Loss of Social Behaviours in Populations of *Pseudomonas aeruginosa* Infecting Lungs of Patients with Cystic Fibrosis

Natalie Jiricny[1], Søren Molin[2], Kevin Foster[1], Stephen P. Diggle[3], Pauline D. Scanlan[1], Melanie Ghoul[1], Helle Krogh Johansen[4], Lorenzo A. Santorelli[1], Roman Popat[5], Stuart A. West[1], Ashleigh S. Griffin[1]*

1 Department of Zoology, Oxford University, Oxford, United Kingdom, 2 Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark, 3 School of Life Sciences, Centre for Biomolecular Sciences, University Park, University of Nottingham, Nottingham, United Kingdom, 4 Institute for International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, 5 Centre for Immunity, Infection and Evolution, University of Edinburgh Ashworth Labs, King's Buildings, West Mains Road, Edinburgh, United Kingdom

Abstract

Pseudomonas aeruginosa, is an opportunistic, bacterial pathogen causing persistent and frequently fatal infections of the lung in patients with cystic fibrosis. Isolates from chronic infections differ from laboratory and environmental strains in a range of traits and this is widely interpreted as the result of adaptation to the lung environment. Typically, chronic strains carry mutations in global regulation factors that could effect reduced expression of social traits, raising the possibility that competitive dynamics between cooperative and selfish, cheating strains could also drive changes in *P. aeruginosa* infections. We compared the expression of cooperative traits - biofilm formation, secretion of exo-products and quorum sensing (QS) - in *P. aeruginosa* isolates that were estimated to have spent different lengths of time in the lung based on clinical information. All three exo-products involved in nutrient acquisition were produced in significantly smaller quantities with increased duration of infection, and patterns across four QS signal molecules were consistent with accumulation over time of mutations in *lasR*, which are known to disrupt the ability of cells to respond to QS signal. Pyocyanin production, and the proportion of cells in biofilm relative to motile, free-living cells in liquid culture, did not change. Overall, our results confirm that the loss of social behaviour is a consistent trend with time spent in the lung and suggest that social dynamics are potentially relevant to understanding the behaviour of *P. aeruginosa* in lung infections.

Introduction

Research at the interface of microbiology and evolutionary biology has revealed that populations of bacterial cells are complex communities of individuals that communicate, compete and cooperate with one another [1,2]. Many basic functions of bacterial cells involve interaction with neighbouring cells through the release of extra-cellular products. Biofilm formation, for example, involves the production of exo-polysaccharides, nutrient acquisition involves the release of degrading enzymes and iron chelators, and cell-to-cell communication involves the release of quorum sensing (QS) signal molecules. Exo-products are often costly to produce, and laboratory experiments show that cells which cheat by avoiding these costs, can outcompete cooperative neighbours, resulting in reduced survival and viability of the population as a whole [3,4,5]. The competitive dynamics of cooperators and cheats have been the subject of detailed study in the lab, but little is known about these dynamics in natural populations. In particular, very little is known about social behaviours during pathogenic infections, despite the fact that

many virulence factors involve the release of exo-products and are, therefore, potentially subject to exploitation [6,7].

The bacterium, *Pseudomonas aeruginosa*, has emerged as a model experimental system for studying social behaviour using strains that either cheat or cooperate to perform a number of important functions. *P. aeruginosa* is also widely studied as an opportunistic pathogen of plants, animals and humans and in particular, as a pathogen causing persistent and frequently fatal lung infections in patients with cystic fibrosis (CF) [8]. There is, however, a lack of studies combining these two fields of research to understand how social evolution influences the behaviour of *P. aeruginosa* as a pathogen [6,9,10].

There is evidence to suggest that a number of traits that have been shown to be cooperative and exploitable in laboratory cultures, may not be expressed, or have reduced expression in strains isolated from the sputa of CF patients with long-term *P. aeruginosa* infections: biofilm formation [11], the siderophore pyoverdin [12], exo-polysaccharide [13,14], pyocins [15]; pyocyanin [16], and quorum sensing signal molecules [17,18,19]. The evidence is inconsistent, however other studies show that the

ability to form biofilms is maintained in chronic isolates [20] and that exo-polysaccharide production is increased in chronic infections [21]. Furthermore, sequence analysis shows that mutations found to accumulate in lung isolates are primarily involved in regulation, making it difficult to assess what phenotypic characteristics are beneficial in the lung environment [18,22,23,24].

The loss of cooperative traits has been shown to confer a competitive advantage over cooperative, wildtype strains in the lab under certain conditions, raising the possibility that the same competitive dynamics are favouring invasion of non-cooperative cheats in the lung [3,5,6,25]. These observations are currently understood, however, to result exclusively from adaptation to the lung environment following colonisation from outside the lung [22,23,24,26]. One difficulty in distinguishing possible explanations arises from the lack of studies that (1) explicitly characterise how putatively social traits change with the duration of time spent in the lung, (2) assess whether there is a general trend across putatively social traits, and (3) determine to what extent this trend is replicated across patients.

In this paper, we report the results of a series of assays designed to provide a general picture of how traits demonstrated to be cooperative in the lab [3,4,5,27] are expressed in strains isolated from different stages of infection. Our sample included isolates from the sputa samples of sixteen patients and eleven isolates from outside the lung (naïve). By treating patients as independent data points, we avoid the common problem of pseudoreplication that results from treating sputa samples or isolates from the same patient as independent data points. As it is not possible without frequent and comprehensive sampling regime to determine the exact length of time a particular genotype has spent in the lung, we estimated this from the clinically defined duration of infection. The strains used in our study were either naïve strains isolated from outside the lung or from three different clinically defined stages of CF lung infection: acute - isolates that have caused a single, acute infection in a child with CF; chronic (<6 months) - isolates that have caused a single, chronic infection for less than six months in an adult with CF; and chronic (>6 months) isolates that have caused chronic infection of more than six month duration in adults with CF (Table S1 in File S1). We assayed three categories of traits shown to be cooperative and exploitable in the lab: (1) the production of extra-cellular products involved in nutrient uptake – protease, elastase and the iron-chelating siderophore, pyoverdin; (2) biofilm formation – a composite trait involving many genes and (3) communication through the production of QS cell-to-cell signalling molecules.

Materials and Methods

Origin of isolates

Our collection of *P. aeruginosa* isolates comprised of 11 harvested from outside the lung, either from the environment or different PA01 strains [28] originally isolated from a burn wound [29,30], six lung isolates from children with CF, four lung isolates from adult CF patients with chronic infections (<6 months duration) and six lung isolates identified as either the transmissible DK-1 or DK-2 strains [22] (Table S1 in File S1) from adult CF patients with chronic infections of >6 months duration (Table S1 in File S1). All clinical isolates were from sputum samples obtained by endolaryngeal suction if the patients are not sputum producers or by sputum cough [31]. Patients were randomly selected from the Copenhagen CF Centre at Rigshospitalet and all have been previously described elsewhere [32,33,34]. The sample size for all statistical analyses was 27.

