Acidic Microenvironment Determines Antibiotic Susceptibility and Biofilm Formation of *Pseudomonas aeruginosa*

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Submitted to the Graduate Faculty of the

Department of Environmental and Occupational Health

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2021

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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University of Pittsburgh, 2021

Pseudomonas aeruginosa (*P. aeruginosa*) can chronically colonize in immunocompromised hosts, especially in cystic fibrosis (CF) lungs, where *P. aeruginosa* persists for decades and frequently exists as biofilms. The impaired function of cystic fibrosis transmembrane conductance regulator (CFTR) leads to abnormal epithelial Cl^- /HCO_3^- transport and acidification of airway surface liquid. It has been reported that the CF lung microenvironment may be more acidic than those in non-CF due to the underlying problems of dysfunctional CFTR and the acidifying extracellular DNA originated from dead bacteria or host immune cells. However, it remains unclear why *P. aeruginosa* versus other pathogens most commonly infects the CF lung. We carried out studies to investigate if lower pH helps *P. aeruginosa* adapt and thrive in the CF-like acidic lung environment in the short term. We also presented evidence that the acidic biofilm lifestyle is selective for *P. aeruginosa vfr* mutations during long-term bacterial evolution studies, which had been identified in human CF respiratory longitudinal studies.

Our results revealed that, during adaptation to the environment, *P. aeruginosa* generally forms more biofilm and generates antibiotic resistance more quickly in acidic conditions. These adverse effects can be reversed by returning the acidic environment to physiologically neutral conditions. Our data provide mechanistic evidence linking the CF-specific acidic microenvironment to the reported emergence of P. aeruginosa *vfr* mutations in clinical CF isolates during long-term evolution.

P. aeruginosa appears to be highly adaptive to the CF-like acidic pH environment. By studying the effects of an acidic environment on bacterial response, we may provide a new therapeutic option in preventing chronic *Pseudomonas aeruginosa* infection and colonization. Specifically, targeting *vfr* may offer a new therapeutic option to eliminate bacterial biofilm formation, the main culprit contributing to the chronic *P. aeruginosa* colonization in CF.

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1.0 Introduction

1.1 Cystic Fibrosis

1.1.1 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder with a lack of cystic fibrosis transmembrane conductance regulator (CFTR) function. CF is a common life-shortening disease that affects multiple organs where cellular CFTR function is required. For example, the defective CFTR function in the pancreas leads to insufficient secretion of digestive enzymes and scarring of the pancreas tissue due to the development of excessive mucus (Kelly & Buxbaum, 2015). The scarring of the pancreas leads to malabsorption and CF-related diabetes because of the disruption of normal insulin production. The defective CFTR also leads to an increased chance of infertility in male CF patients (Sokol, 2001). These CF-related diseases might not impose an immediate threat to patient life with adequate treatments, such as pancreatic enzyme replacement therapy (PERT) for digestive health. But the fatal disease in CF is usually respiratory infections and, eventually, respiratory failure. Since there is no known cure for CF, increasing life expectancy by carefully managing lung disease has been the focus of current CF-related research. CF used to be strictly a pediatric disease, and patients rarely survived into adulthood during the initial identification of the disease in the early 20th century. However, given the current treatment options, such as CFTR-modulator drugs (e.g. Ivacaftor, Lumacaftor) plus aggressive antibiotic treatment, more than 50% of CF patients are 18 years and older (Foundation, 2020). Effective antibiotic treatments and CFTR modulators have shown promising results in promoting the quality of life in

CF and overall survival. Still, respiratory infections, especially *P. aeruginosa* infections, can rarely be eradicated by current therapeutic options, and even after effective CFTR modulator treatments, *P. aeruginosa* remains in the lungs and continue to be one of the most significant factors that contribute to CF mortality.

1.1.2 Current CF treatment options

The primary treatment option for CF is using CFTR modulators to restore normal CFTR function, such as Ivacaftor, Lumacaftor, and Tezacaftor. These newly developed drugs only target specific CFTR mutations that include gating mutations (G1244E, G178R, S549R, G551D), residual function mutations (A455E, E193K, R117C), splice mutations (711+3A→G, 3272-26A→G, E831X), conduction mutation (R117H) and protein processing mutation (F508del). According to the Cystic Fibrosis Foundation 2019 patient registry annual data report, the prevalence of F508del homozygous genotype of people who were seen by healthcare providers in 2019 was 44.4%, and the prevalence for people who carry a heterozygous F508del mutation was 40.9%. The CF patients who carry neither homozygous nor heterozygous F508del mutations were at a prevalence rate of 14.7% (Foundation, 2020). These clinical data suggest that the CFTR modulators have comprehensive coverage on the most prevalent CF genotypes. However, unless the patients have one of these exact CFTR mutations, these drugs will not work for them. For this reason, many CF patients are still left untreated by CFTR modulators. Therefore, we still have to investigate other potential underappreciated CF-related pathological factors for better CF respiratory infection management.

The other main CF treatment method is the use of antibiotics. The thick mucus buildup in the CF lungs reduces the mucociliary clearance of bacteria, induces severe chronic infections, and

results in irreversible lung damage. Even with carefully managed antibiotic treatments, respiratory failure is ultimately inevitable for 80% to 95% of CF patients (Lyczak, Cannon, & Pier, 2002; O'Sullivan & Freedman, 2009). *P. aeruginosa* is the major cause of CF lung damage and deterioration. It is commonly accepted that the intrinsic ability to become drug-resistant (Baqui & Rahman, 1987; Kobayashi, Yamaguchi, & Mitsuhashi, 1972; Richmond, Jack, & Sykes, 1971) is partly the reason why *P. aeruginosa* cannot be eradicated by antibiotics once acquired. For this reason, pediatric CF patients are under constant surveillance for initial lung *P. aeruginosa* colonization (Dickinson & Collaco, 2021). Once *P. aeruginosa* is cultured positive, an aggressive course of antibiotic treatment is given to the patients for the purpose of early eradication. Treatments usually involve nebulized tobramycin or aztreonam (Mogayzel et al., 2014). According to the 2019 CF patient registry, with successful surveillance and antibiotic treatments, 44.8% of pediatric CF patients transition into adulthood without *P. aeruginosa* lung infection (Foundation, 2020). However, after long-term antibiotic treatments, multi-drug resistant (MDR) *P. aeruginosa* starts to accumulate and become difficult to treat in older CF adults (Foundation, 2020).

1.1.3 Acidification of the CF airway

According to the CF Foundation Patient Registry Report, the prevalence of *P. aeruginosa* lung infection gradually increases over time from approximately 20% to 80% in CF patients from age 2 to 45 (Foundation, 2020). This report has led us to two hypotheses: 1. some components of the CF microenvironment favor the colonization and growth of *P. aeruginosa*; 2. *P. aeruginosa* adapts to the CF microenvironment through long-term evolution. Therefore, we looked into the functions of CFTR HCO_3^- secretion and epithelial ATP12A H^+/K^+ transportation. Normal CFTR secretes HCO_3^- across airway epithelia, but this function is impaired in CF (Coakley et al., 2003;

Devor, Bridges, & Pilewski, 2000; Garland et al., 2013; Pezzulo et al., 2012; Poulsen, Fischer, Illek, & Machen, 1994; J. J. Smith & Welsh, 1992; Tang et al., 2016). Shah et al. recently discovered that CF mouse does not display the human CF phenotype and is spared from bacterial lung infection because of the absence of airway ATP12A (Shah et al., 2016). ATP12A is the α subunit of the nongastric H^+/K^+ transporting ATPase that is found in both human and porcine airways. The active secretion of proton from the human and porcine airway actively acidifies the airway surface liquid and therefore leads to host defense abnormalities (Shah et al., 2016) while the airway HCO_3^- secretion is disrupted due to the altered CFTR function. The excess proton is not neutralized, which could explain why the CF porcine airway surface liquid (ASL) is naturally more acidic than non-CF subjects. In mouse, the ATP12A function is absent. The proton transportation is achieved by a much slower V-type ATPase. Without active epithelial H^+ secretion into the ASL, the CF mouse does not have acidified airway and is not prone to bacterial infection (Grubb & Boucher, 1999; Guilbault, Saeed, Downey, & Radzioch, 2007; Shah et al., 2016). They also presented evidence that human and porcine CF epithelia express ATP12A, but there is not enough HCO_3^- secretion to neutralize the excessive H^+ due to the abnormal CFTR function. This mechanism leads to airway acidification and impaired host bactericidal activity against *Staphylococcus aureus* (Pezzulo et al., 2012).

The measurements of CF ASL pH in humans have been inconclusive and controversial. The outcome of *in vivo* pH measurements in humans is dependent on pH probe material (gold or antimony), location (nose or bronchus), and age (neonates, children, or adults) (McShane et al., 2003; A. Schultz et al., 2017). Additionally, some studies reported that they were unable to measure significant pH differences in ASL of children with or without (w/wo) CF (André Schultz et al., 2017).

However, one of the most compelling pieces of evidence of CF ASL acidification was reported by Shah et al. They surgically opened a tracheal window and directly measured the tracheal ASL pH of newborn CF pigs and non-CF pigs. The pH values are approximately 6.9 and 7.2, respectively (Shah et al., 2016). They also tested the ASL pH of CF bronchial epithelial (CFBE) cells and non-CF human bronchial epithelial (HBE) cells seeded on an air-liquid interface (ALI). The pH values are approximately 6.4 in CFBE ASL and 6.9 in HBE ASL, while the basolateral media were supplemented with physiological HCO_3^- (25 mM) (Shah et al., 2016). In addition, extracellular DNA (eDNA) originated from the bacterial biofilm and human immune cells also contribute to the acidification of CF microenvironments, which is evidenced by acidic pH gradients within *P. aeruginosa* biofilms (de los Rios, Wierzchos, Sancho, & Ascaso, 2003; Hidalgo et al., 2009; Hunter & Beveridge, 2005) and acidified CF exhaled breath condensate (Ojoo, Mulrennan, Kastelik, Morice, & Redington, 2005; Tate, MacGregor, Davis, Innes, & Greening, 2002), all of which are difficult to be discredited by the specific study using a direct ASL pH measurement in children, who are usually with less prominent CF phenotype and severity. Cowley et al. reported a pH range of 2.9 to 6.5 in pediatric CF sputum samples (Cowley, Kopf, LaRiviere, Ziebis, & Newman, 2015), which also disputes the notion that acidic pH is not a significant pathophysiological factor in CF. The CF microenvironment is much more complicated than what can be currently measured on human subjects.

Many adverse effects of CF ASL acidification have been reported, such as reduced ASL antimicrobial activity (Abou Alaiwa et al., 2014; Pezzulo et al., 2012) and impaired mucociliary clearance as well as altered ASL and mucus viscosity (Birket et al., 2014; Gustafsson et al., 2012; Hoegger et al., 2014; Quinton, 2008). In addition to these validated concerns, we propose that the acidic CF ASL also contributes to the persistent *P. aeruginosa* lung infection.

Another important aspect of the abnormal CF lung environment is the thick mucus buildup from the faulty epithelial Cl^- ion transfer (Moreau-Marquis, Stanton, & O'Toole, 2008; Worlitzsch et al., 2002), a direct result of abnormal CFTR function. *P. aeruginosa* growth has been found in the thick mucus layer on top of CF lung epithelial cells (Hassett et al., 2002; Worlitzsch et al., 2002). The mucus environment changes *P. aeruginosa* biofilm formation behavior (Yoon et al., 2002) and for this reason, the CF sputum medium, which contains additional mucin and DNA, was developed to mimic this particular condition (Kirchner et al., 2012). Recent studies suggested that the CF lung submucosal glands secrets significantly more acidic mucus upon response to bacterial infections compared to non-CF subjects (Xie et al., 2020). The acidic mucus has higher elasticity and makes mucociliary clearance more difficult. Restoring neutral pH after the formation of acidic mucus strands did not change the detrimental effects of the acidic mucus. This research provided additional evidence that the airway microenvironment acidification is not limited to ASL, but also the mucus secretion. Since CFTR modulators do not target all CF genotypes, the acidic pH microenvironment in the CF lungs is likely an important pathological factor. Investigating the fundamental mechanisms of *P. aeruginosa* adaptation to acidic pH could help discover better treatment options.

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous, rod-shaped, Gram-negative opportunistic pathogen. The genome size of *P. aeruginosa* ranges from approximately 5 to 7 Mbps, which indicates that *P. aeruginosa* is a highly diverse bacterial species. *P. aeruginosa* is aerobic and facultatively anaerobic (Arai, 2011). *P. aeruginosa* is a motile bacterium and adapts three types of motilities:

flagellum mediated swimming motility, flagellum and biosurfactant mediated swarming motility, and pilus mediated twitching motility. *P. aeruginosa* motility is often correlated to different stages of clinical infections and is an important indicator of acute virulence (Drake & Montie, 1988). For example, the flagella modulated swimming motility is an indicator of planktonic growth and decreased biofilm formation tendency, as evidenced by our gene expression data (Fig. 17A). Swarming motility is modulated by both the flagella and the secretion of biosurfactant while twitching motility is achieved by type IV pilus activity. Both motilities have been positively associated to increased virulence factors activation and antibiotic resistance (Overhage, Bains, Brazas, & Hancock, 2008).(Zolfaghar, Evans, & Fleiszig, 2003).

P. aeruginosa could cause human diseases in certain conditions. *P. aeruginosa* is especially dangerous for people who are immunocompromised. According to the CDC, nosocomial infections such as ventilator-associated pneumonia caused by MDR *P. aeruginosa* are now considered a serious threat to public health (CDC, 2019). Long-term implantation of medical catheter is also associated with high incidence rates of *P. aeruginosa* infections (Cole, Records, Orr, Linden, & Lee, 2014). *P. aeruginosa*-induced sepsis is a significant concern for managing patients with large-scale burn wounds (Gonzalez et al., 2016). Most importantly, *P. aeruginosa* is one of the most important virulent pathogens that contribute to CF morbidity and mortality (Clary-Meinesz, Mouroux, Cosson, Huitorel, & Blaive, 1998; Garland et al., 2013; Kreda, Davis, & Rose, 2012; Nakayama et al., 2002), but the interactions between acidified ASL and *P. aeruginosa* have never been addressed.

Many factors contribute to CF airway acidification, and the response of *P. aeruginosa* to this CF-related pathological factor needs to be carefully investigated. Studies demonstrated that when *P. aeruginosa* is under acidic stress, the bacterial outer membrane permeability

(PhoPQ/PmrAB-controlled surface modifications) could decrease and result in decreased antibiotic uptake (Adewoye & Worobec, 1999; Wilton, Charron-Mazenod, Moore, & Lewenza, 2016). Additionally, acidic pH likely modulates aminoarabinose-modified LPS and spermidine, which mask bacterial negative surface charges and block the entrance of aminoglycosides (Ernst et al., 2007; Johnson, Mulcahy, Kanevets, Shi, & Lewenza, 2012; Wilton et al., 2016). Most importantly, the acidic ASL has shown a disruption of the host defense mechanisms, such as the innate host antimicrobial peptides human β-defensin (hBD)-3 and LL37 (Abou Alaiwa et al., 2014). This is one of the fundamental aspects of the detrimental effects of CF airway acidification.

1.3 Current research directions

It is imperative to keep exploring the possibilities of new *P. aeruginosa* prevention and treatment strategies such as acidic pH neutralization, which has been proposed as a CF lung infection treatment (Abou Alaiwa et al., 2016; Ferrera, Capurro, Delpiano, Gianotti, & Moran, 2021). The inhalation of Recombinant human DNase (rhDNase), an approved treatment option for the purpose of reducing CF airway mucus viscosity, could also potentially decrease the airway microenvironment pH by reducing the amount of available eDNA released by host immune cells or dead bacterial cells. It is also important to understand the genetic basis of *P. aeruginosa* adaptation to the CF-specific microenvironment and how to target these essential bacterial gene mutations in order to better manage *P. aeruginosa* chronic colonization.

These future research directions are warranted because, in recent years, significant progress has been made in the development of CFTR-potentiator drugs. Ivacaftor and lumacaftor-ivacaftor combination drugs have shown promising results on CF patients who carry the G551D and

Phe508del mutations. Significant improvement of CF lung function and reduced *P. aeruginosa* infection severity have been reported (Caverly, Zhao, & LiPuma, 2015; Heltshe et al., 2015; Millar, McCaughan, Rendall, Downey, & Moore, 2018; Rowe et al., 2014; Solomon, Marshall, Ramsey, & Rowe, 2015). However, a decline of CF sputum *P. aeruginosa* density after initial Ivacaftor treatment was followed by *P. aeruginosa* persistence in the long-term (Hisert et al., 2017), which could be a result of efficient bacterial adaptation/evolution to the newly established, Ivacaftor-modulated lung microenvironment.

1.4 Public Health Impact

Cystic fibrosis is a progressive genetic disease that causes persistent lung infections and limits the ability to breathe over time. There is currently no known cure for this disease, and it is affecting more than 31,000 people in the United States and more than 70,000 people worldwide. Respiratory failure is the leading cause of death in CF patients, and chronic *P. aeruginosa* lung infection is the predominant cause of CF morbidity and mortality. The mechanisms of CF lung acidification are only discovered in recent years, but no study has addressed the potential correlation between the acidic lung microenvironment and the high prevalence of *P. aeruginosa* infections in CF. This research investigated the possible mechanisms and provided essential guidance for clinical research on better *P. aeruginosa* prevention and treatment.

CF is a relatively rare disease that primarily impacts the Caucasian population, with an incidence rate of approximately 1 per 3200 individuals (Hamosh et al., 1998). However, in public health research, it is not only about promoting health for most people, but all people.

2.0 Short-term *Pseudomonas aeruginosa* response to the acidic environment

2.1 Introduction

The environment in CF lungs is thought to be acidic, as evident by the lower-than-neutral pH value of the airway surface liquid (ASL) in newborn CF pigs and differentiated human and porcine primary epithelial cell cultures, compared to non-CF controls (Pezzulo et al., 2012; Shah et al., 2016). The malfunction of CFTR also leads to elevated Na^+ and Cl^- levels in the airway, inhibiting the natural antimicrobial factors in ASL (Grandjean Lapierre et al., 2017; Zabner, Smith, Karp, Widdicombe, & Welsh, 1998). *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic Gram-negative pathogen that is the most prevalent bacterial species in adult CF lungs and contributes to the associated high mortality rates (Silva Filho et al., 2013). Bacteria expand their population in the natural environment and the host via two different forms. The planktonic form of free-moving bacteria is the typical way bacteria spread themselves at the initial stage of reaching a new environment. In contrast, the accumulated form of bacteria, so-called "biofilm", represents another critical pathogenic mechanism. It is noted that the bacterial biofilm formation in the CF airway likely contributes to the chronic colonization of *P. aeruginosa* (Lyczak et al., 2002) since it is commonly accepted that biofilm formation is a phenotype that is usually associated to chronic infection and increased difficulty to eradication (Moreau-Marquis et al., 2008). This has been further investigated and confirmed by post-mortem dissection of the CF lungs infected by *P. aeruginosa* biofilms (Lam, Chan, Lam, & Costerton, 1980).

