Title: Environmental and genetic factors influencing biofilm structure.

Running title: Factors influencing biofilm structure.

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<A> **INTRODUCTION**

It is increasingly evident that biofilms growing in a diverse range of medical, industrial, and natural environments form a similarly diverse range of complex structures (Stoodley *et al.,* 1999a). These structures often contain water channels which can increase the supply of nutrients to cells in the biofilm (deBeer and Stoodley 1995) and prompted Costerton *et al.,* (1995) to propose that the water channels may serve as a rudimentary circulatory system of benefit to the biofilm as a whole. This concept suggests that biofilm structure may be controlled, to some extent, by the organisms themselves and may be optimized for a certain set of environmental conditions. To date most of the research on biofilm structure has been focused on the influence of external environmental factors such as surface chemistry and roughness, physical forces (i.e. hydrodynamic shear), or nutrient conditions and the chemistry of the aqueous environment. However, there has been a recent increase in the number of researchers using molecular techniques to study the genetic regulation of biofilm formation and development. Davies *et al.* (1998) demonstrated that the structure of a *Pseudomonas aeruginosa* biofilm could be controlled through production of the cell signal (or pheromone) *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL). In this paper we will examine some of the research that has been conducted in our labs and the labs of others on the relative contribution of hydrodynamics, nutrients and cell signalling to the structure and behaviour of bacterial biofilms.

<A> **HYDRODYNAMICS**

The hydrodynamic conditions of an aquatic environment will determine the transport rate of nutrients and planktonic cells to a surface, the shear stress acting on the biofilm and the rate of erosion of cells from the biofilm. The morphology and physical properties of biofilms appear to be strongly influenced by the magnitude of the shear stress under which the biofilm developed. At low, laminar flows individual biofilm microcolonies, although irregular in shape, commonly form isotropic patterns with no obvious directional component to the pattern (Møller *et al.,* 1998, Stoodley *et al.,* 1999b & c, Wolfaardt *et al.,* 1994) (Fig. 1a). However, biofilms grown at higher shear are commonly filamentous with the microcolonies being elongated in the downstream direction (Bryers & Characklis, 1981, McCoy *et al.,* 1981, Stoodley *et al.,* 1999c) (Fig. 1b). The length of the filaments or "streamers" appears to be greatest in turbulent flows with Reynolds numbers (Re) between transition and 17000. At higher Re the biofilm filaments are reduced in length, presumably because of continual shearing off of biofilm material at the tip (Bryers & Characklis, 1981). Other structures such as ripples and dunes have also been reported in pure and defined mixed culture laboratory biofilms that were grown in turbulent flow (Gjaltema *et al.,* 1994, Stoodley *et al.,* 1999d).

<B> **Fluid-like flow of biofilm micro-colonies over the substratum**

In addition to the influence that hydrodynamics have on biofilm morphology we have used digital time lapse microscopy (DTLM) to demonstrate that hydrodynamics can also influence dynamic behaviour in bacterial biofilms (Stoodley *et al.,* 1999d). In this work ripple shaped and round micro-colonies in mixed culture biofilms, grown under turbulent

flow (Re 3600), were transported downstream across the upper and lower surfaces of a square glass flow cell (Fig. 2). Some of the structures appeared to roll across the surface while others appeared to slide. The travel velocity of the micro-colonies across the surface varied with short term variations in the velocity of the bulk liquid. A maximum migration velocity of approximately 1 mm hr^{-1} occurred in the transition region between laminar and turbulent flow. The ripple shaped micro-colonies were also observed to continually detach from the glass surface. These observations support the hypothesis made by Inglis (1993) that ventilator-associated pneumonia may be related to the detachment of biofilm fragments from the walls of tracheal tubes. The biofilms that he observed had distinct wave patterns which led Inglis to hypothesize that the biofilm had been flowing and that this dynamic phenomena may be related to biofilm detachment and dissemination into the lungs. Time lapse movies of biofilms in turbulent flow taken at frame intervals of 0.5 to 1 h over time periods of up to 24 h suggest that biofilms behave like very viscous fluids flowing along channel walls (Stoodley *et al.* 1999d). In addition to flow along channel walls we have also observed similar phenomena around glass beads in a porous media flow cell (unpublished data). These observations are supported by several studies which show that biofilms can behave like viscoelastic liquids (Christensen and Characklis, 1990; Ohashi and Harada, 1994; Stoodley *et al.,* 1999e). Flowing biofilms have important consequences for the dissemination of bacterial infection or contamination since this mechanism allows biofilm bacteria to colonize adjacent clean surfaces without depending on a planktonic phase that is known to be more susceptible to antimicrobial agents (Gilbert and Brown 1995).

