Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations

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Chronic respiratory infection by *Pseudomonas aeruginosa* contributes significantly to the morbidity and mortality associated with cystic fibrosis (CF). Using a series of phenotypic and genotypic tests on collections of 40 isolates per sputum sample, we analysed fluctuations within sputum populations of the *P. aeruginosa* Liverpool epidemic strain (LES) during pulmonary exacerbations. For each of three patients, three sequential sputum samples were analysed: (1) on presentation with exacerbation at the Regional Adult Cystic Fibrosis Unit, Liverpool; (2) a few days into intravenous antibiotic treatment; (3) when the patient had recovered. Fluctuations were observed in morphotype distribution, the production of virulence-associated quorum-sensing-dependent exoproducts (the phenazine compound pyocyanin and the elastase LasA), antibiotic susceptibility profiles and levels of auxotrophy. PCR assays were used to screen isolates for the presence of novel regions of the LES genome (islands and prophages) and to detect free phages. In one patient there was an increase in the prevalence of the LESGI-5 genomic island during the sampling period from 10 to 97.5% carriage. LES phages 2–4 were detected in either the majority or all sputum samples tested, indicating widespread phage activity during the sampling period. The results of this study are indicative that significant fluctuations occur within *P. aeruginosa* populations during short periods of pulmonary exacerbation and intravenous antibiotic therapy.

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INTRODUCTION

Pseudomonas aeruginosa is the most important pathogen infecting the lungs of cystic fibrosis (CF) patients and, once established, infections with *P. aeruginosa* are impossible to eradicate (Hart & Winstanley, 2002). The Liverpool epidemic strain (LES) of *P. aeruginosa* is a particularly successful CF transmissible strain and is widespread in the UK (Scott & Pitt, 2004). First identified in 1996 (Cheng *et al.*, 1996), the LES has been associated with increased morbidity (Al-Aloul *et al.*, 2004), transmission to both the non-CF parents of a CF patient (McCallum *et al.*, 2002) and infection of a pet cat (Mohan *et al.*, 2008). In addition, we have reported previously that some LES isolates exhibit an unusual phenotype characterized by enhanced antimicrobial resistance (Salunkhe *et al.*, 2005) and overproduction of quorum-sensing-regulated products (Fothergill *et al.*, 2007) with potential roles in pathogenicity in CF (Winstanley & Fothergill, 2009).

The genome sequence of an isolate of the LES, LESB58, indicated the presence in its accessory genome of five intact prophages (LES prophages 2–6) and five genomic islands (LESIs1–5). A rat model of chronic lung infection was used to demonstrate the involvement of a novel genomic island (LESGI-5) and novel prophages (LES prophages 2, 3 and 5) in the *in vivo* competitiveness of the strain (Winstanley *et al.*, 2009), implicating regions of the accessory genome in its success as a pathogen.

Exacerbations, described as recurrent episodes of increased pulmonary symptoms, occur periodically in CF (Goss & Burns, 2007). Hospitalization during these periods is common and intravenous antibiotics are generally used, with the aim of reducing symptoms and increasing lung function (Regelmann *et al.*, 1990). It is known that the characteristics of *P. aeruginosa* isolates isolated from CF patients change over long periods of time; mutations leading to mucoid conversion, auxotrophy, loss of motility and loss of virulence are common amongst CF isolates (Smith *et al.*, 2006). However, little is known about the fluctuations within *P. aeruginosa* populations in the CF

Abbreviations: BSAC, British Society for Antimicrobial Chemotherapy; CF, cystic fibrosis; LES, Liverpool epidemic strain.

lung during very short periods of intense selective pressure, such as when intravenous antibiotic therapy is administered during exacerbations.

METHODS

Sputum samples. Sputum samples were collected from adult CF patients at the Regional Adult Cystic Fibrosis Unit in Liverpool. These represented routine samples taken for diagnostic purposes, and the antimicrobial therapy was determined by clinicians. For each patient, three samples were taken: the first at the beginning of the exacerbation, before intravenous antibiotic treatment had commenced (sample A); the second during the exacerbation, whilst antibiotic therapy was being carried out (sample B); the third at the end of the exacerbation when symptoms had resolved (sample C) (Table 1). Exacerbations were defined by a clear set of criteria, including a drop in FEV₁ (forced expiratory volume in 1 s), increased sputum or discoloration, temperature and increased cough (Goss & Burns, 2007). All of the patients studied were chronically infected with the LES. Sputum was treated with an equal volume of Sputasol (Oxoid), incubated at room temperature with shaking at 200 r.p.m. for 15 min, and then cultured on *Pseudomonas* selective agar with CN supplement (Oxoid). Forty single colonies were isolated per sputum sample, ensuring that each different colony morphology type was proportionately represented. Each of these 40 isolates was confirmed as *P. aeruginosa* LES using PCR tests for the amplification of the *P. aeruginosa* *oprL* gene (De Vos *et al.*, 1997) and the two LES markers, PS21 (Parsons *et al.*, 2002) and LESF9 (Smart *et al.*, 2006), before being subjected to a range of phenotypic and genotypic tests (Fig. 1).