Our results demonstrated that *P. aeruginosa* grew under other classes of antibiotics (β-lactam and fluoroquinolone), in addition to the previously published aminoglycosides (Wilton et

al., 2016) also increase the acquired antibiotic tolerance when medium pH is acidic. Currently, known mechanisms could partly explain why acidic pH induces immediate antibiotic tolerance, but the long-term impact of acidic pH on bacterial evolution with antibiotic treatment remained unknown. From the host perspective, the CF lung is burdened with acidic eDNA originated from the immune cells recruited by chronic bacterial infections (Lethem, James, Marriott, & Burke, 1990). In addition, Shah et al. reported evidence that the nongastric H+/K+ adenosine triphosphatase (ATP12A), a proton pump that actively acidifies human and porcine airways, is absent in mice (Shah et al., 2016). This explains why CFTR-deficient mice with no acidic airway epithelium are free from opportunistic respiratory infections.

The evidence of CF airway acidification and the high prevalence of *P. aeruginosa* among CF lung infections prompted us to determine the effects of a CF-like acidic environment on the pathogenic ability of *P. aeruginosa*. Our results indicated that the acidic environment stimulates increased *P. aeruginosa* biofilm formation, promotes faster bacterial evolution towards elevated antibiotic resistance as evidenced by stable genetic mutations, and increases expressions of multiple biofilm/virulence-related genes. Interestingly, based on our observation, *P. aeruginosa* was the only bacterial species from the notorious ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.*) that showed a pattern of increased biofilm formation in acidic conditions (Lin, Pilewski, & Di, 2021). Furthermore, the acidic environment on the apical surface of differentiated primary bronchial epithelial cells isolated from CF patients (CFBEs) results in increased *P. aeruginosa* attachment and bacterial numbers than the physiologically neutral environment on the surface of cultured primary human bronchial epithelial cells from normal subjects (HBEs). These adverse effects of a CF-like acidic environment can be ameliorated by

modulating the acidic environment into physiologically neutral conditions. The varying characteristics and behavior of *P. aeruginosa* in different pH conditions may provide additional treatment targets and options for CF sufferers in preventing chronic *P. aeruginosa* infection and colonization.

2.2 Materials and Methods

Bacterial strains. Clinical *P. aeruginosa* strains were isolated from pediatric CF patients with chronic pulmonary infections at Seattle Children's Research Institute except for AA43 and KK1(Bragonzi et al., 2009), which were from a collection of the CF clinic at Medizinische Hochschule of Hannover, Germany. The lab strains used in this study were *P. aeruginosa* PAO1 (ATCC, BAA-47), UCBPP-PA14 (Rahme et al., 1995), *Staphylococcus aureus* ATCC#49775, *Klebsiella pneumoniae* ATCC#43816, and *Acinetobacter baumannii* ATCC#19606. All other ESKAPE species were collected from patients at the University of Pittsburgh Medical Center or Seattle Children's Research Institute.

Preparation of *P. aeruginosa*. All bacteria were retrieved from -80°C glycerol stock and streaked on tryptic soy agar plates. Single colonies were picked and incubated in tryptic soy broth (TSB) overnight at 37°C in an orbital shaker. The overnight culture was diluted at 1:5 with fresh TSB and incubated for an additional 2 hours for exponential growth. Bacteria were centrifuged at 2,000g for 5 minutes. The pellet was resuspended in 1ml PBS. To ensure reproducible results, bacterial concentration was adjusted to approximately 10^9 CFU/mL, optical density (OD_{500nm}) = 0.5±0.01 in a spectrophotometer.

Bacterial growth inhibition assay (GIA). All GIA studies were performed in 10% TSB diluted in PBS. The 96-well plate was incubated at 37°C in a microplate reader for 18 hours. Optical density (OD) at 570nm was measured every hour with continuous double orbital shaking at 425 cycles per minute. The pH of the bacterial culture was adjusted to 6.0, 6.5, 7.0, and 7.5 using hydrochloride acid (HCl). The starting bacterial concentration in each treatment group was 10^6 CFU/mL. All pH-adjusted media were filter-sterilized by a syringe filter unit (pore size: 0.22μm, Millipore SLGP033RS) to ensure sterility.

Biofilm assay. The crystal violet biofilm staining method developed by O'Toole *et al.* was used in this study with slight modifications (O'Toole, 2011). All biofilm studies were performed in pH adjusted m63 medium supplemented with 1mM $MgSO_4$, 25μM $FeCl_3$, 40mM of D-glucose, and 4mM of L-glutamine. The m63 was supplemented with 10% TSB to facilitate *Staphylococcus aureus* growth. The combination of D-glucose and L-glutamine allows the m63 to maintain its pH after *P. aeruginosa* overnight incubation. The testing bacterial concentrations were $5x10^7$ and 10^6 CFU/ml for biofilm formation and antibiotic biofilm prevention, respectively. After 18 hours of biofilm formation in a humidified 37°C incubator (with or without antibiotic treatment), the supernatant was carefully removed by pipetting, and biofilm attached to the plate was stained with 0.5% crystal violet (20% ethanol + 80% deionized water) solution for 15 minutes. The excessive dye was then rinsed off with water, and 95% ethanol was added to release the dye from the biofilm. OD values were acquired by a microplate reader at 620nm wavelength.

Bacterial evolution in antibiotics. Evolution studies were carried out using the m63 medium (same as biofilm assay). The bead transfer-based biofilm evolution model was described previously (Cooper, 2018; Jiang, Deslouches, Chen, Di, & Di, 2019; Poltak & Cooper, 2011). Briefly, the PA14 ancestor was added to 5mL of pH 6.5/7.5 m63 (media pH adjusted by HCl) with

½ MIC of antibiotic treatment at day 1. After 24 hours, the PA14 biofilm (formed on sterile acrylic beads) was transferred to the next tube of fresh m63 medium with a sterile bead inside. For planktonic culture, 50uL of PA14 overnight culture was transferred to the next tube of fresh m63 medium. The dosage of antibiotics was doubled at the time of each transfer. The groups that survived antibiotic treatment were transferred to the next tube. The groups that could not tolerate the elevated antibiotic concentration were incubated again at the prior concentration without doubling antibiotic concentration.

Bacterial whole-genome sequencing. Bacterial genomic DNA samples were extracted using the Qiagen DNeasy PowerBiofilm Kit. Biofilm attached to beads was dissociated by sonication in sterile PBS before DNA extraction. Planktonic cultures were centrifuged and pelleted before DNA extraction. Library preparation (Baym et al., 2015; Turner, Marshall, & Cooper, 2018) and WGS were performed by the University of Pittsburgh Microbial Genome Sequencing Center using Illumina NextSeq500.

Bioinformatics. Raw sequencing reads were quality filtered and trimmed by Trimmomatic (Bolger, Lohse, & Usadel, 2014) using the following parameters: NexteraPE-PE.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:70. Genetic variants were predicted by *breseq* v0.33.0 (Deatherage & Barrick, 2014). The *Pseudomonas aeruginosa* UCBPP-PA14 reference genome was downloaded from NCBI. The PA14 ancestor clone was sequenced to eliminate background mutations. The sequencing depths of all evolved PA14 populations were at least 125x. Mutation percentage higher than 20% are shown in the figures.

Primary airway epithelial cell cultures. Differentiated primary human bronchial epithelial cells were derived from lungs removed at the time of lung transplantation at the Center for Organ Recovery and Education (Pittsburgh, PA, USA). Cells were prepared using previously

described methods (Liu, Bartlett, et al., 2013; Liu, Di, et al., 2013) approved by the University of Pittsburgh Institutional Review Board. Donor primary human CF and non-CF bronchial epithelial cells were first isolated from donor tissues and propagate under submerged cell culture. Upon confluence, epithelial cells were disassociated and seeded onto a transwell cell culture plate at approximately $2x10^5$ cells/well (Corning, NY). Epithelial cells were maintained in Bronchial Epithelial Cell Growth Medium (Lonza, Basel, Switzerland). The cell culture was changed to air-liquid interface (ALI) by removing the apical medium three days after initial cell seeding and maintained for three weeks for cell differentiation.

Treatment and host defense activity of primary airway epithelial cell cultures. The 4mM ouabain stock solution was prepared and dissolved in DMSO. Ouabain was diluted to 20μM in normal saline and 20μL of the diluted ouabain or DMSO (vehicle control) was added to the primary cell cultures apically and incubated for 2 hours in 37ºC supplied with 5% CO_2. Primary cultured epithelial cells were washed apically with 100μL of PBS 24 hours before the experiment. A total of 10μL of the apical fluid was immediately added onto pH test strips. After pH reading, all remaining apical fluid was removed, and another 20μL of 20μM ouabain or DMSO was added with 50μL of PAO1 suspended in normal saline (10^7 CFU/insert). Cells were then incubated for additional 5 hours for biofilm to form. All apical supernatant was collected for determining the CFU of unattached planktonic bacteria. Biotic biofilm assay was used by counting CFU of bacterial biofilm formed on the epithelial cells. ALI membrane was removed from the filter and sonicated in 2mL of PBS for 30 seconds at 80% amplitude by the DPS-20 dual processing system (130 W; PRO Scientific) to disassociate congregated PAO1 biofilm. After sonication, both planktonic and biofilm samples of PAO1 were plated on tryptic soy agar plates for total CFU counting.

Gene expression. A total of 5 x 10^7 CFU/mL PAO1 was cultured in pH-adjusted (pH=6.0, 6.5, 7.0 and 7.5) DMEM for three hours in 100x15mm round-petri dishes at 37°C. For biofilm RNA samples, supernatant in all petri dishes was discarded, and a thin layer of PAO1 biofilm was scraped off using a sterile cell scraper. The same amount of PAO1 was incubated separately in 5 mL of pH 7.5 DMEM at the same time and served as a planktonic control. Total biofilm and planktonic RNA were extracted as previously published (Casciaro et al., 2019; Cury & Koo, 2007; Lin, Deslouches, Montelaro, & Di, 2018). The cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression results were obtained using the Fast SYBR Green Master Mix (Applied Biosystems) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). $\Delta\Delta C_t$ values were calculated and analyzed using a method previously published (Lin & Di, 2020). The list of primers is listed in Table 1.

Table 1. List of primers used for quantitative real-time PCR.

Gene	Primer sequence	
tolA	Forward	5' - GCG TAA TGG AAT GAG CGT AGA A -3'
	Reverse	5' - CGA ACT GTC GAA AGG CTT GT - 3'
ndvB	Forward	5' - TGT GGA TCG CCT ACG ACT A - 3'
	Reverse	5' - CGG TGA ACA GCA CGA TGA - 3'
mucB	Forward	5'-TTT GCT TGG CAG CCT GAT-3'
	Reverse	5'-GGT GCC TTG GAA ACT GTT CT-3'
exoS	Forward	5'-GAC AGG CTG AAC AGG TAG TG-3'
	Reverse	5'-TTC AGG GAG GTG GAG AGA TAG-3'
exoT	Forward	5'-GCT GAA CAG GTC GTG AAG A-3'
	Reverse	5'-CCG GGA GGT GGA GAG ATA G-3'
fimT	Forward	5'-CGC TTG CAA AGA AGG AAA GG-3'
	Reverse	5'-CCT TCC GCA GAG CAG AAA-3'
fimX	Forward	5'-CCT GGC CTA TAT CCA TCT CAA C-3'
	Reverse	5'-ACT GTT CAC GCA TCA GTC C-3'
rhlA	Forward	5'-CGA GAC CGT CGG CAA ATA C-3'
	Reverse	5'-GCA CCT GGT CGA TGT GAA A-3'
rhlB	Forward	5'-CTC ACG AGA AGT ACG GGA TTC-3'
	Reverse	5'-CTC GGG CAC GTT GAA CT-3'
rplU *	Forward	5'-CGC AGT GAT TGT TAC CGG TG-3'
	Reverse	5'-AGG CCT GAA TGC CGG TGA TC-3'

*The constitutively expressed *rplU* gene served as a reference gene.

Bacterial motility assay. Bacterial swarming and twitching motility assays were performed according to the protocols previously published (Ha, Kuchma, & O'Toole, 2014a; Turnbull & Whitchurch, 2014). M8 medium ($Na_2HPO_4 \cdot 7H_2O$, supplemented with 0.5% casamino acids, 0.2% D-glucose, 1mM $MgSO_4$) was used for agar preparation (0.5% agar for swarming and 1.5% agar for twitching). Agar pH was adjusted to 7.5 and 6.5 by concentrated HCl. The overnight culture of PAO1 was diluted to 10^6CFU/mL. For swarming motility, 2.5μL of diluted PAO1 was dropped

to the center of the agar. For twitching motility, a thin 10μL pipette tip was first dipped into the diluted PAO1, then punctured through the center of the agar. Plates were incubated at 37°C for 24 hours before measurement.

Statistical analysis. Error bars represent mean ± standard error of the mean (SEM). One-way Analysis of Variance (ANOVA) and Tukey's multiple comparisons tests were used to assess the overall change of MIC in the bacterial evolution model. One-way ANOVA and Dunnett's multiple comparisons test were used to assess the change of bacterial gene expressions among various pH conditions. Two-way ANOVA and Dunnett's multiple comparisons test were used to assess the effects of pH and salt on bacterial biofilm formation. The student's *t-test* was used to assess statistical significance between two subjects, such as biofilm formation and antibiotic biofilm prevention in different pH, human ALI ASL pH, and CFU with or without ouabain treatment. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; otherwise not significant (NS).

Data availability. All data generated or analyzed during this study are included in this published article (and its supplementary information files). All sequencing data have been deposited with links to BioProject accession number PRJNA685187 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

2.3 Results

2.3.1 The response of *P. aeruginosa* to CF-like microenvironment

P. aeruginosa gradually increases its presence in the CF airways after the pathological phenotypes of CFTR malfunction appear more obviously with the increased age of the CF sufferers

(Foundation, 2020). It is well-documented that compromised functions of CFTR and the CFTR-modulated HCO_3^- secretion in CF result in an abnormal environment of higher salt (~100±5mM) (Zabner et al., 1998) and lower pH (~6.7±0.3) (Chen et al., 2010; Pezzulo et al., 2012; Shah et al., 2016) in the CF ASL than those of normal subjects.

We first examined the effects of salt concentration and acidic condition on bacterial planktonic and biofilm mode of growth to understand the effects of the CF-like microenvironment on the behavior of *P. aeruginosa*. Two widely used *P. aeruginosa* lab strains (PAO1 and PA14) and two MDR *P. aeruginosa* clinical strains isolated from CF patients (*P.a.* 129-5 and *P.a.* 152-19) were selected for this experiment. The growth curves indicated that various salt concentrations (addition of 50, 100, and 150mM NaCl) and pH conditions (pH= 6.0, 6.5, 7.0, and 7.5 adjusted by HCl) did not result in any noticeable difference in the proliferation rate of planktonic *P. aeruginosa* in all four strains (Fig. 1). There was less biofilm biomass formed with the increasing NaCl concentrations in PAO1. In contrast, the effect of salt concentrations on biofilm formation was not apparent in the other three *P. aeruginosa* strains (Fig. 2). Nonetheless, we observed significant changes in the initial attachment of bacterial biofilm under acidic pH conditions after just 3 hours of incubation (Fig. 2). Except for PA14, all the other three tested *P. aeruginosa* strains showed similar results of increased attachment of bacteria at acidic (pH<7) conditions within each tested NaCl concentration group. These results suggest that the acidic environment likely promoted the initial attachment of *P. aeruginosa*.

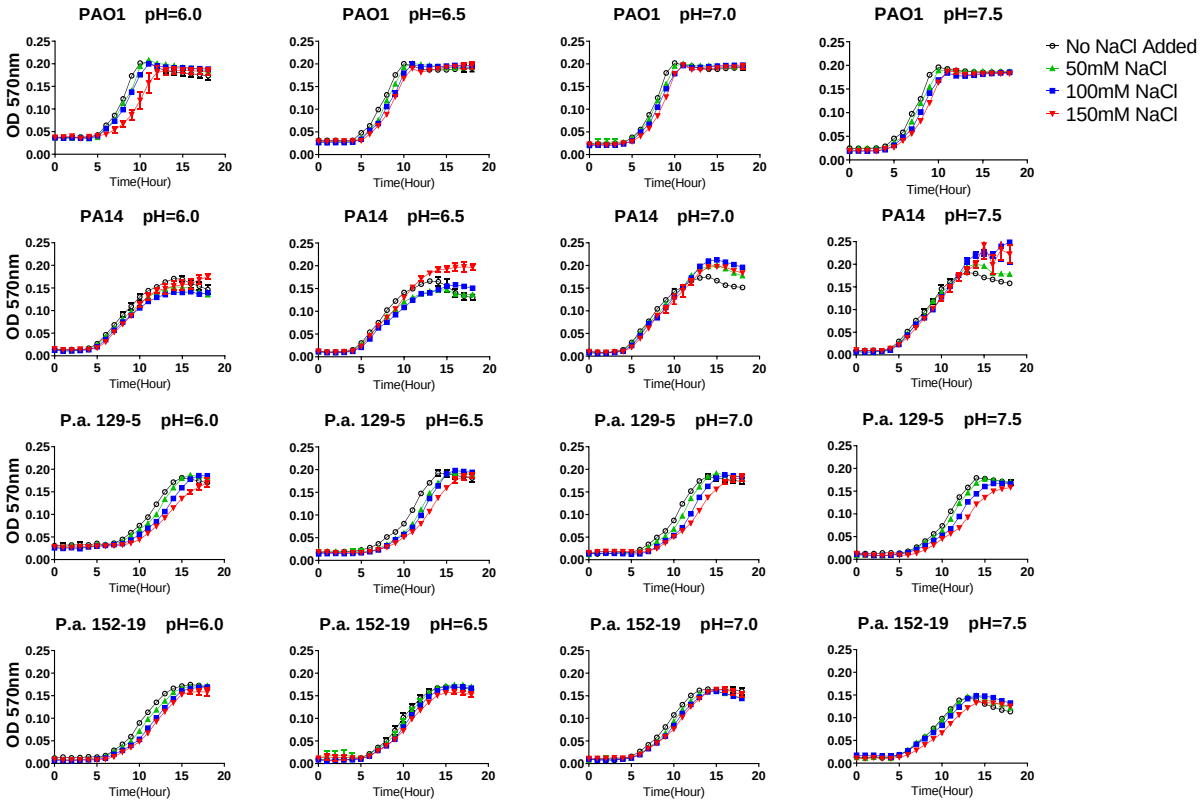


Figure 1. Adjusted salt and pH conditions do not significantly affect the proliferation rate of planktonic *P. aeruginosa*.

PAO1, PA14, *P.a.*129-5 and *P.a.*152-19 were inoculated in 10% TSB (n=3). The salt concentration of the culture media was adjusted by the addition of 50, 100, and 150mM NaCl. No NaCl group (pH=7.5) was used as a control. Optical density at 570nm was measured every hour for 18 hours at 37°C in a microplate reader.