To minimise potential for adaptation to the lab environment following isolation from source, all isolates were stored at $-80C$ until required.

Classification of isolates

We categorised 40 *P. aeruginosa* isolates according to whether they were isolated from the lung and the duration of infection: naïve (external), acute, chronic (<6 months) or chronic (>6 months) for a number of traits (Table S1 in File S1). Where multiple isolates per category were obtained from the same lung, their trait profiles were averaged before analysis to avoid pseudoreplication [35].

Measuring pyoverdin production

We prepared isolate cultures from freezer stock in 200 µL volumes of King's B medium (20 g Proteose peptone N°3, 10 mL Glycerol, 1.5 g $K_2HPO_4.3H_2O$, and 1.5 g $MgSO_4.7H_2O$ per litre) in a 96-well microtitre plate incubated at 37°C, 200 rpm for 24 h. After 24 h we transferred 1 µL of each culture to a 200 µL volume of iron-limited CAA (5 g Casamino acids, 1.18 g $K_2HPO_4.3H_2O$, 0.25 g $MgSO_4.7H_2O$, per litre with 100 µg mL^{-1} human apo-transferrin (Sigma) and 20 mM sodium bicarbonate [36,37] in a 96-well microtitre plate. The apo-transferrin in the CAA binds iron, triggering siderophore production in iron-starved bacteria. We incubated the cultures statically at 37°C for 24 h and then measured their cell density at an absorbance of 600 nm (A_{600}) [36,38], and pyoverdin production in relative fluorescence units (RFU), at an excitation wavelength of 400 nm and an emission wavelength of 460 nm [38] using a fluorimeter (SpectraMax M2, Molecular Devices, UK). We replicated each assay seven times per culture, and calculated the mean pyoverdin production per cell by RFU/A_{600}. We determined siderotypes using multiplex PCR to identify each isolate's ferripyoverdin receptor profile, as described by Bodilis *et al.* [39] (Figure S1 in File S1).

Measuring pyocyanin production

We assayed for pyocyanin production based on the method described by Essar *et al.* [40]. We cultured isolates from freezer stock in 10 mL King's B (KB) media (KB medium: 20 g Proteose peptone N°3, 10 mL Glycerol, 1.5 g $K_2HPO_4.3H_2O$, and 1.5 g $MgSO_4.7H_2O$ per litre) in glass 25-mL vials and incubated these for 24 h at 37°C, 180 rpm. We then vortexed and dispensed 100 µL of each culture into 1 mL cuvettes containing 900 µL dH_2O to measured A_{600} and then sub-cultured all isolates into 10 mL fresh KB in 100 mL conical flasks to maximise pyocyanin production, with final cell densities standardised to an A_{600} value of 1, using 10 mL of sterile KB as the control. Cultures were then incubated at 37°C, 180 rpm for 18 h before measuring A_{600} as above; 10 mL of each culture was transferred into 15 mL Falcon tubes and centrifuged at 1,470 g for 15 min. We then filter-sterilised (0.2 µm pore size) the supernatant and aliquoted 5 mL into a second set of Falcon tubes to which was added 3 mL chloroform before being vortexed for 5 sec and centrifuged at 1,120 g for 10 min. We transferred 2 mL of the bottom extraction layer of chloroform into a new 15 mL Falcon and added 2 mL 0.2 M HCl to each for re-extraction. After vortexing for 5 sec, we centrifuged these extraction layers at 1,120 g for 1 min, transferred 1 mL of each top layer to a 1.5 mL Eppendorf before centrifuging at 15,490 g for 1 min and removed 900 µL of the top layer of each Eppendorf to a cuvette to read the A_{520} of the pyocyanin in acidic solution, using the control as the blank. We then quantified pyocyanin production per culture as µg.mL^{-1} as (A_{520}/
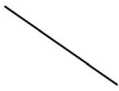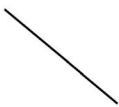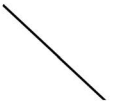
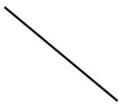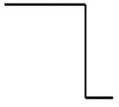| TRAIT	ANOVA/ Non-parametric test	Ordered Heterogeneity test (with Bonferroni critical P-values)	Tests with Merged Groups	Trend
Exo-products				
Pyoverdin	$F_{(1,3)}$ =18.415, **P=1.391^{e-05}** (P<0.0125)	P<0.0125		
Pyocyanin	(ln transformed) $F_{(1,3)}$ =4.9232, P=0.01217, n.s.	n.s.		
Protease	Kruskal-Wallis chi-squared = 12.8516, df=3, **P=0.004969** (P<0.0125)	P<0.0125		
Elastase	(exp transformed) $F_{(1,3)}$ =6.955, **P=0.002935** (P<0.0125)	P<0.0125		
Biofilm formation				
Proportion of cells in Biofilm	(ln transformed) $F_{(1,3)}$ =1.3672, P= 0.2885, n.s.	n.s.	1-3 merged vs 4 t = 1.693, P= 0.1386 n.s.	
Communication				
3-oxo-C$_{12}$-HSL (LC/MS)	(ln (x+1) transformed) Kruskal-Wallis χ-squared = 10.3996, df=3, P=0.01546, n.s.	P<0.0125		
PQS (LC/MS)	$F_{(1,3)}$=4.4192, P=0.01912, n.s.	n.s.	1-3 merged vs 4 t = 4.386, **P=0.0005509** (P<0.0125)	
C$_4$-HSL (LC/MS)	$F_{(1,3)}$= 4.0669, P=0.0252, n.s.	n.s.	Groups could not be merged	
C$_7$-HHQ (LC/MS)	(sqrt transformed) $F_{(1,3)}$=10.996, **P=0.0003640** (P<0.0125)	n.s.	Groups 1-3 merged vs 4; t = -5.0885, **P=0.0002145** (P<0.0125)	

Figure 1. A table summarising statistical analyses and trends in expression over time across all traits.
doi:10.1371/journal.pone.0083124.g001

A_{600})17.072 [41] then multiplied the result by 0.66, as only $^2/_3$ of the chloroform extraction layer was sampled [40,41].

Measuring total protease activity

We dispensed 250 μL Azocasein solution (2% Azocasein: Sigma, 2 mM $CaCl_2$, 40 mM tris-HCl, pH 7.8) into 2 mL Eppendorfs and added 150 μL filter-sterilised supernatant and the control supernatant (as described in the pyocyanin protocol) to each. We vortexed the Azocasein-treated supernatants for 5 sec before incubating them statically at 37°C for 45 min before stopping the reaction by precipitating out the undigested substrate in 1.2 mL trichloroacetic acid (10%) for 15 min and centrifuging at 15,490 g for 10 min. We then added 900 μL of each supernatant to 750 μL 1 M NaOH (1 M) in fresh Eppendorfs, vortexed for 5 sec then transferred 100 μL of each acid-treated supernatant into a cuvette containing 900 μL dH_2O and read A_{440} of each using the control as the blank. We calculated the protease activity of each isolates A_{440}/A_{600} of pre-filtered culture [42,43].

