Revised manuscript

Lytic activity by temperate phages of *Pseudomonas aeruginosa* in long-term cystic fibrosis chronic lung infections

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Running title: Phage dynamics in chronic infection

Abstract

Pseudomonas aeruginosa is the most common bacterial pathogen infecting the lungs of cystic fibrosis (CF) patients. The transmissible Liverpool Epidemic Strain (LES) harbours multiple inducible prophages (LESφ2; LESφ3; LESφ4; LESφ5; and LESφ6), some of which are known to confer a competitive advantage in an *in vivo* rat model of chronic lung infection. We used quantitative PCR (Q-PCR) to measure the density and dynamics of all five LES phages in the sputa of 10 LES-infected CF patients over a period of two years. In all patients, the densities of free LES-phages were positively correlated with the densities of *P. aeruginosa*, and total free phage densities consistently exceeded bacterial host densities 10 - 100 fold. Further, we observed a negative correlation between the phage-to-bacterium ratio and bacterial density, suggesting a role for lysis by temperate phages in regulation of the bacterial population densities. In 9/10 patients, LESφ2 and LESφ4 were the most abundant free phages, which reflects the differential *in vitro* induction properties of the phages. These data indicate that temperate phages of *P. aeruginosa* retain lytic activity after prolonged periods of chronic infection in the CF lung, and suggest that temperate phage lysis may contribute to regulation of *P. aeruginosa* density *in vivo*.

Key Words: Bacteriophage / Cystic fibrosis / *Pseudomonas aeruginosa* / Q-PCR

Subject Category: Microbial population and community ecology

Original Article

Introduction

Cystic Fibrosis (CF) patients are subject to life-long chronic respiratory infections, most commonly with the bacterium *Pseudomonas aeruginosa*. Periodic exacerbation of symptoms occurs throughout the lifetime of CF patients leading to progressive deterioration of lung function. Phage particles have been detected in the sputa of CF patients (Fothergill et al 2011, Ojeniyi et al 1991). Metagenomic analysis of CF sputa has identified >450 viral genotypes, while most viruses were unknown, of those that could be identified viruses the majority were infective against CF pathogens, including many *Pseudomonas* phages (Lim et al 2014). More generally, phages, outnumber eukaryotic viruses both in abundance and diversity in the human virome (Reyes et al 2012), and are known to be present at various body sites including the gastrointestinal (Breitbart et al 2003, Kim et al 2011) and the respiratory tracts (Willner et al 2009). However, the *in vivo* ecological dynamics of temperate bacteriophages and their role during bacterial infections remain largely unknown.

Upon infection of a bacterial cell, a temperate phage can either complete the lytic cycle or integrate into the bacterial chromosome as a prophage, which may subsequently be induced to enter the lytic cycle by a range of bacterial or environmental cues (Ghosh et al 2009, Little 2005). Because lysis is obviously detrimental to the individual host bacterium it is often assumed that integrated prophages will eventually lose their lytic activity, becoming cryptic. While the selective forces and mechanisms driving this remain poorly understood, inactive prophage remnants have been detected in many bacterial species, and are thought to result from an ongoing process of phage decay (Brussow et al 2004). This could be due to the accumulation of mutations for example, to inactivate phage N-anti-terminator genes (Desiere et al 2001) and portal protein genes (Lawrence et al 2001), preventing completion of the replicative cycle. By contrast, lytic

activity may be retained if it enhances bacterial population fitness for example by acting as an anti-competitor strategy or through the release of virulence-related toxins upon lysis (Brown et al 2006, Brussow et al 2004, Willner et al 2009). Over longer evolutionary timescales, the domestication of prophages is thought to be an important process in the evolution of bacteria, leading to the origin of a number of phage-derived traits (e.g., bacteriocins, killer particles etc.;(Bobay et al 2014))

The *P. aeruginosa* Liverpool Epidemic Strain (LES) exhibits increased antibiotic resistance levels compared to other *P. aeruginosa* isolates from CF patients (Ashish et al 2012) and is widespread across the UK (Martin et al 2013). Patients infected with this strain have been shown to suffer greater morbidity than those infected with other strains (Al-Aloul et al 2004, Fothergill et al 2012). The *P. aeruginosa* LESB58 genome contains 5 inducible prophages and transposon mutagenesis of this isolate identified several mutations in prophages encoding LESφ2, LESφ3 and LESφ5 that reduced bacterial competitiveness in a rat model of chronic lung infection (Winstanley et al 2009), suggesting that the phages play a key role in the infection process. We have previously characterised the infection properties of several LES phages *in vitro*. Induction experiments demonstrated that free LESφ2 was produced more rapidly and in higher numbers than LESφ3 and LESφ4 in response to norfloxacin. Each phage was shown to exhibit a different immunity profile and was able to infect a range of susceptible *P. aeruginosa* hosts via the type IV pili (James et al 2012). Due to a lack of suitable acceptor strains, we have thus far been unable to isolate and purify LESφ5 and LESφ6.

