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Original Article

Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis

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Abstract

Background: A diverse array of bacterial species is present in the CF airways, in addition to those recognised as clinically important. Here, we investigated the relative impact of antibiotics, used predominantly to target *Pseudomonas aeruginosa* during acute exacerbations, on other non-pseudomonal species.
Methods: The relative abundance of viable *P. aeruginosa* and non-pseudomonal species was determined in sputa from 12 adult CF subjects 21, 14, and 7 days prior to antibiotics, day 3 of treatment, the final day of treatment, and 10–14 days afterwards, by T-RFLP profiling.
Results: Overall, relative *P. aeruginosa* abundance increased during antibiotic therapy compared to other bacterial species; mean abundance pre-antibiotic 51.0±36.0% increasing to 71.3±30.4% during antibiotic (ANOVA: $F_{1,54}=5.16$; $P<0.027$). Further, the number of non-pseudomonal species detected fell; pre-antibiotic 6.0±3.3 decreasing to 3.7±3.3 during treatment (ANOVA: $F_{1,66}=5.11$; $P<0.027$).
Conclusions: Antibiotic treatment directed at *P. aeruginosa* has an additional significant impact on non-pseudomonal, co-colonising species.

Keywords: Antibiotics; Pulmonary exacerbation; Bacterial diversity; T-RFLP; Relative abundance

1. Introduction

Pseudomonas aeruginosa is commonly cultured from the adult CF lung and chronic infection with this organism is also associated with reduced life expectancy [1]. In patients who are chronically infected, *P. aeruginosa* is commonly the primary target for antibiotic therapy in response to the onset of CF pulmonary exacerbation (CFPE) [2]. In such cases, it is common practise to administer two anti-pseudomonal antibiotics with different modes of action; for example, a β-lactam and aminoglycoside [2].

Whilst often referred to as anti-pseudomonal, antibiotics such as ciprofloxacin, ceftazidime, and tobramycin, are broad spectrum, capable of affecting a wide array of Gram positive and Gram negative bacteria. This is potentially important given the many other bacterial species have been reported in adult CF lower airway secretions in addition to *P. aeruginosa* [3-10].

In the most part, this wider bacterial diversity has only come to light with the recent application of culture-independent, molecular profiling techniques that avoid the often substantial challenge of growing bacteria *in vitro*. As such, with the exception of a relatively small group of species recognised as CF pathogens, the clinical significance of these species is not yet known. It has,

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however, been shown that a substantial proportion of these wider species are capable of altering the virulence of *P. aeruginosa* [11,12]. As such, the impact of antibiotics used to target *P. aeruginosa* infections on non-pseudomonal species might be a contributory factor in treatment outcome.

The culture-independent approach of terminal restriction fragment length polymorphism (T-RFLP) analysis has been shown recently to be informative in tracking changes in the presence or absence of species over periods of antibiotic treatment [9]. By differentiating between bacterial species based on their 16S ribosomal RNA gene sequences, T-RFLP profiling allows *P. aeruginosa* populations to be distinguished from populations of other bacterial species present in the same sample, and their relative abundances assessed. Such analysis alone cannot however report accurately changes in relative bacterial population sizes in response antibiotic treatment. This is because DNA present in bacteria killed by action of antibiotics, or subsequently released into the extracellular environment, will contribute to the signal derived. To prevent this, a photochemical strategy of pre-treating samples prior to DNA extraction with propidium monoazide (PMA) can be used to limit signal to only that from viable bacterial cells [13,14]. The principle is based on membrane integrity as a common sign of viability: PMA does not enter cells with intact membranes, whereas it readily does so in cells with compromised membranes [15]. In this way, the combination of sample pre-treatment and T-RFLP profiling allows shifts in the relative abundance of viable bacteria from different species to be determined.

Here, we applied such a strategy to the analysis of sputum samples from twelve adult CF patients, prior to, during, and following, antibiotic treatment for pulmonary exacerbation.

2. Materials and methods

2.1. Subjects and clinical samples

This study was undertaken with local ethical approval from Southampton and South West Hampshire Research Ethics Committee (06/Q1704/26). Sputum samples were collected prospectively from 14 adult CF subjects at Southampton General Hospital, Southampton, UK. Subjects were selected that were persistently productive of sputum. All subjects provided sputum samples and clinical monitoring information three times per week for at least 12 months.