Simple phenotypic tests. Auxotrophy was determined by testing the ability of isolates to grow on glucose M9 media. Hypermutability was assessed by determining spontaneous mutation rates on Luria agar containing rifampicin (Sigma) (300 µg ml⁻¹) following overnight growth in Luria broth as described by Oliver *et al.* (2000). Colony morphology was assessed on Columbia agar plates (Oxoid).

Exoproduct production assays. Pyocyanin production was determined and cut-off values for overproduction were used as described previously (Fothergill *et al.*, 2007). Overproduction refers to culture supernatant with an absorbance at 695 nm of >0.1 after overnight

growth of a strain in 5 ml Luria broth with shaking at 200 r.p.m. LasA activity was determined using a spectrophotometric assay measuring the lysis of a staphylococcal (*Staphylococcus aureus* NCTC 6571) suspension (Kessler *et al.*, 2004) on a plate reader (Fluostar omega; BMG Labtech). LasA overproduction was defined as a value at the end of the assay of <80 % of the original absorbance at 600 nm.

Determination of antibiotic susceptibility. Antibiotic susceptibilities were determined by disc diffusion tests for six antibiotics (ceftazidime, colistin, ciprofloxacin, meropenem, tazobactam/piperacillin and tobramycin) using current British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Andrews, 2001). The following amounts were used: 85 µg tazobactam/piperacillin, 10 µg meropenem, 10 µg tobramycin, 5 µg ciprofloxacin, 30 µg ceftazidime and 25 µg colistin sulphate (all from Oxoid). The sizes of the zones of inhibition (mm) were also recorded and statistical analysis was performed using SPSS data analysis software.

PCR amplification assays. PCR amplifications were carried out in a total volume of 25 µl. For each reaction, 1.25 U GoTaq polymerase (Promega), 1 × TaqMaster (Helena Biosciences), 300 nM each oligonucleotide primer (Sigma-Genosys; Table 2), 1 × Taq buffer, 2.5 mM MgCl₂ and 100 µM nucleotides (dATP, dCTP, dGTP, dTTP) was used along with 1 µl DNA from boiled suspensions of colonies. PCR amplification was carried out for 30 cycles of 95 °C (1 min), the chosen annealing temperature (2 min) and 72 °C (2 min). Following this, a final extension step of 72 °C for 10 min was carried out. In the case of LES prophages 2, 3, 4 and LESGI-5, additional PCR assays were performed using primers designed for the flanking regions of the insertions, and therefore only if the insertion was not present would the expected band be observed. For PCR amplification to detect free phages directly from sputum samples, the sputum was first treated with Sputasol, diluted (1:10) with sterile distilled water, filter-sterilized (0.2 µm) and DNase I treated as follows: addition of 3.5 U DNase I (Roche), 3.5 µl 10 × DNase I buffer and 3 µl sterile distilled water, followed by incubation for 15 min at room temperature. To stop the reaction 3.5 µl 25 mM EDTA (pH 8.0) was added and the solution was heated to 65 °C for 10 min. Following this, PCR amplification (Table 2) was carried out using 5 µl of the phage preparation. Each PCR amplification reaction was replicated twice.

RESULTS AND DISCUSSION

We investigated the changes in the prevalences of various phenotypes and genotypes amongst *P. aeruginosa* LES populations during short periods of exacerbation and intravenous antibiotic therapy. The total number of *P. aeruginosa* recovered from the sputum samples ranged from 1 × 10³ to 1 × 10⁶ c.f.u. ml⁻¹. However, there was no clear pattern in the change in the number of *P. aeruginosa* recovered from sputum following treatment with antibiotics. For patient I, there was a marked reduction in *P. aeruginosa* between samples A and C (1 × 10⁶ to 3 × 10⁴ c.f.u. ml⁻¹); for patient III, the numbers remained relatively constant, at around 5 × 10⁵ c.f.u. ml⁻¹; for patient II the number of *P. aeruginosa* actually increased over the exacerbation period, from 1 × 10⁴ c.f.u. ml⁻¹ in sample A to 7 × 10⁵ c.f.u. ml⁻¹ in sample C. In order to facilitate multiple testing, we restricted our study to 40 isolates per sputum sample and to 3 CF patients. Whilst we accept that these numbers may not be sufficient to fully represent the variation present, our study was conducted in considerably more depth than would normally be used in

Table 1. Sputum samples used in this study

Sample A was taken at the beginning of the exacerbation when the CF patients were admitted to the Regional Adult Cystic Fibrosis Unit with exacerbation. Sample B was taken during intravenous antibiotic treatment. Sample C was taken at the end of the exacerbation.

