Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation

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Extracellular polymers can facilitate the non-specific attachment of bacteria to surfaces and hold together developing biofilms. This study was undertaken to qualitatively and quantitatively compare the architecture of biofilms produced by *Pseudomonas aeruginosa* strain PAO1 and its alginate-overproducing (mucA22) and alginate-defective (algD) variants in order to discern the role of alginate in biofilm formation. These strains, PAO1, Alg⁺ PAO mucA22 and Alg⁻ PAO algD, tagged with green fluorescent protein, were grown in a continuous flow cell system to characterize the developmental cycles of their biofilm formation using confocal laser scanning microscopy. Biofilm Image Processing (BIP) and Community Statistics (COMSTAT) software programs were used to provide quantitative measurements of the two-dimensional biofilm images. All three strains formed distinguishable biofilm architectures, indicating that the production of alginate is not critical for biofilm formation. Observation over a period of 5 days indicated a three-stage development pattern consisting of initiation, establishment and maturation. Furthermore, this study showed that phenotypically distinguishable biofilms can be quantitatively differentiated.

INTRODUCTION

The most common mode of bacterial growth in nature is the formation on surfaces of organized biofilm communities, held together by a matrix composed of exopolysaccharides (EPS) (Costerton et al., 1987). The EPS matrix has been implicated in maintaining the individual cells and communities of a biofilm in close proximity and in providing a unique microenvironmental niche (Costerton et al., 1994). Much of our understanding of biofilms comes from studies of the opportunistic pathogen *Pseudomonas aeruginosa*, which causes chronic lung infection and is the major cause of morbidity and mortality in patients with cystic fibrosis (CF) (Høiby, 1975). *P. aeruginosa* strains isolated from the lungs of patients, especially with advanced stages of disease, are distinctive because about 85% have a mucoid colony morphology (Fick et al., 1992). In contrast, only 1% of strains isolated from other sites of infection are mucoid (Doggett et al., 1966). These observations suggest that mucoid *P. aeruginosa* cells have a distinct survival advantage in the CF lung environment. This mucoid phenotype is indicative of the overproduction of the EPS alginate, an O-acetylated linear polymer of D-mannuronate and L-guluronate residues (Evans & Linker, 1973). The expression of this polymer confers increased resistance to the host immune response and results in chronic pulmonary infection and poor prognosis for the patient (Baltimore & Mitchell, 1980; Govan & Harris, 1986). Infection with alginate-producing *P. aeruginosa* in CF patients has been associated with an overactive immune response and a poor clinical condition, suggesting that alginate production is a virulence factor (Høiby, 1974; Baltimore et al., 1989; Pedersen et al., 1992). Also, mucoid organisms have been

Abbreviations: CF, cystic fibrosis; CLSM, confocal laser scanning microscopy; EPS, exopolysaccharide.
observed in a biofilm mode of growth in the lungs (Lam et al., 1980). Animal studies support the view that alginate production impedes host immune clearance, contributes to tissue damage and favour survival in the lung (Boucher et al., 1997; Yu et al., 1998; Song et al., 2003).

In recent years, it has become apparent that predicting actual bacterial behaviour in their natural environment, on the basis of experiments done in liquid suspension growth media (i.e. planktonic form), may not always be reliable. As a result, many laboratories have begun to investigate how cells can coordinate their activities and build the complex structures that are found in mature biofilms. The advent of non-destructive techniques, especially the use of live-monitor systems with confocal laser scanning microscopy (CLSM), has greatly increased our understanding and appreciation of the complex architecture of biofilms (Lawrence et al., 1991). Such analyses of biofilms have shown that there is often a three-dimensional distribution of organisms with specific substructures, leading to a model for biofilm structure called the ‘water channel model’ (Costerton et al., 1987). In this model, the biofilm is not just multiple layers of evenly distributed cells, but is composed of many substructures protruding from the substratum to the top of the biofilm. These substructures have void sectors representing channels through which substrate and waste products can move. These substructures, designated mounds, mushrooms and void channels, penetrate from the substratum, and are held together by the EPS matrix (Costerton et al., 1994).