All subjects had a previous history of CFPE. During the study period only 12 of 14 patients experienced at least one exacerbation, and only data from these are presented. The start of a CFPE was defined by the clinician's decision to initiate antibiotic therapy for deteriorating clinical status, broadly based on a range of factors described previously [17]. In turn, the end of CFPE was defined by the decision to cease antibiotic therapy due to stabilisation or improvement in signs and symptoms.

Clinical information and details of antibiotic treatment given during CFPE for these twelve patients are shown in Table 1. A range of antibiotic selections was administered. In addition to antibiotics selected as anti-pseudomonal treatments (ciprofloxacin, colomycin, tobramycin, meropenem, amikacin, gentamicin), antibiotics used included those that potentially would be active against *P. aeruginosa*, but which were not selected for their anti-pseudomonal properties (doxycycline - Patient 3, and clarithromycin - Patient 9), and agents that would not be expected to have an anti-pseudomonal impact (metronidazole, used in conjunction with ciprofloxacin – Patient 10).

Samples were selected retrospectively for analysis that fell approximately 21, 14, 7 days prior to the start of antibiotic treatment for pulmonary exacerbation, and then at day 3 of treatment, on the final day of treatment, and 10–14 days after the end of treatment.

2.2. Clinical assessment

Prospective monitoring of clinically relevant symptoms and spirometry was undertaken at the time of each sputum sample. Lung function (FEV_1) was assessed using a Koko PeakPro home spirometer (Ferraris Cardiorespiratory, Louisville, CO, USA). In addition, four respiratory symptoms were measured using visual analogue scales (VAS), with patients asked to assess levels of breathlessness, cough, sputum production, and general well-being, scored from 0 to 100, representing worst and best respectively.

Table 1
Patient information.

Subject	Age	Gender	Genotype I	Genotype II	FEV_1 (% predicted)	BMI	Diabetic	Antibiotics given for CFPE
1	30	Male	phe508del	Unknown	54.9	29	No	Ciprofloxacin PO
2	45	Female	phe508del	phe508del	40.2	18.5	Yes	Colomycin IV + tobramycin IV
3	30	Female	phe508del	711 + 3A7G	38.2	25	No	Doxycycline PO
4	22	Female	phe508del	phe508del	36.2	19	No	Ciprofloxacin PO, then meropenem IV + amikacin IV
5	55	Male	phe508del	G85E	52.2	24.5	No	Ceftazidime IV + gentamicin IV
6	21	Female	phe508del	phe508del	56.6	19	No	Ciprofloxacin PO
7	22	Male	phe508del	phe508del	16.5	17.9	Yes	Meropenem IV + colomycin IV
8	18	Female	phe508del	phe508del	84	22.5	No	Ceftazidime IV + gentamycin IV
9	24	Female	phe508del	G542X	72.5	23.4	No	Clarithromycin PO
10	20	Male	phe508del	phe508del	26.8	30.4	No	Ciprofloxacin PO + metronidazole PO
11	20	Male	phe508del	phe508del	54.4	21	No	Ceftazidime IV + gentamycin IV
12	23	Male	phe508del	phe508del	53.6	20.7	Yes	Meropenem IV + tobramycin IV

2.3. Sample collection and processing

Samples were refrigerated immediately on collection. A portion of all samples was aseptically removed and submitted for routine CF microbiological assessment at the Health Protection Agency South East laboratory, in accordance with Health Protection Agency standard operating procedures [16]. Samples were then frozen prior to processing and transported by courier in accordance with national guidelines [2,16].

2.4. Propidium monoazide cross-linking

Propidium monoazide (PMA) treatment was performed as described previously [18]. Specifically, PMA was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mmol/L with this added to samples to give a final concentration of 50 μmol/L. Following an incubation period of 30 min in the dark with occasional mixing, samples were light exposed using LED Active Blue equipment (IB—Applied Science, Barcelona, Spain).

