BIOFILM STRUCTURE, BEHAVIOR, AND HYDRODYNAMICS

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9

During the past decade our appreciation for biofilm structural complexity and its significance in natural as well as man-made settings has 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 et al., 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. 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 (Fig. 1).

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 with advantages beyond mere survival—a further development of complex multicellular-like systems. Perhaps it is no wonder that attached biofilms and microbial mats appeared early in the fossil record and have endured about 3.3 billion years to exist in modern environments (Rasmussen, 2000; Westall et al., 2001).

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similar flow determines characteristics regardless of environment.

Chapter links hydrodynamics to "other" (like QS) parameters that influence biofilm growth.

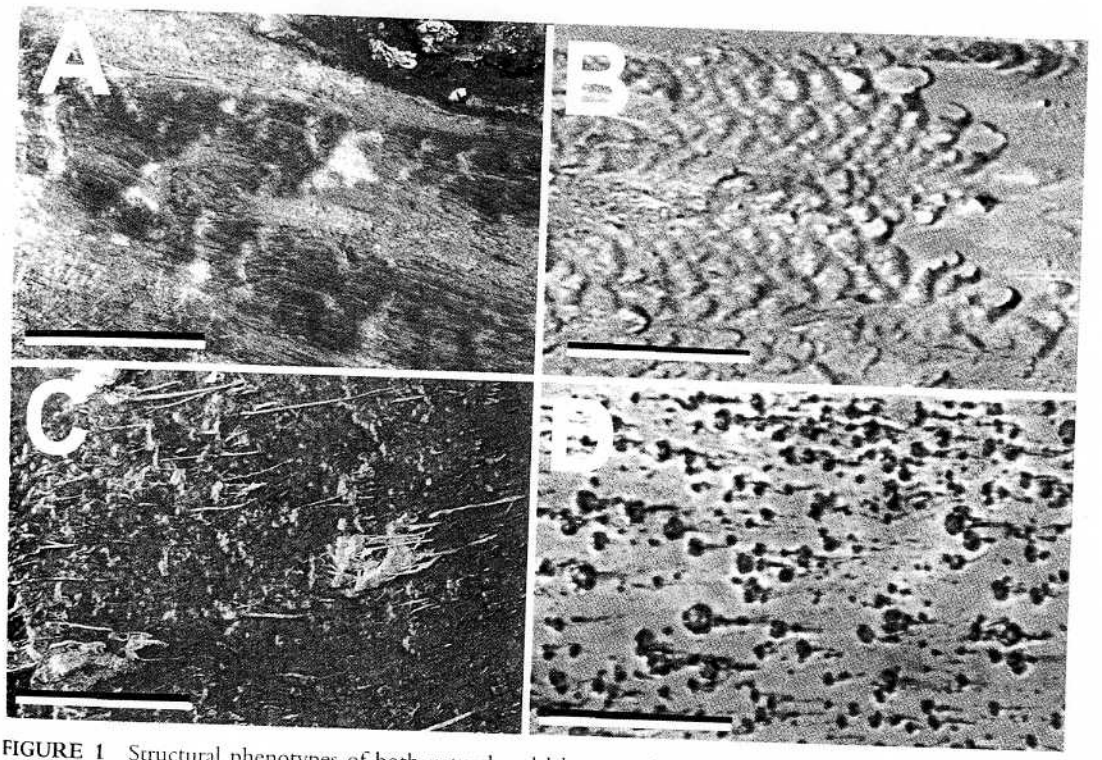

FIGURE 1 Structural phenotypes of both natural and laboratory biofilms are strikingly similar. A microbial biofilm growing in a hot spring in Biscuit geyser basin, Yellowstone National Park, forming ripple structures (A) was similar to the ones formed by mixed-species biofilms in the laboratory (B). A microbial biofilm with streamers in Canary Spring, Yellowstone National Park (C), was similar to streamers formed by a laboratory-grown *P. aeruginosa* biofilm (D). The direction of fluid flow in all panels was left to right. Bars, ~20 cm (A and C) and 200 μm (B and D).