Measuring elastase activity

Methods for measuring elastase production was based on Ohman et al. (1980) [44]. We dispensed 900 μL Elastin Congo-Red (ECR) buffer (0.0275 g ECR:Sigma, 1 mM $CaCl_2$, 100 mM Tris, pH 7.5) into 2 mL Eppendorf tubes and added 100 μL of filter-sterilised supernatant and the control supernatant (as described in the pyocyanin protocol) to each. We then incubated ECR-treated supernatants at 37°C with shaking for 4 h before spinning-down the ECR granules (13,000 rpm for 1 min), transferring 100 μL supernatant off the top of each Eppendorf into 900 μL dH_2O in a cuvette and reading A_{495} of each, using the control as the blank.

Extraction of quorum sensing molecules

QS molecules extraction methods based on Ortori et al [45,46]. Isolates were cultured from freezer stock in 10 mL Luria Broth (LB, Sigma) media in glass 25-mL vials and incubated these for 24 h at 37°C, 180rpm, with 10 mL of sterile LB as the control. Cultures were then vortexed and 100 μL dispensed into 1 mL cuvettes containing 900 μL dH_2O for measuring cell density at A_{600}. We then transferred the 9.9 mL of each culture and the control into 15 mL Falcon tubes and centrifuged these at 1,470 g for 15 min before filter-sterilising (0.2 μm pore size) the supernatants and aliquoting 9 mL of each into a second set of Falcon tubes. We extracted QS molecules from the supernatants by adding 9.5 mL of acidified ethyl acetate (100 μl glacial acetic acid, 1 L ethyl acetate), vortexing these mixtures for 30 s and then centrifuging them for 10 min at 1,470 g to separate the organic and aqueous phases. We then removed 7 mL of the organic phase of each treated supernatant and dried them using a centrifugal evaporator (Jouan, RC10 22) for 2 h at 50°C. We reconstituted the dried samples in 1 mL methanol and aliquoted 500 μl of each into two 1.5 mL Eppendorfs. Samples were stored at −20°C.

Quantifying proportion of the cell population in biofilm

We quantified the proportion of cells growing as a biofilm in a microtitre plate using a BacTiter-Glo assay (BacTiter-Glo[TM] Microbial Cell Viability Assay kit, Promega). Cultures were inoculated from freezer stock and grown overnight, in 3 mL Luria Broth (LB) at 37°C, 200 rpm. We normalised the cultures to 0.05 A_{600} in 3 mL fresh LB, incubated them for 2 h to reach exponential phase, then diluted them to 0.0025 A_{600} in 10 mL 1% Bacto Tryptone media (TB: 10 g Bacto[TM] Tryptone (Becton, Dickinson & Co, Franklin Lakes, NJ, USA) in 1 L distilled H_2O (dH_2O)). We aliquoted eight 150 μL volumes of each culture into a flat-bottomed 96-well microtitre plate (plate A), filling one column with sterile TB as a negative control. Plates were sealed in
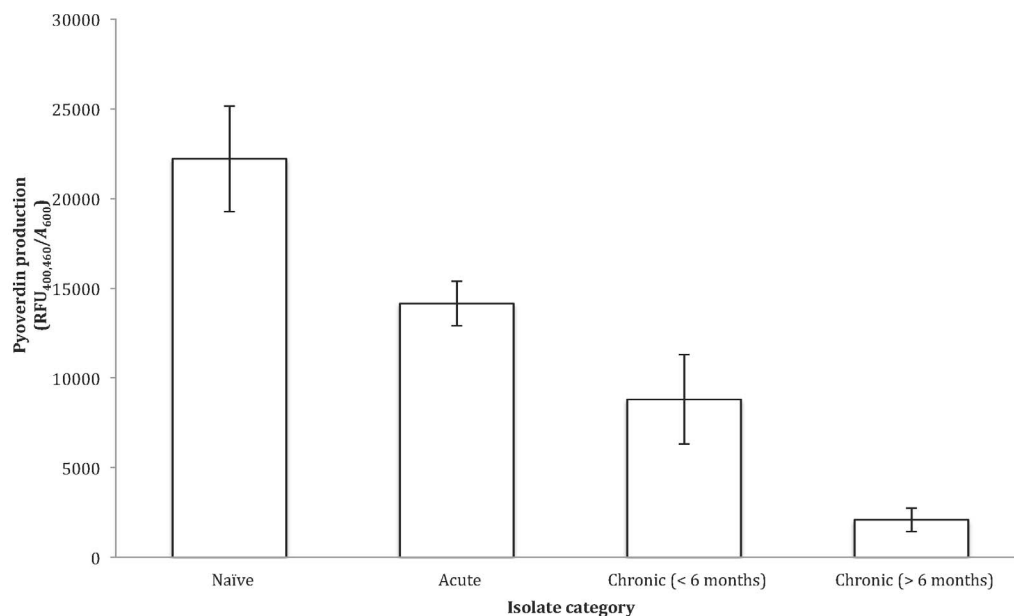

Figure 2. The estimated pyoverdin production per cell of the four *P. aeruginosa* isolate categories. Pyoverdin production calculated as RFU$_{400,460}$/A_{600}. Data shown as untransformed averages of five replicates per isolate per category ±SE.
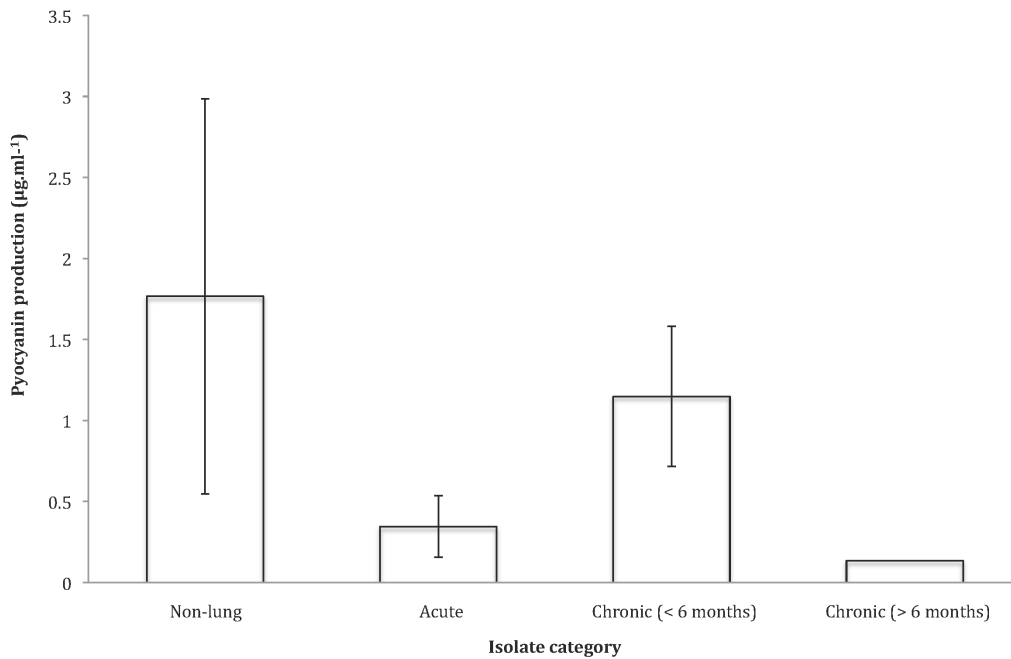doi:10.1371/journal.pone.0083124.g002