<A> **NUTRIENTS AND HYDRODYNAMICS**

At higher nutrient concentrations and loading rates, biofilms tend to be thicker and denser than those grown in nutrient poor environments (Characklis 1990). However, less is known about the influence of nutrient type and concentration on the morphology of bacterial biofilms. Moller *et al.* (1997) reported that the morphology of an established undefined degradative community became more homogeneous when the nutrient source was changed from 2,4,6-trichlorobenzoic acid (2,4,6-TCB) to Trypticase soy broth (TSB) while maintaining a constant carbon loading rate. They noted that the biofilm grown on TSB closely resembled the biofilms that they had previously grown exclusively on glucose and TSB. They hypothesised that that mound shaped microcolonies that they had observed in the biofilms grown on 2,4,6-TCB may be characteristic of growth by their particular community on chlorinated substrates. It is likely that the morphological differences in their biofilms may have occurred due to population shifts in the community in response to changes in the enrichment conditions. Pure culture experiments on *Mycobacterium* spp. growing in laminar flow showed that, although biofilms took longer to accumulate on sterile tap water than on enrichment media, the morphology of the biofilms was similar (Hall-Stoodley *et al.,* 1999).

Stoodley *et al.* (1999c) have also shown that the morphology of an established biofilm can change significantly by varying the carbon concentration. In these experiments the morphology of the microcolonies in a 21 d mixed species biofilm grown under turbulent flow changed from that of ripples and streamers to large, closely packed, mound structures when the concentration of glucose (the sole carbon source) was increased by a factor of 10. The morphology was noticeably different within 10 h. In addition to the

morphological change, there was also a change in the dynamic behaviour of the biofilm. At the low glucose concentration (40 ppm) the biofilm appeared to flow downstream over the glass surface but at 400 ppm glucose the downstream motion of biofilm microcolonies was much less evident. However, microcolonies could be observed to be continually growing and detaching using DTLM (Stoodley 1999f). When the glucose concentration was reduced back to 40 ppm there was a net reduction of biomass and the ripples and streamers began to reform within approximately 48 h.

<A> GENETIC REGULATION OF BIOFILM STRUCTURE

In the preceding sections we have discussed some of the influences of the external environment on biofilm structure. Now we will turn to the influence that the biofilm micro-organisms themselves may have on the structure of the biofilms in which they live.

<B> Cell signalling and quorum sensing

Quorum sensing (QS) is a mechanism used by both Gram-positive and Gram-negative bacteria to regulate their gene expression, and resulting phenotype, as a function of the density of the cell culture (Bassler, 1999). The cell culture density is "sensed" through production of cell signalling molecules which, once a threshold concentration is reached, initiate a signal transduction cascade, resulting in the expression of a number of target genes. In many Gram-negative bacteria these cell signals commonly belong to a family of acylated-homoserine lactones (AHLs). However, cyclic dipeptides (Holden *et al.* 1999) and quinolones (Pesci *et al*. 1999) can also function as signalling molecules. QS in *P. aeruginosa* proceeds through the *lasI*/*lasR* system which is homologuous to the *luxI*/*luxR* system responsible for light production in some marine *Vibrio* species. However, in *P.*