The biofilm architecture of an organism on a surface can be described in terms of the direction of growth, which can be horizontal, vertical or combined. Horizontal biofilm growth occurs when the bacteria colonize an available substratum. However, it can also occur when nutrients are scarce and bacteria are forced to extend the biofilm in order to find a new carbon or nutrient source (Stoodley et al., 1999). In this mode, the biofilm grows parallel to the substratum and spreads horizontally. This increases the surface area covered by the biofilm, with little increase in thickness, leading to confluent growth covering the available surface area. When the bacteria have already colonized the available substratum, the biofilm extends vertically, increasing the thickness of the biofilm. Vertical growth can also be a result of physical barriers when a biofilm has covered the available surface. Differences and combinations of horizontal and vertical biofilm growth determine the different structures, such as mushrooms, towers and channels as described in the water channel model (Costerton et al., 1987). Differences and combinations may occur in different ratios at different times, and at different points in the biofilm, and they are most likely controlled by a combination of gene expression, as well as factors generated by environmental conditions.

P. aeruginosa biofilm cells typically exhibit very slow growth rates relative to planktonic cells grown in liquid cultures (Brown et al., 1988). The difference in physiology of the bacteria in these two modes of growth contributes to the differences in their cellular metabolism (Brown et al., 1988). Differences have been observed in the expressions of protein profiles (Brown & Williams, 1985; Sauer & Camper, 2001), β-lactamase (Gilbert & Brown, 1998), fimbriae (O’Toole & Kolter, 1998), pili (O’Toole & Kolter, 1998), superoxide dismutases (Hassett et al., 1999) and catalases (Hassett et al., 1999). The biofilm mode of growth contributes to increased resistance to hydrogen peroxide (Hassett et al., 1999; Cochran et al., 2000), antibiotics (Hoyle & Costerton, 1991; Ashby et al., 1994; Gander, 1996; Schierholz et al., 1999) and phagocytosis (Jensen et al., 1990).

The formation of an EPS matrix may play an important role in establishing a sustainable biofilm. It was postulated that the expression of alginate genes may be critical to biofilm formation by P. aeruginosa because expression of algC is high in the mucoid strain PA8830 under biofilm conditions (Davies et al., 1993; Davies & Geesey, 1995). However, the product of the algC gene is involved in lipopolysaccharide (LPS) synthesis and rhamnolipid and alginate production (Goldberg et al., 1993; Olvera et al., 1999). As a result, activation of the algC gene could reflect activation of any of the three pathways. Expression of an algD–lacZ fusion was examined in a mucoid derivative of PAO1 called PAO579 (Hoyle et al., 1993). The algD gene promoter drives the 18 kb alginate biosynthetic operon, and thus activation of this promoter indicates expression of alginate. The algD–lacZ activity in adherent cells within a modified Robbins device, compared with cells in the effluent, showed a transient elevation of promoter activity, suggesting enhanced EPS production following adherence. Niven et al. (2001) explored the role of alginate and its O-acetylation in the formation of biofilm using a CF clinical isolate FRD1 and its mutant derivatives. They showed that O-acetylation of alginate was critical for successful biofilm maturation. However, the production of alginate per se may not be critical for strain FRD1 biofilm formation.

Based on the available data, we argued that alginate may not play a role in biofilm matrix formation (Mathee et al., 2002). In this study, we examined the role of alginate production in the formation and architecture of biofilms by comparing the prototype strain of P. aeruginosa, PAO1 (Holloway & Morgan, 1986), to its alginate-overproducing (Alg+) derivative, Alg+ PAOmucA22 (PDO300; Mathee et al., 1999a, b) and to the alginate-defective (Alg−) strain PAOalgD (Garrett et al., 1999).

