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ORIGINAL ARTICLE

The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage

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Mature *Pseudomonas aeruginosa* biofilms undergo specific developmental events. Using a bacteriophage mutant, generated by deletion of the entire filamentous Pf4 prophage, we show that the phage is essential for several stages of the biofilm life cycle and that it significantly contributes to the virulence of *P. aeruginosa in vivo*. Here, we show for the first time that biofilms of the Pf4 phage-deficient mutant did not develop hollow centres or undergo cell death, typical of the differentiation process of wild-type (WT) *P. aeruginosa* PAO1 biofilms. Furthermore, microcolonies of the Pf4 mutant were significantly smaller in size and less stable compared with the WT biofilm. Small colony variants (SCVs) were detectable in the dispersal population of the WT biofilm at the time of dispersal and cell death, whereas no SCVs were detected in the effluent of the Pf4 mutant biofilm. This study shows that at the time when cell death occurs in biofilms of the WT, the Pf4 phage converts into a superinfective form, which correlates with the appearance of variants in the dispersal population. Unexpectedly, mice infected with the Pf4 mutant survived significantly longer than those infected with its isogenic WT strain, showing that Pf4 contributes to the virulence of *P. aeruginosa*. Hence, a filamentous prophage is a major contributor to the life cycle and adaptive behaviour of *P. aeruginosa* and offers an explanation for the prevalence of phage in this organism.

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Introduction

Bacteriophage have been suggested to be one of the most abundant biological agents on the planet, estimated at a total of 1×10^{31} (Rohwer and Edwards, 2002), compared with $2–6 \times 10^{30}$ bacterial cells (Whitman *et al.*, 1998), outnumbering prokaryotes by approximately 10:1. Given this numerical dominance, it is not surprising that phage play important roles in the biosphere despite being unable to replicate independently. For example, it has been suggested that phage are the major cause of bacterial death in the environment and thus, bacteriophage are thought to have a significant impact on nutrient cycling due to bacterial lysis (Wilhelm and Suttle, 1999). Bacteriophage also play important roles in the transfer of genetic material between bacteria and clearly represent an important factor in bacterial evolution. Furthermore, the strong selective pressure of phage-mediated lysis can drive evolution of the target population, so that infection-resistant mutants may become dominant in the population. For example, Brockhurst *et al.* (2005) have shown that phage mediate changes in the bacterial population, controlling the emergence of different variants. The selective pressure of lytic bacteriophage has also been shown to drive the

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appearance of mutator phenotypes in the host, which have the potential to escape infection (Pal *et al.*, 2007).

Selective pressures are also likely to operate on lysogenic phage, which integrate themselves into the host genome. This relationship is not neutral, where the integrated phage can represent a genetic, for example replicative, burden to the host. Thus the prophage must either confer a selective advantage on the host or it risks accumulating mutations that render it defective. For example, the pyocins of *P. aeruginosa* appear to be remnants of phage tail spike genes, and thus have been maintained because they provide a competitive advantage by killing off sensitive bacteria (Nakayama *et al.*, 2000). It has also been shown that the lambda encoded gene products, Bor and Lom respectively, are important as virulence factors for attachment or immune system evasion during infection (Tinsley *et al.*, 2006). Thus, based on their abundance and effects on the prokaryotic community, it is clear that viruses play a significant role in bacterial survival, activity and evolution.

Bacteria predominantly live in high-density communities, called biofilms, where there are likely to be significant interactions between bacteriophage and their host. This may be particularly important if bacteriophage play active roles in the biofilm life cycle or if they mediate bacterial diversity during biofilm development. For example, it has recently been shown that filamentous phage can be isolated from biofilms of *P. aeruginosa* during the dispersal phase of the biofilm life cycle and that the appearance of the phage correlated with the appearance of small colony variants (SCVs) (Webb *et al.*, 2003, 2004). The appearance of dispersal variants has been linked to increased adaptability of the dispersal population (Boles *et al.*, 2004; Mai-Prochnow *et al.*, 2004; Purevdorj-Gage *et al.*, 2005; Koh *et al.*, 2007). Small colony variants are particularly important during chronic infection of the lungs of cystic fibrosis patients, where the appearance of SCVs correlates with poor lung function and increased resistance to antibiotic therapies (Haussler *et al.*, 1999). It was further shown that the addition of purified phage to biofilms of *P. aeruginosa* resulted in the induction of cell death within microcolonies and the emergence of SCVs, linking the phage to both phenomena (Webb *et al.*, 2003, 2004). This is supported by the observation that the Pf4 genes are some of the most strongly induced genes in the biofilms of *P. aeruginosa* (Whiteley *et al.*, 2001). In our earlier attempt to make a Pf4 deletion strain, it was observed that the replicative form (RF) was retained and that infective Pf4 phage could be recovered from biofilm supernatants (Webb *et al.*, 2003). Thus, it is possible that the RF of the phage could rescue the defective chromosomal prophage. In this study, we have generated a defined Pf4 chromosomal deletion mutant of the entire Pf4 prophage genome. Deletion of the chromosomal Pf4 prophage resulted in loss of the RF of the phage, and we confirmed that the Pf4 mutant did not produce Pf4 phage. Using this mutant, we showed that the phage plays an important role in biofilm development of *P. aeruginosa* PAO1, the formation of small colony variants as well as the structural integrity of the biofilm. These effects are manifested at the time when the phage becomes capable of superinfection, and we propose that the formation of the superinfective form is essential for key biofilm stages including autolysis, microcolony maturation and stability, and dispersal as well as for the formation of morphotypic variants. Finally, we show that the phage mutant was less virulent than the wild type (WT), highlighting the overall significance of the phage in the biofilm developmental life cycle and the ability of *P. aeruginosa* to infect a host.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* strains were maintained on Luria–Bertani (LB) medium (Bertani, 1951), either in the broth or on the LB plates supplemented with 1.5% w/v agar when necessary. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ml^{-1} ampicillin, 100 μg ml^{-1} carbenicillin or 30 μg ml^{-1} gentamicin. The *P. aeruginosa* Pf4 knockout mutant (PAO1ΔPf4) was grown in LB medium supplemented with 30 μg ml^{-1} gentamicin. Cultures were incubated overnight at 37 °C at 200 r.p.m.

