Pseudomonas aeruginosa Enhances Production of a Non-Alginate Exopolysaccharide during Long-Term Colonization of the Cystic Fibrosis Lung

Holly K. Huse1,2, Taejoon Kwon2, James E. A. Zlosnik3, David P. Speert3, Edward M. Marcotte2, Marvin Whiteley1,2*

1 Section of Molecular Genetics and Microbiology, University of Texas at Austin, Austin, Texas, United States of America, 2 Institute of Cellular and Molecular Biology and Center for Systems and Synthetic Biology, University of Texas at Austin, Austin, Texas, United States of America, 3 Department of Pediatrics and Center for Understanding and Preventing Infection in Children, The University of British Columbia, Vancouver, British Columbia, Canada

Abstract

The gram-negative opportunistic pathogen Pseudomonas aeruginosa is the primary cause of chronic respiratory infections in individuals with the heritable disease cystic fibrosis (CF). These infections can last for decades, during which time P. aeruginosa has been proposed to acquire beneficial traits via adaptive evolution. Because CF lacks an animal model that can acquire chronic P. aeruginosa infections, identifying genes important for long-term in vivo fitness remains difficult. However, since clonal, chronological samples can be obtained from chronically infected individuals, traits undergoing adaptive evolution can be identified. Recently we identified 24 P. aeruginosa gene expression traits undergoing parallel evolution in vivo in multiple individuals, suggesting they are beneficial to the bacterium. The goal of this study was to determine if these genes impact P. aeruginosa phenotypes important for survival in the CF lung. By using a gain-of-function genetic screen, we found that 4 genes and 2 operons undergoing parallel evolution in vivo promote P. aeruginosa biofilm formation. These genes/operons promote biofilm formation by increasing levels of the non-alginate exopolysaccharide Psl. One of these genes, phaF, enhances Psl production via a post-transcriptional mechanism, while the other 5 genes/operons do not act on either psl transcription or translation. Together, these data demonstrate that P. aeruginosa has evolved at least two pathways to over-produce a non-alginate exopolysaccharide during long-term colonization of the CF lung. More broadly, this approach allowed us to attribute a biological significance to genes with unknown function, demonstrating the power of using evolution as a guide for targeted genetic studies.

Introduction

Chronic infections are an especially difficult healthcare problem because pathogens persist in the host despite therapeutic treatment. Due to a genetic defect, individuals with the heritable disease cystic fibrosis (CF) are prone to chronic, fatal respiratory infections caused by the Gram-negative opportunistic pathogen Pseudomonas aeruginosa [1]. During chronic infection, P. aeruginosa undergoes adaptation in the CF lung, which is thought to enhance the fitness of this bacterium in vivo [2,3]. Therefore, identifying fitness traits in chronic CF strains is important for designing novel treatment strategies, but this problem is challenging because animal models that recapitulate chronic P. aeruginosa CF infections do not currently exist.

One means of overcoming this challenge is to utilize an evolutionary approach to identify beneficial adaptations. Chronic CF infections are well suited for evolutionary studies because they are typically dominated by a single P. aeruginosa strain allowing clonal, chronological isolates to be sampled over decades-long time periods [4]. Beneficial adaptations can be determined by 1) identifying parallel genotypes and phenotypes that arise within multiple P. aeruginosa lineages, a strong indicator of adaptive evolution and 2) using whole-genome sequencing to identify evidence for positive selection. Indeed, phenotypic studies have shown that P. aeruginosa frequently undergoes parallel changes in vivo, one of the most
well understood being conversion to the mucoid phenotype [5]. This phenotype is characterized by over-production of the exopolysaccharide alginate, which enhances biofilm formation [6]. Prevalence of mucoid isolates is on average reported as ~41% [4,7,8], though one study reported 80% [9]. These data suggest that other important adaptive traits likely arise in vivo. Additionally, genome sequencing has demonstrated adaptation occurring during the first 40,000 of up to 200,000 generations in vivo [2,3]. This initial period of adaptation is followed by a longer period of genetic drift and negative selection [3], suggesting that adaptive traits are likely to arise during initial phases (~40,000 generations) of chronic infection.