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are shown in Table 1. The prototypic non-mucoid P. aeruginosa strain PAO1 (Holloway & Morgan, 1986) and its isogenic derivatives were used. These derivatives were the mucoid strain Alg+ PAOmucA22 (PDO300) and Alg− PAOalgD. The Alg+ PAOmucA22 strain carries the mucA22 allele, resulting in the constitutive production of alginate (Mathee et al., 1999a, b). The Alg− PAOalgD is a non-mucoid strain carrying a deletion in the biosynthetic gene algD (Garrett et al., 1999). The algD gene encodes GDP-mannose dehydrogenase that was proposed to commit metabolic sugar intermediates to alginate production (Deretic et al., 1987; Roychoudhury et al., 1989).
Table 1. Strains and plasmids used in this study

<table>
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<tr>
<th>Strains and plasmids</th>
<th>Relevant genotype and characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>CC118 Δpir</td>
<td>Δ(ara-leu) araD ΔlacX74 galE galK pheA20 thi-1 rpi-1 rpoB argE(Amp) recA thi pro hsdRM+ RP4-2-Tc::Mu-Km::Tn7 Δpir</td>
<td>Herrero et al. (1990)</td>
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<td>MT102</td>
<td>F− thi araD139 ara-leuΔ7679 Δ(lacIOPZY) galU gal K r− m− SmR</td>
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<td>PAO1</td>
<td>Prototypic non-mucoid wild-type strain</td>
<td>Holloway &amp; Morgan (1986)</td>
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<td>PDO300</td>
<td>PAO1mucA22</td>
<td>Mathee et al. (1999b)</td>
</tr>
<tr>
<td>PAOalgD</td>
<td>PAO1algD::xylE aacC1, GmR</td>
<td>Garrett et al. (1999)</td>
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<td>PAO1-mini-Tn5-Pa1/04/03-gfpmut3−T0−T1, TelR</td>
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<td>This study</td>
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<td>KMD230</td>
<td>PDO300-mini-Tn5-Pa1/04/03-gfpmut3−T0−T1, TelR</td>
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<td>J. B. Andersen, unpublished</td>
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<td>pMH305</td>
<td>pUCP22 Not-derived GFP-cloning vector, ApR GmR</td>
<td>This study</td>
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**Media and growth condition.** *Escherichia coli* and *P. aeruginosa* were routinely cultured in LB broth (10 g tryptone, 5 g yeast extract and 5 g NaCl l−1). Low-salt LB broth contained 2.5 g NaCl l−1. LA/PIA, used in triparental matings, was a 1:1 mixture of *Pseudomonas* isolation agar (Difco) and LB agar. All incubations were carried out at 37°C. The strains of *P. aeruginosa* were grown as biofilms in plexiglass flow chambers (Fig. 1) with modified EPRI media (Davies et al., 1998).

Antibiotics, when used, were at the following concentrations per ml unless indicated otherwise: ampicillin at 50 μg, kanamycin at 30 μg, tetracycline at 20 μg for *E. coli*; carbenicillin at 300 μg, tetracycline at 60 μg and tellurite at 10 μg for *P. aeruginosa*.

**DNA manipulations.** General DNA manipulations were performed as described previously (Maniatis et al., 1982). Plasmids were isolated from *E. coli* using columns and procedures as described by the manufacturer (Qiagen). Triparental matings were used as described (Goldberg & Ohman, 1984) to mobilize plasmids from *E. coli* HB101 to *P. aeruginosa* using the conjugation helper plasmid pRK600 (Kessler et al., 1992). Transconjugants following triparental matings were selected on LA/PIA with appropriate antibiotics.

**Electroporation.** Electroporation of *P. aeruginosa* was performed using a procedure described previously (Mathée et al., 1997). Briefly, 0.2 ml of a *P. aeruginosa* PAO1 overnight culture was used to inoculate 20 ml fresh LB broth and was incubated at 37°C with shaking. When the culture reached an OD600 of 0.6–0.8, the cells were pelleted by centrifugation (10 000 r.p.m., 10 min at 4°C) and washed with 20 ml SMH buffer (300 mM sucrose, 1 mM MgCl2, 1 mM HEPES, pH 7.0). This procedure was then followed by two more washes with 10 ml cold SMH buffer and the pellet was resuspended in 1 ml cold SMH. Plasmid DNA (1–2 μg) was added to a 100 μl aliquot of these competent cells, which were then mixed and incubated on ice for 1 min. The bacteria–DNA mixture was transferred to a chilled electroporation cuvette (Bio-Rad Gene Pulser/E. coli Pulser cuvette) with a 0.2 cm electrode gap. Electroporation was performed at 800 Q, 25 μF and 8 kV (cm gap)−1 on a Gene Pulser electroporator (Bio-Rad). Immediately after electroporation, 900 μl cold SOC medium (2.0% tryptone, 0.5% yeast extract, 10 mM MgCl2, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO4, 20 mM glucose) was added to the cells and incubated on ice for 30 min, followed by another 30 min incubation at 37°C. Samples (200 μl) of the electroporated cells were spread onto LB agar plates containing the appropriate antibiotics followed by incubation for ~36 h at 37°C.