In this study, we used culture-independent quantitative PCR (Q-PCR) to follow the ecological dynamics of all five active LES phage populations in 188 expectorated sputum samples from ten long-term LES-infected patients over a period of 28 months. To our

knowledge this represents the first longitudinal study of a bacterial pathogen and its temperate phages in a human chronic infection.

Materials and Methods

Patients and Samples

188 sputum samples were collected from 10 LES-infected CF patients, for diagnostic purposes, over a period of more than two years (January 2009 to May 2011). The details of each patient and the sampling rationale have been described previously (Fothergill et al 2010, Mowat et al 2011a). All patients had long-term LES infections (duration at beginning of the study ranged from >5-to- >10 years). Table 1 and Figure S1 describe the number and dates of acquired samples from each patient that were analysed for density of LES bacteria and LES phages. 98 samples were collected during routine visits when each patient was well (stable) and 90 samples were collected during periods of acute exacerbated symptoms of respiratory infection (acute). Sputa obtained during exacerbation periods included samples taken before and during aggressive intravenous antibiotic treatment (Table 1, Figure S1). The criteria for diagnosing exacerbations were physician-based and have been described previously (Mowat et al 2011a). Briefly, patients were considered to be undergoing an exacerbation if they showed signs of reduced lung capacity, increased sputum production and discoloration, increased temperature, cough, dyspnea and malaise (Goss and Burns 2007). Where known, antibiotics administered to patients during the study period are listed in Table 1. However, detailed information of the antibiotics administered during each exacerbation is very incomplete. Thus we were unable to fully assess the effect of different antibiotics on phage induction *in vivo*. This study was approved by the local research ethics committee (REC reference 08/H1006/47).

Detection of viable phage particles by plaque assay

We were unable to accurately determine phage densities by culture-dependent techniques for several reasons: (a) we lacked acceptor strains for LESφ5 and LESφ6; (b) culturable phages were indistinguishable by plaque morphology; and (c) sputum samples were routinely frozen upon collection which reduced phage viability. However, to confirm the presence of viable phage particles in sputum samples, we quantified the density of culturable phages in ten sputum samples from 3 LES-infected patients (CF3, CF4 and CF7). Sputum samples (50 μl) were treated with sputasol (200 μl) and incubated at 37 ºC for 1 h, with shaking at 200 r.p.m. Treated samples were diluted with sterile phosphate buffered saline. A rifampicin-resistant mutant (PAO1-rif) was created by successive passage in increasing rifampicin concentrations (method described by(James et al 2001)). This enabled enumeration of active phages capable of infecting PAO1 directly from un-filtered sputum. Briefly, mid-exponential phase PAO1-rif (OD_{600} 0.5; 100μl) was added as an indicator host to treated sputum samples (400 μl). Rifampin (300 mg ml^{-1}) was incorporated in the soft agar overlay (5 ml; 0.4 % [w/v] LB agar) to select for the indicator host and incubated overnight at 37°C before the plaques were counted. This method only provides confirmation that active *P. aeruginosa* phages are present in the sputa. It does not accurately reflect abundance and does not discriminate between phages.

Real-time Q-PCR

To overcome the limitations of culture-dependent methods, we have developed and validated a simple quantitative (Q)-PCR protocol to measure the density of each individual LES phage (James et al 2012). Each sputum sample was treated with an equal volume of Sputasol (Oxoid, Basingstoke) and incubated at 37°C for 30 min, with shaking at 200 r.p.m. DNA was prepared from each treated sputum sample (400 μl) using the "Bacterial and Virus DNA extraction kit" (Qiagen, Valencia, CA, USA) and the automated QIAsymphony machine (QIAGEN; pathogen

complex 200 protocol). The protocol yielded 0.3 – 0.9 μg μl^{-1} DNA. Each sample was diluted 1:100 with sterile distilled H_2O. The number of DNA copies of each LES phage in sputum and bacterial culture samples was quantified from extracted DNA. The number of specific copies detected for each phage was compared to a concentration gradient of known standards (James et al 2012). For each LES phage (LESφ2 - LESφ6), two specific primer sets were used to quantify i) prophage and ii) total copies (10 primer sets in total). Differentiation between total phage and prophage copies, allowed free-phage densities to be calculated as the difference between these values as previously described (James et al 2012). Bacterial host density was quantified using primers specific for *P. aeruginosa* (PS21-6F1/PS21-6R1 and gyrPA-F1/gyrPA-R1) (Fothergill et al 2013). All primer sequences and targets are listed in Table S1.