We previously used an evolutionary approach to identify P. aeruginosa gene expression traits likely undergoing adaptive evolution in vivo [10]. P. aeruginosa was chronologically sampled from 3 CF patients, ranging from the first infecting bacterium (the ancestor) to ~40,000 generations post-infection. By comparing gene expression profiles of early and late isolates sampled from multiple patients, we identified 24 parallel gene expression changes that occurred over time within each lineage. These data strongly indicate that these gene expression traits are undergoing adaptive evolution and therefore benefit the bacterium during chronic infection.

Of these 24 genes, 15 were up-regulated in chronic isolates compared to their ancestor; however, the phenotypic consequences associated with these gene expression changes were unclear. Several of these genes were previously associated with biofilm formation, a sessile mode of growth prominent in the CF lung presumably due to its protective effects against antibiotics and host defenses [11-14]. We therefore hypothesized that a portion of these genes would promote enhanced biofilm formation. To test this hypothesis, we performed a gain-of-function screen to determine the impact of these up-regulated genes on biofilm formation. Our results reveal that expression of 4 genes and 2 operons increases biofilm formation via enhancing levels of the P. aeruginosa exopolysaccharide Psl. One of the genes, the transcriptional regulator phaF, enhances Psl levels via a post-transcriptional mechanism, while the other genes enhance Psl production but do not affect either psl transcription or translation. Finally, we show that within the first ~40,000 generations of chronic infection, Psl production increases in ~72% of chronic CF isolates compared to their ancestor. These data indicate that enhanced production of Psl is an important adaptation during the first ~40,000 generations of P. aeruginosa growth in the CF lung and that multiple phases evolved to impact this trait.

Materials and Methods

Bacterial strains and growth media

The bacterial strains used in this study are listed in Tables S3 and S4. Escherichia coli was routinely grown on Luria-Bertani medium at 37°C. P. aeruginosa strains were grown at 37°C on tryptic soy broth/agar medium, morpholinepropanesulfonic acid (MOPS)-buffered medium (50 mM MOPS [pH 7.2], 93 mM NH4Cl, 43 mM NaCl, 3.7 mM KH2PO4, 1 mM MgSO4, and 3.5 μM FeSO47H2O) supplemented with 0.5% glucose and 0.5% casamino acids, or a previously described Synthetic Cystic Fibrosis Medium (SCFM) [15]. When applicable, antibiotics were used at the following concentrations: 10 μg/ml and 25 μg/ml gentamicin for maintenance and selection, respectively, in E. coli and 50 μg/ml and 100 μg/ml gentamicin for maintenance and selection, respectively, in P. aeruginosa.

DNA and plasmid manipulations

The primers used in this study are listed in Table S5. Standard methods were used for DNA and plasmid manipulations.

Strain construction

A non-polar deletion of pslA in PAO1 was constructed using allelic replacement and sacB counter-selection as previously described [16]. pslA-lacZ transcriptional and translational fusions were a generous gift from Dr. Yasuhiko Irie and were introduced onto the chromosome at the attB site as previously described [17]. Allelic replacement and chromosomal integrations were confirmed via PCR analyses and DNA sequencing.

Microarray analyses

The empty vector control strain and the phaF over-expression strain were grown in 250 ml flasks in 25 ml MOPS minimal media supplemented with 0.5% glucose and 0.5% casamino acids. RNA was prepared from cells grown to OD600=1.0 in MOPS minimal medium, and 5 μl was used to inoculate 95 μl media (final OD600=0.05) in a 96-well polystyrene microtiter dish (NUNC). Each well contained a 3 mm borosilicate glass bead for aeration. Microtiter dishes were sealed with parafilm and incubated at 37°C with 250 r.p.m. shaking. Staining with 0.1% crystal violet was performed at 12 h, followed by washing and dye solubilization with 33% acetic acid. Absorbance was measured at 620 nm. Strains were grown in octuplicate and at least 3 biological replicates were performed.

Microarray analyses

The empty vector control strain and the phaF over-expression strain were grown in 250 ml flasks in 25 ml MOPS minimal media supplemented with 0.5% glucose and 0.5% casamino acids. RNA was prepared from cells grown to OD600=0.9-1.0. Clinical isolates and PAO1 were grown in SCFM and RNA was prepared from cells grown to OD600=0.4-0.5 [10]. RNA extraction, cDNA synthesis, hybridization, and downstream data analyses were performed as previously described [10,15]. Genes were considered differentially expressed if they exhibited a greater than 2-fold change and a false discovery rate (FDR) < 0.05.