aeruginosa, instead of light production, high cell densities in stationary phase batch cultures can result in the production of virulence factors and secondary metabolites (Jones *et al.,* 1993, Latifi *et al.,* 1995, 1996, Winson *et al.,* 1995). The QS cascade in *P. aeruginosa* is activated by the cell signalling molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) whose synthesise is directed by *lasI*. At high concntrations OdDHL binds with a transcriptional activator (the LasR protein) which further upregulates *lasR* and *lasI* in addition to a number of other genes including *lasB* resulting in the production of elastase and other virulence factors (Pesci & Iglewski, 1997). The LasR-OdDHL complex also upregulates *rhlI* which produces another signalling molecule. *N*-butanoyl-L-homoserine lactone (BHL). BHL binds to RhlR and this complex upregulates the *rhl* regulon resulting in the production of rhamnolipid (Pearson *et al.* 1997). Whiteley *et al.* (1999) have identified between 39 and 270 genes that are controlled by OdDHL and BHL activated QS mechanisms in *P. aeruginosa*.

It was anticipated that QS may play a role in the development of biofilms which also exhibit high cell densities (Williams & Stewart, 1994). Davies *et al.* (1998) strengthened this hypothesis when they reported that the cell signal OdDHL was required for *P. aeruginosa* JP1, a *lasI* mutant (defective in the production of OdDHL) to develop the structurally complex biofilms which were formed by the parental wild type (WT) PAO1 cells.

<B> **Cell signalling and hydrodynamics**

However, unlike suspended batch cultures, biofilms usually do not grow in completely mixed closed systems and transport through biofilm micro-colonies appears to be mainly through diffusion (Bryers & Drummond, 1998; deBeer at al. 1997). In this case it is not

only the cell density that is important for the build up of cell signalling molecules to concentrations at which QS mechanisms are activated, but also the production rate of signals, the rate of transport through the biofilm, the shape and dimensions of biofilm structures and the mass transport conditions outside the biofilm. The experiments by Davies *et al.* (1998) were conducted under very low laminar flows (Reynolds number = 0.17). It is possible that under higher flows cell signals may be diluted before they can reach QS concentrations within biofilm micro-colonies. To investigate this further we grew biofilms using *P. aeruginosa* PAOR, a *lasR* mutant (Latifi *et al.*, 1996), and the parental WT (PAO1) strain under laminar (Re = 120) and turbulent (Re = 3600) flow (Stoodley *et al.,* 1999b). Production of OdDHL was suppressed in the PAOR mutant as demonstrated by biosensor assay (Winson *et al.*, 1998) which showed that OdDHL concentration in the spent medium was below detection limits (approx. 10^{-3} nM). We also used *P. aeruginosa* PAN067 (Jones *et al.,* 1993), a mutant deficient in the production of *N*-butanoyl-L-homoserine lactone (BHL), another *N*-acyl homoserine lactone (AHL) which has been implicated in biofilm cell signalling (Davies *et al.,* 1998). BHL synthesis is directed by *rhlI*. In our experiments we found that both the WT and the two mutant strains formed complex structures and it was the hydrodynamics that had the greatest influence on the observed microcolony structure (Fig. 3). In laminar flow the microcolonies of both the mutant strains (PAOR and PAN067) and their parental strains were circular in shape but in turbulent flow they formed elongated streamers (Fig. 3e & f). The influence of the inability to produce AHLs on biofilm formation was more subtle than found by Davies *et al.* (1998) and appeared to be related more to the *rates* of growth and detachment than the ability to form complex structures (Stoodley *et al.,* 1999b).

Clearly further work is required to determine how the hydrodynamic conditions may influence QS mechanisms in biofilms, particularly those grown in well mixed, open environments.