Q-PCR reactions (25 μl) contained 1 μM each primer pair and 1X Rotorgene-SYBR green super-mix (Qiagen). All primer sets were used with the same cycling conditions: 95 °C for 10 min; followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. Phage DNA copy numbers were quantified from DNA samples (1 μl) in triplicate using a Rotorgene cycler (Qiagen). Q-PCR data were analyzed using Rotorgene Q series software 1.7 (Qiagen).

Sputasol induction experiments

To test for any potential induction properties of sputasol, DNA was extracted from LESB58 cultures grown to mid-exponential phase in Luria broth (LB) (James et al 2012) treated (in triplicate) with an equal volume of sputasol or LB for 30 min. DNA was prepared from each culture using a DNA mini kit (Qiagen) and phage densities estimated by Q-PCR as described for the sputum samples. No effect of sputasol on phage induction was observed (Figure S2).

Statistical analysis

To model phage densities (or phage-to-bacterium ratios), we fitted linear mixed effects models with Maximum Likelihood using the R package nlme (Pinheiro and Bates 2000) with and without temporal autocorrelated errors (an ARMA(1) model). Models with temporal autocorrelated errors were significant improvements over those without, and therefore we present only these models below. We included a random effect for patient ID and fixed effects for time, exacerbation, bacterial load and the interaction between bacterial load and exacerbation. We compared full models with and without temporally autocorrelated errors using a Likelihood Ratio Test, and then used a backwards stepwise process to remove non-significant fixed effects until the minimum adequate model was identified. Models analysing normalised variables gave similar results to those analysing non-normalised data and are presented in the supplementary information (Table S2a).

Results

Dynamics of total free-phage abundance

Plaque-assays confirmed the presence of viable particles of the subset of culturable phages in all sputum samples that were tested (range $1.25 \times 10^2 – 1.39 \times 10^4$ p. f. u. ml^{-1}). Free-LES phage DNA was detected in all patient sputa ($9.35 \times 10^4 – 5.54 \times 10^9$ copies per μl) and, in general, total free-phage density (i.e. the sum of all the free LES phages present in the sample) exceeded that of *P. aeruginosa* within each sample (mean range 11-fold to 90-fold) (Figure 1 and Table S3). We observed a positive linear relationship between total free-phage density and bacterial density (Figure 2a & Table S2b; bacterial coefficient 0.607 +- 0.054, LRT = 85.9, d.f. = 1, $p < 0.001$), consistent with on-going lytic activity by the temperate phages, but no effect of time or exacerbations on total free-phage densities. Further, we observed a negative linear relationship between the phage-to-bacterium ratio and bacterial density (Figure 2b & Table S2c

187 bacterial coefficient -3.206+- 0.484, LRT = 108.4, d.f. = 1, $p < 0.001$), suggesting a role for
188 phage lysis in regulation of bacterial densities. Time and exacerbations had no significant effect
189 on phage-to-bacterium ratios. It is perhaps surprising that exacerbations were not associated with
190 either a change in phage densities or a change in the phage-to-bacterium ratio (Figure 3), given
191 that these episodes are associated with the administration of high-dose intravenous antibiotics.
192 However, it should be noted that these patients were all subject to variable cocktails of
193 antibiotics over several years irrespective of exacerbations (Table 1). Moreover, clinical data on
194 antibiotic use in these patients was too incomplete to be used in analyses, and therefore effects
195 of particular antibiotics on phage dynamics may have been missed.
196

197 **Abundance heirarchy among individual phages within lungs**

198 Next, we considered each phage individually, observing a general hierarchy of free-phage
199 densities, though the precise patterns were clearly influenced by the fact that the LES
200 populations for each patient did not all share the same prophage complement (Table 1). Figure 4
201 illustrates the free-phage densities of individual LES phages for each of the patients. In the
202 majority of patients (CF2-CF9) similar free-phage dynamics were observed in that the density of
203 free LESφ2 was consistently higher than that of the other LES phages, closely followed by
204 LESφ4 (Figure 4). A positive correlation was observed between LESφ2 and LESφ4 densities in
205 these patients (Table S4). The dynamics observed in samples from patient CF10 (Figure 3)
206 exhibited a change in the hierarchy of free phage, with considerably higher free LESφ4 densities
207 observed. Despite consistent carriage of LES prophage 3, very little free LESφ3 was detected in
208 patients CF2 - CF10. However, higher levels of free LESφ3 (3.29×10^7 μl^{-1}) were observed in all
209 sputa from patient CF1 (Figure 4), whose *P. aeruginosa* were the only populations not to carry
210 prophage 2 (Table 1). We showed previously that LES populations exhibit genotypic diversity,

211 including variation in carriage of LES prophages. In particular, the carriage of LES prophage 5
212 was not consistent in all individuals of a given LES population (Fothergill, *et al.,* 2010). In this
213 study, prophage 5 was intermittently detectable in the sputum from patients 7 (up to 10^5 copies
214 μl^{-1}) and 10 ($10^2 – 10^4$ copies μl^{-1}). This explains the low density of free LESφ5 in these patients.
215 Free copies of LESφ6 were not detected in the majority of sputum samples. Where free copies
216 were detected, the density was lower than the host bacterial load ($6.7 \times 10^3 – 1 \times 10^7$ copies μl^{-1}).

