cepacia* (Riedel *et al.*, 2001). Interestingly, these two species of bacteria are capable of forming mixed biofilms in the lungs of CF patients and perhaps the ability of these organisms to communicate with each other may facilitate successful colonization of a host lung. In the environment where two species commonly encounter each other, the ability to sense signal molecule production and interfere with the normal communication of the other species would provide an important survival advantage. A soil microorganism *Bacillus subtilis* produces an enzyme called AiiA which inactivates the HSL autoinducer of another soil habitat *Erwinia carotovora*, rendering the latter avirulent (Dong *et al.*, 2000).

In general, laboratory based findings of QS in bacterial species are helpful; however, more research is needed to understand the importance of such systems in a natural ecosystem in the presence of resident microbial communities.

Quorum Sensing in Biofilm Establishment and Structural Development

Microbial biofilms may be defined as populations of microorganisms that are concentrated at an interface (usually solid/liquid) and typically surrounded by an extracellular polymeric slime matrix (EPS) (Costerton *et al.*, 1995). Biofilms appear to be more resistant to chemicals and biocides as compared to their planktonic counterparts and also better able to evade host immune defence such as phagocytes or antibodies. The resistance of biofilms confers the ability of the cells to thrive in varied and often harsh environmental conditions such as inside host organisms, surface of the pipelines, dental unit water lines, catheters, ventilators and medical implants, causing tremendous problems in both industry and medicine. From the sessile, matrix-bound community, planktonic cells can be continuously shed from the biofilm (Bryers 1988, Stoodley *et al.*, 2001), also the recently discovered shear mediated downstream movement of intact biofilms along the surface (Stoodley *et al.*, 1999a), could all lead to contamination, or in humans to a systemic and often times chronic infection. It is now estimated that about 65% of all nosocomial infections are biofilm-related (Archibald *et al.*, 1997). Life threatening infection caused by *Pseudomonas aeruginosa* biofilms in Cystic Fibrosis (CF) patients is one of many examples. Since biofilm is of concern enormous effort has been directed toward understanding the mechanisms of biofilm establishment and development. Biofilm establishment and development is a dynamic multifactorial process governed by both environmental and genetic control systems. Although many bacteria are capable of forming biofilms much of what we know today about biofilms is gained through experiments largely based on pure cultures of *Pseudomonas spp.* The current

model of biofilm establishment describes several steps starting from free-floating planktonic cells attaching on the surface followed by growth into complex pillar like structures intervened with water channels, which eventually may disperse via detachment of individual cells into the bulk fluid. The shape and structure of the biofilm is an important aspect of the biofilm life cycle as it may determine mass transport of solutes such as antimicrobials or nutrients, biofilm detachment, and energy losses in industrial pipelines (Stewart 1998, Stoodley *et al.*, 1997). Although the underlying mechanisms that define biofilm structural phenotype have not been completely characterized, previous studies have demonstrated that multiple environmental cues such as fluid flow (Vieira *et al.* 1993, Stoodley *et al.*, 1999a), carbon source (Moller *et al.*, 1997, Wimpenny and Colosanti 1997, Stoodley *et al.*, 1999a) and surfaces (Dalton *et al.*, 1994) all play important roles in biofilm process. Also, key physiological functions such as twitching motility mediated via type IV pili (O'Toole and Kolter 1998), the global carbon metabolism regulator, Crc (O'Toole *et al.*, 2000) and stationary phase σ factor RpoS (Heydorn *et al.*, 2000b) were shown to be important in biofilm initial formation of microcolonies and growth. Subsequently, QS, has also been shown to be involved in differentiation of the *Pseudomonas aeruginosa* biofilms into a mature biofilm consisting of mushroom-shaped microcolonies interspersed with water channels (Davies *et al.*, 1998). Since QS is a concentration dependent phenomenon, it is not likely to occur during initial stages of biofilm formation but rather in later stages when the cell density is high. This was demonstrated in a study where detailed quantitative analysis of *Burkholderia cepacia* biofilm structures formed by wild-type and mutant strains showed that the QS was not involved in the regulation of initial cell attachment, but rather

controlled the maturation of the biofilm (Huber *et al.*, 2001). QS has also been linked to biofilm structure in oral bacteria that initiate dental plaque formation *Streptococcus gordonii* (Loo *et al.*, 2000), *Streptococcus mutans* (Li *et al.*, 2002), *Salmonellae spp.* (Prouty *et al.*, 2002) and in the opportunistic pathogen *Aeromonas hydrophilia* (Lynch *et al.*, 2002). The potential role of QS in biofilm development has been demonstrated in *in situ* studies where functional autoinducers were detected in biofilms existing in river sediments (McLean *et al.*, 1997), urinary catheters (Stickler *et al.*, 1998) and recently in the sputum of CF patients (Erickson *et al.*, 2002, Middleton *et al.*, 2002 Wu *et al.*, 2000 and Singh *et al.*, 2000). It has also been shown that QS expression in *Pseudomonas putida* biofilm coincided with marked changes in biofilm morphology which switched from consisting of microcolonies to thick distinct mushroom structures with intervening water channels (De Kievet *et al.*, 2001). The expression of QS gene was substantial at the substratum where bacterial cell density and signal accumulation is expected to be high.

Although many studies pinpoint to the direct role of QS in controlling the biofilm structural development, it is increasingly evident that QS-biofilm structure relationship is more complicated. Given the many genes and pathways involved in biofilm formation, the redundancy and overlap of regulatory pathways in biofilm development, it is highly likely that biofilm would behave differently and may not use same mechanism under different environmental condition and QS is not an exception. To complicate the matter, bacterial biofilms often form a variable complex heterogeneous pattern even over small distances of less than a millimetre, making comparison of isogenic variants to the wild type (WT) parent difficult and possibly resulting in apparently contradictory results. For example recently it was reported that wild type *P. aeruginosa* biofilm was structurally

flat (Heydorn *et al.*, 2002, Nivens *et al.*, 2001) and resembled the QS mutant biofilm described in Davies *et al.*, (1998) study. Also, De Kievet *et al.*, (2001) have reported that under static condition with glucose as a carbon source the biofilm developed into a thick multilayer but with citrate it formed only a sparse monolayer. This structural difference was attributed in part to *pilA* gene, responsible for twitching motility, regulated by global regulator of carbon metabolism, Crc (O'Toole *et al.*, 2000). Interestingly in a flow through system the difference in medium composition did not significantly affect the structural development, suggesting the QS regulating factor(s) in biofilm is differentially expressed under different environmental conditions (De Kievet *et al.*, 2001). Since QS is a concentration dependent phenomenon it will be influenced by mass transfer processes or liquid flow surrounding the biomass. The flow condition may influence the concentration of signal molecules and thus affect QS mechanism in the biofilm.

Purevdorj *et al.*, (2002) have demonstrated that a mutation in the QS pathway only had a limited effect on biofilm structure but that flow dynamics had a much more pronounced effect. These findings indicate that the QS may not be the determining global system in the biofilm development, and other environmental factors such as hydrodynamics, carbon source, mass transfer etc. may also play role in this dynamic process. More studies needed to elucidate the importance of QS in the biofilm structural development and many questions are still remaining to be resolved. What QS controlled genes are involved in the biofilm development? To what extent and in what environmental conditions does QS play significant role in the biofilm development and is there a system where the biofilm development is independent of QS?

Methods of QS Study in the Biofilms

Advancement in methods technology such as improvements in fluorescent molecular techniques, design of suitable systems for biofilm cultivation, more sophisticated microscopy and digital imaging systems have enabled non-destructive, direct analysis of biofilms *in vitro*, which have greatly benefited our understanding of biofilm structural development as a complex community.

Flow Cells

Naturally occurring biofilms are often found in places where it is difficult to access, making direct analysis impossible. However, biofilm study is possible through culturing in a relatively simple laboratory model system. At the present there are no standardized methods of cultivating biofilms and different laboratories utilize numerous different methods and devices for their biofilm studies. Flow cells are one of the most commonly used reactors systems. For their simplicity they are proven to be useful in growth and in-situ visualization of biofilms. Flow cells allow control of flow velocity as well as the loading rate of nutrients or other test compounds. Multi-channel flow cells exhibit efficiency by producing experimental data in replicates as well as convenience of side by side running of the control and test biofilms. Traditional transmission light microscopy, scanning confocal laser or epifluorescent microscopy all can be used to monitor in situ biofilm growing in glass flow cells. There are many types of flow cell (Hall-Stoodley *et al.*, 1999a, Palmer *et al.*, 1999, Zinn *et al.*, 1999) and they can be either once through or recirculating. In once through system, nutrients or testing compounds are pumped through the flow cell into the waste container at the effluent end (Palmer *et al.*,

1999). In this system, the flow rate may not be adjusted independently of residence time and the experiments are largely limited to the low flow conditions due to the cost and time associated with media preparations. In re-circulating system the flow cells are incorporated into re-circulating loop attached to a mixing chamber. This system allows the flow rate to be independent of the nutrient flow rate, allowing high flow experiments without impractical volumes of media. Stoodley *et al.*, (this volume) provides a detailed description of the flow cell and associated results with the system.

The role and function of the QS systems in autoinducer producing microorganisms can be determined via several different methods such as utilization of reporter gene and isogenic variants of parental strain for structural comparison.

Reporter Genes

The direct and temporal quantification of specific gene products within biofilm bacteria is now possible by combining flow cell with fluorescent based reporter technology. The green fluorescent protein (GFP) originally derived from jellyfish *Aequorea victoria* (238 amino acids), has been mutated to improve the fluorescence intensity of the reporter protein and chromophore formation kinetics (Cormack *et al.*, 1996, Heim *et al.*, 1995). GFP has become an important visual marker of in-situ gene expression because it is non-toxic and does not require a special substrate or cofactor for detection. It has been used in many different areas of protein tracking and expression analysis such as in studies of the starvation gene expression (Wolfaardt *et al.*, 1996), induction of meta-pathway promoter (Moller *et al.*, 1998), to monitor chitinase gene

expression (Stretton *et al.*, 1998) as well as in QS regulated gene expression studies (De Kievit *et al.*, 2000, Heydorn *et al.*, 2000).

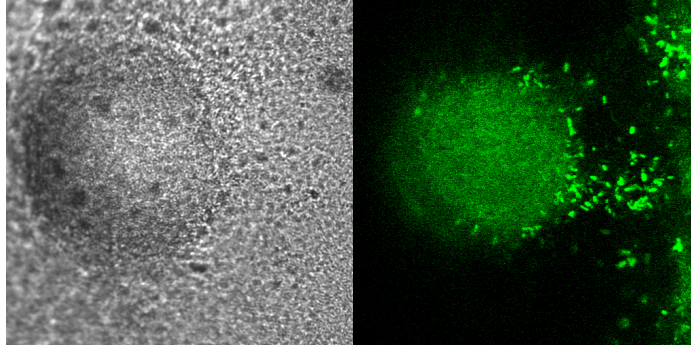


Figure 2.2. *P. aeruginosa* pMH 516 *plasB::gfp* biofilm in the flow cell expressing the GFP (40x objective lens). Transmitted and Scanning Laser Confocal Microscopy (right) image.

One disadvantage of using GFP is that the wild-type and original mutated GFP were very stable, with half lives greater than 24 hours (Tombolini *et al.*, 1997). This means that the intracellular pool of GFP may not immediately decrease after reduction of gene expression, but rather through dilution by cell proliferation. To increase temporal resolution of gene expression, mutants producing unstable GFP have been created via addition of tale-specific protease (Andersen *et al.*, 1998). This allowed analysis of downshift in gene expression. By using fusions of GFP with a half-life as short as 40 minutes, De Kievit *et al.*, (2001) were able to analyze real-time temporal and spatial expression of the two QS – genes *lasI* and *rhII* in *P. putida* biofilm. Another disadvantage is the dependency of fluorescence on oxygen. Care must be taken not to misinterpret heterogeneity in oxygen distribution in the biofilm with heterogeneity in gene expression. This can be tested by using a constitutively expressing mutant which produces the same fluorophore as a control. Luciferase activity is also used to monitor gene expression in

biofilms. Luciferase catalyzes the oxidation of reduced flavin (FMNH₂) to form intermediate peroxide, which reacts with a long-chain aldehyde to give blue-green luminescence emitting at 490 nm (Woodland 1996). The bioluminescence activity can be measured by X-ray or photographic films, by visual or microscopic observations and by luminometer or a scintillation counter in chemiluminescence mode. Although luciferase displays good temporal response, similar to GFP it requires oxygen and is limited to specific hosts for expression e.g., *Escherichia coli* and *Vibrio parahaemolyticus*.

Quorum Sensing Mutants

To study the contribution of QS regulated genes in biofilm development QS mutants were generated in *P. aeruginosa* which consequently has one of the best characterized QS systems outside of the photo-luminescent marine vibrio. Null mutations in the R-proteins (transcriptional activator), the autoinducer synthases (*lasI*, *rhII* and *lasIrhII*) and mutants of QS regulated products have been generated which has led to many important discoveries in the field.



Figure 2.3. A bright field microscopic image of *P. aeruginosa* biofilm growing in the flow cell (10x objective lens, the flow direction is from left to right). 3 day old *P. aeruginosa* Pao1 wild type (WT) (left) and *lasI* null mutant Pao1-JP1 strain (right). In our system only subtle differences in the structure were observed between the WT and the mutant biofilm.

To ensure that the mutant phenotype is the direct result of the specific gene, complementation experiments by providing the copy of wild-type gene on a plasmid or in case of autoinducers, by exogenous addition of signal molecules to the system should be conducted. However, there are few downfalls associated with the usage of gene knock out mutants. Since the mutants are created by insertionally inactivating the gene by replacing the antibiotic resistance cassette, it is possible that the cassette is excised from a chromosome, restoring the wild-type genotype. However, the problem can be circumvented by deleting the portion of gene of interest. Also the chance of cross-over event can be high if there is at least 1 kb of chromosomal DNA flanking each side of the cassette (De Kievet and Iglewski, 2000). Although not usually reported in the literature it is widely known that false positive or negative results in the mutant strains can also result due to polar mutation.

Biofilm Structural Analysis

Although biofilm structure has been extensively studied, at the present there are few standardized methods available for quantification. Most of the previous studies were largely qualitative relying on visual interpretations of the biofilm images. However, biofilm structural parameters that can be readily measured by microscopic techniques include thickness, thickness variability, (roughness), and surface area coverage (Stewart *et al.*, 1993, Murga *et al.*, 1995). Fractal dimension of activated sludge-biofilms (Hermanowics *et al.*, 1995) and density, porosity, specific surface area and mean pore radius of waste water biofilms (Zhang and Bishop 1994) were also measured.

Recently more complicated software programs for systematically quantifying biofilm images have been developed. These include Image Structural Analysis (ISA) which was developed at the Center for Biofilm Engineering to (www.erc.montana.edu/CBEssentials-SW/research/ImageStructureAnalyzer/default.htm), and COMSTAT which was developed at the Danish Technical University in Lyngby (<http://www.im.dtu.dk/comstat>).

ISA. ISA extracts information from 2 dimensional biofilm images based on 9 different textural and dimensional parameters for statistical comparison (Purevdorj *et al.*, 2002, Yang *et al.*, 2000). Calculated biofilm cell cluster dimensions include porosity (surface area cover), microcolony length and width, average diffusion distance (equivalent to an average diameter), maximum diffusion distance (maximum distance from the interior of the cluster to the edge), and fractal dimension (a measure of the roughness of the biofilm cell clusters). These parameters were calculated from automatically thresholded binary images to remove subjectivity from the analysis (Yang *et al.*, 2001). Briefly, each image is automatically analysed to find the threshold region which has the most influence on porosity. In these region small changes in threshold result in large variations in dimensional parameters and a threshold value outside of this region is selected based on the same criteria for all images. ISA also calculates three textural parameters from the grey scale images which describe the microscale heterogeneity of the image. These parameters are textural entropy (a measure of randomness between individual pixels), angular second moment (a measure of directional repeating patterns in the biofilm), and inverse difference moment (a measure of spatially repeating patterns). ISA was deigned to analyze larger scale biofilm patterns in 2D grey

scale images and is, therefore, useful for lower power images taken with conventional bright field or epi-fluorescence microscopy.

COMSTAT. COMSTAT was developed to analyse high-resolution 3D confocal image stacks (Heydorn *et al.*, 2000). Prior to quantification, the image stacks are thresholded, which results in a three-dimensional matrix with a value of ONE in positions where the pixel value is above or equal to the threshold value (biomass), and ZERO when the pixel values are below the threshold value (background). Confocal images are less sensitive to thresholding and automatic thresholding is not available. Therefore, manual thresholding is required and it is advised that a fixed threshold value for all image stacks is used and that the operator is not varied (Heydorn *et al.*, 2000). It also features a function where noise is automatically removed from the background, by eliminating biomass pixels that are not connected to the substratum. In general COMSTAT comprises ten image analysis features for biofilm structural quantification which include bio-volume (the overall volume of cells in the biofilm – EPS is not included if it is not specifically stained), the area occupied by bacteria at different heights in the biofilm, thickness and roughness, identification and distribution of micro-colonies at the substratum, micro-colony volume, fractal dimension, average and maximum diffusion distance, and surface to volume ratio.

