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Filamentous bacteriophages are associated with chronic *Pseudomonas* lung infections and antibiotic resistance in cystic fibrosis

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Data materials and availability: The genomic data analyzed in this study are deposited in the Sequence Read Archive under accession ERP004853. Further detail about accession codes for individual isolates are included in the Supplementary Materials of the original publication by Marvig *et al.* (40). All data associated with this study are present in the paper or in the Supplementary Materials.

Abstract

Filamentous bacteriophage (Pf phage) contribute to the virulence of *Pseudomonas aeruginosa* infections in animal models, but their relevance to human disease is unclear. We sought to interrogate the prevalence and clinical relevance of Pf phage in patients with cystic fibrosis (CF) using sputum samples from two well-characterized patient cohorts. Bacterial genomic analysis in a Danish longitudinal cohort of 34 patients with CF revealed that 26.5% ($n = 9$) were consistently Pf phage positive. In the second cohort, a prospective cross-sectional cohort of 58 patients with CF at Stanford, sputum qPCR analysis showed that 36.2% ($n = 21$) of patients were Pf phage positive. In both cohorts, patients positive for Pf phage were older, and in the Stanford CF cohort, patients positive for Pf phage were more likely to have chronic *P. aeruginosa* infection and had greater declines in pulmonary function during exacerbations than patients negative for Pf phage presence in the sputum. Last, *P. aeruginosa* strains carrying Pf phage exhibited increased resistance to antipseudomonal antibiotics. Mechanistically, in vitro analysis showed that Pf phage sequesters these same antibiotics, suggesting that this mechanism may thereby contribute to the selection of antibiotic resistance over time. These data provide evidence that Pf phage may contribute to clinical outcomes in *P. aeruginosa* infection in CF.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR), an anion transport protein present in mucosal surfaces (1). In the CF lung, disrupted chloride and bicarbonate transport result in the accumulation of tenacious secretions in the airways (2, 3). This, in turn, sets off a vicious cycle of infection, inflammation, and tissue damage resulting in severe and progressive obstructive pulmonary disease with substantial morbidity and mortality (4, 5). It is now well established that lung disease starts very early in the life of patients with CF, primarily as a consequence of mucus obstruction and bacterial infection (6, 7). Despite substantial therapeutic advancements in the medical management of CF, progressive airway obstruction, measured as a decline in forced expiratory volume in 1 s (FEV_1), still predicts mortality (8).

Pseudomonas aeruginosa is a common pathogen recovered from CF airway secretions (9). Infection with *P. aeruginosa* likely starts as an intermittent infection progressing to a chronic stage with eventual conversion to a biofilm-entrenched state (10). These communities of bacteria encased in extracellular matrix allow *P. aeruginosa* to effectively adapt to the airway environment and persist despite strong immune response and antibiotic therapy (11, 12). Infection with *P. aeruginosa* typically begins in childhood. By age 3 years, 95% of children with CF have serologic evidence of intermittent *P. aeruginosa* infection (13). Antibiotic eradication protocols are now the standard of care when *P. aeruginosa* is first detected; however, successful eradication is variable and not sustained (14–16). As a result, nearly 60% of patients with CF have chronic *P. aeruginosa* infection when reaching adulthood (17, 18).

Chronic endobronchial infection with *P. aeruginosa* in patients with CF is associated with worsening lung function and increased mortality (19–22). Despite prolonged suppressive

antibiotic therapy for those patients with CF and chronic *P. aeruginosa* infection, episodic exacerbations with increased symptomatology, decompensation in lung function, and progression of lung disease are still commonly observed (15, 19, 20, 23).

Filamentous bacteriophage, Pf phage, has previously been described in *P. aeruginosa* biofilms (24–26), and we recently reported that Pf phage promotes the formation of *P. aeruginosa* biofilms (27). In particular, we have shown that polymers present in the dense, viscous sputum that is characteristic of CF drive the self-assembly of Pf phage into a highly ordered liquid crystal via depletion attraction, a cohesive force that operates between crowded, like-charged elements in environments where sufficient ionic strength exists to screen their repulsive forces (27, 28). The capsid of Pf phage has a dense negative charge, as do most host and bacterial polymers, such as DNA, mucin, actin, and glycosaminoglycans (29–31). This crystalline architecture promotes the adhesiveness and viscosity of *P. aeruginosa* biofilms (27). Moreover, antibiotics have difficultly penetrating these lattice-like networks, leading to antibiotic tolerance (27, 28). These effects are highly concentration dependent, with higher Pf phage concentrations promoting increasingly pathogenic *P. aeruginosa* phenotypes (27).

In light of these effects, we have proposed that Pf phage could contribute to the pathogenesis of *P. aeruginosa* biofilm infections (28). Unlike lytic phage used in phage therapy, Pf phage are lysogenic and typically do not lyse (kill) their bacterial hosts. Consistent with a pathogenic role, Pf phage has been shown to contribute to the virulence of *P. aeruginosa* infections in animal models of acute lung infection (32, 33). Further, we reported that Pf phage was abundant in a small cohort of 10 patients with CF with advanced lung disease, being found at concentrations averaging 10^7 copies of Pf phage/ml of sputum (27). Pf phage is produced by *P. aeruginosa* isolates from CF sputum (34–36), and the Pf prophage (phage DNA integrated into the *P. aeruginosa* genome) has been found in *P. aeruginosa* isolates collected from patients with CF and in other clinical settings (25, 37, 38). However, much remains unknown regarding the prevalence of Pf phage within the CF population and the clinical implications of its presence.

We hypothesized that Pf phage is widespread in the CF patient population and that higher Pf phage concentrations are associated with features of advanced lung disease in CF. To test this hypothesis, we explored the prevalence of Pf prophage in *P. aeruginosa* isolates in an online dataset and in a previously described cohort of Danish patients with CF. We then performed a prospective cross-sectional cohort study at the Stanford CF Center to evaluate Pf phage concentrations in sputum and clinical outcomes in patients with CF.

RESULTS

Pf phage is present in more than half the strains cataloged within the Pseudomonas Genome Database

Because Pf is incorporated as a prophage, its carriage can be detected within genomic sequences of *P. aeruginosa* (37, 39). Exploiting this, we first interrogated the prevalence of Pf phage in a large collection of *P. aeruginosa* genetic sequences, the Pseudomonas Genome Database (www.pseudomonas.com). This is an internet- and community-based genome

project that contains *Pseudomonas* genome sequences and their annotations. We queried the database for the presence of the Pf phage *coaB* coat protein gene as a marker for carriage of Pf phage. We found evidence of Pf phage in 1159 of 2226 *P. aeruginosa* sequences (52.1%) (Fig. 1A). Together, these data indicate that Pf phage is widespread within *P. aeruginosa* but that not all isolates harbor this bacteriophage.

Pf phage is present in a Danish CF cohort and is associated with older patient age

We then evaluated the prevalence of Pf phage within 474 *P. aeruginosa* whole-genome sequences collected from a cohort of 34 Danish patients with CF previously described (40). These patients were enrolled upon their first respiratory culture that grew *P. aeruginosa* and followed longitudinally, with one *P. aeruginosa* isolate collected and sequenced at each visit. We interrogated these whole-genome sequences for the presence of Pf phage coaB coat protein.

