Extensive diversification is a common feature of *Pseudomonas aeruginosa* populations during respiratory infections in cystic fibrosis

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Received 12 February 2013; received in revised form 28 March 2013; accepted 3 April 2013

Available online 1 May 2013

**Abstract**

**Background:** Populations of the Liverpool Epidemic Strain (LES) of *Pseudomonas aeruginosa* undergo extensive diversification in the cystic fibrosis (CF) lung during long-term chronic infections.

**Methods:** We analyzed sets of 40 isolates from the sputa of five CF patients, each chronically infected with a different non-LES strain of *P. aeruginosa*. For each sample (two per patient), diversity was assessed by characterizing nine phenotypic traits.

**Results:** All *P. aeruginosa* populations were highly diverse, with the majority of phenotypic variation being due to within-sample diversity.

**Conclusions:** Maintenance of diverse populations in the CF lung is a common feature of *P. aeruginosa* infections.

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**Keywords:** *Pseudomonas aeruginosa*; Cystic fibrosis; Population biology

**1. Introduction**

During chronic lung infections of cystic fibrosis (CF) patients *Pseudomonas aeruginosa* adapts by accumulating mutations associated with phenotypic adaptations, leading to populations of *P. aeruginosa* composed of multiple clones with differing antimicrobial susceptibility profiles [1,2]. Previously [3], we analyzed *P. aeruginosa* sputum populations from ten CF patients each infected with an important transmissible strain, the Liverpool Epidemic Strain (LES) [4,5], and showed that LES populations were highly diverse and dynamic during CF infections [3].

Studies using sequential isolates have demonstrated the accumulation of particular mutations that indicate adaptation to the CF lung [6–9]. A recent study of strain DK2, infecting multiple patients in Denmark, suggested that after the accumulation of mutations during the early stages of infection, a homogeneous population of DK2 emerged [10], appearing to contradict our observations.

Here, we examined the diversity of *P. aeruginosa* populations in five adult CF patients each chronically infected with a different non-LES strain of *P. aeruginosa*. To test whether extensive diversification is a feature unique to the LES, or common to *P. aeruginosa* infections of CF patients in general, we compared LES and non-LES populations from matched chronically infected adult CF patients.

**2. Materials and methods**

**2.1. Patients and samples**

Sputum samples were collected for routine diagnostic purposes from adult CF patients (CF20–CF24, Table 1) chronically infected (>5 years) with different non-LES strains of *P. aeruginosa* in
2009–2010. Strains were genotyped using an ArrayTube system [11] and identified according to the hexadecimal code generated by this method as genotypes 2F82 (CF21), 2C1A (CF22; Midlands 1 strain [12]), 0F1A (CF23), C80A (CF24) and AF9A (CF25).

Samples taken during periods of exacerbation (defined as previously [13]) were sub-divided into two categories: beginning of an exacerbation (acute 1), before intravenous antibiotic treatment had commenced; typically 3–7 days after admission (acute 2). For each patient included in this study, one acute 1 and one acute 2 sample was used. The five patients chronically infected with non-LES strains were matched with five LES-infected patients (Table 1). Comparison data for LES-infected patients was taken from a previous study [3]. As far as possible, patients with similar age, lung function (FEV1) and BMI were selected. This study was approved by the Local Research Ethics Committee (REC reference 08/H1006/47).

### Results

#### 3.1. Overall P. aeruginosa phenotypic diversity

High phenotypic diversity was apparent in the P. aeruginosa populations from each of the chronically infected CF patients. Based on the nine phenotypic traits analyzed, 400 isolates taken from 10 sputum samples from non-LES-infected patients comprised a total of 75 distinct phenotypic sub-types. The number of sub-types present within each patient is shown in Table 1, along with the frequency of each characteristic measured. When the data from LES-infected and non-LES-infected patients were analyzed together, there were 152 sub-types of P. aeruginosa present in total, of which 97 were found only in LES-infected patients, 76 were found only in non-LES-infected patients, and 12 were shared between the two groups (see Supplementary Fig. 1). There was a mean of 10 sub-types per set of 40 isolates for samples from non-LES-infected patients, compared to 10 for samples from LES-infected patients.