AHL Detection in the Biofilm Studies

Since the first discovery of AHLs in naturally occurring biofilms (McLean *et al.* 1997), quantitative data for AHLs have been reported for a number of signal producing

bacteria including *Pseudomonas fluorescens* (Shaw *et al.*, 1997), *Agrobacterium tumefaciens* (Zhu *et al.*, 1998) and *Pantoea stewartii* (Beck von Bodman *et al.* 1998). In the systems examined so far both the transcriptional activator R proteins and the AHL molecules are remarkably similar (Fuqua *et al.*, 1996), all AHLs for these organisms being identified as N-acylated derivatives of L-homoserine lactone, which is unique to gram negative organisms. Specificity of these signal molecules is conferred by the length and the nature of the substitution at carbon 3 of the acyl-side chain (Pearson *et al.*, 1994, Passador *et al.*, 1996). Although the transcriptional activator R protein is specific, some infidelities however, do occur (Pearson *et al.*, 1994). Also it is now known that one type of bacteria can produce more than one type of acyl-HSL, which can also pose problem during the detection process. Potential candidates for AHL detection are *P. aeruginosa* *rhlI* and *lasI* QS systems, which mediate via 4 and 12 carbon AHLs, respectively; and *Agrobacterium tumefaciens* system which mediates via 8 carbon AHL system. The bacterial strains carrying genes encoding the R-proteins activated by either 4, 8 or 12 carbon AHLs, target gene fused with *lacZ* (encodes β -galactosidase, an enzyme which hydrolyzes o-Nitrophenyl- β -d-Galactoside, ONPG, yielding o-nitrophenol that absorbs light at 420 nm (Miller *et al.*, 1992)) or another reporter gene are needed for bioassay. For the indicator strain, whether *E. coli* strain harboring *lasR* and *lasI-lacZ* on a multiple copy plasmid. *P. aeruginosa* autoinducer synthase mutant deficient in the production of 3-oxo-C₁₂-HSL and C₄-HSL, harboring *lasI-lacZ* fusion plasmid can be used. Overexpression of R-proteins in the indicator strain may increase the sensitivity of the bioassay to noncognate AHLs. Currently there are two methods to assay autoinducer presence in the sample. First one described by McLean *et al.*, (1997), plating both the

indicator strain and the biofilm sample side by side on an agar plate. If the autoinducers are present in the biofilm sample, they will diffuse through the agar to the indicator strain resulting in the blue color. The second method, instead of solid agar, liquid culture is used, co-incubating the sample with indicator strain. AHL activity is detected with a standard B-galactosidase assay. So far the majority of quantitative studies have been focused on planktonic cultures and these quantitative bioassays are limited in both analyte separation and detection. Depending on the bioindicator the detection is limited to those acyl-HSLs to which the bioindicator responds and the signals must be present at the levels detectable by the reporter. In addition other non – AHL components in the extract may also interfere with the bioassay. Previous attempts to quantify the AHL concentration in the biofilm that is not based on a bioassay include high-performance liquid chromatography (HPLC) (Reimann *et al.*, 1997) and more sensitive the gas chromatography – mass spectrometry (GC-MS) (Charlton *et al.*, 2000). The latter study determined the presence of diverse range of 3-oxo AHLs in *P. aeruginosa* biofilm at the levels well above those required for upregulation of QS. However, the spatial distribution of the signal molecules in the biofilm, namely whether they are localized in the biofilm extracellular matrix or limited to within the cell, still remains to be resolved.

Concluding Remarks

The QS system allows both species specific and interspecies cell-cell communication to coordinate the behaviour of a prokaryotic community. It is proven to play an important role in survival of the population by facilitating the colonization of hosts, defense against competitors, adaptation to varying physical conditions, cellular

differentiation, and probably holds evolutionary importance as it directs individual cells in the population to act as a multi-cellular unit. At the present discovery of new pathways and mechanisms of cell-cell communication systems as well as their roles in physiological functions of variety different microorganisms is ongoing process. While many studies have demonstrated the importance of QS in biofilm structural development, there are studies that do contradict this phenomenon. It is increasingly apparent that the QS and biofilm structure relationship is far more complicated and may depend on or act together with other environmental cues, which remains to be determined. Further work is needed to elucidate the relative contribution of QS in the biofilm structural development.

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CHAPTER 3

INFLUENCE OF HYDRODYNAMICS AND CELL SIGNALING ON THE
STRUCTURE AND BEHAVIOR OF *PSEUDOMONAS AERUGINOSA* BIOFILMS.Summary

Biofilms were grown from wild type *Pseudomonas aeruginosa* PAO1 and the cell signaling *lasI* mutant PAO1-JP1 under laminar and turbulent flows to investigate the relative contribution of hydrodynamics and cell signaling on biofilm formation. By visual comparison hydrodynamics significantly affected biofilm structure while the influence of the cell signaling mutation was more subtle. For non subjective comparison morphological features of the biofilm were quantified using Image Structure Analyzer (ISA) software. Multivariate analysis demonstrated that both cell signaling and hydrodynamics significantly ($P < 0.000$) influenced biofilm structure. Both biofilms in turbulent flow formed streamlined patches, which in some cases developed ripple-like wave structures which flowed downstream along the surface of the flow cell. The structural morphology and dynamic behavior of the JP1 mutant biofilm did not significantly differ from the WT biofilm. In laminar flow both biofilms formed flat monolayers interspersed with small circular micro-colonies although ripple-like structures also formed in 4 out of 6 WT biofilms. The ability of cell signaling mutants to form biofilms in high shear flow demonstrates that signaling mechanisms are not required for the formation of strongly adhered biofilms. Similarities between biofilm morphology in WT and mutant biofilms suggests that dilution of signal molecules by mass transfer

effects in faster flowing systems may mollify the dramatic influence of signal molecules on biofilm structure reported in previous studies.

Introduction

Cells in bacterial biofilms are often less susceptible to host immune responses and antibiotics than cells grown in suspension (Nickel *et al.*, 1985). Biofilms may also provide a protective environment for pathogens, which, when released from the biofilm, may result in contamination of drinking water and medical fluids in delivery devices such as dialysis machines, venous catheters, dental water lines, and airway ventilators. Life-threatening infection caused by *Pseudomonas aeruginosa* in cystic fibrosis patients is a well-known example (Govan and Deretic 1996). Since biofilm formation in itself can be considered a virulence factor, it is important to understand the mechanisms which influence biofilm accumulation, structure, and behavior. Both hydrodynamics and cell signaling have been found to influence the structure of *P. aeruginosa* PAO1 biofilms. Stoodley *et al.*, (1999a) reported that, under conditions of low-shear laminar flow, the biofilm consisted of a monolayer of cells with mound-shaped circular microcolonies but under high-shear, turbulent flow conditions, the biofilm formed filamentous streamers. Davies *et al.* (1998) found that N-3-oxo-dodecanoyl homoserine lactone (OdDHL), a cell signal molecule involved in quorum sensing (QS) (reports regarding putative regulatory QS pathways and the role of QS in pathogenicity can be found elsewhere (Passador *et al.*, 1993; DeKievit and Iglewski 2000; Miller and Bassler 2001), was required for the differentiation of biofilms into complex mushroom- and tower-like structures, which they described as characteristic of normal biofilms. However, Heydorn *et al.*, reported in a

recent study (2002) that in their system, the wild-type (WT) PAO1 biofilm was relatively flat and there was no statistically significant difference in structure between the WT and the *lasI* JP1 mutant. Additionally, Heydorn *et al.* reported that the structure of laboratory-grown biofilms is often highly variable (2000). A complicating factor in the role of QS in biofilm formation is the possible effect that an overlying flowing fluid will have on the concentration of signal molecules within a biofilm. QS is not solely a function of high cell density but is more directly related to high signal concentrations. In closed-system batch cultures, these tend to coincide in the stationary phase. However, in open, flowing systems in which the bulk water is continually refreshed, it is possible for diffusible signal molecules to be washed out of the biofilm. Even though mass transport studies demonstrate that diffusion is the principal transport mechanism within cell clusters (DeBeer and Stoodley 1995), the removal of signal molecules from the flowing bulk fluid surrounding the cell clusters increases the concentration gradient across the biofilm-bulk liquid interface, driving the diffusive flux of signal molecules out of the biofilm. The signal concentration in the cell clusters in an open system would, therefore, be expected to depend on the production rate, the rate of diffusion through the biofilm, and the hydrodynamic conditions of the bulk liquid. An understanding of the effects of QS on biofilm structure and behavior under different flow conditions has important applications in industry and medicine.

The goal of the study was to investigate the role of QS in biofilm structure and behavior under different flow regimens. We grew biofilms from the WT *P. aeruginosa* PAO1 strain and the OdDHL-deficient JP1 mutant strain under conditions of either turbulent (high shear) or laminar (low shear) flow. Digital time-lapse microscopy

(DTLM) was used to quantify biofilm accumulation, structural morphology, and dynamic behavior. The influence of QS and flow on biofilm structure was assessed by univariate and multivariate analysis of spatial and textural image parameters, which were quantified using Image Structure Analyzer (ISA) software developed for biofilm analysis.

Materials and Methods

Bacterial Strains and Nutrients

Biofilms were grown from WT *P. aeruginosa* PAO1 (Holloway *et al.*, 1979) and JP1, a $\Delta lasI::tet$, *lasI* null mutant derived from PAO1 which does not produce the QS signal OdDHL (Pearson *et al.*, 1997). Luria-Bertani (LB) broth (1/50 strength, 4 g/liter) was used as the growth medium for biofilms. Full-strength LB broth was used to grow the flask cultures (grown with 24 h of shaking) used for initial inoculation.

Biofilm Reactor System

Biofilms were grown in two glass tube flow cells, 20 cm long and 3 mm in width and height (Friedrick & Dimmock, Millville, N.J.), which were incorporated in parallel positions into a recirculating continuous-culture system. The flow velocity (u) was maintained at $u = 0.033$ m/s in one flow cell for laminar flow and at $u = 1.0$ m/s in the other flow cell for turbulent flow. At these flow velocities, the Reynolds (Re) numbers were 100 and 3,000, respectively. The Re number is a comparative indicator of hydrodynamic conditions in different flowing systems. Flow velocity was measured with

in-line flow sensors (McMillan model 101T Flo-Sensor, serial no. 3724 and 3835; Cole-Parmer, Niles, Ill.). The flow cells were positioned in a polycarbonate holder, which was mounted on the stage of an Olympus BH2 upright microscope so that the biofilm could be imaged in situ without interrupting flow (Figure 3.1).

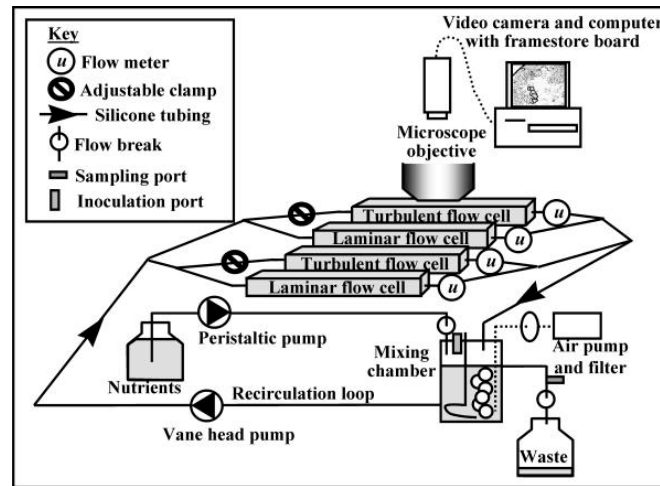


Figure 3.1. Schematic representing the main components of the flow system and orientation of flow cells in respect to the microscopic objective.

A septum-sealed sampling port was positioned between two flow breaks in the effluent line. The system, including a mixing chamber designed for aseptic aeration and nutrient addition, is described in detail elsewhere (Stoodley *et al.*, 1999c). Independent triplicate experiments (runs 1, 2, and 3) were run for 6 days, each system consisting of side-by-side laminar and turbulent flow cells in duplicate. Under operating conditions, the water temperature in the reactor system was 23°C, and all experiments were performed at this temperature.

Reactor Sterilization

The reactor system, except the thermally sensitive flow sensors, was autoclaved at 121°C for 15 min. The sensors were sterilized with 70% ethanol for 15 min, NaOCl solution for 15 min, and 70% ethanol for 30 min (Stoodley *et al.*, 1999c). The sterility of the reactor system was confirmed by plating 0.1-ml aliquots of effluent onto LB agar (LA).

Inoculum and Media

The reaction mixture, containing 1/50-strength LB broth, was inoculated with 1 ml of an overnight LB broth (20 g/liter; 37°C) shake flask culture of PAO1 and JP1. The reactor was initially run as a recirculating batch culture for 24 h, to allow attachment, before being switched to continuous culture mode. The system was switched to continuous culture mode by delivering 1/50-strength LB broth to the mixing chamber via peristaltic pump (serial no. 7553-80; Cole-Parmer). The influent flow rate was maintained at 4.3 ml/min, giving a dilution rate of 0.025 h⁻¹ (hydraulic residence time = 40 min). This rate was above that of washout (the growth rates of PAO1 and JP1 on LB broth were 0.15 ± 0.01 h⁻¹ (*n* = 3) and 0.10 ± 0.02 h⁻¹ (*n* = 3), respectively) to minimize suspended growth and encourage biofilm growth. Effluent samples were taken periodically to monitor the detached population and to confirm culture purity. The JP1 effluent was plated on both LA and LA with tetracycline (50 µg/ml) to confirm the culture purity and integrity of the mutant. Daily comparisons showed no significant difference (*P* > 0.05).

Biofilm Cell Concentration

At the end of each experiment, the flow cells were aseptically separated from the system and 2-cm-thick sections were cut (using a diamond knife) from the inlet, middle, and outlet of the flow cells. The sections of glass tubes were sonicated for 5 min and vortexed in test tubes with 5 ml of Ringer's solution to remove biofilm cells (Stoodley *et al.*, 1999c). This procedure was repeated three times. A serial dilution was prepared, and six 10- μ l aliquots were plated onto LA and LA plates with tetracycline (50 μ g/ml). The plates were incubated at 37°C for 24 h.

Microscopy

The developing biofilm was visualized in situ by using transmitted light and 5 \times , 10 \times , and 50 \times objective lenses with an Olympus BH2 microscope. Images were collected using a COHU 4612-5000 charge-coupled device camera (Cohu, Inc., San Diego, Calif.) and captured with a VG-5 PCI framestone board (Scion Inc., Frederick, Md.). The Scion image software was used to collect time-lapse sequences and for image enhancement and analysis. A 1-mm-long graticule with 10- μ m divisions was used to calibrate length measurements. For the ripple dimensions, the length was defined as the longer dimension, running perpendicularly to the flow, and the width was defined as the shorter dimension, running parallel to the flow. On days 5 and 6, the distance traveled by individual ripples over a 16-h monitoring period was also measured, at hourly intervals. Linear regression was used to calculate the average ripple travel velocity over this time period. The biofilm thickness and surface area coverage were measured on each day at five random locations in the biofilm area for each flow cell (Stoodley *et al.*, 1999c).

ISA. The ISA software package, which was developed by the Biofilm Structure and Function Research Group at the Center for Biofilm Engineering (www.erc.montana.edu/CBEssentials-SW/research/ImageStructureAnalyzer/default.htm), was used to quantify nine spatial and textural parameters from individual biofilm images for statistical comparison (Yang *et al.*, 2000). The ISA software was operated in a MATLAB 6.1 program (The MathWorks). Low-power images, taken with a 10× lens objective, were used to quantify the larger-scale biofilm structures and patterns, which could not be seen at higher power magnifications. The calculated biofilm cell cluster dimensions were horizontal run length (average length of the cell clusters, which in our setup were parallel with flow direction), vertical run length (average length of the cell clusters perpendicular to flow), average diffusion distance (average distance from the cells in the cluster to the nearest interstitial space, similar to an equivalent radius), and maximum diffusion distance (maximum distance from the interior of the cluster to the edge). The fractal dimension (a measure of the roughness of the biofilm cell clusters) and porosity (the proportion of void areas) were also quantified. These parameters were calculated from automatically thresholded binary images to remove subjectivity from the analysis (Yang *et al.*, 2001). ISA also calculates three textural parameters from the gray scale images which describe microscale heterogeneities in the image: textural entropy (a measure of randomness between pixels in the biofilm image), angular second moment (a measure of directional repeating patterns of pixels), and inverse difference moment (a measure of spatially repeating patterns of pixels).