The fundamental importance of biofilm formation by microbes is possibly revealed by the numerous redundancies and overlaps of pathways involved in biofilm development, as suggested by gene knockout 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 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, that 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). 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 inte-

gral component of modern day prokaryotic life. To control them against their renowned resilience we may be fighting more than 3 billion years of evolutionary adaptation for survival on surfaces in hostile environments.

HISTORICAL ASPECTS OF BIOFILM STRUCTURE

Some of the earliest evidence of biofilm documentation can be traced back to van Leeuwenhoek's crude description of microorganisms on tooth surfaces in the 1680s to the observation by Heukelekian and Heller (1940) that growth and activity for some marine bacteria were substantially enhanced by surface incorporation, and three years later to the report 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 and coworkers (1987, 1994, 1999, 2001) have extended the concept of prokaryotic life in biofilms to a wide variety of microbial ecosystems ranging from the natural to the man-made, including humans themselves, 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 they 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) (Fig. 2).

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 challenges in biofilm structural studies and are, perhaps, one of the main causes of the 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 biofilm architecture and to 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 stresses.

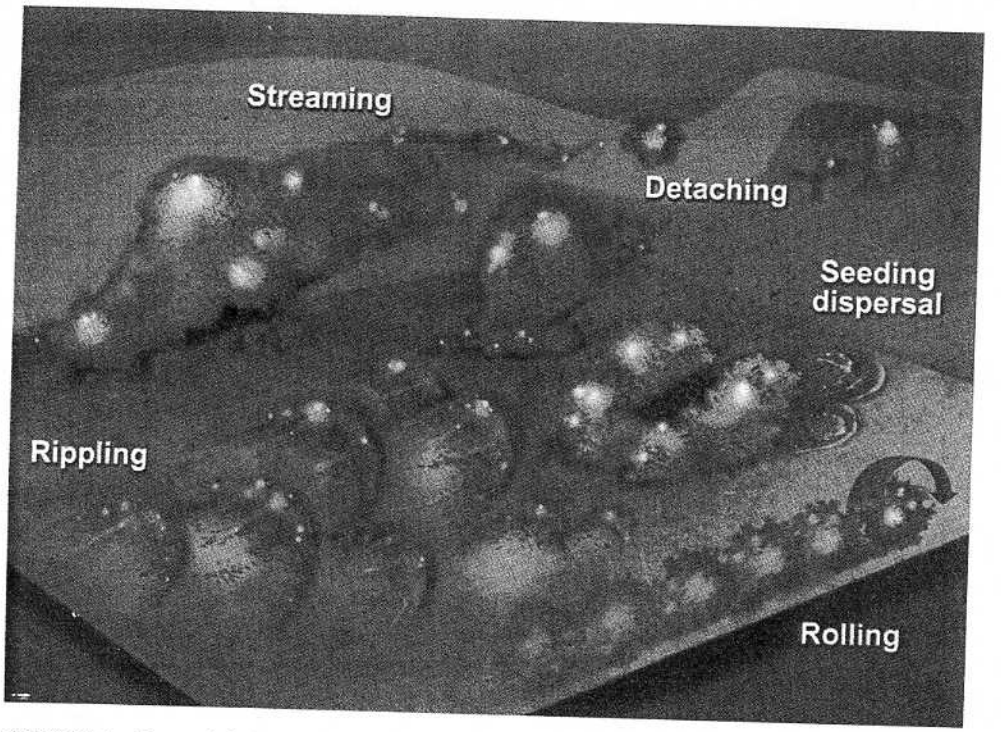

FIGURE 2 Dynamic behavior and associated structural phenotypes of bacterial biofilms in flowing fluids. The various dynamic processes are discussed in the text. Schematic by P. Dirckx, Center for Biofilm Engineering, Montana State University, 2003.