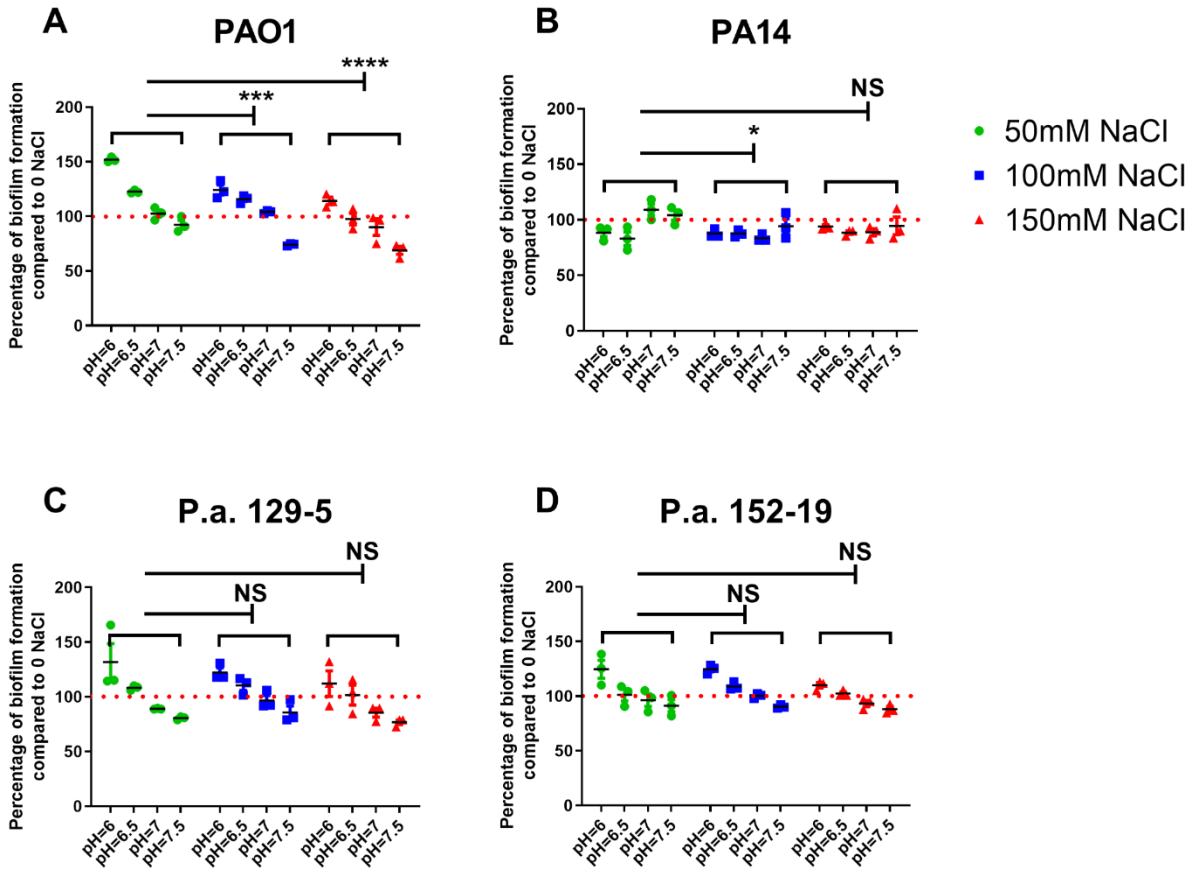


Figure 2. Elevated NaCl concentrations have minimal effect on P. aeruginosa biofilm formation but acidic pH promotes *P. aeruginosa* biofilm formation.

(**A** to **D**) Effect of NaCl (50, 100 and 150mM) and pH (6.0, 6.5, 7.0 and 7.5) on *P. aeruginosa* biofilm formation compared to positive controls (n=3). Bacteria were incubated for 3 hours at 37°C in 96-well microplates. The crystal violet staining method was used to quantify the biofilm/biomass attachment. The red dotted lines denote the (+) control of biofilm formation at pH 7.5 without additional NaCl. Data are mean ± SEM. Two-way ANOVA was used for statistical analysis. ***p*<0.01; ****p*<0.001; *****p* <0.0001; NS: not significant.

2.3.2 Increased *P. aeruginosa* antibiotic tolerance under acidic pH conditions

It is known that common antibiotic treatments such as inhaled tobramycin and ceftazidime in CF respiratory infections do not guarantee respiratory *P. aeruginosa* eradication (Foundation, 2020; Mayer-Hamblett et al., 2012). Thus, we sought to determine if the CF-like acidic environment enhanced bacterial tolerance to antibiotics. Three different classes of commonly used

standard-of-care antibiotics, including ceftazidime (β-lactam), ciprofloxacin (fluoroquinolone), and tobramycin (aminoglycosides), each with a distinct antibacterial mechanism, were tested against the same four bacterial strains of *P. aeruginosa* at their respective MICs (at physiologically neutral pH 7.5) under different pH conditions. Our results indicated that the acidic pH environment alone does not notably change the *P. aeruginosa* proliferation rate (Fig. 3A). However, acidic conditions increased bacterial tolerance to all three clinically used antibiotics, as demonstrated in the elevated growth curves compared to pH 7.5 when the same dosage of each antibiotic was used in all varying pH conditions (Fig. 3B). In all cases, *P. aeruginosa* grown under the acidic conditions (pH=6.0 & pH=6.5) demonstrated higher tolerance to the antibiotic treatments as their growth curves were closer to those of the "no antibiotic treatment" controls. The slope of the growth curve gradually decreases as the pH value increases. To determine if the increased bacterial growth in lower pH conditions was due to permanent degradation or temporary inactivation of antibiotics in the acidic environment, we pre-incubated the antibiotics in physiologically neutral pH 7.5 or acidic pH 6.0 for 5 hours before the bacterial growth kinetic experiments. Interestingly, the antibiotics pre-exposed to pH 6.0 and pH 7.5 showed similar growth inhibition curves when the growth inhibition assay (GIA) was performed at the pH 7.5 condition (Fig. 4). The results indicated that antibiotics pre-exposed to acidic conditions (pH=6.0) did not lose their drug potency and regained their antibacterial activity after returning to the physiologically neutral condition (pH=7.5). These data suggested that *P. aeruginosa* could quickly become more tolerant to antibiotic treatment in an acidic environment.

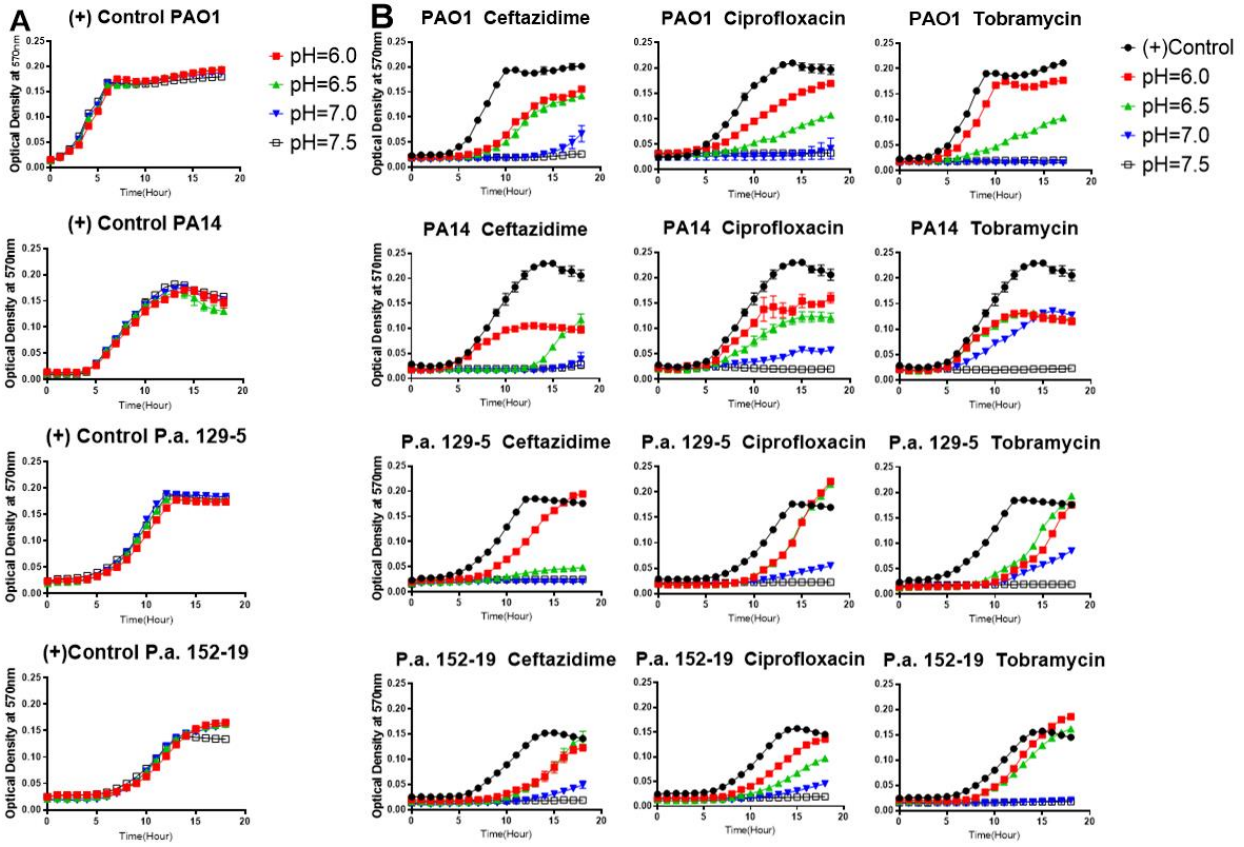


Figure 3. Acidic pH conditions impair the antimicrobial activity of standard-of-care antibiotics against *P. aeruginosa*.

(A) *P. aeruginosa* growth curve in pH adjusted, antibiotic-free medium; **(B)** *P. aeruginosa* growth curve in pH adjusted medium, supplemented with ceftazidime, ciprofloxacin, and tobramycin at the respective MIC for each antibiotic at pH 7.5. Optical density at 570nm was measured every hour for 18 hours at 37°C in a microplate reader. A pH-adjusted 10% TSB medium was used in this experiment. By limiting the total nutrient, a complete growth curve from exponential phase to stationary phase was able to be measured within 18 hours without sample drying or bacterial overgrowing. A closely fitted lid was placed on the microplate to prevent liquid evaporation. No samples were placed near the edge of the 96-well microplate to prevent any drying effect during overnight incubation. Results are mean ± SEM from three independent experiments with bacteria grew in duplicates for each condition.

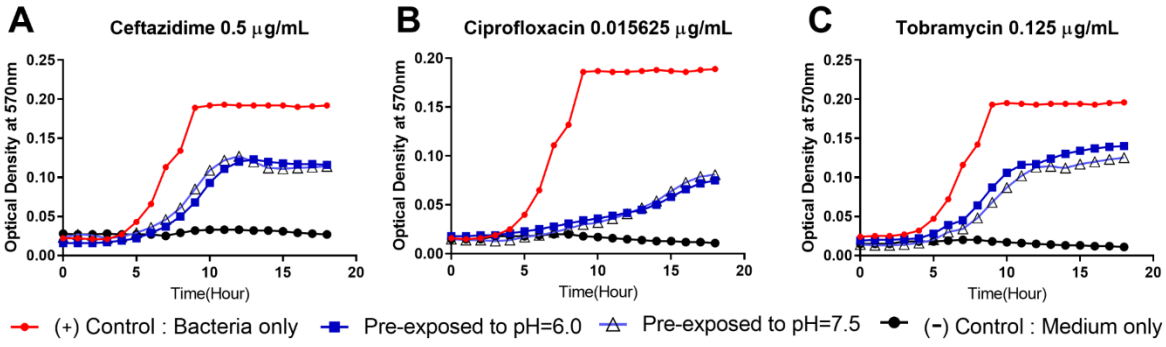


Figure 4. Antibiotics restore their antimicrobial activity in physiological pH conditions after prior exposure to an acidic environment.

Antibiotics (ceftazidime, ciprofloxacin, and tobramycin) were pre-exposed to pH = 6.0 or 7.5 in 10% TSB for 5 hours, the pH for all exposed antibiotics was then re-adjusted back to 7.5 before the antibiotics were added into planktonic *P. aeruginosa* (PAO1) culture for GIA at pH 7.5. The applied treatment concentration for each antibiotic was selected using less than their respective MIC to demonstrate the partial inhibition of bacterial growth. Optical density at 570nm was measured every hour for 18 hours at 37°C in a microplate reader. Results are mean ± SD from two independent experiments with bacteria grew in duplicates for each condition.

2.3.3 Increased *P. aeruginosa* antibiotic resistance under acidic pH conditions

To further explore if acidic pH promotes *P. aeruginosa* antibiotic resistance, we carried out studies to investigate bacterial evolution with antibiotics in acidic and neutral pH conditions. Both biofilm and planktonic *P. aeruginosa* were grown under the treatment of ceftazidime, ciprofloxacin, and tobramycin, three of the most commonly used antibiotics in treating CF respiratory infection. The reference strain of *P. aeruginosa* (PA14) was selected because we sought to focus on investigating the pH effect without concerns of horizontally transferred plasmids or bacteriophages that could exist in clinical strains. The *P. aeruginosa* biofilm was generated by growing PA14 on acrylic beads and transferred daily to a new culture tube with a new sterile bead in the existence of antibiotics (Fig. 5A). The planktonic culture served as a control of the biofilm evolution model in contrasting the different growth modes (Fig. 5B).

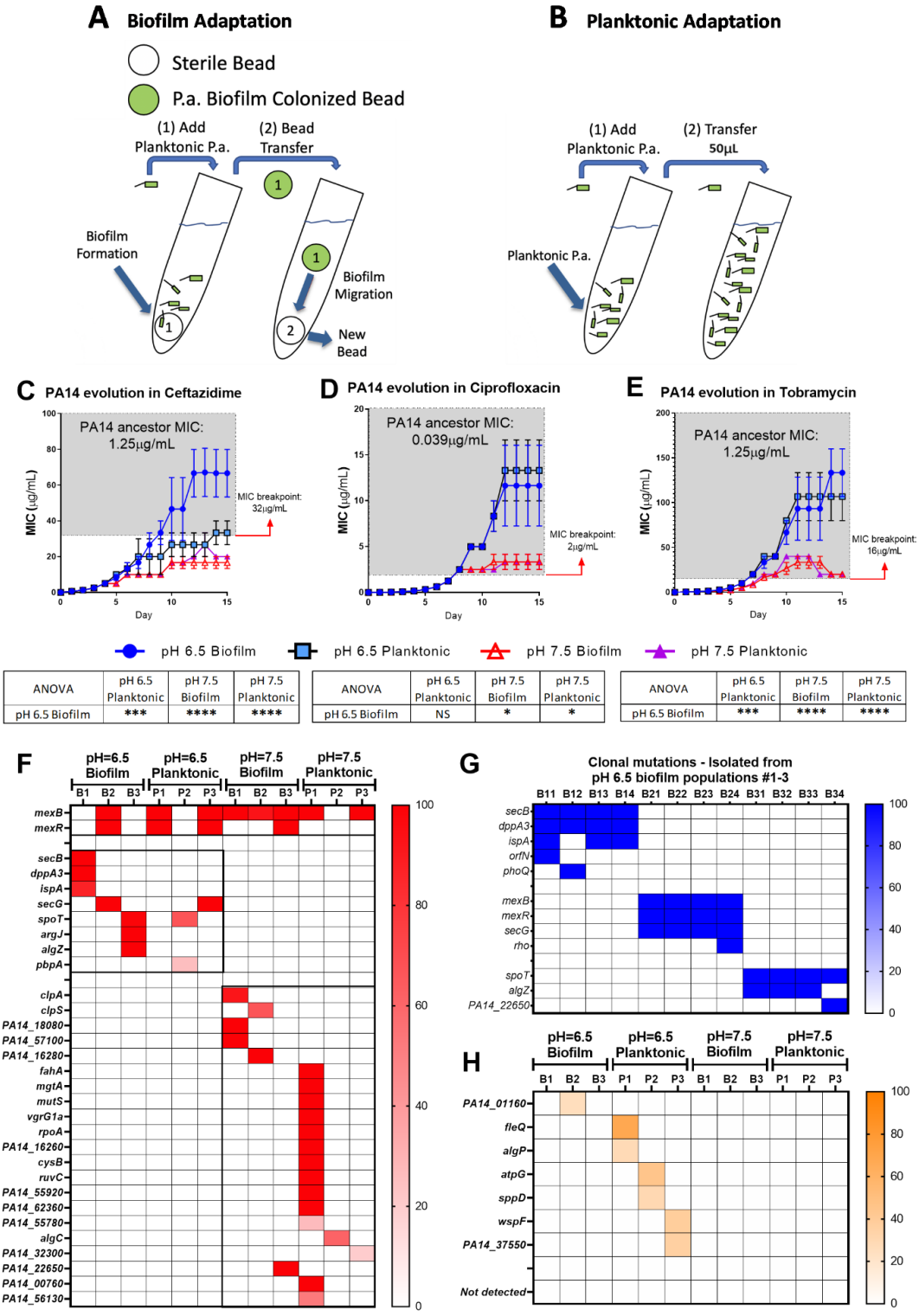


Figure 5. Acidic pH promotes faster accumulation of adaptive resistance of *P. aeruginosa* against antibiotics compared to pH 7.5.

(A and B) Schematics of the *P. aeruginosa* PA14 antibiotic adaptation experiment: biofilm on beads (A) and planktonic bacteria (B) were transferred to fresh m63 media (pH=6.5/7.5, adjusted by HCl) every 24 hours; (C-E) The PA14 biofilm/planktonic cultures were treated with ½ MIC at day 1. Antibiotic concentrations were doubled after every bead/planktonic transfer (n=3). The grey boxes on figures (C-E) denote MIC values that are considered antibiotic-resistant according to the guideline "MIC Breakpoints for *Pseudomonas aeruginosa"*, published by The Clinical and Laboratory Standards Institute. (F) PA14 population mutations were identified after 15 days of evolution with ceftazidime. Each bacterial lifestyle/pH was evolved in triplicate populations. Biofilm (B) /planktonic (P) populations were labeled as B1, B2, B3, and P1, P2, P3, respectively. (G) Clonal mutations detected after 15 days of evolution with ceftazidime. Clones were labeled from number 1 to 4 following their population number. For example, B11 represents biofilm population B1, clone #1; (H) Mutations from PA14 evolution without antibiotics for 15 days. Color scale bars represent mutation frequency, which ranges from 0 to 100%. Data are mean ± SEM. One-way ANOVA was performed by comparing the combined day 14 and 15 MIC values. * p<0.05; *** p<0.001; **** p<0.0001; NS: not significant.

The new MIC of each PA14 culture condition was determined daily by the survival of each population after 24 hours of incubation in a fresh medium containing doubled antibiotic concentrations. After 15 days of continuous growth and evolution, PA14 populations acquired increased levels of resistance against all three antibiotics. The acidic environment significantly increased the MIC required for the tested antibiotics to kill *P. aeruginosa* (Fig. 5C-E), regardless of the antibiotic killing mechanisms.

Acidic pH environment induces immediate antibiotic tolerance of *P. aeruginosa* in planktonic growing conditions up to 8-fold compared to a neutral pH environment (Table 2).

Table 2. Effects of acidic pH on *P. aeruginosa* antibiotic tolerance in MIC changes.

MIC data were acquired using the GIA assay. Results are averaged MICs from two independent GIA experiments.

