The Role of Hydrodynamics and AHL Signalling Molecules as Determinants of the Structure of Pseudomonas aeruginosa Biofilms

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ABSTRACT

The ability of two Pseudomonas aeruginosa PA01 wild type strains and two quorum sensing mutants to form biofilms in a recirculating continuous-culture system was examined. Biofilms were grown under laminar and turbulent flow in parallel glass flow cells for between 9 and 12 d. One mutant, PANO67, is deficient in the production of BHL, however, it does produce OdDHL whereas a lasR lasI mutant does not produce OdDHL but is capable of producing BHL. The accumulation of biofilm biomass was estimated from the surface cover and the average microcolony thickness. The amount of biomass increased initially at a higher rate in the wild type strains than in the two quorum sensing mutants and a maximum was reached between 2 and 7 d after which there was some detachment of biofilm microcolonies. However, the biomass of the mutant strains steadily increased so that by the end of the experiments the mutant biofilms had a greater volume of biomass than the wild type strains. The results suggested no marked difference in the structure of the mutant biofilms compared to the wild type biofilms. However, the flow conditions had a profound influence on biofilm structure. Biofilms grown in turbulent flow consisted of filamentous streamers, while those grown in laminar flow consisted of a mono-layer of cells interspersed with circular microcolonies.

INTRODUCTION

Pseudomonas aeruginosa produces diffusible chemical signals (*N*-acyl homoserine lactones; N-AHLs) which regulate the production of virulence determinants and secondary metabolites in a cell density dependent manner known as quorum sensing (Salmond *et al*. 1995). Two quorum sensing regulons have been identified in which the LuxR homologues, LasR and VsmR (also termed RhlR) are activated by *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL) respectively (Winson *et al*. 1995).

It has been suggested that quorum sensing may play a specific role in the development of biofilms since this environment can demonstrate high cell densities (Williams and Stewart, 1994). There are a multitude of problems associated with biofilm colonisation ranging from medical infections (Bonten *et al*. 1996) to the fouling of industrial components (Stoodley *et al*. 1998). Biofilms often form complex structures consisting of cell clusters, discrete aggregates of microbial cells in a slime extracellular polymeric substances (EPS) matrix, surrounded by channels through which water can flow (Costerton *et al*. 1995; Stoodley *et al*. 1994). The structure may determine important properties of a biofilm such as mass transfer of antimicrobial agents and nutrients, biofilm detachment, and energy losses in pipelines (Stoodley *et al*. 1997). However, the factors involved in determining these structures are poorly understood. In a recent study on the role of cell signalling in *Ps. aeruginosa* biofilms Davies *et al*. (1998) reported that OdDHL was required for the development of structurally complex biofilms which were formed by wild type cells.

The goal of this project was to determine the role of quorum sensing and hydrodynamic conditions in the formation of *Ps. aeruginosa* biofilms. In order to study the role of N-AHLs and hydrodynamics in biofilm development, we used a flow system in which biofilms could be grown in parallel glass flow cells under both laminar and turbulent flow. The developing biofilms were observed *in situ* using digital image analysis microscopy.

METHODS

Biofilm flow system

Biofilms were grown in parallel glass flow cells (Fig. 1), one under laminar flow (Reynolds number (Re) = 120) and one under turbulent flow (Re = 3600). The hydrodynamics of the flow system have been characterised and described elsewhere (Stoodley *et al*. 1999). *Ps. aeruginosa* biofilms were grown on a minimal salts medium with glucose (400 mg l^{-1}) as the sole carbon source (Stoodley *et al*. 1994). The developing biofilms were imaged using bright field microscopy and images captured digitally with an image analysis system as described elsewhere (Stoodley *et al*. 1999). Changes in biofilm structure over time were qualitatively documented through photomicrographs and time lapse imaging. Image analysis was used to quantify various parameters related to structure including extent of surface coverage and cell cluster thickness. The total amount of biomass per unit area of surface was estimated from the average surface area covered (n = five 0.18 mm^2 fields) and the average microcolony thickness (n=5 micro-colonies). These measurements are described in detail by Stoodley *et al*. (1999).