217

218 **Discussion**

219 The levels of free LES phages detected in all patients throughout this study suggest an active
220 lytic cycle that may be promoted by the presence of H_2O_2 or DNA damaging antibiotics in the
221 CF lung (Fothergill et al 2011, McGrath et al 1999). Surprisingly, we observed no effect of
222 patient exacerbation on total free-phage density, although this is consistent with previous studies
223 showing that neither fluctuations in *P. aeruginosa* populations (Mowat et al 2011a), nor in the
224 wider bacterial population (Fodor et al 2012), show any relationship with the exacerbation period
225 in chronically infected patients, despite the use of high level intravenous antibiotic therapy. It is
226 known that particular antibiotics can induce phage lysis, and it is possible that different
227 antibiotics regimes may have influenced differential induction of phages between patients.
228 Indeed, we have shown previously that LES induction varies in response to different antibiotics
229 (Fothergill et al 2011). Unfortunately, because records of antibiotic treatments for these patients
230 were very incomplete, we were unable to explicitly test for effects of particular antibiotics in this
231 study. This would in any case be difficult because of the extensive and varied use of antibiotics in
232 this group of patients (Table 1), which was not restricted to periods of exacerbation.

233 Our data do however suggest that on-going phage lysis may play a role in regulating
234 bacterial density in the CF lung. Treatments which induced the lytic cycle of temperate phages

could therefore offer a promising alternative or addition to standard antibiotic therapies which in themselves often do not successfully reduce *P. aeruginosa* densities in long-term chronically infected patients (Foweraker 2009, Mowat et al 2011b). Several studies have demonstrated effective phage-antibiotic synergism in the reduction of bacterial numbers *in vitro* and *in vivo* (Comeau et al 2007, Hagens et al 2006, Knezevic et al 2013). However, this strategy would need to be considered with caution. Antibiotic therapies that induce stx phages of Shiga-toxigenic *E. coli* have been shown to increase expression of shiga toxin genes that are encoded in the late region of the phage genome and thus increase cytotoxic damage and exacerbate symptoms (Matsushiro et al 1999). Although we have not identified any obvious virulence factors encoded in the late gene region of the LES phages (Winstanley, *et al.,* 2009, James, *et al.,* 2012), we cannot ignore the possibility that the lytic cycle might induce upregulation of virulence genes.

We demonstrate here that LESφ2 was the most abundant free phage in 9-out-of-10 LES-infected patients. The hierarchy of free LES phage in patient sputa was also observed in our previous studies of LES phage induction in *in vitro* bacterial cultures (James et al 2012). This suggests therefore that LESφ2 is generally more readily induced or exhibits a more efficient lytic cycle than the other phages both *in vitro* and *in vivo*. In the sputa of patient CF1, who was infected by a LES that lacked prophage 2, LESφ3 reached far higher abundances than observed in other patients, suggesting potential suppression of LESφ3 lysis by LESφ2 *in vivo*. In accordance with our previous *in vitro* observations of co-induction of lysis by prophages, we observed a degree of synchronisation of free-phage dynamics *in vivo*, suggesting that the phages may be responding to shared signals, which could include a wide variety of human host, bacterial and environmental triggers(Little 2005). It is exceptionally difficult to disentangle to drivers of microbial dynamics *in vivo* due to the complexity of host microenvironments; future studies using laboratory models of the infection environment alowing the constituent drivers to be decomposed will be necessary to elucidate this (Fothergill et al 2014, Wright et al 2013).

The long-term maintenance of intact, active temperate phages in the LES genome despite substantial cell lysis suggests some selective or competitive advantage *in vivo*, consistent with previous work highlighting a loss of competitiveness observed following the introduction of mutations to some LES prophage regions (Winstanley et al 2009). One possibility is that free-phage particles produced by a subpopulation of LES could kill competing bacteria (Brown et al 2006). Indeed, LESφ2, LESφ3 and LESφ4 are capable of infecting and lysing other clinical *P. aeruginosa* isolates (James et al 2012). Thus frequent induction of the lytic cycle may enhance the competitive ability of LES by promoting superinfection, which has been observed clinically (McCallum et al 2001), and preventing invasion of the lung by other strains of *P. aeruginosa*. Alternatively the prophages may contain accessory genes that contribute directly to LES fitness in the CF lung, which are only expressed during the lytic cycle, as observed for other pathogens (Wagner et al 2001).