**Construction of green fluorescent protein (GFP) reporter strains for biofilm architecture.** Architectural analysis of biofilms requires a reporter system for the visualization of cells and structures within a biofilm without the use of disruptive techniques. GFP from the jellyfish...
**RESULTS AND DISCUSSION**

**Alginate production is not critical for biofilm formation**

We examined the role of alginate production on biofilm growth and architecture by comparing the growth of the prototypic non-mucoid, alginate-inducible strain PAO1, which has the capacity to produce alginate (Holloway & Morgan, 1986), to its isogenic alginate-overproducing Alg\(^+\) PAOmucA22 (Mathee et al., 1999a, b) and alginate-defective Alg\(^-\) PAOalgD (Garrett et al., 1999) derivatives. All strains carried a chromosomal gene for GFP (tagged with GFP) allowing biofilm development to be easily monitored using CLSM (Fig. 2). For this, we used an experimental set-up that permitted not only the usual qualitative analysis, but also quantitative description of biofilm formation. The flow cells used in our study were constructed using computer-driven instrumentation, with each flow cell having three identical flow chambers that allowed a direct comparison of a set of strains in a single unit (Fig. 1). For each biofilm, the CLSM images were taken at seven random spots.

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**Flow cell experiments**. Biofilms were grown at room temperature in three-channel flow cell chambers with individual channel dimensions of 1 × 4 × 40 mm. The original flow cells and bubble-traps of the biofilm experimental system introduced by Caldwell (Wolfaardt et al., 1994) were modified extensively and machining was computerized to standardize every unit (Fig. 1; K. Mathee and B. Gallick, unpublished). Use of these flow cells has now been described in several publications (Heydorn et al., 2000, 2002; Hentzer et al., 2001, 2002). The flow cell system was assembled and prepared as described previously (Christensen et al., 1999). The substratum consisted of a microscope coverslip. Cultures for inoculation of flow cells were prepared as follows. A single colony of each strain was used to inoculate test tubes containing

**Quantitative analysis of biofilms using Community Statistics (COMSTAT) software**. Images obtained by CLSM were processed using COMSTAT (Heydorn et al., 2000). This image analysis program includes several features for quantifying three-dimensional biofilm image stacks. In this study, we analysed three quantities to define the biofilm architecture. The quantities, selected on the basis of their biological and physical significance, were: (i) biomass (measurement of overall volume of the biofilm, not including pores and voids inside the biofilm), thus providing an estimate of the biomass in the biofilm, (ii) mean thickness (measurement of mean thickness of the biofilm (including the pores and voids inside the biofilm), thus providing a measure of the spatial size occupied by the biofilm), and (iii) substratum coverage (measurement of surface area covered by the biofilm). Since some of the quantities displayed an exponential increase, all of the quantities were log-transformed before further statistical analysis.

**Statistical analysis**. The results computed using COMSTAT and BIP were analysed using the statistical software package SPSS (version 10.0 for Windows).
Qualitative analysis reveals that alginate production is not critical for biofilm formation but contributes to distinct biofilm architecture

All three strains formed biofilms. The PAO1 biofilm on day 1 was mostly composed of single cells and small clusters of no more than three or four bacterial cells, with no apparent organization (Fig. 2a). There was some movement of cells, suggesting a transient attachment. By day 3, PAO1 spread horizontally as a confluent layer of cells covering the entire available substratum, with a uniform increase in thickness without noticeable architectural structures (Fig. 2b). By day 5, this biofilm was mainly composed of a relatively uniform and dense architecture, except for the occasional presence of mounds and channels (Fig. 2c). The latter may be due to the vertical growth of the biofilm once a confluent layer has covered the available substratum.

