Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis

Sylvia M. Kirov,¹ Jeremy S. Webb,^{2†} Che Y. O'May,¹ David W. Reid,¹ Jerry K. K. Woo,² Scott A. Rice² and Staffan Kjelleberg²

Correspondence

Sylvia M. Kirov

S.M.Kirov@utas.edu.au

¹School of Medicine, University of Tasmania Clinical School, 43 Collins St, Hobart, Tasmania 7001, Australia

²School of Biotechnology and Biomolecular Sciences and Centre for Marine Biofouling and Bio-innovation, University of New South Wales, Sydney, NSW 2052, Australia

Intractable biofilm infections with *Pseudomonas aeruginosa* are the major cause of premature death associated with cystic fibrosis (CF). Few studies have explored the biofilm developmental cycle of *P. aeruginosa* isolates from chronically infected individuals. This study shows that such clinical isolates exhibit biofilm differentiation and dispersal processes similar to those of the better-studied laboratory *P. aeruginosa* strain PAO1 in the glass flow-cell (continuous-culture) biofilm model, albeit they are initially less adherent and their microcolonies are slower to develop and show heterogeneous, strain-specific variations in architecture. Confocal scanning laser microscopy combined with LIVE/DEAD viability staining revealed that in all CF biofilms bacterial cell death occurred in maturing biofilms, extending from the substratum to the central regions of mature microcolonies to varying degrees, depending on the strain. Bacteriophage activity was detected in the maturing biofilms of all CF strains examined and the amount of phage produced paralleled the degree of cell death seen in the biofilm. Some CF strains exhibited 'seeding dispersal' associated with the above phenomena, producing 'hollowing' as motile cells evacuated from the microcolony interiors as has been described for strain PAO1. Moreover, morphotypic cell variants were seen in the biofilm effluents of all CF strains. For those CF strains where marked cell death and seeding dispersal occurred in the microcolonies, variants were more diverse (up to five morphotypes) compared to those of strain PAO1 (two morphotypes). Given that variants of strain PAO1 have enhanced colonization traits, it seems likely that the similar biofilm dispersal events described here for CF strains contribute to the variability seen in clinical isolates and the overall persistence of the *P. aeruginosa* in the CF airway.

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INTRODUCTION

Cystic fibrosis (CF), the most common inherited lethal genetic disease in Caucasian populations, results from mutations in the CF transmembrane conductance regulator (CFTR) gene. These mutations result in defective cyclic-AMP-regulated chloride channel activity that impairs pancreatic, pulmonary and intestinal functions (Wilschanski *et al.*, 1995). In the lung, mucus secretions become depleted of paraciliary fluid in the lower respiratory tract, impairing the clearance of inhaled

microbes, resulting in chronic infections and an exaggerated inflammatory response that leads to progressive deterioration in lung function and ultimately premature death (Lyczak *et al.*, 2002; Saiman & Siegel, 2004). The opportunistic bacterial pathogen *Pseudomonas aeruginosa* is the most important agent involved in chronic lung infection in CF patients (Burns *et al.*, 2001; Lyczak *et al.*, 2002). The CF lung environment favours conversion of this organism from a motile, planktonic form to a mucoid, sessile biofilm mode of growth (Costerton *et al.*, 1999; O'May *et al.*, 2006; Worlitzsch *et al.*, 2002). Recognized stages in biofilm development elucidated in experimental model systems include initial attachment, the formation of multicellular structures ('microcolonies'), and the maturation of microcolonies into thick, three-dimensional structures encased in an exopolymeric matrix and interspersed with water channels (Stoodley *et al.*, 2002). In the

[†]Present address: School of Biological Sciences, University of Southampton, Southampton SO17 1BJ, UK.

Abbreviations: AHLs, *N*-acylhomoserine lactones; CF, cystic fibrosis; HAQs, 4-hydroxy-2-alkylquinolines; HHQ, 4-hydroxy-2-heptylquinoline; HSL, homoserine lactone; LCV, large-colony variant; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing; SCV, small-colony variant.

CF lung mucus, large aggregates of mucoid *P. aeruginosa* biofilm communities (growing as per the later stages described in biofilm model systems) have been described (Worlitzsch *et al.*, 2002). In this form the organism becomes impossible to eradicate with existing therapies and is refractory to host defences (Costerton *et al.*, 1999; Hassett *et al.*, 2002).