We found that 211 of the total 474 *P. aeruginosa* isolates (44.5%) were positive for Pf phage (Fig. 1B). Twenty-one of the 34 patients [61.8%; 95% confidence interval (CI), 43.6 to 77.8%] had at least one isolate containing Pf phage at any time during the study. Nine of the 34 patients (26.5%; 95% CI, 12.9 to 44.4%) were consistently Pf phage positive in all samples collected. Twelve (35%) had a mixture of isolates with and without Pf phage over the study period. Thirteen of the 34 patients (38.2%; 95% CI, 22.2 to 56.4%) were consistently Pf phage negative over the course of the study period (Fig. 2A).

Patients who were consistently Pf phage positive were significantly older than patients who never had Pf phage detected in their isolates (19.1 years versus 13.9 years; $P = 0.046$) (Fig. 2B). Moreover, subject age was directly correlated to the proportion of isolates with Pf phage detected for each patient ($r = 0.5098$, $P = 0.048$) (Fig. 2C).

These data confirm the finding that Pf phage is widespread within *P. aeruginosa* sequences and suggest there may be a tendency for *P. aeruginosa* strains that produce Pf phage to dominate in the sputum of individual patients with CF over time.

Pf phage are abundant in *P. aeruginosa* lung infections in a Stanford CF cohort

To further interrogate the role of Pf phage in *P. aeruginosa* infections in CF, we collected sputum samples from a total of 76 patients with CF followed at the Stanford CF center from December 2016 to February 2018. Patient characteristics are described in Table 1. The methodology involved in screening and enrolling these patients is detailed in Materials and Methods.

We first assessed the prevalence of *P. aeruginosa* infection in this cohort. We found that in our 76 patients, 58 had *P. aeruginosa* detected by qPCR analysis of the sputum. Forty-eight of those grew *P. aeruginosa* on concurrent respiratory culture. Of the remaining 10 patients that had *P. aeruginosa* detected by qPCR but did not grow *P. aeruginosa* on their respiratory culture, 9 had remote history of *P. aeruginosa* on previous respiratory cultures and 1 had transferred care to Stanford University, and previous records were unavailable for review. Of the samples with no *P. aeruginosa* detected by qPCR, 10 patients had a history of *P.*

aeruginosa but were not culture positive at present and 8 had never grown *P. aeruginosa* from their sputum (Table 1 and Fig. 3A).

We then examined the presence or absence of Pf phage in these individuals using our previously published qPCR-based assay (27). The prevalence of Pf phage was 36.2% (21 of 58; 95% CI, 24.0 to 49.9%) in patients with *P. aeruginosa* infection and 27.6% (21 of 76; 95% CI, 18.0 to 39.1%) in all patients (Table 1). There was no Pf phage detected in any *P. aeruginosa*–negative samples. Pf phage concentrations averaged 4.4×10^9 copies/ml with a range from 1.3×10^6 to 3.1×10^{10} copies/ml (Fig. 4A). The presence of Pf phage in sputum was not associated with clinical characteristics such as FEV_1, body mass index (BMI), or copy number of the CFTR mutation F508del (Table 1 and fig. S1).

Pf phage is highly correlated with both the bacterial burden and chronicity of *P. aeruginosa* lung infection in the Stanford CF cohort

We next asked whether Pf phage correlated with bacterial burden in CF lung infections in the Stanford CF cohort. The sputum burden of *P. aeruginosa* was greater in patients positive for Pf phage (7.8 ± 1.1 log copies/ml) than patients negative for Pf phage (6.4 ± 2.0 log copies/ml) ($P = 0.01$) (Table 1 and Fig. 3B). Across individual patients, the amount of Pf phage and the amount of *P. aeruginosa* were closely correlated; however, the copy numbers of Pf phage DNA consistently averaged 1 to 2 logs higher than *P. aeruginosa* DNA copies (Fig. 4A). *P. aeruginosa* carry only a single integrated copy of Pf phage within their genomes (a prophage). The concentration of Pf phage reported in Fig. 4A, fig. S2, and Table 1 reflect the deduction of prophage from the total phage burden (raw Pf phage concentration by qPCR with the raw *P. aeruginosa* concentration by qPCR subtracted). Together, these data indicate that Pf phage are highly abundant in the sputum of infected patients and that these phage outnumber their bacterial hosts by 10 to 100×.

We next asked whether the presence of Pf phage was correlated with the chronicity of *P. aeruginosa* lung infections as defined by Leeds criteria, a well-validated and clinically useful set of criteria used to identify patients with CF who have progressed to a chronic infected stage with *P. aeruginosa* (41). A patient is categorized as having chronic infection if >50% of the respiratory cultures in the past 12 months have growth of *P. aeruginosa.* Patients positive for Pf phage were more likely to meet Leeds criteria (21 of 21) than patients negative for Pf phage (13 of 37) ($P = 0.002$) (Fig. 4B).

Together, these data indicate that, among patients with CF, the presence of Pf phage is highly correlated with both the bacterial burden and chronicity of *P. aeruginosa* lung infection.

Pf phage is more prevalent in older patients in the Stanford CF cohort

We observed that patients positive for Pf phage in the Stanford CF cohort were, on average, older (33.4 ± 10.9 years) than patients negative for Pf phage (25.9 ± 11.0 years) ($P = 0.006$) (Fig. 4C). Consistent with this, only 1 of 11 pediatric patients (9.1%; 95% CI, 0.2 to 41.3%) were Pf phage positive, whereas 20 of 46 (43.5%; 95% CI, 28.9 to 58.9%) adult patients were Pf positive ($P = 0.03$) (Fig. 4D). Moreover, in our recently published study, samples from 10 of 10 (100%; 95% CI, 69.2 to 100%) patients with CF who were undergoing lung transplant evaluation were Pf phage positive (27). Consistent with the results shown for the

Danish CF cohort, these data confirm in a second independent cohort that the presence of Pf phage is associated with older patient age.

Pf phage is associated with decreased FEV_1 in the Stanford CF cohort

We next asked whether the absence or presence of Pf phage was associated with worse pulmonary disease in the Stanford CF cohort. Overall, we did not observe significant differences in FEV_1% predicted on the day of sputum collection between patients positive for Pf phage and patients negative for Pf phage infected with *P. aeruginosa* ($P = 0.57$) (Table 1 and fig. S1A). However, within patients positive for Pf phage, there was a modest inverse correlation between the concentration of Pf phage present in their sputum and their FEV_1 value at the time of sampling ($r = -0.35$, $P = 0.01$) (fig. S2).

We also asked whether pulmonary exacerbations had a more detrimental effect in FEV_1 in the setting of Pf phage presence by comparing the pulmonary function of patients during a pulmonary exacerbation (defined as a combination of a flare in symptomatology, treatment with increased airway clearance, and antibiotic therapy) to the pulmonary function when not in exacerbation. We found that the drop in FEV_1 during exacerbation was significantly greater in patients positive for Pf phage (Fig. 5, $P = 0.03$). Together, these data suggest that Pf phage burden in the airways is associated with a decrease in FEV_1 during exacerbations.

Clinical *P. aeruginosa* isolates from patients positive for Pf phage exhibit increased antibiotic resistance in the Stanford CF cohort

Given that older patients were more likely to be Pf phage positive, we suspected that strains producing Pf phage may have a selective advantage over strains that do not. We therefore examined the phenotypes and antibiotic resistance patterns in clinical isolates from patients positive and negative for Pf phage, as described in Table 2. These data are for the predominant clinical isolate in the same sputum samples used to assess Pf phage status.