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 the 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, high-shear environments. In the evolutionary perspective microbial survival in these nonpermissive 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; P. Stoodley, 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 permits flow along surfaces while remaining attached (Körgstens et al., 2001; Towler et al., 2003; Klapper et al., 2002; Stoodley et al., 1999d; M. Winston, C. J. Rupp, A. Vinogradov, B. W. Towler, H. Adams, and P. Stoodley, Proc. ASCE 16th Eng. Mechanics Conf. Am. Soc. Civil Eng., paper 548, 2003). 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., 2003). Coaggregation is observed primarily during the stationary growth phase in intergeneric, intrageneric, and intraspecies manner via lectin-saccharide interaction (Rickard et al., 2000, 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.

EPS (SLIME) MATRIX

One of the key factors in the biofilm structure is the extracellular polymeric substance or extracellullar polysaccharides (EPS) that 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), the EPS comprises 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, hydrophobic, fucose-rich EPS regions contained both positive and negative charges, suggesting the existence of specific functional regions within complex biofilm communities (Moller et al., 1997; Wolfaardt et al., 1994). Stoodley et al. (2001) demonstrated that 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 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 overexpression of alginate itself (Henzter et al., 2001) produced structurally more differentiated biofilms than 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 and hydrogen bonding to occur between the closely pulled neighboring polymers (Flemming et al., 2000). Although 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 the 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, 1998; Lawrence et al., 1991; Nivens et al., 2001; Henzter et al., 2001). While many environmental conditions and the inherent nature of the 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 among 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) 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 conditions, the thickness of the boundary layer decreases and results in an increased rate of nutrient diffusion into the biofilms. This was shown by the increased biofilm density observed in the high-shear compared with the 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 et al., 1991; Caldwell et al., 1992; Moller et al., 1997). De Kievit et al. (2001) have shown that in a static condition *P. aeruginosa* biofilms form 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. The global regulator of carbon metabolism, CsrA, was shown to have a profound effect on the biofilm formation in *Escherichia coli* (Jackson et al., 2002) and 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 the intricate interplay between nutrient composition and hydrodynamics in defining biofilm growth and subsequent architecture, as well as the importance of the consideration of these factors in the experimental setup.

QUORUM SENSING

Davies et al. (1998) reported the requirement for quorum sensing (QS) molecules in the maturation of *P. aeruginosa* biofilm into complex mushroom structures. Subsequently, other researchers have also investigated links between QS and biofilm formation in hopes of identifying a "magic bullet" for biofilm control. For instance, QS has been linked to biofilm structure in oral bacteria that initiate dental plaque formation: *Streptococcus gordonii* (Loo et al., 2000), *S. mutans* (Li et al., 2002), *Salmonella* spp. (Prouty et al., 2002), and the opportunistic pathogen *Aeromonas hydrophilia* (Lynch et al., 2002). Also, a study based on detailed quantitative analysis of *Burkholderia cepacia* biofilm structures formed by WT and mutant strains showed that QS was not involved in the regulation of initial cell attachment, but rather controlled the maturation of the biofilm (Huber et al., 2001). 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 patients with cystic fibrosis (Singh et al., 2000). It has also been shown that QS expression in *Pseudomonas aeruginosa* biofilms coincided with marked changes in biofilm morphology, which switched from consisting of flat micro-