Although the antibiotic resistance of such infections is well documented, the reasons for it are not fully understood. The mechanisms proposed all reflect the multicellular nature of the biofilm communities. They include the delayed penetration of the agent into the biofilm (exopolymer blocks diffusion or even actively binds agents), the slower growth rate of at least some biofilm bacteria (antibiotics target activities such as cell division and nutrient uptake in growing bacterial cells), and the altered microenvironments (such as anaerobiosis and acidic waste products in the biofilm interior that might directly antagonize the action of an antibiotic or cause physiological changes in the bacteria that lower antibiotic uptakes) (Stewart & Costerton, 2001). Antibiotic-resistant 'persister' cell phenotypes that forfeit rapid propagation to ensure population survival in the presence of lethal factors can also be generated in biofilms (Lewis, 2005). More recently, intensive biofilm research of a number of model organisms, including the laboratory strain *P. aeruginosa* PAO1, in *in vitro* biofilm models has identified a common biofilm developmental cycle or series of adaptive responses involving an active dispersal mechanism, termed 'seeding dispersal', that itself is associated with the generation of genetic diversity in biofilm communities, increasing the chances of survival of the organism (Boles *et al.*, 2004; Hentzer *et al.*, 2005; Koh *et al.*, 2007; Mai-Prochnow *et al.*, 2004; Purevdorj-Gage *et al.*, 2005; Webb *et al.*, 2003).

Seeding dispersal is characterized by 'hollowing' in mature microcolonies caused by highly motile cells departing the microcolony interior, leaving behind a stationary cluster of 'wall' cells. Hollowing is associated with cell death in a subpopulation of the interior cells. For *P. aeruginosa* PAO1, these cell death and dispersal events have been linked to the conversion of a prophage to a superinfective lytic form observed at the same time and location as the accumulation of reactive oxygen and nitrogen species (RONS) (Barraud *et al.*, 2006; Webb *et al.*, 2003). Significantly, cells of *P. aeruginosa* PAO1 released by seeding dispersal exhibit phenotypic and functional diversification. Thus, some biofilm-derived variants show an increased ability to disseminate, whereas others manifest accelerated biofilm formation (Kirisits *et al.*, 2005; Webb *et al.*, 2004). Antibiotic resistance has also been linked to phenotypic variation and biofilms (Drenkard & Ausubel, 2002).

Clearly it is important that more is learnt of the dispersal mechanism and its relevance to clinical isolates if improved methods to deal with chronic *P. aeruginosa* biofilm infections in CF are to be found. Although seeding dispersal has been well documented in the laboratory strain PAO1

(Purevdorj-Gage *et al.*, 2005), there have been few published studies of the biofilm biology of CF clinical *P. aeruginosa* strains. Lee *et al.* (2005) examined a selection of non-mucoid CF isolates and demonstrated that such strains had highly variable biofilm architecture consistent with our and many other published observations that *P. aeruginosa* isolates with different properties (e.g. decreased motilities and virulence factor expression, increased antibiotic resistance) evolve within the CF respiratory tract (Head & Yu, 2004; Mahenthiralingam *et al.*, 1994; O'May *et al.*, 2006; Oliver *et al.*, 2000). Lee *et al.* (2005) did not examine mucoid isolates or dispersal in their CF strain biofilms. A mucoid CF strain examined by Purevdorj-Gage *et al.* (2005) was, however, found to be 'seeding dispersal negative', leading to speculation that this mechanism may in fact not be relevant in the clinical setting (Purevdorj-Gage *et al.*, 2005). Here, we expand on our previously published comment on this finding (Kirov *et al.*, 2005) and document experiments that show that seeding dispersal does occur in CF strain biofilms. Moreover, we present evidence that such dispersal events may well be important in the overall persistence of *P. aeruginosa* in the CF lung by facilitating the release of multiple dispersal cell variants.