Statistical Analysis

Statistical comparisons of thickness and manually measured parameters from Scion Image were analyzed by analysis of variance (ANOVA) using Minitab software (version 13.3; Minitab, Inc., State College, Pa.). Data were reported as means \pm 1 standard error. Univariate ANOVA and multivariate ANOVA (MANOVA) were used to statistically compare quantified parameters from ISA. Data from biofilm images taken on days 4 and 5 were pooled to increase statistical rigor. A two-factor (*P. aeruginosa* strain and flow rate) additive ANOVA and MANOVA calculation was performed on the nine-variable matrix. Differences were considered significant for *P* values of <0.05 .

Results

Biofilm Development, Morphology, and Behavior

Biofilms grown under laminar flow. Within 24 h of the inoculation period, the biofilms for both the PAO1 and JP1 strains consisted of a sparse layer of cells (Figure 3.2). By day 3, circular microcolonies approximately 15 μm thick had developed in both biofilms. By this time, there was a monolayer of cells between the colonies such that the surface area coverage had reached 100% in both biofilms (Figure 3.3). The thickness and surface area coverage did not change significantly ($P > 0.05$), and at the end of the run (day 6), the PAO1 and JP1 biofilms were $17.5 \pm 0.7 \mu\text{m}$ and $19.6 \pm 3.7 \mu\text{m}$ thick, respectively. The average surface coverage of the PAO1 biofilm had decreased slightly to $87.3 \pm 12.7\%$, whereas the JP1 biofilm remained at $100 \pm 0\%$.

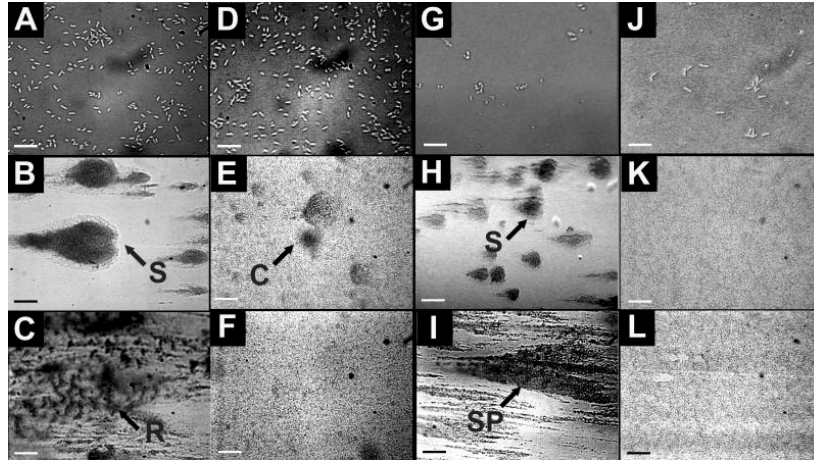


Figure 3.2. *P. aeruginosa* PAO1 and JP1 biofilms grown in turbulent and laminar flow cells. PAO1 biofilm in turbulent (A-C) and laminar flow cells (G-I), JP1 biofilm in turbulent (D-F) and laminar (J-L) flow cells on days 1, 3 and 6 (scale bars = 20 μm , 100 μm and 100 μm , respectively). Both PAO1 and JP1 strains in turbulent flow by day 3 had formed similar streamlined patchy biofilms. In laminar flow, both PAO1 and JP1 formed a flat monolayer of cells with occasional circular colonies. C - circular colony, R - ripple structure, S – streamers and SP - streamlined patches. The flow direction is from right to left on each panel.

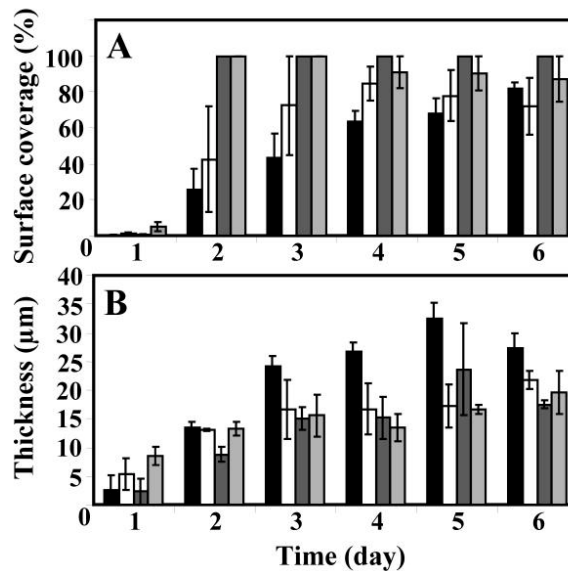


Figure. 3.3. Biofilm development and accumulation measured by surface area coverage (A) and thickness (B) over the course of the experiments. PAO1 turbulent (open bars), PAO1 laminar (bars with left hatching), JP1 turbulent (solid bars) and JP1 laminar (gray bars). Error bars are 1 standard error (SE) $n=15$.

There was no significant difference between the measurements of daily thickness (all P values of >0.06) and surface area coverage (all P values of >0.19) of the PAO1 and JP1 biofilms on any of the 6 days. In runs 1 and 2, the PAO1 biofilms formed highly organized ripple-like structures (regularly spaced ridges running perpendicular to the flow direction) (Figure 3.4, panels A to C).

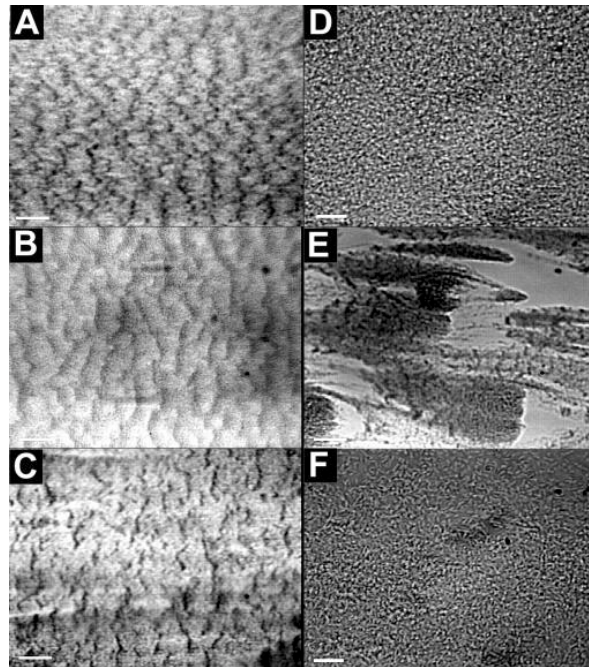


Figure 3.4. Ripple structures formed in PAO1 and JP1 biofilms. PAO1 biofilm ripple structures in the biofilms growing in the turbulent flow cell (A) and the laminar flow cell (B) taken at days 4 and 5, respectively. The ripples were aligned perpendicularly to the flow direction (indicated by arrow), scale bar = 200 μm . JP1 biofilm ripple structures in the turbulent flow cell taken on day 6 (C), scale bar = 200 μm . The ripple structures were much less evident under higher magnification (panel D and F image in same field as in panel A and C, respectively). Scale bar = 20 μm . Patchy PAO1 biofilm structures in turbulent flow cell, Run 3, day 5. Scale bar = 100 μm (E).

The ripples were only evident when the biofilm was viewed under low-power magnification (10 \times) and were not apparent when viewed with higher (50 \times) magnification objectives (Figure 4D and F). Although ripple motion was not apparent in real time,

DTLM showed that the ripples were traveling at a constant downstream velocity of $0.51 \pm 0.06 \mu\text{m/h}$ ($n = 17$) (Figure 3.5).

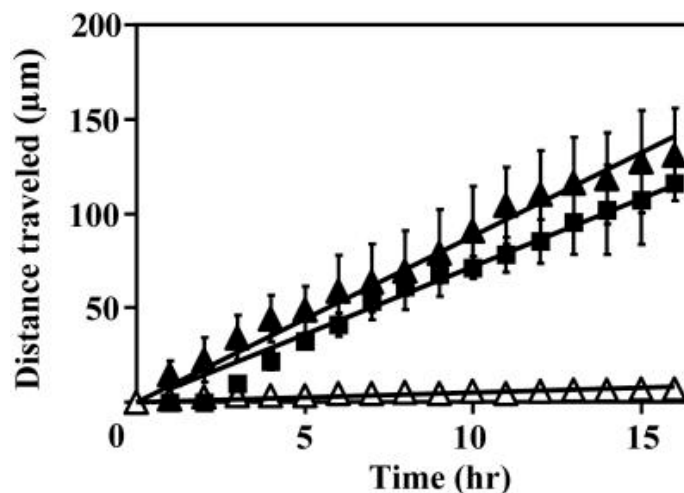


Figure 3.5. Downstream transport of PAO1 ripples that formed in laminar (open triangles) and turbulent (solid triangles) flow and in the turbulent JP1 biofilm (solid squares) over a 16 h monitoring period. Measurements were made on day 5 of Run 1 for turbulent biofilms and day 6 of Run 1 for the laminar biofilm. The position of five different ripples were used to calculate the mean distance traveled during each hour, error bars = 1 S.D. The solid linear regression curves were used to determine the average ripple travel velocity.

It appeared that the individual ripples moved over an underlying layer of bacterial cells that were more firmly attached to the surface. Ripple structures were not seen in any of the JP1 biofilms.

Biofilms grown under turbulent flow. The mean surface coverage of both PAO1 and JP1 biofilms steadily increased over the growth period and by day 6 reached $82.1 \pm 3.4\%$ and $72.2 \pm 1.4\%$, respectively. The JP1 biofilm was generally thicker than the PAO1 biofilm and after 6 days was $27.2 \pm 2.6 \mu\text{m}$ thick compared to $21.7 \pm 15.8 \mu\text{m}$. However, in similarity to the biofilms grown in laminar flow, there were no statistical

differences between the daily thickness (all P values of >0.05) and surface coverage (all P values of >0.05) measurements over the course of the experiment. Morphologically, both biofilms formed large streamlined patches and filamentous streamers (Figure 2). Between the streamers, the substratum was covered with single cells, whose arrangement ranged from a sparse covering to a confluent monolayer. In some areas, the thicker patches of biofilms appeared to have joined to form a continuous layer. All three of the PAO1 biofilms and two of the JP1 biofilms also formed ripple structures similar to those seen in the PAO1 biofilms grown in laminar flow. Between days 3 and 5 of growth, the ripples formed both in the streamlined patches and in the more extensively covered areas. These ripples had higher contrast, making them easier to visualize than those that developed in laminar flow. However, the ripple dimensions in the PAO1 biofilms were not significantly different from those that formed in laminar flow (length, $P = 0.45$; width, $P = 0.79$; and spacing, $P = 0.35$). The ripples that formed in the JP1 turbulent biofilm were smaller than those in the PAO1 biofilm (all length, width, and spacing P values, <0.001). DTLM from run 1 on day 5 showed that the ripples traveled downstream at an average velocity of $8.7 \pm 1.6 \mu\text{m/h}$ ($n = 17$; $r^2 = 0.97$) in the PAO1 biofilm and $7.2 \mu\text{m/h}$ ($n = 17$; $r^2 = 0.98$) in the JP1 biofilm (Fig. 5). In run 3, the PAO1 biofilm ripples were traveling at an average velocity of $14 \mu\text{m/h}$ ($n = 15$; day 6). These velocities were more than 10 times those measured in the PAO1 biofilms grown in laminar flow conditions ($0.51 \mu\text{m/h}$, $r^2 = 0.61$, $n = 17$). Video time lapse movies showing the biofilm ripple structures flowing downstream are available at the following website:

www.erc.montana.edu/Res-Lib99-SW/Movies/default.htm.

ISA and Statistical Analysis

ISA results and univariate ANOVA results from ISA are shown in Table 3.1.

	<i>PAOIL</i>	<i>PAOIT</i>	<i>JPIL</i>	<i>JPIT</i>	<i>ANOVA</i>	
					<i>(P values)</i>	
N	20	28	12	41	Strain	Flow
Por	0.62±0.12	0.60±0.09	0.66±0.07	0.62±0.08	0.219	0.152
ADD (um)	6.49±6.53	18.71±18.90	2.38±0.39	9.99±5.30	0.003	0.000
MDD (um)	64.94±65.71	80.00±55.35	58.46±36.60	77.57±39.43	0.016	0.002
HRL (um)	10.75±8.06	17.29±10.16	15.48±9.12	20.93±8.33	0.178	0.000
VRL (um)	8.47±6.05	11.19±7.28	10.32±7.33	12.27±4.60	0.029	0.000
FD	1.53±0.12	1.37±0.11	1.56±0.08	1.34±0.12	0.536	0.000
En (x10 ⁻³)	4.26±1.37	3.93±1.13	2.83±1.19	0.99±1.23	0.032	0.105
TE	8.31±0.35	8.40±0.27	8.96±0.51	8.47±0.61	0.015	0.128
Hom	0.12±0.03	0.16±0.03	0.10±0.03	0.17±0.06	0.796	0.000

Table 3.1. Parameters from ISA analysis of low power images taken from days 4 and 5 of *P. aeruginosa* WT (PAO1) and mutant (JP1) strains grown at laminar (L) and turbulent (T) flow. ANOVA columns shows P values from univariate analysis for comparisons between *P. aeruginosa* strain and flow regime. Shaded values indicate significant difference (P<0.05).

The five dimensional parameters showed that the cell clusters in the biofilms grown under turbulent flow were larger than those grown under laminar flow. These differences were highly significant between the different flows for all of the parameters

($P < 0.002$), while between the WT and mutant biofilms, three of the five dimensional parameters were significantly different. There was a significant difference between bacterial strains in two of the three textural parameters and between the different flow rates in one of the three textural parameters. MANOVA showed that both strain and flow had a significant influence on the ISA parameters (both P values, <0.001), but there was not a significant univariate interaction between flow and strain ($P = 0.091$). Recalculation using an additive model with 1 degree of freedom also yielded P values of <0.001 for both flow and strain.

Biofilm Cell Concentration

The average biofilm cell concentration in the three PAO1 runs was similar ($P = 0.28$) in both turbulent and laminar flow cells ($1.2 \pm 0.1 \times 10^7$ CFU/cm² [$n = 54$] and $8.1 \pm 0.7 \times 10^6$ CFU/cm² [$n = 61$], respectively). In the JP1 biofilms, there was also no significant difference ($P = 0.79$) between cell concentration in the turbulent and laminar flow cells ($2.1 \pm 0.9 \times 10^6$ and $1.7 \pm 0.6 \times 10^6$ CFU/cm², respectively). Differences were not significant between the PAO1 and JP1 biofilms grown in turbulent flow ($P = 0.09$) but were significant between the PAO1 and JP1 biofilms grown in laminar flow ($P = 0.01$).

Effluent Cell Concentration

After 3 days, the average effluent concentration in the three runs in the PAO1 and JP1 biofilms increased to $8.9 \times 10^7 \pm 3.4 \times 10^7$ CFU/ml and $1.6 \times 10^8 \pm 2.4 \times 10^8$

CFU/ml, respectively. Over the remaining 3 days of the experiment, these concentrations varied no more than 1 order of magnitude.

Discussion

Biofilm structure

There was little difference in the accumulation rates of the WT and QS mutant biofilms grown under either laminar or turbulent conditions, as estimated by thickness, surface area coverage, or viable biofilm cell concentration values. After 6 days, the thickness of all the biofilms had stabilized at approximately 20 μm and the surface area coverage was over 80%. We noted few visual differences between the WT and mutant biofilms, in contrast to more obvious differences between biofilms grown under the two flow regimens. Biofilms grown under laminar flow consisted predominantly of a thin layer of cells interspersed with distinct circular microcolonies. In some cases in the WT biofilm, ripple-like ridges running perpendicularly to the flow appeared in the biofilm. The ripples were only apparent at lower magnification. Similar structures have been reported in mixed-species laboratory biofilms grown in turbulent flow (Stoodley *et al.*, 1999b), and in river water biofilms (Neu and Lawrence 2001). The ripples consisted of densely packed bacteria. In turbulent flow, both WT and mutant biofilms developed streamlined patches, which were tapered in the downstream direction. Ripple structures formed in each of six flow cells with WT biofilms but in only four out of six of the mutant biofilms, demonstrating the inherent variability commonly encountered in flow cell studies. Manual measurement of ripple dimensions revealed that the WT ripples were

significantly larger than the ripples formed in the QS mutant ($P < 0.05$). Ripples also formed in four out of six of the WT biofilms grown under laminar flow but in none of the mutant biofilms. It is possible that this variability may be explained by subtle differences in the composition and cohesiveness of the extrapolymeric substance matrix formed by the different strains under the two different growth conditions.