Psl immunoblots

Psl immunoblots were carried out as previously described with minor modifications [19]. Stationary phase cultures were diluted to a final OD600=0.05 in 5 ml MOPS minimal media supplemented with 0.5% glucose and 0.5% casamino acids or
SCFM. Cultures were grown for approximately 16 h, and either 10 OD equivalents (volume [ml] = 10/culture OD$_{600}$) or whole cultures were harvested. Cell pellets were re-suspended in 100 μl of 0.5 M EDTA, boiled at 100°C for 30 minutes, and centrifuged. The supernatant fraction was treated with proteinase K (final concentration 5 mg/ml) for 1 h at 60°C, followed by inactivation for 30 minutes at 80°C. Samples were stored at 4°C for Psl immunoblotting. Pelleted lysate was re-suspended in 1 ml 6 M urea and boiled for 1 h at 100°C. Protein concentration was measured via Bradford assay. 

**Results**

**Over-expression of up-regulated genes enhances biofilm formation**

Recently we identified 24 *P. aeruginosa* gene expression traits undergoing selection within the CF lung [10]. Fifteen of the 24 genes were up-regulated in chronic isolates compared to the ancestor (defined as the *P. aeruginosa* isolate initially establishing the chronic infection), and their roles during chronic infection are unknown. Some of these genes are predicted to be part of an operon and have been associated with biofilm formation in previous studies [11,13,14]. To test the hypothesis that some of these up-regulated genes/operons contribute to biofilm formation, we used a genetic approach to test for gain-of-function phenotypes. Each gene or operon was introduced into the laboratory strain *P. aeruginosa* PAO1 on a low-copy plasmid and expressed from the arabinose-inducible pBAD promoter [21]. This genetic system is relevant for studying gain-of-function phenotypes because 1) the pBAD promoter exhibits minimal expression without arabinose inducer [21], allowing us to avoid non-specific cellular responses caused by gene over-expression; 2) the PAO1 genome is sequenced and pathways impacting biofilm formation are well characterized; and 3) microarray analyses showed that expression of the 15 up-regulated genes are comparable between PAO1 and ancestral clinical isolates (Table S1). We conducted our experiments without arabinose since this resulted in an approximate 3-4 fold increase in expression, levels similar to those found in chronic clinical isolates (Table S2). Expression of PA1106, PA1323-24, PA1592, PA3691-92, phaF (PA5060), and PA5178 enhanced biofilm formation relative to the empty vector control strain (Figure 1A). While the phenotype is modest, similar increases in biofilm formation have been reported [22]. As expected, a negative control strain unable to initiate attachment, PAO1 ΔpflA pJN105, showed a significant biofilm defect (Figure 1A). Among the genes and operons that enhanced biofilm formation, only PA3692 (lptF) and phaF have known or proposed functions. PA3692 is involved in adhesion to lung epithelial cells, while phaF is a transcriptional regulator of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* [23,24]. The remaining biofilm-enhancing genes have hypothetical functions.

**Enhanced biofilm formation of *P. aeruginosa* requires Psl**

We next sought to determine the mechanism governing enhanced biofilm formation. Although multiple pathways are important for *P. aeruginosa* biofilm formation, we obtained an important clue to the mechanism when one of the adapted genes (phaF) was over-expressed in the *P. aeruginosa* laboratory strain PA14. Surprisingly, this strain did not produce more biofilm (Figure S1). Examination of the PAO1 and PA14 genomes revealed that PA14 does not encode the genetic machinery required for production of the exopolysaccharide Psl. Since Psl is important for biofilm formation in *P. aeruginosa*, we hypothesized that enhanced biofilm formation of PAO1 over-expressing phaF, and potentially the other
adapted genes, involves Psl production. To test this hypothesis, we expressed the adapted genes/operons in a PAO1 strain that lacks the ability to produce Psl (ΔpslA) and tested these strains for biofilm formation (Figure 1B). As in PA14, phaF expression did not enhance biofilm formation in ΔpslA. Notably, the other 5 strains also did not form more biofilm than the empty vector control strain, ΔpslA pJN105 (Figure 1B), suggesting that these 6 genes/operons enhance biofilm formation in a Psl-dependent manner.