METHODS

Bacterial strains and culture media. *P. aeruginosa* strain PAO1 was originally isolated from a burn wound (Holloway, 1955). The Tasmanian clinical strains of *P. aeruginosa* (strains 3A, 18A, 32A and 75A) included in this study were isolated from CF sputum samples of chronically infected individuals, as previously described (O'May *et al.*, 2006). The Australian mainland 'clonal' CF isolate (strain MC1A) was obtained from the Royal Children's Hospital Melbourne, Australia (Armstrong *et al.*, 2002). These strains were all genotypically distinct by PFGE with restriction enzyme *SpeI* and random amplified polymorphic DNA (RAPD) assay (Mahenthiralingam *et al.*, 1996). The CF isolates were mucoid on initial isolation and had functional properties (decreased motilities and adherence, impaired biofilm formation in *in vitro* models especially under anaerobic conditions) typical of *Pseudomonas* 'biofilm phenotypes' isolated from chronically infected individuals (O'May *et al.*, 2006). The strains were further characterized for virulence determinants (LasA protease, LasB elastase and pyocyanin) using methods described elsewhere (Diggle *et al.*, 2002). They were also examined for quorum-sensing (QS) signalling molecules [*N*-acylhomoserine lactones (AHLs) and 4-hydroxy-2-alkylquinolines (HAQs)]. In brief, these were identified following extraction from overnight LB₁₀ broth cultures, thin-layer chromatography and detection with *luxCDABE* reporter strains prepared at the Centre for Biomolecular Sciences, University of Nottingham, UK (Diggle *et al.*, 2003; Middleton *et al.*, 2002). Strains were stored at -80 °C in 30% (v/v) glycerol and subcultured from storage onto Luria-Bertani (LB₁₀) medium [tryptone 10 g l⁻¹, NaCl 10 g l⁻¹, yeast extract 5 g l⁻¹ and 15 g l⁻¹ agar], for the experiments below. Bacterial inocula for the biofilm experiments were grown in LB₁₀ broth (~16 h, 37 °C, with shaking) and standardized (OD₆₁₀) so that an inoculum of 0.5 ml contained ~10⁹ bacteria ml⁻¹. Biofilms (continuous-culture flow-cell experiments) were cultivated in M9 minimal medium containing 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 100 µM CaCl₂ and 5 mM glucose.

Biofilm flow-cell (continuous-culture) system. Biofilms were grown at room temperature in three-channel flow cells with

individual channel dimensions of $0.3 \times 4 \times 40$ mm, as described elsewhere (Moller *et al.*, 1998; Webb *et al.*, 2003). Following inoculation with bacteria prepared as described above, the channels were inverted for 2 h with no flow, to allow bacterial adherence. This time was extended beyond the standard 1 h because of decreased adherence abilities of CF strains to glass compared to strain PAO1 (O'May *et al.*, 2006). Media flow (mean velocity of 0.2 mm s^{-1}) was then started and biofilm development followed for up to 8 days, as described below. Three sets of flow cells were run in parallel in each experiment, and duplicate or triplicate channels of each strain were examined in at least two experiments.

Microscopy. Adhesion and microcolony development of all strains were initially followed daily by bright-field microscopy (Leica DMLB Epifluorescent microscope) and representative fields (minimum five per chamber) were recorded by photography. In brief, individual flow cells were sealed by clamping the in-flow and out-flow tubing to each channel of the flow cell. The flow of M9 medium was then temporarily stopped and individual flow channels were examined directly on the microscope stage. Microcolonies and mature biofilms (days 6–7) were subsequently stained using the BacLight LIVE/DEAD viability kit reagents, SYTO 9 and propidium iodide (Molecular Probes), as described elsewhere (Webb *et al.*, 2003), and examined with a confocal laser scanning microscope (Olympus LSMGB-200). Replicate image stacks (red and green scans) were recorded at $2 \mu\text{m}$ intervals through the biofilm at random positions in the flow cell. Captured images were subsequently merged to obtain three-dimensional images of live and dead cells within individual microcolonies.

Analysis of colonies from biofilm effluent. Spent culture medium (~ 1 ml) emerging from individual flow cells was 'aseptically' collected into sterile tubes. Serial dilutions (10^{-2} – 10^{-10}) of each effluent sample in M9 medium were spread plated onto LB₁₀ agar and detached/dispersed biofilm cells quantified and examined for morphotypic colony variants. Plates were incubated at 37°C and colonies were observed daily for up to 3 days. Total c.f.u. per ml of each effluent was determined.

Bacteriophage assay. Effluent samples were also examined for the bacteriophage activity at chosen intervals using a top-layer agar method (Eisenstark, 1967). In brief, aliquots ($20 \mu\text{l}$) were serially

diluted (10^{-2} – 10^{-8}) in SM (phage) buffer ($180 \mu\text{l}$) (Hitch *et al.*, 2004). Phage titres (p.f.u. ml^{-1}) were determined by testing a $10 \mu\text{l}$ aliquot of each dilution on sectors of LB agar plates overlaid with ~ 3 ml of a top agar of LB₁₀ 0.8% (w/v) agar seeded with an overnight *Pseudomonas* strain PAO1 LB broth culture. Effluents of selected strains were also tested on agars seeded with CF strain 3A. Phage activity was recorded after overnight incubation of the plates at 37°C . Each effluent was assayed on duplicate dilutions.