Patient	Sample	Date	Antibiotic treatment
I	A	12/01/2006	Meropenem/colistin
	B	16/01/2006	
	C	20/01/2006	
II	A	03/02/2006	Meropenem/colistin
	B	06/02/2006	
	C	17/02/2006	
III	A	11/04/2006	Ceftazidime/colistin
	B	13/04/2006	
	C	21/04/2006

Fig. 1. Processing of the sputum samples and tests performed.

the analysis of sputum samples in diagnostic laboratories. All of the isolates tested were identified as the *P. aeruginosa* LES by virtue of PCR-positive assays for *oprL* and the two LES markers (PS21 and LESF9).

Changes in the phenotype of *P. aeruginosa* LES populations during the exacerbations

For patients I and II, there was an increase in the prevalence of isolates exhibiting overproduction of pyocyanin and LasA during the exacerbation period (Table 3). Conversely, isolates from patient III showed a very different trend, beginning with a higher level of overproducers (37.5%), which gradually decreased over the exacerbation period (to 15%). In all samples, pyocyanin-negative isolates could also be identified, with the level in the population ranging from 20 to 67.5%. There was strong concordance between levels of LasA activity and pyocyanin production (Table 3).

There was a general trend in the changes in colony morphologies. At the beginning of the exacerbation, in all three patients, the majority of isolates were green, smooth and non-mucoid (Fig. 2). However, the prevalence of this morphotype decreased over the exacerbation period and the occurrence of both white, mucoid isolates and smooth, non-mucoid, white/straw-coloured isolates increased. A very low level of mucoid translucent isolates was also detected in samples IB and IIB. In all samples, the majority of isolates were non-mucoid rather than exhibiting the mucoid phenotype commonly associated with chronic infection (Fig. 2). All of the pyocyanin overproducers had

green pigmented colony morphology, suggesting that the colour observed was at least in part due to the high levels of pyocyanin produced. However, many of the other isolates were also pigmented and it was unclear whether this was due to the production of pyocyanin or other pigmented products such as pyoverdine.

Changes in the antibiotic susceptibility profiles were detected in all of the patients during the exacerbation period (Table 3). The overall prevalence of resistance to tobramycin was very different between patients I and II, but varied little between the three samples taken for each patient. In contrast, there was a decrease in the prevalence of isolates from patient III with resistance to tobramycin over the sampling period. The LES populations in all three patients showed some decrease in the prevalence of resistance to ceftazidime during the exacerbation period, with the greatest decrease observed in patient III (from 97.5% resistance to 27.5% resistance). It is interesting to note that, during this period, patient III was treated with ceftazidime. In contrast, the two patients treated with meropenem (patient I and II) showed a greater prevalence of resistance to meropenem at the end of the treatment compared to the beginning (Table 3).

The change in prevalence of resistance to tazobactam/piperacillin was patient specific and no overall trend could be observed. This varied from very little change for patients I and III, to a marked decrease in the prevalence of resistance for patient II. In all three patients, the prevalence of isolates with resistance to ciprofloxacin increased during the sampling period (Table 3).

Table 2. Oligonucleotide primers used in this study

'Circular genome' indicates that these PCR assays target the phage in its circularized genomic form only and will not yield amplicons with linear prophage DNA inserted into the bacterial genome.