Compared with the prototypic non-mucoid PAO1, the Alg⁺ PAOmucA22 cells behaved differently. On day 1, these cells showed a tendency to stay together and form compact aggregations of cells (Fig. 2d). This biofilm appeared to grow both vertically and horizontally from the moment of initial attachment. On day 3, the Alg⁺ PAOmucA22 biofilm formed distinct microcolonies, growing as cell clusters with limited horizontal spreading and with some structures such as mushrooms and mounds. A confluent layer could be observed at certain sections (Fig. 2e). In contrast to day 1, there was an increase in the number of microcolonies as well as in thickness. On day 5, the Alg⁺ PAOmucA22 cells almost completely covered the substratum with mounds and mushrooms of varying thicknesses and showed a significant increase in thickness as compared to day 3 (Fig. 2f).

The Alg⁻ PAOalgD strain behaved differently from the other two strains discussed above. These cells formed filamentous clusters (Fig. 2g). As a result, the thickness of the Alg⁻ PAOalgD biofilm varied from being composed of a single cell (2 μm) to cell clusters several cells thick (8 μm). On day 3, the Alg⁻ PAOalgD biofilm showed extensive heterogeneous structures, microcolony mounds and mushrooms of thicknesses varying from 10 to 40 μm (Fig. 2h). The Alg⁻ PAOalgD biofilm on day 5 had a very heterogeneous...
architecture, covering most of the available surface with varying thicknesses and architecture from confluent to distinct structures (Fig. 2i).

This qualitative analysis showed that, when inoculated in a flow cell with a constant supply of fresh medium, the three P. aeruginosa strains, PAO1, Alg+ PAOmucA22 and Alg− PAOAlgD, successfully attached to the surface and established biofilms. If alginate production were critical for biofilm formation, then Alg− PAOAlgD should not have formed any biofilm. However, Alg− PAOAlgD did form a biofilm that shared characteristics with both PAO1 and Alg+ PAO mucA22. It started growing from single cells and cell clusters, and continued to develop into a heterogeneous biofilm. The Alg− PAOAlgD biofilm formed structures that protruded from the base layer of cells; these structures were less defined than those of Alg+ PAO mucA22.

One of the algT alleles used had a single base change, resulting in a missense mutation that made four to five times less algT/U transcripts, whereas the other, an algT::Tn insertional mutant, made no algT/U transcripts in planktonically grown cells (DeVries & Ohman, 1994). All of these strains formed biofilms with varying thickness and architecture, suggesting that, in the mucoid CF strain FRD1, O-acetylation of alginate was more critical for successful biofilm maturation than alginate itself (Nivens et al., 2001). This was the most detailed study performed so far comparing the biofilm of mucoid FRD1 to non-mucoid strains, although the non-mucoid strains had double mutations.

This study, using three isogenic strains, overtly demonstrated that alginate production was not critical for biofilm formation. All three strains could deposit on a flow-cell surface within an hour and maintain themselves against a constant flow of medium. Qualitative analysis showed that the three P. aeruginosa strains, PAO1, Alg+ PAOmucA22 and Alg− PAOAlgD, matured over time into visibly distinguishable biofilms. Our analysis is further supported by a recent work in which the authors, using the same strains as described here, demonstrate that alginate was not required for biofilm formation (Wozniak et al., 2003). These authors also concluded that PAO1 and Alg− PAOAlgD biofilms were similar in structure, although their quantitative analysis of their day 2 biofilm suggests otherwise (Wozniak et al., 2003). In fact, our analysis shows that biofilm differentiation is more prominent after day 3 (Fig. 2).