**Up-regulated genes induce Psl production**

If Psl is required for enhanced biofilm formation in the over-expression strains, each should correspondingly produce more Psl. We therefore used immunoblot analyses to measure Psl production in all 14 of our over-expression strains. First, Psl anti-sera specificity was demonstrated with a strain that cannot produce Psl, served as a negative control. While fold change in biofilm formation is reported for clarity, statistics were performed on absorbance values for empty vector control and over-expression strains assayed on the same day. At least 3 biological replicates were performed in octuplicate. *, P value < 0.001 by Student’s t test. Error bars represent standard error of the mean, n=3. (B) Fold change in biofilm formation of ΔpslA over-expression strains. Bars represent the average fold change in biofilm formation compared to the empty vector control strain, ΔpslA pJN105 (dashed line). Statistics and error bars are reported as in A. (C) Psl production in PAO1 pJN105 (top) and PAO1 over-expression strains (bottom). The top panels represent Psl production in the wild-type empty vector control strain, PAO1 pJN105, while the bottom panels represent Psl production in the over-expression strains, or as in the last bottom panel, the negative control strain ΔpslA pJN105. At least 3 biological replicates were performed.

do: 10.1371/journal.pone.0082621.g001

**Figure 1. Biofilm formation and Psl production in over-expression strains**. (A) Fold change in biofilm formation of wild-type PAO1 over-expression strains. Bars represent the average fold change in biofilm formation compared to the empty vector control strain, PAO1 pJN105 (dashed line). ΔpslA pJN105, a strain that cannot produce Psl, served as a negative control. While fold change in biofilm formation is reported for clarity, statistics were performed on absorbance values for empty vector control and over-expression strains assayed on the same day. At least 3 biological replicates were performed in octuplicate. *, P value < 0.001 by Student’s t test. Error bars represent standard error of the mean, n=3. (B) Fold change in biofilm formation of ΔpslA over-expression strains. Bars represent the average fold change in biofilm formation compared to the empty vector control strain, ΔpslA pJN105 (dashed line). Statistics and error bars are reported as in A. (C) Psl production in PAO1 pJN105 (top) and PAO1 over-expression strains (bottom). The top panels represent Psl production in the wild-type empty vector control strain, PAO1 pJN105, while the bottom panels represent Psl production in the over-expression strains, or as in the last bottom panel, the negative control strain ΔpslA pJN105. At least 3 biological replicates were performed.

do: 10.1371/journal.pone.0082621.g001

**phaF regulates Psl production post-transcriptionally**

Although over-expression of 6 adapted genes/operons enhanced biofilm formation via increased Psl production, it was unclear whether these genes/operons acted within the same or different pathways. *P. aeruginosa* regulates Psl both transcriptionally and post-transcriptionally [17,25], allowing us to test whether these genes/operons affect Psl levels via the same pathway. We constructed strains containing previously characterized chromosomal pslA-lacZ transcriptional or
translational fusions [17] and each over-expression construct. Compared to the empty vector control strain, none of the over-expression strains exhibited altered pslA transcription in either exponential or stationary phase (Figure 2A). Similarly, overexpression of PA1106, PA1323-24, PA1592, PA3691-92, and PA5178 did not affect pslA translation at either growth stage (Figure 2B). However, overexpression of phaF induced an ~5-fold increase in pslA translation during stationary phase (Figure 2B). Indeed, microarray analyses verified that pslA transcription did not change in the phaF over-expression strain (Table S2). These data suggest that these genes/operons are affecting Psl levels via at least 2 distinct pathways, as phaF is a positive regulator of psl translation and the remaining 5 genes/operons affect Psl production via other mechanisms.