Little is known about the consequences for the human host of the presence of large numbers of phage in the lung. However, high titre phage preparations have recently been found to interact with the immune system *in vivo* (Letkiewicz et al 2010). It has also been suggested that, following adherence to mucous, some phages may act as a form of innate host immunity enhancing host defences against bacterial pathogens (Barr et al 2013). Our findings of high free-phage abundances in CF lungs highlight the urgent need for research into the interaction of phages with host immunity, particularly in CF where dysfunctional immune responses contribute to pathological processes.

Supplementary information is available on The ISME journal website

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We declare that there are no competing commercial interests in relation to this submitted work

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Figure Legends

Figure 1: Longitudinal dynamics of total free-phage density and *P. aeruginosa* density in ten CF patients

Q-PCR assays were used to enumerate free LES phage (dotted line) and *P. aeruginosa* (solid line) densities from the sputa of 10 LES-infected CF patients (CF1-CF5 left to right top row, and CF6-CF10 left to right bottom row) over a two year period. Samples were obtained from patients both during stable periods (black symbols) and during exacerbation of symptoms

(red symbols). The dotted line represents the mean values of all free LES phages (2,3,4,5 and 6) for each patient. The density of free-phage copies of each LES phage was calculated by subtracting prophage copies from total phage copies in each case.

Figure 2: Relationships of phage density and phage-to-bacterium ratio with bacterial density.

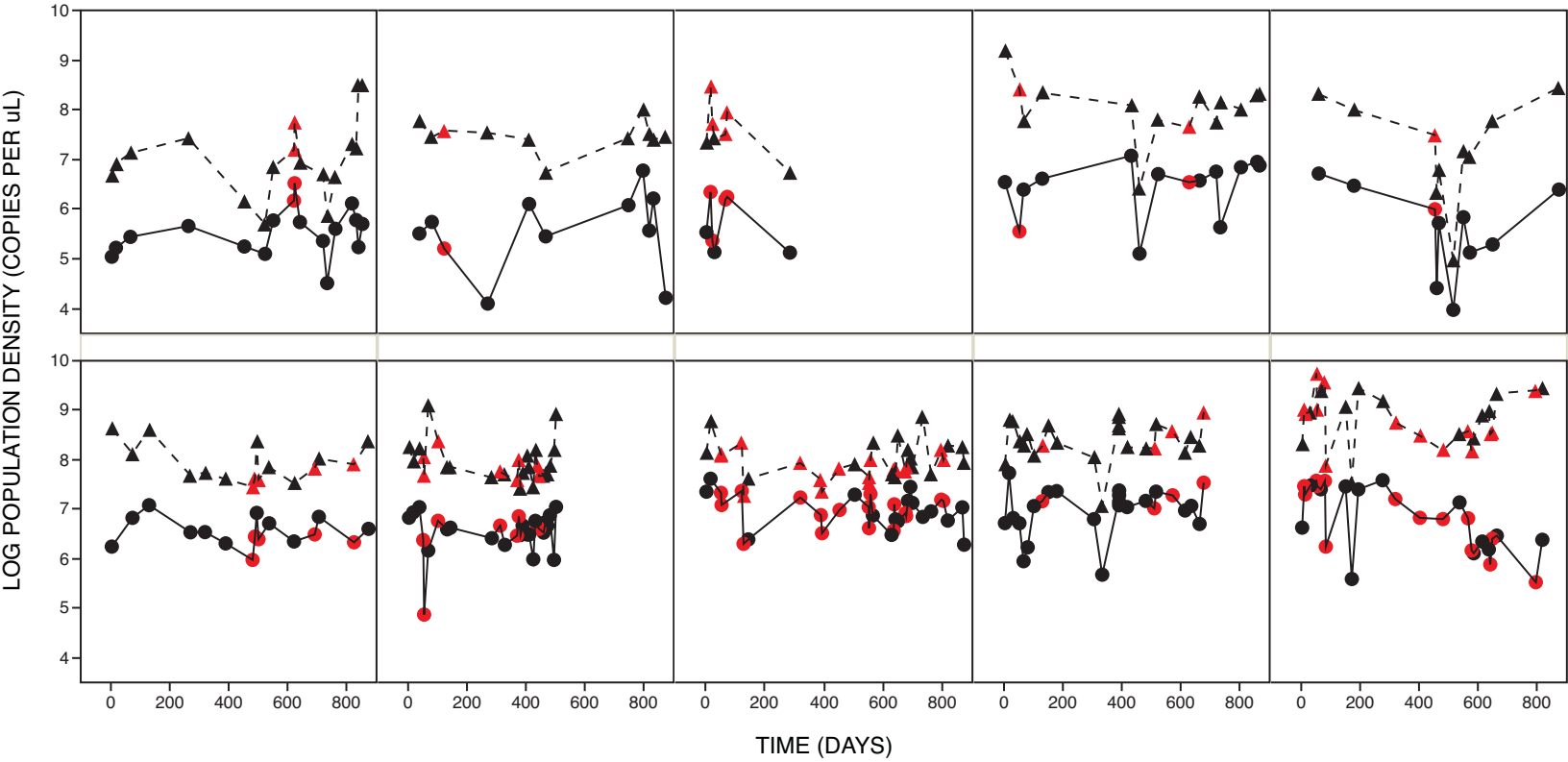
Datapoints represent sputum samples; patient identity is indicated by colour (see visual key for details); regression lines indicate significant relationships between variables. Panel A (upper) shows the positive relationship between log10 phage density and log10 bacterial density; panel B (lower) shows the negative relationship between phage-to-bacterium ratio and log10 bacterial density.