Structural Analysis of Biofilm Images and Statistical Comparisons

The ISA image analysis package was used to nonsubjectively quantify various spatial and textural parameters of the biofilm images, allowing us to statistically assess the influence of cell signaling and flow on biofilm structure. As noted by Heydorn *et al.*, (2000; 2001), the inherent heterogeneity common in biofilm structure makes qualitative comparisons difficult. To overcome this difficulty, the Danish group developed the software package COMSTAT, which is based on single-cell resolution in a three-dimensional volume collected by confocal microscopy. ISA quantifies two-dimensional grayscale images, and although ISA does not incorporate three-dimensional information, it is useful for quantifying the shapes and dimensions of biofilm microcolonies and patterns which are visible at the larger scales (i.e., at the millimeter level). It can be used with images collected by conventional bright-field or fluorescence-based microscopy. Although visually it was difficult to distinguish structural differences between the WT and QS mutant biofilms in comparison to the more obvious differences due to flow regimes, MANOVA of the ISA data revealed significant structural differences due to both strain and flow. While the parameters quantifying size dimensions were easily interpretable, textural information was also useful for the statistical comparison of data

sets, even though physical interpretation of the data was less obvious.

OdDHL is not Required for the Formation of Strongly Attached, Cohesive Biofilms

Although none of our biofilms developed the distinct mushroom structures which formed in the PAO1 biofilms in the study of Davies *et al.* (1998), both the WT and mutant strains did form significant biofilms in both laminar and turbulent flow. Unlike the results of the Davies study, which suggested that, in the absence of OdDHL, there was no true biofilm formation but only a loose accretion of cells, in our flow system, which was operated at much higher shear stresses, the biofilms must have been strongly adhered and cohesive to remain attached. Our data suggest that cell signaling is not required for biofilm formation but possibly plays a role in the structural heterogeneity of the biofilm. What is becoming apparent is that biofilm structure is highly sensitive to growth conditions. In the present study, both the WT and mutant biofilms grown in laminar flow were more similar to the flat WT biofilms described by Hentzer *et al.*, (2001) and Heydorn *et al.*, (2000; 2001), and interestingly, to the cell signaling JP1 mutant biofilm in the study of Davies *et al.* than were those grown in turbulent flow conditions. Hentzer *et al.*, (2001) attributed the structural differences between the PAO1 WT biofilm in their study and the WT biofilm in the Davies study to differences in the growth medium used. In the present study, we used the same nutrients (2% LB broth) as Hentzer *et al.*, (2001), which may explain why our biofilms were also relatively flat. These data challenge the ever-growing acceptance of a generalized, normal biofilm structure as consisting of mushrooms and towers.

Hydrodynamic Effects on Mass Transfer, Cell Signaling, and Biofilm Structure

Hydrodynamic conditions can also strongly influence biofilm structure. In a previous study of the influence of cell signaling and hydrodynamics on the structure of *P. aeruginosa* biofilms, Stoodley *et al.* found that hydrodynamic conditions had a greater influence than null mutations on structure in the cell signaling regulators *lasR* and *rhlIR* (Stoodley *et al.*, 1999a). In the study of Davies *et al.* (1998), the biofilms were grown at a Re of 0.17. The calculated wall shear stress value, however, was comparable to that calculated for our laminar flow cell (approximately 0.1 Nm^{-2}), suggesting that differences in observed WT biofilm structure were more likely related to rates of mass transfer than to shear-related detachment (which may be expected to result in flatter, rather than mushroom-shaped, biofilms). It is possible that in our flow system, the higher rates of mass transfer, even in our laminar flow cell, had a dilution effect on signaling molecules. In the absence of QS-inducing concentrations, it may be expected that there would be little difference between the WT and QS mutant biofilms. In addition to influencing structure, hydrodynamic conditions also influence biofilm density (Viera *et al.*, 1993) and strength (Beyenal and Leewandowski 2002; Stoodley *et al.*, 1999a), which in turn may be expected to influence the diffusion of nutrients and signals through the biofilm.

Movement of Biofilm Ripples over Solid Surfaces

DTLM revealed that the ripples traveled downstream along the channel walls of the glass. This movement was not noticeable in real time. Although there were no

significant differences in the morphologies of the ripples grown in the two flow regimens, the velocity of the ripples in the turbulent flow cell was 8.7 $\mu\text{m}/\text{h}$, approximately 10 times faster than that of those that formed under laminar flow. Although these velocities appear low, they may represent a large downstream flux of microorganisms. For example, if half of the approximately 10^7 CFU of biofilm cells/ cm^2 were traveling in ripples at 8.7 $\mu\text{m}/\text{h}$, the downstream flux (which is dependent on the tube geometry) would be approximately 5×10^3 CFU $\text{cm}^{-1} \text{h}^{-1}$. The flux in the laminar flow cell would be 10-fold less. With the exception of detachment events, biofilms are often depicted as immobilized layers in which the cell clusters remain at the same location on the substratum (Costerton *et al.*, 1999; Palmer *et al.*, 1997). The present study demonstrates that biofilms can move along solid surfaces while remaining attached to those surfaces. The only previous reports of traveling ripples in bacterial communities have been for the myxobacteria (Sager and Kaiser 1994; Shimkets and Kaiser 1982). However, we believe that there are some fundamental differences in the mechanisms of migration described in those reports from those reported here. For *Myxococcus xanthus*, a species of gliding bacteria, ripples are formed because of the coordinated motility of individual cells, which is controlled by signaling molecules (Sager and Kaiser 1994). These ripples are not flow dependent and can occur on solid surfaces in quiescent air or water. In our biofilms, the migration velocity and ripple structure varied as a function of the fluid shear stress (Stoodley *et al.*, 1999b), suggesting that this behavior was a physical phenomenon. The apparent flow of biofilms may be related to the fluid-like properties reported for mixed- and pure-culture PAO1 biofilms (Stoodley *et al.*, 1999c) and to the hydrogel nature of the polymer matrix (Flemming *et al.*, 2000; Sutherland 2001). Nevertheless, shear-mediated migration of

biofilms represents a previously unrecognized mechanism for dissemination in flowing systems and may have important consequences for contamination and infection in industrial or clinical environments.

Flowing biofilms have been implicated in ventilator-associated cases of pneumonia (Inglis 1993). In that clinical study, 23 of 50 tracheal tubes were found to contain biofilms with what were termed “wave-like” patterns. This led Inglis *et al.* (Inglis *et al.*, 1989; Inglis 1993) to infer that the biofilm had been flowing along the tube and that this flow may be related to dissemination into the lungs. Our studies directly demonstrated that this phenomenon is possible. Unlike dissemination via detached planktonic cells, surface-associated transport allows the spread of entire biofilm structures, presumably with preserved resistance to various antibiotics and chemical disinfectants (Stewart and Costerton 2001). The flow of biofilms along pipe walls may also be an important consideration in infection from venous catheters (Donlan *et al.*, 2001), dental unit water lines (Putnins *et al.*, 2001), or dialysis machines (Man *et al.*, 1998). Further work is required to determine the significance of surface-associated biofilm transport in the dissemination of microbial pathogens in both clinical and industrial settings.

Concluding Remarks

In agreement with previous studies (Heydorn *et al.*, 2000, Stoodley *et al.*, 1999a), our results suggest that QS alone is not necessarily required for biofilm formation and that other factors of the growth environment, such as nutrients and hydrodynamic conditions, can play a role of equal if not greater significance in determining the biofilm

structure. The relative contribution of each of these interlinked factors under different growth conditions has yet to be established. However, until a connection is made that relates the formation of specific biofilm structures to biofilm virulence in both clinical and industrial contexts, interference in cell signaling pathways may not be the magic bullet for biofilm control it was initially thought to be.

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CHAPTER 4

THE INFLUENCE OF HYDRODYNAMICS ON THE ONSET OF LAS QUORUM
SENSING IN *PSEUDOMONAS AERUGINOSA* BIOFILMS.Introduction

Pseudomonas aeruginosa is known to utilize two main hierarchical Quorum Sensing (QS) systems, *las* and *rhl* to sense its population density and coordinate the expression of specific genes that may contribute to biofilm development (Davies *et al.*, 1998), pathogenic (Pearson *et al.*, 2000; Wu *et al.*, 2001), as well as symbiotic relations with its animal (Parsek and Greenberg 2000; Williams *et al.*, 2004) and plant (Bauer and Mathesius 2004) hosts. Fully functional signals employed by *las* and *rhl*, *N*-(3-oxododecanoyl)-**L**-homoserine lactone (OdDHL) and *N*-(butanoyl)-**L**-homoserine lactone (BHL) respectively (collectively referred to as autoinducers), have been detected in the biofilms isolated from wide range of environmental (Cabrol *et al.*, 2003), industrial (Bruhn *et al.*, 2004) and clinical (Stickler *et al.*, 1998; Erickson *et al.*, 2002; 1998, Singh *et al.*, 2000) settings demonstrating an important implications of QS in the life cycle of this microorganism, in general. Recent evidence suggests however, that in some environmental conditions QS may not be a sole effector utilized by the biofilms to regulate the expression of genes and traits that are under its control and that along with QS itself they could be controlled by independent pathways of both environmental and cellular origin such as media composition (DeKievit *et al.*, 2001), oxygen availability, alternative sigma factor, RpoS (Shcuster *et al.*, 2004), RpoN (Thompson *et al.*, 2003) and

a global virulence regulator Vfr (Beatson *et al.*, 2002) . In previous studies we have demonstrated that hydrodynamics, played an important role on biofilm structural development and that biofilm morphological differences attributed to the difference in the flow regimen were far more pronounced than those resulted from deficiency in the QS system alone (Stoodley *et al.*, 1999; Purevdorj *et al.*, 2002,). Since QS performance is dependent on accumulation of threshold autoinducer concentration, we hypothesized that the flow dynamics in the bulk fluid surrounding the biofilm, which also determines mass transfer characteristics, would play an important role in QS system and thus the genes and phenotypes that are under its control. In this study we wished to investigate the onset and expression pattern of *lasB* (coding for elastase expression), during the *in vitro* biofilm growth and development under varying hydrodynamic conditions. Elastase, one of the strongest virulence factors produced by *P. aeruginosa* (Woods *et al.*, 1982), is controlled by *las* QS circuitry (Pearson *et al.*, 1997; Passador *et al.*, 1993; Gambello and Iglewski 1991) and has been implicated with tissue-damaging activity (Bejarano *et al.*, 1989) as well as pro-inflammatory responses in the lungs of Cystic Fibrosis patients (Azghani *et al.*, 2002). Understanding of elastase expression via QS pathway in biofilms particularly in the flowing liquid environments is not only medically relevant but also contributes to our fundamental understanding of how the external environment can modulate the phenotypic response of biofilms. To address our hypothesis we tracked the spatial and temporal expression of the *las* system in PAO1 biofilms grown at different flow rates using a *lasB* green fluorescence protein (GFP) reporter construct PAO1 (pMH516) and confocal microscopy (CM). Additionally the concentration of the bioactive signal (OdDHL) in the surrounding bulk fluid before and after the onset of GFP production was

measured by utilizing an *E. coli* reporter construct (Pearson *et al.*, 1994). The onset of GFP expression and signal concentration produced by the flow cell grown biofilms were contrasted to the results from planktonic samples cultured in a closed, batch, system. The effect of hydrodynamics on QS expression in the biofilms is discussed in light of planktonic and biofilms control experiments presented herein.

Materials and Methods

Reporter Construct

A Gfp-transcriptional reporter fusion vector (pMH487) featuring an RNaseIII cleavage site between the multiple cloning site and the promoterless *gfp* gene was constructed to ensure independent translation of the *gfp* gene (Linn and St- Pierre, 1990). The primers RNaseIII_Fwd (5'-GCATCTAGACTCGAGTAACTAACTAGCGATCCCGA-3') and RNaseIII_Rev (5'-GCCGGCATGCTGTTTCCTGTGTGATAAAA-3') were allowed to hybridize and digested with *KpnI* and *SphI* and ligated into the corresponding sites of pMH305 (Hentzer *et al.*, 2002) to yield pMH487. The *P. aeruginosa lasB* promoter was excised as a 350 bp of *KpnI-XbaI* fragment from pMRP24-*lasB* and ligated into the corresponding sites of pMH487. The resulting vector, pMH510 containing the *lasB-gfp* reporter fusion on a *Pseudomonas* shuttle vector, was electroporated into *P. aeruginosa* PAO1 to yield strain MH516.

Bacterial Strains and Growth Media

Biofilms were grown from the reporter *P. aeruginosa* PAO1 which carried pMH516 plasmid encoded gene fusion between *lasB* and green fluorescent protein (GFP) genes. Luria-Bertani (LB) full strength broth (20 g/L) was used as the growth medium for biofilms and the 24 hour shake flask cultures, which were used for initial inoculation. LB agar (LA) was used for culturing the effluents for Colony Forming Unit (CFU) counts as well as culture purity checks. To verify that flow cell conditions were suitable for GFP production, biofilms were grown from *P. aeruginosa* PAO1 with plasmid pMF 230 (Nivens *et al.*, 2001) encoded constitutive GFP under the same conditions. The plasmids pMF230 and pMH509 were maintained on a selective LA with 300 µg/mL carbenicillin and 40 µg/mL gentamicin, respectively. Growth curve analysis revealed there were no differences in the doubling time between the GFP tagged and Wild Type (WT) PAO1 strains (2.3 hours).

Batch Culture Experiments

A sterile 250 mL Erlenmeyer flask containing 100 mL of full strength LB was inoculated with 100 µL of overnight non-fluorescent cells culture of pMH516, ($OD_{600} = 0.4$). The flask culture was incubated on a shaker incubator at room temperature. Samples were taken for OD_{600} , confocal imaging and OddHL measurements every hour till cells entered exponential phase, during which the sampling was performed every 40 minutes. When the cells entered the stationary phase, the sampling intervals were taken once in every 2 hours and then after 23 hours during the late exponential phase.

OddHL Measurements

Sampling for ODDHL assay. One mL biofilm bulk fluid which were collected in the effluent were filtered through the 0.2 μ m filter into sterile vial and stored at -6 °C freezer until the assay time. For the planktonic culture 1 mL was aspirated from the flask culture at each sampling time points and was similarly filtered through the 0.2 μ m filter into sterile vial and stored at -6 °C freezer until the assay time.

β -galactosidase assay by *E. coli* reporter. Bioactive OddHL activity was detected by utilizing an *E. coli* MG4/pKDT17 indicator strain, as described elsewhere (Pearson *et al.*, 1994), by standard β -galactosidase assay (Miller 1972).

OddHL concentration. The specific units of β -galactosidase measured in the samples were converted into OddHL concentration by using a formula obtained from the standard curve. A dose-responsive curve was generated by reacting 10 μ L of known amount of OddHL concentrations (1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M) with the reporter *E. coli* and by plotting the OddHL concentrations over the specific units of β -galactosidase. [OddHL] = 0.0023 x (β -galactosidase specific units) – 0.3011; $R^2 = 0.9857$).

Batch system. The onset of reporter GFP expression in the planktonic culture of *P. aeruginosa* pMH 516 occurred during the early log phase when the cell concentration reached $OD_{600} = 0.6$. The fluorescence intensity increased until the cells entered stationary phase ($OD_{600} = 1.8$) and leveled during the late stationary phase. Concentration of bioactive signal in the system increased from initial zero or below

detection level to a maximum 17 nM during the mid logarithmic growth phase ($OD_{600} = 0.8$) and decreased to about 5 nM during the late stationary phase (Figure 4.1)

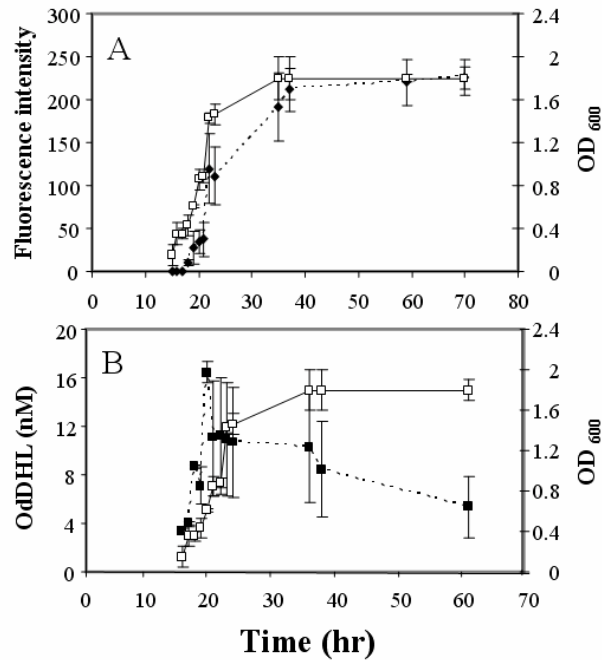


Figure 4.1. *P. aeruginosa* pMH 516 batch culture growth curve. A) fluorescence intensity (FI) of individual cells (■) and OD_{600} (□), B) OdDHL concentration (■) and OD_{600} (□). Each point on the primary axis is a mean \pm S.D. of $n=30$ from 2 individual experiments and $n=4$ from 2 individual experiments for the secondary axis.

