Influence of flow on the structure of bacterial biofilms

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ABSTRACT

Bacteria attached to surfaces in biofilms are responsible for the contamination of industrial processes and many types of microbial infections and disease. Once established, biofilms are notoriously difficult to eradicate. A more complete understanding of how biofilms form and behave is crucial if we are to predict, and ultimately control, biofilm processes. A major breakthrough in biofilm research came in the early 1990's when confocal scanning laser microscopy (CSLM) showed that biofilms formed complex structures which could facilitate nutrient exchange. We have recently found that biofilms growing in turbulent flow can also be temporally complex. Structures such as cell clusters and ripples can migrate downstream along solid surfaces. Further, biofilm viscoelasticity allows the biofilm to structurally deform when exposed to varying shear stresses.

Biofilm accumulation

Biofilms have been defined in numerous ways by various researchers. These definitions are usually constructed to be inclusive of the many environments in which biofilms are found and the disciplines that the subject covers. Characklis and Marshall defined a biofilm as consisting of "cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin" [3]. Costerton et al. supplied a broader definition of biofilms as "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces" [4]. But what is it that physically distinguishes biofilm cells from their planktonic counterparts? Some salient features include the association with a surface, a high population density, and the presence of an extracellular polysaccharide (EPS) glycocalyx. However, it may be argued that it is structural organization which best distinguishes biofilm cultures from conventional well mixed suspended cultures [13]. Characklis [2] identified up to 8 different processes describing the progression of biofilm accumulation. These can be condensed into three main steps. First, the attachment of cells to a surface; second, cell growth and EPS production; and third, detachment of single cells (erosion) or large pieces of biofilm (sloughing) from the surface.

Complexities in biofilm structure revealed by confocal microscopy

Over the last decade the development of improved imaging techniques, such as confocal scanning laser microscopy (CSLM), has allowed biofilms to be studied in the fully hydrated state. The combination of CSLM and fluorescent molecular staining has revealed detailed information on the three dimensional structure of biofilms and the spatial arrangement of microbial species within the biofilm [10, 11]. The use of flow cells and time lapse image

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analysis has also allowed biofilm development to be studied under flowing conditions [14]. The emerging picture of biofilm structure is one of complexity. It is now widely reported that biofilms composed of mono or mixed cultures from the laboratory, industry or the environment are generally heterogeneous and consist of cell clusters, discrete aggregates of microbial cells in an EPS matrix, surrounded by interstitial voids and open channels connected to the bulk liquid [1, 6, 8].

Biofilm structure and mass transfer

Microelectrode techniques combined with CSLM allowed the study of the effects of structural heterogeneity on local hydrodynamics and mass transport. In a flowing system it was found that oxygen concentrations in the voids were significantly higher than in the adjacent cell clusters, suggesting that the voids could act as transport channels [6]. However, this effect was dependent on the flow rate of the bulk liquid. At relatively low flows the mass boundary layer (MBL) was parallel to the substratum [7]. In this case fluxes were perpendicular to the substratum and the surface exchange area was equal to that of the substratum, as expected for the "planar" biofilm model. However, at high flows the MBL became convoluted and followed the irregular biofilm surface. In this case fluxes were perpendicular to the convoluted biofilm surface and the surface exchange area was greatly increased. Liquid flow and particulate transport through the biofilm channels was also directly confirmed and quantified by particle tracer studies [12]