We found that more patients who are Pf phage positive have mucoid strains of *P. aeruginosa*, a marker of adaptation to the airway environment (58.3% versus 85.7%) ($P = 0.03$) (Fig. 6A). Further, patients who are Pf phage positive have *P. aeruginosa* strains that exhibit increased resistance to the antibiotics aztreonam ($P = 0.0499$), amikacin ($P = 0.0009$), and meropenem ($P = 0.002$), as determined by the Kirby Bauer disk diffusion method in our clinical laboratory (Table 2 and Fig. 6B). When comparing mucoid versus nonmucoid isolates, there were no differences in antibiotic resistance patterns (table S3). Because antibiotic therapy is an important component of CF care, these data suggest that an association with heightened antibiotic resistance may contribute to the link between Pf phage status and increased lung disease pathogenicity in CF.

Pf phage sequesters antimicrobials

We previously reported that Pf phage spontaneously assemble into a liquid crystalline structure in the presence of polymers abundant in CF sputum and that these liquid crystals sequester antibiotics and prevent their diffusion through biofilms, thereby contributing to increased antibiotic tolerance (bacterial survival despite the presence of antibiotics) (27, 28).

This may over time lead to selection for fixed antibiotic resistance (the ability to grow at elevated concentrations of antimicrobial drugs due to heritable resistance mechanisms).

To test specifically whether this crystalline organization prevents diffusion of the antibiotics identified in Table 2, we used our previously described flow chamber system (27). Briefly, DNA, Pf phage, and an antibiotic were combined on one side of a microfilter apparatus, and then, the effluent was collected on the other side of the filter. This effluent was then added to antibiotic susceptible *Escherichia coli* to functionally assess antibiotic concentrations. Using this system, we observed that aztreonam ($P < 0.05$), amikacin ($P < 0.0001$), and meropenem ($P < 0.05$) were all sequestered by Pf phage–DNA complexes, whereas ciprofloxacin was not (Fig. 7).

DISCUSSION

Pf bacteriophage are prevalent in 36.2 to 52.1% of *P. aeruginosa* isolates, including in two independent cohorts of patients with CF, which is similar to that reported by Knezevic *et al.* (25), who reported that 56% of 241 clinical isolates carried Pf prophage. In the prospective Stanford CF cohort reported here, Pf phage was associated with chronic *P. aeruginosa* infection, worse lower pulmonary function during exacerbation, antibiotic resistance, and, in both Stanford and Danish CF cohorts, older patient age. Together, these observations suggest that Pf phage might contribute to poor clinical outcomes in patients with CF. Although bacteriophage are known to encode genes for toxins and other factors involved in acute infections (42), these findings suggest that a bacteriophage might also play a role in the pathogenicity of CF in humans.

Our data from both the Stanford and Danish CF cohorts together suggest that either patients with CF acquire Pf phage–producing strains of *P. aeruginosa* or the Pf phage–negative *P. aeruginosa* become infected with Pf phage as patients age and their disease progresses. This suggests that Pf carrying *P. aeruginosa* strains have a selective advantage over those that do not—an observation that may be explained by our observations of increased antibiotic resistance.

In light of the data presented here and our previously published work (27), we hypothesize that Pf phage promote the structure and function of *P. aeruginosa* biofilms in ways that drive the development of antibiotic resistance and chronic infection (fig. S3). This model is consistent with work by ourselves and other groups identifying Pf phage as a virulence factor and implicating Pf phage in the pathogenesis of *P. aeruginosa* lung infections in animal models (24, 25, 27, 28, 32, 33). Although lytic phage parasitize their bacterial hosts (43–46), our data suggest that Pf phage, a lysogenic phage, instead contributes to chronic *P. aeruginosa* infections.

We report that clinical isolates of *P. aeruginosa* collected from patients positive for Pf phage exhibit increased resistance to amikacin, meropenem, and aztreonam (Table 2 and Fig. 6B). Of note, in these studies, *P. aeruginosa* was grown in planktonic form (not as a biofilm). Therefore, our findings are indicative of resistance and not tolerance because there would be no biofilm or liquid crystal present in the assay, and the survival is related to genetic

mutation conferring resistance. The association between sputum Pf phage and antibiotic resistance may be of high relevance to CF, given that the current standard of care in the United States for chronic lung infection is the use of inhaled topical antibiotics. Amikacin and aztreonam are all antibiotics commonly used to control *P. aeruginosa* infections in CF. Specifically, inhaled aztreonam, which reaches high concentrations in the airway, is one of the antibiotics that are routinely used as long-term therapy to control chronic *P. aeruginosa* infection. We propose that the tolerance conferred by the presence of Pf phage has allowed for selection of heritable antibiotic resistance genes as is reflected in the antibiotic susceptibilities measured in our isolates. These data provide a plausible mechanism for how *P. aeruginosa* strains that produce Pf phage might have a selective advantage and be disproportionately represented in the lungs of older patients with CF.

There was no difference in resistance to ciprofloxacin between Pf phage–positive and Pf phage–negative isolates. This was expected given our previous work showing ciprofloxacin was not sequestered by Pf phage (27), perhaps because of its neutral molecular structure. Whereas we saw that resistance to the aminoglycoside antibiotic amikacin was associated with the presence of Pf phage, there was no association with resistance to tobramycin, which contrasts to our previous report of sequestration of this antibiotic in vitro (27). This difference indicates to us that there may be additional mechanisms driving the development of tobramycin resistance beyond Pf phage–mediated sequestration (47), such as selective pressure from the high incidence of its chronic use among patients with CF. Alternatively, in addition to providing tolerance, bacteriophage can carry antibiotic resistance genes, as demonstrated in CF sputum (48), and this could provide an additional mechanism for influencing antibiotic resistance patterns. If the findings reported here are validated in larger studies and independent cohorts, a diagnostic test for the presence of Pf phage may have future implications for clinical care.

These studies together with the available animal data (32, 33) provide evidence of a role for Pf phage in the pathogenesis of *P. aeruginosa* chronic lung infections. Although in our cohort the overall FEV_1 values, BMI, and other metrics of CF disease are not different in patients positive for versus patients negative for Pf phage, we did find a strong interactive effect with pulmonary exacerbations because the largest impact in FEV_1 was among patients positive for Pf phage. Unfortunately, there is currently no ideal animal model for studying chronic pulmonary infection, making these associations difficult to evaluate experimentally.

In addition to promoting chronic infection by promoting antibiotic resistance, bacteriophage has been described as interacting with the mammalian immune system (49). Although this could have different effects on *P. aeruginosa* infection either favoring chronic infection or clearance, we recently reported that Pf phage suppresses local immunity in association with mouse and human wound infections. Studies are underway to examine the impact of Pf phage on immunity in patients with CF.

This study has several limitations. The methods used to collect and sequence samples in the datasets included in these analyses were highly heterogeneous. The Danish cohort and the Stanford cohort are CF specific, whereas the Pseudomonas Genome Database is not. Moreover, in these CF cohorts, only a single, dominant strain was sampled for each time

point, whereas in patients with CF, there are often multiple *P. aeruginosa* lineages present (50–52). Our prevalence figures therefore may capture only a small part of the ecology of Pf phage–positive and Pf phage–negative *P. aeruginosa* strains. The cross-sectional design of our Stanford CF cohort does not permit us to definitively establish the role of Pf phage in the progression of CF lung disease. Future, prospective longitudinal studies will be necessary to tease apart the contribution of Pf phage to disease progression in individuals. It will also be critical to evaluate the relationships between Pf phage and specific antibiotic resistance mechanisms, host genetic modifiers, and associations with other microorganisms typical of polymicrobial lung infections in CF. Our ongoing work aims to further investigate Pf phage pathogenicity through a larger prospective longitudinal study with repeated sampling, including the investigation of changes at the individual level in association with episodes of exacerbation.