Biofilm Culture Flow Cell System

Biofilms were grown in 1mm x 1mm square glass capillary (Friedrick & Dimmock®, Millville, NJ, USA) flow cells, which were incorporated into a once through flowing system (Stoodley *et al.*, 1999). A sterile nutrient medium was pumped through the system via a peristaltic pump at flow rates (Q) of = either 1.0, 0.1 and 0.005 ml/min. At these flow rates flow was laminar with Reynolds numbers of 16, 1.6, and 0.08 and wall shear stresses (τ_w) of 134, 13.4 and 0.7 mPa along the center of the lumen

respectively. Since the inoculum was injected directly into the flow cells through the septum on a “Y” fitting immediately upstream of the inlet the colonized volume in the viewing area was 0.14 ml and the hydraulic residence times were 0.14, 1.4, and 28 min. for each of the flow rates respectively. The flow cells were positioned in a polycarbonate holder which was mounted on the stage of an Olympus BH2 upright microscope or a confocal (Leica Microsystems, Inc.) so that the biofilm could be imaged *in situ* without interrupting flow. A septum sealed inoculation port was positioned upstream of the flow cell through which the initial load of bacterial cells were introduced to the system. Triplicate flow cell experiments with LB medium were run for *P. aeruginosa* pMH509 at each flow rate between 1 and 8 days. The growth period was for 5 days and the flow was turned off and the biofilm was monitored for a further 3 days. Under operating conditions the water temperature in the reactor system was 23 °C and all experiments were performed at this temperature.

Inoculum

The reactor containing full strength LB media was inoculated with 1 ml of 37 °C shake flask culture of microorganisms with $OD_{600} = 0.4$ at which the cells were nonfluorescent, i.e. there was no reporter activity. The flow system was allowed to incubate for 30 min, to permit attachment, before switching to continuous culture which was achieved by delivering full strength LB to the mixing chamber via peristaltic pump (Ismatek, Cole Parmer).

Reactor Sterilization

The reactor system was autoclaved at 121 °C for 15 minutes. The sterility of the reactor system was confirmed by plating 0.1 ml aliquots of effluent onto LA. Pure culture was confirmed through visual examination of colonies grown by plating 0.1 ml aliquots of effluent onto solid LA on a daily basis. Effluent from the reporter and GFP control biofilms were plated onto LA plates and LA plates with selective antibiotics to confirm the plasmid maintenance. There was no statistical difference between the Colony Forming Units (CFU) counted between LA and LA plates with gentamicin and carbenicillin ($P > 0.05$). The biofilms were grown without a selective marker pressure and therefore to confirm the plasmid maintenance the biofilm and confirm the plasmid was intact over the course of the experiments.

Microscopy

The developing biofilms in the flow-through system were nondestructively examined by Confocal Scanning Laser Microscopy (CSLM) and Olypmus BH2 microscope with a long range 10X, 50X and short range 100X objective lenses. Transmitted images were collected using a COHU 4612-5000 CCD camera (Cohu, Inc., San Diego, CA. USA) and captured with a Scion VG-5 PCI framestone board (Scion Inc. Frederick, MD). The Scion image software was used to collect time-lapse sequences and for image enhancement and analysis. A 1 mm graticule with 10 μm divisions was used to calibrate length measurements. The biofilm thickness and surface area coverage were measured on each day at five random locations in the biofilm area for each flow cell

experiment (Stoodley *et al.*, 1999). The reporter and control GFP in biofilm, planktonic and detached cell populations were examined by Confocal Scanning Laser Microscopy (CSLM) by exciting the GFP with 488-nm line of an argon laser.

Fluorescence Intensity of Cells

CSLM images of planktonic and OddDHL induced cells were collected by X100 power oil immersion lens. The average fluorescent intensity (FI) of individual cells were measured using Image J software (available at www.rsb.info.nih.gov/ij/) program and the results are reported as a mean \pm standard deviation S.D. of n=30 (n= number of individual cells) for each cell population.

Exogenous Addition of OddDHL to the Reporter Biofilms

As a positive control for the induction of *lasB* expression and detection of GFP synthetic OddDHL was dissolved from a powder form into 1 ml of 95 % ETOH and was added to LB for a final concentration of 10 μ M (Davies *et al.*, 200). LB + OddDHL was then delivered to 3-day old flow cell biofilms grown in 1 ml/min with a sterile syringe via the inoculation port and GFP production was monitored with confocal microscopy.

Constitutive GFP Expression in the Biofilm Culture Flow Cell System

For a positive control to demonstrate that we could detect GFP expression under our flow cell conditions (i.e. sufficient oxygen for GFP folding) we grew biofilms from isogenic PA01 biofilm carrying the pMF203 plasmid which constitutively expresses GFP

(Nivens *et al.*, 2001). Flow cell experiments with *P. aeruginosa* pMF 230 were run in duplicate for 1 and 0.005 ml/min flow regimens 14 days and GFP production was continuously monitored throughout the experimental time period.

Results

Biofilm Flow Cell System

Biofilm development. Immediately after inoculation PA pMH 509 cells readily colonized the glass flow under all three flow rates. After 5 days at the flow rates of 1ml/min and 0.1 ml/min the biofilms developed into circular microcolonies interspaced by a monolayer of single cells (Figure 2a and b).

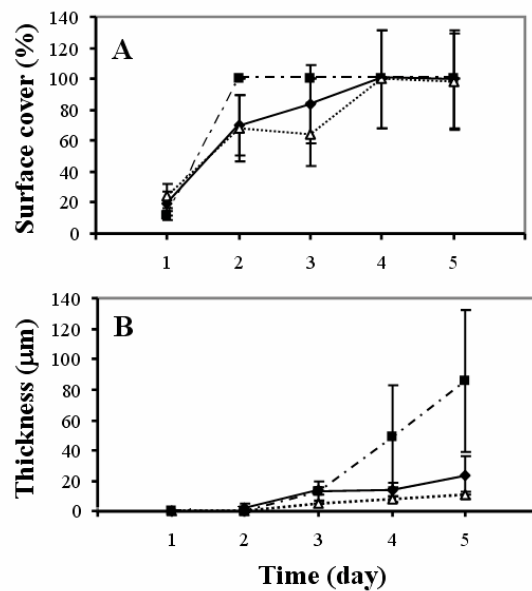


Figure 4.2. Biofilm accumulation quantified by surface area coverage (A) and thickness (B) of the *P. aeruginosa* pMH516 biofilm grown at 1ml/min (■), 0.1 ml/min (◆) and 0.005 ml/min (Δ), each point on the graph is a mean \pm S. D. of at least n=10 from 2 individual experiments.

However, at the lower flow rate of 0.005 ml/min the biofilm never developed more than a thin monolayer of cells (Figure 4.5c).

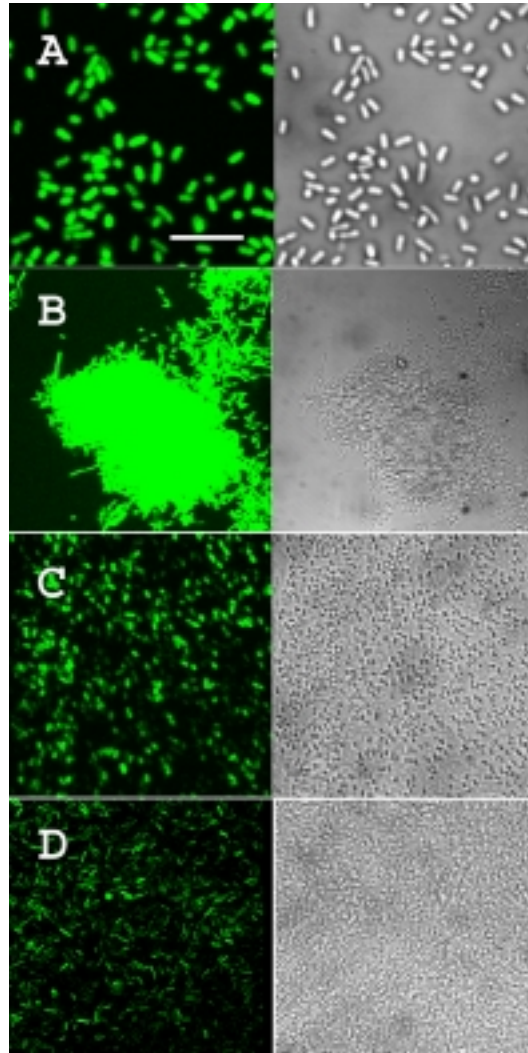


Figure 4.5. GFP fluorescence reporter activity indicates QS induction in individual planktonic and biofilm cells. A) planktonic pMH 516 cells in the batch culture during late logarithmic growth phase. B) pMF230 biofilm on day 7 with constitutive GFP expression (positive control for GFP detection). C) Five day old pMH 516 biofilm grown at 0.005 ml/min, 6 hours post-stagnation. D) Five day old biofilm grown at 1 ml/min, 10 hours post-stagnation. Image taken in a monolayer area between biofilm cell clusters. Scale bar = 20 μm on all panels except panel A. Images on the left are individual slices from a CSLM stack and those on the right are the corresponding transmitted light images.

Biofilm accumulation estimated by daily measurements of surface cover and thickness showed that initially by day 2, 1 ml/min biofilms covered the entire surface of the glass surface and about 60% in 0.1 and 0.005 ml/min grown biofilms (Figure 4.2a). However, by the end of experimental run time all the biofilms covered almost entire surface of the flow cells, in general. While the depth of the biofilms was about the same up to day 3 ($P>0.05$), the biofilm in 1ml/min gradually increased and resulted in significantly thicker ($80 \pm 20 \mu\text{m}$) biomass than those in 0.1 ml/min ($20 \pm 10 \mu\text{m}$) and 0.005 ml/min ($10 \pm 2 \mu\text{m}$) (Figure 4.2b), $P< 0.05$.

GFP reporter activity in the biofilm bulk. Throughout the growth period (with continuous flow of the liquid media), there was no reporter activity indicating that the *las* QS system was not induced. (Figure 4.3a).

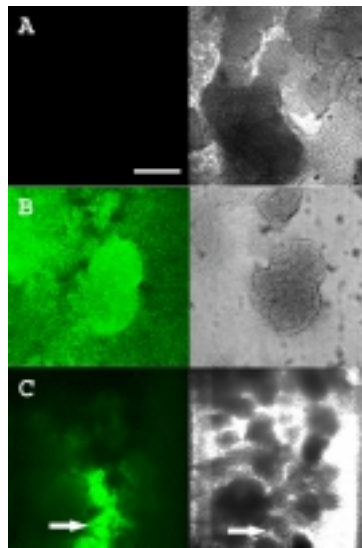


Figure 4.3. Reporter GFP expression in the PA pMH516 biofilms. Images on the left are maximum projections of the CSLM stacks showing GFP reporter activity and the images on the right are the corresponding transmitted light images. A) There was no reporter activity during flow (in this case 1 ml/min) but GFP was expressed (6 hr) after stagnation, (B). C). Reporter activity indicated that cell signaling was heterogeneous in the biofilm. In some cases the reporter activity appeared to be concentrated in the clusters while in others was localized in the monolayer regions between clusters (indicated by the white arrow in Panel C). The scale bar = 40 μm in panels A and B, and 100 μm in C.

However, after stopping the flow (stagnation phase) GFP reporter activity was detected (Figure 4.3b). The lag period for the onset of GFP in the stagnant biofilms varied between the replicate experiments as well as between the flow rates (Figure 4.4).

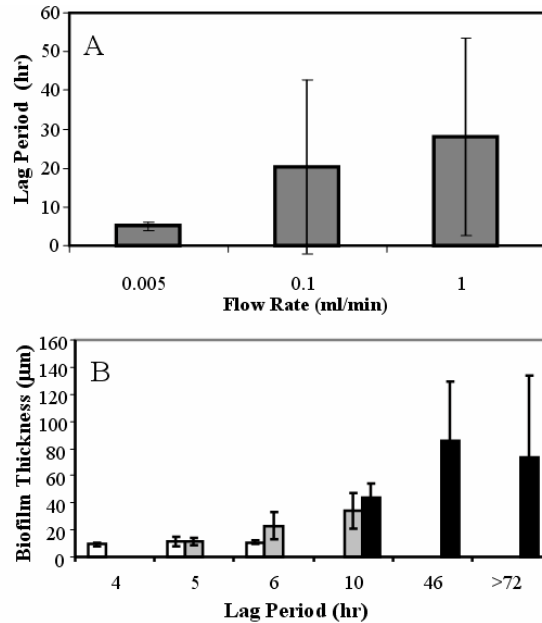


Figure 4.4. The lag period for GFP induction in the stagnant biofilms as a function of flow rate (A) and the thickness (B) in 3 individual experiments for 0.005 ml/min (□), 0.1 ml/min (▒) and 1 ml/min (■) grown biofilms. While the reporter activity wasn't detected in the flow cells during the growth phase induction was observed after stagnation. Each point on panel A is a mean \pm S. D. of 3 individual experiments. i.e. the lag period was the period taken to detect reporter activity after flow was switched off and a mean \pm S. D of 5 individual measurements on panel B.

Biofilms cultivated at 0.005 ml/min expressed GFP after either 4, 5, and 6 hours post stagnation in the 3 replicates while GFP was detected in biofilms grown at 0.1 ml/min. after 5, 10, and 46 hours. The biofilms grown at 1ml/min showed reporter activity after 10 and 46 hours in replicates 1 and 2, but in the third replicate GFP reporter activity was still not detected after 3 days post stagnation. Monitoring was stopped at this time due to dissolution and detachment of the biofilm. While the expression of GFP was relatively uniform in the thinner biofilms ($< 50 \mu\text{m}$), in the thicker biofilms reporter

activity was heterogeneous (Figure 4.3c).

Concentration of OddDHL in the biofilm effluent. The concentration of bioactive signal OddDHL in the effluent of the biofilms grown at 1 ml/min were below detection level for the first 3 days and increased from 0.1 nM on day 4 to 1 nM on day 5 (Figure 6a).

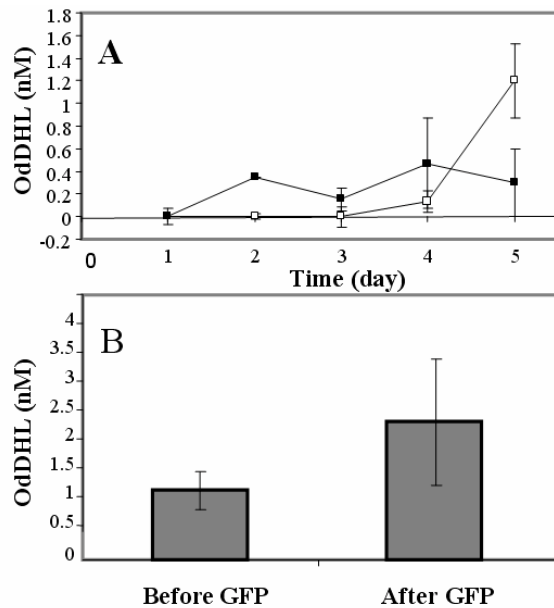


Figure 4.6. A) The OddDHL concentration in the effluent of 1 ml/min (□) and 0.005 ml/min (■) over the course of the experimental period. B) OddDHL concentration in biofilm bulk fluid before stagnation (no reporter activity in the biofilms) and after GFP expression in the stagnation phase (day 5 biofilms grown at 1 ml/min). Each point on the graph is a mean \pm S.D. of $n=6$ measurements from 2 individual experiments.

The OddDHL in the effluent of the biofilm grown at 0.005 ml/min was always lower than 0.5 nM (Figure 4.6b). The signal concentration has increased to 2.5 ± 1.5 nM during the onset of GFP reporter activity but was lower than the concentration required for the batch induction at the time of GFP production (Figure 4.6b).

Control Experiments

During the PA01 pMF 230 biofilm growth and development we have observed radial expansion as well as detachment and subsequent re-growth of the biofilm micro colonies were evident during which we saw the expression of brightly fluorescent GFP (Fig 4b, fluorescent intensity throughout experimental time was 235 ± 13 , compared to batch planktonic $FI=225 \pm 21$, $P>0.05$). Also in two out of 3 replicate experiments we were able to induce the expression of GFP in the flow cell grown biofilms by exogenous addition of the 10 μ M OdDHL signal molecule in the absence of flow (Figure 4.7).

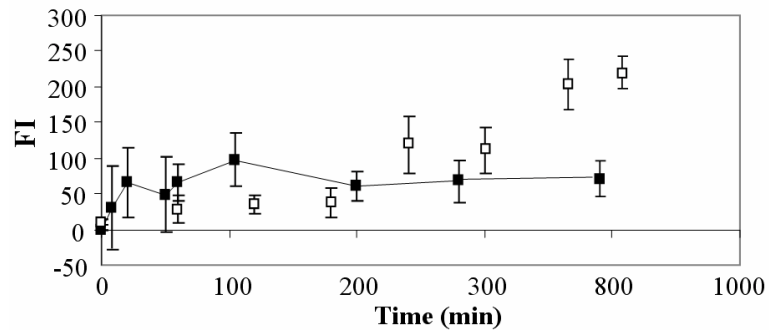


Figure 4.7. GFP fluorescence intensity over time by individual cells in planktonic batch (□) and in 3 day old biofilms induced by exogenously added OdDHL (10 μ M) (■). Each point on a graph is a mean \pm S.D. of $n=30$ cells from 2 individual experiments.