2.5. DNA extraction, PCR amplification, and T-RFLP profile analysis

Nucleic acid extractions were performed on 250 μL portions of PMA-treated sputum. Guanidium thiocyanate–EDTA–sarkosyl (500 μL) and phosphate buffered saline, pH 8.0 (500 μL) were added to samples. Cell disruption was performed using a Fastprep-24 instrument (MP Biomedicals Europe, Illkirch, France) at 6.5 m/s for 60 s, followed by incubation at 90 °C for 1 min and −20 °C for 5 min. Cell debris was pelleted by centrifugation at 12,000*g* for 2 min at 4 °C. Precipitation of DNA from supernatant was achieved by the addition of NaCl (to a final concentration of 0.5 mol/L) and polyethylene glycol (to a final concentration of 15% v/v), with incubation at 4 °C, for 30 min. DNA was pelleted by centrifugation at 12,000*g* at 4 °C for 2 min and resuspended in 300 μL of sterile distilled water. Samples were heated at 90 °C for 30 s and vortexed. Next, 0.3 mL of Tris-buffered phenol (pH 8.0) was added to each sample before the tubes were vortexed vigorously. After centrifugation at 12,000*g* for 10 min, supernatants were transferred to fresh microcentrifuge tubes. A further 0.3 mL of Tris-buffered phenol (pH 8.0)–chloroform–isoamyl alcohol (25:24:1) was added, and the mixture was vortexed vigorously. After centrifugation at 12,000*g* for 10 min, DNA was precipitated by the addition of an equal volume of isopropanol, a 0.1 volume of 10 M ammonium acetate, and 1 μL GenElute linear polyacrylamide (Sigma–Aldrich, Gillingham, UK), with incubation at −20 °C for 1 h. DNA was pelleted by centrifugation at 12 000*g* for 5 min and washed three times in 70% ethanol. Pellets were air dried and resuspended in 50 μL sterile distilled water. DNA extracts were quantified using a Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK).

16S rRNA gene amplification was performed using the universal oligonucleotide primers, 8f700IR (5′-700IR-AGAGT TTGATCCTGGCTCAG-3′) and 926r (5′-CCGTCAATTCATTT GAGTTT-3′) as described previously [3]. The constituents of the PCR reaction mixture were as follows: 25 μL of Sigma readymix REDTaq (Sigma–Aldrich), each primer at a final concentration of 0.2 μM, 50 ng of DNA template, made up to a final reaction volume of 50 μL with nuclease-free water. Cycling conditions comprised an initial denaturation at 94 °C for 2 min, followed by 32 cycles of denaturation at 94 for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min, with a final extension step at 72 °C for 10 min.

Approximately 20 ng of each PCR product was digested to completion with 1 U of the restriction endonuclease *Cfo*I (Sigma–Aldrich) for 5 h at 37 °C. Approximately 10 ng of digested PCR product was denatured at 95 °C for 1 min and separated by length using a 25 cm SequagelXR denaturing polyacrylamide gel (National Diagnostics, Hessle, UK), containing 8.3 M urea and formamide 10% v/v. Electrophoresis was performed at 55 °C and 1,200 V on an IR2 automated DNA sequencer (LI-COR Biosciences, Lincoln, US).

T-RF band lengths and volumes were determined by comparison to marker microSTEP 15a (700 nm) (Microzone, Lewes, UK) using Phoretix one-dimensional advances software (version 5.10; Nonlinear Dynamics, Newcastle upon Tyne, UK). T-RF band volume was expressed as a percentage of the total band lane volume. The resolution of T-RFLP bands was over the region of 50 to 950 bases, with a detection threshold of 0.1% of total profile signal.

Each of the 72 samples collected was analysed by T-RFLP profiling. The number of T-RF bands detected in each sample (species richness), and their relative abundance, was determined. Further, the relative proportions of all profiles that represented T-RF bands derived from *P. aeruginosa* (defined as those of 155 bases) and T-RF bands from other species, were calculated.

2.6. Statistical analysis

One-way ANOVA tests (including, *F*-ratio, degrees of freedom, and significance (*P*)) and summary statistics were calculated using Minitab software (version 14.20, Minitab, USA).

3. Results

Results of routine diagnostic microbiology for the period preceding CFPE are shown in Table 2. The presence of *P. aeruginosa* in all subjects was confirmed by PCR, as described in a previous study involving the same patient group [19].

Spirometry data for the patient group are shown in Fig. 1a. A general trend of decreasing FEV_1 from baseline values was observed in the period prior to the start of antibiotic treatment, with FEV_1 recovery to pre-treatment levels observed in response to therapy, and extending beyond its cessation. In keeping with observed FEV_1 data, summed VAS symptom scores increased in the period prior to the initiation of antibiotic therapy, and decreased in the period during, and following treatment (Fig. 1b).

T-RFLP profiling was performed on all sputum samples following PMA treatment. Mean species richness for each subject ranged from 1.2 to 9.0 species, with a mean richness for the sample set as a whole of 5.5 species (SD 3.5 species). T-RFLP profiles showed relative levels of *P. aeruginosa* to be highly variable between individual subjects (Supplementary

Table 2
Conventional diagnostic microbial. Scant growth is denoted by "+," moderate growth by "++" and heavy growth by "+++."