Name	Sequence 5'→3'	Size (bp)	Target gene	Reference
PAL1	ATGGAAATGCTGAAATTCGGC	504	<i>oprL</i>	De Vos <i>et al.</i> (1997)
PAL2	CTTCTTCAGCTCGACGCGACG			
PS21F	AAGCAGGCCAGCGTGTCTA	364	PS21 (LES marker)	Parsons <i>et al.</i> (2002)
PS21R	AAAACGTAGCAAGCAGTG			
LESF9F	AACACTTGCTCCATCTGC	431	LESF9 (LES marker)	Smart <i>et al.</i> (2006)
LESF9R	CACGATATCCAGCAAGAC			
LES1F	TCGGCGTAATGTCTCTA	392	LES prophage 2	Smart <i>et al.</i> (2006)
LES1R	TGAAGCCGACGATGGAAG			
4138up	TAGCCAGCGCATCAGCAT	998	<i>tyrS</i> and <i>PALES_07881</i> (<i>PA4139</i>) flanking	This study
4139up	TTCATCGAAGCTCAGTTC		regions of LES prophage 2	
LES1out	GACTCAGGAGATCACGAA	425	LES prophage 2 (circular genome)	This study
LES1down	CAAGGGTGCGAGAGTATC			
LES1nestF	TTTGGTGATGATCGGCTTAGC	289	LES prophage 2 (circular genome); using	This study
LES1nestR	TGTGGAAGCGATCAGTCT		nested primers	
LESE7F	TTGATGATGATGCGCGGA	273	LES prophage 3	Smart <i>et al.</i> (2006)
LESE7R	TCGGTCATTTTGGGCGTG			
3663	CGTAATTGGTCTATCTGG	363	<i>PALES_13721</i> (<i>PA3663</i>) and <i>PALES_13201</i>	This study
3664	TTTGATGCCGTAGAGAAC		(<i>PA3664</i>) flanking regions of LES	
			prophage 3	
Cluster6up	TTCGGCATCTGGTCGTTTC	532	LES prophage 3 (circular genome)	This study
Cluster6dn	GCTGCCAGGCTTCATGAA			
Clus6nestF	GGATCGACGTGGCATAATCTG	410	LES prophage 3 (circular genome); using	This study
Clus6nestR	ACGATTCTCCGGCATGCAGCG		nested primers	
D3112cIF	GACAGTTACGCCTATATC	383	LES prophage 4 cI repressor gene	This study
D3112cIR	ATGATGTCAACTGCTTCC			
3464dn	ACTTCAGCGAGAGCAACCA	722	LES prophage 4 cI repressor and flanking	This study
D3112cIout	GGAAGCAGTTGACATCAT		gene <i>PALES_15481</i> (<i>PA3464</i>)	
D3112nestF	TGTATGACGCTCGGGTGA	339	LES prophage 4 cI repressor gene; using	This study
D3112nestR	TCCTGCTTAGAGACGAAC		nested primers	
LESD3cIF	ATGAAAAAGCCGTAAGA	490	LES prophage 5 cI repressor gene	This study
LESD3cIR	GCCATTCCCGCTTAAAAG			
D3nestF	TCTACAAGAAGCGAGTCA	399	LES prophage 5 cI repressor gene; using	This study
D3nestR	GTAGCTCGAATCTCAACA		nested primers	
PS1F	ACAGAAATATTCGAAGCAG	338	LES genomic island-5	Smart <i>et al.</i> (2006)
PS1R	ACAAGAGCCTAACACCAC			
831F	TGCAATACCTGACCACCA	310	<i>oruR</i> and <i>PALES_44861</i> (<i>PA832</i>) flanking	This study
831R	CAATGATGGTGCAGTTGC		regions of LES genomic island-5	

To determine whether the changes in resistance observed using the accepted BSAC cut-off values were due to significant shifts in the resistance of the population or simply fluctuations of inhibition zone size around the breakpoint, the zone size produced by each isolate to the six antibiotics was measured and used to calculate a mean for each sputum sample. The change in the mean zone size was monitored across the three samples to determine whether the population, as a whole, was becoming more or less resistant to the antibiotics. There was very good agreement between the trends in the changes in resistance as determined by using breakpoints compared to the change in mean zone sizes. As the prevalence of resistance increased, the mean zone size decreased. Examples of significant shifts in the mean zone size were observed for

each of the antibiotics; however, for ceftazidime, meropenem and tobramycin these changes were significant between the first and final sample in all three patients ($P<0.05$). Measuring the zone of inhibition also gave an indication of more subtle differences in the resistance. For example, the LES populations in both patient I and patient II would be classed as ceftazidime resistant using the breakpoint cut-off value (23 mm). However, the population in patient II appeared to be much more resistant, with a mean zone size of only 5 mm by sample C, whereas the LES population from patient I had a mean zone size of 20 mm.

The prevalence of auxotrophic mutants amongst the LES populations from two patients (I and III) increased from

Table 3. Phenotypic characteristics of the 40 isolates taken from each sputum sample

Values indicate the percentage of the 40 isolates with each characteristic. Sputum samples were taken: at the beginning of an exacerbation (sample A), during the exacerbation and antibiotic therapy (sample B) and at end of the exacerbation (sample C).