Psl production is enhanced in *P. aeruginosa* chronic clinical isolates

The results described above suggest that over-expression of PA1106, PA1323-24, PA1592, PA3691-92, phaF, and PA5178 induce Psl production in strain PAO1. In our previous study, chronic *P. aeruginosa* CF isolates from 3 patients expressed all of these genes at higher levels than their ancestor [10] (Table S3). Therefore, we hypothesized that these chronic isolates would increase Psl production relative to their ancestor strain. To test this hypothesis, we used anti-Psl immunoblotting to compare Psl production in *P. aeruginosa* chronic isolates relative to their ancestors. In 18 chronic *P. aeruginosa* isolates collected from 4 different patients, 13 (~72%) produced more Psl than their corresponding ancestor (Figure 3). This phenotype was expected for at least 5 isolates that showed small colony formation on agar plates (small colony variant; SCV; indicated in Figure 3), but only 4 showed enhanced Psl production compared to the ancestor. Since enhanced Psl production occurred in over 70% of the strains isolated during the first ~40,000 generations, we propose that enhanced production of Psl is important during adaptation to the CF lung environment.

Discussion

Since animal models do not manifest chronic *P. aeruginosa* respiratory infections typical of human CF patients, adaptations that improve *in vivo* fitness are difficult to identify. To overcome this challenge, we used an evolutionary approach to identify gene expression traits undergoing natural selection and then characterized the phenotypes these genes impacted. Our studies are similar to *in vitro* microbial evolution experiments that have utilized transcriptomics and genomics to identify beneficial adaptations that arise during standard laboratory growth conditions [26,27]. The strength of these approaches lies in analyzing chronological isolates that have undergone selection, thereby allowing evolution to direct more targeted genetic studies. Here, this powerful approach not only allowed us to identify *P. aeruginosa* traits that are clinically significant, but also illuminated the biological significance of genes with unknown functions.

*P. aeruginosa* chronic CF infections are characterized by an ~40,000 generation adaptation period followed by genetic drift and negative selection [2,3]. Due to the high incidence and association of mucoidy with worsening disease symptoms, many studies have focused on this phenotype [5,9,28]. Indeed, alginate over-production leads to highly structured biofilms with increased antibiotic resistance properties [6]. Here we propose that a non-alginate exopolysaccharide, Psl, is equally important as alginate during adaption to the CF lung. This is based on our data showing that the 6 genes/operons undergoing parallel evolution *in vivo* enhance Psl production, and over 70% of chronic clinical isolates produce more Psl than their ancestor strains during the first ~40,000 generations *in vivo*. Furthermore, recent studies have shown that Psl is essential for biofilm formation even in mucoid strains [29], and small colony variants, characterized as over-producing Psl, can undergo positive selection during chronic infection [30].
While PA1106, PA1323-24, PA1592, PA3691-92, and PA5178 enhance Psl production via an unknown mechanism, phaF positively regulates ps1 translation. These data suggest a model where at least 2 distinct pathways are undergoing adaptive evolution resulting in enhanced Psl production. One possible explanation for these findings is that each pathway has additive fitness benefits, thereby allowing selection to act on them separately. Indeed, in vitro evolution experiments have shown that multiple genes affecting the same trait can gain mutations that impact fitness [31,32]. While the benefit of enhanced Psl production in vivo is unknown, it may be important for antibiotic resistance or for avoiding the host immune response, which has been demonstrated in vitro [33,34].

Since the goal of this study was to identify P. aeruginosa phenotypes undergoing selection in vivo, we did not fully investigate the molecular mechanisms of enhanced Psl production in the PAO1 over-expression strains. PA1106, PA1323-24, PA1592, PA3691-92, and PA5178 did not affect either ps1 transcription or translation, while phaF enhanced ps1 translation. One explanation for enhanced Psl production in the PA1106, PA1323-24, PA1592, PA3691-92, and PA5178 over-expression strains is that they have high levels of the small signaling molecule bis-(3',5')-cyclic-dimeric-guanosine monophosphate (cyclic-di-GMP), which can correlate with increased Psl production without a corresponding increase in ps1 transcription [35]. Additionally, phaF over-expression may increase levels of two activators of ps1 translation, the small non-coding RNAs RsmZ and RsmY. However, Northern blot analyses demonstrated that neither RsmZ nor RsmY increased expression in the phaF over-expression strain relative to the empty vector control strain (Figure S3). Therefore, we hypothesize that phaF is acting independently of RsmZ and RsmY, and we are currently investigating alternative mechanisms of action.