GFP production was evident within 10 ± 5 minutes of signal addition and continuously increased for the next 3 hours, which was approximately 10 times faster than the lag period for GFP onset in the biofilms by stagnation alone.

Discussion

In this study we examined QS onset and its spatio-temporal expression pattern *in situ* biofilms grown under different flow regimens. We utilized *P. aeruginosa* pMH, a PAO1 strain carrying a plasmid encoded gene fusion encompassing a GFP reporter for expression of the *lasB* promoter region which allowed nondestructive real time imaging of QS expression at a single cell level. The expression of the *lasB* reporter complex is tightly controlled by *lasI/lasR* (Gambello and Iglewski 1991) system and as such has been utilized for monitoring las/rhl QS in *P. aeruginosa* (Hentzer *et al.*, 2002). Control experiments with planktonic batch cultures demonstrated that the reporter was sensitive to QS induction with OdDHL concentrations as low as 10 nM. The GFP fluorescence intensity of individual cells, as well as the concentration of endogenously produced OdDHL in the bulk fluid, increased in a cell density dependent manner, reaching a maximum brightness (FI = 225) and signal concentration (17 nM) during late (35 h) logarithmic growth. This was in agreement with previous findings (Hentzer *et al.*, 2002; Brumlik & Storey, 1992).

During the biofilm growth and development in the flowing system, however, we did not observe the onset of the las/rhl QS system as indicated by lack of reporter activity over the full five days of the experiment, despite an increase in adhered biomass shown by daily surface area coverage and thickness measurements. At the higher flow rates of 0.1 and 1 ml/min ($\tau_w = 13.4$ and 134 mPa) the biofilms grew thicker and consisted of distinct microcolonies interspersed by a sparse monolayer of single cells. At the lowest flow rate of 0.005 ml/min ($\tau_w = 0.7$ mPa) the biofilm did not form distinguishable microcolonies but remained a thin homogenous layer. These findings are consistent with

previous work in which we reported that PAO1 biofilms grown at a τ_w of 5.09×10^3 mPa were heterogeneous and consisted of a patchy pattern of ripple and streamer formations while biofilms grown at 9 mPa were flat and undifferentiated (Purevdorj *et al.*, 2002). Interestingly, in the same study using an OddDHL knockout (JP1) we reported that cell signaling had only a subtle influence on biofilm development and was not required for the formation of structurally differentiated biofilms in the high shear flows (Stoodley *et al.*, 1999; Purevdorj *et al.*, 2002). Our findings presented here, strengthen our previous conclusions that 1) cell signaling is not necessarily required for biofilm formation in *P. aeruginosa* and 2) that the hydrodynamic conditions of the growth environment will have a significant impact on QS based cell signaling systems. In our flow system the absence of GFP expression can not be attributed to oxygen limitation or loss of reporter construct, since we demonstrated constitutive GFP production in the control pMF230 biofilms, and confirmed the plasmid maintenance by plating the detached and biofilm cell population on a selective marker. Low OddDHL levels in the biofilm bulk effluent as well as the immediate induction of reporter activity with exogenous OddDHL addition to the non-fluorescent biofilms further supports our hypothesis that the signal molecules were being removed by the continuous flow before accumulation of inducible levels were achieved, even at the lowest flow rate of 0.005 ml/min. However, we point out that the reactor geometry, as well as flow rate determines the overall hydrodynamics. Our results are in contrast to De Kievit *et al.* (2001) who did see expression of *lasI* (using a GFP reporter) in flow cell grown *P. aeruginosa* biofilms on the monitored days 4, 6 and 8. It appears unlikely that this difference can be attributed to hydrodynamics since their *Re* (approximately 0.6) and system residence time (6.5 min) were both within our range and

their shear stress (0.4 mPa) was similar to ours at our lowest flow rate of 0.005 ml/min. (0.7 mPa). It is more likely that the discrepancy is attributable to differences in nutrient source since they used FAB-citrate medium whereas we used LB. Indeed, in static experiments they found that when grown on FAB-citrate medium there was little difference between biofilms formed from wild type (WT) and signaling knockout mutants in contrast to marked differences when grown on a M9 - glucose medium. The main difference between these media, in addition to the carbon source, is that FAB also contains ethylene diamine tetra acetate (EDTA) and CaCl_2 both of which may influence biofilm structure through cross-linking interactions in the extracellular polymeric substance (EPS) slime matrix. Regardless of specific mechanisms it is apparent that nutrient source can have a profound influence on cell signaling and biofilm structure.

While there was no GFP activity in the biofilms within the range of tested flow regimens down to 0.005 ml/min, GFP was expressed in all biofilms after the flow was switched off. The lag periods of induction of *lasB* by GFP reporter activity was highly variable and ranged from 4 to 46 hours. The length of the lag period was positively correlated with flow rate but also with biofilm thickness, since biofilms grown at higher flow rates were thicker. Flow rate will influence two parameters important to attached biofilms exposed to flowing fluids. The physical parameters (shear stress, and drag and lift forces) acting on the biofilm, and mass transfer, which governs the transport of solutes both into (i.e. exogenous nutrients) and out of (i.e. endogenous products such as metabolites and signaling molecules) the biofilm (Stoodley *et al.* 1999 – Biofouling reference). Data from Peyton (1996) showed that over a shear stress range of 1.44 to 2.97 Pa there was no

trend between shear stress and biofilm thickness but there was a linear increase of thickness with substrate loading rate (S_1), where for our system:

$$S_1 = QC/A \quad [1]$$

where Q = nutrient flow rate (L^3/T), C = substrate concentration (M/L^3) and A = reactor surface area (L^2). Similarly in our system operating under a range of low shear stresses it is possible that the increase in fluid flow had a negligible effect on detachment but stimulated growth through increased nutrient delivery (Stoodley *et al.* 1999, Beyenal & Lewandowski 2004).

Interestingly, the signal concentration in the stagnant bulk fluid (2.2 nM) was still below our estimated threshold concentration in the planktonic fluid (10 nM) as well as 20 nM in the biofilm (Hentzer *et al.*, 2002), at the time of reporter activity, suggesting that the critical concentration of signal OdDHLs was localized within the biofilm clusters.

Our current study once again demonstrates importance of hydrodynamics in modulating the biofilm structure and also expression of QS pathway in *P. aeruginosa*. It is possible that the individual cells have evolved pathways to co-ordinate group behavior not only as a function of cell density but also to external growth conditions which would contribute to its well known ability to thrive in diverse range of environments. Growth conditions should be considered as a possible interfering factor when QS is used as a method for biofilm control.

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CHAPTER 5

PHENOTYPIC DIFFERENTIATION AND SEEDING DISPERSAL IN NON-MUCOID
AND MUCOID *PSEUDOMONAS AERUGINOSA* BIOFILMS.Summary

There is growing evidence that *Pseudomonas aeruginosa* biofilms exhibit a multi-cellular developmental life cycle analogous to that of the myxobacteria. In non-mucoid PAO1 biofilms cultured in glass flow cells we describe the differentiation of bacterial microcolony into an interior motile, swarming, phenotype and a non-motile surrounding, “wall phenotype” which formed a fruiting body-like shell. After differentiation the interior cells swarmed out of the microcolony from local break out points and spread over the wall of the flow cell. A critical microcolony diameter of approximately 100 μm was required for differentiation suggesting that regulation was related to cell density and mass transfer conditions. We term this phenomenon “seeding dispersal”. We found that nascent PAO1 microcolonies growing in grooves scratched onto an agar surface exhibited seeding dispersal, while those on the smooth surface did not. This could be explained by the significantly higher cell packing density in the grooves, suggesting the presence of a short-range intercellular signal analogous to C-factor protein required for fruiting body differentiation in *Myxococcus* spp. Using the flow cell and the scratched agar techniques to screen for seeding dispersal in mutations known to be associated with biofilm structure we found that the production of rhamnolipid (*rhlA*⁻), las/rhl quorum

sensing (QS) signals (PAO1-JP1 and PAO1-JP2), and pilus (*pilA*) were not required for seeding dispersal. However, flagellar motility (*flhM*) and the mucoid cystic fibrosis (CF) isolate, *P. aeruginosa* FRD1, did not exhibit seeding dispersal. The clinical significance of seeding dispersal in mucoid and non-mucoid strains as well as mucoidy conversion in CF is also discussed.

Introduction

The proliferation and persistence of bacterial biofilms on various surfaces have been well documented in both *in vitro* and *in vivo* settings and modern microscopic and molecular techniques have revealed that biofilm formation is a complex multifactorial process regulated by both genetic and environmental factors (Stoodley *et al.*, 2002). Although much is known about the initial stages of biofilm development very little is known about the mechanisms governing the detachment process (Stewart 1993; Stewart *et al.*, 2000). Detachment has recently been shown to play an important role in shaping the morphological characteristics and structure of mature biofilms (Picioreanu *et al.*, 2001; Stewart 1993; Van Loosdrecht *et al.*, 1995; Van Loosdrecht *et al.*, 1997) which further extends the implication of this process in biofilm function and behavior in general. Earlier studies have characterized two main types of detachment processes; erosion (the continual detachment of single cells and small portions of the biofilm) and sloughing (the rapid, massive loss of biofilm) (Bryers 1998; Charaklis 1990; Stoodley *et al.*, 2001). However, these types of detachment were generally thought of in terms of passive, shear dependent processes. Only recently has detachment been considered as an active dispersal mechanism. A number of laboratories have observed the “hollowing” out

of microcolonies by cells actively leaving the interiors (Sauer *et al.*, 2002) and the remaining “hollow mounds” have been noted in *Pseudomonas putida* biofilms (Tolker-Nielsen *et al.*, 2000). Based on previous reports the suggested mechanisms for this particular hollowing process in the biofilm clusters differ from one biofilm species to another. For example, Kaplan *et al.* (2003a and b) have shown that “non-motile” Gram negative bacteria *Actinobacillus actinomycetecomitans*, grown statically on agar released individual cells from within the biofilm colony via enzymatic activity of β -hexosaminidase. On the other hand transmission electron micrographs (TEM) of *Staphylococcus epidermidis* cultured on the agar plates suggested that the hollowing process may have occurred through the process of cell lysis (Stewart, P., personal communication). Also Webb *et al.* (2003) have demonstrated that the cells interior of *P. aeruginosa* clusters lysed via prophages, which resulted in the formation of a structurally differentiated biofilm cluster.

In previous experiments (Purevdorj-Gage & Stoodley, 2003, preliminary results) we observed highly motile areas inside clusters of non-mucoid *P. aeruginosa* PA01. We hypothesized that this behavior was linked to the dispersal process and resulting hollow mounds. It was the goal of this work to quantify the progression and dimensions of biofilm colonies as well as to demonstrate the actual seeding dispersal process in the flowing system, all of which to our knowledge have not been reported elsewhere. The biofilms of non-mucoid *P. aeruginosa* PA01, isogenic rhamnolipid deficient strain and *P. aeruginosa* FRD1, a mucoid isolate from a cystic fibrosis (CF) patient, strains were grown in a once through flow system and Digital Time Lapse Imaging Microscope (DTLM) and Scanning Laser Confocal Microscope (SLCM) were used for visualization

and quantification. We developed a rapid screen for seeding motility in *P. aeruginosa* by adapting a technique used by Kim & Kaiser (1990). They found that they could induce contact dependent expression of genes involved during the fruiting body formation in the non motile *Myxococcus* spp. colonies by allowing them to develop within the fractured zones of agar. Microscopic observations revealed that these zones artificially created a high density environment which maximized a transfer of a short range membrane bound “C-factor” signaling. By growing colonies on roughened agar poured on a microscope slide we investigated the effects of various isogenic PA01 mutants deficient in flagella, rhamnolipid and global Quorum Sensing (QS) las/rhl systems on seeding dispersal since these are recognized factors associated with *P. aeruginosa* biofilm structural development (Davey *et al.*, 2003, Davies *et al.*, 1998). Finally, the clinical significance of seeding dispersal in mucoid and non-mucoid strains as well as mucoidy conversion in CF is discussed in our conceptual model describing the *P. aeruginosa* life cycle.

Materials and Methods

Bacterial Strains and Media

Luria-Bertani (LB) full strength broth (20 g/l) was used as the growth medium for biofilms and the 24 hour shake flask cultures, which were used for initial inoculation. Appropriate selective antibiotics were used for the various mutants. Biofilms were grown from the *P. aeruginosa* strains, listed in Table 1.

Strain/plasmid and genotype	Phenotype and description	Swarmin g dispersal	Source
<i>P.aeruginosa</i> PA01	Wild type	+	Pearson <i>et al.</i> , 1998
<i>P.aeruginosa</i> PA01-JP1, <i>lasI</i> ::Tn10, Tc ^r	QS ⁻	+	Pearson <i>et al.</i> , 1998
<i>P.aeruginosa</i> PA01-JP2, Δ <i>lasI</i> ::Tn10, Tc ^r ; Δ <i>rhlI</i> ::Tn501, Hg ^r	QS ⁻	+	Pearson <i>et al.</i> , 1998
<i>P.aeruginosa</i> PA01, <i>rhlA</i> ::Tn5, Gm ^r	Rhamnolipid ⁻	+	O'Toole, & Kolter 1998.
<i>P.aeruginosa</i> , PA01, <i>fliM</i> ::Tn5B30 Tet ^r	Flagella ⁻	-	Klausen <i>et al.</i> 2003
<i>P.aeruginosa</i> FRD1, CF patient isolate, <i>mucA22</i> Alg ⁺	Mucoid	-	Ohman <i>et al.</i> , 1981
pMF36 with <i>mut2</i> GFP, Ca ^r	Constitutive GFP	+	Nivens <i>et al.</i> , 2001
<i>P.aeruginosa</i> PA01, <i>pilA</i> ::Tn5B30 Tel ^r ,	Pili ⁻	+	Klausen <i>et al.</i> 2003

Table 5.2. A list of strains, their sources and descriptions.

Biofilm Culture Flow Cell System

Biofilms were grown in 1mm x 1mm square glass capillary (Friedrick & Dimmock®, Millville, NJ. USA) flow cells, which were incorporated into a once through flowing system (Stoodley *et al.*, 1999). A sterile nutrient medium was pumped through

the system via a peristaltic pump at flow rate of = 1.0 ml/min. At this flow rate flow was laminar with a Reynolds number of 16 and a shear stress of 0.3 Pa along the center of the lumen. The flow cells were positioned in a polycarbonate holder which was mounted on the stage of an Olympus BH2 upright microscope so that the biofilm could be imaged *in situ* without interrupting flow. A septum sealed inoculation port was positioned upstream of the flow cell through which the initial load of bacterial cells were introduced to the system. Triplicate flow cell experiments were run for 5 days for *P. aeruginosa* PA01, FRD1 and duplicate experiments for *rhlA* mutant with two replicate flow cells for each experiment. Under operating conditions the water temperature in the reactor system was 23 °C and all experiments were performed at this temperature.

Reactor Sterilization

The reactor system was autoclaved at 121 °C for 15 minutes. The sterility of the reactor system was confirmed by plating 0.1 ml aliquots of effluent onto LB agar (LA). Pure culture and conversion of mucoidy (for *P. aeruginosa* FRD1) flow cell experiments was confirmed through visual examination of colonies grown by plating 0.1 ml aliquots of effluent onto solid LA on a daily basis. Effluent from the *rhlA* mutant biofilm were plated onto LA plates and LA plates with selective antibiotic (70 µg/ml gentamicin) to confirm integrity of the mutation. There was no statistical difference between the Colony Forming Units (CFU) counted between LA and LA plates with gentamicin ($P > 0.05$).

Inoculum and Media

The reactor containing full strength LB was inoculated with 1 ml of an overnight full strength LB 37 °C shake flask culture of microorganisms. The flow system was allowed to sit for 30 min, to permit attachment, before switching to continuous culture which was achieved by delivering full strength LB to the mixing chamber via peristaltic pump (Cole Parmer #7553-80). Effluent samples were taken periodically to monitor the detached population and to confirm culture purity.

Microscopy

The developing biofilm was visualized *in situ* by using transmitted light and 5X, 10X and 50X objective lenses with an Olympus BH2 microscope. Images were collected using a COHU 4612-5000 CCD camera (Cohu, Inc., San Diego, CA. USA) and captured with a Scion VG-5 PCI framestone board (Scion Inc. Frederick, MD). The Scion image software was used to collect time-lapse sequences and for image enhancement and analysis. A 1 mm graticule with 10 µm divisions was used to calibrate length measurements. The biofilm thickness and surface area coverage were measured on each day at five random locations in the biofilm area for each flow cell experiment (Stoodley *et al.*, 1999).