Figure 3. Pyocyanin production of the four *P. aeruginosa* isolate categories. Data shown as untransformed averages of five replicates per isolate per category ±SE.
doi:10.1371/journal.pone.0083124.g003

an airtight container and incubated at room temperature (RT) for 24 h. BacTiter-Glo (BTG) substrate and buffer was thawed and mixed before being left for a further 1 h 30 min to allow time for trace amounts of ATP in the reagent to burn-off – maximising the assay's sensitivity. After 24 h incubation we removed 40 µL of supernatant from each well of plate A and diluted these 1:2.5 in a 96-well plate containing 60 µL dH$_2$O (plate B). We then transferred 66 µL of the plate B dilutions to a black 96-well microtitre plate (plate C), and added 33 µL of BTG. We then washed plate A three times in distilled water (dH$_2$O) to remove the remaining supernatants, dried the plate on paper towels and loaded each well with 50 µL equilibrated BTG, quickly followed by 100 µL Phosphate Buffered Saline (PBS: dH$_2$O). We then placed plate A on a 750 rpm shaking platform for 5 min and then transferred 100 µL of each well of plate A to a black 96-well plate (plate D). We then read the luminescence per well of plates D and E immediately (t_1) in counts per minute (CPM) on a Top-Count.NXT^{TM} Microplate Scintillation & Luminescence counter. A second CPM reading was taken of both plates 2–4 h later (t_2). The two CPM readings were used to calculate the total luminescence emitted by the supernatant and biofilm populations of each culture, and these totals were used to back-calculate signal strength of each population at time 0 when the BTG was first added to each well ([CPM]$_0$). [CPM]$_0$ was calculated as:

$$\left(\frac{(CPM_1 - CPM_2)}{In(CPM_1/CPM_2)} \right) * e(kt_1)$$

where $k = \ln(CPM_1/CPM_2)/(t_2\text{-}t_1)$.

We then corrected the [CPM]$_0$ values for dilution factors and calculated the proportion of cells in a microtitre culture existing in biofilm as: biofilm [CPM]$_0$/(biofilm [CPM]$_0$ + planktonic[CPM]$_0$), using the average for the 8 replicates of each isolate for further analysis.

Quantification of QS signal molecules by liquid chromatography/mass spectrometry (LC/MS)

We prepared reconstituted organic extracts for isolates as described above. After sonicating for 5 min followed by centrifuging at 9,500 *g* for 10 min, we transferred extracted supernatant (described above) of each into a glass insert within a high performance liquid chromatography (HPLC) vial and sealed these with screw caps. For the liquid chromatography (LC) analysis of QS molecules *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C$_{12}$-HSL), *N*-butanoyl-L-homoserine lactone (C$_4$-HSL), 2-heptyl-4(1*H*)-quinolone (C$_7$-HHQ), and 2-heptyl-3-hydroxy-4(1*H*)-quino-lone (PQS), we used a mobile phase run through a pre-primed and equilibrated HPLC Agilent instrument, series 1200, with an Ascentis Express C18 column (150×2.1 mm internal diameter, 2.7 µm particle size). We then cleaned the Agilent HPLC system and recalibrated it into the positive ion mode to run full mass spectrometry (MS) scans on the samples for the same QS molecule range. We then used the Bruker DataAnalysis programme version 3.3 to interpret the LC/MS data. The programme matched LC retention times and MS peak spectra to the 10 µM QS standards injected at intervals throughout both assay methods to produce an LC/MS profile for each isolate.

Statistical analyses

We tested for significant differences with time spent in the lung (as estimated by source of isolate) using a one-way ANOVA analysis. We normalised residuals prior to analyses: data from biofilm and pyocyanin assys were transformed by natural log (ln(*x*)), and data from the elastase and the C$_7$-HHQ assays by exponential (exp(*x*)) and square root (sqrt(*x*)) respectively. We performed Kruskal-Wallis tests for non-parametric data sets to test for significant differences in production of protease and 3-oxo-C$_{12}$-HSL. We log-transformed 3-oxo-C$_{12}$-HSL data by (ln(*x*+1)) to normalise residuals. Ordered heterogeneity (OH) tests were used to test for the occurrence of a trend in the production levels of each
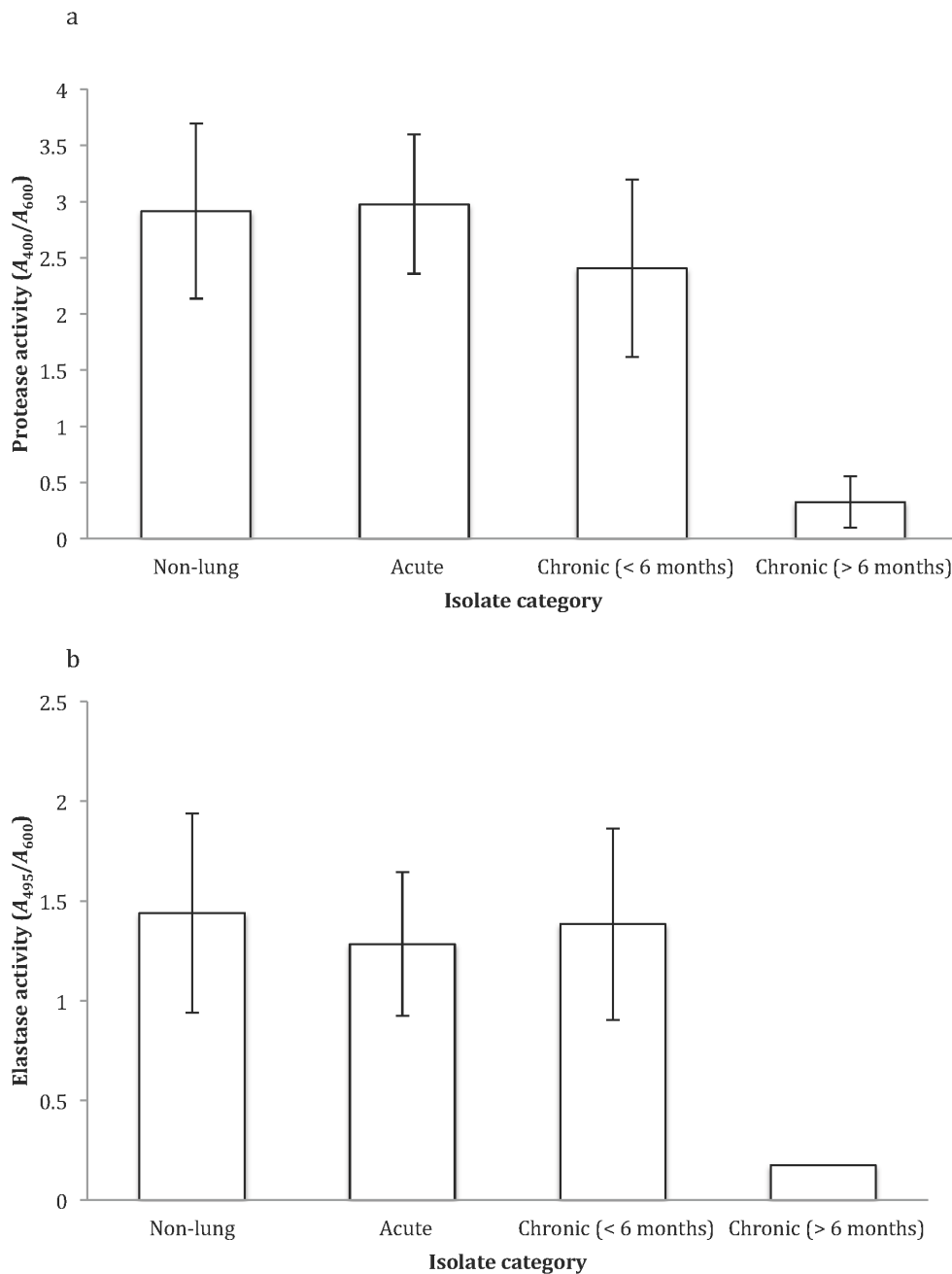
a