The influence of hydrodynamics on biofilm structure and behavior

For an immersed surface the hydrodynamic conditions will determine the rate of transport of cells and nutrients to the surface, as well as the magnitude of shear forces acting on a developing biofilm. In turn biofilm structure will influence momentum, mass, and heat transfer which will effect, respectively, energy losses in pipelines, biotransformation reactions, and heat exchanger efficiency. We have used time lapse microscopic imaging to observe pure and mixed culture biofilms growing in square glass tubing under laminar (Reynolds number (Re, based on flow cell geometry) = 120) and turbulent flow (Re 3600) (Fig. 1). The associated average flow velocities (u) in the flow cells were 0.033 and 1 m s^{-1} respectively. The transition point between laminar and turbulent flow occurred at Re 1200 [15]. *Pseudomonas aeruginosa* PAO1 was used for growing pure culture biofilms and a four member community composed of *Klebsiella pneumoniae* (ca. 90% composition by CFU/cm^2) *P. aeruginosa* (7%), *P. fluorescens* (1.5%) and *Stenotrophomonas maltophilia* (1.5%) was used to grow mixed species biofilms. The biofilms were grown on a minimal salts media with glucose (40 mg/l for the mixed species or 400 mg/l for *P. aeruginosa*) as the carbon source [16].

Under the low laminar flows (which are more commonly used for biofilm flow cell studies) the cell clusters tend to be approximately circular in shape. However, in turbulent flow the biofilms look very different. The larger cell clusters become elongated in the downstream direction to form filamentous "streamers" which can oscillate rapidly in the flow [14, 16]. More recent experiments with the mixed species biofilms show the appearance of ripple structures after approximately nine days of flow cell operation when flow was turbulent.