<B> **Biofilm structures formed through twitching motility**

In addition to cell signalling mechanisms by which biofilm structures form through growth, time lapse imaging has shown that micro-colonies can also form from the co-ordinated movement of single attached cells to specific loci on a surface (Dalton *et al.,* 1996). In *P. aeruginosa* such co-ordinated motion has been shown to be associated with type IV pili-mediated twitching motility (Semmler *et al.,* 1999). O'Toole and Kolter (1998) have shown that this type of motility is important for the formation of biofilm structures in the initial stages of biofilm development. However, since these studies are generally limited to the first few hours of biofilm development it is not clear how twitching motility may influence the long term structural arrangements of biofilms.

<A> **DISCUSSION**

A more complete understanding of biofilm development and behavior is essential if we are to predict, and ultimately control, biofilm processes. The use of confocal microscopy has documented some of the structural complexities of different types of biofilms while time lapse imaging is starting to reveal some of the dynamic behaviors occurring in biofilms.

<B> **Biofilm development and behavior: nature or nurture ?**

Clearly, both environment and genotype have been shown to play a role in biofilm development and behavior but it is not so clear how the environmental conditions determine which factors dominate. Shear is one environmental condition we have studied that appears to be of fundamental significance. There are others yet to be elucidated including nutrients and surface type to name a few. For example, the aggregation of single cells into micro-colonies in the initial stages of biofilm formation in low shear environments appears to be controlled at the genetic level (O'Toole & Kolter, 1998), while the downstream motion of biofilm micro-colonies in high shear flow appears to be a physical phenomenon related to the magnitude of the shear and the material properties of the biofilm EPS (Stoodley *et al.,* 1999d,f). Likewise, in low shear flows cell signaling has been shown to play a significant role in the determination of biofilm structure (Davies *et al.,* 1998) while in high shear, the structures that develop appear to be shaped by the external shear and drag forces acting on the growing biofilm (Stoodley *et al.,* 1999b).

Increasingly, researchers are using genetic techniques to identify the role that individual genes may have on the phenotype of the individual cells and consequently the overall development of bacterial biofilms (O'Toole *et al.,* 1999). This approach has been advanced by the use of microtitre plates to assess biofilm accumulation. This technique allows rapid screening of large numbers of constructed mutants necessary for genetic analysis. However, these experiments are generally limited to studying biofilms in non flowing, batch culture environments and in the very initial stages (hours) of biofilm development. In contrast, the microscopic monitoring of biofilms growing in flow cells allows long term (days to months) experiments under flowing continuous culture

conditions. However, this technique is limited by the number of replicates per experiment and in the total number of experiments that can be conducted.

An obvious approach is to use microtitre plates for rapid screening and then use flow cells to look at the longer term influence of a particular mutation on biofilm growth and behavior in a flowing system. Presently, the construction of mutants deficient in specific phenotypes thought to be important for biofilm formation is proceeding at a much faster pace than can be studied in long term flow cell experiments. To clear this backlog will require the development of biofilm flow cell systems capable of accommodating large numbers of replicates so that the influence of a particular mutation on biofilm development can be systematically assessed. It is only by the study of both the environmental *and* genetic influences on biofilm development that we will be able to begin to piece together how different biofilms behave in the real world, outside of the laboratory.

<A> **ACKNOWLEDGEMENTS**

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FIGURE CAPTIONS

Fig. 1. Ten day old *Pseudomonas aeruginosa* PAN067 (Jones *et al.,* 1993) biofilm grown under laminar (A) and turbulent flow (B) at Reynolds numbers of 120 and 3600 respectively (Stoodley *et al.,* 1999b). The laminar grown biofilm was composed of single cells and small microcolonies (labeled "C") while the turbulent grown biofilm microcolonies formed elongated streamers ("S") in the downstream direction. The biofilm was stained with the LIVE/DEAD Bac Light^{TM} Bacterial Viability kit (Molecular Probes). Although not seen in this greyscale image, approximately 98% of the cells were viable (green). Scale bar = 50 μm.