In conclusion, we report that Pf phage is abundant in *P. aeruginosa* in multiple settings and that higher concentrations of Pf phage are associated with increased patient age, chronic *P. aeruginosa* infection, lower FEV_1 during pulmonary exacerbations, and increased antibiotic resistance profiles in a cohort of patients with CF. Together with the available in vitro and animal model data, these findings implicate Pf phage in the pathogenesis of CF lung disease and specifically in establishing chronic infection with *P. aeruginosa*. Our study identifies carriage of Pf phage as a potential biomarker for risk of antibiotic resistance and a potential therapeutic target for treatment of chronic *P. aeruginosa* infections in CF.

MATERIALS AND METHODS

Study design

We investigated the association between Pf phage and *P. aeruginosa* infections in CF in several sample sets. First, we investigated the prevalence of Pf phage in a large, public database of *P. aeruginosa* genomic sequences. Second, we determined the incidence of Pf phage in a population of well-characterized Danish patients with CF as to their temporal acquisition of *P. aeruginosa*. Third, we performed a prospective, cross-sectional cohort study in patients seen at the Stanford CF Center. In this group, we examined the associations between Pf status and patient demographics, patient clinical data, and microbiological strain attributes, as detailed below. In addition, we performed in vitro studies of Pf phage–mediated antibiotic sequestration.

Analysis of Pseudomonas Genome Database

Using the BLAST function on the Pseudomonas Genome Database website, we interrogated all *P. aeruginosa* strains for the sequence of the mature coaB major coat protein. We considered a sequence positive for Pf phage if there was a >80% identical match for the mature *coaB* sequence. This sequence was chosen as is necessary for mature phage production and contributes the anionic charge responsible for liquid crystal formation (27).

Analysis of Danish CF cohort

The whole-genome sequences from 474 isolates from 34 patients from the Copenhagen Cystic Fibrosis Center at the University Hospital, Rigshospitalet, Denmark described by

Marvig *et al.* (40) were provided by the authors. In addition, they provided demographic information. We used Geneious Pro (Biomatters Limited) to interrogate the sequences for the presence of the coaB major coat protein.

The age of the patient at the last isolate of *P. aeruginosa* collected was used. Three patients were excluded from analysis of age because the ages were not available.

Subjects in Stanford CF cohort

This prospective, cross-sectional cohort study was conducted at the Stanford Cystic Fibrosis Center in Palo Alto, California. Patients were screened for study participation in a consecutive and unbiased manner. Eligible patients were defined as patients with confirmed diagnosis of CF, ability to expectorate, and ability to reliably perform pulmonary function testing. Patients who had received lung transplantation were excluded. Patients were identified either during hospital admission for treatment of a pulmonary exacerbation or at a routine clinic visit. Both pediatric and adult patients were recruited for participation. Patient data are reported in Table 1 and fig. S1.

Clinical data from the Stanford CF cohort

Clinical and laboratory data were extracted from the electronic medical record for each patient encounter. Pediatric age was defined as <18 years of age. Pulmonary exacerbation was defined as a change in clinical status that led to the patient being treated with antibiotics outside of the patient's baseline regimen (such as chronic azithromycin therapy or chronic inhaled antibiotics) and an increase in airway clearance therapy regimen. Leeds criteria were used to define chronic *P. aeruginosa* infection (>50% of sputum cultures with *P. aeruginosa* in the preceding 12 months) (41, 53). FEV_1 data recorded were from spirometry collected on the day of sputum sample collection (±7 days).

Sample acquisition and processing from the Stanford CF cohort

Expectorated sputum was collected from subjects in sterile specimen cups and frozen at −80°C. At a later date, samples were thawed and 200 μl of aliquot underwent mechanical homogenization by bead beating (MagNA Lyser Instrument, Roche) for 60 s at 6500 rpm. DNA extraction was performed with the QIAamp DNA Mini Kit by Qiagen as per the manufacturer's protocol for tissue. DNA was eluted by adding 200 μl of elution buffer AL used.

Characterization of *P. aeruginosa* isolates in the Stanford CF cohort

On the day of sample collection, an additional sputum sample was sent to the clinical laboratory as per routine CF clinical care. In the clinical laboratory, respiratory cultures were performed as per Cystic Fibrosis Foundation recommendations (54). Briefly, respiratory samples from patients with CF are cultured on routine media [blood, chocolate, MacConkey, and colistin and nalidixic acid (CNA) agar] and *Burkholderia cepacia* Selective Agar (Remel) and BD BBL CHROMagar Staph aureus (Becton Dickinson). CF pathogens are identified by matrix-assisted laser desorption/ionization–time-of-flight, and antibiotic susceptibility testing is performed per Clinical & Laboratory Standards Institute recommendations. As per Stanford University Clinical Microbiology Laboratory protocol, a

single representative clone is chosen for antibiotic susceptibility profiling. If multiple clonal phenotypes are observed, then a representative sample of each is tested. Antibiotic resistance is determined by the Kirby Bauer disc diffusion method. If there was not a sample sent to the clinical laboratory on day of sample collection, then susceptibilities were included from sputum sample within ±1 week. Isolates of *P. aeruginosa* are determined to be mucoid by visual identification based on colony morphology by the microbiology laboratory technicians.

Quantification of sputum *P. aeruginosa* and Pf phage by qPCR in the Stanford CF cohort

Pf phage and *P. aeruginosa* in sputum were quantified by qPCR. Two microliters of the DNA extracted from sputum samples was used as a template in 20-μl qPCR reactions containing 1× SensiFAST Probe Hi-ROX (catalog no. BIO-82020, Bioline), 200 nM probe, and 2 nM forward and reverse primers. Cycling conditions were as follows: 95°C for 2 min, (95°C for 15 s, 60°C for 20 s) × 40 cycles on a StepOnePlus Real-Time PCR system (Applied Biosystems). To quantify Pf, the primers [Pf-Conserve-F (TTCCCGCGTGGAATGC) and Pf-Conserve-R (CGGGAAGACAGCCACCAA)] targeting *PA0717*, a gene that is conserved across diverse strains of Pf phage (Pf1, Pf4, and Pf5), were used together with a PA0717 probe (AACGCTGGGTCGAAG). For *P. aeruginosa* quantification, primers targeting the 50*S* ribosomal subunit gene *rpIU* [rpIU-F (CAAGGTCCGCATCATCAAGTT) and rpIU-R (GGCCCTGACGCTTCATGT)] were used together with an rpIU probe (CGCCGTCGTAAGC). For the standard curves, the sequence targeted by the primers and probe was inserted into a pUC57 plasmid (Genewiz), and 10-fold serial dilutions of the plasmid were used in the qPCR reactions. Using these methods, we determined that the lower limit of detection in our qPCR assay for the RPIU gene of *P. aeruginosa* is 10^2 copies/ml and for the Pf phage conserved gene is 10^3 copies/ml. All samples were run in duplicate. The reported Pf phage concentrations represent the measured Pf phage with the *P. aeruginosa* concentration subtracted to account for detection of prophage DNA contained in the genome of *P. aeruginosa*.