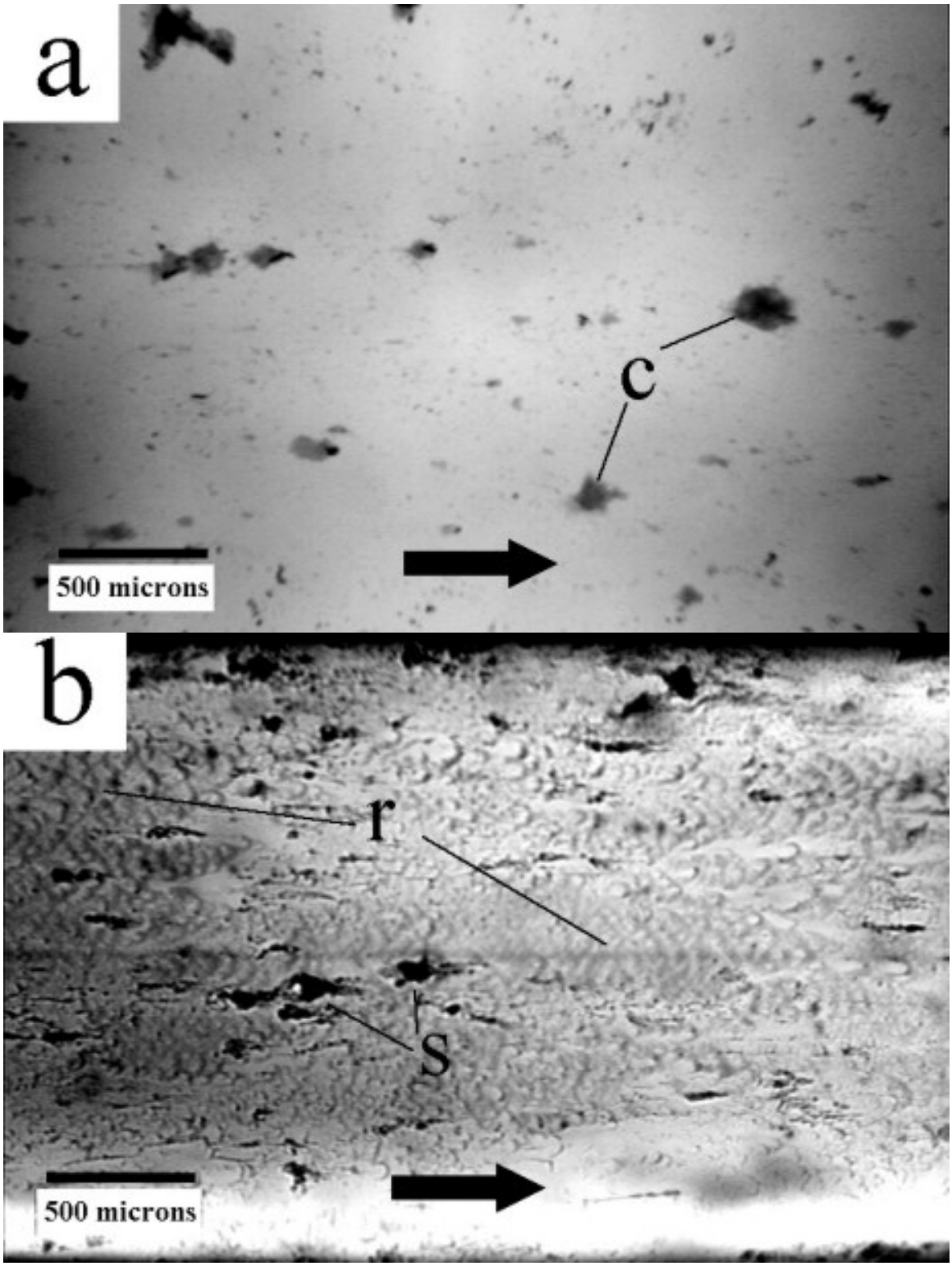

Fig. 1. Mixed species biofilm growing in **a**) laminar (Re 120) and **b**) turbulent (Re 3600) flow. The biofilms were grown for 15 days. In the laminar flow cell the biofilm consisted of approximately circular shaped cell clusters separated by void areas. Two of the larger cell clusters are labeled (c). In the turbulent flow cell the biofilm was also heterogeneous but formed elongated streamers (s) and ripple beds (r). Bulk liquid flow was in the direction indicated by the arrow.

Temporal complexity in biofilms: long and short term effects of hydrodynamics

Surface transport of biofilm structures and the formation of biofilm ripples

We have found that hydrodynamic drag not only influences shape of biofilm structures but also the temporal behavior of biofilms. We have discovered that, in the mixed species biofilms growing in turbulent flow (u = 1 m s^{-1}, Re 3600), small cell clusters (dia. ca. <20 µm) migrated downstream on the upper surface of the flow cells at a velocity of ca. 15 µm h^{-1} [16]. Further, the biofilms formed micro-scale migratory ripples. The morphology and migration velocity of the ripples were a function of the Re in the flow cell (Fig. 2). The biofilm streamers did not migrate downstream.