Sample	Overproduction (%) [*]		Antibiotic resistance (%) [†]						AUX (%)	HYP (%)
	Pyocyanin	LasA	TOB	CEF	MEM	TZP	CIP‡	CT		
IA	10	10	22.5	97.5	12.5	17.5	7.5 (70)	0	0	5
IB	32.5	30	20	77.5	85	25	50 (45)	0	30	10
IC	27.5	27.5	20	67.5	55	27.5	57.5 (40)	0	57.5	10
IIA	5	7.5	100	100	37.5	90	57.5 (42.5)	0	7.5	7.5
IIB	47.5	47.5	95	97.5	27.5	37.5	42.5 (57.5)	0	2.5	10
IIC	12.5	12.5	97.5	92.5	77.5	45	92.5 (7.5)	0	2.5	5
IIIA	37.5	37.5	95	97.5	97.5	5	2.5 (80)	0	0	5
IIIB	22.5	22.5	2.5	37.5	40	12.5	52.5 (42.5)	0	32.5	15
IIIC	15	15	40	27.5	27.5	0	17.5 (42.5)	0	42.5	5

AUX, Auxotrophy; HYP, hypermutability (based on spontaneous resistance to rifampicin).

^{*}Overproduction of pyocyanin refers to isolates giving an absorbance at 695 nm of >0.1; overproduction for LasA activity refers to a value at the end of the assay of <80 % of the original absorbance at 600 nm for *S. aureus* suspensions.

[†]The antibiotics tested were tobramycin (TOB), ceftazidime (CEF), meropenem (MEM), tazobactam/piperacillin (TZP), ciprofloxacin (CIP) and colistin (CT).

[‡]The percentage found to have intermediate resistance to ciprofloxacin is shown in parentheses.

undetectable to 57.5 and 42.5 %, respectively, during the sampling period (Table 3). In contrast the prevalence of auxotrophic mutants in samples from patient II remained low throughout. Isolates exhibiting mutation rates compatible with the hypermutable phenotype were identified in all of the samples, but prevalence levels varied little (5–15 %; Table 3).

In our study, all three patients were treated with dual intravenous antibiotic therapy of colistin and ceftazidime (patient III) or colistin and meropenem (patients I and II). Colistin has been shown to have an additive effect when used in conjunction with either ceftazidime or meropenem (Guzel & Gerceker, 2008). However, serious questions have been raised generally about the levels of antibiotics reaching the lung during therapeutic intervention in CF (Moriarty *et al.*, 2007). This could have consequences with respect to the development of resistance as well as the efficacy of therapy. In this study, changes were observed in the *P. aeruginosa* LES antibiotic susceptibility profiles during therapy. Although it is difficult to discuss general trends in a study of only three patients, whereas the two patients treated with meropenem showed an increase in the prevalence of isolates with resistance to meropenem, the patient treated with ceftazidime showed an overall decrease in the prevalence of isolates with resistance to ceftazidime. Hence, although one might expect an increase in the occurrence of isolates showing resistance to a therapeutic agent, this was not the case for all three patients in our study, indicating that levels of antibiotics reaching the target population may not be sufficient to select for a resistant subpopulation.

Interestingly, in all three patients, the proportion of isolates resistant to ciprofloxacin increased during therapy. Ciprofloxacin resistance occurs via two major resistance mechanisms: firstly, due to mutations in the DNA gyrase or topoisomerase genes; secondly, through activation of efflux pumps with ciprofloxacin being a substrate for the pumps MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM. Both forms of resistance mechanisms can occur simultaneously during antibiotic treatment and this can result in the development of highly resistant isolates (Nakajima *et al.*, 2002; Niga *et al.*, 2005). Interestingly, it has been reported that treatment with drugs other than ciprofloxacin is a significant risk factor in the development of resistance to ciprofloxacin (Hyatt & Schentag, 2000). However, we have no direct evidence that the increase in ciprofloxacin resistance is the result of increased mutations rather than selection for a subpopulation characterized by resistance to ciprofloxacin. It is interesting to note that no LES isolate was completely sensitive or completely resistant to all of the six antibiotics tested, and that the widespread use of colistin to treat LES-infected patients within our clinic appears justified by the lack of resistance observed amongst LES isolates in this study.