RESULTS

Development of CF strain biofilms: decreased adherence and strain-specific microcolony architecture

The developmental sequence of five CF strain biofilms was followed daily by bright-field microscopic observations. As we have reported previously, the CF strains were significantly less adherent to glass than strain PAO1 and non-CF isolates of *P. aeruginosa* (O'May *et al.*, 2006). Thus, after 2 h incubation on the flow-cell glass coverslips, only the Melbourne clonal strain, MC1A, showed comparable adherence to strain PAO1 (data not shown). Strain 32A, an auto-aggregating strain that settled in static broth culture, exhibited small clusters of adherent cells as well as individual attached bacteria. Twenty-four hours post-inoculation, strain PAO1 had formed a layer of cells covering the entire surface. By contrast, the CF strains showed little (strain MC1A) or no change in surface coverage compared to the 2 h time point. After this initial 24 h lag, however, biofilm growth and microcolony development occurred for all strains. By day 5 post-inoculation, each CF strain had its own distinctive and reproducible microcolony architecture. Microcolonies for individual strains varied in their frequency of occurrence, size and structural features as summarized in Table 1 and

Table 1. Flow-cell biofilm analyses (5–7 days post-inoculation)

Data were obtained from two or three experiments.

Strain	Microcolony morphology	Mature microcolony diameter (μm)*	No. of morphological variants in biofilm effluent†
PAO1	Flat biofilm ($\sim 11 \mu\text{m}$ thick)	Microcolonies not seen‡	2
3A	Concentric ring (wall and central cells) to 'doughnut' forms with central hole	117.7 ± 49.1 ($n=22$)	3–5
18A	Outer wall cells surrounding structured, cellular centre	50.7 ± 26.1 ($n=17$)	2–5
32A	Distinctive 'slime' layer surrounding microcolonies with wall cells and structured, compact cellular centre	69.6 ± 35.7 ($n=14$)	2
75A	Flat, 14 – $17 \mu\text{m}$ thick, occasional large microcolonies only	62.0 ± 13.1 ($n=3$)§	2
MC1A	Flat, $\sim 11 \mu\text{m}$ thick, numerous small microcolonies with structured wall cells and cellular centre	41.3 ± 19.1 ($n=8$)	2

*Values are the mean $\pm$ SD; n , number of mature microcolonies from which measurements were taken (bright-field and confocal microscopy).

†Colonies were examined after 48 h incubation at 37°C .

‡Where microcolonies have been observed for strain PAO1 under similar experimental conditions, their mean diameter was 75.6 ± 4.7 at 5 days (Webb *et al.* 2003; unpublished observations).

§This strain grew predominantly as a flat biofilm.

illustrated in Fig. 1 (bright-field microscopy) and Fig. 2 (confocal microscopy). Some strains (strains 3A, 18A and 32A) formed isolated microcolonies, while for others (strains 75A and MC1A) the microcolonies emerged over time from the mat of cells covering the glass surface, as described in published studies of strain PAO1 (Webb *et al.*, 2003). Microcolony sizes for all strains varied with their stage of development. The ones shown (Fig. 2) and measured (Table 1) were those at the upper size limit and/or (for strain 3A) the ones in which central hollowing (described below) was evident (henceforth referred to as 'mature microcolonies'). The mean height $\pm$ SD of such microcolonies for strains 3A, 32A and 75A at days 6–7 (determined by confocal analysis of a combined total of 14 microcolonies of these strains) was $34.5 \pm 9.7 \mu\text{m}$. Microcolonies of strains 18A ($n=6$) and MC1A ($n=5$) were shorter and less variable, at $21.7 \pm 0.8 \mu\text{m}$ and $13.0 \pm 1.41 \mu\text{m}$, respectively. In the experiments reported here, strain PAO1 grew rapidly, covering the flow cell and forming flat biofilms. Microcolony formation as described in previously published studies was not observed.

Strain 3A formed the largest microcolonies of the CF strains studied here. This strain's characteristic 'doughnut' appearance (central hollowing) was observed at day 4 (Figs 1a and 2b). By days 5–6, highly active motile bacteria filled the central area (Fig. 3a, arrow). The size of the voids

increased with further biofilm development and motile cells were seen breaking out from the outer microcolony structure (Fig. 3b, c, arrows). These observations mirror those of seeding dispersal described for strain PAO1 (Purevdorj-Gage *et al.*, 2005). In contrast, strain 32A had a characteristic encapsulated appearance (large, acellular, amorphous layer surrounding the microcolony) and essentially maintained a dense cellular interior (wall and central cells) over the period of the experiment (Figs 1c, 2e and 3d). Central motile cells were, however, occasionally observed even in the larger microcolonies of this strain. Strain 18A formed numerous, compact microcolonies over the glass surface (Figs 1b and 2c). Generally, these were of smaller size than the microcolonies of strains 3A and 32A. Strain 75A grew as a multilayered, flat biofilm by day 4 from which occasional tall ($62.0 \pm 13.1 \mu\text{m}$) microcolonies emerged from day 5 (Fig. 2a, d). Strain MC1A also initially grew as a flat biofilm that subsequently formed numerous small microcolonies from day 5 onwards (Figs 1d and 2f). Central voids were not apparent by bright-field microscopy for strains 18A, 75A and MC1A at day 5 (e.g. Fig. 1b, d).