Construction of a Pf4 deletion vector

A genomic knockout of the entire Pf4 prophage was generated by cloning the genomic regions flanking the Pf4 prophage into the suicide vector pEX18-ApGW (Choi and Schweizer, 2005). The ends of the Pf4 prophage were amplified using two sets of primers, Pf4-UpF-GWL and Pf4-UpR-GM (Table 2), which amplify a 300 bp region from position 785011 to 785310 and primers Pf4-DnF-GM and Pf4-DnR-GWR, which amplify a 300 bp region corresponding to positions 797748–798047 of the published PAO1 genome (Stover *et al.*, 2000) (http://www.Pseudomonas.com/). Figure 1 shows a schematic representation of primer binding sites and the final deletion construction. At the same time, a 1053 bp PCR fragment was generated using primers GmF and GmR (Table 2), which amplify the gentamicin cassette plus the flanking FRT region from plasmid pPS856 as described (Hoang *et al.*, 1998). These primers have incorporated sequences that allow for the amplified fragments to be joined by splicing overlap extension PCR (SOE PCR) (Horton *et al.*, 1990). Three PCR cycles were performed without the addition of primers to join the two Pf4 fragments to the FRT–gentamicin–FRT cassette, after which

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	*Genotype and/or phenotype*	*Reference or origin*
Strains		
E. coli		
DH5-α	F$^-$ 80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1 tonA*	Wilhelm and Suttle (1999)
P. aeruginosa		
PAO1	Wild-type (WT)	Lab stock
Δ*lasR-rhlR*	PAO1, double deletion mutation of *lasR-rhlR*	Whitchurch *et al.* (2002)
PAO1ΔPf4	PAO1, Δ(Pf4): Gm^R	This study
Plasmids		
pPS856	Ap^R, Gm^R; 0.83-kb blunt-ended *Sac*I fragment from pUCGM ligated into the *Eco*RV site of pPS854	Tinsley *et al.* (2006)
pDONR221	Km^R; Gateway donor vector	Rohwer and Edwards (2002)
pEX18ApGW	Ap^R; Gateway destination vector	Rohwer and Edwards (2002)
pEX2.5	pEX18ApGW gene replacement vector carrying a Gm^R cassette flanked by a 300 bp Pf4 upstream fragment and a 300 bp Pf4 downstream fragment (*attB1*-'Pf4-FRT-Gm-FRT-Pf4'-*attB2*); ColE1 replicon; *sacB* Gm^R Ap^R Cb^R	This study

Abbreviations: *att*, attachment site; Ap^R, ampicillin resistance; Cb^R, carbenicillin resistance; FRT, Flp recombinase target; Gm^R, gentamicin resistance; Kn^R, kanamycin resistance; 'Pf4, upstream of the Pf4 phage gene cluster; Pf4', downstream of the Pf4 phage gene cluster (refer to Table 2 for details); Sm^R, streptomycin resistance.

Table 2 Primers used in this study

Primer number	*Primer*	*Sequence (5′-3′)*[a]	*Chromosomal position*	*Amplified product*
1	Pf4-UpF-GWL	tacaaaaaagcaggctTCTGGGAATACGACGGGGGC	785011	5′ of *attL*
2	Pf4-UpR-GM	tcagagcgcttttgaagctaattcgGATCCCAATGCAAAAGCCCC	785310	3′ of *attL*
3	Pf4-DnF-GM	aggaacttcaagatccccaattcgCGTCATGAGCTTGGGAAGCT	797748	5′ of *attR*
4	Pf4-DnR-GWR	tacaagaaagctgggtTGGCAGCAGACCCAGGACGC	798047	3′ of *attR*
5	GmF	CGAATTAGCTTCAAAAGCGCTCTGA		Gentamicin cassette with FRT sites
6	GmR	CGAATTGGGGATCTTGAAGTTCCT		From pPS856
7	GWL-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT		
8	GWR-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT		
9	RF-F	AGCAGCGCGATGAAGCAAT	797111–797129	3′ of *attL*
10	RF-R	TAGAGGCCATTTGTGACTGGA	785538–785518	5′ of *attR*
11	Pre-Pfk-F	GTGGTTGCTTCCCTGTTCAT	784716–784735	596 bp 5′ *attL*
12	Pre-Pfk-R	ACTTCCATATCCGCGAAGTG	798259–798240	513 bp 3′ of *attR*
13	GendelF	ATCTTTCCCGGGCTTTACG	785227–785245	85 bp 5′ of *attL*
14	GendelR	GCAATTGGCAAAGTGTTCGA	786046–786027	3′ of *attL*
	M13 F	GTAAACGACGGCCAGT		
	M13 R	AAACAGCTATGACCATG		

[a]Sequences in lower case are common sequences for overlap amplification with either the *attB* primers or the Gm primers. Sequences in uppercase are gene specific.

time, primers GWL-attB1 and GWR-attB2 (Table 2) were added and the entire region was amplified. Note that the latter two primers also incorporate *att* sites at the ends of the PCR fragment. The reaction mixture consisted of 1 × *Pwo* buffer, 5% DMSO, 200 μM dNTPs and 0.05 U μl^{-1} *Pwo* polymerase (Roche, Sydney, NSW, Australia). Cycle conditions were: 94 °C hot start for 2 min, after which the polymerase was added, followed by three cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min. After the three cycles, primers GWL-attB1 and GWR-attB2 were added for the second PCR stage, which consisted of: 94 °C for 30 s, 56 °C for 30 s, 68 °C for 5 min for a total of 25 cycles followed by a final 10 min step at 68 °C. The gel purified PCR fragment was then cloned into plasmid pDONR221 using the clonase method (Invitrogen, Thornton, NSW, Australia) and subsequently transferred into pEX18ApGW as described (Choi and Schweizer, 2005) to generate plasmid pEX2.5. The construct and correct insertion into pEX18-ApGW was confirmed by sequencing from the M13 primer sites that are located outside the cloning site.

Generation of a Pf4 chromosomal deletion in P. aeruginosa PAO1

The pEX2.5 suicide vector was purified using the Wizard *Plus* Minipreps DNA purification systems

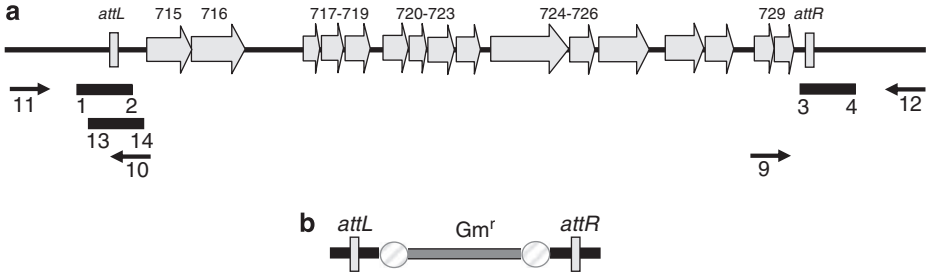

Figure 1 Construction of the Pf4 chromosomal deletion and location of primers. (**a**) The genomic organization of the Pf4 prophage in *P. aeruginosa* PAO1 is shown. The integration sites, *attL* and *attR* are shown, along with open reading frames, shown as thick arrows. Numbers above the arrows indicate gene numbers (adapted from Webb *et al.*, 2004). Solid lines indicate amplification products and numbers indicate primers, as listed in Table 2. Small arrows indicate individual primers and are also numbered with reference to Table 2. (**b**) Organization of the ΔPf4 genomic region subsequent to deletion of the prophage. Black lines indicate the amplified 3′ and 5′ regions, which are ligated to the FRT sites (hatched circles), recognized by the Flp recombinase, and the gentamicin resistance cassette (grey line, Gm^r). The figures are not drawn to scale.