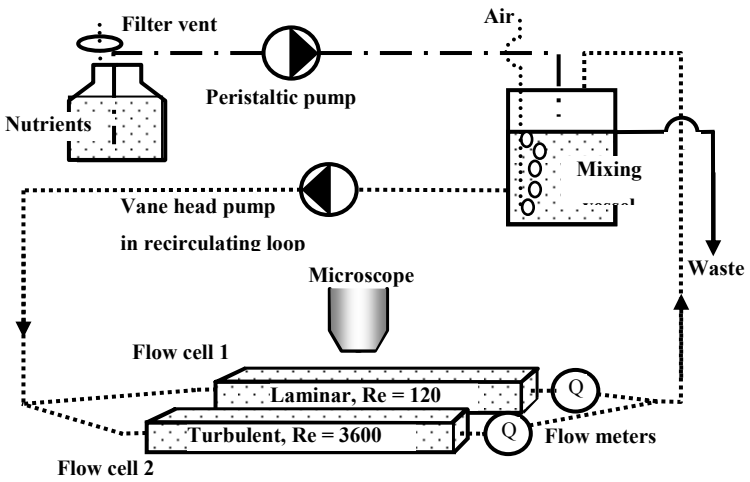

Figure 1 Biofilm reactor system consisting of two glass flow cells which were incorporated into a re-circulating loop attached to a mixing vessel to which nutrients and air were added. Flow was laminar in one flow cell (flow velocity (u) = 0.033 m/s but turbulent in the other (u = 1 m/s). The flow cells were held in a polycarbonate holder and positioned on the microscope stage for *in situ* imaging (Stoodley *et al*. 1999).

Strains and inoculation procedures

The biofilms formed by the following *Ps. aeruginosa* strains were investigated: PANO67 (Jones *et al*. 1993; Latifi *et al*. 1995) and its isogenic PAO1 wild type (PANO67 WT) strain (Holloway collection), and a *lasR lasI* mutant (kindly provided by M. Bally, Marseille) and its isogenic WT strain (*lasR/I* WT). PANO67 strain is a chemically induced strain deficient in the production of BHL, however it does produce OdDHL. The *lasR lasI* mutant does not make OdDHL but is capable of producing BHL. One ml of a late log phase batch culture was inoculated directly into the mixing chamber of the flow system with flow in the recirculating loop. Initially the flow system was run as a batch culture for 24 h to allow attachment of the bacterial cells to the substratum. Nutrient flow was then turned on and the system was operated as a chemostat. The dilution rate (D) was maintained at 0.74 h^{-1} to wash out suspended cells ($\mu_{(max)}$ for *Ps. aeruginosa* growing aerobically on glucose is 0.4 h^{-1}) and encourage biofilm growth.

Detection of OdDHL in the effluent

One litre of effluent from the WT PA01 and the *lasR/I* mutant was extracted with dichloromethane (700:300 supernatant/dichloromethane). Dichloromethane was removed by rotary evaporation and the residue reconstituted in 200 µl distilled water. This extract was added to wells cut in Nutrient Agar plates seeded with a bioluminescent *E. coli*-based sensor (*E. coli* containing pSB1075), incubated overnight at 30°C and then bioluminescence was monitored using a Berthold LB980 imaging system (Winson *et al*.1998).

RESULTS

The four strains of *Ps. aeruginosa* examined were all capable of forming biofilms in both laminar and turbulent flows. In the laminar flow cell the biofilms initially consisted of micro-colonies surrounded by a monolayer of singe cells (Fig 2). In the turbulent flow cell the microcolonies were elongated in the downstream direction to form filamentous streamers (Fig 2). Single cells were sparsely attached in the void spaces between the streamers. Similar structures have been reported in a mixed species biofilm growing under turbulent flow

(Stoodley *et al*. 1998).

Biofilms grown in laminar flow

After 4 d the WT biofilms growing in the laminar flow cells began to detach leaving patches in which few attached cells remained. Between 4 and 6 d the biomass had reached a maximum of approximately 3 cm^3 of biofilm per m^2 of substratum (Fig. 3). Further detachment resulted in a net decrease in biomass so that at the end of the experiment there was 0.3 and 1.6 cm^3 of biofilm m^{-2} in the PANO67 WT and the *lasR/I* WT biofilms respectively. However, although the accumulation rate of the mutant biofilms was initially lower it remained relatively constant so that after 9 d there was 1.6 cm^3 of biofilm m^{-2} in the PANO67 strain and 3.7 cm^3 of biofilm m^{-2} in the *lasR/I* strain. These values were between 2 to 3 times that of the isogenic WT strains.