Cell death and seeding dispersal occurs in CF strains

Viability staining (*BacLight* LIVE/DEAD kit, Molecular Probes) and confocal analysis (day 7 post-inoculation)

Fig. 1. Representative microcolonies of CF *P. aeruginosa* strains (a) 3A, (b) 18A, (c) 32A and (d) MC1A cultivated in the flow-cell system at 5 days post-inoculation (bright-field microscopy). Each strain shown had its own distinctive and reproducible microcolony architecture (Table 1).

Fig. 2. Confocal photomicrographs of CF *P. aeruginosa* strain microcolonies and biofilm formation following viability staining (BacLight LIVE/DEAD kit, Molecular Probes) at 7 days post-inoculation. Strains shown are: (a) 75A (microcolony), (b) 3A, (c) 18A, (d) 75A (flat biofilm), (e) 32A and (f) MC1A. Images are *x-y* plane views through the microcolonies approximately one-third from the base. Green fluorescent cells are viable whereas red fluorescent cells are dead. Arrows indicate central cell death in a microcolony of strain 75A, central and peripheral cell death and hollowing in a microcolony of strain 3A, and seeding dispersal associated with central cell death in microcolonies of strain 18A.

revealed cell death in the maturing biofilms and microcolonies of all strains and evidence of seeding dispersal development (Fig. 2). The spatial organization and degree of bacterial death and lysis varied between strains but was always most marked at the base of the biofilm or microcolony and least apparent at the top of the microcolony. For strain 75A, extensive killing was seen in the centres of its relatively rare microcolonies and appeared identical to that reported for strain PAO1 microcolonies (Fig. 2a, arrow) (Webb *et al.*, 2003). Large patches of cell death (greatest at base) were also observed even in the flat, undifferentiated portions of the biofilm for this strain (Fig. 2d). A similar pattern of lysis was observed in the flat PAO1 biofilms seen in these experiments (not shown). For strains 18A and MC1A, central cell death was also observed within microcolonies. At 7 days this death was more pronounced in strain 18A than in strain MC1A, but central voids were occasionally seen in the larger microcolonies of both strains. Seeding dispersal in strain 18A is evident in Fig. 2(c) (arrows). Strain 3A showed cell death not only from the mature microcolony base upward but also from the microcolony edges inwards as well as central cell death and hollowing (Figs 2b and 3c, arrows). A subpopulation of viable cells was also seen in the central hollow space of the microcolony. Seeding dispersal in association with the cell death and central hollowing was frequently observed for this strain (Fig. 3b, c, arrows). In contrast, the degree of cell death in strain 32A microcolonies was less than that seen in all of the above strains and,

although centralized cell death was observed, hollowing and seeding dispersal were rarely observed for this strain in the 8 day experimental period (Fig. 3d).

Phage titre in the effluent of mature CF strain biofilms

Biofilm effluents were examined for viable bacteria (c.f.u.) and bacteriophage (p.f.u.) at days 4, 5, 6 and 7 post-inoculation (one to three experiments). Phage titres were quantified by titration of effluents using a top-layer agar method (usually with strain PAO1 as target). Effluents of strains 3A, 18A and 32A, also examined on CF strain 3A as target, yielded essentially identical phage titres to those obtained on strain PAO1. Between days 4 and 7 phage titres were detected in the effluents from all CF strains as for strain PAO1. For strain PAO1, they exceeded 10^9 p.f.u. ml⁻¹ throughout (days 4–7). Phage titres of the CF strains were $>10^6$ p.f.u. ml⁻¹ at days 4 and 5 post-inoculation and reached levels $>10^9$ at day 7, with the exception of strain 32A. For this latter strain, titres were more variable, but they were reproducibly 2–5 logs lower (e.g. $<10^5$ p.f.u.) on day 7.