Ceftazidime MIC (μg/mL)				
NO.	Strain	pH 6.5 average MIC	pH 7.5 average MIC	MIC fold increase from pH 6.5 to 7.5
1	PAO1	1.875	0.9375	2
2	PA14	2.5	1.25	2
3	116-37	2.5	0.9375	3
4	71-75	0.625	0.15625	4
5	KK1	2.5	1.25	2
6	AA43	5	2.5	2
7	40-21	2.5	1.25	2
8	151-40	5	2.5	2
9	129-5	5	1.25	4
10	109-10	2.5	1.25	2
11	480-1	0.625	0.15625	4
12	82-9	2.5	1.25	2
13	152-19	5	2.5	2
14	80-37	2.5	1.25	2

Ciprofloxacin MIC (μg/mL)				
NO.	Strain	pH 6.5 average MIC	pH 7.5 average MIC	MIC fold increase from pH 6.5 to 7.5
1	PAO1	0.0390625	0.0390625	1
2	PA14	0.07813	0.0390625	2
3	116-37	0.07813	0.0390625	2
4	71-75	0.15625	0.1171875	1
5	KK1	0.03906	0.0390625	1
6	AA43	0.15625	0.078125	2
7	40-21	0.07813	0.0390625	2
8	151-40	0.9375	0.625	2
9	129-5	0.07813	0.0390625	2
10	109-10	0.15625	0.05859375	3
11	480-1	0.01464845	0.0146485	1
12	82-9	0.07813	0.0390625	2
13	152-19	0.07813	0.0390625	2
14	80-37	0.0390625	0.0390625	1

Tobramycin MIC (μg/mL)				
NO.	Strain	pH 6.5 average MIC	pH 7.5 average MIC	MIC fold increase from pH 6.5 to 7.5
1	PAO1	2.5	0.9375	3
2	PA14	3.75	1.25	3
3	116-37	1.25	0.3125	4
4	71-75	2.5	0.625	4
5	KK1	5	1.25	4
6	AA43	10	1.875	5
7	40-21	1.25	0.3125	4
8	151-40	2.5	1.25	2
9	129-5	0.625	0.625	1
10	109-10	10	2.5	4
11	480-1	0.9375	0.3125	3
12	82-9	5	0.625	8
13	152-19	1.25	0.625	2
14	80-37	5	0.9375	5

Furthermore, we observed a 32- to 512-fold MIC increase in the acidic pH-treated biofilm populations after 15 days of evolution (Fig. 5C-E). Therefore, we hypothesized that when under antibiotic pressure, certain naturally occurring genetic mutations that could potentially favor bacterial survival during PA14 proliferation will be selected, and the ceftazidime treated PA14 populations were selected for further analysis. Whole-genome sequencing (WGS) of the bacteria was performed in every testing bacterial population to identify the high-frequency mutations (Fig. 5F). WGS of four randomly picked single colonies from each of the pH 6.5 biofilm populations (B1-B3) were then carried out to directly determine the evolved genotypes in correspondence to the MIC-increasing phenotypes (Table 3). Common mutations on the efflux pump (*mexB* and *mexR*) known to induce ceftazidime resistance (Sanz-Garcia, Hernando-Amado, & Martinez, 2018) were identified in half of pH 6.5 populations and five out of six pH 7.5 populations (Fig. 2F). The *mexB* and *mexR* mutations conferred pH 6.5 population B2 ceftazidime resistance a 64-fold increase (Fig. 5C), which was subsequently confirmed by clonal genotype (Fig. 5G) and MIC (Table 3) of selected individual clones.

Table 3. *P. aeruginosa* PA14 clonal MIC from the ceftazidime evolution study.

Population	Clone number	Clonal MIC (Fold increase)		pH-induced fold increase
		pH 6.5	pH 7.5	
Biofilm pH 6.5 population 1 (B1)	B11	64	4	16
	B12	80	4	20
	B13	64	4	16
	B14	94	6	15.7
Biofilm pH 6.5 population 2 (B2)	B21	96	10	9.6
	B22	96	24	4
	B23	96	24	4
	B24	128	32	4
Biofilm pH 6.5 population 3 (B3)	B31	6	4	1.5
	B32	4	4	1
	B33	6	4	1.5
	B34	8	4	2

*Ceftazidime MIC against PA14 ancestor: 1.25μg/mL

Interestingly, three out of six evolved bacterial populations under the pH 6.5 conditions (biofilm and planktonic) generated antibiotic resistance to ceftazidime via efflux pump-independent mechanisms. For instance, the combined mutations in *secB* and *dppA3* resulted in an acidic pH-inducible 64-fold MIC increase in the pH 6.5 biofilm population B1 (Fig. 5C) and 64 to 94-fold MIC increase in individual clones (Fig. 5G, Table 3). Mutations on *spoT*, *argJ*, and *algZ* potentially contributed to a 32-fold MIC increase in the pH 6.5 biofilm population B3 but in the selected individual clones only showed mild MIC increase (4 to 8-fold increase, Table 3), which indicated a common discrepancy in MIC due to bacterial lifestyle difference. The sequencing results indicated that the mutations generated from PA14 bacterial populations evolved without antibiotics (Fig. 5H) do not overlap with mutations identified under antibiotic selection (Fig. 5F-G), which suggests that acidic pH condition is a critical factor in promoting the evolution of drug-resistance when antibiotics are present.

2.3.4 Acidic conditions promote biofilm formation of *P. aeruginosa* clinical isolates and compromise biofilm prevention activities of antibiotics

We further evaluated fourteen *P. aeruginosa* strains to test the hypothesis that acidic pH promotes bacterial biofilm formation and impairs antibiotic efficacy. These include twelve clinical *P. aeruginosa* isolates obtained initially from CF patients and two standard lab strains (PAO1 and PA14). In Fig. 6A, the CF-like, acidic pH 6.5 condition alone stimulated more mature biofilm formation from approximately 71% (10/14) of all *P. aeruginosa* strains compared to pH 7.5. The difference is significant and more noticeable when 1x MIC of ceftazidime, ciprofloxacin, and tobramycin was used in *P. aeruginosa* biofilm prevention. In Fig. 6B-D, fourteen *P. aeruginosa* strains were treated with antibiotics using their respective MIC dosages (Table 2) in pH 6.5 and 7.5 conditions. The majority of antibiotic-treated *P. aeruginosa* strains formed more biofilm after 18 hours in the pH 6.5 condition compared to the pH 7.5 condition. These biofilm data are in accordance with our growth kinetic studies and the PA14 evolution studies (Figs. 3 and 5). All experiment results showed a similar pattern that ceftazidime, ciprofloxacin, and tobramycin were generally less effective in inhibiting *P. aeruginosa* growth and preventing biofilm formation at acidic pH conditions. All other five species of ESKAPE pathogens except *P. aeruginosa* conversely demonstrated decreased biofilm formation under acidic pH stress (Fig. 6E).

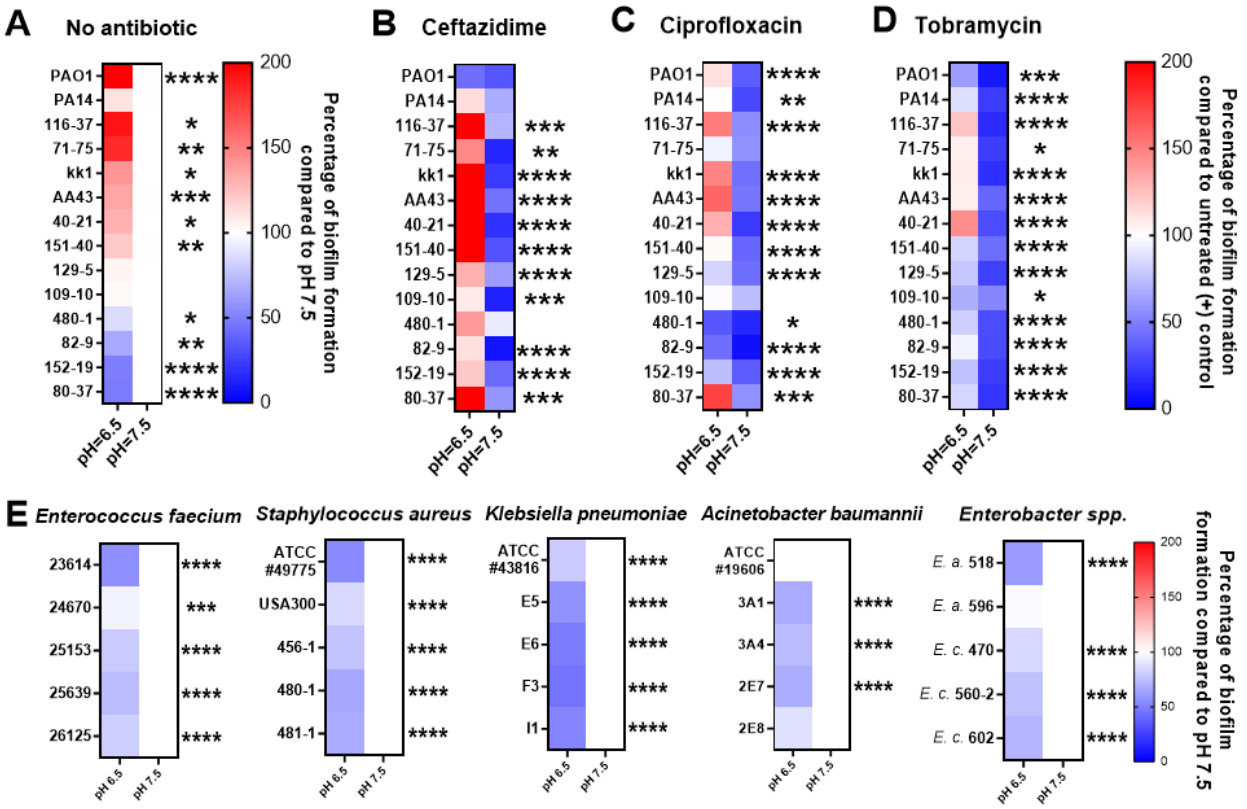


Figure 6. Acidic pH modulates ESKAPE pathogens biofilm formation and impairs antibiotic biofilm prevention against *P. aeruginosa.*

All *P. aeruginosa* strains (2 lab strains and 12 clinical strains) were incubated in pH adjusted m63 medium for 18 hours. The crystal violet staining method was used to quantify biofilm formation (n=6). (A) *P. aeruginosa* biofilm formation without antibiotic treatment. Biofilm formation in pH 7.5 served as a positive control. (B-D) *P. aeruginosa* biofilm formation was treated by ceftazidime, ciprofloxacin, and tobramycin at the concentrations of 1x MIC of each *P. aeruginosa* strain (MIC in normal pH 7.5 condition). The percentage of antibiotic-treated biofilm formation was calculated by comparing it to each untreated *P. aeruginosa* strain in pH 7.5. (E) Biofilm formation of other bacterial species of the ESKAPE bacterial pathogens. Five isolates from each of the other ESKAPE species were grown at pH 6.5 and 7.5 (as a control) in biofilm mode. *E.a.* and *E.c.* are abbreviations for *Enterobacter aerogenes* and *Enterobacter cloacae*, respectively. The color scale bars represent the percentage of biofilm formation compared to appropriate control groups. Red scale: increased biofilm formation (100% - 200%); white: no change (100%); blue scale: decreased biofilm formation (0 – 100%). Data were collected from three independent experiments. Unpaired *t-test* was used for statistical analysis between each pH 6.5 and pH 7.5 conditions. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001; otherwise not significant.

2.3.5 Neutralization of acidic pH in CF epithelial cells restores impaired host defense

Acidic pH impairs important host defense mechanisms such as the ASL antibacterial activities of antimicrobial peptides, including β-defensin-1, -3, and LL-37 (Abou Alaiwa et al., 2014; Johansson, Gudmundsson, Rottenberg, Berndt, & Agerberth, 1998; Nakayama et al., 2002). Our results demonstrate that the acidic pH in the CF-like microenvironment increases bacterial tolerance/resistance against antibiotics and enhances biofilm formation, which may promote *P. aeruginosa* colonization. To simulate the airway microenvironment, we further determined if the neutralization of the acidic ASL of differentiated human bronchial epithelial cells that were maintained under air-liquid interface (ALI) culture could help alleviate *P. aeruginosa* infection (Fig. 7A). The primary human bronchial epithelial (HBE, non-CF) cells and CF bronchial epithelial (CFBE) cells were used. We tested ouabain, an ATP12A inhibitor that inhibits H^+ secretion by human epithelial cells, raising the pH value of cultured epithelial cells (Shah et al., 2016). Without any treatment, the ASL of cultured CFBE cells was more acidic than non-CF HBE cells (Fig. 7B). However, ouabain successfully elevated the pH value of CFBE ASL from approximately 6.7 to 7.6. The increased pH value reflected neutralized ASL of CFBE, which consequently resulted in decreased CFU of *P. aeruginosa* (PAO1) grew on top of human epithelial cells (Fig. 7C). The ouabain modulation of pH in ASL of HBE was minimal, which did not result in significant changes of *P. aeruginosa* CFU (Fig. 7C). Ouabain itself does not display any bactericidal activity at the treatment concentration of 20μM (Fig. 8).

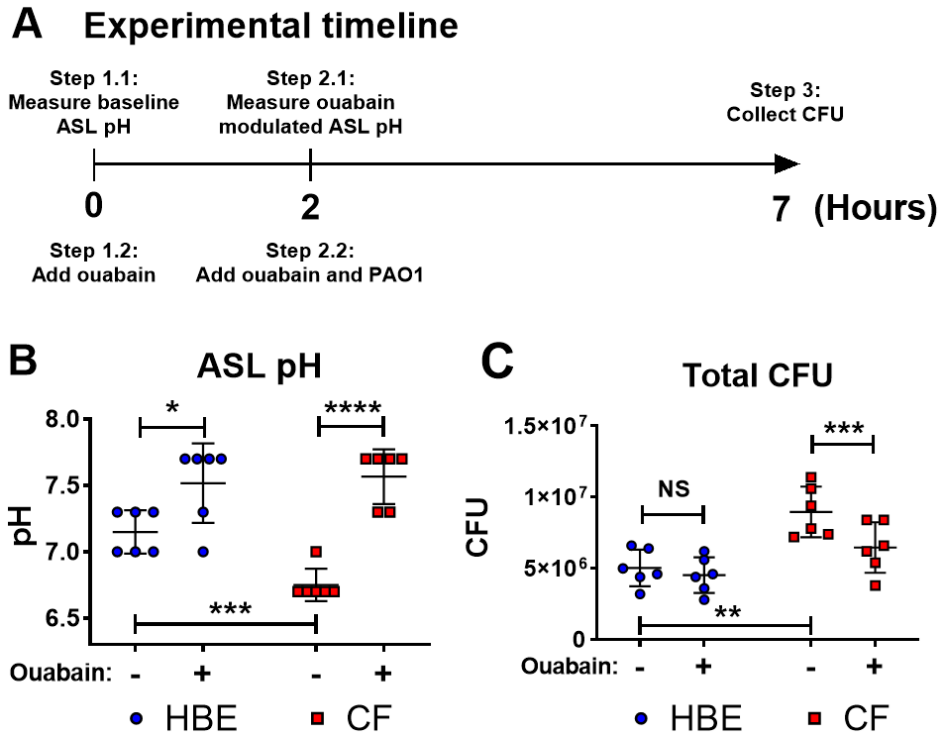


Figure 7. Ouabain helps restore host defense activities by reversing the acidic pH to neutral pH in differentiated human bronchial epithelial cell cultures.

(**A**) Experimental timeline. pH values were measured on the apical side of air-liquid interface cultured cells before and after two hours of treatment with/without 20μM ouabain; PAO1 was then added to the apical side of cultured cells with ouabain or DMSO solvent control for additional 5 hours. (**B**) Effect of ouabain treatment on pH values in non-CF (HBE) and CF epithelial cells. The change of pH in the epithelial apical wash was measured by narrow range pH test strips (pH 6-7.7, resolution 0.3 pH unit; n=6). (**C**) Effect of ouabain treatment on *P. aeruginosa* CFU in non-CF (HBE) and CF epithelial cells. PAO1 was incubated on the apical side of cultured HBE and CF epithelial cells in the existence of 20μM ouabain or DMSO (solvent control). Biofilm and planktonic CFU were obtained by plated on agar plates for total PAO1 CFU (n=6). Data were collected from two independent experiments. Unpaired *t-test* was used for statistical analysis. Data are mean ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; NS: not significant.

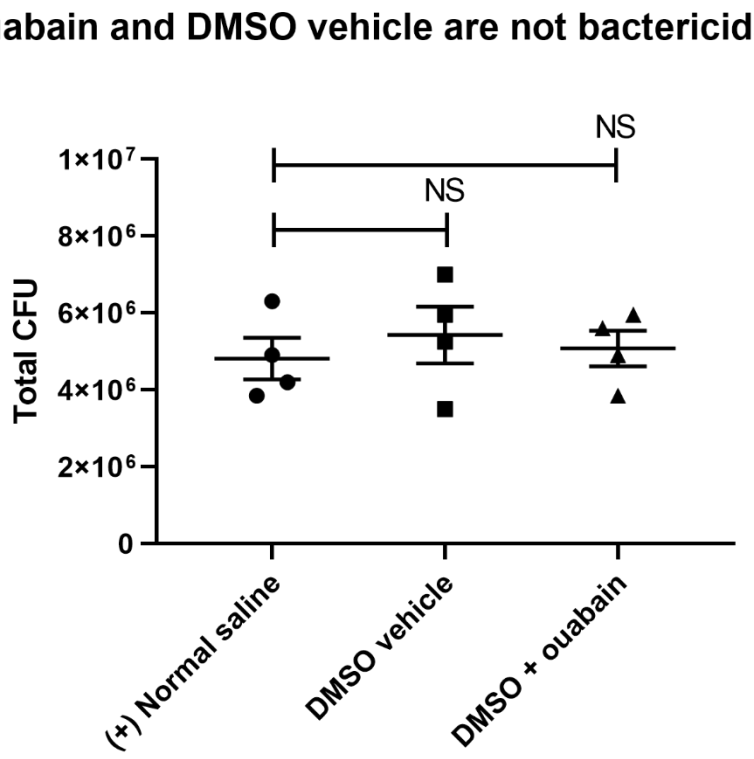


Figure 8. Ouabain treatment was not bactericidal to *P. aeruginosa* PAO1.

A total volume of 50µL of 2 x 10^8 CFU/mL PAO1 and 20µL of 20µM Ouabain or DMSO (solvent control) were incubated in normal saline for 5 hours at 37°C (n=4). Data are mean ± SEM from two repeated experiments. Student's *t*-tests were used for statistical analysis. NS: not significant.

2.3.6 Bacterial gene expressions are modulated by acidic pH

To evaluate if acidic microenvironment-enhanced *P. aeruginosa* infection in CF is regulated through increased bacterial biofilm formation at the transcriptomic level, we determined the gene expression of a panel of biofilm/virulence-related genes (Fig. 9A) in association with the observed biomass changes. Multiple biofilm-related genes such as *tolA*, *ndvB*, *rhlA*, and *rhlB* were all significantly increased in the acidic conditions of biofilm formation.