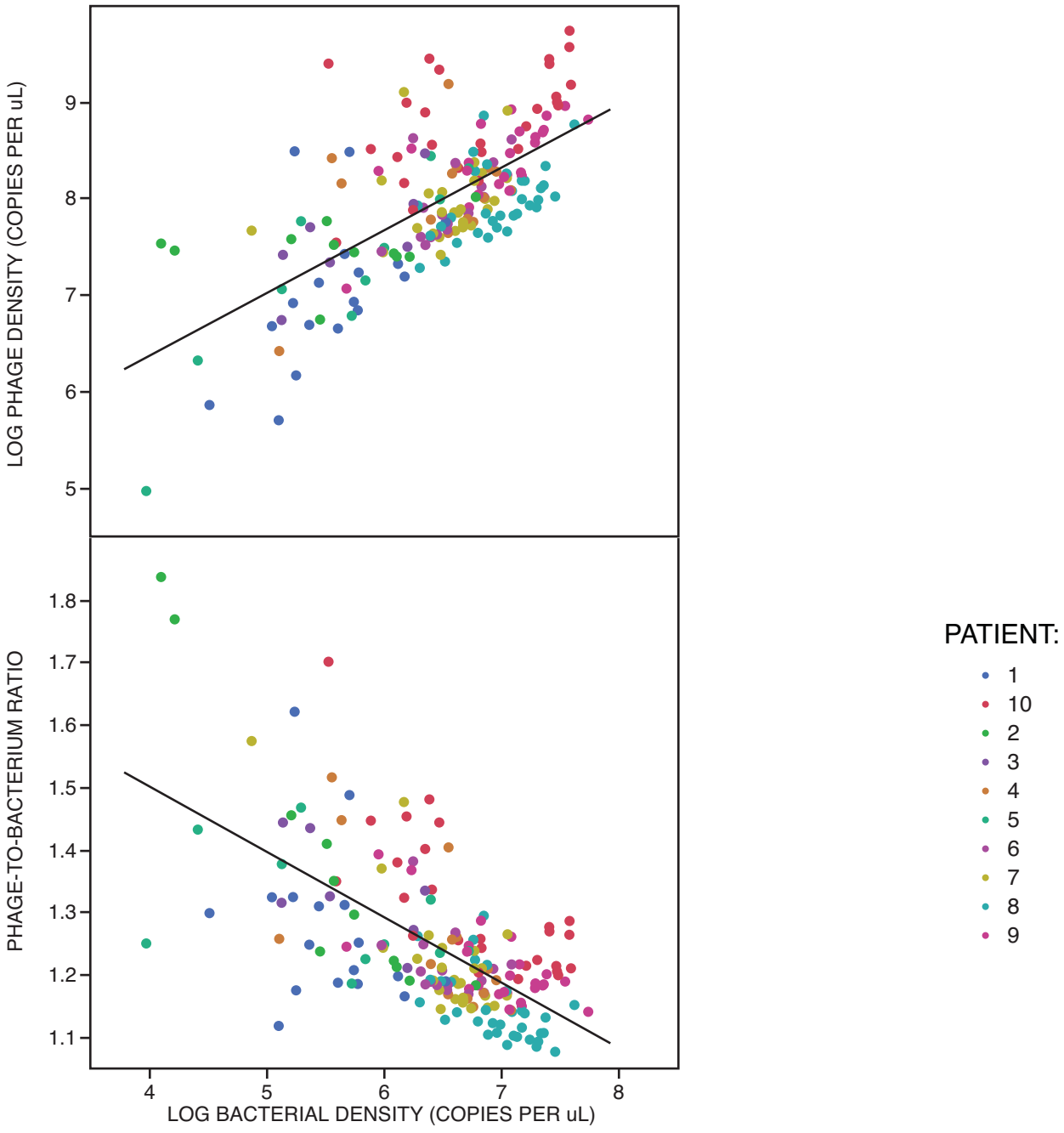
Figure 3: Phage density and phage-to-bacterium ratio are not affected by exacerbations