Antibiotic sequestration assay

Sequestration experiments were performed by placing indicated concentrations of antibiotics with or without the mixture of Pf phage and DNA (Pf4, 10^{11} plaque-forming units/ml; DNA, 1.6 mg/ml) in a 2-kDa (0.5-ml volume) slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific). Cassettes were dialyzed against 30-ml LB broth at 4°C overnight. Antibiotic concentrations are consistent with measured concentrations in the lung for both intravenous and inhaled dosing of antibiotics (55–58). LB broth outside the cassette was collected and used to quantify the amount of antibiotics by measuring the bactericidal effect. Briefly, log-phase *E. coli* DH5a culture was obtained by growing single colony in LB broth for 2 to 4 hours. The bacteria from 500 μl of the log-phase culture were spin down and mixed with 500 μl of the broth collected outside the cassette. The mixture was incubated at 37°C for 1 to 2 hours, and the number of viable bacteria was quantified by counting colony forming units (CFU) after plating serial dilutions. The CFUs for the groups with Pf phage were normalized to corresponding groups without Pf phage to obtain the degree of antibiotic sequestration.

Study approval

This study was approved by the Stanford University Institutional Review Board. Before inclusion in the study, informed consent was obtained from each subject for sputum collection and biobanking.

Statistics

Wilcoxon ranked sum was for analysis used to evaluate differences in concentration of Pf phage across continuous variables and Pearson's χ^2 or Fisher's exact test across categorical variables. Mann-Whitney test was used for comparison of FEV_1 values when well versus during exacerbation and comparison of patient age and phage status. For correlations with the concentration of Pf phage, Kendall correlation coefficients with log Pf phage were used. For correlations of patient age with proportion of isolates being Pf phage positive, Pearson correlation was used. For comparison of proportions of antibiotic resistance across phage and mucoid status, Pearson's χ^2 was used. For comparison of degree of antibiotic sequestration, Student's *t* test was used. For all tests, an alpha level of 0.05 was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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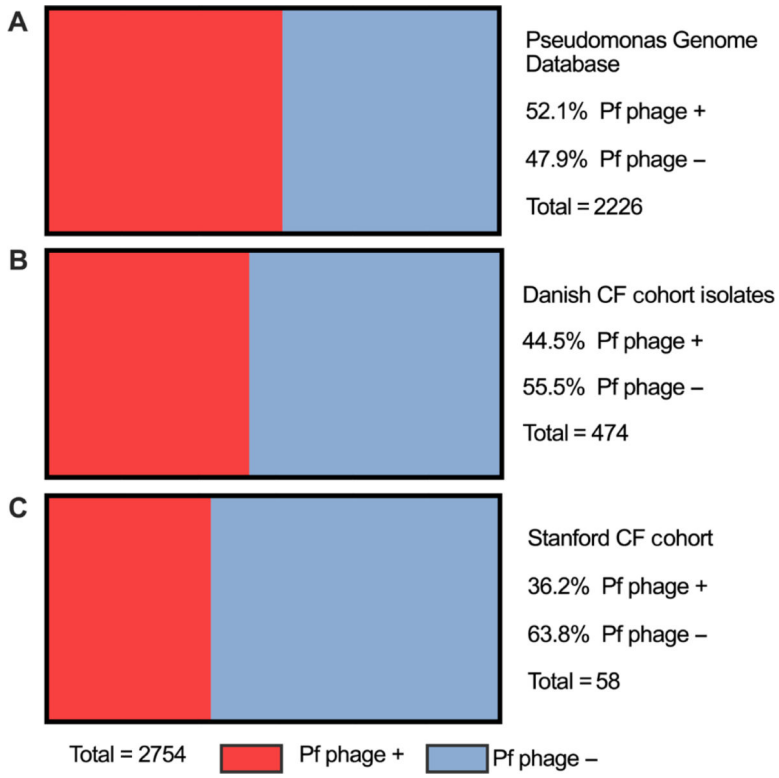

Fig. 1. Pf phage is prevalent in the Pseudomonas Genome Database, a Danish CF cohort and the Stanford CF cohort.

(**A**) The prevalence of the detection of the Pf prophage in *P. aeruginosa* logged in the Pseudomonas Genome Database, an internet- and community-based genome project containing the genome sequences and annotations of both clinical and environmental isolates of *P. aeruginosa*. (**B**) The prevalence of the detection of Pf prophage in *P. aeruginosa* isolates from the Danish CF cohort previously described by Marvig *et al.* (40). Four hundred seventy-four *P. aeruginosa* whole-genome sequences were collected from a cohort of 34 Danish patients with CF who were followed longitudinally. There are multiple isolates from each patient represented in this figure. (**C**) The prevalence of Pf phage detected by quantitative polymerase chain reaction (qPCR) in the sputum of patients also with *P. aeruginosa* in their sputum from the Stanford CF cohort. Each patient contributed only one sputum sample.