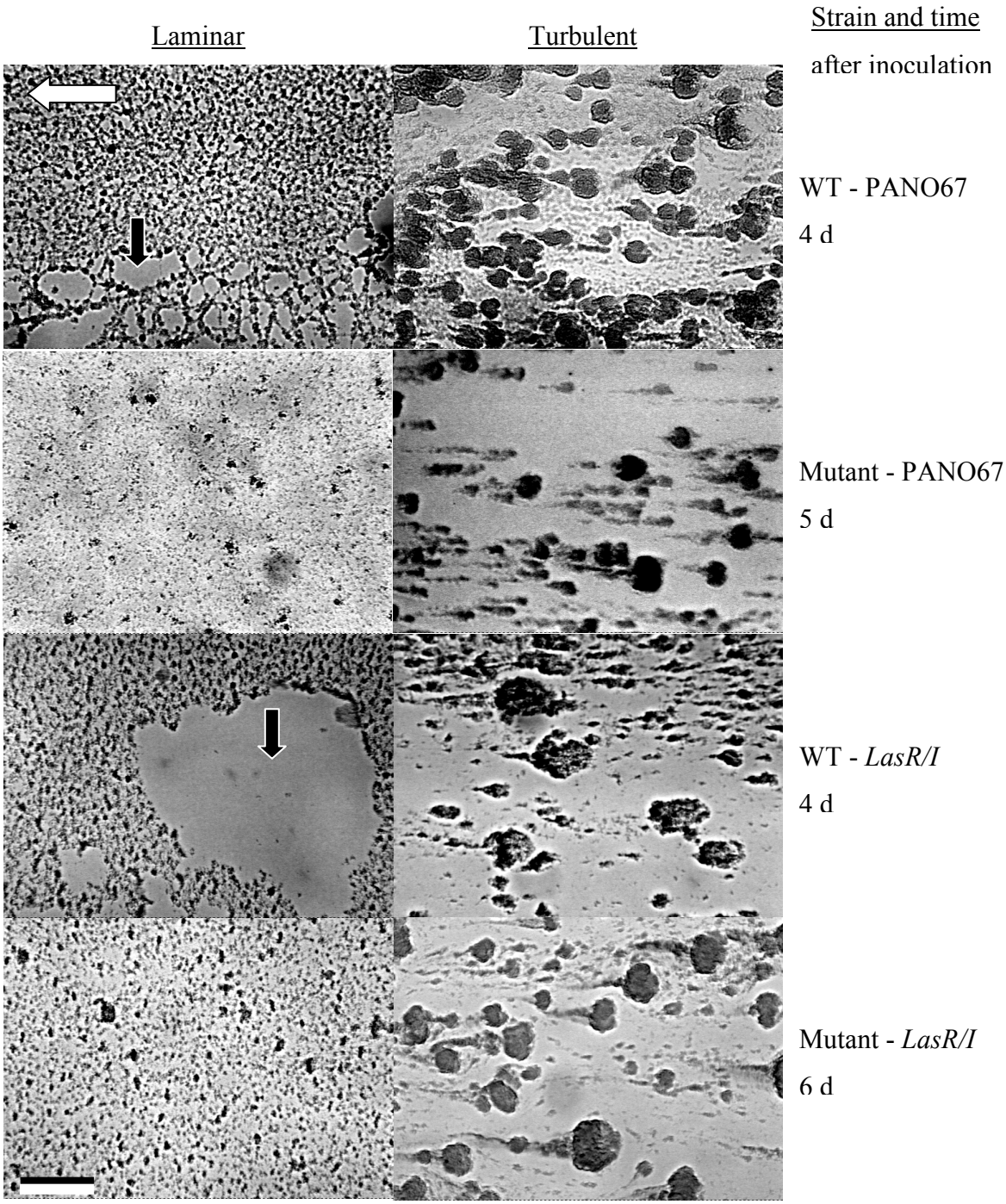

Figure 2 Structure of the 4 strains of biofilm grown under laminar and turbulent flow. The biofilms grown in laminar flow were composed of circular microcolonies while the biofilms grown in turbulent flow were composed of filamentous streamers. Clear patches where biofilm had sloughed from the WT biofilms growing in the laminar flow are indicated by the black arrows. The white arrow indicates flow direction, scale bar = 100 μm.