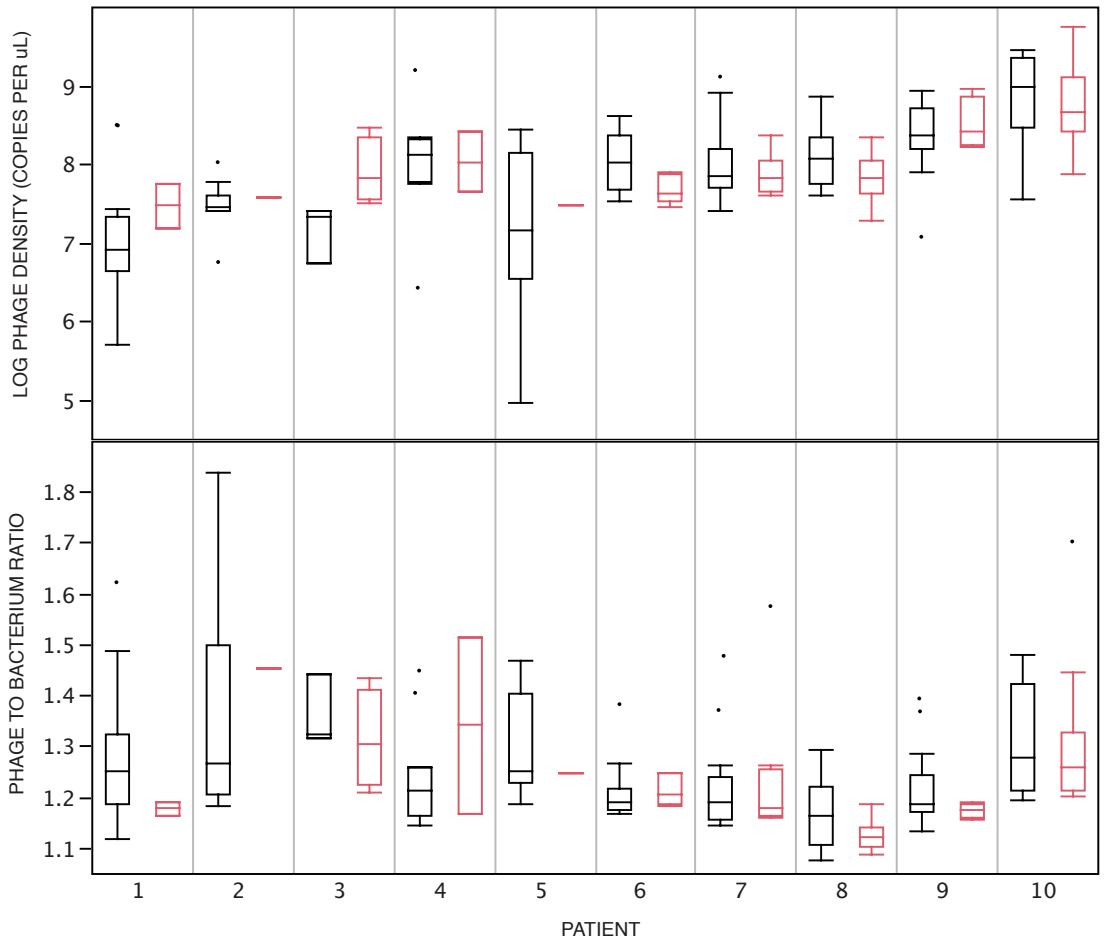
Outlier box-plots display phage density (upper panel) or phage-to-bacterium ratio (lower panel) in sputa from patients during stable periods (black) and exacerbations (red).

Figure 4: Densities of individual LES phage types in patient sputa exhibit hierarchical trends.

The free-phage densities, calculated for each individual LES phage in the ten CF patients analysed (CF1-CF5 left to right top row, and CF6-CF10 left to right bottom row). Each line represents one LES phage type; LESφ2 (blue); LESφ3 (cyan); LES φ4 (pink); LES φ5 (green); LES φ6 (orange); *P. aeruginosa* (black circles). All Q-PCR assays were performed in triplicate and mean values are presented. The density of free-phage copies of each LES phage was calculated by subtracting prophage copies from total phage copies in each case.