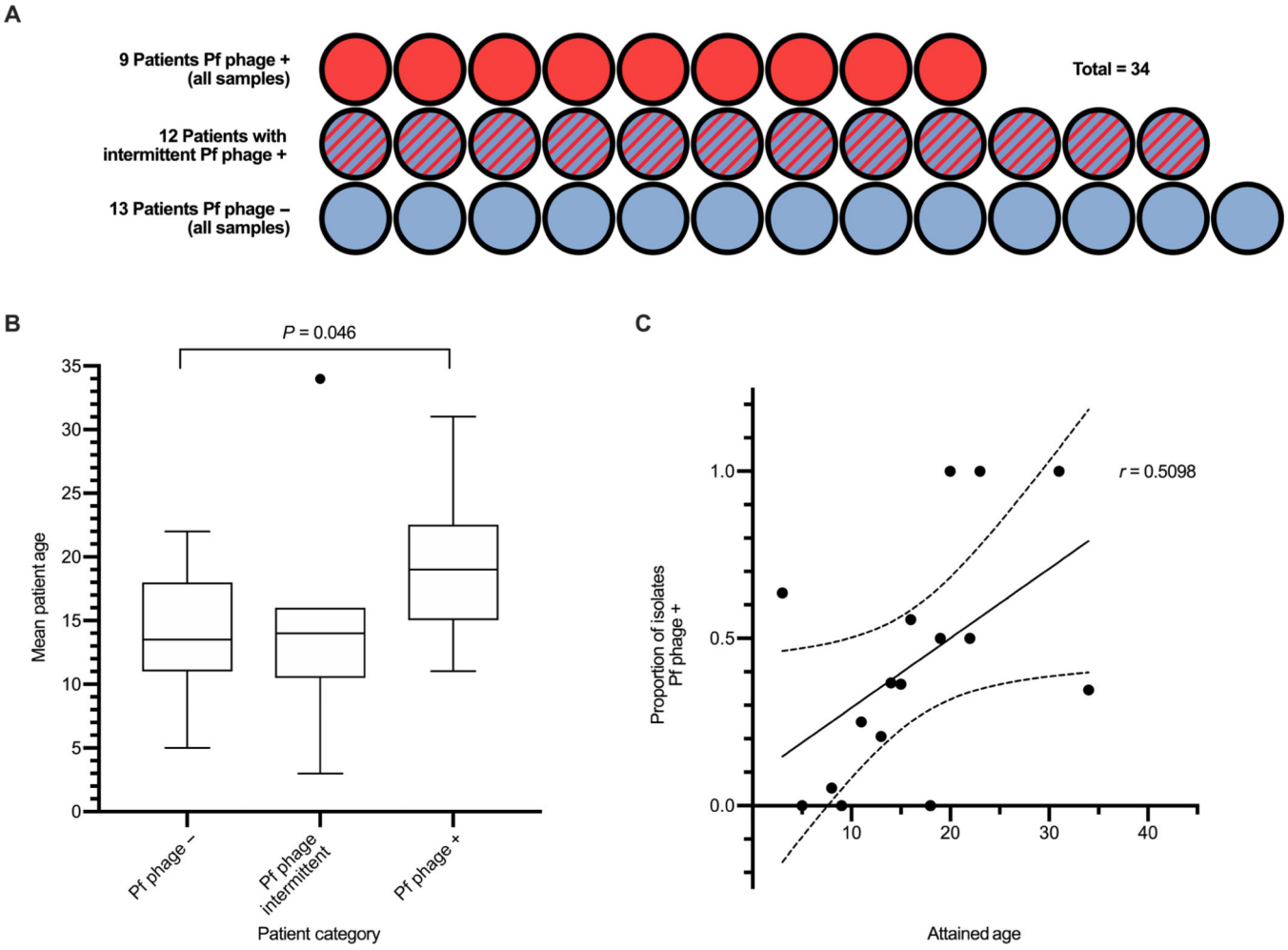

Fig. 2. In a Danish CF cohort, the prevalence of Pf phage rises with increasing patient age.

(**A**) Patients were categorized as having all isolates with the Pf prophage detected (Pf phage +), intermittently having the Pf prophage detected (Pf phage intermittent), or all isolates with no Pf prophage detected (Pf phage −). (**B**) Mean age of patients with CF in each category. Age was unavailable for three subjects ($n = 12$, $n = 9$, $n = 9$). Boxes represent interquartile range (IQR) with whiskers showing 1.5 × IQR and dots as outliers. Comparison was made by Mann-Whitney test. The outliers on graph were included in analysis. Data are available in table S1. (**C**) Correlation between subject attained age at the date of the last isolate versus the proportion of total isolates in which Pf prophage was detected($r = 0.5098$, $P = 0.048$). Data for $n = 31$ patients are included with each dot representing a specific age category and assessed by Pearson correlation.

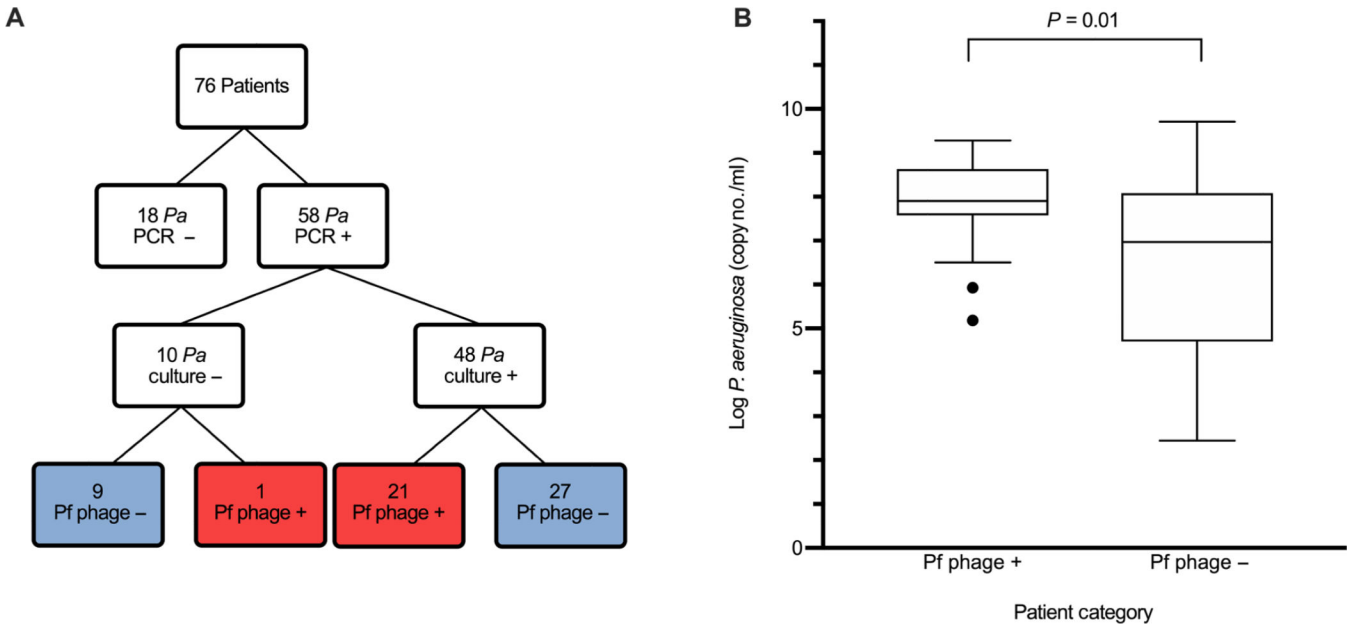

Fig. 3. *P. aeruginosa* concentration is higher in patients with Pf phage detected in the sputum within the Stanford CF cohort.

(**A**) Patients enrolled at the Stanford CF Center with detection of *P. aeruginosa* by both qPCR and respiratory culture and detection of Pf phage by qPCR. Of the 10 patients with *P. aeruginosa* detected by PCR but not grown on respiratory culture, 9 had history of *P. aeruginosa* on previous respiratory culture and 1 patient had no previous records available. *Pa*, *P. aeruginosa*. (**B**) *P. aeruginosa* concentrations in patients with Pf phage–positive sputum and Pf phage–negative sputum ($P = 0.01$). Boxes represent IQR with whiskers showing 1.5 × IQR and dots as outliers. Comparison was done by Wilcoxon ranked sum. Outliers on graph were included in analysis.