Variant morphotypes in biofilm dispersal effluents

Effluents from strain PAO1 and all CF strain biofilms contained $>10^7$ c.f.u. ml⁻¹ (days 5–7 post-inoculation). In

Fig. 3. Comparison of cell death and dispersal for representative 'mature' microcolonies of strains 3A and 32A at 7 days post-inoculation. Strain 3A (seeding dispersal 'positive'): (a) mature microcolonies showing the highly motile central cellular interior (arrow), central hollowing and the formation of 'doughnut' microcolonies, (b) seeding dispersal (arrows) from mature microcolonies (bright-field microscopy), and (c) seeding dispersal (arrows) (viability staining and confocal microscopy, x - y plane view, approximately one-third from the base of the microcolony). Strain 32A (seeding dispersal 'negative'): (d) no evident hollowing or seeding dispersal from the microcolony despite some central cell death (viability staining and confocal microscopy, x - z plane composite, 2 μ m sections).

contrast to the homogeneous colony morphologies of each of the inoculum strains, at least two colony morphotypes were grown from the biofilm effluents of all strains (Fig. 4). For *P. aeruginosa* strain PAO1 these were large (>4 mm diameter) colonies, sometimes with an irregular edge, like the inoculum strain, and small-colony (2 mm diameter) variants (SCVs), as previously reported (Webb *et al.*, 2004). The CF strains also showed these two morphotypes in the biofilm effluents. However, the situation was the reverse of that seen with strain PAO1. Despite all strains being mucoid on initial isolation, only strains 3A and 18A retained their mucoidy on subculture, and the other CF biofilm inoculum strains (strains 32A, 75A and MC1A) grew predominantly as small colonies with a translucent edges and yellow centres (Fig. 4b, c, d, e, f, insets). These latter CF inoculum strains gave rise to differing proportions of large-colony variants (LCVs), as well as SCVs. LCVs dominated the effluents of strains 75A and MC1A, but were less common in the effluents from strain 32A (Fig. 4d, e, f). Strains 3A and 18A showed a greater

heterogeneity of morphotypic variants, with up to five morphotypes isolated (Table 1; Fig. 4b, c). These strains were also the ones where significant seeding dispersal had been observed, as described above. On subculture on LB₁₀ agar, these biofilm-generated variants were stable and did not revert to the parental morphotypes or convert into other morphotypes over at least two passages (data not shown).

Seeding dispersal and QS signals

CF strains 3A and 18A, which consistently showed seeding dispersal and had the greatest range of morphotypic variants, produced AHLs [3-oxo- C_{12} -homoserine lactone (3-oxo- C_{12} -HSL) and C_4 -homoserine lactone (C_4 -HSL)], and HAQs [*Pseudomonas* quinolone signal (PQS) and its biosynthetic precursor 4-hydroxy-2-heptylquinoline (HHQ)] signalling molecules. Strains 32A, 75A and MC1A, which showed less cell death and morphotypic variation, did not produce 3-oxo- C_{12} -HSL. Strain MC1A

Fig. 4. Morphotypic colony variants seen in the flow-cell biofilm effluents at days 5–7 post-inoculation (Table 1). (a) *P. aeruginosa* strain PAO1, (b–f) CF inoculum strains 3A, 18A, 32A, 75A and MC1A, respectively. Insets show the essentially uniform morphologies of the initial corresponding wild-type inoculum strains. (The wild-type CF strains 3A and 18A retained mucoidy while strains 32A, 75A and MC1A, mucoid on initial isolation, grew as small colonies with a translucent edge and yellow centre.) Bar, ~5 mm.

was in addition C₄-HSL negative, while strain 32A, which showed the lowest phage titres and least cell death, was also negative for PQS. Virulence factor production also correlated with this signalling pattern. Thus strains 3A and 18A remained mucoid, produced pyocyanin and were weakly positive for LasA and LasB activities, whereas the other three strains had lost these virulence capacities.

DISCUSSION

CF strain biofilm development parallels that of strain PAO1

There is little published information about the biofilm biology of clinical *P. aeruginosa* strains and how it compares to that of the more commonly studied laboratory model strain PAO1. In this study, isolates from chronically infected CF individuals were grown in a continuous-culture flow-cell model under conditions (e.g. M9 minimal

medium, standardized inocula) used to investigate seeding dispersal in strain PAO1 (Webb *et al.*, 2003, 2004). The flow-cell system allowed for the slower developmental sequence of CF strain biofilms to be followed (8 day experimental period). It was found that the clinical isolates, despite their differences in adhesion and biofilm growth kinetics, and quite distinct microcolony morphologies, exhibited biofilm differentiation events analogous to those described for strain PAO1 (Webb *et al.*, 2003). In particular, it was established that CF strains showed seeding dispersal as the biofilms matured. This active dispersal mechanism, not previously described in clinical strains, also showed a temporal association with the production of lytic bacteriophage and the generation of morphotypic variant dispersal cells as described for strain PAO1. The reproducibility of the differences seen with the CF strains suggests that each of these strains becomes locked into its own pattern of development. Overall, the results confirm that insights gained from studies of strain PAO1 biofilm differentiation and dispersal have clinical

relevance. A noteworthy difference, however, was that morphotypic variants recovered from those CF strain biofilms showing marked seeding dispersal were much more heterogeneous than the variants recovered from strain PAO1.