Diversity within each population with regards to a number of other phenotypic markers was also observed. It has been reported that during periods of exacerbation there is evidence of oxidative damage in the CF lung (McGrath *et al.*, 1999). Endogenous oxidative stress is thought to generate diversity in *P. aeruginosa* populations by causing dsDNA breaks. The mutagenic repair of such breaks then leads to diversity (Boles & Singh, 2008). The role of

Fig. 2. Prevalence of colony morphotypes in the sputum samples. Percentage values are indicated for the following four colony morphology types: green, non-mucoid, smooth (G, NM, Sm); white, mucoid, opaque (W, M, O); translucent mucoid (Tr, M); straw-coloured, non-mucoid, smooth (S, NM, Sm). Results for three sputum samples per patient are shown, representing samples taken at the beginning of the exacerbation but before intravenous antimicrobial therapy (white bars) (sample A), samples taken during the exacerbation and antibiotic treatment (grey bars) (sample B), samples taken at end of exacerbation (black bars) (sample C).

oxidative stress was reinforced by experiments showing that the addition of antioxidants reduces DNA damage and bacterial diversity (Boles & Singh, 2008). In this study, in all three patients, the colony morphology showed a consistent shift from a smooth, pigmented morphotype to an increase in white mucoid isolates and a general increase in the diversity of the bacteria with respect to morphology. Conversion into a mucoid phenotype during CF lung infection with *P. aeruginosa* has been widely reported. Mucoid isolates are thought to be more resistant to phagocytosis (Cabral *et al.*, 1987) and antibiotics (Govan & Fyfe, 1978). Furthermore, mucoid conversion and selection has also been associated with the presence of

reactive oxygen species, which can be released by the host immune system (polymorphonuclear leukocytes) (Mathee *et al.*, 1999).

It has also been suggested that the use of certain antibiotics can affect the quorum-sensing system (Skindersoe *et al.*, 2008), potentially leading to increases in exoproducts, including pyocyanin, that may contribute to the presence of reactive oxygen species (Winstanley & Fothergill, 2009). We detected LES isolates with the previously reported pyocyanin overproduction phenotype (Fothergill *et al.*, 2007) in each of the sputum samples tested. The physiological relevance of this phenotype in the CF lung is unknown but it could potentially lead to increased oxidative effects in patients infected with the LES.

In previous studies, the level of hypermutability amongst CF isolates has ranged from 18 to 54.4 % (Montanari *et al.*, 2007; Ciofu *et al.*, 2005). It has been shown that hypermutable isolates can be prevalent at high levels in chronic infections, such as the CF lung, compared to acute infections, where prevalence is less than 1 % (Oliver *et al.*, 2000). The presence of hypermutable isolates has been associated with accelerated resistance to antimicrobials, especially during exposure to antibiotics (Oliver, 2004; Plasencia *et al.*, 2007). However, hypermutable isolates have been shown to have increased susceptibility to colistin (Macia *et al.*, 2007), and therefore colistin could be an ideal choice in dual therapy to counteract any selection for resistance. In this study, the prevalence of hypermutable isolates remained constant throughout the sampling period (5–15 %), suggesting that any changes in antibiotic susceptibility or other phenotypes were not caused by the expansion of this subset of the LES population.

Changes in the genotype of the *P. aeruginosa* LES populations during exacerbations

PCR assays were performed using oligonucleotide primers for sequences specific to the four LES prophages that are not found in *P. aeruginosa* strain PAO1 (LES prophage 2, 3, 4 and 5) and the novel genomic island, LESGI-5 (Table 4). None of the LES isolates tested in this study contained LES prophage 5. However, all of the other LES prophage sequences assayed (from LES prophages 2–4) were detected in all of the LES isolates tested from patients I and II. Likewise, in patient III, LES prophage 2–4 sequences were detected in all isolates tested prior to therapeutic intervention (sample A). However, loss of LES prophages was detected in subsequent samples from patient III (Table 4). In sample C from patient III, LES prophages 2, 3 and 4 were all absent from the genomes of two isolates (Table 4). Prophage deletions were confirmed by PCR amplification using primers flanking the prophages in the genome of the LES.

In patient I, the prevalence of LESGI-5 amongst isolates increased from a level of 10 to 97.5 % over the sampling period. However, in the other two patients the prevalence of LESGI-5 was high throughout (97.5–100 %).

Table 4. Prevalence of LES prophages and LESGI-5 amongst LES isolates

The percentage of PCR isolates positive for the presence of LES prophages and LESGI-5 sequences amongst sets of 40 LES isolates are shown for sputum samples taken at the beginning of an exacerbation (sample A), during the exacerbation and antibiotic therapy (sample B) and at end of the exacerbation (sample C).