(Promega Inc., Sydney, NSW, Australia) and electroporated into *P. aeruginosa* PAO1 as described (Choi and Schweizer, 2005). Six millilitres of an overnight culture grown in LB medium was harvested in four 2 ml tubes by centrifugation at 16 000 *g* for 2 min at room temperature and washed twice with 1 ml of 300 mM sucrose. Pellets from the four tubes were combined together and resuspended to a final volume of 100 µl of 300 mM sucrose. Five hundred nanograms of the pEX2.5 suicide vector DNA was mixed with 100 µl of electrocompetent cells in an electroporation cuvette. Electroporation was performed using a GenePulserXcell electroporator (Bio-Rad, Hercules, CA, USA) at 25 µF, 200 Ohm and 2.5 kV. One millilitre of LB medium was added immediately after electroporation, the cells were transferred to a 2 ml tube and incubated for 2 h at 37 °C with constant shaking at 200 r.p.m. The culture was concentrated to 100 µl and plated onto LB agar supplemented with 30 µg ml^{-1} of gentamicin (LB + Gm30) and incubated at 37 °C for 24 h. Transformant colonies were patched onto LB + Gm30 and LB + Cb200 (LB agar supplemented with 200 µg ml^{-1} of carbenicillin) plates to differentiate single-crossover mutants from double-crossover mutants. Transformants that were gentamicin resistant and carbenicillin sensitive were considered to be putative double-crossover deletion mutants.

Confirmation of the deletion strain

Colonies of putative double-crossover mutants were resuspended in 50 µl of sterile water and incubated at 100 °C for 7 min. Cell debris was removed by centrifugation at 16 000 *g* for 2 min and the supernatant was transferred to an ice-cold 1.5 ml tube. Five microlitres of the supernatant was used as the template DNA for PCR: 2 mM $MgCl_2$, 1 × AmpliTaq PCR buffer, 2.4 µM of each primer (Sigma Genosys Pty Ltd, NSW, Australia) (Table 2), 0.4 µM dNTPs and 0.02 U µl^{-1} of AmpliTaq Polymerase (Applied Biosystems, Foster City, CA, USA). PCR cycling conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C to 60 °C for 30 s depending on the primer set used (Table 2) and 72 °C for 2 min; with a final extension at 72 °C for 10 min. PCR products were visualized via gel electrophoresis on a 2% (w/v) agarose gel.

Biofilm formation and assessment

Biofilms were cultivated in flow cells (channel dimensions, 1 × 4 × 40 mm), as described by Moller *et al.* (Moller *et al.*, 1998) with some modifications. The flow cell was sterilized in 10% (v/v) bleach for 4 h, thoroughly rinsed in sterile milliQ water and connected to sterile silicon tubing (Silastic laboratory tubings). The flow cell system was flushed with sterile medium (M9 minimal salts medium with glucose: Na_2HPO_4, 47.8 mM; KH_2PO_4, 22 mM; NH_4Cl, 6.8 mM; NaCl, 18.7 mM; $CaCl_2$, 100 µM; $MgSO_4$, 2 mM or 5.6 mM glucose) for 4 h at a flow rate of 6 ml h^{-1} before the inoculation of bacterial culture. One millilitre of an overnight culture of PAO1 (WT) or PAOΔPf4 mutant was injected into the flow cell, and incubated for 1 h without flow at room temperature in an inverted position. The medium flow was resumed at a flow rate of 6 ml h^{-1}. Three independent experiments in triplicate were conducted for 7 days. Biofilms were stained with the LIVE/DEAD *Bac*Light viability probes (Molecular Probes Inc., Eugene, OR, USA) and visualized using an Olympus FV1000 confocal scanning laser microscope (Olympus Optical Co. Ltd, Tokyo, Japan) with argon-ion laser excitation at 488 nm or 594 nm and emission filters at 522–535 nm or 605–632 nm to visualize the live, green fluorescent cells and the dead, red fluorescent cells, respectively. The confocal scanning laser microscopy images were analysed using ImageJ version 1.36b (http://rsb.info.nih.gov/ij/) and statistical analysis was performed using Prism 4.03 for Windows (GraphPad Software Inc., USA).

Phage add-back to biofilms

Because the integration site for the Pf4 is removed during mutant construction, it was not possible to

perform genetic complementation. Therefore, functional complementation was performed by adding superinfective Pf4 phage to biofilms of the WT and the Pf4 mutant. Superinfective Pf4 phage were collected from the effluent of 5- to 6-day-old biofilms of the WT *P. aeruginosa*. Phage particles were concentrated by the addition of an equal volume of phage precipitation buffer (2 M NaCl, 4% polyethylene glycerol (m.w. 8000)) and incubated at 4 °C for 8 h. Precipitated phage was then concentrated via centrifugation at 15 000 *g* for 20 min. The pellet containing phage particles was resuspended in 3 ml of SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris HCl (pH 7.5)). Superinfection and phage titre were determined by quantification of plaque forming units (p.f.u.) on lawns of both the WT and Pf4 mutant. Biofilms of the WT or the ΔPf4 strains were allowed to develop in the flow cell for 3 days, after which time, the medium was changed from M9 medium to M9 medium supplemented with filter sterilized Pf4 (1×10^6 p.f.u. ml^{-1}) for 24 h. Viability of the phage treated biofilms along with the negative controls was determined by LIVE/DEAD staining. Biofilms were stained with the LIVE/DEAD BacLight viability stain (Invitrogen, USA) for 1 h before visualizing with a confocal scanning laser microscope, Olympus LSMGB200 (Olympus Optical Co., Japan); viable cells would be stained green and non-viable cells would be stained red.