Role of microcolony size, phage induction and cell signalling in seeding dispersal

The observation that seeding dispersal was most commonly seen in CF strains that produced large microcolonies (e.g. strain 3A, with mature microcolony diameters of >100 µm) is consistent with that of Purevdorj-Gage *et al.* (2005), who reported that a microcolony diameter of ~80 µm was required for seeding dispersal in strain PAO1. Microcolonies of the other CF strains studied in this investigation rarely reached this size. In those microcolonies that did reach this size, central motile cells and seeding dispersal were observed. Cell death was, however, seen in the biofilms/microcolonies of all strains as they matured. Although strain PAO1 grew as a flat biofilm in this series of experiments, cell death associated with phage activity and dispersal variant production were nevertheless observed (in patches in the biofilm not in discrete microcolonies). Interestingly, CF strain 75A also formed very flat biofilms similar to strain PAO1, although they were slower to develop. It also rarely formed microcolonies in these experiments. However, the microcolonies that were occasionally seen with this strain had an identical appearance and pattern of central cell death to those previously reported for strain PAO1 (Webb *et al.*, 2003). Biofilm structural maturation has been shown to be dependent on environmental conditions (Klausen *et al.*, 2003; Shrout *et al.*, 2006). Hence, even minor (uncontrolled) differences in conditions could alter the pattern of biofilm development, resulting in flat biofilms especially for the faster-growing, motile strain PAO1, as is not uncommonly observed (Hentzer *et al.*, 2001; Heydorn *et al.*, 2002).

All CF strains showed bacteriophage activity and the phage titres reflected the occurrence of cell death within the biofilms. For instance, strain 32A, with the least death in microcolonies, had significantly lower phage titres than the other strains. To our knowledge, phage production has not previously been documented in relation to CF strain biofilm dispersal, as here. It is, however, now well established that Pf-like filamentous prophages are common among diverse *P. aeruginosa* clinical isolates. Pf1-like prophage sequences (detected by PCR with phage-specific primers) were present in 8 of 11 CF isolates obtained from different laboratories worldwide (Webb *et al.*, 2003). Additionally, another recent study found that *P. aeruginosa* isolates from urinary tract catheters and from CF sputa contained Pf1-like phage sequences in their genomic DNA (Mooij *et al.*, 2007). The fact that phage activity from all CF strains was detected on strain PAO1 suggests that these filamentous phages have a broad host specificity in *P.*

aeruginosa. This is supported by the fact that titres of three CF strains tested on CF strain 3A were identical to the titres obtained on strain PAO1. It is also possible that isolates may contain multiple phages, any one of which could cause plaquing of strain PAO1 (Tan *et al.*, 2007).

Purevdorj-Gage *et al.* (2005) found that *las/rhl*-mediated QS was required for seeding dispersal to occur in *P. aeruginosa* PAO1 and our results also support the conclusion that these processes are linked. The loss of QS signal production is a feature of isolates from chronic infections (Hentzer *et al.*, 2005). It has recently been reported that genetic adaptation in the CF lung over time commonly results in mutations in the *lasR* gene, the elimination of which results in the loss of many acute virulence determinants (Nguyen & Singh, 2006; Smith *et al.*, 2006). Consistent with these reports, several of the clinical strains (strains 32A, 75A and MC1A) studied here were unable to produce one or more of the QS signal molecules and showed loss of acute virulence determinants. QS-negative (*lasR*) mutants of *P. aeruginosa* PAO1 are also reportedly significantly more resistant to cell lysis and death than the wild-type (Heurlier *et al.*, 2005). Thus, the loss of AHL signals could possibly affect the degree of cell death and the evacuation of cells through the microcolony wall for these strains. While there did not appear to be a correlation between QS and phage production, strain 32A, which had the lowest phage titres, was also negative for PQS. Interestingly, PQS has been linked to *P. aeruginosa* autolysis (phage activity) (D'Argenio *et al.*, 2002). Inactivation of *lasR* results in the accumulation of the PQS precursor, HHQ (Deziel *et al.*, 2004). Thus, a LasR-regulated balance of HAQs may limit *P. aeruginosa* autolysis (D'Argenio *et al.*, 2007).