b

Figure 4. The production of ezymes protease (a) and elastase (b) of the four *P. aeruginosa* isolate categories. Protease and elastase activity calculated as A_{440}/A_{600} and A_{495}/A_{600}, respectively. Data shown as untransformed averages of five replicates per isolate per category ±SE.
doi:10.1371/journal.pone.0083124.g004

trait across the four isolate categories. We merged data that showed no significant differences in production across categories, naïve to chronic (confirmed by Tukey's HSD (honestly significant difference) test); Figure 1) and repeated the above analysis on merged data sets. When the merged data resulted in only two categories, we performed a t-test to check for significant differences, and no OH tests would be required in such cases. To correct for multiple comparisons being applied to the same dataset, the Bonferroni correction was applied to all analyses, with each test on exo-product traits requiring P-values of $P < 0.0125$ (0.05/4 tests) and each test of signal molecule production requiring $P < 0.0125$ (0.05/4 tests).

Binomial GLMs were used to check whether there is a significant representation of siderotypes I, II or III in each isolate category. All analysis was implemented in R statistical software (http://www.R-project.org) (Summary of all tests and significance levels reported in Figure 1).

Results

Secretion of exo-products

There was a significant negative correlation between the production of pyoverdin, and stage of infection, with the least productive strains isolated from patients with chronic infections
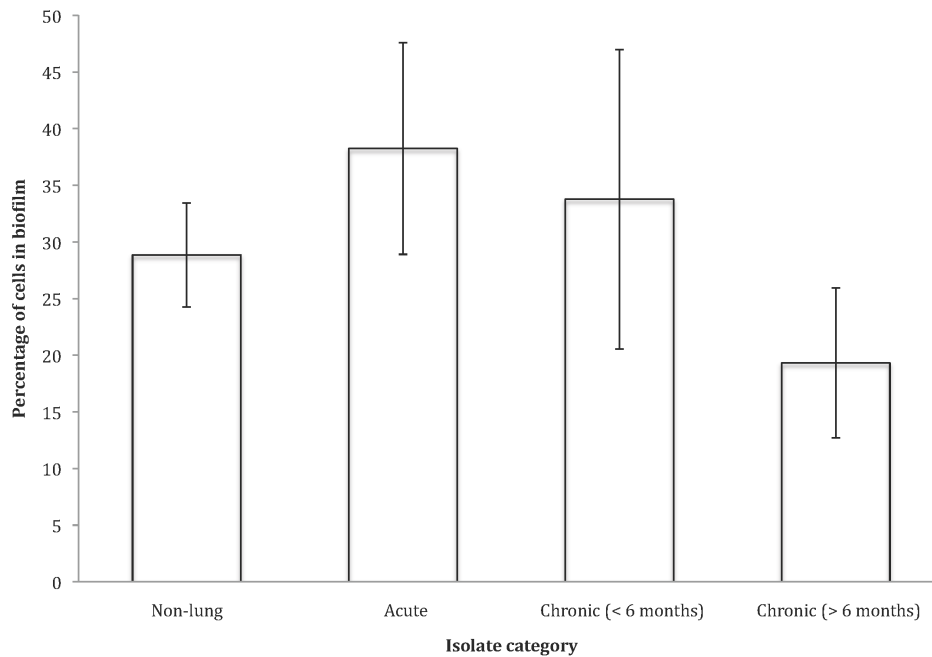
Figure 5. The percentage of cells in microtitre culture existing in biofilm of the four *P. aeruginosa* isolate categories. Data shown as untransformed averages of five replicates per isolate per category ±SE.
doi:10.1371/journal.pone.0083124.g005

(>6 months) (OH test: $F_{(1, 3)} = 18.415$, $P = P = 0.000014 <$ Bonferroni (0.0125) Figure 1, Figure 2). Pyocyanin production was not significantly correlated with duration of infection (Figure 1, Figure 3). OH test (ln transformed): $F_{(1, 3)} = 4.92$, $P = 0.01 >$ Bonferroni (0.0125)). Both protease and elastase production were significantly reduced with increasing duration of infection (Figure 1, Figures 4a and 4b): protease – Kruskall Wallis $\chi^2 = 12.85$, $p = 0.005 <$ Bonferroni (0.0125); elastase - OH test (exp transformed), $F_{(1, 3)} = 6.96$, $p = 0.003$, Bonferroni (0.0125).

Biofilm Formation

There was no significant difference in the proportion of cells in biofilm, relative to growing in suspension, across categories (OH test (ln transformed): $F_{(1, 3)} = 1.37$, $P = 0.29 >$ Bonferroni (0.05) Figure 1, Figure 5), suggesting that the propensity of cells to form biofilm is not reduced with duration of infection.