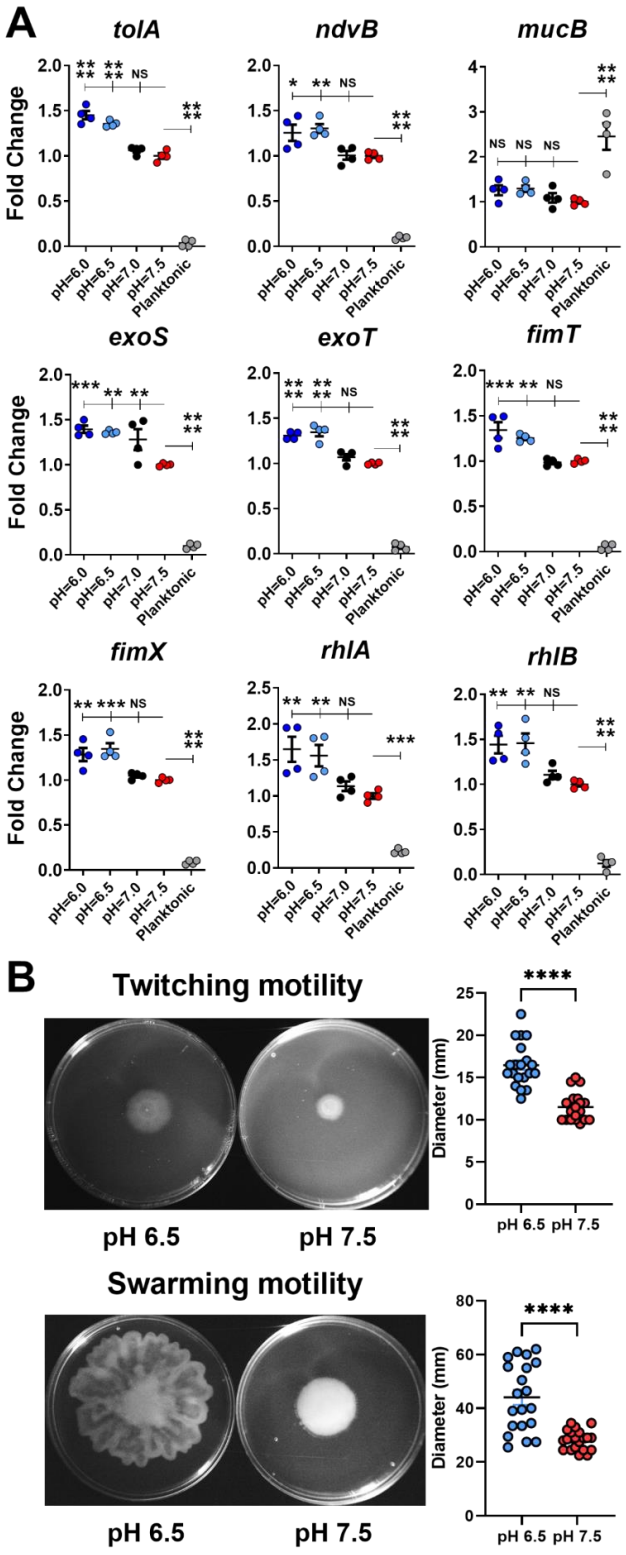


Figure 9. Acidic pH conditions increase biofilm/virulence-related gene expression of *P. aeruginosa.*

(A) *P. aeruginosa* PAO1 biofilm was cultured in pH adjusted DMEM for 3 hours. Physiologically related biofilm condition in pH 7.5 was used as a control for biofilm growth. Planktonic form of *P. aeruginosa* inoculated in pH 7.5 was also included for comparison (n=4). (B) PAO1 motility assays. PAO1 swarming and twitching motility assays were performed on pH adjusted M8 agar (n=20). Data were representative of two independent experiments. Results are mean ± SEM. One-way ANOVA and Dunnett's multiple comparisons test were used for gene expression statistical analysis. Student's *t*-tests were used for bacterial motility assay statistical analysis * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; NS: not significant.

Additionally, the expressions of several virulence-associated genes such as *exoS, exoT, fimT,* and *fimX* were also significantly increased in acidic environments (pH 6.0 and 6.5) compared to in physiological conditions (pH 7.0 and 7.5). Interestingly, the planktonic-associated mucB gene expression did not vary among all acidic and physiological biofilm-forming pH conditions. However, its expression differed significantly between the biofilm and planktonic forms of *P. aeruginosa*.

The *rhlA* and *rhlB* controlled rhamnolipid biosurfactant synthesis (Caiazza, Shanks, & O'Toole, 2005), and their gene expression data were validated by a bacterial swarming motility assay (Fig. 9B). *P. aeruginosa* (PAO1) displayed significantly increased swarming diameter in acidic agar compared to pH 7.5 agar. Similarly, the results from the follow-up twitching motility assay agreed with the elevated expression *fimT* and *fimX* in acidic pH conditions (Fig. 9B).

2.4 Discussion and Conclusions

P. aeruginosa is the most dominant bacterial pathogen associated with CF disease severity and mortality. The CF microenvironment resulted from a CFTR malfunction may have an unwanted effect in contributing to the infection and colonization of *P. aeruginosa*. However, the underlying pathogenic mechanisms remain to be elucidated. In this study, we investigated the CF-like acidic microenvironment on the behavior of *P. aeruginosa*. We demonstrated that multiple *P.*

aeruginosa clinical isolates significantly increased their biofilm formation and antibiotic tolerance/resistance under acidic conditions. Remarkably, this pathological factor of acidic pH also activated a series of biofilm- and virulence-related genes of *P. aeruginosa*.

Under the selection pressure in an experimental condition with gradually increased antibiotic concentrations, we demonstrated that *P. aeruginosa* adapted to the antibiotics quickly and evolved significantly faster in pH 6.5 than in pH 7.5, which eventually acquired strong antibiotic resistance in only 15 days (approximately 99 generations, estimated at 6.6 generations/day (Flynn et al., 2016)). In contrast to the PA14 ancestor, evolved PA14 progeny bacteria demonstrated an indisputable ability to survive and adapt to highly stressful antibiotic treatments, while acidic pH significantly expedited this process. Of note, all of the frequently detected *mexB* mutations are single nucleotide polymorphisms (SNPs). These SNPs are not necessarily loss-of-function mutations, which would allow *P. aeruginosa* to maintain the MexAB-OprM efflux pump activities against ceftazidime. On the contrary, mutations on the *mexR* gene, a bacterial efflux pump repressor, are sometimes indels, which could lead to unsuppressed efflux pump activities. Both mechanisms likely contributed to the elevated PA14 ceftazidime resistance. The ancestral PA14 did not increase its biofilm production under acidic pH without antibiotic pressure (Fig. 2, Fig. 6A). Therefore, the changes of MIC in this PA14-based bacterial evolution model were less likely influenced by acidic pH-enhanced biofilm formation and more likely a true adaptation to environmental pH and antibiotics.

Our findings suggest that the acidic CF microenvironment likely plays a critical role in facilitating *P. aeruginosa* colonization despite the antibiotic treatment. Interestingly, *P. aeruginosa* appears to be the only bacterial species among the notorious MDR ESKAPE pathogens that displayed significantly increased biofilm formation under a CF-like acidic environment (Fig.

6E), which may explain the high prevalence of *P. aeruginosa* in adult CF population when their airways are expected to be more acidic. Our data provide an important link between the worsened CFTR function-associated acidic microenvironment and enhanced *P. aeruginosa* biofilm formation supported by the evidence of *P. aeruginosa* biofilm-related phenotypic/genetic changes and multiple microenvironment acidification factors.

It has been reported that the lowered airway pH is associated with impaired host defense mechanisms (Abou Alaiwa et al., 2014; Adewoye & Worobec, 1999; Johansson et al., 1998; Nakayama et al., 2002; Shah et al., 2016; Tang et al., 2016; Zabner et al., 1998). By neutralizing the acidic environment via inhibiting ATP12A, the apical pH value increased on the differentiated human primary airway epithelial cells (CFBE and non-CF HBE). The neutralized pH not only can potentially restore the antimicrobial activity of the naturally secreted host defense factors by airway epithelial cells such as SPLUNC1 (Di et al., 2013; Walton et al., 2016) but also may prevent biofilm formation and/or pH-induced drug-tolerance/resistance of *P. aeruginosa*. These observations could provide potential therapeutic insights. For instance, there are several means to neutralize the acidic CF lung environment. By inhibiting ATP12A, the secretion of H^+ is inhibited, and therefore pH is elevated (Fig. 7B). The extracellular H^+ could also be neutralized by commonly used substances, such as sodium bicarbonate (Dobay et al., 2018; Wilton et al., 2016) or hydroxide salts. The increased bacterial susceptibility to host factors after raising the acidic pH to neutral pH suggests an alternative approach in treating CF chronic infection induced by *P. aeruginosa*. Recent studies showed evidence that CFTR modulators, such as ivacaftor and lumacaftor-ivacaftor combination, increased the lung function of several genotypes of human CFTR mutations (Caverly et al., 2015; Rowe et al., 2014) and decreased *P. aeruginosa* lung culture positivity rates (Heltshe et al., 2015). The increased CFTR activity reduces sweat Cl^-, which may subsequently result in

elevated ASL pH (Abou Alaiwa et al., 2018). However, the CFTR genotypes that are not covered by the CFTR modulators continue to be a challenge for *P. aeruginosa* management.

In addition to the efforts to neutralize the acidic host environment, it is also vital to examine the complex response of *P. aeruginosa* to acidic pH and how this response leads to antibiotic tolerance/resistance. Our gene expression results provided mechanistic insight into pH-related pathways in pathogenic *P. aeruginosa*. Acidic conditions at pH values of 6 and 6.5 significantly increased the expression of *tolA*, activated in biofilms (Whiteley et al., 2001). Its product is responsible for aminoglycoside-resistance in *P. aeruginosa* by decreasing its permeability and blocking the entrance of antibiotics (Bryan, Haraphongse, & Van den Elzen, 1976; Bryan & Kwan, 1983; Bryan, Nicas, Holloway, & Crowther, 1980; MacLeod et al., 2000; Rivera, Hancock, Sawyer, Haug, & McGroarty, 1988). The *ndvB* gene encodes for a glucosyltransferase that is required for the synthesis of cyclic-b-(1, 3)-glucans (Bhagwat, Gross, Tully, & Keister, 1996). Cyclic glucans can physically interact with tobramycin and therefore eliminate antibiotics before reaching their targeted site of action (Mah et al., 2003). In acidic conditions, there was a noticeably higher *ndvB* gene expression in *P. aeruginosa* biofilm compared to the pH 7.5 of physiological control. Planktonic form of PAO1 expressed significantly less *ndvB* than any biofilm group. The elevated expression of *ndvB* could be one factor contributing to the acidic pH-induced antibiotic resistance in *P. aeruginosa* (Figs. 3, 5, and 6). Both *rhlA* and *rhlB* are required for rhamnolipid synthesis (Deziel, Lepine, Milot, & Villemur, 2003), a biosurfactant that contributes to the swarming motility of *P. aeruginosa* to promote biofilm colonization. Rhamnolipids are known to interfere with phagocytosis (McClure & Schiller, 1996) and normal tracheal ciliary function (Read et al., 1992). The expression of these virulence factors was increased under the acidic pH environment. Both *fimT* and *fimX* are responsible for the biogenesis and functioning of type IV pili

and twitching mobility of *P. aeruginosa* (Huang, Whitchurch, & Mattick, 2003) and are crucial during biofilm formation (Chiang & Burrows, 2003; O'Toole & Kolter, 1998). *P. aeruginosa* injects cytotoxins (e.g. ExoS and ExoT) into host cells by utilizing the type III secretion system (Barbieri & Sun, 2004). Acidic pH conditions activated *exoS* and *exoT* will likely lead to delayed wound healing, impaired phagocytosis, and spread of *P. aeruginosa* (Hauser, 2009). The *mucB* gene is a negative regulator of the sigma factor AlgU. Inactivation of this gene in acidic conditions will facilitate the conversion of *P. aeruginosa* into alginate-producing mucoid forms (Boucher, Schurr, Yu, Rowen, & Deretic, 1997; Martin, Schurr, Mudd, & Deretic, 1993).

There are limitations to this study. We used the m63 medium for bacterial evolution experiments. M63 is a minimal medium that has been used for studying *P. aeruginosa* in cystic fibrosis (Wolfgang, Jyot, Goodman, Ramphal, & Lory, 2004). We used D-glucose and 4mM L-glutamine as the only nutrition sources in the m63 medium to maintain the pH consistency in simulating the CF-like acidic microenvironment. The synthetic cystic fibrosis sputum medium (SCFM) was developed to mimic the selective nutrient environment in the CF lungs (Palmer, Aye, & Whiteley, 2007). However, we did not select SCFM for this study because it uses other amino acids, such as L-arginine, which causes an alkaline pH by releasing ammonia through deamination. While SCFM is a valuable tool in general CF-related studies, it remains an artificial medium and affects the acidic impact of cultivating *P. aeruginosa*. Therefore, we believe m63 is a more appropriate medium that consistently maintains acidic pH even after overnight bacterial culture. Regarding the sequencing results, although the pH-specific genes are not individually investigated in this study, they are viable candidates that could guide future research that focuses on the mechanisms of pH-mediated bacterial drug resistance.

This study was designed to explore the mechanisms of initial *P. aeruginosa* colonization and how the CF-specific lung microenvironment might contribute to this adverse health outcome. *P. aeruginosa* colonization in respiratory tracts is a chronic process, and the bacteria likely adapt and evolve in the CF lungs for decades. Longitudinal studies of the CF *P. aeruginosa* genotypes have illustrated that *P. aeruginosa* undergoes extensive genomic DNA mutations to survive in the CF lungs (E. E. Smith et al., 2006). Although many mutated genes have been identified, there was no connection between any previously identified mutations to the acidic pH environment. Our WGS data provided direct evidence that correlates acidic pH-promoted bacterial drug resistance to short-term bacterial evolution. More studies are needed to target this aim and explore the role of acidic pH in long-term *P. aeruginosa* evolution/adaption to resolve the persistent biofilm formation and chronic colonization suffered by CF patients.

3.0 Long-term *Pseudomonas aeruginosa* response to the acidic environment

3.1 Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative opportunistic pathogen that often causes chronic infections in immunocompromised people. It is common to find *P. aeruginosa* chronically colonized in the respiratory tracts of cystic fibrosis (CF) patients. According to the recent Patient Registry from the Cystic Fibrosis Foundation (Foundation, 2020), the prevalence of respiratory *P. aeruginosa* infection rises accordingly when CF individuals age, accompanied by the increased CF disease severity. Consequently, *P. aeruginosa* gradually becomes the most prevalent pathogen among CF patients older than 25 years old (Foundation, 2020). Abundant evidence suggests that the insufficient cystic fibrosis transmembrane conductance regulator (CFTR) function leads to acidified airway surface liquid and impaired host-defense mechanisms (Pezzulo et al., 2012; Shah et al., 2016; Zabner et al., 1998). Additionally, extracellular DNA (eDNA), one of the major components of the bacterial biofilm matrix (Matsukawa & Greenberg, 2004; Okshevsky & Meyer, 2015), also acidifies the biofilm microenvironment (Wilton et al., 2016), as evidenced by the pH gradient observed in the *P. aeruginosa* biofilm (Hunter & Beveridge, 2005). We previously demonstrated that the CF-like environment with acidic pH promotes *P. aeruginosa* biofilm formation and increases both immediate antibiotic tolerance and long-term antibiotic resistance to tobramycin, ciprofloxacin, and ceftazidime (Lin et al., 2021) through acquired genetic mutations. A question that remains to be further investigated is if chronic exposure of inhabited bacteria to acidic pH environments impacts *P. aeruginosa* evolution.

Human longitudinal studies of isolated microbes had been carried out to identify bacterial pathogens' genetic mutations in CF patients (E. E. Smith et al., 2006). Many of the observed genetic mutations were linked to the typical *P. aeruginosa* phenotypes associated with chronic lung infections, such as the loss of acute virulence (*exsA*) (Vakulskas, Brady, & Yahr, 2009), the rise of mucoid biofilm phenotypes (*wspF*) (Borlee et al., 2010), and the generation of multidrug resistance resulted from long-term antibiotic treatments (*mexA*) (Poole et al., 1996). However, these changes are not unique to CF and do not fully explain why *P. aeruginosa* preferentially colonizes in CF airways. For example, the *lasR* mutation is often found in clinical *P. aeruginosa* isolates originated from chronic obstructive pulmonary disease (COPD) patients (Zhao et al., 2020). Therefore, it is crucial to precisely determine how a CF-like host environment interacts with the bacterial pathogens that may have promoted chronic *P. aeruginosa* colonization. We hypothesized that CF acidic pH conditions contribute to the rise of selected genetic mutations, resulting in long-term P. *aeruginosa* adaptation and colonization in the CF airways. To test our hypothesis, we performed bead-based (Cooper, 2018; Poltak & Cooper, 2011) biofilm evolution studies using *P. aeruginosa* strain PA14 in varying CF-related conditions, identified genetic variations in bacterial populations and individual clones by whole genome sequencing (WGS), and characterized the selected mutations potentially associated with promoting bacterial colonization in CF.

Our data suggested that the major global virulence factor regulator (*vfr*) played a role in the PA14 biofilm ecological system. Through positive regulation, *vfr* is a significant controller of the *P. aeruginosa* type III secretion system (T3SS) (Marsden et al., 2016), type IV pili (Beatson, Whitchurch, Sargent, Levesque, & Mattick, 2002; Dasgupta, Ashare, Hunninghake, & Yahr, 2006), and the *las* quorum-sensing system (Albus, Pesci, Runyen-Janecky, West, & Iglewski,

1997; Schuster, Lostroh, Ogi, & Greenberg, 2003; Wagner, Bushnell, Passador, Brooks, & Iglewski, 2003). The Vfr protein is directly activated by the secondary messenger 3',5'-cyclic adenosine monophosphate (cAMP) (Fuchs et al., 2010), which our results demonstrated an increased bacterial intracellular cAMP production upon acidic pH stimulation. *P. aeruginosa* with the mutated *vfr* gene conforms to many of the representative phenotypes of chronically colonized *P. aeruginosa* clinical isolates. Most importantly, in acidic conditions, the vfr mutated clone showed enhanced biofilm fitness than the PA14 ancestor strain. The *vfr* mutations had been identified in human longitudinal studies of CF patients (Markussen et al., 2014; E. E. Smith et al., 2006) but the potential causative CF-pathophysiological factors remained unknown. This study is the first to report potential associations between *vfr* mutations and the detrimental effect of CF airway acidification. The loss-of-function mutations on the *vfr* gene are indicated by mucoid bacterial phenotypes that occurred after this acute to chronic genetic adaptation event, in which *P. aeruginosa* could subsequently persist in the CF respiratory tract for life (Hogardt & Heesemann, 2013). Identifying the primary driving force of *vfr* mutation provides new insights into the relationship between environmental stress in the CF microenvironment and the related bacterial adaptation. Our approaches using laboratory bacterial evolution and precise WGS provide mechanistic insight into chronic colonization of *P. aeruginosa* in CF and identify a potential novel target for effective treatment strategies.

3.2 Materials and Methods

Bacterial strains and growth conditions. The frozen bacterial stock was streaked on tryptic soy agar (TSA, MP Biomedicals, 091010617) plates and inoculated at 37°C overnight. A

single clone was picked and incubated in tryptic soy broth (TSB, MP Biomedicals, 091010717) overnight in a shaking incubator at 37°C and 225 rounds per minute (RPM). The overnight bacterial culture was diluted in fresh TSB at 1:5 ration and incubated for another 3 hours until exponential growth was reached. Bacterial culture was spun down at 2000x*g* for 5 minutes. The pellet was dissolved in sterile PBS. Bacterial concentration was adjusted to approximately 10^9 CFU/mL at optical density (OD) 500_{nm}=0.5±0.05 using a spectrophotometer.