Quantification of phage
Phage plaque assays were performed using a modified version of the top-layer agar method as described earlier by Webb *et al.* (Webb *et al.*, 2003). One millilitre of culture was collected and centrifuged at 16 000 *g* for 5 min and filter sterilized using a 0.2 μm filter (Acro-disk, Pall Co., West Chester, PA, USA). The cell-free supernatant was serially diluted and spotted onto an LB medium top layer containing 0.8% (w/v) agar seeded with 1×10^8 cells ml^{-1} of *P. aeruginosa* PAO1 (WT) or the PAO1ΔPf4 mutant. The number of plaques formed after 18 h of incubation at 37 °C, was determined using a Leica ZOOM 2000 dissecting microscope (Leica Microsystems, North Ryde, NSW, Australia) and p.f.u. per ml for each sample were calculated.

SDS treatment of biofilms
To assess the stability of WT and *P. aeruginosa* PAO1 and the PAO1ΔPf4 mutant biofilms to SDS treatment, 4-day-old biofilms were fed with M9 medium containing 0.01% SDS for 2 h. Confocal scanning laser microscopy images were acquired before and after the SDS treatment. As a further control, the *P. aeruginosa lasR/rhlR* quorum sensing deficient double mutant (Beatson *et al.*, 2002), earlier shown to be less stable during SDS treatment than the WT (Davies *et al.*, 1998; Hentzer *et al.*, 2003; Allesen-Holm *et al.*, 2006), was also included. The confocal scanning laser microscopy images were analysed using ImageJ version 1.36b.

Infection by the wild-type and Pf4 mutant
Survival experiments comparing WT and the Pf4 mutant were performed using the nasal aspiration mouse model of acute pneumonia (Comolli *et al.*, 1999). Briefly, bacteria were grown for 17 h in MINS medium (Nicas and Iglewski, 1984) at 37 °C with shaking, diluted 1:100 in MINS medium 3 h before infection, concentrated by centrifugation and then re-suspended in phosphate-buffered saline. Bacterial concentrations were determined by measuring optical densities and were verified by viable counts on LB plates. Using a pipette tip, approximately 7×10^7 c.f.u. of bacteria in 50 μl phosphate-buffered saline were instilled into the nares of 6- to 8-week-old BALB/c mice anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg ml^{-1}) and xylazine (20 mg ml^{-1}). For each survival experiment, five mice each were infected with mutant or WT bacteria and then monitored for viability over 7 days. Severely ill mice were sacrificed and scored as dead. Results from three separate experiments were pooled. Statistical significance was assigned by the log-rank test. All experiments were approved by the Northwestern University Animal Care and Use Committee.

Results

Isolation of a Pf4 knockout
Transformants of *P. aeruginosa* that had been electroporated with the suicide Pf4 deletion vector pEX2.5 were screened on LB plates for gentamicin resistance and carbenicillin sensitivity to identify putative double-crossover mutants. One clone was identified and selected for further verification by colony PCR. Primers Pre-Pfk-F and Pre-Pfk-R bind to positions 784716–784735 and 798259–798240 (Table 2), which lie outside of the Pf4 genomic region and are not expected to generate a product from either a WT or a single-crossover, but would generate a 2.2 kb product in a double-crossover mutant (also see Figure 1). Primers GendelF and GendelR bind to positions 785227–785245 and 786046–786027 (Table 2) and will only generate a PCR product in the WT or in a single-crossover but not in the double-crossover mutant. One set of primers, GWL-attB1 and Pre-Pfk-R, was chosen that would amplify an internal fragment of the suicide vector in the event of a single-crossover insertion. Lastly, primers RF-F and RF-R (Table 2) were designed to specifically amplify an 839 bp region of the extra chromosomal RF of the Pf4 filamentous phage. Analysis of the PCR products from the WT as a control and the putative Pf4 mutant showed the expected banding patterns for the WT and a double-crossover, respectively (data not shown). Interestingly, although the RF of the Pf4 filamentous phage,

which is an extragenic, circular copy of the phage genome, could be detected in the WT, no product was observed in the putative mutant, suggesting that the putative mutant had been spontaneously cured of the RF (data not shown). To confirm that the pEX2.5 suicide vector had not integrated into the PAO1 genome and to confirm a double-crossover knockout insertion, the PCR fragment generated using primers Pre-Pfk-F and Pre-Pfk-R was purified. Sequence analysis identified the expected flanking sequences disrupted by the gentamicin cassette (data not shown) and no vector sequences were detected, indicating that the clone was a true Pf4 deletion mutant.

The Pf4 mutant does not produce infective phage

Overnight cultures of both *P. aeruginosa* WT and the Pf4 mutant were grown in LB at 37 °C. Ten microlitres of the cell-free supernatants were then spotted onto soft-agar lawns of either the PAO1 WT or the mutant to detect plaque formation, indicative of phage infection. Supernatant from the WT produced a zone of clearing on the Pf4 mutant lawn, but not when plated onto the WT lawn, suggesting that the WT supernatant contains active Pf4 phage (Figure 2). This shows that the WT is resistant to re-infection by the Pf4 phage. In contrast, supernatant from the mutant did not produce clearing zones on either lawn (Figure 2). Hence the mutant, designated *P. aeruginosa* PAO1ΔPf4, was confirmed to be deficient for the presence of Pf4 sequences and phage.

Maturation, cell death and fitness of ΔPf4 mutant biofilms

Biofilm development by the ΔPf4 mutant was compared with the developmental process of the parental WT in flow cell biofilms over a 7-day period. By day 3, biofilms of both the WT and the ΔPf4 mutant had begun to form, where the WT had formed microcolonies with a diameter of $41.75 \pm 2\,\mu m$ whereas microcolonies of the mutant were $17 \pm 1.7\,\mu m$ in diameter (Figures 3a and b). By day 5 (Figures 3c and d), microcolonies of the WT had expanded to $74.5 \pm 4.6\,\mu m$ whereas the ΔPf4 mutant formed microcolonies that were $51.9 \pm 2.7\,\mu m$. The most striking observation was that on day 5 the microcolonies of the WT had begun to show areas of hollowing (Figure 3c), and some microcolonies of the WT had regions of dead cells in their centres (data not shown) as has been commonly reported for PAO1 (Webb *et al.*, 2003, 2004). In contrast, the ΔPf4 mutant biofilm did not undergo cell death or hollowing of the microcolonies (Figure 3d). By day 7, microcolonies of the WT biofilm had expanded to an average size of