Subject	Culture 1	Culture 2	Culture 3	Culture 4
1	*Pseudomonas spp.* (++)	Oral flora (+)		
2	*P. aeruginosa* (+++)	*Pseudomonas spp.* (+++)	Oral flora (++)	*A. fumigatus* (+++)
3	*P. aeruginosa* (+++)	MRSA (*S. aureus*) (+)	Oral flora (+++)	
4	*Pseudomonas spp.* (+++)	Oral flora (+++)	*P. aeruginosa* mucoid (+++)	*A. fumigatus* (+++)
5	*Pseudomonas spp.* (+++)	Yeasts (+)	Oral flora (+++)	*P. aeruginosa* (+++)
6	*P. aeruginosa* (+++)	Mucoid *P. aeruginosa* (+++)		
7	*Pseudomonas spp.* (+++)	*Pseudomonas spp.* (+++)	*Pseudomonas spp.* (+++)	Oral flora (+++)
8	*Pseudomonas spp.* (+++)	Oral flora (+++)	Yeasts (+)	*A. fumigatus* (+)
9	*P. aeruginosa* (+++)	*Pseudomonas spp.* (+++)	Yeasts (+)	*A. fumigatus* (+)
10	*Pseudomonas spp.* (+++)	*Pseudomonas spp.* (+++)	*Pseudomonas spp.* (+++)	Oral flora (+++)
11	mucoid *P. aeruginosa* (+++)	*Pseudomonas spp.* (+++)		
12	*Pseudomonas spp.* (+++)	*Pseudomonas spp.* (+++)	Oral flora (+++)	

Fig. 1a). However, when pre- and during mean percentage relative abundance of *P. aeruginosa* was compared within individual subjects, only one subject (Patient 5), displayed a decrease in relative abundance following initiation of antibiotic treatment (Supplementary Fig. 1b). In addition, bacterial taxa richness (excluding *P. aeruginosa*) was also highly variable between subjects (Supplementary Fig. 1b). However, within individual subjects, only subject (Patient 8) showed an increase in bacterial richness following antibiotics. The overall pattern observed was a decrease in mean richness of bacterial taxa detected during treatment (Supplementary Fig. 1b). For illustrative purposes, an example of changes observed in relative T-RF band intensity over the six time points from one patient (Patient 9) is shown in Supplementary Fig. 2.

When the patient group was analysed as a whole, the abundance of *P. aeruginosa* relative to non-pseudomonal species was seen to increase as a result of antibiotic therapy (Fig. 2a). Whilst the difference in relative *P. aeruginosa* abundance was not significant at any one time-point (ANOVA: $F_{5,50}=1.02$; $P=0.413$), the increase in relative abundance was significant during antibiotic therapy when compared to the pre-treatment period (ANOVA: $F_{1,54}=5.16$; $P<0.027$); where the mean abundance pre- was 51.0±36.0% increasing to 71.3 ±30.4% during treatment (Fig. 3a). Further, the overall mean bacterial taxa richness was observed to decrease after the initiation of antibiotic therapy treatment (Fig. 2b). Again, no single time point showed a significant difference in mean bacterial taxa richness (Fig. 2b) (ANOVA: $F_{5,62}=0.99$; $P=0.431$); however, the decrease in richness was significant when the overall pre-treatment and during treatment data were compared (ANOVA: $F_{1,66}=5.11$; $P<0.027$), where mean richness pre-treatment was 6.0±3.3 decreasing to 3.7±3.3 during treatment (Fig. 3b).

4. Discussion

Antibiotic therapy for pulmonary exacerbations in CF is associated with both positive clinical outcomes [20-22] and a reduction in the number of viable bacteria present in airway secretions [14]. However, the possibility that the beneficial effects that result from antibiotic therapy are contributed to by their impact on species other than those being targeted, has been raised [23]. These non-targeted bacteria represent a wide array of species, and commonly include facultative and obligate anaerobes more traditionally associated with the oral cavity [3-10].