Clinical strain dispersal variants show greater diversity than those of strain PAO1

Biofilm growth has been shown to lead to the emergence of colony morphotypic variants in a variety of species (Kirisits *et al.*, 2005; Koh *et al.*, 2007; Mai-Prochnow *et al.*, 2006; Webb *et al.*, 2004). SCVs produced by strain PAO1 have significant functional differences from the wild-type strain (e.g. enhanced attachment, accelerated biofilm development) (Kirisits *et al.*, 2005; Webb *et al.*, 2004). Further studies are needed to investigate the functional significance of CF strain variants and the exact mechanism(s) by which they are generated. In particular, it will be of interest to determine whether there are CF strain variants that are better adapted to colonization and/or survival (e.g. by contributing to antibiotic resistance) under conditions found in the CF airway. The *in vitro* observations to date are consistent with the high degree of phenotypic variation widely reported in *P. aeruginosa* isolates from CF sputa despite the fact that most individuals are colonized with one (or relatively few) genotypic clone(s) of the organism (Foweraker *et al.*, 2005; VanDevanter & Van Dalfsen, 2005). It seems likely that *in vivo* the generation of biofilm

dispersal variants represents a key adaptive response that enables protection against antibiotics and host defences, favours recolonization in new niches and ensures continued biofilm growth in pulmonary infection. This study identified variants by colony morphology but morphologically identical clones may show functional variation (Mai-Prochnow *et al.*, 2006). Indeed, we have preliminary evidence from studies of substrate utilization profiles (Biolog Microplates) that this is the case for the dispersal cells of some CF strains.

There are a variety of mechanisms by which variant production may be facilitated in maturing biofilms. Reversible phenotypic and functional variation (e.g. phase variation and persister cells) has been reported in *P. aeruginosa* (Deziel *et al.*, 2001; Drenkard & Ausubel, 2002; Lewis, 2005) and phase variation observed for some of the CF strains examined in this study (unpublished observations). Thus, LCVs of strain 32A were motile and expressed virulence factors (LasA, LasB and pyocyanin, as well as QS signal molecules) while SCVs of strain 32A and the 32A inoculum strain were non-motile and did not express these virulence factors. Such phenotypic switching could also be observed after serial passage and other culture manipulations of this and other strains from this study (unpublished data).

Biofilm growth also facilitates heritable diversification through genetic exchange and mutagenesis. Two recognized mechanisms that accelerate the latter are the stress-inducible genes, usually part of the SOS regulon, that generate genetic variability in times of stress such as occurs in ageing biofilms and regulator genes (e.g. *mutS* of the bacterial DNA mismatch repair system), whose functional loss increases the rate of genetic variability and gives rise to 'hypermutable/mutator' strains (Barraud *et al.*, 2006; Moyano *et al.*, 2007; Sanders *et al.*, 2006; Smania *et al.*, 2004). Adaptations will be selected for and may be linked to hypermutator mutations if they confer a selective advantage (Hall & Henderson-Begg, 2006; Montanari *et al.*, 2007). The association between multidrug antimicrobial resistance and hypermutability in chronic CF infection has been well documented (Maciá *et al.*, 2004, 2005; Oliver *et al.*, 2000, 2004). Other common genes targeted in chronic infection include the *lasR* and *mexZ* genes (Smith *et al.*, 2006). Loss of *lasR* confers a growth advantage with particular carbon and nitrogen sources as well as increased β -lactamase activity and resistance to ceftazidime, a widely used β -lactam antibiotic in CF (D'Argenio *et al.*, 2007). Loss of *mexZ* upregulates the multidrug-efflux pump, MexXY-OprM, that is associated with resistance to the aminoglycoside tobramycin, also commonly used in CF treatment (D'Argenio *et al.*, 2007). While selection of *mutS* mutations with adaptive responses such as those above may be one mechanism by which CF strains give rise to the greater diversity of biofilm dispersal progeny compared to strain PAO1, there are likely other as-yet-unrecognized genes that are also important in causing hypermutability. A recent study found that the level of *mutS* mutators in multidrug-resistant epidemic

strains (strains likely to be particularly well adapted to CF airway survival) was low (13 %, 2/15) (Kenna *et al.*, 2007).

In summary, this paper reports new insights into how biofilm differentiation and dispersal might contribute to the generation of diversity in CF *P. aeruginosa* isolates and the persistence and intractable nature of airway infections with this organism. These insights have significant implications for ongoing research and the development of improved treatment strategies for chronic *Pseudomonas* infections in CF. Early and aggressive therapy to prevent and delay colonization, conversion to mucoidy and biofilm growth, and the selection of mutator strains is clearly desirable, as others have concluded (Kenna *et al.*, 2007). This study, however, suggests that successful treatment is only likely to be accomplished if disease management also includes the targeting of the dominant dispersal variants and/or the mechanisms by which they are generated. Further research into these areas is thus clearly warranted.

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