Intuitively, one would expect some sort of progression of biofilm characteristics along the lines of Alg− PAOAlgD > PAO1 > Alg+ PAO mucA22. The Alg− PAOAlgD biofilm appeared to have a pattern of development that lay between that of PAO1 and Alg+ PAO mucA22. Indeed, we do not know if any extracellular polysaccharide or other compound is involved. It is possible that bacterial LPS may be an important component of the Alg− PAOAlgD matrix. Alternatively, the secreted mannose–rhamnose polysaccharide may play a role in the Alg− PAOAlgD biofilm (Kocherova et al., 1989). A recent chemical analysis of the biofilm matrix showed the presence of an unknown amino sugar that may form the predominant matrix component (Wozniak et al., 2003). It is possible that Alg− PAOAlgD may overexpress the non-alginate polysaccharide and/or LPS to compensate for the lack of alginate production, giving rise to the intermediate architecture. If this is true, it is likely that infection with Alg− PAOAlgD is more severe compared with PAO1. In fact, we showed that animals infected with Alg− PAOAlgD had a significantly higher bacterial load and a more severe pathology compared with animals infected with PAO1 (Song et al., 2003).

Although alginate production may not be required for biofilm formation in vitro on a glass surface, it undeniably affects the biofilm architecture. The Alg+ PAOmucA22 biofilm phenotype is presumably due to alginate production and/or the absence of twitching motility (data not shown). In a parallel study, Alg− PAOmucA22 was shown to exhibit a highly structured architecture compared to PAO1 (Hentzer et al., 2001). In fact, it was shown that alginate-dependent microcolony formation does contribute to antibiotic resistance against antibiotics and biocides, both in vivo and in vitro (Evans et al., 1991; Hoyle & Costerton, 1991; Hentzer et al., 2001). In vitro analyses show that alginate aids in bacterial adherence to human cells (Doig et al., 1987; Marcus & Baker, 1985; Ramphal & Pier, 1985), protects the bacteria from host defences such as lymphocytes, phagocytes, the ciliary action of the respiratory tract, antibodies and complement (Pedersen et al., 1990) and aids in biofilm growth (Lam et al., 1980).

Quantitative analysis to assess the biofilm heterogeneity

It is clear from qualitative observations that the three strains make biofilms, and that these biofilms differ in structure. We attempted to make a more quantitative assessment of our many microscope images, taking advantage of the variety of analyses in the BIP and COMSTAT software packages (Heydorn et al., 2000; Ji, 2000). Quantitative analysis of images requires reproducible results with a standardized experimental system, as described earlier. For each biofilm, the CLSM images were taken at seven random spots across the length of the flow cell (approx. 6 mm apart). All quantities computed were tabulated and analysed using SPSS, a standard statistical software package. For each bacterial strain and for each of the three times considered (days 1, 3 and 5), each computed quantity was shown as a range of values (the bar indicates a 99 % confidence interval) of mean (Figs 3 and 4).

Both COMSTAT and BIP compute a number of measures such as roughness coefficient, mean diffusion distance, textural en-
tropy and areal porosity that can be used as indicators of biofilm heterogeneity (Heydorn et al., 2000; Ji, 2000). The textural entropy (Fig. 3a) is a measure of the extent to which the biofilm organization varies. Thus, it should increase with increased heterogeneity and the values close to zero should correspond to biofilms that are least heterogeneous. Areal porosity (Fig. 3b) measures the ratio of the void area to the total image, and thus should decrease as the biofilm extends itself on its surface. The diffusion distance (Fig. 3c) measures the distance over which the substrate has to diffuse from the void space to reach bacteria within the clusters. Thus, the value should decrease with increased biofilm heterogeneity. The results of applying these measures to our data are shown in Fig. 3.