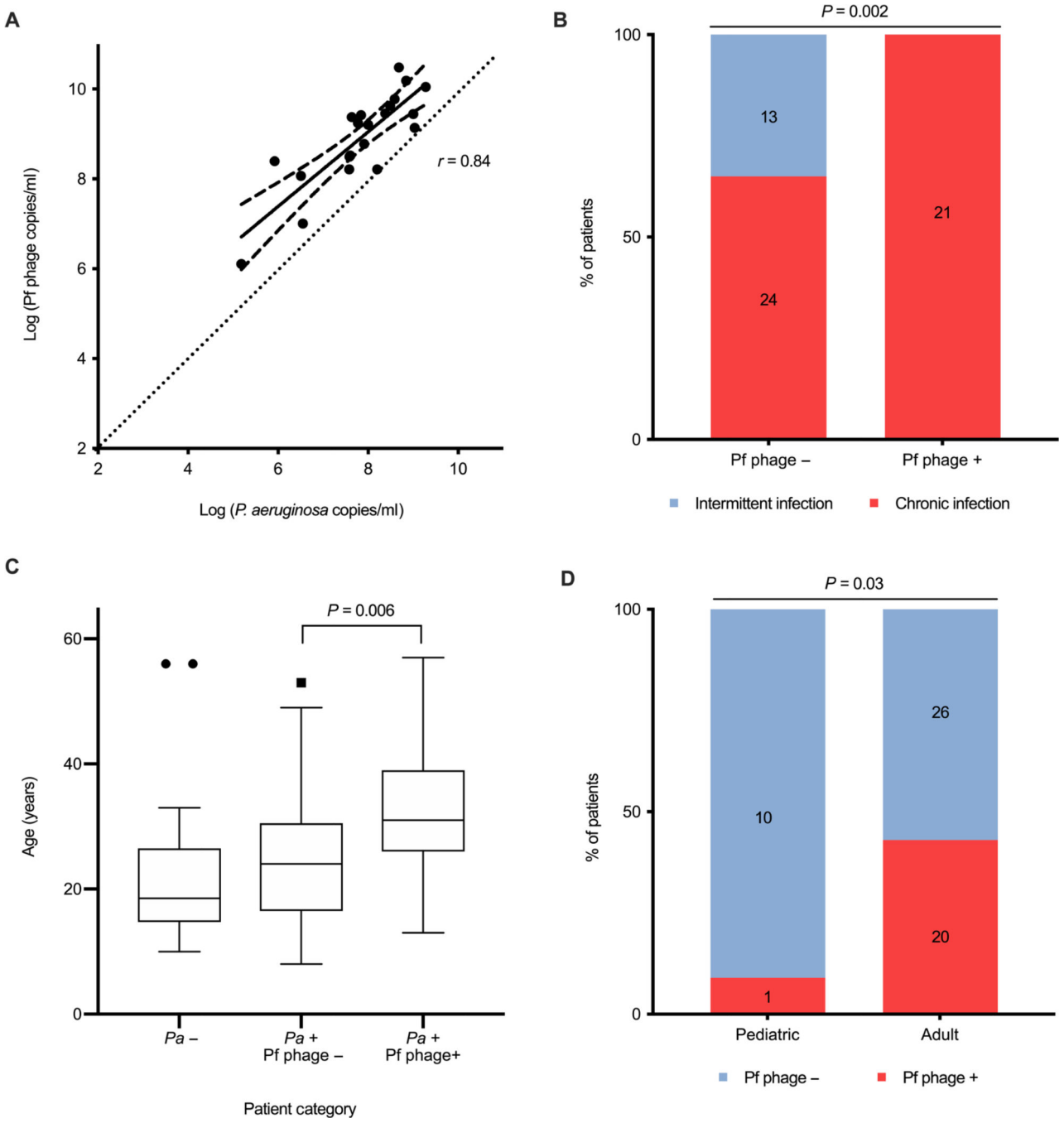

Fig. 4. Pf phage concentration correlates with *P. aeruginosa* burden, chronic infection and patient age in the Stanford CF cohort.

(**A**) Correlation between the concentrations of Pf phage and *P. aeruginosa* in patients positive for Pf phage ($r = 0.84$, $P < 0.001$). Dotted line, line for 1:1 correlation for reference. All samples were run in duplicate. The area between dashed lines represents the 95% CI. Correlation calculated by Kendall correlation. (**B**) Percentage of patients meeting the Leeds criteria for chronic *P. aeruginosa* infection in Pf phage–positive and Pf phage–negative samples ($P = 0.002$). Comparison by Pearson's χ^2 was used. (**C**) Age of patients with *P.*

aeruginosa–negative sputum, *P. aeruginosa*–positive and Pf phage–negative sputum, and *P. aeruginosa*–positive and Pf phage–positive sputum. Boxes represent IQR with whiskers showing 1.5 × IQR and dots as outliers. Comparison was done by Wilcoxon ranked sum. (**D**) Prevalence of Pf phage in adult versus pediatric patients with CF infected with *P. aeruginosa* ($P = 0.03$). Comparison by Pearson's χ^2. Outliers on graph were included in analysis.

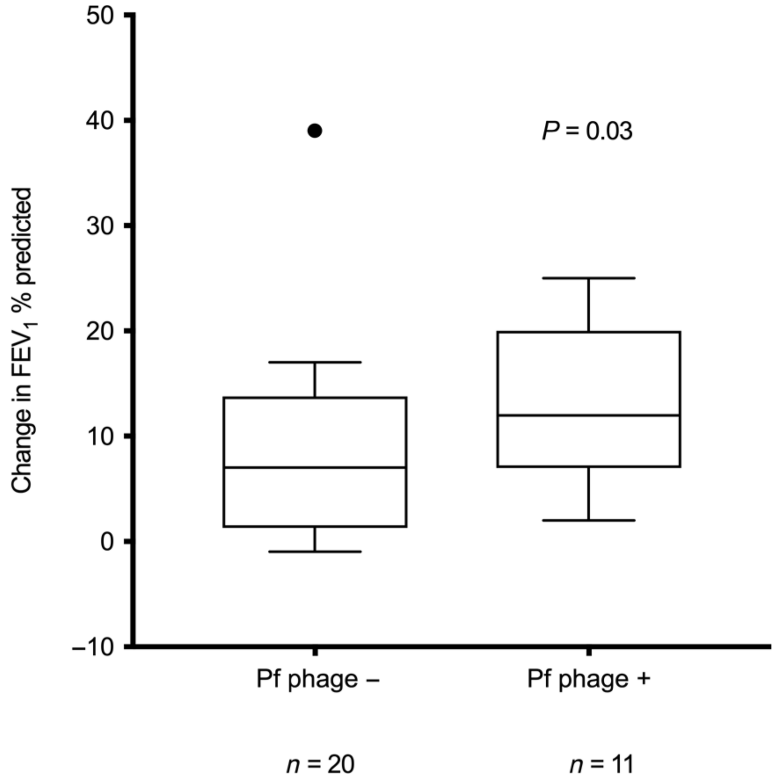

Fig. 5. Patients positive for Pf phage have reduced lung function during exacerbations in the Stanford CF cohort.

The difference between FEV_1% predicted at time of sampling and at presentation of subsequent exacerbation in patients with Pf phage–positive sputum and in those with Pf phage–negative sputum ($P = 0.03$). Only patients who were enrolled while at baseline health and who had exacerbation after enrollment were included in this analysis ($n = 11$ patients positive for Pf phage, $n = 20$ patients negative for Pf phage). These data include only patients with *P. aeruginosa* detected in their sputum. Boxes represent IQR with whiskers showing 1.5 × IQR and dots as outliers. Comparisons were made by Mann-Whitney test. Outlier on graph was included in analysis. Data are available in table S2.