Fig. 2. Bacterial biofilm microcolony (outlined in white) moving downstream along the upper surface of a glass flow cell at a velocity of approximately 12 μm hr^{-1} (Stoodley *et al.,* 1999d). The microcolony moved over the top of the surrounding monolayer of single cells. The bulk liquid velocity was 1ms^{-1} in the direction shown by the arrow. The elapsed time between each panel was 50 min. Scale bar = 10 μm.

Fig. 3. Influence of cell signaling and hydrodynamics on biofilm structure after 6 days growth. A) *P. aeruginosa* PAO1 grown under laminar flow (Re 120). The biofilm was composed of a monolayer of single cells interspersed with circular shaped microclonies (labeled "MC"). Some void areas "V" were devoid of cells. B) *P. aeruginosa* PAO1 grown under turbulent flow (Re 3600). The microcolonies "MC" were elongated in the downstream direction to form streamers "S". C) *P. aeruginosa* PAOR, a *lasR* mutant

(Latifi *et al.*, 1996) grown under laminar flow. The biofilm was similar in morphology to the parental PAO1 strain. D) *P. aeruginosa* PAOR grown under turbulent flow. Again, the biofilm morphology was similar to the parental PAO1 strain grown under the corresponding flow velocity. E) Low magnification image of same PAOR biofilm shown in panel "C" showing overall pattern of the biofilm grown in laminar flow. F) Low magnification image of same PAOR biofilm shown in panel "D" showing the influence of increased shear on biofilm morphology. The biofilm microcolonies formed elongated "streamers". An void area caused by localized sloughing detachment is indicated "v". All biofilms were grown on a minimal salts media with glucose (400 ppm) as the sole carbon source. The black arrow indicates the direction of bulk fluid flow in all panels. Scale bar in panels A,B,C, and D = 10 μm, in panels E and F = 500 μm

FIGURES

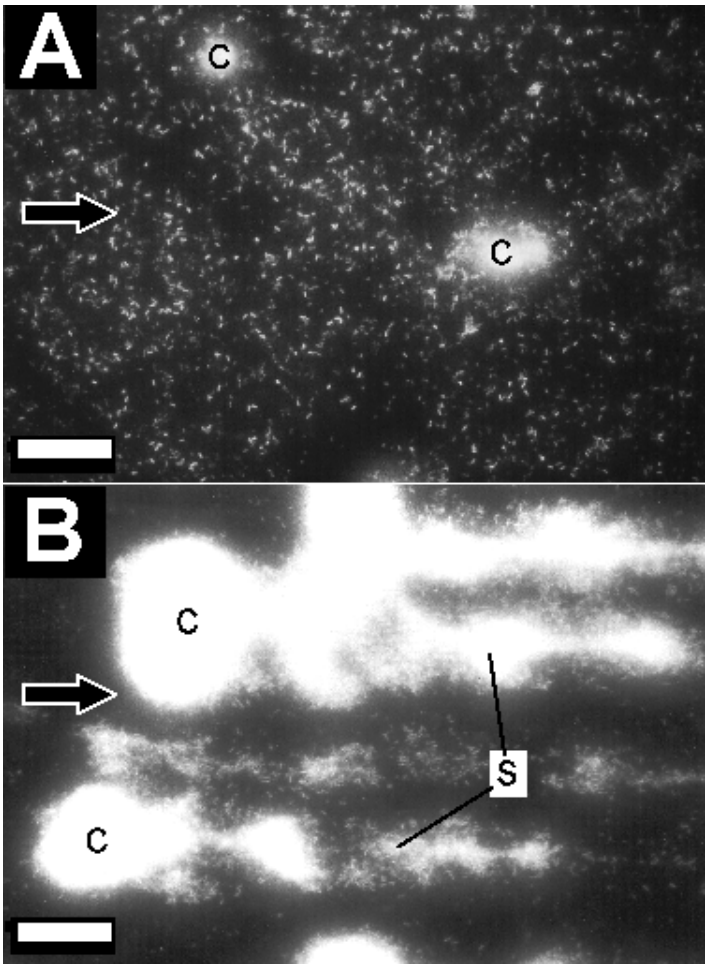

Fig. 1.