The textural entropy values (Fig. 3a) on days 1 and 3 were significantly higher for Alg$^+$ PAO$mucA22$ and Alg$^-$ PAO$algD$ than PAO1. They then levelled off by day 5, although they appeared to increase with time for PAO1. Similarly, the areal porosity (Fig. 3b) decreased and then levelled off for Alg$^+$ PAO$mucA22$ and Alg$^-$ PAO$algD$, whereas it appeared to decrease exponentially for PAO1 even on day 5. These data (Fig. 3b) appeared to behave in a manner that was exactly opposite to that of the textural entropy analysis (Fig. 3a). Diffusion distance (Fig. 3c) analysis showed an interesting pattern, Alg$^+$ PAO$mucA22$ started high and reduced over time, reaching the same value as that of the day 1 PAO1 biofilm (leveling off). PAO1 and Alg$^-$ PAO$algD$ showed similar trends and values, and these increased with time. The confidence interval for diffusion distance increased for strains Alg$^-$ PAO$algD$ and PAO1, and decreased for Alg$^+$ PAO$mucA22$.

Qualitative analysis clearly showed the distinct architecture conferred by alginate production in the Alg$^+$ PAO$mucA22$ strain. Quantitative analyses of day 1 biofilms showed that the textural entropy, mean diffusion distance and areal porosity were similar in all three strains, but slightly elevated in Alg$^+$ PAO$mucA22$ (Fig. 3). The increase in size of the confidence intervals is surprising because one would expect this value to decrease in size with increasing maturity of the biofilm. However, it may represent the increasing variability (~complexity) in structure as the biofilm matures. This is supported by Fig. 3(a), where the textural entropy increases with time. The textural entropy analysis that measures the biofilm heterogeneity was perhaps the most meaningful in explaining the qualitative data. The least heterogeneity was observed in the PAO1 biofilm, which was thicker and denser and was depicted by the rapid and continuous loss of areal porosity (Fig. 3b). Areal porosity shows uniformly small confidence intervals (high confidence) throughout the time period except, quite strangely, for PAO1 on day 5. In a parallel study, Alg$^+$ PAO$mucA22$ was shown to exhibit a highly structured architecture compared to PAO1 (Hentzer et al., 2001). This is reflected in the fact that Alg$^+$ PAO$mucA22$ is the only strain whose biofilm showed a decrease in mean diffusion distance over time. The other strains exhibited the predicted behaviour of an increase in mean diffusion distance over time. It is intuitive to observe a decrease in areal porosity along with an increase in mean diffusion distance as a function of time, as was observed with the Alg$^+$ PAO$mucA22$ biofilm.
Qualitative and quantitative analyses reveal distinct developmental cycle of biofilm

We observed a three-stage developmental pattern for biofilms over a period of 5 days: initiation, establishment and maturation. Qualitative analysis was validated by quantitative measurements such as biomass, substratum coverage and mean thickness using the COMSTAT software. Biomass measures the overall volume of the cells (Fig. 4a). For strain PAO1, biomass increased sharply over the entire time period. It showed an exponential increase over this time period as seen by the linearity of the growth of the log-transformed values. The quantities for strains Alg⁺ PAMucA22 and Alg⁻ PAOalgD also increased, but levelled off by day 5. The mean thickness of the biofilm (Fig. 4b), which provided a measure of the spatial size of the biofilm, was positively correlated ($r = 0.993$) to the biomass for the three strains. The substratum coverage (Fig. 4c) measures the amount of area occupied by the biofilm, and this quantity also showed a correlation to the biomass. Thus, all three strains followed a similar pattern as was observed in the biomass analysis. Both the Alg⁺ PAOalgA22 and Alg⁻ PAOalgD biofilms reached a plateau by day 5, while the PAO1 biofilm appeared to continue to increase. Overall, these data suggest that the principal structures of the Alg⁺ PAMucA22 and Alg⁻ PAOalgD biofilms were built during the first 3 days of development, with a slight detachment of structures from days 3 to 5 of development.