Bacterial evolution. Bacterial evolution studies were based on a previously described method (Poltak & Cooper, 2011). The m63 medium was used in all evolution studies. The medium was supplemented with 0.4% L-arginine, 25μM $FeCl_3$, and 1mM MgSO4. Medium pH was adjusted using HCl. Although liquid culture pH change is inevitable with rapid bacterial growth, this buffered m63 medium can maintain an acidic pH for up to 18 hours (Fig. 10), at which time the bacterial culture has already reached the stationary phase. Polystyrene beads were sterilized by autoclave using a gravity cycle (121°C for 25 minutes, 15psi, 30-minute drying time).

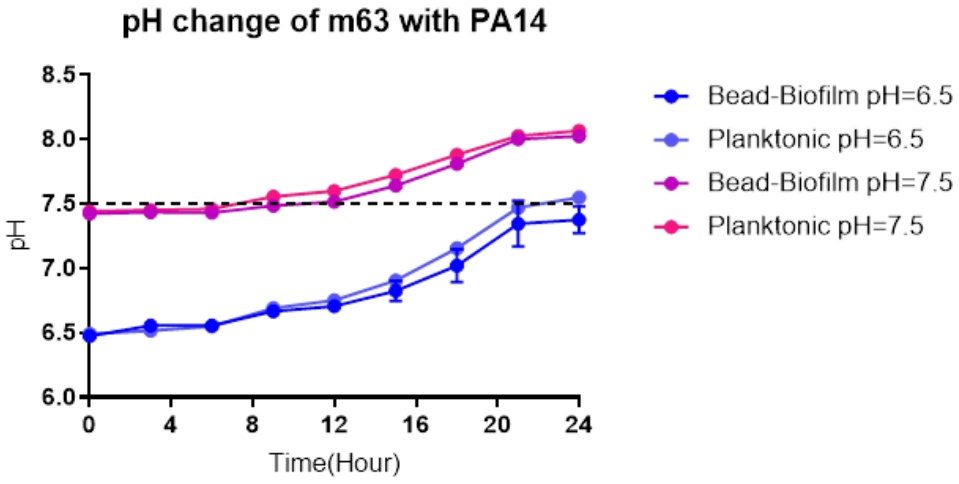


Figure 10. Change of m63 medium pH during 24 hours of incubation.

Media pH was measured using a 70% ethanol sterilized pH probe every 2 hours (n=2).

Phenotypic characterization. TSA was supplemented with 40μg/L Congo Red and 20μg/L Coomassie Brilliant Blue G-250 before autoclave. At various time points of the evolution study, bacterial populations were diluted and plated on TSA plates for morphological statistics. Before plating, biofilm on beads was sonicated in cold PBS for 15 seconds at 80% amplitude using a DPS-20 dual processing system sonicator (PRO Scientific) (130 W). Planktonic bacteria were vortexed for 10 seconds and plated on TSA directly after appropriate dilution. TSA plates were then placed in a 37°C incubator for 24 hours followed by 48 hours at room temperature.

Whole-genome sequencing. Total DNA from the individual bacterial population/clone was extracted using the MO BIO PowerSoil DNA Isolation Kit. Population DNA was extracted after 40 days of evolution. To find a clone that only contains the *vfr* I44S mutation, 4 individual clones were randomly picked and sequenced from the mixed biofilm pH 6.5 population 1 at day 40. The library preparation (Baym et al., 2015; Turner et al., 2018) and WGS were performed by the University of Pittsburgh Microbial Genome Sequencing Center using an Illumina NextSeq500.

Bioinformatics. Raw sequencing data were quality filtered and trimmed by Trimmomatic v0.38 (Bolger et al., 2014). Trimmomatic parameters: NexteraPE-PE.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:70. Variants calling was performed using *breseq* v0.33.0 (Deatherage & Barrick, 2014). The PA14 ancestor was also sequenced before variant calling to detect background mutations. The reference genome *Pseudomonas aeruginosa* UCBPP-PA14 was downloaded from NCBI, genome assembly accession number GCF_000014625.1. Population/ clonal sample sequencing depths are at least 125x/ 30x respectively. Mutations reported in this study are indels or nonsynonymous SNPs above the 10% *breseq* threshold.

Sanger sequencing and PCR genotyping. The *vfr* I44S genomic DNA was amplified by the following *vfr* primers: Forward: 5' – CAGCTCGCCGAAGAAATCAC – 3'; Reverse: 5' – CTACACCGCAAAGAGCACCA – 3'. The PCR products were purified using the GENECLEAN® III Kit (MP Biomedicals, 111001600) and sent for Sanger sequencing at Eurofins Genomics. The Δ*vfr* genomic DNA was amplified using the following primers: F1 forward 5' - TGGCGGGTGATCTTGATCTG - 3'; F2 forward 5' - CGATGAGGATGGTGACCGAA - 3'; R1 reverse 5' - CGCCTTGTACGTCAGGCATA - 3'.

Gene expression assays. PA14-WT and Δ*vfr* were added to pH-adjusted m63 media at 10^6CFU/mL x 10mL each. Bacteria were incubated in a shaking incubator at 37°C and 225 RPM for 6 hours. Bacteria were then spun down at 4°C for 5 minutes. The supernatant was discarded and the tubes were put on ice. Bacteria total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen 74106). Possible genomic DNA contamination was eliminated by Qiagen RNase-Free DNase Set (Qiagen, 79254) according to the product protocol. RNA samples were adjusted to 100±2ng/μL. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). The gene expression results were obtained using the Fast SYBR™ Green Master Mix (Applied Biosystems, 4385612) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). Data analysis and $\Delta\Delta C_t$ were calculated using a previously described method (Lin et al., 2018; Lin & Di, 2020). The constitutively expressed genes *rplU* and *proC (Alqarni, Colley, Klebensberger, McDougald, & Rice, 2016)* were used as reference genes. A list of primer sequences can be found in Table 4.

Table 4. Primer sequences for qPCR and Sanger sequencing.

Gene	Primer sequence	
vfr	Forward	5'- TGGCGGGTGATCTTGATCTG -3'
	Reverse	5'- CGCCTTGTACGTCAGGCATA -3'
cyaB	Forward	5'- ATTCGTGCTCGACGTAGCTG -3'
	Reverse	5'- GGCTATTGCACGGTGGGTAA -3'
exsA	Forward	5'- ATGCAAGGAGCCAAATCTCT -3'
	Reverse	5'- ATGTTCCAATGACAAGACGT -3'
lasR	Forward	5'- ATGGCCTTGGTTGACGGTTTTCT -3'
	Reverse	5'- GGCGCTCCACTCCAATTTTC -3'
rhlA	Forward	5'- CGAGACCGTCGGCAAATAC -3'
	Reverse	5'- GCACCTGGTCGATGTGAAA -3'
fimX	Forward	5'- CCTGGCCTATATCCATCTCAAC -3'
	Reverse	5'- ACTGTTCACGCATCAGTCC -3'
rplU *	Forward	5'- CGCAGTGATTGTTACCGGTG -3'
	Reverse	5'- AGGCCTGAATGCCGGTGATC -3'
proC *	Forward	5'- CAGGCCGGGCAGTTGCTGTC -3'
	Reverse	5'- GGTCAGGCGCGAGGCTGTCT -3'

* The constitutively expressed *rplU* and *proC* served as reference genes.

Bacterial cAMP quantification. The enzyme-linked immunosorbent assay (ELISA) cAMP Parameter Assay Kit was purchased from R&D Systems (KGE002B). PA14-WT, Δ*vfr, vfr* I44S overnight cultures were prepared as described above. Each strain was adjusted to the same CFU number (10^9CFU/10mL/tube) and then incubated in a shaking incubator at 37°C and 225 RPM for 6 hours. Bacterial samples were spun down at 2000x*g*, 4°C for 10 minutes. All supernatant was discarded, and the cell lysis buffer provided by the cAMP ELISA kit was used to

dissolve the bacterial pellet. After three cycles of freeze-thaw, the cell lysate was transferred into 2mL microcentrifuge tubes with screw caps and 0.1g of 0.1mm diameter fine glass beads. Vortex the tubes for 10 minutes in a 4°C refrigerator. Cell lysates were spun down at 2000x*g*, 4°C for 10 minutes. The supernatant was transferred into a new tube on ice. The cAMP quantification ELISA was performed according to the manufacturer's protocol. The final cAMP results were normalized by the total protein concentrations of each sample (DC Protein Assay, BIO-RAD, 5000111).

Bacterial motility assays. Bacterial swimming (Ha, Kuchma, & O'Toole, 2014b) and swarming (Ha et al., 2014a) assays were performed using M8 media with 0.3% and 0.6% agar, respectively. After autoclave, both swimming and swarming M8 media were supplemented to the final concentrations of 0.5% casamino acids, 0.2% glucose, and 1mM $MgSO_4$. A bacterial twitching assay was performed on TSA plates. All tested bacterial strains were diluted to the same concentration (OD_{500}=0.5±0.05) before plating. All plates were incubated in a 37°C incubator for 24 hours followed by 48 hours at room temperature.

Fitness assay. The m63 and SCFM (Palmer et al., 2007) media were used in the fitness assay. Before the competitive fitness assay, PA14-WT, and PA14 *vfr* I44S were diluted to 10^9CFU/mL, 25µL of each dilution was mixed into two pH conditions and two lifestyles. One sterile polystyrene bead was added to each biofilm condition. After a 24-hour incubation at 37°C, the beads were sonicated, diluted, and plated on TSA plates supplemented with congo red and coomassie blue. The mucoid phenotype of *vfr* mutants can be distinguished from the hypervirulent PA14 ancestor, which demonstrates larger twitching zones. Planktonic fitness assay was performed without the presence of plastic bead. Both biofilm and planktonic tubes were prefilled with 5mL of fresh m63 or SCFM media. The selection rate *r* was calculated using the following

formula as previously published (K. B. Harris, K. M. Flynn, & V. S. Cooper, 2021; Travisano & Lenski, 1996):

$$r = \frac{\ln[\frac{A(day_1)}{A(day_0)}] - \ln[\frac{B(day_1)}{B(day_0)}]}{1\ day}$$

Cytotoxicity assays. CF41o- and CFWT cell lines were cultured in Minimum Essential Media (MEM, Gibco, 41090101) supplemented with 10% Fetal Bovine Serum (Sigma, 12103C). For CFWT cells, puromycin (InvivoGen, ant-pr-1) was added to a final concentration of 0.5μg/mL. All cells were incubated in a 37°C incubator supplemented with 5% CO_2.

Cell viability test. A total of $4x10^5$/mLx100μL cells were seeded onto each well of 96-well cell culture plates (Greiner, 655160). Plates were incubated overnight to allow cells to attach. Cells were treated with 100μL of medium with or without bacteria ($5x10^3$CFU/mL). Incubate the 96-well plate for 3 hours. Add 10μL of TetraZ™ Cell Proliferation Kit buffer (BioLegend, 424501) to each well and incubate for 2 more hours to allow the color to develop. Measure OD_{450} in a microplate reader to quantify viable cells. Since the Cell Proliferation Kit buffer has negligible toxicity on mammalian cells, we considered the total bacterial treatment time to be 5 hours.

Transepithelial electrical resistance (TEER) measurement. A total of $5x10^5$ cells were seeded onto each Transwell® polyester membrane cell culture inserts (Corning, 3470). Add 350μL of medium to the basolateral chamber. Change culture to ALI after 3 days by removing the apical medium. After 7 days, cells were ready to be treated by bacteria. Bacteria were diluted to 10^7 CFU per 100μL PBS and added to the apical side of ALI inserts. TEER was measured using the Millicell® ERS-2 Voltohmmeter (Millipore Sigma, MERS00002) every hour for 5 hours.

Mouse survival study. Animal experiments were carried out by strictly following protocol #20077642 approved by the University of Pittsburgh, Institutional Animal Care and Use Committee. All mice were purchased directly from Charles River Laboratories. Female CD-1 mice were 5 weeks old at the time of this study. All bacterial strains were adjusted to similar CFU numbers (approximately 10^7CFU/50μL/mouse, a lethal dose of PA14-WT against the CD-1 mouse strain). Bacteria were delivered by intratracheal instillation (I.T.). Animals were censored from the study when determined to be in the moribund stage.

Statistical analysis. Data are mean ± SEM. The student's t-test was used to determine statistical significance in qPCR, ELISA, and bacterial motility studies. One-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test were used for statistical analysis in cytotoxicity studies. The Log-rank (Mantel-Cox) test was used in mouse survival studies. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; otherwise not significant (NS).

3.3 Results

3.3.1 PA14 displayed distinct phenotypes under different pH conditions

A single PA14 ancestor clone was picked and continuously cultured in different lifestyles (grew as planktonic or biofilm on beads with daily transfer) and pH (6.5 and 7.5) for 40 consecutive days (Fig. 11A). During this period, PA14 went through approximately 264 generations of continuous evolution (~6.6 generations/day) (Flynn et al., 2016). A total of five distinct phenotypes were recorded (Fig. 11B<i>). PA14 ancestor showed a non-mucoid phenotype at the beginning of the evolution. The four new morphologies (M1 to M4) all showed elevated biofilm formation

ability on the air-liquid interface of the culture tubes (Fig. 11B<ii>). The small colony variants (SCVs, named M1 in this study) were observed within 5 days of incubation (Fig. 11B<iii>). M1 (and similar phenotypes) quickly increased in all test conditions but disappeared by day 15 in the pH 6.5 groups. Interestingly, the M3 phenotype gradually dominated the entire acidic biofilm population in all three independent replicates approaching nearly 100% after 25 days, which indicated that the acidic biofilm condition is highly selective for *P. aeruginosa* with M3 phenotype. Acidic pH 6.5 planktonic groups were served as a control to biofilm and showed clones with more random phenotypes due to the nature of the liquid-only transfer of bacterial suspension. Various pH conditions induced striking differences in the percentage of different PA14 phenotypes (M1 persistence in pH 7.5 conditions and M3 dominance that was only observed in pH 6.5 conditions). These observations warranted examining the genetic evolution within these bacterial populations. Of note, it has been reported that *P. aeruginosa* morphology and genotype may not often correlate (Katrina B Harris, Kenneth M Flynn, & Vaughn S Cooper, 2021). Therefore, WGS on the entire mixed evolved population would provide more direct evidence.

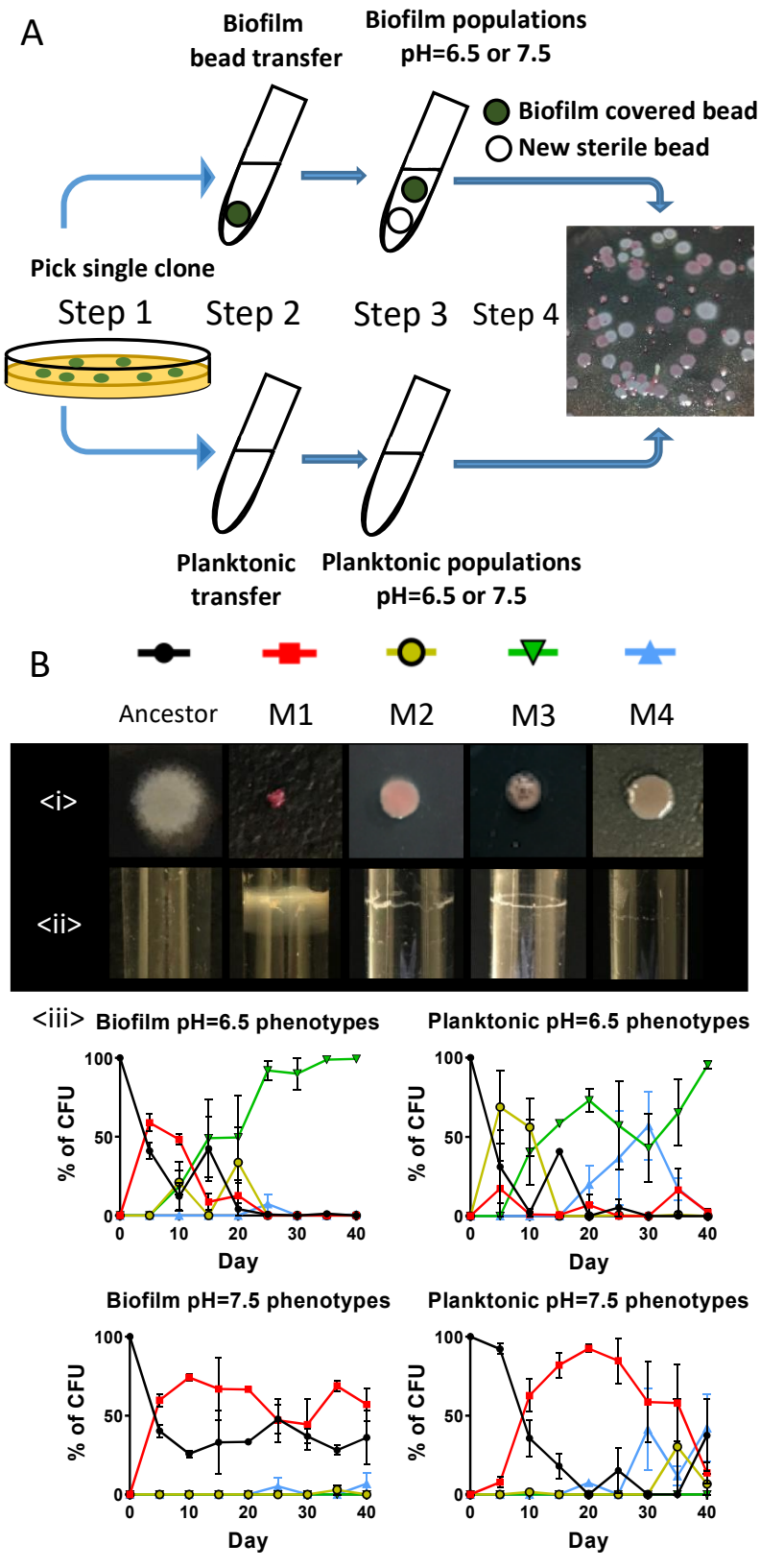


Figure 11. Bacterial evolution model and change of bacterial phenotypes.

(A). Schematic of the bacterial evolution models. Step 1: Frozen PA14 ancestor was streaked on an agar plate. Step 2: A single PA14 colony was picked and cultured in m63 medium overnight. Liquid PA14 culture was added to culture tubes with (biofilm) or without (planktonic) beads. The sterile bead was covered with PA14 biofilm after overnight incubation. Planktonic cultures were served as a control to biofilm. Step 3: (1) For biofilm populations, all liquid culture medium was removed, the old biofilm-covered bead was transferred into a new tube with a new sterile bead. PA14 went through the biofilm life cycle of dispersal and migration. (2) For Planktonic populations, 50µL of the overnight culture was transferred into 5mL of fresh medium. Both bead and planktonic transfers were performed every 24 hours. Step 4: An example of evolved PA14 colonies in a mixed population on an agar plate. **(B). PA14 morphological change after 40 consecutive days of evolution.** (i) A panel of PA14 morphologies of an ancestor colony and four new colonies isolated from all populations. (ii) Isolated clones were incubated overnight and displayed different biofilm formation abilities on clear culture tubes near the air-liquid interface. (iii) Each evolved population was plated on agar plates in triplicates (n=3) every 5 days for 40 days. The relative percentage (represented as relative abundance in fitness) of each clone type is shown in percentage.