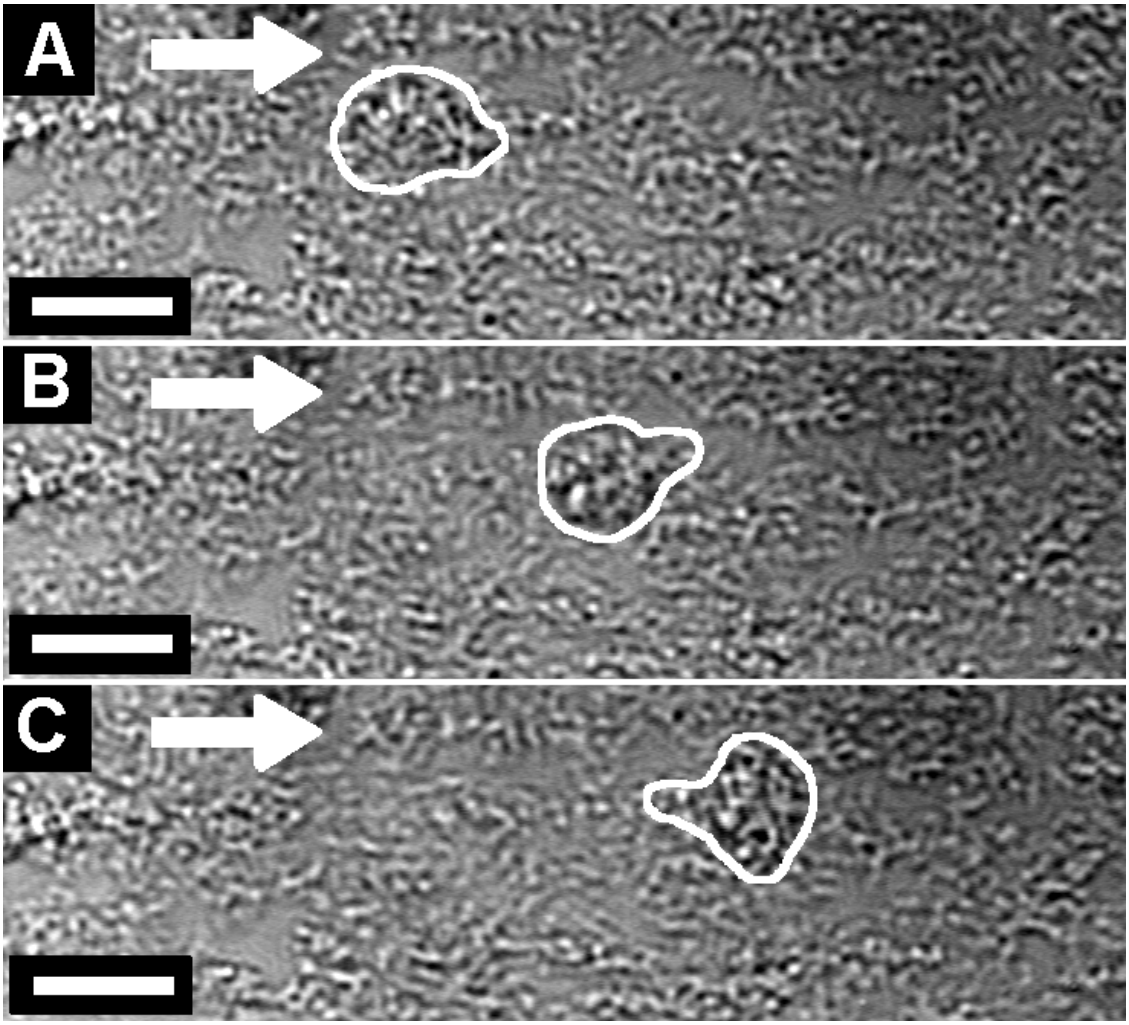

Figure 2