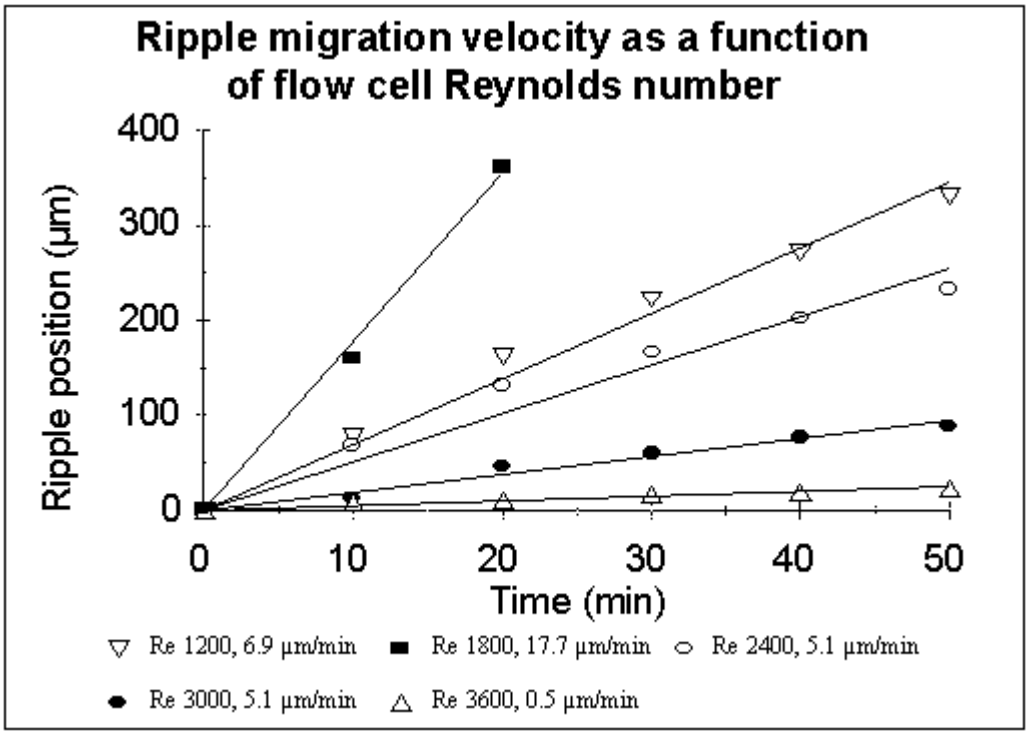

Fig. 2. Migration of biofilm ripples at different flow cell Reynolds numbers. The mixed species biofilm was grown for 18 days at Re 3600 prior to the experiment. The downstream position of individual ripples was recorded at 10 minute intervals. The ripple velocity (reported in the legend after the Re) is the slope of the regression line (shown as solid lines) associated with each data set. A maximum migration velocity occurred at Re 1800.

The ripples travelled over the top of the base layer of single cells and smaller cell clusters (dia. <10 µm) at a velocity of approximately 30 µm min^{-1}. A maximum migration velocity of approximately 1000 µm h^{-1} occurred at u = 0.5 m s^{-1} (Re 1800). These exciting discoveries give new insights into biofilm behavior and add to our understanding of how surfaces are colonized by bacteria.

Biofilm rheology

We have also developed a non invasive technique to investigate biofilm rheology by measuring structural deformations of mixed species [16] and pure culture *P. aeruginosa* biofilms caused by short term changes in flow rate. Biofilms were grown in either constant laminar (Re 120) or turbulent flow (Re 3600) for one week or more. Selected biofilm cell clusters or streamers were then monitored microscopically as the fluid shear stress was varied (by changing the flow rate) over time periods of seconds to minutes. Relative changes in cluster or streamer length (here length is defined as the longitudinal length dimension parallel to the flow) were used to calculate the strain (Fig. 3a).

However, although the strain may be measured with reasonable accuracy the shear force acting on the biofilm can only be roughly estimated. In the absence of a direct