3.3.2 Bacterial WGS revealed pH and lifestyle-specific mutations

Bacterial DNA was extracted from 12 populations (two pH conditions x two lifestyles x triplicate populations). The PA14 ancestor served as the reference genome. After 40 consecutive days of evolution, we identified *vfr* mutations in two out of three biofilm pH 6.5 populations (Fig. 12). In biofilm pH 6.5 population 1 (B1), the nonsynonymous *vfr* I44S mutation was identified, and this mutation is among the three total mutations detected in this population. In biofilm pH 6.5 population 3 (B3), a single-base deletion at position 717,593 of the PA14 genome was detected. This deletion occurred after the 65th base of the 645-base open reading frame of the *vfr* gene (*vfr*-Δ1bp, coding 65/645). This frameshift resulted in a nonsense mutation that leads to the appearance of a stop codon resulting in early termination of Vfr protein translation (Fig. 13), which makes it a loss-of-function mutation. This 1-basepair deletion was the only detectable mutation in this population. The *vfr* mutations appeared to be specific to the acidic biofilm condition and were not detected in all other experimental conditions. We suspected that the *vfr* I44S mutation would also demonstrate a loss-of-function phenotype. Therefore, we acquired a known *vfr* functional knockout clone, Δ*vfr*, from the PA14 transposon library (Liberati et al., 2006) (the functional ablation of vfr with a DNA fragment insertion was confirmed by PCR-based genotyping, Fig. 14)

and focused on comparing the phenotypes of *vfr* I44S to Δ*vfr* throughout the subsequent study. Several ATP synthase mutations (Fig. 12, pH 6.5 Planktonic P1-P3) explicitly appeared from the planktonic pH 6.5 populations. Other commonly known biofilm-associated mutations such as *wspF* were also observed in our study in the pH 7.5 biofilm groups (Fig. 12, pH 7.5 Biofilm B3), which was expected because most previous laboratory-based bacterial evolution studies were performed in neutral pH culture media (McElroy et al., 2014). Combining the phenotypic and genotypic changes that are specific to the acidic biofilm condition, we selected *vfr* as the target gene for subsequent PA14 functional studies in the CF-like acidic biofilm condition.

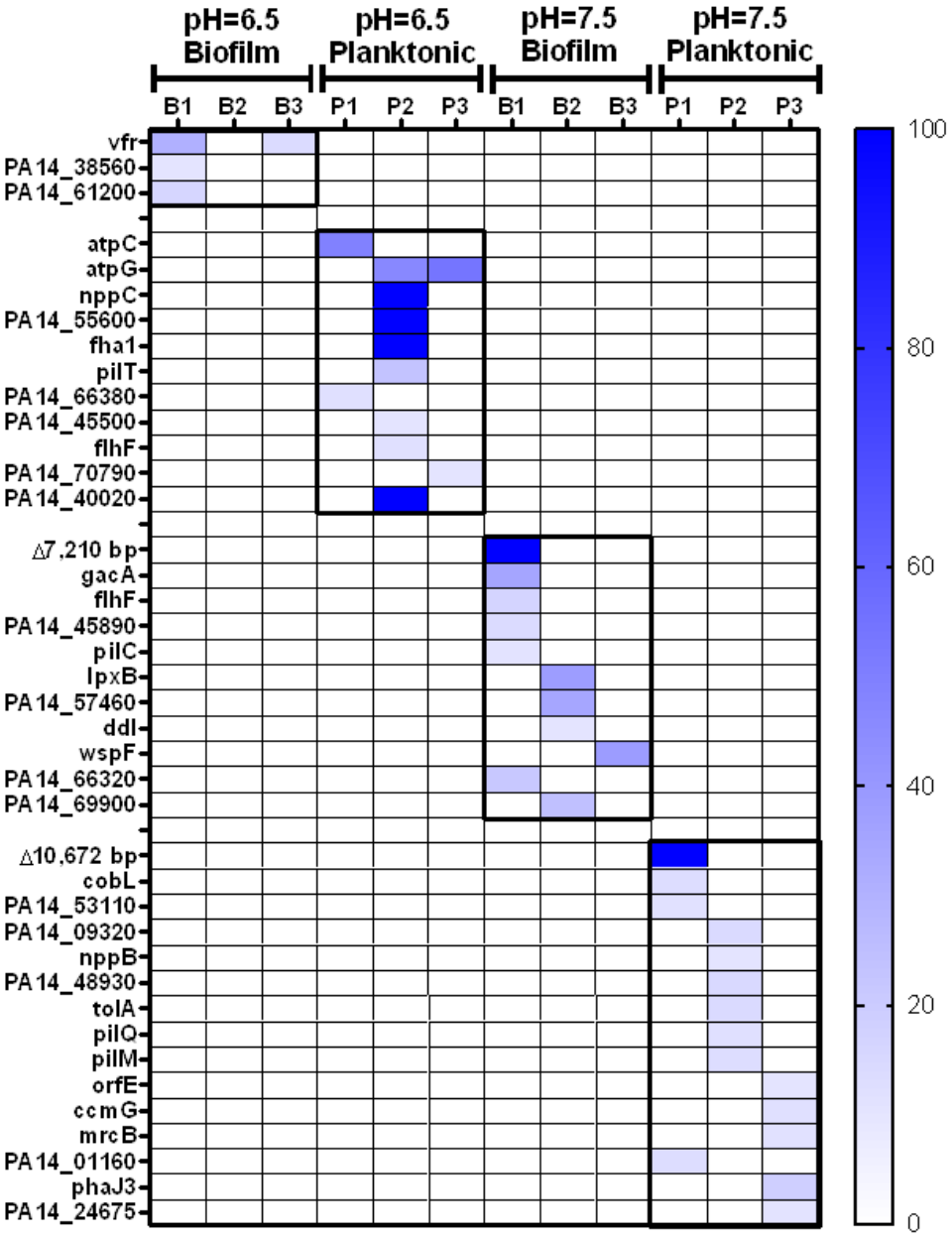


Figure 12. Whole population sequencing results revealed distinct evolutionary trajectories among different bacterial lifestyles and media pH.

Three populations of each culture condition were sequenced at 125x coverage for all 12 populations after 40 days of continuous evolution. Nonsynonymous mutations and insertions/deletions were grouped by bacterial lifestyle (biofilm or planktonic) and pH (6.5 or 7.5). Mutation frequencies below 10% were excluded. The blue scale bar represents the percentage of mutations detected from each population. Genes deleted from the Δ7,210 bp genotype: PA14_45890, PA14_45910, PA14_45920, PA14_45930, *lasI*, *rsaL* and *lasR*. Genes deleted from the Δ10,672 bp genotype: PA14_66320, *msrA*, PA14_66340, PA14_66350, PA14_66380 and PA14_66400.

PA14 Vfr wildtype protein sequence

M	V	A	I	T	H	T	P	K	L	K	H	L	D	K	L	L	A	H	C	1-20
H	R	R	R	Y	T	A	K	S	T	I	I	Y	A	G	D	R	C	E	T	21-40
L	F	F	I	I	K	G	S	V	T	I	L	I	E	D	D	D	G	R	E	41-60
M	I	I	G	Y	L	N	S	G	D	F	F	G	E	L	G	L	F	E	K	61-80
E	G	S	E	Q	E	R	S	A	W	V	R	A	K	V	E	C	E	V	A	81-100
E	I	S	Y	A	K	F	R	E	L	S	Q	Q	D	S	E	I	L	Y	T	101-120
L	G	S	Q	M	A	D	R	L	R	K	T	T	R	K	V	G	D	L	A	121-140
F	L	D	V	T	G	R	V	A	R	T	L	L	D	L	C	Q	Q	P	D	141-160
A	M	T	H	P	D	G	M	Q	I	K	I	T	R	Q	E	I	G	R	I	161-180
V	G	C	S	R	E	M	V	G	R	V	L	K	S	L	E	E	Q	G	L	181-200
V	H	V	K	G	K	T	M	V	V	F	G	T	R	Stop						201-215

PA14 Vfr Δ1 (65/645) protein sequence

M	V	A	I	T	H	T	P	K	L	K	H	L	D	K	L	L	A	H	C	1-20
H	P	A	A	T	P	Q	R	A	P	S	S	M	P	A	I	A	A	K	R	21-40
C	S	S	S	S	R	V	R	S	P	S	S	S	R	T	T	T	A	A	K	41-60
Stop																				61

Figure 13. Protein sequence translated from the vfr Δ1bp, coding (65/645) clone.

The Δ1bp indel mutation leads to a 9.8% Vfr protein sequence similarity to the PA14-WT Vfr (underlined). The frameshift mutation leads to a stop codon after the 60th residue.

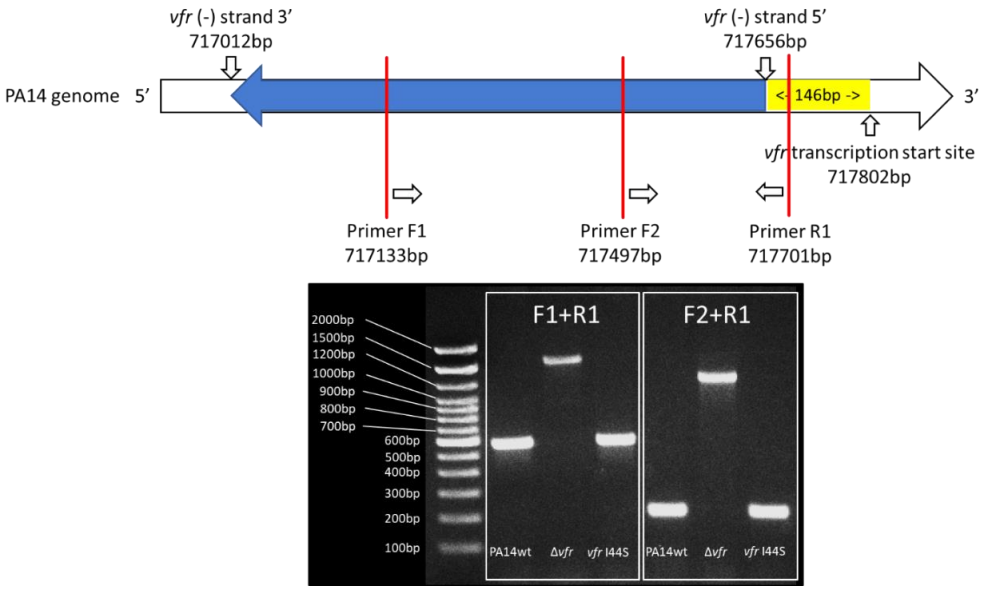


Figure 14. PCR amplification located a large insertion within the Δvfr genome.

The transposon insertion is located between 717497bp – 717701bp of the PA14 genome.

3.3.3 The *vfr* gene is crucial for the function of PA14 acute virulence

To investigate how acidic pH prepares PA14 for biofilm colonization and how acute bacterial virulence is regulated in acidic pH, RNA was extracted from both PA14 wild-type (PA14-WT) and PA14 Δ*vfr* strains that had been incubated in pH 6.5 or 7.5 media for 6 hours in planktonic culture. The bacterial gene expression results (Fig. 15A) revealed that, compared to pH 7.5, acidic pH stimulated higher expression of PA14-WT acute virulence factors such as *vfr* (Fuchs et al., 2010; Suh et al., 2002), *cyaB* (Fuchs et al., 2010), *exsA* (Vakulskas et al., 2009), *exoS* (Hauser, 2009) and *lasR* (Gambello, Kaye, & Iglewski, 1993). Δ*vfr* showed loss of acute virulence (*vfr, exsA, exoS,* and *lasR*) but possessed intact activities of the quorum-sensing system (*rhlA*) (Mukherjee, Moustafa, Smith, Goldberg, & Bassler, 2017). The secondary messenger cAMP is required for Vfr to bind to the promoter regions of many downstream acute virulence genes (Wolfgang, Lee, Gilmore, & Lory, 2003). The *cyaB* gene encodes adenylyl cyclase, which is the enzyme that synthesizes cAMP, expressed higher in acidic conditions of PA14-WT but was decreased in Δ*vfr* (R. S. Smith, Wolfgang, & Lory, 2004). We suspected that acidic pH stimulated the production of intracellular cAMP in *P. aeruginosa*. The increased availability of cAMP could consequently promote Vfr activation (Fuchs et al., 2010). We next determined if the intracellular cAMP concentrations of *P. aeruginosa* were increased using the enzyme-linked immunosorbent assay (ELISA).

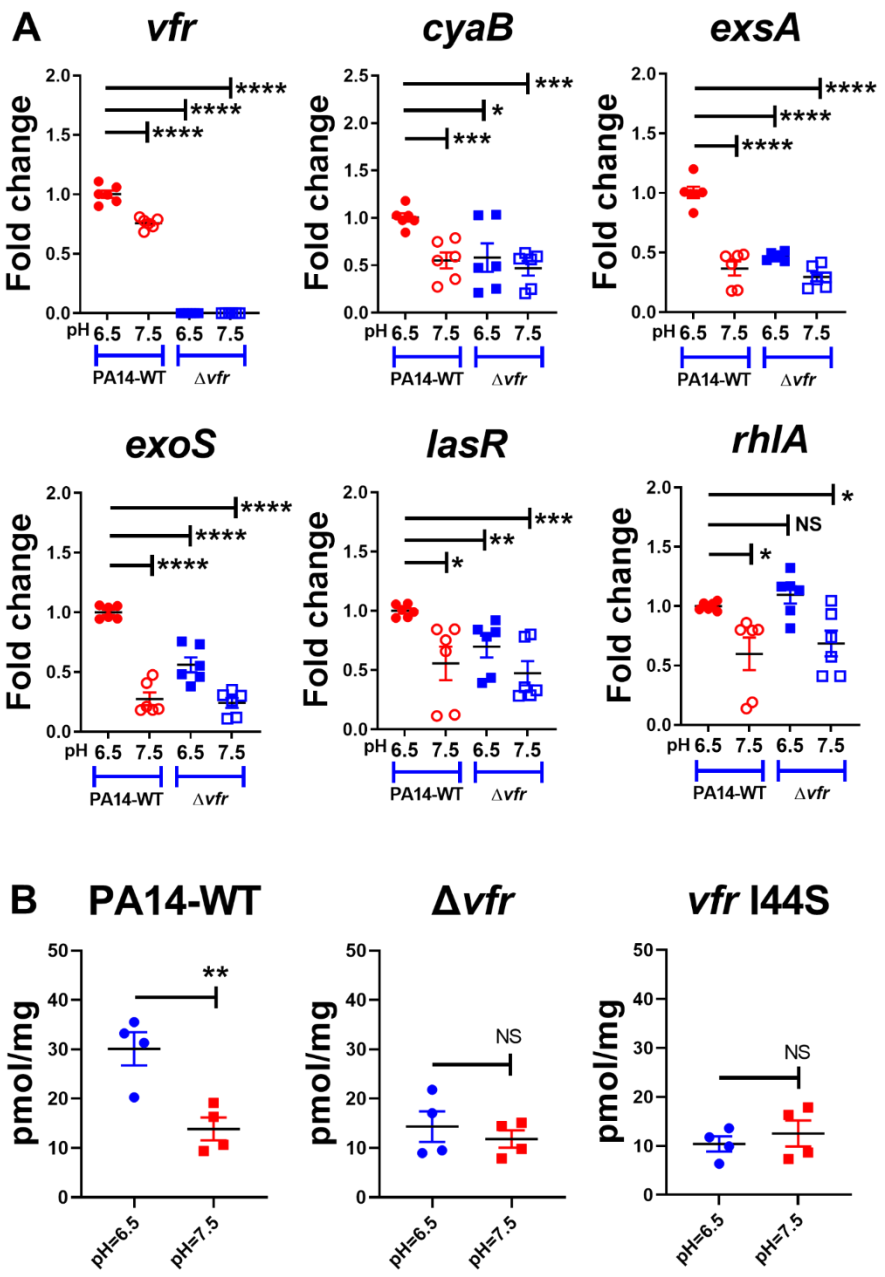


Figure 15. *P. aeruginosa* PA14 response to different pH measured by qPCR and ELISA.

(A) PA14 gene expression under different pH conditions. Planktonic PA14-WT and PA14 Δ*vfr* strains were incubated in pH 6.5 and 7.5 m63 media for 6 hours before RNA extraction. Data are mean ± SEM from three independent experiments with biological replicates (n=6). **(B) Acidic pH increases intracellular cAMP concentrations of PA14-WT strains.** The intracellular concentration of *P. aeruginosa* cAMP was measured by ELISA after 6 hours of planktonic incubation in pH 6.5 and 7.5. The cAMP concentrations were normalized by total protein quantification. Data are mean ± SEM from two independent experiments with biological replicates (n=4). The student's t-test was used for all statistical analyses. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; NS=not significant.

3.3.4 Acidic pH stimulates cAMP production

WGS data indicated *vfr* mutations in the acidic biofilm population 1. We randomly picked up and sequenced four single colonies from this group to carry out additional studies. Clone 1 was confirmed to contain only the *vfr* I44S mutation by Sanger sequencing (Fig. 16) and WGS (Table 5). The representative clone 1 with only a single mutation in *vfr* was used for all subsequent studies to avoid the possibility of contributing effects by any other mutations that are not directly related to the Vfr functions. In the PA14-WT genotype, acidic pH increased intracellular cAMP concentrations (Fig. 15B). Such a response to acidic pH was not observed when *vfr* mutations exist.

Table 5. Detected mutations of the four randomly picked single clones from the mixed biofilm pH 6.5 population 1 on day 40.

Predicted mutations						
	evidence	position	mutation	annotation	gene	description
Clone 1	RA	717,527	A→C	I44S (ATC→AGC)	*vfr*	cyclic AMP receptor like protein
Clone 2	RA	333,405	A→C	Y449D (TAT→GAT)	*PA14_RS01525* ←	GGDEF domain containing protein
Clone 3	RA	333,386	C→T	G455D (GGT→GAT)	*PA14_RS01525* ←	GGDEF domain containing protein
	RA	717,527	A→C	I44S (ATC→AGC)	*vfr*	cyclic AMP receptor like protein
Clone 4	RA	333,386	C→T	G455D (GGT→GAT)	*PA14_RS01525* ←	GGDEF domain containing protein
	RA	717,527	A→C	I44S (ATC→AGC)	*vfr*	cyclic AMP receptor like protein
	RA	3,719,154	T→C	N486S (AAC→AGC)	*ppsA*	phosphoenolpyruvate synthase

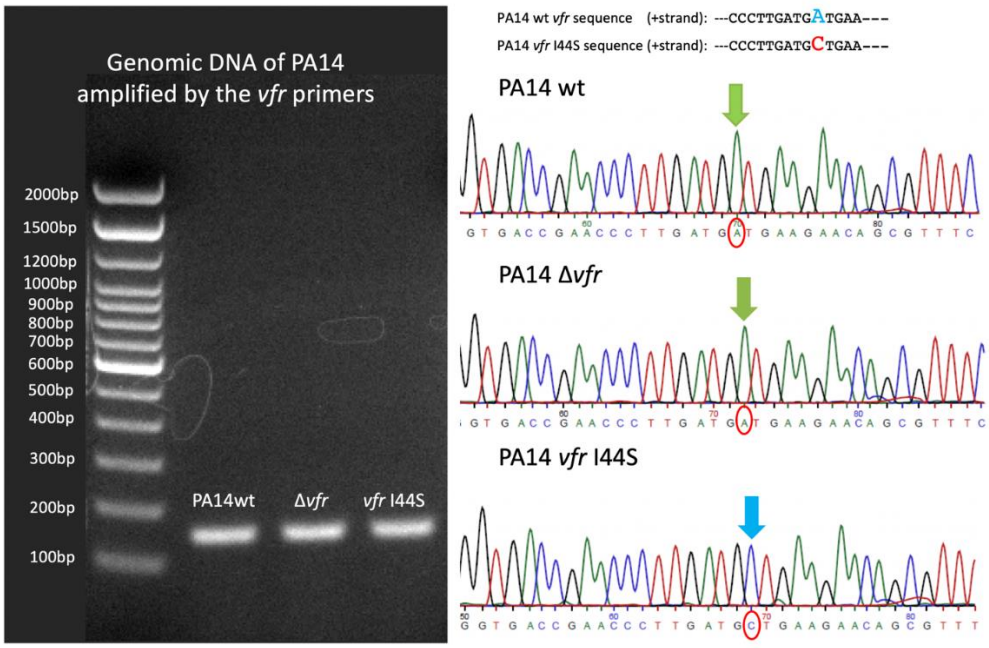


Figure 16. PCR followed by Sanger sequencing confirmed the vfr I44S SNP.