Supporting Information

Figure S1. Biofilm formation of PAO1 and PA14 strains carrying the phaF expression construct. (A) Biofilm formation of PAO1 strains carrying the phaF expression construct. (B) Biofilm formation of PA14 strains carrying the phaF expression construct. Biofilm formation was tested in the empty vector control strain (black bar) and the phaF over-expression strain (white bar) as described in the Materials and Methods. At least 2 biological replicates were performed in octuplicate. *, P value < 0.001 by Student’s t test compared to the empty vector control strain. Error bars represent standard error of the mean, n ≥ 16.

Figure S2. Psl anti-sera specificity. Psl anti-sera specificity was tested on PAO1 control strains grown as described in the Materials and Methods. Psl (strain WFPA800, a deletion of the ps1 promoter in PAO1), pBAD-ps1 (strain WFPA801, ps1 promoter is replaced with the pBAD promoter in PAO1), PAO1 pJN105 (the wild-type empty vector control strain). See Table S4 for more strain information.

Figure S3. RsmY and RsmZ expression in the empty vector control (PAO1 pJN105) and phaF over-expression strain. Strains were grown in MOPS buffered minimal media supplemented with 0.5% glucose and 0.5% casamino acids for ~16 hours starting from OD=0.05. 15 μg total RNA was separated on a 10% polyacrylamide-8 M urea gel, transferred to nitrocellulose, and probed for RsmY or RsmZ (see Table S5 for probe sequences). Two biological replicates were performed, and a representative is shown. PAO1 pJN105: empty vector control strain; PAO1 phaF: phaF over-expression strain.

Table S1. Expression of the 15 up-regulated genes in ancestors from 3 CF patients (A1, B1, and C1) compared to PAO1.

Figure 3. Psl production in clinical isolates from 4 patients with CF. Strains were grown in SCFM for ~16 hours and Psl was extracted from whole cultures. Psl extracts were normalized to equal protein concentrations for each patient and subjected to immunoblot blot analyses as described in the Materials and Methods. Each isolate is centered on its corresponding number of in vivo generations (Table S3). Isolates at time 0 are the ancestor. At least 3 biological replicates were performed, and red boxes indicate isolates that produced more Psl on average than the ancestor. SCV: small colony variant.

doi: 10.1371/journal.pone.0082621.g003
We would like to thank Dr. Daniel Wozniak for his gift of Psl antiserum and P. aeruginosa strains WFP800 and WFP801.

References

1. Lyczak JB, Cannon CL, Pier GB (2002) Lung infections associated with cystic fibrosis. Clin Microbiol Rev 15: 194-222. doi:10.1128/CMR.15.2.194-222.2002. PubMed: 11932230.
2. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR et al. (2006) Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103: 8487-8492. doi:10.1073/pnas.0602138103. PubMed: 16687447.
3. Yang L, Jeltsbak L, Marvig RL, Damkiaer S, Workman CT et al. (2011) Evolutionary dynamics of bacteria in a human host environment. Proc Natl Acad Sci U S A 108: 7481-7486. doi:10.1073/pnas.1018249108. PubMed: 21518885.
4. Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Speert DP (1996) Random amplified polymorphic DNA typing of Pseudomonas aeruginosa isolates recovered from patients with cystic fibrosis. J Clin Microbiol 34: 1129-1135. PubMed: 8727889.
5. Doggett RG (1989) Incidence of mucoid Pseudomonas aeruginosa from clinical sources. Appl Microbiol 18: 936-937. PubMed: 29484207.
6. Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S et al. (2001) Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function. J Bacteriol 183: 5395-5401. doi:10.1128/JB.183.18.5395-5401.2001. PubMed: 11514525.
7. Mahenthiralingam E, Campbell ME, Speert DP (1994) Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis. Infect Immun 62: 596-605. PubMed: 8300217.
8. Wilder CN, Allada G, Schuster M (2009) Instantaneous within-patient diversity of Pseudomonas aeruginosa quorum-sensing populations from cystic fibrosis lung infections. Infect Immun 77: 5631-5639. doi:10.1128/IAI.00755-09. PubMed: 19865523.
9. Holby N (1975) Prevalence of mucoid strains of Pseudomonas aeruginosa in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. Acta Pathol Microbiol Scand Suppl 83: 549-552. PubMed: 812334.
10. Huse HK, Kwon T, Zlosnik JE, Speert DP, Marcello EM et al. (2010) Parallel evolution in Pseudomonas aeruginosa over 39,000 generations in vivo. mBio 1(4): e00199-10. doi:10.1128/mBio.00199-10. PubMed: 2086684.
11. Müskens M, Di Fiore S, Dötsch A, Fischer R, Häussler S (2010) Genetic determinants of Pseudomonas aeruginosa biofilm establishment. Microbiology 156: 431-441. doi:10.1099/mic.0.033290-0. PubMed: 19850623.
12. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ et al. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762-764. doi:10.1038/35037627. PubMed: 11047825.
13. Waite RD, Paccanaro A, Papakonstantinopoulou A, Hurst JM, Saeq M et al. (2006) Clustering of Pseudomonas aeruginosa transcriptomes from planktonic cultures, developing and mature biofilms reveals pslA-lacZ transcriptional and translational fusion constructs were a generous gift from Dr. Yasuhiro Irie and Dr. Matthew Parsek. Thank you to Peter Jorth for help with experiments and members of the Whiteley lab for helpful discussions and critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: HKH MW. Performed the experiments: HKH. Analyzed the data: HKH TK. Contributed reagents/materials/analysis tools: HKH TK JEAZ. Wrote the manuscript: HKH MW. Provided and genetically typed microbial samples: JEAZ DPS.