Biofilms grown in turbulent flow

Under turbulent flow the PANO67 WT biofilm accumulated most rapidly and after 7 d had increased to 10.1 cm^3 of biofilm m^{-2} (Fig. 3). However, between 7 and 8 d many of the streamers detached so at that the end of the experiment (9 d) only 1.9 cm^3 of biofilm m^{-2} remained. Although the accumulation rate of the PANO67 mutant was initially lower than

the WT no significant detachment events were observed. After 9 d there was 5.2 cm^3 of biofilm m^{-2}. The *lasR/I* WT formed a patchy biofilm in which some areas were heavily colonised with streamers but there were other areas in the flow cell which showed very little biofilm. After 5 d there was 1.8 cm^3 of biofilm m^{-2} and by day 12 further detachment had reduced this to 0.4 cm^3 of biofilm m^{-2}. By contrast the *lasR/I* mutant biofilm continued to accumulate and after 12 d had increased to 16 cm^3 of biofilm m^{-2} (Fig. 3).

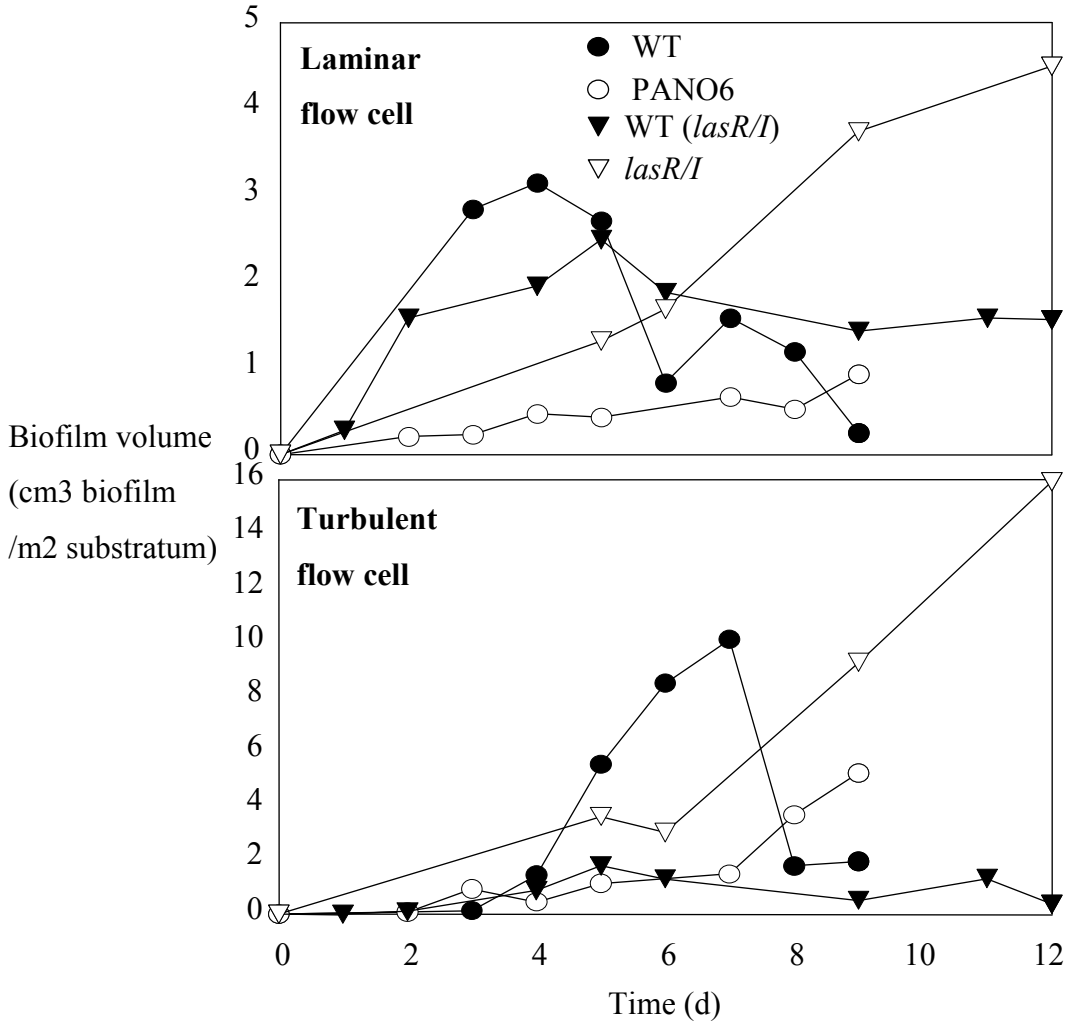

Figure 3 Biofilm accumulation for the two *Ps. aeruginosa* PAO1 WT and mutant strains growing under laminar and turbulent flow.