Rheometry Experiments

To investigate the role of biofilm viscosity on seeding dispersal PA01 and FRD1 biofilms were grown on LA plates for 48 hrs at 36 °C. The colonies were aseptically

scraped off the agar surface and used for rheometry measurements after Towler *et al.* (2004). Two individual agar plates were used for each individual measurement. A total of 8 replicate measurements from PA01 and 7 from FRD1 were performed for statistical comparisons. The viscosity was measured from parallel plate shear creep tests using a TA Instruments AR 1000 Rheometer (info@tainst.com) in which the accumulated strain in response to an applied shear stress of 0.5 or 1 Pa was measured over time.

Motility Assay

To investigate the role of motility in the seeding dispersal we used a motility assay at different agar viscosities. Sterile LA plates with varying concentrations of the agar (0.3%, 1.5%, 3% and 5%) were prepared. Equal concentrations of bacterial cultures (OD₆₀₀ nm) were stab inoculated into the agar plates with a toothpick. The plates were inverted and incubated at 36° C for 16 hours. The radius of the zone of spreading from the stab was then measured. Twelve replicate measurements were performed for each testing strain.

The “Seeding Dispersal” Rapid Screen Assay

The surface of a LA poured on a microscope coverslip was aseptically scored by parallel strokes with sterilized Al₂O₃ sandpaper. An aliquot of an overnight bacterial culture (adjusted to OD 0.1) was spread plated over the scored and non-scored surfaces using sterilized hockey sticks. The plates were incubated at 37°C. Motility within the grooves was assessed with low power (10x objective lens) light microscopy and high power confocal (63x objective lens) microscopy to visualize individual cells and pattern

of organization after 8 hours of incubation. For cell density and packing quantifications, *P. aeruginosa* PA01 with plasmid pMF230 (Nivens *et al.*, 2001) encoded constitutive Green Fluorescent Protein (GFP) was used. Confocal Scanning Laser Microscopy was used for cell visualization by exciting the GFP with 488-nm line of an argon laser. The plasmid was maintained on a selective LA with 300 $\mu\text{g/ml}$ carbenicillin. Growth curve analysis revealed there was no difference in the doubling time between the GFP tagged and Wild Type (WT) PA01 strains. The cells cultured on the scored and non-scored agar surfaces as well as on the surface of glass flow cell (after day 2, when distinct microcolonies were observed,) were counted and the surface areas of the colonies were measured using Scion Image software (Scion Inc., Frederick, Md.) (Figure 5.1)

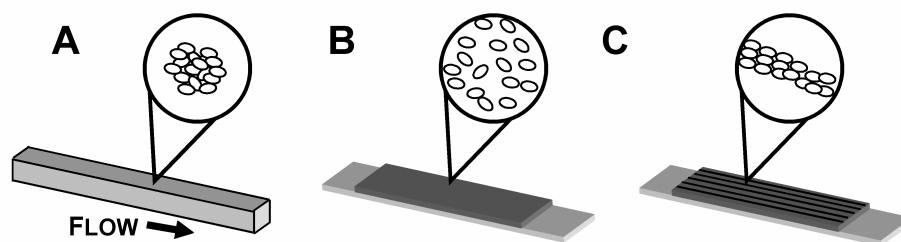


Figure 5.1. A schematic representation of flow cell (A), smooth (B) and roughened (C) agar mounted on microscopy cover slips. Microscopic observations of the cell organization in each system are shown in circles on each panel.

A 1-mm-long graticule with 10- μm divisions was used to calibrate measurements. The ratio of cells per unit area measured on flat and grooved agar, as well as flow cells ($n=5$) were statistically compared by univariate ANOVA Minitab software (version 13.3; Minitab, Inc., State College, Pa.). The data is reported as a mean \pm standard deviation (S.D.) of 10 individual measurements for each surface type. For quantification of cell alignment the number of cells per chain that were in end-to-end contact were quantified

for colonies growing on the smooth and grooved agar. Univariate ANOVA was used to statistically compare 20 replicate measurements for each surface. Differences for all the statistical comparisons were considered significant for P values of <0.05 .

Results

Flow Cell PAO1 and FRD1 Biofilms

Twenty four hours post inoculation both PAO1 and FRD1 biofilms consisted of a sparse single layer of cells. By the second day PAO1 has formed 20 ± 4 ($n=5$) μm thick biofilms with hemispherical microcolonies which were 70 ± 30 ($n=30$ individual clusters) μm in diameter. The FRD1 biofilm was 16 ± 3 μm thick with similarly shaped clusters 30 ± 7 ($n=30$ individual clusters) μm in diameter. Between the colonies there was a monolayer of cells so that the surface area coverage at this time had reached 100% in both biofilms. Throughout the remainder of the experimental run time the thickness and surface area cover did not change in PAO1 biofilm (P values > 0.05), and by day 5 it was 20 ± 2 μm thick and covered the entire surface of the glass flow cell. There were no significant differences between PAO1 and FRD1 daily thickness (all P values > 0.05) or surface area coverage measurements on any of the 5 days (all P values > 0.05), Figure 5.2.

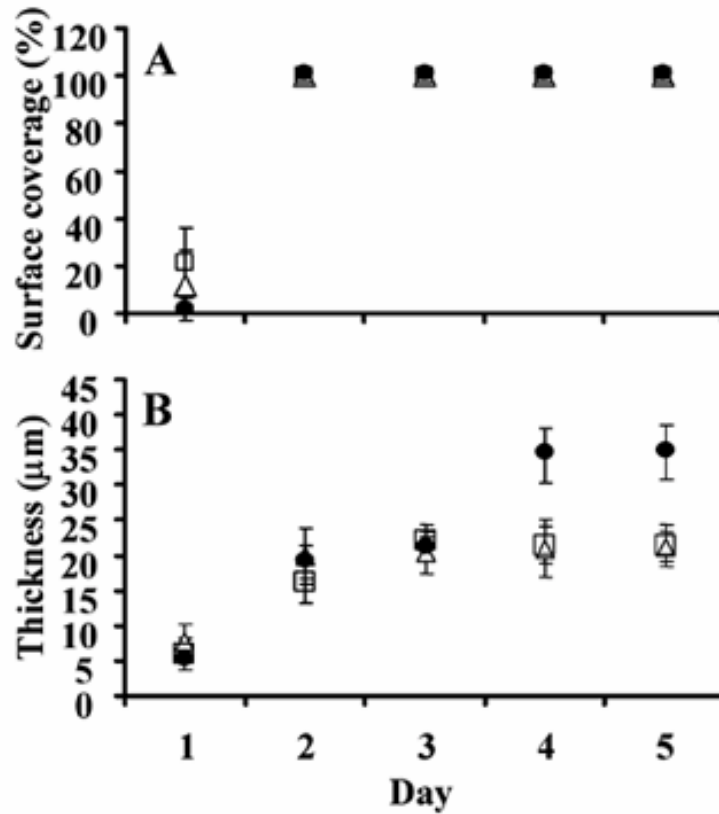


Figure 5.2. PA01 (open triangle), FRD1 (open square) and *rhlA* mutant (filled circle) biofilm development revealed by the surface coverage (A) and thickness (B) over time. Error bars are 1 standard deviation (n=5).

Similarly, by day 5 the average cluster diameter in FRD1 biofilm reached $120 \pm 50 \mu\text{m}$, which was similar to those measured in PA01 (P values > 0.05). The visual organization of the cell clusters was also similar in PA01 and FRD1 biofilms up to day 2. However, by day 3 we observed a prominent difference in the appearance of the mature clusters formed by two biofilm types (Fig. 5.3).

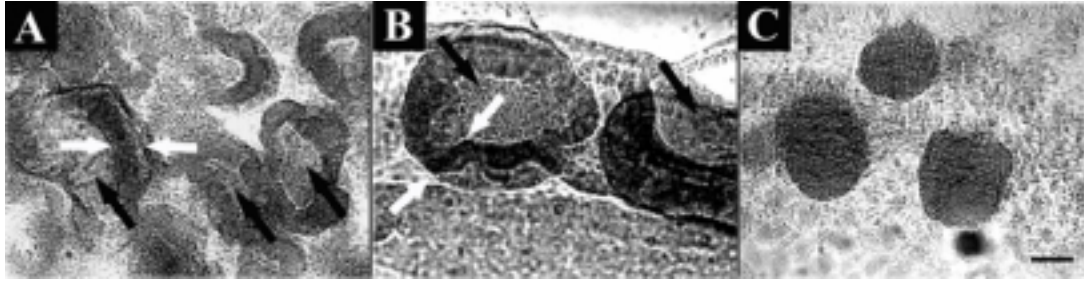


Figure 5.3. PA01 (A), *rhlA* mutant (B) and FRD1 (C) biofilms cultivated in flow cell system. The hollow mounds were evident in 5 day old mature PA01 biofilm clusters and 2 day old *rhlA* mutant biofilms. White arrows indicate the distinct walls of nonmotile cells and black arrows indicate void areas on individual clusters (A, B). FRD1 biofilm clusters do not display the same structural characteristics. The scale bar = 20 μm and the flow direction was from left to right.

The larger PA01 biofilm clusters had developed a hollow interior surrounded by a distinct shell-like wall of nonmotile cells in all 3 replicate experiments but the FRD1 clusters were consistently homogeneous and showed no evidence of hollowing. On closer inspection of the PA01 biofilm, we observed that on day 3 the clusters had differentiated into two distinct cell phenotypes; highly motile cells in the interior of the clusters and cells forming an outer cluster “wall” which remained stationary (Figure 5.4).

In some cases we observed the process of cells swimming out of clusters from local “breakout” points in the walls which were at random locations and independent of the flow direction (Figure 5.5).

With continuous monitoring of the clusters, from which the cells had evacuated, we noted that the remaining cluster walls did not exhibit further expansion and the central region of the cluster did not refill with new cell growth but remained as hollow mounds over the course of the experiment (Figure 5.6).

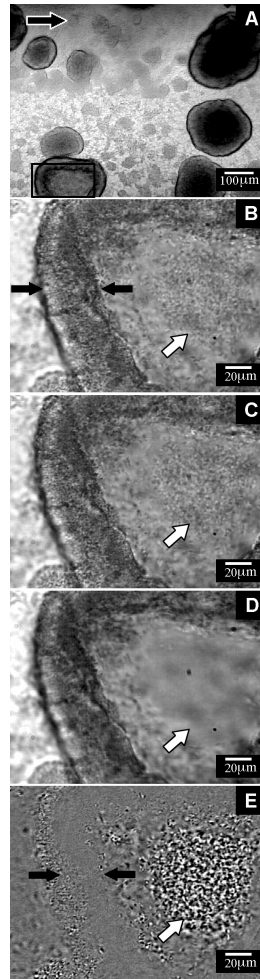


Figure 5.4. The evidence of motile cells within 3 day old biofilm cluster. Low power magnification of the biofilm clusters, higher power magnification of the cluster area surrounded by the black rectangle is shown on B and C at different time points (1 s apart). An image of the same cluster obtained by averaging the real time lapse images (30 frames) (D) and by subtracting B from C (E). The motile cells are blurred in the averaging process (D) whereas motile cells are evident and immobile cells are canceled out during the subtraction (E). The black arrows indicate the distinct walls of the hollow mound which is shown by the white arrow.

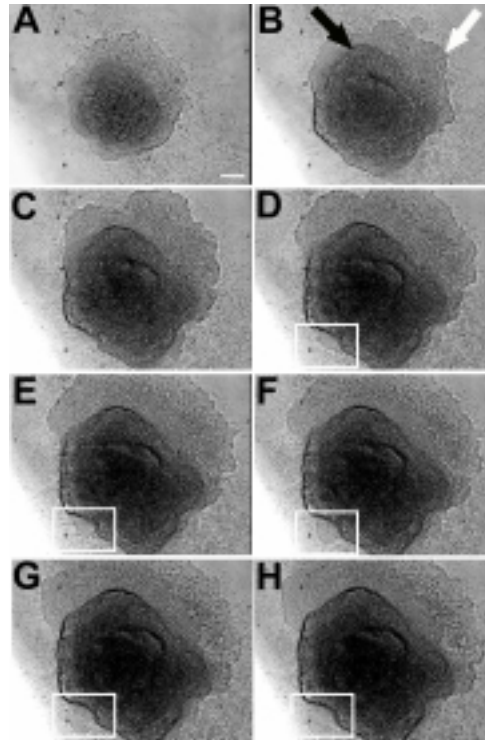


Figure 5.5. Time-lapse sequence depicting seeding dispersal in a 3 day old PA01 biofilm cluster. The time interval between the images was 15 minutes with the exception of 3 hr between frames “A” and “B”. The black arrow shows the immobile cluster wall whereas the white arrow indicates the dispersing cells. Another swarming and dispersal area is outlined by the white rectangle. Scale bar = 20 μm .

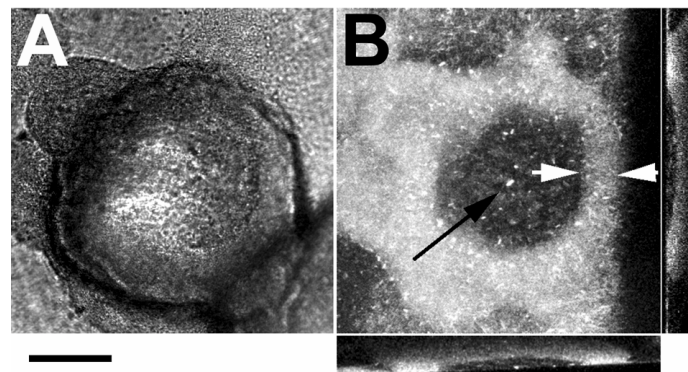


Figure 5.6. A hollow mound formed by Green Fluorescent Protein (GFP) tagged *P.aeruginosa* Pa01 (day 5). A transmitted (A) and the respective combined stacks of confocal image of the biofilm cluster expressing GFP (B). C and D are the cross sections of the cluster. A black arrow indicates the hollow mound and two white arrows indicate the walls of the cluster. The scale bar = 40 μm .

Quantification of Developmental Progression of Cell Clusters Exhibiting Seeding Dispersal in PA01

During the first two days of the biofilm growth $95 \pm 0.05\%$ ($n = 3$ independent experiments) of all the clusters present in PA01 biofilms appeared homogeneous without any obvious void areas, however, by day 3 as many as $46 \pm 20\%$ clusters had developed hollow mounds at the central regions of the biofilm cluster and increased up to $70 \pm 20\%$ by day 5 (Figure 5.7a).

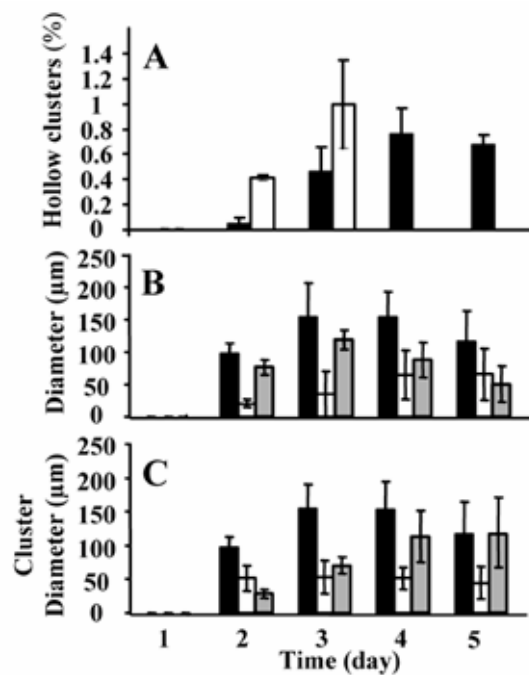


Figure 5.7. Development of microcolonies in the biofilms. (A) Percentage of the empty clusters in PA01 (black bars) and *rhlA* mutant (open bars) over time (mean and 1 S.D. from 3 independent experiments from PA01 and 2 from *rhlA* mutant). (B) The development of the hollow mounds in PA01, as revealed by the diameter of the hollow cluster (black bars), the diameter of empty area within the cluster (open bars) and the thickness of the wall (grey bars). Each point on the graph is a mean and S.D. of at least 20 individual clusters. (C) Cluster diameter (μm) of PA01 clusters which had developed hollow mounds (black bars) compared with those that had not yet emptied (open bars). FRD1 cluster diameters are also shown (grey bars). Each point in the graph is a mean and 1 S. D. from an n of between 20 and 30 clusters from at least 5 individual images.

With a closer analysis of these mounds, we determined that the void areas within the biofilm clusters gradually increased over time, but the thickness of the outer walls stayed roughly the same (Figure 5.7b). Interestingly, the cluster measurement data shows that there was a significant difference between the average diameters of the hollow and non-hollow clusters ($P < 0.05$) indicating that clusters which were hollow were at least 100 μm thick, while clusters which were homogeneous were less than 100 μm in diameter (Figure 5.7c).