Our aim was to investigate the relative impact of antibiotics on *P. aeruginosa* and non-pseudomonal species in patients during treatment for pulmonary exacerbation. In most cases, where patients are chronically infected with *P. aeruginosa*, antibiotics tend to be selected for their anti-pseudomonal activity. It might be expected therefore that a greater impact would be observed on *P. aeruginosa* populations as compared to populations of other, non-pseudomonal species that are

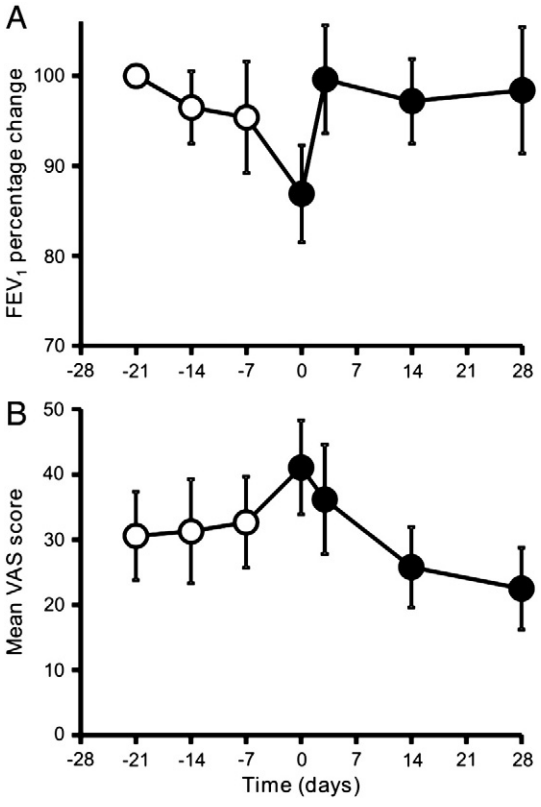

Fig. 1. The (A) relative change in forced expiratory volume in the first second (FEV1), and (B) summed VAS scores, before during and following initiation of treatment for cystic fibrosis pulmonary exacerbation (CFPE) in twelve subjects. Error bars represent 95% confidence interval around the median.

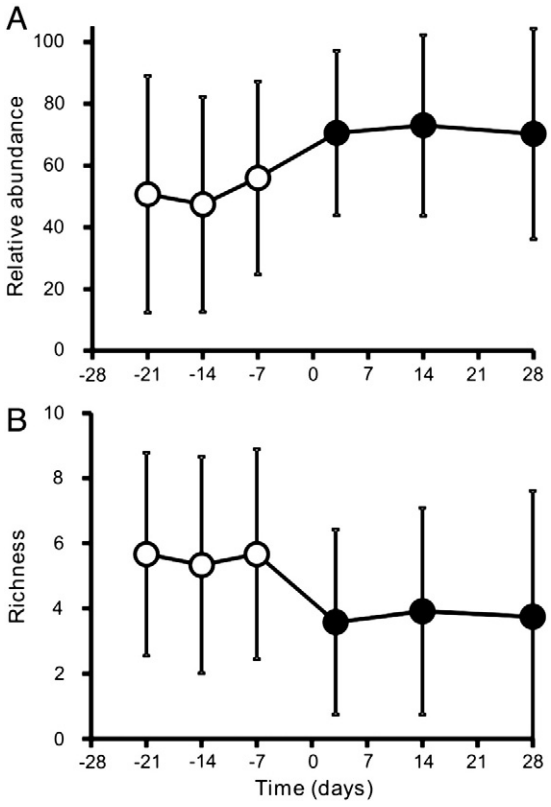

Fig. 2. The (A) mean relative abundance (%) of *Pseudomonas aeruginosa* and (B) mean bacterial taxa richness (excluding *P. aeruginosa*) over the time series. Open circles denote pre-IV treatment and closed circles denote during/post IV treatment. Error bars represent the standard deviation of the mean ($n=12$).

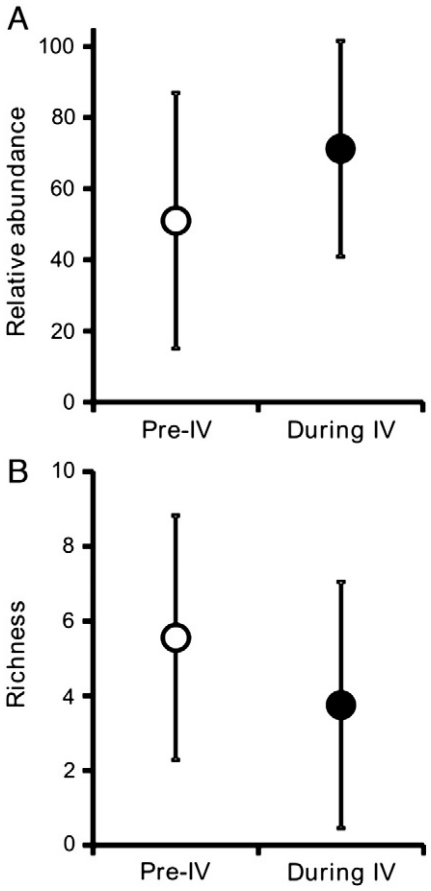

Fig. 3. The (A) mean relative abundance (%) of *Pseudomonas aeruginosa* and (B) mean bacterial taxa richness (excluding *P. aeruginosa*) pre- and during IV treatment. Error bars represent the standard deviation of the mean ($n=36$).