Patient	Sample	Prophage 2 (%)	Prophage 3 (%)	Prophage 4 (%)	Prophage 5 (%)	LESGI-5 (%)
I	A	100	100	100	0	10
	B	100	100	100	0	65
	C	100	100	100	0	97.5
II	A	100	100	100	0	97.5
	B	100	100	100	0	100
	C	100	100	100	0	100
III	A	100	100	100	0	100
	B	100	97.5	100	0	100
	C	92.5	95	95	0	100

Overall, less variation was observed with respect to the genotypic markers targeted in this study compared to the phenotypic characteristics. LES prophages 2, 3 and 4 were identified in every sputum sample, but there was an example of loss of prophages from isolates within the population in one of the patients over the course of the study.

Further screening of multiple sputum samples for prevalences of LESGI-5 and auxotrophy

Following the results obtained from the screening of isolates from the three patients (I, II and III), we decided to carry out further screening to study changes in the prevalence of two markers for which considerable variation had been observed: carriage of LESGI-5 and the prevalence of auxotrophic mutants. Nine further patients were selected and equivalent samples at the beginning and end of an exacerbation were analysed (equivalent to samples A and C) to determine if any changes had occurred over this period (Table 5). In five out of nine patients, the prevalence of LESGI-5 was 100% in both samples. In patients IV, V and X there was a small increase in prevalence between samples A and C (range 2.5–12.5%). In one patient a small decrease in prevalence was observed (2.5%; equivalent to one isolate from 40).

The prevalence of LESGI-5 carriage amongst isolates changed dramatically over the three samples for patient I, with an increase from 10 to 97.5%. This striking shift could suggest positive selection for this genomic island during the period of the study. We sought evidence that this selection may occur in other patients. However, our efforts were generally confounded by the high levels of carriage occurring prior to antimicrobial therapy in many of the other patients tested. It is interesting to note that a mutation in this genomic island leads to an impaired ability to establish chronic infection in the rat chronic lung infection model (Winstanley *et al.*, 2009), suggesting a possible role for this island in virulence. However, further investigation is needed in order to elucidate the precise role

of this island, and we cannot rule out the possibility that the carriage of LESGI-5 by a subpopulation in patient I was not a significant factor in the selection of this subpopulation.

The prevalence of auxotrophic mutants in samples from each of the additional patients was highly variable (0–90%). In samples from two patients (IX and XII) no change was observed. In samples from four patients the prevalence decreased, whereas in samples from three patients the prevalence increased (Table 5). However, percentage changes greater than 30% were only observed for three of the additional patients tested (VI, VII and XI).

Overall, in 5 out of the 12 patients studied there was a notable increase in the prevalence of auxotrophic mutants during the sampling period. The amino acid concentration

Table 5. Prevalence of LESGI-5 and auxotrophic mutants amongst LES isolates

The percentage of PCR isolates positive for the presence of auxotrophic mutants and LESGI-5 amongst sets of 40 LES isolates from 9 CF patients are shown for sputum samples taken at the beginning of an exacerbation (sample A) and taken at end of the exacerbation (sample C).

Patient	LESGI-5		Auxotrophy	
	Sample A (%)	Sample C (%)	Sample A (%)	Sample C (%)
IV	87.5	92.5	32.5	30
V	0	12.5	60	52.5
VI	100	100	10	47.5
VII	100	100	0	32.5
VIII	100	97.5	0	7.5
IX	100	100	0	0
X	0	2.5	12.5	0
XI	100	100	90	52.5
XII	100	100	72.5	72.5

of sputum is high, especially during infective exacerbations, and the concentration correlates with pulmonary disease severity (Thomas *et al.*, 2000). It has been proposed that auxotrophy may be an advantage due to the down-regulation of some metabolic pathways as the production of amino acids, in terms of energy, is expensive. This may explain the increases in the proportion of auxotrophic mutants observed in some patients.

Detection of LES phages directly from the sputum of CF patients

PCR assays were performed directly on Sputasol-treated, diluted, filter-sterilized and DNase I-treated sputum samples from patients I–III. Original testing using oligonucleotide primers specific for the LES phages produced only very weak amplicons and therefore nested primers were used to increase sensitivity. For the novel LES phages 2 and 3, primers for detection of the circular form of the genome were used. PCR assays for the *oprL* and 16S rRNA genes were used on all samples to ensure that there was no contamination with genomic DNA. LES phage 2 was detected in all of the sputum samples except one (IIIA), and LES phage 3 was detected in all samples except two (IB and IIIA). LES phage 4 was detected in every sample, whereas LES phage 5 was absent in all the samples except one (IIA) (Table 6).