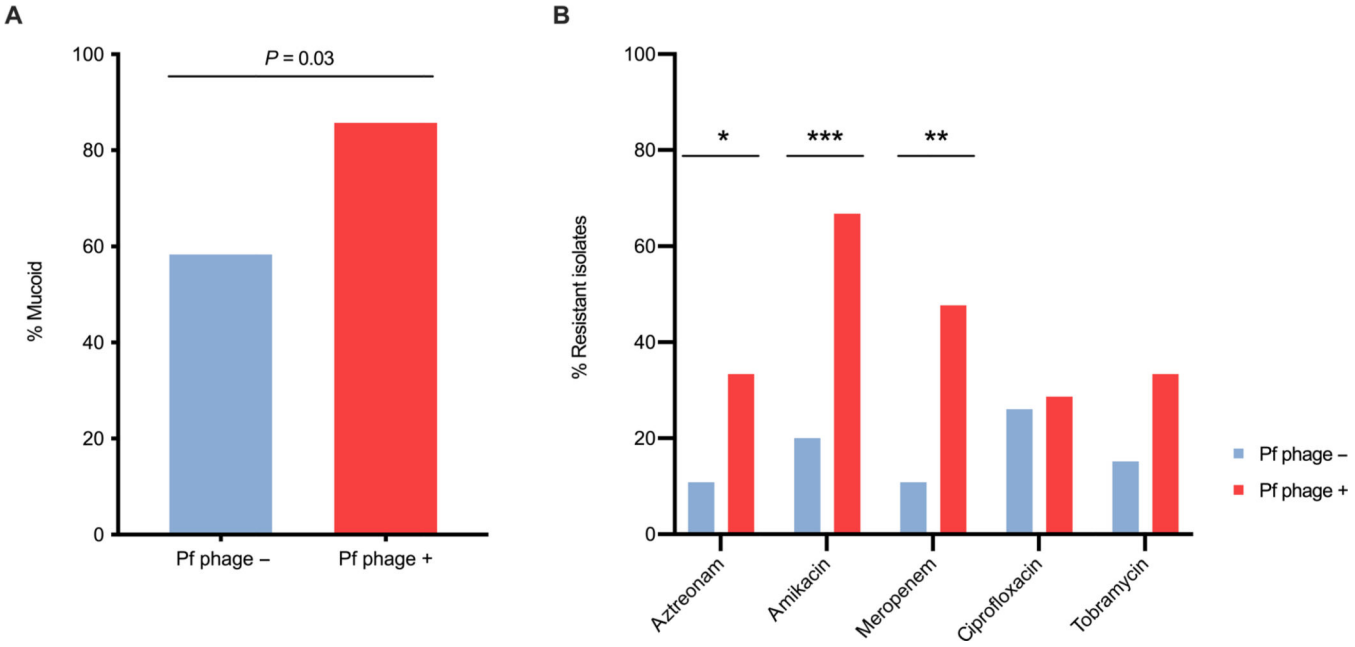

Fig. 6. Clinical isolates of *P. aeruginosa* from patients with Pf phage–positive sputum exhibit increased antibiotic resistance and are more likely to be mucoid.

(**A**) Proportion of isolates that display mucoid phenotype in isolates from Pf phage–negative sputum and Pf phage–positive sputum ($P = 0.03$). Comparison by Pearson χ^2 test. (**B**) The proportion of isolates that were resistant to antibiotics listed. $n = 28$ Pf phage–negative samples, $n = 20$ Pf phage–positive samples. Comparison was made by Pearson's χ^2 test. *$P = 0.0499$, **$P = 0.002$, ***$P = 0.0009$.

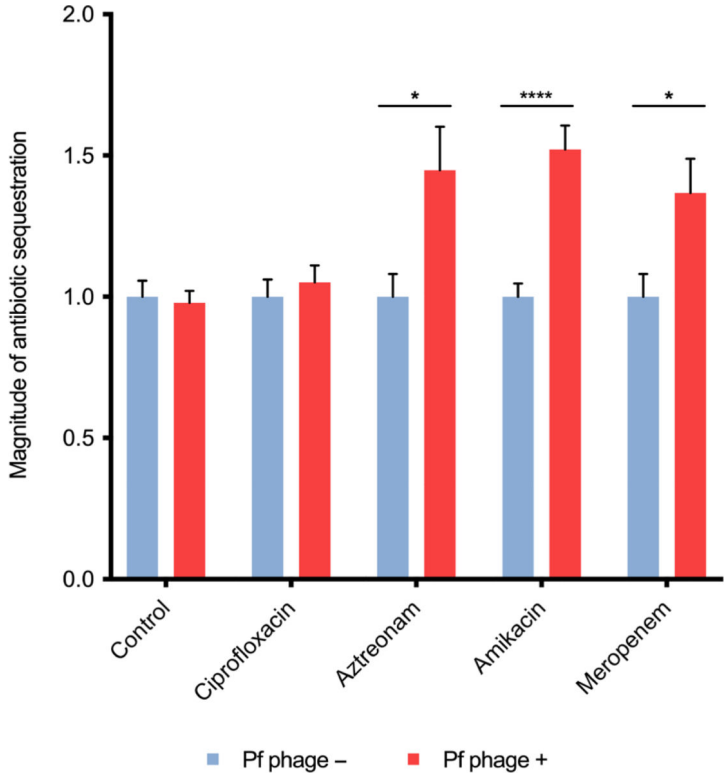

Fig. 7. Antibiotics aztreonam, amikacin, and meropenem can be sequestered by Pf phage.

The magnitude of sequestered antibiotic (bacterial proliferation normalized to control conditions) is displayed for each antibiotic when run through dialysis cassette containing either Pf phage and DNA or DNA alone (control). Samples were run in triplicate ($N = 2$ experiments). Error bars indicate mean ± SEM. Comparisons were made by Student's t test with an α level of 0.05. ****$P < 0.0001$, *$P < 0.05$.

Table 1.

Patient characteristics and microbiologic data in the Stanford CF cohort.

Wilcoxon ranked sum was used for analysis of continuous values, and Pearson's χ^2 was used for categorical values. *P* values for comparison of the two right columns, *P. aeruginosa*-positive Pf phage – patients versus *P. aeruginosa*-positive Pf phage-positive.

	***P. aeruginosa*-negative by qPCR**	***P. aeruginosa*-positive by qPCR**		***P***
		Pf phage-negative	**Pf phage-positive**	
Subjects, *n*	18	37	21	—
Sex, % male	72.2%	51.4%	52.4%	0.94
Age, years (±SD)	23.2 ± 13.5	25.9 ± 11.0	33.4 ± 10.9	0.006
FEV_1, % predicted (±SD)	50.3 ± 24.0	54.1 ± 20.1	58.7 ± 26.5	0.57
BMI, *Z* score (±SD)	−0.16 ± 1.0	0.8 ± 0.85	0.09 ± 0.9	0.37
CFTR genotype				
Homozygous F508del, *n* (%)	5 (27.8%)	17 (46.0%)	11 (54.5%)	0.52
Heterozygous F508del, *n* (%)	8 (44.4%)	14 (37.8%)	5 (23.8%)	
P. aeruginosa, copies/ml (range)	0	2.2×10^8 (2.8×10^2–5.1×10^9)	3.2×10^8 (1.5×10^5–1.9×10^9)	0.01
Pf phage, copies/ml (range)	0	0	4.4×10^9 (1.3×10^6–3.1×10^{10})	—

Table 2.

Phenotype and resistance patterns from respiratory culture from patients in the Stanford CF cohort.

Antibiotic resistance was assessed in clinical *P. aeruginosa* isolates cultured from the same sputum samples in which Pf phage status was assessed in our cohort. $N = 28$ Pf phage–negative samples and 20 Pf phage–positive samples. Comparison by Pearson's χ^2 test.

	***P. aeruginosa* + by qPCR**		***P***
	Pf phage −	**Pf phage +**	
Mucoid strain	58.3%	85.71%	**0.03**
Ciprofloxacin	12 (33%)	6 (29%)	0.71
Tobramycin	7 (19%)	7 (33%)	0.24
Aztreonam	5 (14%)	7 (33%)	**0.0499**
Amikacin	8 (22%)	14 (67%)	**0.0009**
Cefepime	7 (19%)	8 (38%)	0.12
Ceftazidime	7 (19%)	5 (23%)	0.70
Meropenem	4 (11%)	10 (48%)	**0.002**
Piperacillin-tazobactam	7 (19%)	5 (24%)	0.70