Presence of OdDHL in effluents

The extract of the effluent from the *las R/I* WT PAO1 gave rise to a zone of light around the well cut into the agar indicating that OdDHL was present in the spent medium. However, the extract derived from the *lasR/I* mutant did not induce a light reaction indicating that there was no OdDHL in the effluent and that the mutation was intact.

DISCUSSION

As flow conditions may modulate the effect of *N*-AHLs due to their freely diffusible nature, biofilms were characterised under both high (turbulent) and low (laminar) flow conditions.

The biofilms formed were viewed using bright field microscopy and the amount of biomass accumulating over time assessed by percent surface cover and microcolony thickness. Both wild type and mutant strains formed biofilms with complex structures.

Generally the volume of biomass increased initially at a higher rate in the wild type strains than in the two quorum sensing mutants. However, between 2 and 7 days there was significant detachment from the WT biofilms which was not observed in the mutant strains. As a result after 9 days the mutant biofilms had more attached biomass than the WT strains. Dalton *et al*. (1996) have observed cyclical colonisation by marine *Vibrio* and *Pseudomonas* species in which microcolonies repeatedly formed and dispersed over periods between 1 and 2 d. We have also seen evidence of cyclical colonisation in a four species biofilm which was grown in the same flow system as described in the present study (Stoodley *et al*. 1999). In this case a biofilm growing in laminar flow increased in surface coverage to approximately 70% after 5 d, the surface cover then reduced to 45% after 12 d, before increasing again. It is possible that the WT *Ps. aeruginosa* strains used in this study have a similar progression of cyclical colonisation with a cycling period on the same order as our 12 d monitoring period. If this is the case it appears that both BHL and OdDHL may play a role in the dispersion of micrcolonies and the absence of these AHLs may interrupt the dispersal cycle. It is possible that AHLs may regulate *algL* which codes for alginate lyase in *Ps. aeruginosa*, the increased production of which has been shown to result in increased detachment of biofilm cells (Boyd and Chakrabarty 1994).

In a recent study on the role of cell signalling in *Ps. aeruginosa* biofilms Davies *et al*. (1998) reported that OdDHL was required for the development of the complex structure formed by wild type cells. The lack of complex structure in a *lasI* mutant biofilm lacking OdDHL was described as failure to form the characteristic microcolonies composed of groups of cells separated by water channels observed in the wild type biofilm. Instead the mutant biofilm formed a flatter, thinner non-cohesive biofilm. Our study found no marked difference in the structural complexity of the *lasR lasI* mutant biofilm as compared to the wild type biofilm but did find differences in the progression of colonisation of the substratum between the WT and mutant strains. The experiments carried out by Davies *et al*. (1998) were conducted in a continuous-culture once-flow-through system with a glass substratum, similar to the set-up used in the present study, however, their Reynolds number was 0.17, over 700 times lower than in our laminar flow cell (Re 120). The calculated wall shear stresses were comparable however, (approximately 0.1 Nm^{-2}) suggesting that differences in observed biofilm structure were more likely related to rates of mass transfer than shear-related detachment. Since quorum sensing is a concentration dependent phenomenon it is likely to be strongly influenced by mass transfer which in turn is highly dependant on the hydrodynamics. There are other differences in the experimental set ups; in our system we used glucose to give 160 mg C l^{-1} while Davies *et al*. used lactate and succinate to give 28 mg C l^{-1}. Differences in surface to volume ratios between the two systems would also result in differing loading rates.

Our results showed that the hydrodynamics had a profound influence on the structure of the biofilms formed. Turbulent flow gave rise to a biofilm structure consisting of filamentous “streamers” while the biofilms formed under laminar flow consisted of smaller circular shaped microcolonies. The influence of AHLs on biofilm formation were more subtle and did not appear to influence the complexity of the structures although the prescence of AHLs did influence the progression of colonisation. Taken together these results suggest that the relative contribution of cell signalling to the overall structure of biofilms growing in aqueous environments during the early stages of colonisation will be determined largely by the hydrodynamic conditions.

Our results suggest that AHL’s play a role in the accumulation of biofilms but the situation appears to be more complex than that suggested by Davies *et al*. (1998) who

concluded that biofilm structure is dependant on OdDHL. As yet ours is a preliminary study and further work is required to elucidate the relative contribution of AHLs to the formation of bacterial biofilms.

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