Communication

We found evidence for reduced production of two out of four quorum sensing signal molecules that we assayed with increased duration of infection (Figure 1, Figure 6a–6d): 3-oxo-C_{12}-HSL showed a significantly negative trend with increased duration of infection when assayed using LC/MS (ordered heterogeneity test $P <,0.001$ Bonferroni (0.0125)). We also found that PQS was significantly reduced in strains isolated from chronic infections (>6 months) relative to isolates in the other three categories (t = 4.386, p = 0.0006, Bonferroni (0.0125). In contrast, C_4-HSL showed no trend and the production of C_7-HHQ in strains isolated from patients with chronic infections (>6 months) was significantly greater than isolates from other categories (t = 2.27, P = 0.0002, Bonferroni (0.0125); Figure 1).

Discussion

We examined a range of social traits in *P. aeruginosa* strains isolated from outside and inside the lungs of cystic fibrosis patients

that were estimated to have been infected for different lengths of time. We found that: (1) pyoverdin, total protease and elastase production were reduced with increased duration of infection (Figures 1, 2, and 3); (2) the ability of cells to form biofilm showed no significant change with increased duration of infection (Figure 5); and (3) while the production of the QS signals 3-oxo-C12-HSL and PQS declined with increased duration of infection (Figures 6a and 6c), the production of C4-HSL did not change and HHQ increased (Figure 6b and 6d). Overall, these results suggest that a number of *P. aeruginosa* social traits, but not all, are lost over time, during the lung infections of cystic fibrosis patients. These changes could occur as a result of evolution within a lineage, or by displacement of one by another lineage – for example, chronic infections of >6 months are typically caused by one of two strains known to be transmissible between patients in the Danish CF community [22] (Table S1 in File S1).

The extent to which cooperative traits are vulnerable to exploitation is expected to depend on several factors and these factors are likely to vary widely between traits. For example, the ability of one cell to exploit a public good produced by another cell will depend on the diffusion distance of the molecule, the spatial structuring of the environment, and the ability of cells to produce exo-products which can only be utilised by cells of the same genotype. Specificity is likely to vary between traits: it has been shown that members of different species benefit from information obtained from signalling systems, the production of beta-lactamases. On the other hand, it has been shown that strains of *P. aeruginosa* produce pyoverdin molecules which produce iron-complexes that can only be taken up by cells of the same strain. High levels of diversifying selection at *pvd* syntheses loci are consistent with the possibility that this is a mechanism to prevent exploitation. Evidently, therefore, it is extremely challenging to make predictions about whether a population of cooperative bacteria is vulnerable to invasion by cheats. We would argue, however, that the possibility that interspecific competitive dynamics are at least partially responsible for the phenotypic patterns
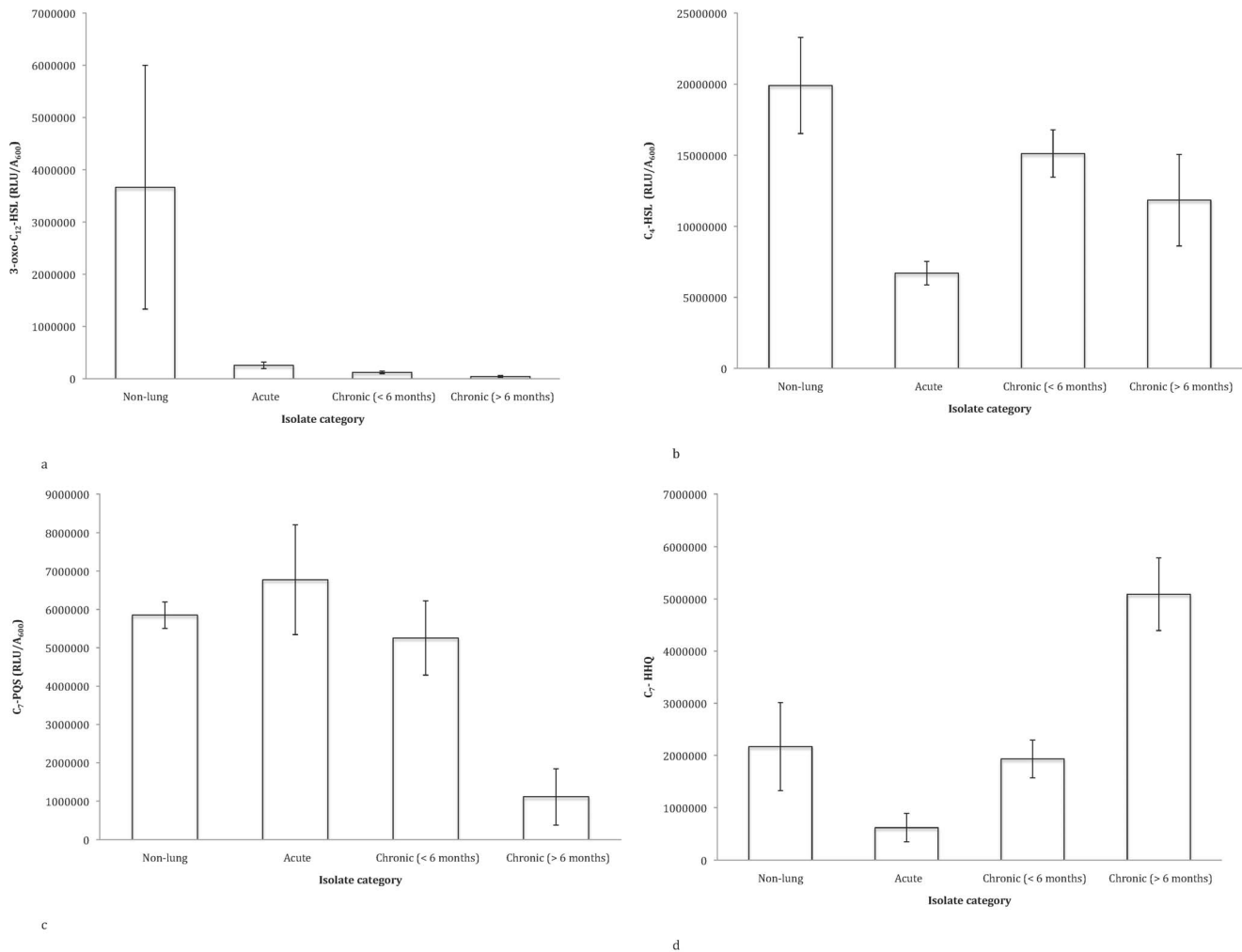
Figure 6. Relative production of (a) 3-oxo-C12-HSL and (b) C_4-HSL (c) PQS and (d) C_7-HHQ interpreted from LC/MS peak readings.
Data shown as untransformed averages of single replicates per isolate per category ±SE.
doi:10.1371/journal.pone.0083124.g006

described in this paper is important to consider. No natural population of organisms has yet been discovered to have evolved entirely in response to its environment, without the additional influence of intra- and inter-specific interactions.