Involvement of Rhamnolipid Biosurfactant in Seeding Dispersal

By day 2 the *rhlA* mutant biofilm covered the entire surface of the flow cell. However, during the last two days of the experimental run, its thickness reached 35 ± 5 μm (Figure 5.2), which was significantly greater than that measured in both PA01 and FRD1 biofilms ($P < 0.05$). Seeding dispersal and resulting hollow biofilm mounds (Figure 5.3b) with structural dimensions similar to PA01 (data not shown) were also evident in the *rhlA* mutant biofilm. While the hollow mounds in PA01 biofilms persisted until the end of the experimental run time, the hollow mounds in *rhlA* mutant had flattened into a homogeneous monolayer by days 4 and 5 (Figure 5.7a).

Rapid Assay for Dispersal and Involvement of Global QS System

We wished to develop a rapid screening assay for the seeding dispersal phenotype since the flow cell method is relatively time consuming. We did not observe this differentiation and dispersal on a regular smooth agar surfaces but we did discover however, that the cells growing in the biofilm microcolonies cultured in the glass flow

system were more densely packed (0.16 ± 0.03 cells/ μm^2) than those that on the agar surfaces (0.11 ± 0.02 cells/ μm^2 , $P=0.002$). In attempts to simulate the dense packing we adapted a method utilized by Kim & Kaiser (1990) where *Myxococcus* spp. was cultured on an agar surface which was scored to create microscopic grooves (5-10 μm wide). In accordance with Kim & Kaiser's observation the *P. aeruginosa* PA01 nascent colonies that grew in the grooves displayed dense packing (0.27 ± 0.04 cells/ μm^2) and a distinct pattern of cell organization, in which their long axes were parallel to the axis of the groove, maximizing cell to cell contact (Figure 5.8).

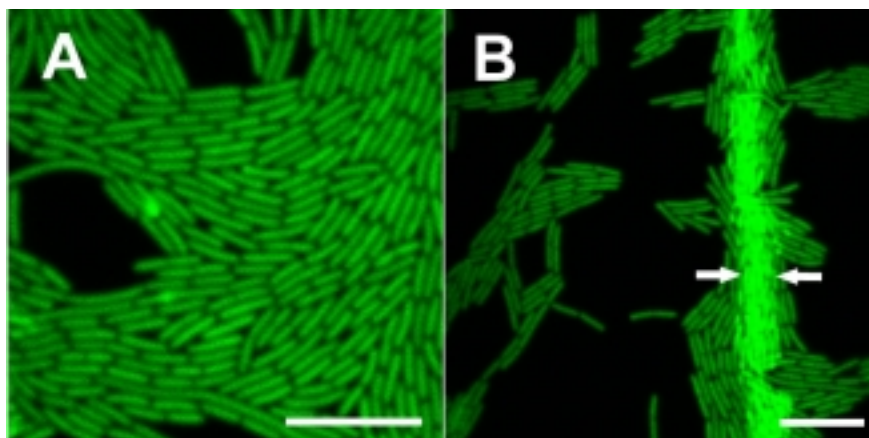


Figure 5.8. GFP tagged *P.aeruginosa* PA01 cells visualized by confocal microscopy. Cell organization and density on a flat surface (A) and on a groove (B) are indicated by white arrows. The scale bars are 10 μm .

Interestingly, we also observed significantly higher number of cells that were connected in chains in an end-to-end fashion than those nascent colonies that formed on a regular agar surface (12 ± 2.26 cells/chain, and 3.6 ± 1.23 cells/chain respectively, $P<0.000$). Eight hours post initial inoculation we have observed coordinated swarming of PA01 WT cells within these microscopic grooves but not in the colonies cultured on the smooth surfaces. Subsequently we utilized this method of culturing to screen various

isogenic mutant derivatives of *P. aeruginosa* PA01 parent strain for seeding dispersal (Table 5.1). Using this method *P. aeruginosa* PA01 WT and rhamnolipid deficient strains displayed a positive result for seeding dispersal whereas FRD1 was negative, which was consistent with flow cell observations. Furthermore, the flagella deficient strain was negative and QS mutants as well as pili mutant were positive in this assay (Table 5.1).

Motility Assay and Material Properties of PA01 and FRD1 Biofilms

In order to address the ability of different strains to utilize different modes of motility we performed a motility plate assay for PA01, isogenic *rhlA* and *fliM* mutants (as a negative control) and FRD1 on the varying concentration of agar (0.3%, 1.5%, 3% and 5% agar). On 0.3% (viscosity = 3.1×10^5 Pa.s) agar the swimming motility zone measured in *fliM* mutant and FRD1 strains were significantly less than that of PA01 and *rhlA* mutant (all P values > 0.05; data not shown) and none of the strains were able to spread over the plate surface with an agar concentration of $\geq 1.5\%$ (viscosity = 1.4×10^6 Pa.s). Since the agar viscosity has had an important role in motility of our test strains we wished to determine if the “increased” viscosity associated with FRD1 may partly explain its deficiency in the swimming motility. We performed creep curve tests in order determine the difference in biofilm viscosity between mucoid and non-mucoid *P. aeruginosa* by culturing FRD1 and PA01 biofilms on the surface of agar plates. Contrary to our expectation we found that the FRD1 colonies were significantly less viscous ($4.8 \pm 0.02 \times 10^5$ Pa.s. Mean \pm S.D; n=7) compared to the PA01 colonies ($5.7 \pm 0.2 \times 10^5$ n=8) (P = 0.014).

Discussion

Phenotypic Differentiation in the “Seeding Dispersal” Behavior

In this work we have documented a phenotypic differentiation in *P. aeruginosa* PA01 biofilm microcolonies which strongly suggests that this organism exhibits functional co-ordinated multicellular behavior associated with biofilm dispersal. We term this phenomenon “seeding dispersal”.

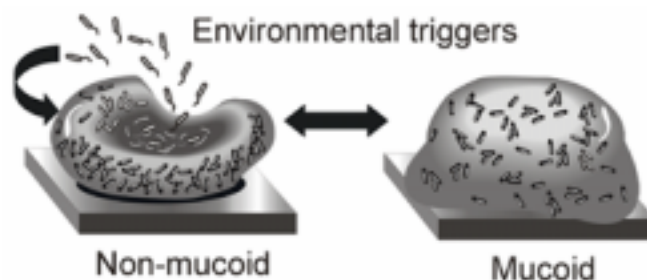


Figure 5.9. Conceptual model for *P. aeruginosa* life cycle. A non-mucoid or environmental isolate of *P. aeruginosa* biofilm cluster undergoes spatial and temporal differentiation, where the cells in the middle of the cluster triggered by the close proximity of neighboring cells become motile and swim out of the cluster in a coordinated manner. This process allows the detached cell population to colonize a new environment. The environmental non-mucoid biofilm can convert into the mucoid or alginate overproducing phenotype triggered by the environmental conditions in the CF lung. Mucoidity is known to confer an increased level of protection to the biofilm cells against a variety of harmful agents such as antibiotics and the host immune defense. Since mucoid isolates lack flagella, these biofilms are unable to disperse through seeding dispersal. With changes in the local environment the biofilm can revert back to non-mucoid, motile state capable of seeding dispersal.

There is also evidence for social behavior in *Escherichia coli* (Pratt & Kolter, 1998; Reisner *et al.*, 2003) and *Vibrio cholerae* (Watnick & Kolter, 1999) biofilms, suggesting that such behavior may be more common within the proteobacteria and not just a special phenomenon associated with myxobacteria (Kaiser 1998; 2003). By

studying seeding dispersal in mucoid and non-mucoid strains, as well as in rhamnolipid deficient and cell-cell signaling mutant strains, we have been able to propose a conceptual model for the role of this “seeding dispersal” in *P. aeruginosa* biofilm development (Figure 5.9).

By quantifying the spatial dimensions of the seeding microcolonies we have determined that there was a “threshold diameter” above which clusters differentiated into hollow mounds ($\geq 100 \mu\text{m}$ in our system), indicative of a spatio-temporal development. Moreover, while void areas within the hollow mounds gradually increased with radial expansion of the clusters, the thickness of the outer wall remained the same, suggesting that the transfer process of exogenous solutes such as nutrients, O_2 etc., into diffusion limited regions, namely interior, of the biofilm colonies was important in regulating this phenomenon. This detachment is then expected to depend upon the culturing conditions such as flow, nutrient concentration, the level of resident microbial activity etc., and its underlying mechanism may vary from one experimental set up to another.

Role of Rhamnolipid Surfactant Production in the Seeding Dispersal

The *rhlA* mutant biofilm was able to detach via the seeding dispersal process and formed hollow structural mounds similar to the PA01 biofilm colonies (Figure 5.2b). The dispersal process and structural dimensions of these hollow mounds were statistically comparable to the ones measured in PA01 biofilms (data not shown). Although both PA01 and *rhlA* mutant biofilms covered the entire surface of the glass flow cells, *rhlA* mutant has resulted in significantly thicker biofilm by the end of the experimental run,

which was consistent to Davey *et al.* findings (2003). While the hollow differentiated mounds persisted in PA01, the hollow colonies of *rhlA* mutant biofilm differentiated earlier in the biofilm growth and by day 4 formed a flat homogeneous biomass. This transient nature of differentiation and dispersal in *rhlA* mutant could therefore be a potential cause for missing this process in other experimental settings as well. Generally, *P. aeruginosa* can utilize three different modes of motility; flagella mediated swimming when the fluid film is sufficiently thick, swarming with flagella in the relatively thin fluid layer and twitching on the solid surfaces also by using pili (Rashid & Kornberg, 2000). Since in our study, the rhamnolipid deficient biofilm was able to differentiate into the hollow mounds, we conclude that this process doesn't occur via twitching or the swarming motility described in the literature since both processes are known to require rhamnolipid (Deziel *et al.*, 2003; Rashid & Kornberg, 2000). Unless there is other yet to be identified surfactants produced by *P. aeruginosa*, the seeding differentiation should occur via flagella dependent swimming in a coordinated manner. This is further supported by the negative result of *fliM* mutant and positive result of *pilB* mutant in our rapid plate assay.

Role of Global QS in the Seeding Dispersal

The PA01 *las/rhl* cell signaling mutants were positive for seeding dispersal phenotype, suggesting that this differentiation and dispersal process is not dependent on accumulation of acylated homoserine lactone (acyl-HSL) signaling molecules in a cell density dependent manner. This however does not exclude the possible role of another yet unidentified signaling pathways such as short range contact mediated signaling,

analogous to C-factor involved in the fruiting body formation in myxobacteria (Kim & Kaiser, 1990) as well as activity of QS linked gene products which can be activated via stringent response, independent of cell density (Van Delden *et al.*, 2001).

Clinical Significance of Seeding Dispersal in *P. aeruginosa*

It is a well known fact that chronically infected CF patients frequently harbor mucoid variants of *P.aeruginosa* (Høiby *et al.*, 2001). We wished to investigate a clinical relevance of this dispersal phenomenon by analyzing biofilms grown from CF isolate mucoid *P.aeruginosa* FRD1 strain. Interestingly in all three replicate flow cell experiments, FRD1 biofilms did not form hollow structures despite the fact that the mean cluster size as well as general biomass accumulation did not significantly differ between the two biofilms (all values for $P > 0.05$). The rapid assay in FRD1 was also negative which confirmed our observation in the flow system. Initially we speculated that inability of FRD1 to undergo the differentiation and dispersal could be due to its “high” viscosity. (In general FRD1 biofilms are commonly perceived to be a more viscous counterpart of wild type strain due to its voluminous slimy nature). The rheometry results however showed that FRD1 biofilms were less viscous than that an environmental non-mucoid PA01 ($P=0.014$). Since the result from our dispersal screening assay for flagella mutant was negative and mucoid FRD1 strain was deficient in swimming motility we speculate that its inability to undergo differentiation and subsequent dispersal could be due to the fact that it is deficient in flagellum function. In fact the variants of *P. aeruginosa* CF isolates are commonly known to have negative control of flagellum expression (Garrett *et al.*, 1999). It is thought that in the human host, the cells might benefit from loss of the

flagella due to its cost of synthesis (Macnab 1996), immunogenicity (Stanislavsky & Lam, 1997) and for its ability to acquire phage infections (Webb *et al.*, 2003).

Subsequently this active seeding dispersal process may not be the method utilized in the nonmotile variants of clinical CF isolates as speculated by others (Webb *et al.*, 2003) but rather a transmission mechanism in the environmental strains of *P. aeruginosa*, which have a functional flagellum.

Rapid Plate Assay

Flow cell grown biofilm microcolonies displaying seeding dispersal, had significantly higher cell densities than those grown on conventional agar plates. By considering this observation we induced a seeding dispersal in *P.aeruginosa* by culturing the cells within microscopic abrasions (grooves) that were artificially created on a surface of solid agar. Confocal microscopy images revealed that the cells that settled and have multiplied within these grooves but not on a smooth surface displayed a distinct cellular organizational pattern which maximized cell-to-cell contact and subsequently simulated an environment of high density and cell compaction observed in our flow system. This was also consistent with the observations made earlier by Kim & Kaiser (1990) where a similar developmental surface was used to artificially align *Myxococcus* spp. cells to achieve contact mediated expression of genes. As suggested by the authors these particular organization allows maximal cell to cell contact and rescues the expression of the developmental genes in *Myxococcus* spp., which otherwise are transcribed at higher cell densities (Kim & Kaiser, 1990). In agreement with flow cell data, the rapid assay indicates that close proximity between the neighboring cells is important in regulating the

process. We were able to use this developmental surface to screen for various isogenic mutants of *P. aeruginosa* PA01 and demonstrated results which corroborated our observations in the flow cell. We point out that this screening assay is limited to screening the seeding phenotype in cells and original flow cell approach should be utilized to study the entire differentiation process in seeding microcolonies.

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CHAPTER 6

CONCLUSIONS AND SUMMARY

In previous three chapters we demonstrated experimentally that hydrodynamics can have significant influence on biofilm structural development, behavior and expression of Quorum Sensing. As a global regulator known to coordinate the collective behavior in *P. aeruginosa*, Quorum Sensing has drawn a significant amount of attention for its therapeutic potential against nuisance biofilms as well as a key approach to reveal fundamental biofilm behavior as an integrated group. A unifying conclusion that can be drawn from our studies is that QS is not a sole global regulator governing biofilm structural maturation and that the expression of QS linked genes including virulence factor *lasB* and those that may contribute to biofilm morphology are significantly influenced by hydrodynamic condition. In parallel to relevant research findings a choice of media composition appears to be equally as important as hydrodynamics which altogether with other yet unidentified factors may have lead to major discrepancies in the field.

As it was mentioned in chapter 1 a challenge in biofilm research remains to provide meaningful quantitative numbers to biofilm structure in order to be able to predict function and behavior in diverse environments. Our study presented in chapter 3 represents one of the first attempts to address this challenge by quantifying the biofilm structural parameters and demonstrated statistically significant differences that can be attributed to difference in the flow dynamics. Physiological implication of these differences should be addressed in future studies.

In chapter 3 we described the development of traveling ripple-like structures in wild type *P.aeruginosa* PAO1 and that the speed with which they traveled were positively correlated with passing flow velocity. As it was mentioned before the formation of these ripple like structures are also documented in other microbial species growing in different in vitro and in vivo systems (Inglis 1993; Murga *et al.*, 1995, Okabe 1998; Stoodley *et al.*, 1999) but the mechanisms underlying this structural formation remains speculative. It was proposed that these ripples form in order to prevent erosion from structural deformation or as a result of cells skipping the zones of reduced mass transfer, or due to shear induced visco-elastic properties of biofilms. Intriguingly, Park *et al.*, (2003) have recently demonstrated that *E. coli* form traveling waves in response to starvation and subsequently collapse into smallest confining structures by forming high density clusters which is then believed to expedite QS mediated response. These findings in addition to our observations in *P. aeruginosa* demonstrate that formation of traveling ripples is not limited to myxobacteria and may represent an important multicellular trait evolved as a part of adaptation process in a natural habitat.

Formation of hollow biofilm shells as a result of seeding dispersal that we documented in the previous chapter is another example of collective behavior which we hope will provide a baseline for further investigation of biofilm dissemination mechanisms in general and perhaps a discovery of new communication system independent of las/rhl long range diffusible signals. Interestingly, a clinical mucoid isolate *P. aeruginosa* FRD1 did not display seeding dispersal in our system, suggesting that the microorganism as versatile as it is, can display not only genotypic but functional differences associated with different phenotypic states and therefore care must be taken

not to derive clinically oriented research conclusions solely based on environmental strains.

Finally, our results strengthen a current notion of multi-cellularity and coordinated behavior in prokaryotes as well as a dynamic network of overlapping pathways and dedicated cellular mechanisms that promote biofilm mode of existence in a model *P. aeruginosa*.

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