present in the lower airways. Were this to be true, a decrease in the relative abundance of *P. aeruginosa* would be seen. Further, given that in most cases, eradication of *P. aeruginosa* is not realistic in chronically infected adult patients, it is likely that there would be little change in the number of species detected. However, in contrast to such expectations, the analysis performed here suggests that the opposite is true (when the patient group is considered as a whole). Instead, an increase in the relative abundance of *P. aeruginosa* was observed, with a decrease in the total number of species detected.

An explanation for this observation is that, whilst absolute numbers of both *P. aeruginosa* and non-pseudomonal species decrease as a result of antibiotic therapy, the relative decrease in non-pseudomonal numbers is greater. In turn, this leads to an increase in relative *P. aeruginosa* abundance. Further, the reduction in viable non-pseudomonal bacterial numbers results in fewer species being detected, and hence a decline in species richness.

These findings are not without precedent. Using standard, selective cultures, anti-pseudomonal antibiotics have been shown to have more significant antimicrobial effects on *Haemophilus influenzae* than on *P. aeruginosa in vivo* [24]. T-RFLP has been used previously to investigate the impact of antibiotic therapy on bacterial community composition during CFPE. However, Tunney et al. reported little impact on overall bacterial diversity using such an approach, in contrast to the findings set out here [9]. A likely reason for this discrepancy is the effect of the steps taken here to limit the contribution of DNA from dead bacterial cells, namely PMA treatment of samples prior to DNA extraction. The contribution of DNA in non-viable bacterial cells an in the extracellular matrix has been shown previously to contribute significantly to amplifiable DNA from CF sputum [13,14]. We recognise that any delay in processing a sample following expectoration may lead to a decrease in its viable bacterial content. This is particularly true of anaerobic species and is most marked in the hour immediately following collection (data not shown). However, by employing a consistent sample handling schedule here, any such impact would have been constant across the sample set, and as such, could not explain the findings that we report.

The explanation for the reported observations set out above is supported by the fact that *P. aeruginosa* has a naturally high tolerance of antibiotics compared to many other species [25]. It is, for example, able to secrete a protective exopolysaccharide matrix, grow as micro-aggregates or biofilms that confer protection against antibiotics [26], and form sub-populations of "persister" cells that are highly antibiotic tolerant [27]. In addition, *P. aeruginosa* is able to adapt phenotypically to antibiotic exposure to become more even more resistant, for example, by antibiotic-mediated up-regulation of cyclic-di-GMP levels [28] and alginate synthesis [29,30]. Further, hypermutable forms of *P. aeruginosa*

are common in CF lung infections [31], a factor that increases the likelihood of strains developing resistance, particularly given the protracted periods for which they colonise the CF airways, and their repeated exposure to antibiotics.

It should be noted that an increase in the abundance of *P. aeruginosa* relative to non-pseudomonal species and a decrease in total bacterial richness were not observed for all patients. The relative impact of antibiotic treatment on these two groups will depend on a number of factors, including the level of antibiotic resistance and antibiotic tolerance exhibited by the *P. aeruginosa* strain(s) present in the patient's airways, the non-pseudomonal species that are present and their susceptibility to the antibiotics, type of antibiotics administered, and the patient's treatment history. However, the periods of treatment studied here were not selected on the basis of the antibiotics used, but were instead a random sample from the wider patient group. As such, the phenomenon of increasing relative *P. aeruginosa* abundance and decreasing bacterial richness might be a widespread consequence of antibiotic therapy for CFPE as commonly administered.

A reduction in the wider bacterial diversity relative to *P. aeruginosa* in the CF airways as a result of antibiotic therapy may not be of clinical significance, and may only represent a side-effect of therapy whose primary benefit is derived from its direct impact on *P. aeruginosa*. However, sufficient evidence of the modulation of *P. aeruginosa* virulence by non-pseudomonal species exists that the possible contribution of such an effect to the beneficial impact of antibiotic therapy cannot be discounted. As such, further investigation of whether a direct relationship exists is now warranted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2012.05.008.

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