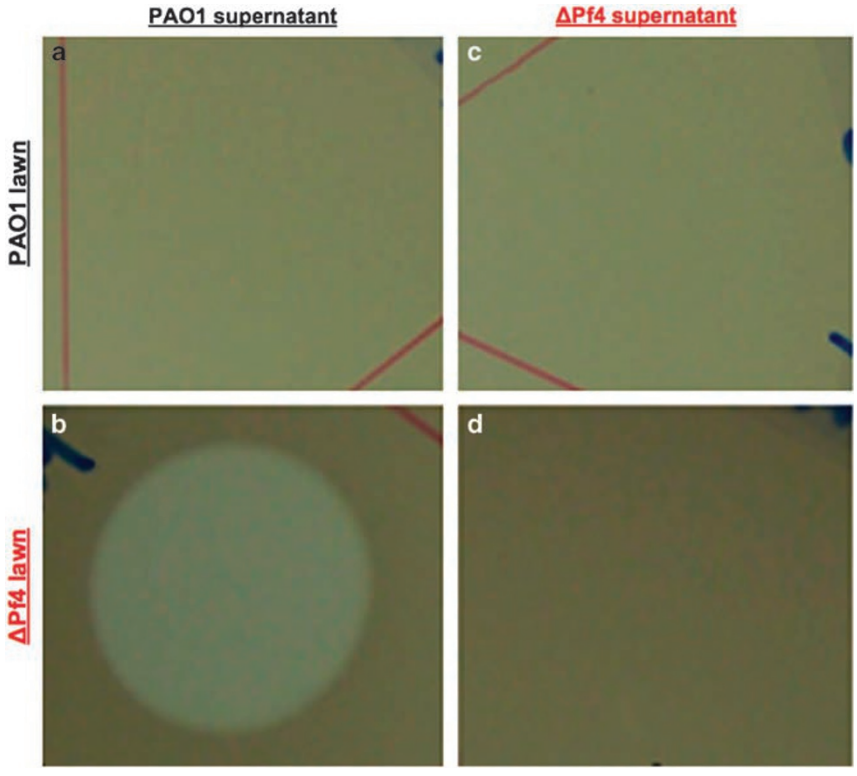

Figure 2 Detection of Pf4 phage in the supernatant of overnight cultures. Supernatants from overnight cultures of the *P. aeruginosa* PAO1 were spotted onto soft-agar lawns of the wild-type (WT) PAO1 (**a**), and the Pf4 mutant (**b**) and supernatants from overnight cultures of the Pf4 mutant were spotted onto either lawns of the WT PAO1 (**c**) or the ΔPf4 mutant (**d**).

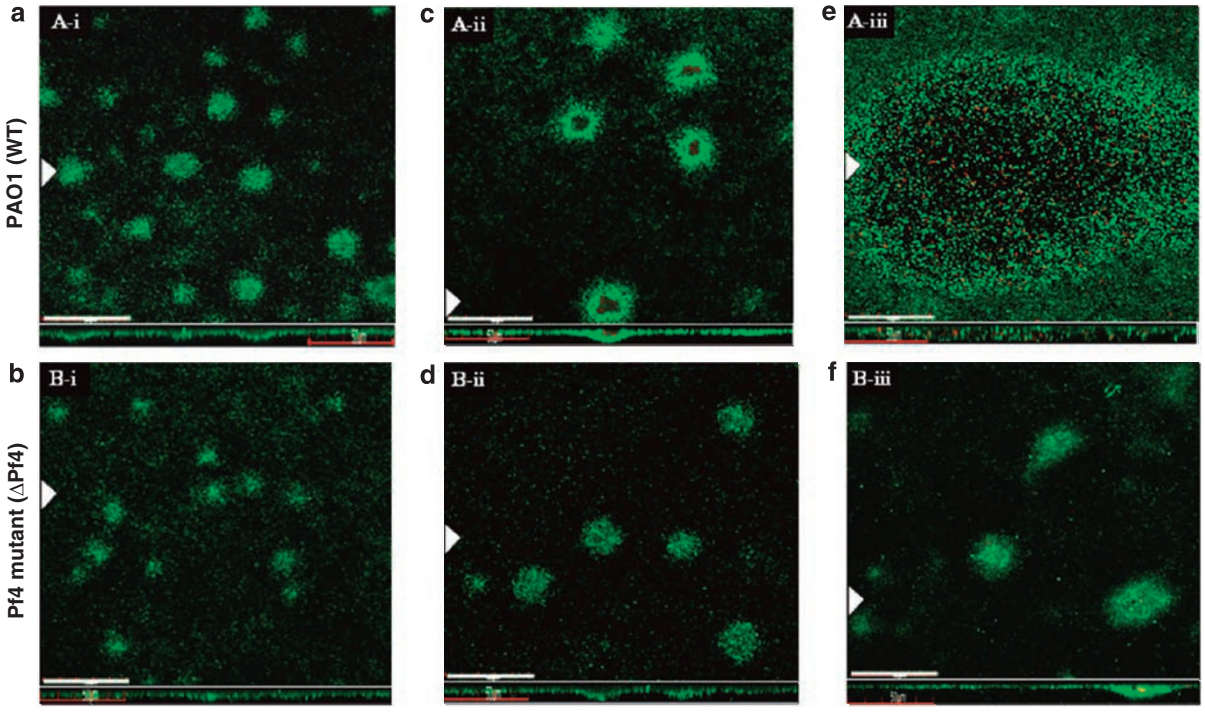

Figure 3 Biofilm formation by WT *P. aeruginosa* PAO1 and its isogenic ΔPf4 mutant. Confocal microscope images of *Bac*Light, LIVE/DEAD (Molecular Probes, Eugene, OR, USA) stained biofilms were collected on days 3, 5 and 7 for the WT and the ΔPf4 mutant. Magnification, × 400; Bars, 50 μm. Upper panels represent X–Y scans, whilst the bottom segments, separated by a line, show the biofilm in the X–Z plane.

478 ± 18 μm in diameter (Figure 3e). These large microcolonies were not entirely empty, where a number of viable cells could be observed in the centres along with dead cells (Figure 3e), whereas the mutant microcolonies were not significantly larger, 52.2 ± 5.5 μm, than those observed on day 5 (Figure 3f) and no centralized cell death or hollowing was observed. The growth rate and final yield of the WT and the Pf4 mutant were identical when grown planktonically in the biofilm medium (M9 + glucose) at room temperature indicating that deletion of the Pf4 region did not alter growth under the conditions tested (data not shown) and therefore, it is unlikely that the differences in microcolony size is the result of growth defects. Because the phage integration site was deleted in the generation of the Pf4 mutant it was not possible to complement the Pf4 mutant. Therefore, we opted to exogenously add back the phage to confirm its effects on the biofilm. Re-infection of the Pf4 mutant biofilm with purified, superinfective phage restored biofilm killing (data not shown), showing that the lack of cell death in the Pf4 mutant biofilm is not due to resistance to Pf4 infection but rather can be ascribed to the absence of Pf4 activity in the mutant. These data therefore suggest that the Pf4 phage plays an essential role in the formation of hollow colonies and the process of cell death within the microcolonies. Moreover, the data suggest that microcolony expansion in the WT requires the presence of the phage for the microcolonies to expand beyond 50 μm in diameter. Because the ΔPf4 mutant biofilm was composed of smaller microcolonies that did not appear to undergo cell death, we compared the stability of the WT and the mutant biofilms. As shown in Figure 4, the ΔPf4 mutant biofilm was less resistant than the WT strain when treated for 2 h with 0.01% SDS. Analysis of the percentage coverage of the biofilms remaining after treatment revealed that the WT biofilm was unaffected by treatment (106% remained) whereas the ΔPf4 mutant biofilm was reduced by 61%, which was similar to the control strain ΔLasR/RhlR (59% reduction). The ΔLasR/RhlR strain was included here as a positive control as it was shown earlier that QS-deficient mutants were sensitive to SDS treatment (Allesen-Holm *et al.*, 2006).