In interpreting our observations, it is important to consider that the traits we measured are unlikely to vary independently from one another. Many of the trends we observed suggest the accumulation of *lasR* QS mutants with increasing duration of infection, a finding in common with previous studies [17,18,24]. We found a decrease in total protease, elastase and the autoinducer 3-oxo-C_{12}-HSL, the production of which are absent or greatly reduced in *lasR* mutants [47,48]. We also noted a decrease in PQS and an increase in HHQ. Again an accumulation of *lasR* mutants could explain these results. The production of HHQ is regulated by the *pqsABCDE* operon and conversion of HHQ into PQS is via PqsH, which is under the control of LasR [49]. We did not observe a trend in production of either the *rhl* QS signal molecule C_4-HSL or the *rhl*-dependent phenazine pyocyanin. It is known that activity of the *rhl*-system is only delayed in a *lasR* mutant and both C4-HSL and pyocyanin have been shown to be produced in the absence of LasR [50,51]. Complex interactions between loci emphasises the importance of studying phenotypic as well as genetic patterns as

the presence or absence of a gene may not necessarily effect phenotype and therefore exposure to selection [52].

A remaining challenge is to characterise the selective environment experienced by bacterial cells living in the lung environment. It is likely that the phenotypes of bacterial cells living inside the lung are largely determined by selection for antibiotic resistance, but the lung is a complex environment with multiple niches, which change during the course of an infection [22]. It is clear, however, that not all of the patterns we observed in this study are easily explained by adaptation alone. For example, production of the iron-chelator, pyoverdin, is predicted to decrease with availability of free iron in the environment, but we find that pyoverdin production is relatively high in isolates from acute infections where iron levels have been shown to be highest [53]. Similarly, production of the QS signal molecule PQS is specifically involved in evasion of the immune system by triggering defence mechanisms against polymorphonuclear phagocytes, and suppressing the activation of T-cells [54] but we find that PQS is reduced with duration of infection. Competition between cooperative and cheating strains, selecting for mutations that reduce investment in cooperative behaviours, could provide an additional explanation for observed changes in behaviour.

A primary aim of research into the behaviour of *P. aeruginosa* in the CF lung is to improve treatment strategies. Potentially the most important difference between a community shaped by adaptation to the lung and a community shaped by social interactions is the effect on transmission between patients. Cheats are expected to be poor at establishing a novel infection but have resistance to invasion from wild-type cooperative strains that will quickly be exploited. The highly transmissible Liverpool Epidemic Strains LES, has been characterised as having highly up-regulated quorum sensing systems but show similar patterns of down-regulation in strains isolated from chronic infections [55]. This suggests that cheating could reduce transmission but also raises questions about where highly transmissible strains originate from and how they are able to persist in populations dominated by QS mutants that have been shown to be competitively superior in the lab [3]. Taking social interactions into account could not only help explain the phenotypes observed in chronic infections but also why infections seem to be initiated by highly cooperative strains.

Supporting Information

File S1   Supporting information includes list of isolates in Table S1 and section on siderotype analysis. Data from siderotype analysis presented in Figure S1. Section 1: Categorisation of *P. aeruginosa* isolates; Table S1. Section 2: Results of siderotype analysis; Figure S1.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: NJ KF SPD SAW ASG. Performed the experiments: NJ PDS MG LAS RP. Analyzed the data: NJ MG. Contributed reagents/materials/analysis tools: SM KF SPD HKJ SAW ASG. Wrote the paper: NJ SM KF SPD PDS MG HKJ LAS RP SAW ASG.