Although LES prophage 5 was not detected in any of the isolates, this phage was detected in one of the sputum samples. We have reported previously that LES prophage 5 is carried by a minority of isolates of the LES and have noted that other LES prophages can also be unstable (Winstanley *et al.*, 2009). However, our observations here suggest that even when LES prophage 5 cannot be detected in a screen of isolates, the phage may nevertheless be present within the LES population in sputum, albeit at a very low level. Our inability to detect greater variation in the carriage of prophages may be due to the fact that only 40 isolates were analysed per sample.

Our observations with respect to free phages in CF patient sputum samples are consistent with the possibility that antimicrobial therapy contributes to phage induction. A previous study has suggested that antibiotics commonly used in the treatment of CF patients can cause phage induction in bacteria (Rolain *et al.*, 2009). Furthermore, hydrogen peroxide has also been shown to induce lysogenic prophages (Selva *et al.*, 2009). The mobility of these bacteriophages could allow genetic information to be shared amongst isolates in the population. Natural phages from *P. aeruginosa* have been found to transduce antibiotic resistance (Blahova *et al.*, 2000). In addition, the phage F116 (sharing some similarity with LES prophage 2 and 3) has been found to transduce resistance to imipenem, cefotaxime, kanamycin and carbenicillin (Blahova *et al.*, 1994). Our observations, coupled with those reported in a previous study implicating LES phages in the competitiveness of the strain in a rat chronic respiratory infection

Table 6. Presence of free LES phages in CF patient sputum samples

The presence of four *P. aeruginosa* LES phages was determined using PCR assays performed directly on Sputasol-treated, diluted, filter-sterilized and DNase I-treated sputum samples representing samples taken at the beginning of the exacerbation (sample A), samples taken during the exacerbation and antibiotic treatment (sample B), samples taken at the end of the exacerbation (sample C). Nested primers were used. +, PCR-positive; –, PCR-negative.

Patient	Sample	Phage 2	Phage 3	Phage 4	Phage 5
I	A	+	+	+	–
	B	+	–	+	–
	C	+	+	+	–
II	A	+	+	+	+
	B	+	+	+	–
	C	+	+	+	–
III	A	–	–	+	–
	B	+	+	+	–
	C	+	+	+	–

model (Winstanley *et al.*, 2009), suggest that the general role of bacteriophages in infections in the CF lung merits further investigation.

Consequences of population diversity for routine diagnostic practice

This study questions routine diagnostic laboratory practice whereby one or two isolates are chosen according to their colony morphology and antimicrobial susceptibility testing is carried out on these isolates. Our study shows that each population carries a large amount of variation in antimicrobial susceptibility and other phenotypes, and that this includes differences between isolates sharing the same colony morphology. Variations in antimicrobial susceptibility results for sputum samples both within and between laboratories have been observed by Foweraker *et al.* (2005). Furthermore, it has been widely reported that antibiotic susceptibility tests are poorly predictive of clinical outcomes in chronic *P. aeruginosa* infection in CF, using either aerosolized or intravenous therapy (LiPuma, 2001; Smith *et al.*, 2003). It has also been shown that reducing the number of routine tests has no impact on short-term clinical outcomes (Etherington *et al.*, 2008). Therefore, in the context of CF sputum samples, it is difficult to defend the practise of choosing one or two isolates to represent a whole population of *P. aeruginosa*, and basing therapeutic decisions on the antibiotic susceptibilities derived from this.

In addition, we cannot claim that a sputum sample is entirely representative of the lung population. However, sputum analysis is widely used in medical microbiology for respiratory pathogens, and especially in the context of CF. Hence, we contend that the approach we have taken is

enough to give us a snapshot of the LES populations in the CF lung environment.

Overall, our observations indicate that changes occur in the make-up of the *P. aeruginosa* LES populations during short periods of intense selective pressure, demonstrating the versatility and adaptability of this organism. Each set of 40 isolates was a mixture of LES subtypes; phenotypic variability was found within each sputum sample for all three patients. Every sample contained variation with respect to colony morphology, virulence factor production and antimicrobial susceptibility profiles. Even though our study tested only three patients, it seems likely that similar variations within *P. aeruginosa* are a general feature of chronically infected CF patients. A larger study, following sequential samples from multiple patients, should enable an improved understanding of the dynamics of evolving *P. aeruginosa* populations. We conclude that the 'metacommunity' needs to be considered when dealing with both the study and the treatment of chronic *P. aeruginosa* infection in CF patients.

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