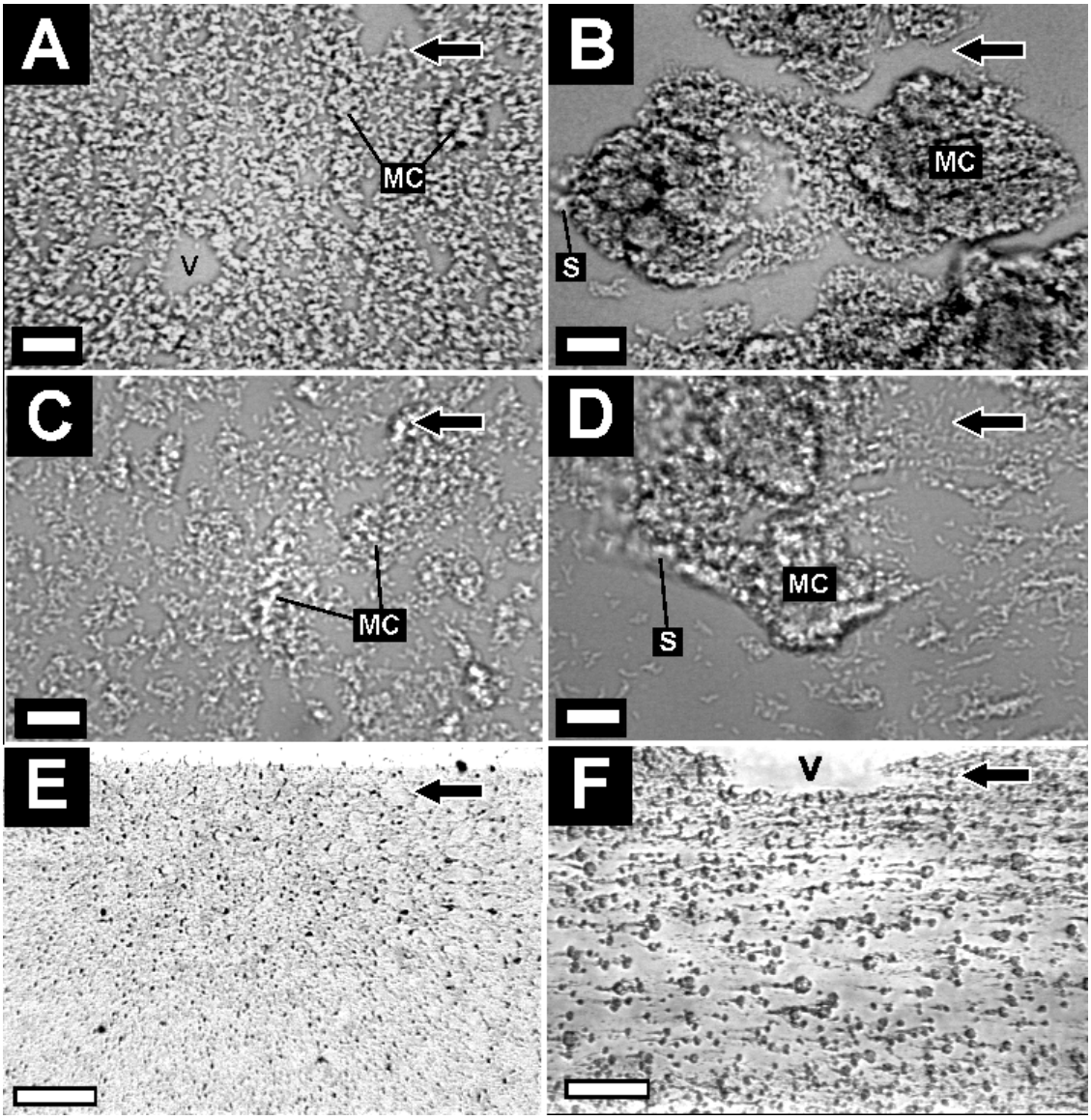

Figure 3.