### Table 1

Patient details and phenotypic characteristic exhibited by sputum isolates from patients chronically infected with P. aeruginosa.

| Patient | Patient characteristics | Total nSub | Mutations (%) | Antibiotic resistance (%) | Colony morphology type (%) |
|---------|-------------------------|------------|---------------|----------------------------|---------------------------|
| Patient | Age (y) | FEV1 (%) | BMI | n | HM | Aux | TOB | COL | CEF | CIP | MER | TAZ | GM | GNMS | Mtr | MWO | StNMS | RM |
| Non-LES | CF20 | M | 26 | 60 | 23 | 80 | 22 | 25 | 95 | 61 | 0 | 75 | 42 | 20 | 17.5 | 2 | 48 | 0 | 2 | 48 | 0 |
| | CF21 | M | 30 | 43 | 15 | 80 | 15 | 61 | 10 | 0 | 0 | 25 | 50 | 20 | 0 | 0 | 47 | 0 | 3 | 50 | 0 |
| | CF22 | M | 21 | 38 | 16 | 80 | 8 | 0 | 100 | 0 | 0 | 2 | 5 | 0 | 0 | 0 | 2 | 76 | 0 | 0 | 22 | 0 |
| | CF23 | M | 21 | 38 | 21 | 80 | 23 | 0 | 71 | 2 | 0 | 31 | 21 | 34 | 24 | 0 | 0 | 0 | 25 | 75 | 0 |
| | CF24 | M | 22 | 54 | 20 | 80 | 16 | 22 | 0 | 0 | 0 | 61 | 90 | 46 | 0 | 0 | 76 | 0 | 24 | 0 | 0 |
| | All (mean) | 24 | 43 | 19 | 400 | 16.8 | 22 | 55 | 13 | 0 | 39 | 42 | 24 | 8 | 1 | 49 | 0 | 11 | 39 | 0 |

#### Abbreviations:
- FEV1: forced expiratory volume in 1 s
- BMI: body mass index
- HM: hypermutable phenotype
- Aux: auxotrophy
- TOB: tobramycin
- COL: colistin
- CEF: ceftazidime
- CIP: ciprofloxacin
- MER: meropenem
- TAZ: tazobactam/pipericillin
- GM: green mucoid
- GNMS: green non-mucoid smooth
- Mtr: mucoid transparent
- MWO: mucoid white opaque
- RM: red mucoid
- StNMS: straw coloured non-mucoid smooth

*nSub indicates the total number of different sub-types for each set of 80 isolates.*
3.2. Sub-type variation in individual patients

Hierarchical analysis of variance was performed on LES and non-LES groups separately to estimate the proportion of phenotypic variation attributable to (i) variation among patients, (ii) variation among samples within patients, and (iii) variation among isolates within samples. In both groups, the greatest contribution to overall diversity was due to phenotypic diversity between isolates within samples (LES-infected patients, 83%; non-LES infected patients, 81%). Overall, the LES and non-LES infections exhibited equivalent levels of diversity within a single sputum sample (Fig. 1a) and an equivalent degree of correlation between different sputum samples taken from the same patient (Fig. 1b).

3.3. Frequency of phenotypic traits

There were differences between the groups (non-LES-infected versus LES-infected patients) with respect to some of the phenotypes tested. For example, there was a far greater range in prevalence of isolates exhibiting the hypermutability phenotype amongst the non-LES samples [Non-LES (0–61%), LES (2–12%)]. Susceptibilities to commonly used antibiotics varied considerably within patient samples, with the exception of colistin (Table 1).

4. Discussion

Previous studies, tending to focus on small numbers of sequential isolates [6–8], have concluded that P. aeruginosa adapts in specific ways, including loss of virulence. Yet, it has been demonstrated that concurrent pairs of isolates can also vary considerably from each other [16], and we have shown previously that some strains exhibit dynamic turnover of sub-types exhibiting different phenotypic and genotypic characteristics [3]. It has been suggested that some strains reach an evolutionary plateau, characterized by low phenotypic variations [10], leading us to wonder whether the extensive diversification seen with chronic LES infections may be a particular feature of this strain. Here we analyzed samples from multiple adult CF patients infected with non-LES P. aeruginosa strains and compared them with equivalent samples from LES-infected patients. Our observations suggest that the extensive diversity reported previously is a widespread feature of P. aeruginosa populations in the lungs of CF patients who have been chronically infected for long periods. For both groups (LES and non-LES), the greatest contribution to the diversity in sub-types observed was within individual sputum samples, rather than because of variation between patients, even though the non-LES group patients were each infected with a different strain.

The wider range of frequencies of some of the phenotypic traits measured between the LES-infected and the non-LES-infected groups is likely to be due to strain-specific variations in the non-LES group. Hence, although the extent of diversity was not different between the two groups, the actual phenotypes contributing to the variations differed. It should be noted that the isolates were not randomly selected. However, the selection criteria were the same for each sample.

This study provides further evidence that the CF lung, which constitutes a spatially heterogeneous environment with multiple discrete ecological niches, is able to sustain multiple divergent sub-types of P. aeruginosa simultaneously, and that this is a common feature of these kinds of infections.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2013.04.003.

Acknowledgements

We thank Paul Roberts for the assistance with collection and archiving of isolates. Funding: This work was supported by a
Wellcome Trust grant 093306 [to S.P. and C.W.] and by the Dr Hadwen Trust for Humane Research.

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