Appearance of phage and colony variants during biofilm development

A hallmark of the differentiation process typical of *P. aeruginosa* biofilms is the generation of phage and the simultaneous appearance of variants, such as SCVs, in the dispersal population. Biofilms were formed for the WT and the ΔPf4 mutant, and effluent was collected to determine phage titre and to identify and enumerate SCV formation to determine whether the ΔPf4 mutant was affected in the shedding of SCVs during dispersal. In these experiments, cell-free biofilm effluent was serially diluted and 10 μl drops were plated onto soft-agar lawns of

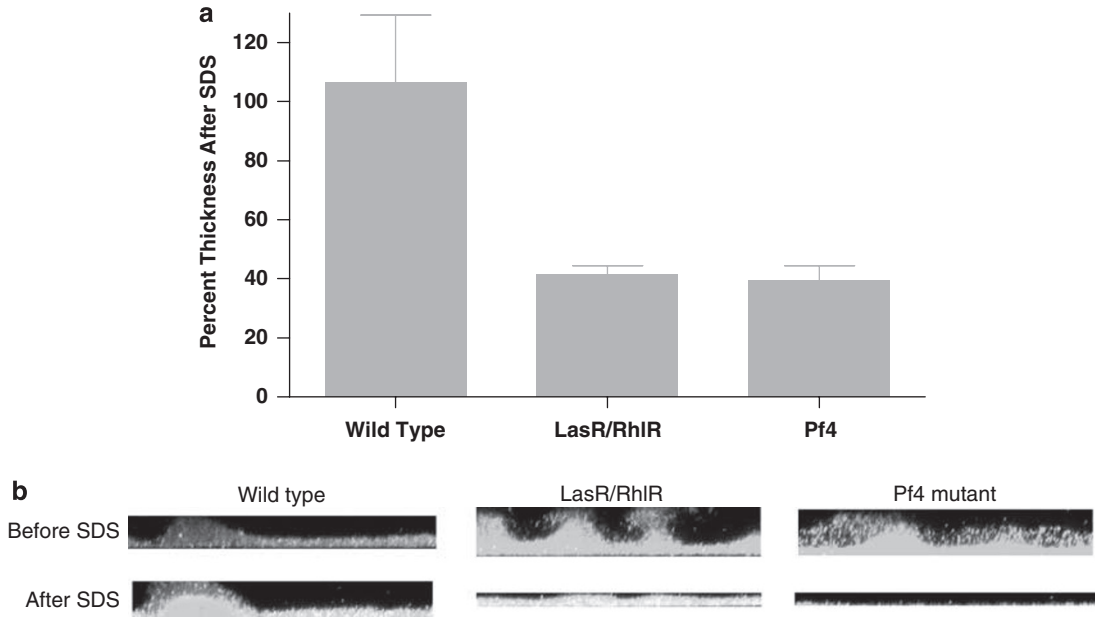

Figure 4 The Pf4 mutant biofilm is less stable than the WT. Biofilms of *P. aeruginosa* PAO1 WT, a quorum sensing mutant and the Pf4 mutant were formed in flow cells for 4 days in M9 minimal medium, at which time, they were treated with 0.01% SDS (in M9 medium) under flow conditions for 2 h at room temperature. Biofilms were stained with the *Bac*Light LIVE/DEAD staining reagents (Molecular Probes, Eugene, OR, USA) and imaged using confocal scanning laser microscopy (CSLM). Images were analysed to determine coverage in the X–Z plane (thickness) and normalized against the untreated controls, bars represent standard errors (**a**). CSLM images (X–Z plane) of the treated and untreated biofilms are shown in (**b**).

both the WT and the ΔPf4 mutant to quantify phage titre over a 5-day period. At the same time, unfiltered biofilm effluent was serially diluted and plated onto LB agar to determine the percentage of SCVs formed. As expected, when cell-free effluent from the WT was serially diluted and spotted onto the WT lawn, no plaque formation was observed for first 3 days of biofilm development and plaques were observed from day 4 onwards (Figure 5). Plaque forming units increased from 1×10^7 ml^{-1} on day 4 to 1×10^8 ml^{-1} by day 5. However, when the same effluent samples were spotted onto the ΔPf4 mutant lawns, plaques were detected from day 1 and increased from 1×10^6 ml^{-1} on day 1 to 1×10^7 ml^{-1} on day 4, after which time, the p.f.u per ml counts were not significantly different on either lawn (Figure 5). Based on detection of plaques on the Pf4 mutant lawn, it is clear that the Pf4 phage is present from the very initial phase of biofilm formation, but that it can not cause plaque formation on the WT lawn until the time at which cell death and dispersal are observed in the microcolonies (see below). The observation that the phage can only infect the WT from day 4 onwards, suggests that the Pf4 phage has converted into a superinfective form and that the WT cells are no longer protected from Pf4-mediated infection and lysis.