LOG POPULATION DENSITY (COPIES PER uL)
10
9
8
7
6
5
4
0
200
400
600
800
TIME (DAYS)


LOG PHAGE DENSITY (COPIES PER uL)
9
8
7
6
5
PHAGE-TO-BACTERIUM RATIO
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
4
5
6
7
8
LOG BACTERIAL DENSITY (COPIES PER uL)
PATIENT:
1
10
2
3
4
5
6
7
8
9


LOG PHAGE DENSITY (COPIES PER uL)
9
8
7
6
5
PHAGE TO BACTERIUM RATIO
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
2
3
4
5
6
7
8
9
10
PATIENT

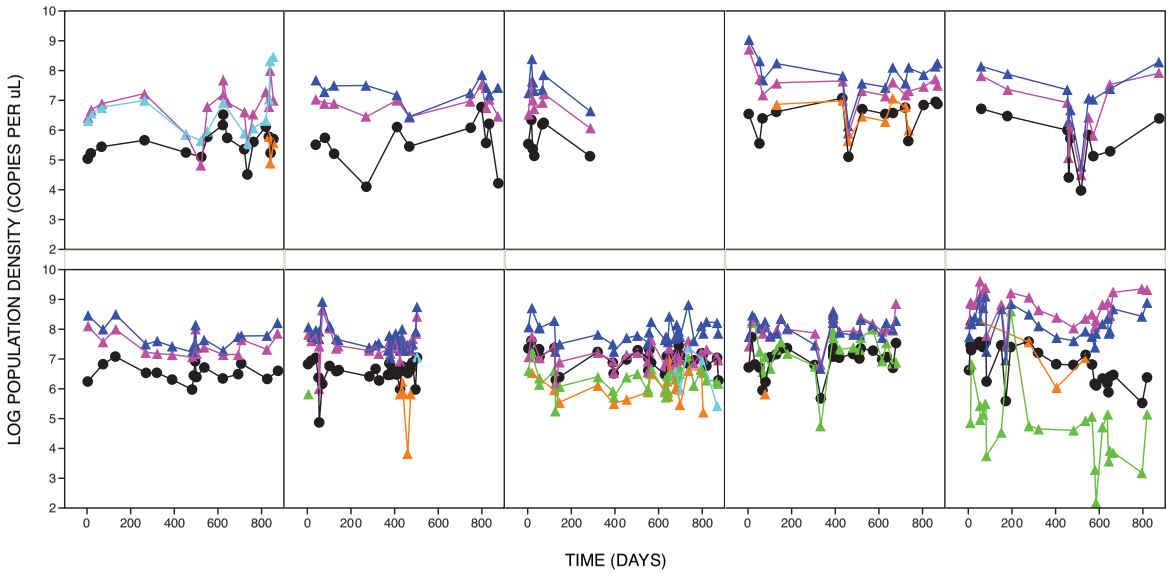

LOG POPULATION DENSITY (COPIES PER uL)
TIME (DAYS)
logbacteria
logphage6
logphage5
logphage4
logphage3
logphage2

Table 1: Patient LES-phage complement and sputum sample summary

Patient	φcomplement	Total	Stable	Exacerbation	Antibiotics
CF1	3, 4, 6	17	14	3	TOB (N), AZT (O)
CF2	2, 3, 4, 6	11	9	2	CEPH (O), CEF (IV), COL (IV)
CF3*	2, 3, 4, 6	7	1	6	AZT (O), COL (N), CEF (IV), COL (IV)
CF4	2, 3, 4, 6	14	10	4	CEF (IV), COL (IV)
CF5	2, 3, 4, 6	10	7	3	
CF6	2, 3, 4, 6	16	9	7	AZT (O), COL (N)
CF7*	2, 3, 4, 5*, 6	28	11	17	AZT (O), COL (N), CEF (IV), COL (IV), MER (IV), FOS (IV)
CF8	2, 3, 4, 5, 6	33	8	25	CEPH (O), FOS (IV), MER (IV), CEF (IV)
CF9	2, 3, 4, 5, 6	25	19	6	AZT (O), COL (N), TOB (N), MER (IV), COL (IV)
CF10	2, 3, 4, 5*, 6	27	10	17	MER (IV), COL (IV)
Total		188	98	90	

Table 1: CF patient sputum contained LES variants that harboured different phage complements. Stable samples were collected during periods of relative patient health. Exacerbation samples were collected during periods of reduced lung function and hospitalisation of patients, who underwent antibiotic treatment for which data is incomplete. Antibiotics used during the period of the study are shown (where known): CEPH, cephadrine; CEF, ceftazidime; COL, colomycin; FOS, fosfomycin; MER, meropenem; TOB, tobramycin; AZT, azithromycin. Route of administration is indicated in brackets: O, oral; N, nebulised; IV, intravenous (used during exacerbations only). * Patients CF3 and CF7 died before completion of this study.