colonies to thick distinct mushroom structures with intervening water channels (De Kievit et al., 2001). The expression of QS genes was substantial at the substratum where bacterial cell density and signal accumulation is expected to be high. Although all these studies point to the direct role of QS in controlling biofilm structural development, in light of the most recent evidence, it is apparent that QS-biofilm structure relationships are intricate. For example, in other experiments it was reported that WT *P. aeruginosa* biofilm was structurally flat (Heydorn et al., 2002; Nivens et al., 2001) and resembled the QS mutant biofilm described in the study of Davies et al. (1998). Hentzer et al. (2001) attributed this difference to differing nutrient conditions. Also, De Kievit et al. (2001) have reported that under static conditions with citrate as a carbon source there was no structural difference between the parent strain and the signaling mutant biofilms. In a flowthrough 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 external conditions, including the flow and media composition (De Kievit et al., 2001). Since QS is a concentration-dependent phenomenon it is influenced by mass transfer processes or the liquid flow surrounding the biomass. The flow conditions may influence the concentration of signal molecules and thus affect the QS mechanism in the biofilm. Stoodley et al. (1999b) and Purevdorj et al. (2002) demonstrated that a mutation in the QS pathway only had a limited effect on biofilm structure but that flow dynamics had a more pronounced effect. Ongoing studies confirm the importance of hydrodynamics in the QS mechanisms in biofilms. For instance, *P. aeruginosa* carrying plasmid-encoded gene fusion between the green fluorescent protein (GFP) and *elastase B* (a virulence factor controlled by the QS circuit) was cultured in the glass flow cell to determine the onset of QS expression in the biofilm, which is theoretically coupled with GFP production. While we have not detected GFP expression in flowing systems, its expression was obvious in stagnancy after the flow was eliminated (B. Purevdorj and P. Stoodley, unpublished results). These findings indicate that QS, a regulator of many virulence genes in clinically relevant bacteria, depends on the hydrodynamic condition of the bulk media surrounding the biofilm and that QS is not necessarily required for biofilm formation.

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 long-term survival in either natural (Nickel et al., 1994) or man-made settings of industrial and medical importance (Zottola and Sasahara, 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 are known to cause biofilm detachment via either erosion of single cells or sloughing of large aggregates of biomass (Bryers, 1988; Stoodley et al., 2002b). Currently biofilm structure is thought of primarily in terms of growth and accumulation via polymer production; however, it is becoming clear that the 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 et al., 2001). Reports of the "hollowing" out of microcolonies by cells actively leaving the interiors are being noted by several 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

"nonmotile" *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 (P. Stewart, personal communication). However, in this case transmission electron micrographs suggest that the hollowing may have occurred through lysis of cells within the colony. In ongoing experiments in our laboratory we have observed the differentiation of *P. aeruginosa* biofilm clusters into distinct phenotypes of nonmotile cells forming rigid walls surrounding the hollow structure with occasional free swimming cells in the center of the cluster (Fig. 3).

From our studies we have observed that these structures have resulted from the release of individual or the eruption of aggregates of cells from the interior portion of the cluster back to the bulk medium surrounding the biomass. This seeding dispersal process may provide the detached cells with the chance to explore more favorable conditions elsewhere. Although the mechanisms of seeding dispersal and the role of hydrodynamics in this processes 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 seeding dispersal could be one of the major dispersal mechanisms in the biofilm life cycle.

ROLE OF MOTILITY IN BIOFILM FORMATION AND STRUCTURE

So far 80% of all known bacteria are motile (Aizawa, 1996) and can move over surfaces via twitching motility mediated by type IV pili and by flagellum-based swarming motility (Costerton et al., 1999; Harshey, 1994). In the biofilm developmental process, type IV pili-mediated twitching, and flagellum-based motility were shown to play an important role in biofilm initiation by surface aggregation in *P. aeruginosa* (O'Toole and Kolter, 1998) and in *Aeromonas* spp. biofilms (Gavín et al., 2002). Motility has been shown to influence the adhesion of bacteria to various surfaces in flowing systems (Korber et al., 1994; Mueller, 1996) and in 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 studies by De Kievit et al. (2001) where type IV pili were important during static biofilm growth and in very-low-flow or intermittently flowing environments but not in constantly flowing, high-shear systems. The authors suggested that the cells subjected to the shear force have limited surface movement via type IV twitching motility, and initial