When cell-free biofilm effluent from the ΔPf4 mutant was spotted onto either the WT or the mutant lawn, no plaques were observed for days 1–5 (data not shown). Small colony variants were also quantified in the biofilm effluents for both strains. Small colony variants appeared in the WT

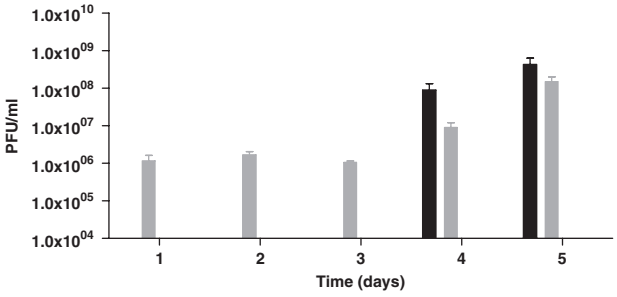

Figure 5 Phage titre of biofilm effluents from *P. aeruginosa* PAO1 and the ΔPf4 mutant biofilms from days 1 to 5. Biofilm effluent was filtered through a 0.2 μm filter to eliminate bacterial cells and serial dilutions were spotted onto soft-agar lawns of either the PAO1 WT or the Pf4 mutant to quantify the number of phage in the effluent (p.f.u. ml^{-1}). The phage titre of *P. aeruginosa* PAO1 biofilm effluent that were determined on the PAO1 lawn or the Pf4 mutant lawn are shown as black and grey bars, respectively.

biofilm at day 5, which correlated with the timing of conversion of the Pf4 into the superinfective form (Figure 5) and autolysis within the microcolonies and constituted approximately 10% of the total colony forming units (c.f.u.) observed. In contrast, no SCVs were detected in the effluent of the ΔPf4 mutant at the time when the phage converted into the superinfective form.

The Pf4 mutant is less virulent than the wild-type strain

To determine whether deletion of the Pf4 phage affected the virulence of *P. aeruginosa*, a mouse

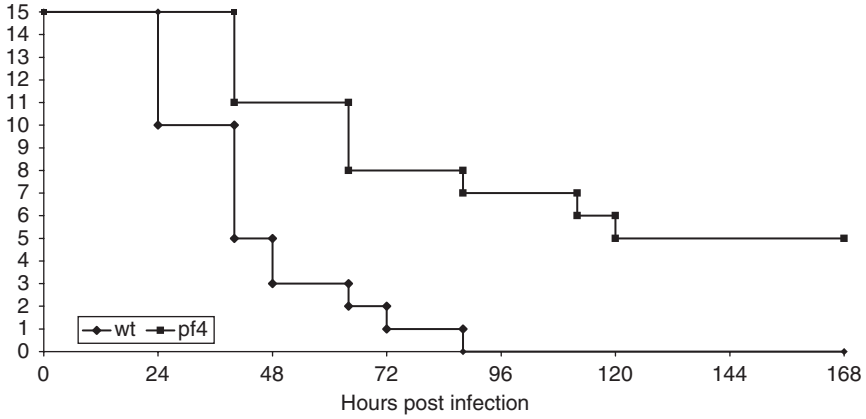

Figure 6 The Pf4 mutant is less virulent than the WT *P. aeruginosa* PAO1. BALB/c mice were infected with approximately 7×10^7 c.f.u. of either the WT PAO1 or the Pf4 mutant and were monitored over a period of 168 h to determine survival. Three independent experiments were performed and the results were pooled. Results are presented as the number of surviving mice at different time points post infection.

model of acute pneumonia was used to assess the *in vivo* virulence of the ΔPf4 mutant strain. Mice were inoculated with approximately 7×10^7 c.f.u. of bacteria by nasal aspiration and monitored for survival over the subsequent 7 days. None of the 15 mice infected with WT bacteria survived to day 7. In contrast, 5 of the 15 mice infected with the ΔPf4 mutant survived for the duration of the experiment (Figure 6). In terms of mean survival times, mice infected with WT bacteria survived 42.7 h (95% confidence interval: 32.9 h to 52.4 h), whereas mice infected with the ΔPf4 mutant survived 100.8 h (95% confidence interval: 74.0 h to 127.6 h), indicating that the ΔPf4 mutant was attenuated in its ability to cause acute pneumonia ($P<0.001$, log-rank test).

Discussion

This study shows that the filamentous phage Pf4 plays an essential role in the biofilm life cycle, mediating mutations (variant formation), and for the first time, shows that the Pf4 plays an important role in the structural integrity of the biofilm (autolysis, microcolony size and stability), and virulence. Filamentous phage have been found associated with 73% (8 of 11) of isolates from cystic fibrosis (CF) patients (Webb *et al.*, 2003) and filamentous phage activity was present in 100% (5 of 5) of CF isolates (Kirov *et al.*, 2007). Thus, the phage-mediated effects observed are likely to be conserved across *P. aeruginosa* strains.

Phage are numerically dominant compared with prokaryotes (Whitman *et al.*, 1998; Rohwer and Edwards, 2002), contribute to the control of bacterial numbers by lysis and mediate gene transfer, which impacts on bacterial evolution. It is known that phage can carry foreign genes that may expand the host's phenotypic capacity. In addition to phage-mediated gene transfer, that is transduction, bacteriophage can also affect bacterial evolution directly by influencing endogenous mutation rates, resulting in single nucleotide changes. For example, bacteriophage P1 can affect mutation rates through the expression of the *hot* gene, which can either stabilize or destabilize the proofreading subunit of DNA polymerase III (Chikova and Schaaper, 2006). More generally, Pal *et al.* (2007) reported that growth of *Pseudomonas fluorescens* in the presence of the lytic phage gave rise to mutator strains at a high frequency. Mutator strains are now recognized as making significant contribution to the infectivity of a range of human pathogenic bacteria, including *Escherichia coli*, *Haemophilus influenza*, *Helicobacter pylori*, *Neiserria meningitidis*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Bucci *et al.*, 1999) reviewed in Hall and Henderson-Begg, 2006. Both clinical and environmental isolates of *P. aeruginosa* show mutator phenotypes (Kenna *et al.*, 2007). As a result of these effects on the host genome, bacteriophage can influence bacterial phenotypes. Phage-mediated variation has significant medical implications, where it has been shown that phage can mediate conversion of *P. aeruginosa* into a mucoid phenotype, which has been associated with poor outcomes for CF patients (Miller and Rubero, 1984; Hoiby *et al.*, 2001). Brockhurst *et al.* (2005) (Webb *et al.*, 2004) have shown that the presence of bacteriophage drives the selection for smooth, phage-resistant morphotypes of *P. aerugionsa* and that the addition of phage to planktonic cultures of *P. aeruginosa* leads to the formation of colony variants.