Acknowledgements

We would like to thank Dr. Daniel Wozniak for his gift of Psl antiserum and P. aeruginosa strains WFP800 and WFP801.

Table S2. Expression of the ptoF over-expression strain compared to the empty vector control strain.

Table S3. Clinical isolates analyzed in this study.

Table S4. Strains and plasmids used in this study.

Table S5. Primers and probes used in this study.

Pseudomonas aeruginosa Adaptation in the CF Lung
segregation of polyhydroxyalkanoate granules in *Pseudomonas putida* KT2442. Mol Microbiol 79: 402-418. doi:10.1111/j.1365-2958.2010.07450.x. PubMed: 21219460.

25. Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102: 14422-14427. doi: 10.1073/pnas.0507170102. PubMed: 16186483.

26. Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK et al. (2009) Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. Nature 461: 1243-1247. doi:10.1038/nature08480. PubMed: 19838166.

27. Cooper TF, Rozen DE, Lenski RE (2003) Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli*. Proc Natl Acad Sci U S A 100: 1072-1077. doi:10.1073/pnas.0334340100. PubMed: 12538876.

28. Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60: 539-574. PubMed: 8840766.

29. Ma L, Wang S, Wang D, Parsek MR, Wozniak DJ (2012) The roles of biofilm matrix polysaccharide PsI in mucoid *Pseudomonas aeruginosa* biofilms. FEMS Immunol Med Microbiol 65: 377-380. doi:10.1111/j.1574-695X.2012.02934.x. PubMed: 22309106.

30. Malone JG, Jaeger T, Manfredi P, Dötsch A, Blanka A et al. (2012) The YfBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. PLoS Pathog 8: e1002760. PubMed: 22719254.

31. Notley-McRobb L, Ferenci T (1999) The generation of multiple co-existing mal-regulatory mutations through polygenic evolution in glucose-limited populations of *Escherichia coli*. Environ Microbiol 1: 45-52. doi:10.1046/j.1462-2920.1999.00003.x. PubMed: 11207717.

32. Wong A, Rodrigue N, Kassen R (2012) Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS Genet 8: e1002928. PubMed: 23028345.

33. Mishra M, Byrd MS, Sergeant S, Azad AK, Parsek MR et al. (2012) *Pseudomonas aeruginosa* PsI polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. Cell Microbiol 14: 95-106. doi:10.1111/j.1462-5822.2011.01704.x. PubMed: 21951860.

34. Yang L, Hu Y, Liu Y, Zhang J, Ulstrup J et al. (2011) Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environ Microbiol 13: 1705-1717. doi:10.1111/j.1462-2920.2011.02503.x. PubMed: 21605307.

35. Roy AB, Petrova OE, Sauer K (2012) The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. J Bacteriol 194: 2904-2915. doi:10.1128/JB.05346-11. PubMed: 22493016.