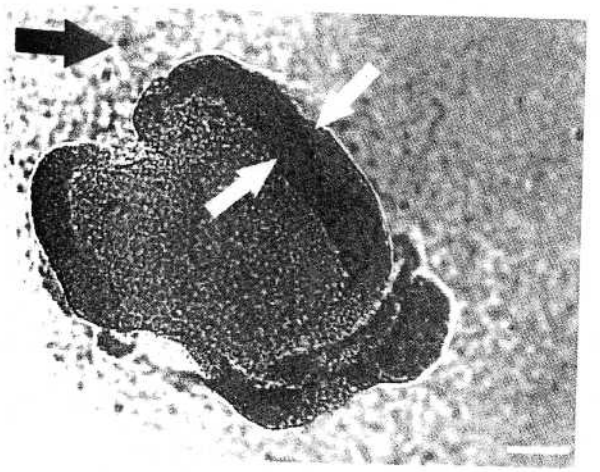

FIGURE 3 A structurally specialized *P. aeruginosa* biofilm cluster. The cluster is composed of an outer layer of nonmotile cells that form a "wall" (indicated by the white arrows). In the interior of the cluster cells swim about rapidly before flowing out, leaving the cluster empty. The flow direction is indicated by the black arrow. Bar, 20 μm.

microcolony formation results 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 several different microorganisms (O'Toole and Kolter, 1998). Although there is some evidence suggesting that motility enhanced and strengthened adhesion at high-flow compared with low-flow conditions (McLaine and Ford, 2002), the dependence of flagellar motility on fluid velocity is yet to be fully characterized.

In summary there are a myriad of factors that influence biofilm development and structure. However, the interaction between hydrodynamics and each of these parameters is poorly characterized. Although in this work we have discussed the influence of hydrodynamics and shear as the major factors that are involved in 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.

QUANTIFICATION OF BIOFILM STRUCTURE

Despite the fact that biofilm structure has been extensively studied, at present few standardized methods are available for quantification. Many studies comparing the effects of a genetic or environmental perturbation on biofilm formation are largely qualitative, relying on visual interpretations of the biofilm images. However, quantification based on structural parameters, such as thickness, thickness variability (roughness), and surface area coverage (Stewart, 1993; Murga et al., 2001), fractal dimension of activated sludge biofilms (Hermanowicz et al., 1995), and density, porosity, specific surface area, and mean pore radius of wastewater biofilms (Zhang and Bishop, 1994), has been used for statistical comparison. Recently, more complex software programs for systematic quantification of biofilm images have been developed. These include image structural analysis (ISA), which was developed at the Center for Biofilm Engineering (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 quantitative information from two-dimensional biofilm images based on nine 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). In brief, each image is automatically analyzed to find the threshold region that has the most influence on porosity. In this 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). Although it is not yet clear how some of the structural parameters of the image relate physically to the biofilm they still may be useful for statistical comparison. ISA was designed to analyze larger-scale biofilm patterns in two-dimensional grey-scale images and is, therefore, useful for lower power images taken with conventional bright-field or epifluorescence microscopy.

COMSTAT

COMSTAT was developed to analyze high-resolution three-dimensional 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 greater than or equal to the threshold value (biomass), and *zero* when the pixel values are less than 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 should be used and that the operator should not be 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 10 image-analysis features for biofilm structural quantification, which include biovolume (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 microcolonies at the substratum, microcolony volume, fractal dimension, average and maximum diffusion distance, and surface-to-volume ratio.

CONCLUDING REMARKS

In this chapter we have discussed the roles of hydrodynamics and shear in 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 laboratory and the observations of biofilms growing in the natural environment demonstrate the importance of hydrodynamics and shear in biofilm structural development. Moreover, survival strategies acquired by biofilms to suit a wide range of environmental shears now assist in the successful colonization of medical and industrial settings and, indeed, human beings themselves. 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 mechanical stresses (both normal and 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 more than 3 billion years during which prokaryotes have adapted survival strategies to remain on surfaces in flowing fluids.

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