We recently reported that CF clinical strains of *P. aeruginosa* produce superinfective phage and that biofilms of these isolates also generate SCVs as shown earlier for *P. aeruginosa* PAO1 (Webb *et al.*, 2003; Kirov *et al.*, 2007). This suggests that phage generally may play a significant role in biofilm development and the generation of phenotypic variants in clinically relevant strains. For example, Kirov *et al.* (2007) showed that high phage titres in

biofilms of clinical strains correlate with biofilm cell death and an increase in the types and overall percentage of variants (Kirov *et al.*, 2007). Like the mucoid variants, other variants, such as the SCVs are clinically relevant as they commonly show increased antibiotic resistance and biofilm formation (Haussler, 2004). Mooij *et al.* (2007) observed no correlation between the production of the Pf5 filamentous phage and SCV formation in *P. aeruginosa* PA14 (Mooij *et al.*, 2007). However, these authors did not observe superinfective phage. In contrast, we have repeatedly observed the conversion of the Pf4 into the superinfective form at the time when SCVs become detectable in the biofilm supernatant. This suggests that superinfection may be a key process in the formation of SCVs by phage activity. One key difference between the Pf5 and Pf4 bacteriophage is that the former does not appear to contain a toxin–antitoxin gene pair; it has been proposed that such addiction molecules are involved in the formation of persister cells (Lewis, 2005) and we have observed that overexpression of the *parE* gene (putative toxin) from a plasmid introduced into *P. aeruginosa* leads to an increased formation of SCVs (Lau and Kjelleberg, unpublished data). Because such variants are generally slow growing and metabolically inactive, they exhibit enhanced resistance to drug therapy. However, the phenotype is revertable and hence, they represent a population that is capable of initiating infection once the antibiotic pressure is relieved. Thus, their contribution to chronic infection is potentially very important. In conjunction with the observation that bacteriophage have been found in the sputum of patients suffering from chronic lung infections (Hoiby *et al.*, 2001), these data suggest that phage-mediated variation plays a significant role in chronic infections *in situ*.

This is the first report to show that a filamentous phage plays a role in the virulence of *P. aeruginosa*. One mechanism by which phage may contribute to virulence, is through the provision of virulence determinants. For example, virulence can be transmitted between strains of *V. cholerae* by the CTX phage that carries the genes coding for cholera toxin (CT) and the RTX toxin (Faruque *et al.*, 2003; McLeod *et al.*, 2005). Within the genome of the Pf4 bacteriophage is a pair of genes that encode a toxin–antitoxin (T–A) system (Webb *et al.*, 2004), however, such genes have not been associated with virulence or toxicity to mammalian cells and therefore it is unlikely that the T–A genes of Pf4 are directly toxic in the mouse assay used here. Furthermore, T–A genes have not been detected in the genomes of other filamentous phage in *P. aeruginosa* (Hill *et al.*, 1991; Mooij *et al.*, 2007). With the exception of the Pf4 encoded gene PAO726, no other genes were identified as having homology with known virulence factors (unpublished observation). PA0726, which is conserved in Pf1 (Webb *et al.*, 2004) and Pf5 (Mooij *et al.*, 2007), shares significant homology to the zonula occludens toxin (Zot) encoded by the filamentous CTX phage of *V. cholerae* (Fasano *et al.*, 1991). Zot disrupts the intercellular tight junctions between cells in the small intestine and has been suggested to synergize the effects of CT in causing diarrhoea (Fasano *et al.*, 1991; Di Pierro *et al.*, 2001). However, *zot* mutants of *V. cholerae* were not affected in a pulmonary infection model and Zot was shown to disrupt tight junctions in the small intestine, but not in the colon (Di Pierro *et al.*, 2001; Fullner *et al.*, 2002). Hence, Zot toxin activity appears to be tissue specific and suggests that the Pf4 encoded *zot* homologue is unlikely to act as a virulence factor here. It has been suggested that Zot has an additional function, where the C-terminal region harbours the toxin activity and the N-terminal domain plays an essential role in phage assembly (Di Pierro *et al.*, 2001).

It has been estimated that 60% of all infections are biofilm related (Costerton *et al.*, 1999; Davies, 2003), and there is substantial evidence that biofilms play an important role in the persistent infections of CF lungs by *P. aeruginosa*. Biofilm formation contributes to infection by protecting the bacteria from the host immune defence as well as mediating antibiotic resistance. Some of the reduced susceptibility of biofilm cells has been attributed to the exopolysaccharide matrix, which may act as a physical barrier to protect the encased cells (reviewed in Hall-Stoodley *et al.*, 2004). Therefore, it is possible that the reduced virulence of the ΔPf4 mutant may be linked to the effects on biofilm formation or maintenance. Very clearly, biofilms of the ΔPf4 mutant develop abnormally compared with the WT, where it fails to undergo the process of autolysis and makes smaller microcolonies. Centralized cell death has been linked to the production of variants, which as noted above, are important in the infection process. Furthermore, this process of cell death, dispersal and variant formation has been observed in many other strains of *P. aeruginosa*, arguing that this is a stage of development that is conserved amongst *P. aeruginosa* strains generally (Kirov *et al.*, 2007).

Alternatively, it is possible that the ΔPf4 biofilms are less stable than the WT. Indeed, we have shown that biofilms of the ΔPf4 mutant were less stable than the WT when challenged with surfactant stress (Figure 4). The ΔPf4 mutant was as sensitive to SDS as a quorum sensing *lasR-rhlR* mutant. It has been shown that QS controls the secretion of extracellular DNA, and that extracellular DNA makes a significant contribution to the EPS of *P. aeruginosa* as well as contributing to the matrix of biofilms (Whitchurch *et al.*, 2002; Bockelmann *et al.*, 2007). Biofilms of QS mutants are more susceptible to disruption by surfactants than the WT strain (Davies *et al.*, 1998; Hentzer *et al.*, 2003). It has been proposed that biofilm stability is related to the QS-controlled secretion of extracellular DNA into the biofilm matrix (Allesen-Holm *et al.*, 2006). Thus, it is

possible that the Pf4 phage, either as free DNA released into the biofilm matrix, or as viral particles, also enhance biofilm stability. Webb *et al.* (2004) observed that SCVs, which were isolated from biofilms at the time when superinfective phage could be detected, have high densities of a surface-associated material that reacts specifically with Pf4 directed antibodies. These surface-associated phage may serve to cross-link cells within the biofilm.

We submit that bacteriophage make a significant contribution to the biofilm life cycle and the virulence of *P. aeruginosa*. These effects may be mediated by multiple mechanisms, where the prophage encodes potential virulence factors, phage may contribute to microbial evolution through the formation of phenotypic variants, for example SCVs, and phage particles may physically enhance the stability of biofilms. These data show for the first time that the bacteriophage Pf4 plays a key role in structural features of the biofilm and in particular, this study is the first to clearly link phage production with centralized cell death and subsequent hollow colony formation. Furthermore, this is the first demonstration that a prophage in *P. aeruginosa* contributes to infection. Bacteriophage are widely disseminated and numerically dominant in the biosphere. Moreover, they display rapid evolution and the capacity to carry out introduction of new genes into their hosts. Therefore, it is highly likely that phage may play similar roles in mediating ecological adaptation and virulence in other bacterial hosts and thus play significant roles in the evolution of bacterial species.

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