In fact, the growth pattern of Alg⁺ PAMucA22 and Alg⁻ PAOalgD strains is reminiscent of the planktonic bacterial growth cycle, showing lag, exponential and stationary phases. We hypothesized that Alg⁺ PAMucA22 and Alg⁻ PAOalgD would form mature biofilms by day 5, whereas wild-type PAO1 would continue to develop. In a recent study, Sauer et al. (2002) examined 12-day PAO1 biofilms and reported five distinct stages of biofilm development, of which the two initial stages are reversible attachment and irreversible attachment. The latter stage is accompanied by loss of twitching motility and a change in the pattern of protein expression (Sauer et al., 2002). Interestingly, both the attachment and detachment stages were observed in our day 1 PAO1 and Alg⁻ PAOalgD biofilms, but not in the Alg⁺ PAMucA22. Similarly, other studies have shown that motility is not a prerequisite for successful biofilm development (Heydorn et al., 2002; Sauer et al., 2002). In our study, depending on the strains, the biofilm was established by day 3 and matured by day 5. In fact, the PAO1 biofilms studied by Sauer et al. (2002) appeared to mature by day 6, implying that it levelled off later than the other strains.

In addition to mounds and mushrooms, doughnut-shaped structures were also observed in several places across the Alg⁺ PAMucA22 and Alg⁻ PAOalgD biofilms on day 3 (Fig. 5), but not in PAO1 biofilms. These doughnut-shaped structures had a cylindrical hollow centre and a ring of bacterial cells. Live microscopy observations showed that the hollow centre inside the doughnut was filled with motile bacteria where cells darted about vigorously and disappeared into the void space, generating the ‘doughnut’ structures. In contrast to day 3, on day 5, the hollow centres of the doughnuts were occupied and no movement was detected. These hollow centres are due to the detachment of cells or cell aggregates from the established biofilms (Stoodley et al., 2001). A
similar phenotype was observed in a 9-day-old PAO1 biofilm and was dubbed the dispersion stage by Sauer et al. (2002).
Since this phenomenon appeared to be a post-maturation process, it supports our hypothesis that Alg$^+$ PAOmucA22 and Alg$^-$ PAOalgD mature earlier than PAO1. It was clear that the biofilm developmental curve that includes initiation, establishment, maturation and dispersion was similar to lag, exponential, stationary and death phases of planktonic growth curves. Although these strains have similar growth patterns in planktonic cells (data not shown), they show distinct patterns in their biofilm mode of growth.

In summary, the quantitative measures of these three strains undoubtedly support the qualitative descriptions given above. Among the computed measures used in this study, biomass, textural entropy, mean diffusion distance and areal porosity quantify information from within the biofilm, whereas the substratum coverage quantifies surface information. Both COMSTAT and BIP were able to evaluate successfully architecture and morphology features to differentiate the cell distribution within the biofilms of the three bacterial strains. The increased use of quantitative measures among biofilm researchers will improve our understanding and interpretation of biofilm growth, environmental and genetic influences.

We have evaluated the role of alginate in biofilm development. Indisputably, the biofilm mode of growth contributes to P. aeruginosa persistence in lungs of CF patients. The detachment of cell aggregates from the mature biofilm (which results in doughnut structures) that can be transported elsewhere and will subsequently establish new focal points of infection is indeed a health concern (Stoodley et al., 2001). It is likely that the physiology of the aggregates is such that they are better at resisting antibiotics and host immune responses. Although the in vitro analysis argues against the alginate requirement, it does not rule out its expression in the lungs of CF patients. In fact, all the available data to date agree that the appearance of mucoid microcolonies in CF patients’ lungs provides the bacteria with a distinct survival advantage, suggesting that alginate is the major virulence factor involved in chronic infection in CF patients. Using the same three isogenic strains, we demonstrated that alginate protects the cells against the host immune response and impedes host

Fig. 5. Doughnut structures. Alg$^+$ PAOmucA22 and Alg$^-$ PAOalgD gave rise to circular doughnut-shaped structures (denoted by arrow heads). Such structures were not observed in the wild-type PAO1 biofilm.
immune clearance in a mouse model of acute lung infection (Song et al., 2003). Early infection by non-mucoid variants supported by a non-alginate matrix may ensure that the biofilm is compact and would thus better resist environmental stresses. The intermediate biofilm architecture seen in Alg– PAO1algD correlates with its intermediate infectious phenotype in the acute animal infection model, and is suggestive that the alternate biofilm matrix may contribute to the bacterial pathogenesis, especially during the initial infection before the genotypic conversion to constitutively producing mucoid strains (Song et al., 2003).

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