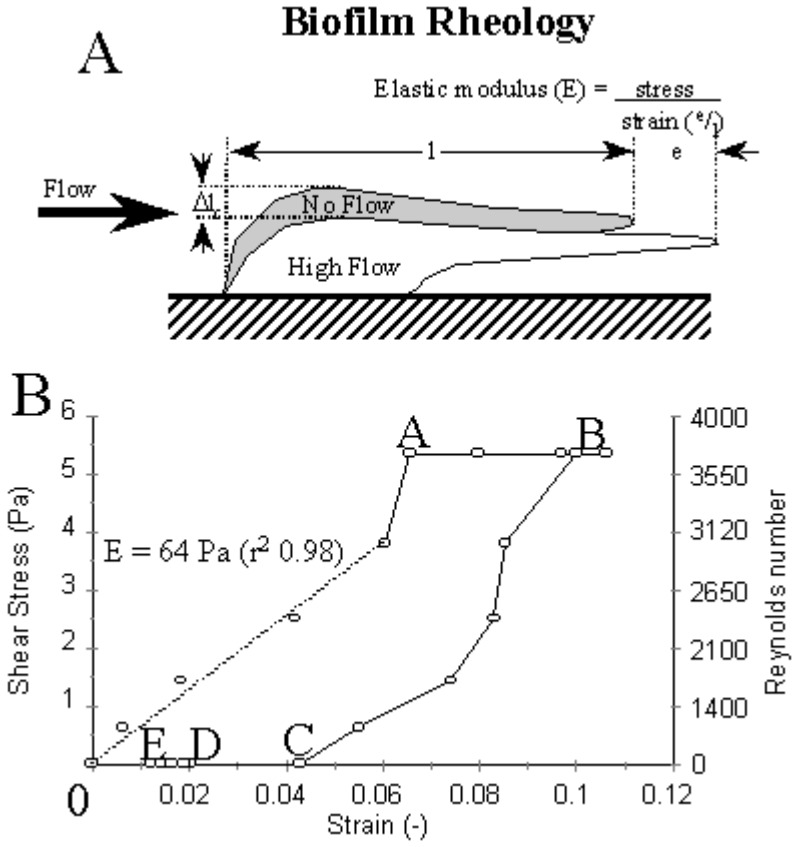

Fig. 3. Biofilm rheological properties were determined by monitoring structural deformations caused by an imposed fluid shear stress. **A**) When flow was turned off the biofilm streamers contracted in the downstream direction and expanded in thickness. The strain was calculated as the ratio between the length extension (e) caused by an imposed fluid shear to the length at zero flow (l). **B**) Stress-strain curve for a streamer in a *P. aeruginosa* PA01 biofilm grown at Re 120. The curve has four distinct regions. OA shows the immediate elastic deformation to increasing shear. The linear portion of the curve (dashed line) was used to estimate the longitudinal elastic modulus, E. AB shows the elongation (creep) of the streamer when held at an elevated shear for 22 minutes. BC shows the immediate elastic recovery when the shear stress was incrementally removed. CD shows the creep recovery over 15 minutes when the flow was turned off. The streamer length had not changed in the last 5 minutes of the experiment. EO is the permanent set that occurred during viscous flow. Note that the Reynolds number on the secondary Y-axis is a non-linear scale.

measurement, which would be technically extremely difficult to obtain in situ, we used the theoretical wall shear stress associated with a smooth wall for a given Re. This simplification assumes that the shear stress on the monitored structures is not influenced by surrounding structures on the surface and that the shear force is constant over the surface of the cluster or streamer. Since clusters and streamers have irregular shapes we might expect that the shear forces will in fact vary at different locations on their surface.

Using this technique we conducted simple stress-strain and transient rheological tests on biofilm structures (Fig. 3b). On switching off the flow, which had been continuous during biofilm development, there was an immediate contraction in cluster or streamer length. When the u was returned to growth flow velocity (u_g) the structures demonstrated elastic behavior and immediately stretched back to the original length. In polymers such an elastic response occurs when individual polymer strands are stretched in alignment to the shear force. When u was increased past u_g the cell clusters became elongated in the downstream direction. The cell clusters continued to elongate over time (creep) demonstrating viscous flow. When u was reduced back to u_g there was an immediate elastic contraction followed by a time dependent viscous recovery. However, when the recovery had stabilized (after approximately 10 minutes) the cell clusters had not completely returned to their original length.

In polymers such a permanent set (or gel) occurs when intermolecular forces, such as hydrogen bonds and Van der Waals attractions, between polymer residues and side chains are broken and the polymer strands begin to flow past each other. When the shear is removed these bonds reform causing the polymer to set in the new position. The permanent set suggests that the biofilm was behaving as a viscoelastic liquid, not a viscoelastic solid. The thickness of the cell clusters (l_c) also varied as a function of flow rate. When flow was turned off the l_c was 38% greater than when u = 1.5 m s^{-1}. From stress strain curves performed at 28^{o}C we estimated that the biofilm had a modulus of elasticity in the longitudinal direction of ca. 60 N m^{-2}. This is a similar value to that reported by Denny [5] for hydrated pedal slug mucus. The ability to flex with the flow may allow the biofilm to withstand intermittent periods of high shear (as would be expected in turbulent flow) and remain attached in a similar way to that employed by many benthic macro-organisms [9].

Concluding remarks

As with most scientific disciplines, the closer we look at biofilms and biofilm processes, the more complex the picture becomes. Recent developments in imaging techniques combined with microelectrodes and molecular labeling have revealed much about the structural complexity of biofilms. However, to date most of the detailed studies have been on relatively young biofilms growing in laminar flows, hence only a part of the picture has been revealed. Now time lapse imaging is beginning to reveal the temporal complexity of biofilm behavior. The ability of biofilms to adapt their morphology to different environmental conditions may help explain their tenacious nature and recalcitrance to control.

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