References

1. Nadell C, Xavier J, Foster K (2009) The sociobiology of biofilms. FEMS Microbiology Reviews 33: 206–224.
2. West SA, Diggle SP, Buckling A, Gardner A, Griffins AS (2007) The social lives of microbes. Annual Review of Ecology Evolution and Systematics 38: 53–77.
3. Diggle SP, Griffin AS, Campbell GS, West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. Nature 450: 411–U417.
4. Jiricny N, Diggle SP, West SA, Evans BA, Ballantyne G, et al. (2010) Fitness correlates with the extent of cheating in a bacterium. Journal of Evolutionary Biology 23: 738–747.
5. Griffin AS, West SA, Buckling A (2004) Cooperation and competition in pathogenic bacteria. Nature 430: 1024–1027.
6. Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, et al. (2009) Quorum Sensing and the Social Evolution of Bacterial Virulence. Current Biology 19: 341–345.
7. Buckling A, Harrison F, Vos M, Brockhurst MA, Gardner A, et al. (2007) Siderophore-mediated cooperation and virulence in *Pseudomonas aeruginosa*. Fems Microbiology Ecology 62: 135–141.
8. Govan J, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. MIcrobiological Reviews 60: 539–574.
9. Köhler T, Buckling A, van Delden C (2009) Cooperation and virulence of clinical *Pseudomonas aeruginosa* populations. Proceedings of the National Academy of Sciences of the United States of America 106: 6339–6344.
10. Rumbaugh KP, Trivedi U, Watters C, Burton-Chellew MN, Diggle SP, et al. (2012) Kin selection, quorum sensing and virulence in pathogenic bacteria. Proceedings of the Royal Society B - Biological Sciences 279: 3584–3588.
11. Lee B, Haagensen JAJ, Ciofu O, Andersen JB, Hoiby N, et al. (2005) Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Journal of Clinical MIcrobiology 43: 5247–5255.
12. De Vos D, De Chial M, Cochez C, Jansen S, Tümmler B, et al. (2001) Study of pyoverdine type and production by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. Archives of Microbiology 175: 384–388.
13. Hancock R, Mutharia L, Chan L, Darveau R, Speert D, et al. (1983) *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infection and Immunity 42: 170–177.
14. Spencer DH, Kas A, Smith E, Raymond C, Sims EH, et al. (2003) Whole-genome sequence variation among multiple isolates of *Pseudomonas aeruginosa*. Journal of Bacteriology 185: 1316–1325.
15. Römling U, Fiedler B, Bosshammer J, Grothues D, Greipel J, et al. (1994) Spidemiology of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis. Journal of Infectious Diseases 170: 1616–1621.
16. Carlsson M, Shukla S, Petersson A, Segelmark M, Hellmark T (2011) Pseudomonas aeruginosa in cystic fibrosis: pyocyanin negative strains are associated with BPI-ANCA and progressive lung disease. Journal of Cystic Fibrosis 10: 265–271.
17. Salunkhe P, Smart C, Morgan J, Panagea S, Walshaw M, et al. (2005) A cystic fibrisos epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. Journal of Bacteriology 187: 4908–4920.
18. Smith E, Buckley D, Wu Z, Saephimmachak C, Hoffman L, et al. (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proceedings of the National Academy of Sciences of the United States of America 103: 8487–8492.
19. Wilder C, Allada G, Schuster M (2009) Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. Infection and Immunity 77: 5631–5639.
20. Lee B, Schjerling C, Kirkby N, Hoffmann N, Borup R, et al. (2011) Mucoid *Pseuodmonas aeruginosa* isolates maintain the biofilm formation capacity and the gene expression profiles during the chronic lung infection of CF patients. APMIS 119: 263–274.
21. Hogardt M, Heesemann J (2013) Microevolution of *Pseudomonas aeruginosa* to a chronic pathogen of the cystic fibrosis lung. Current Topics in Microbiology and Immunology 358: 91–118.
22. Folkesson A, Jelsbak L, Yang L, Krogh Johansen H, Ciofu O, et al. (2012) Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nature Reviews Microbiology 10: 841–851.
23. Yang L, Holm Rau M, Yang L, Høiby N, Molin S, et al. (2011) Bacterial adaptation during chronic infection revealed by independent component analysis of transcriptomic data. BMC Evolutionary Biology 11: 184.
24. Yang L, Marvig R, Damkiær S, Workman C, Holm Rau M, et al. (2011) Evolutionary dynamics of bacteria in a human host environment. Proceedings of the National Academy of Sciences of the United States of America 108: 7481–7486.
25. Harrison F, Browning LE, Vos M, Buckling A (2006) Cooperation and virulence in acute *Pseudomonas aeruginosa* infections. BMC Biology 4: 21.
26. D'Argenio D, Manhong W, Hoffman L, Kulasekara H, Déziel E, et al. (2007) Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. Molecular Microbiology 64: 512–533.
27. Rainey PB, Rainey K (2003) Evolution of cooperation and conflict in experimental bacterial populations. Nature 425: 72–74.
28. Klockgether J, Munder A, Neugebauer J, Davenport C, Stanke F, et al. (2010) Genome diversity of *Pseudomonas aurugisoa* PA01 laboratory strains. Journal of Bacteriology 192: 1113–1121.
29. Holloway B (1955) Genetic recombination in *Pseudomonas aeruginosa*. Journal of General Microbiology 13: 572–581.
30. Holloway B (1975) Genetic organization of *Pseudomonas*. In: Clarke P, Richmond M, editors. Genetics and biochemistry of *Pseudomonas*. London: John Wiley & Sons Ltd.
31. Johansen H, Norregard L, Gotzche P, Pressler T, Koch C, et al. (2004) Antibody response to Pseudomonas aeruginosa in cystic fibrosis – a marker of therapeutic success? Pediatric Pulmonology 37: 427–432.
32. Hansen S, Rau M, Johansen H, Ciofu O, Jelsbak L, et al. (2012) Evolution and diversification of *Pseudomonas aeruginosa* in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. . ISME 12: 31–45.
33. Rau M, Hansen S, Johansen H, Thomsen L, Workman C, et al. (2010) Early adpative developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrisos hosts. Environmental Microbiology 12: 1643–1658.
34. Jelsbak L, Johansen H, Frost A-L, Thøgeren R, Thomsen L, et al. (2007) Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis. Infection and Immunity 75: 2214–2224.
35. Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecological Monographs 54: 187–211.
36. Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160: 47–56.
37. Meyer J, Neely A, Stintzi A, Georges C, IA H (1996) Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infection and immunity 64: 518–523.
38. Ankenbauer R, Srioyosachati S, Cox C (1985) Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infection and Immunity 49: 132–140.

39. Bodilis J, Ghysels B, Ossayande J, Mattijs S, Pirnay J, et al. (2009) Distribution and evolution of ferri-pyoverdine receptors in *Pseudomonas aeruginosa*. Environmental Microbiology 11: 2123–2135.

40. Essar D, Eberly L, Hadero A, Crawford I (1990) Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. Journal of Bacteriology 172: 884–900.

41. Kurachi M (1958) Studies on the biosynthesis of pyocyanine. II. Isolation and determination of pyocyanine. Bulletin of the Institute for Chemical Research, Kyoto University 36.

42. Ayora S, Gotz F (1994) Genetic and biochemical properties of an extracellular neutral metalloprotease from *Staphylococcus hyicus* subsp. *hyicus*. Molecular and General Genetics 242: 421–430.

43. Hentzer M, Riedel K, Rasmussen T, Heydorn A, Andersen J, et al. (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by halogenated furanone compound. Microbiology 148: 87–102.

44. Ohman DE, Cryz S, Iglewski B (1980) Isolation and characterization of *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. Journal of Bacteriology 142: 836–842.

45. Ortori C, Atkinson S, Chhabra S, Cámara M, Williams P, et al. (2007) Comprehensive profiling of N-acylhomoserine lactones produced by *Yersinia* pseudotuberculosis using liquid chromatography coupled to hybrid quadrupole-linear ion trap mass spectrometry. Analytical and Bioanalytical Chemistry 387: 497–511.

46. Ortori C, Dubern J, Chhabra S, Cámara M, Hardie K, et al. (2011) Simultaneous quantitative profiling of N-acyl-L-homoserine lactone and 2-alkyl-4(1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS. Analytical and Bioanalytical Chemistry 399: 497–511.

47. Passador L, Cook J, Gambello M, Rust L, Iglewski BH (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science 260: 1127–1130.

48. Gambello M, Kaye S, Iglewski BH (1993) LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. Infection and Immunity 61: 1180–1184.

49. Dubern J, Diggle SP (2008) Quorum sensing by 2-alkyl-4-quinolines in *Pseudomonas aeruginosa* and other bacterial species. Molecular Biosystems 4: 882–888.

50. Diggle SP, Winzer K, Chabra S, Worrall K, Cámara M, et al. (2003) The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Molecular Microbiology 50: 29–43.

51. Dekimpe V, Déziel E (2009) Revisiting the quorum-sensing hierarchy in *Pseudomonas aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors. Microbiology 155: 712–723.

52. Barrett R, Hoekstra H (2011) Molecular spandrels: tests of adaptation at the genetic level. Nature Reviews Genetics 12: 767–780.

53. Reid D, Carroll V, O'May C, Champion A, Kirov S (2007) Increased airway iron as a potential factor in the persistence of *Pseudomonas aeruginosa* infection in cystic fibrosis. European Respiratory Journal 30: 286–292.

54. Fletcher M, Heeb S, Chhabra S, Diggle SP, Williams P, et al. (2010) 2-alkyl-4(1H)-quinolone signalling in *Pseudomonas aeruginosa*. In: Ramos J, Filloux A, editors. Molecular microbiology, infection and biodiversity. London: Springer. pp. 29–57.

55. Mowatt E, Paterson S, Fothergill J, Wright E, Ledson M, et al. (2011) *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. American Journal of Respiratory and Critical Care Medicine 183: 1674–1679.