The PCR products of the *vfr* primers were purified for Sanger sequencing. The electropherogram confirmed the SNP (adenine to cytosine) in the *vfr* I44S genome.

3.3.5 Δ*vfr* changes *P. aeruginosa* swimming, swarming, and twitching motility

Bacterial motility affects their biofilm-forming ability. Vfr regulates bacterial virulence by suppressing *P. aeruginosa* swimming and swarming motilities (Dasgupta, Ferrell, Kanack, West, & Ramphal, 2002) and upregulates twitching motility (Beatson et al., 2002; Dasgupta et al., 2006). We examined whether a loss-of-function mutation in *vfr* will break the balance in *P. aeruginosa* motility systems. The PA14-WT showed limited swimming and swarming motility due to the intact Vfr function (Fig. 17). The loss of functional *vfr* (Δ*vfr* and *vfr* I44S) significantly increased swimming and swarming motilities. On the other hand, the twitching mobility in both Δ*vfr* and *vfr* I44S was decreased when compared with the original PA14-WT clone.

The similar phenotypes between Δ*vfr* and *vfr* I44S suggest that the laboratory-generated *vfr* I44S mutation leads to a disruption of Vfr function similar to those with the Δ*vfr* mutation.

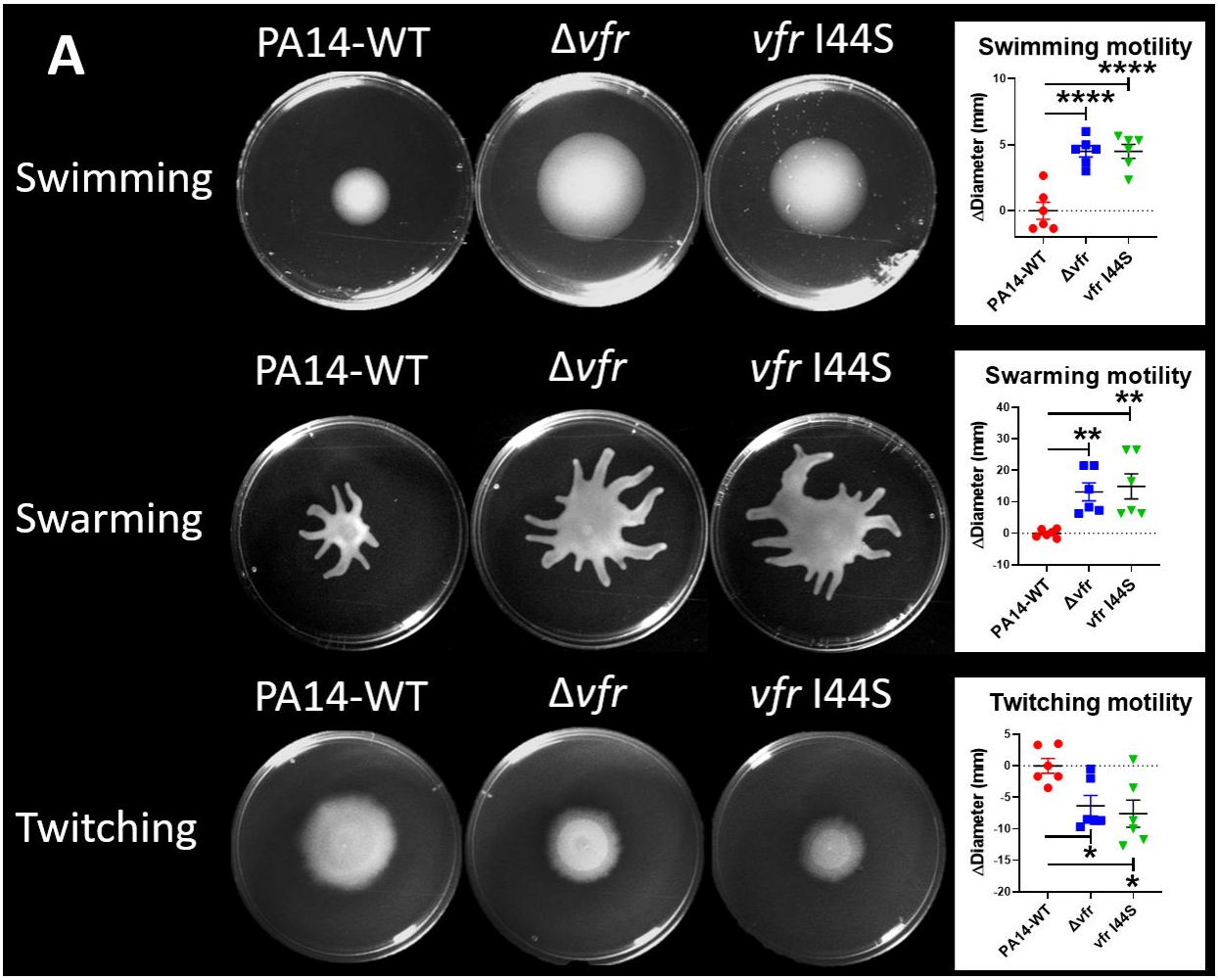


Figure 17. *P. aeruginosa* PA14 motility and fitness. (A) PA14 swimming, twitching, and swarming motilities.

The same CFU of different PA14 genotypes were delivered on agar plates. Photos were taken from representative agar plates. Data are mean ± SEM from three independent experiments (n=6). One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analysis. **(B)** and **(C) Bacterial fitness between PA14-WT and Δ*vfr* or *vfr* I44S.** Bacterial fitness was tested for three consecutive days in biofilm and planktonic lifestyles, pH 6.5 and 7.5 m63 media. Results are mean from three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; NS=not significant.

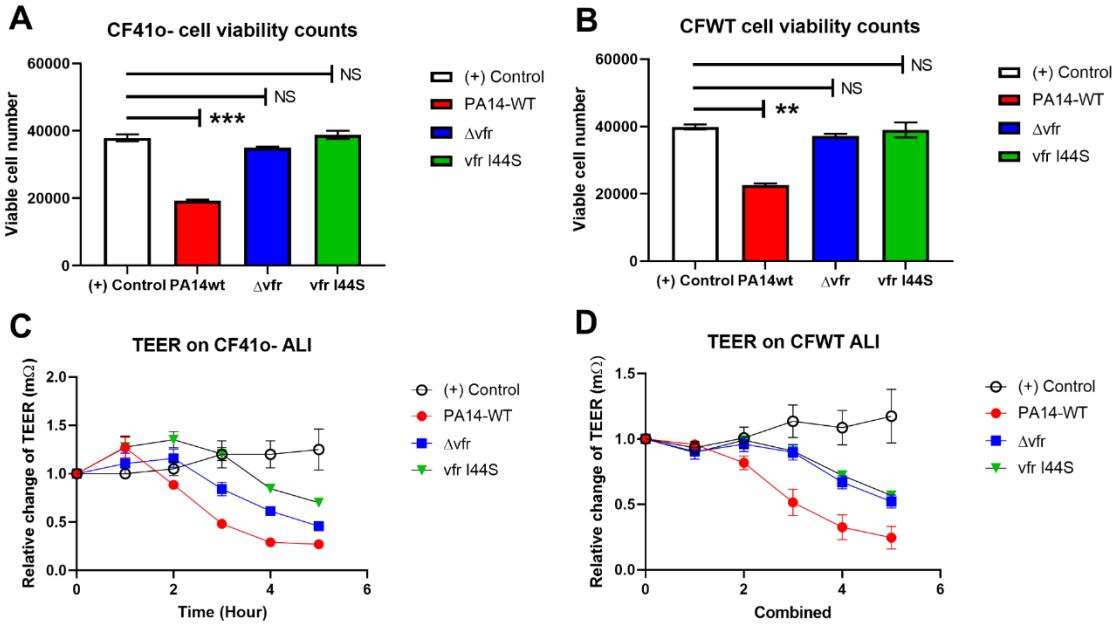
3.3.6 The loss of Vfr function confers better biofilm fitness compared to PA14-WT

To evaluate the fitness of the bacteria after acquiring *vfr* mutation, an equal number of colony-forming units (CFU) of both PA14-WT and Δ*vfr* or *vfr* I44S were grown in different pH and lifestyles with the same conditions used in Fig. 11. The percentage change in each phenotype

was surveyed every 24 hours for three consecutive days. Clones Δvfr and *vfr* I44S showed a similar mucoid phenotype on agar plates as clone M3 (Fig. 11B<i>) and were easily distinguished from the PA14-WT ancestor. The fitness study showed that *vfr* mutants outcompete the PA14-WT ancestor bacteria mostly in biofilm conditions (Fig. 17B-C). The *vfr* mutated clones also benefited more in acidic pH, which was the selecting growing environment that the *vfr* I44S mutant evolved from the PA14 ancestor. We suspect that the *vfr* I44S genotype was detectable by WGS (Fig. 12) because this *vfr* mutation is beneficial to the long-term survival of *P. aeruginosa* in an acidic biofilm environment.

3.3.7 The *vfr* I44S clone showed decreased cytotoxicity on human epithelial cells

It has been reported that *P. aeruginosa* clinical isolates obtained from chronically infected CF lungs usually lose their acute virulence (Hogardt & Heesemann, 2013). We compared the cytotoxicity of different PA14-WT and *vfr* mutants on CF bronchial epithelial cells (CF41o-) and the comparing (CFWT) cells with corrected expression of the functional CFTR to resemble normal epithelial cells. PA14-WT significantly decreased epithelial cell viability to approximately 50% while Δ*vfr* and *vfr* I44S showed minimal effects on reducing cell viability (Fig. 18A-B). When measuring bacterial disruption to the human epithelial cell tight junction by the transepithelial electrical resistance (TEER) assay, our results indicated that PA14-WT disrupted the epithelial cell tight junction at the fastest speed, probably due to the type III secretion system (T3SS) activity while Δ*vfr* and *vfr* I44S mutants showed lesser and slower effects (Fig. 18C-D). These results confirmed the functional role of Vfr in promoting acute bacterial virulence on human epithelial cells during initial infection because the cAMP-vfr signaling pathway directly controls T3SS (Fuchs et al., 2010).



ANOVA	(+) control	PA14-WT	Δ*vfr*	*vfr* I44S
PA14-WT	****	n/a	***	****
Δ*vfr*	****	***	n/a	**
vfr I44S	****	****	**	n/a

ANOVA	(+) control	PA14-WT	Δ*vfr*	*vfr* I44S
PA14-WT	****	n/a	*	**
Δ*vfr*	****	*	n/a	ns
vfr I44S	****	**	ns	n/a

Figure 18. *P. aeruginosa* PA14 Δvfr showed decreased cytotoxicity compared to PA14-WT.

(A) and **(B)**. Cell viability after 5 hours of PA14 infection. Control is medium only (without bacteria). **(C)** and **(D)**. Polarized airway epithelial cells on ALI were treated with PA14. TEER was measured every hour for 5 hours. Positive (+) control is PBS only (on apical sides). Data are mean ± SEM from two independent experiments with biological replicates (n=4). One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analysis. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; NS=not significant.

3.3.8 Vfr mutants showed decreased PA14 acute virulence in vivo

To determine the pathological consequence of *vfr* mutation in respiratory infection, CD-1 mice were infected with PA14-WT and *vfr* mutated strains by intratracheal instillation into the mouse lungs. The inoculated bacterial CFU numbers were determined based on a lethal dose for the PA14-WT strain. The first moribund mouse with PA14-WT infection was observed at 12 hours post-infection (Fig. 19). All 10 mice of the PA14-WT group became moribund and were censored from the study from hour 12 to hour 22. The Δ*vfr* strain appeared to be substantially less virulent

to the host (R. S. Smith et al., 2004) and showed 70% survival. The *vfr* I44S group also showed in vivo virulence similar to the Δ*vfr* group with an 80% survival rate. The survival data further validated our hypothesis that *P. aeruginosa* (PA14), when evolved in CF-like acidic biofilm conditions, loses its acute virulence by mutating the *vfr* gene to facilitate chronic colonization.

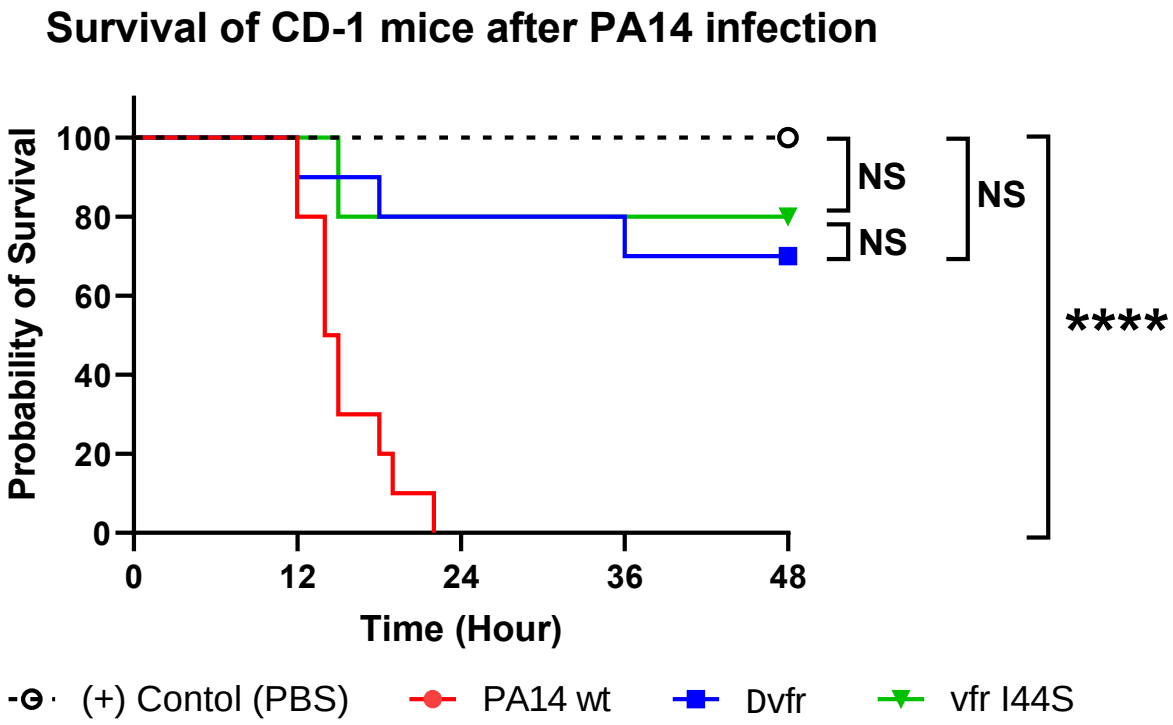


Figure 19. In vivo survival studies indicated reduced acute virulence in vfr mutated *P. aeruginosa* PA14 genotypes compared to PA14-WT.

CD-1 mice were infected with PA14-WT, Δ*vfr*, and *vfr* I44S clones by intratracheal instillation at 10^7CFU/50µL/mouse. The Kaplan-Meier survival curves were generated from 10 mice per group. Mice that survived longer than 48 hours were censored. The Log-rank (Mantel-Cox) test was used to compare bacterial groups to the no infection (+) control (PBS) group (n=10). **** $p<0.0001$; NS=not significant.

3.4 Discussion and Conclusions

P. aeruginosa is one of the most studied pathogens in CF due to its high prevalence and frequently elevated drug resistance to many antibiotics. In recent years, the mechanisms of CFTR dysfunction-induced acidification of airway surface liquid (ASL) are beginning to be elucidated. The fact that *P. aeruginosa* outcompetes all other microorganism species and dominantly exists in the CF lungs posts a vital question regarding if the acidic CF lung microenvironment preferentially promotes *P. aeruginosa* colonization.

Our investigations revealed that *P. aeruginosa* biofilm could quickly adapt and evolve in a pathophysiological-like acidic environment. Studies have shown that acute virulence-associated gene and protein expression such as T3SS correlates to worsened clinical outcomes (Coburn, Sekirov, & Finlay, 2007). It has also been proposed that T3SS makes bacteria detectable by the host and increases the chance of eliciting an immune response (Mahenthiralingam, Campbell, & Speert, 1994), which is not beneficial to the long-term survival of *P. aeruginosa*. By decreasing the *vfr* gene expression and its associated functions, *P. aeruginosa vfr* mutants conveniently gained better fitness than its ancestor by suppressing their virulence factors. While PA14-WT is burdened with increased cAMP levels and overproduction of acute virulence factors (Fig. 2A), *vfr* mutants lack response to cAMP. *P. aeruginosa* likely utilizes such a survival strategy to evade host immune response and allocate more resources to mucoid biofilm production with better swimming and swarming activities. In clone *vfr* I44S, the hydrophobic, 44th amino acid isoleucine (I) is located near the hydrophobic core of the Vfr protein that binds cAMP (Cordes, Worzalla, Ginster, & Forest, 2011). During PA14 evolution in acidic biofilm conditions, the hydrophobic isoleucine mutated to the hydrophilic serine (S), which could alter cAMP binding affinity to *vfr* I44S. New studies could be designed to further explore this potential mechanism.

To focus on the acidic pH effect on PA14 evolution, we used the minimal m63 medium supplemented with L-arginine as the sole carbon and nitrogen source, which has been reported to promote biofilm formation (Bernier, Ha, Khan, Merritt, & O'Toole, 2011) and has been used for pH effect on PA14 evolution (Lin et al., 2021). But we also acknowledge that the synthetic cystic fibrosis medium (SCFM) has a better replication of the average CF lung nutritional components. Therefore, we tested the biofilm fitness of the two *vfr* mutants, Δvfr and *vfr* I44S against their common ancestor PA14-WT in both m63 and SCFM. The selection rate *r* indicated that *vfr* mutants maintained an approximately 0.5 natural logs advantage in biofilm fitness over their ancestor in both media (Fig. 20). The *vfr* mutations favor acidic biofilm fitness regardless of medium composition. This partly explain why *vfr* mutants were detected from human longitudinal studies.

Bacterial motility is another important aspect of *P. aeruginosa* pathogenesis. It has been discovered that Vfr upregulates twitching motility (Dasgupta et al., 2002) and inhibits swimming motility (Beatson et al., 2002; Dasgupta et al., 2006). Swarming requires both swimming motility and the secretion of biosurfactants. As we demonstrated in Fig. 2A, the *P. aeruginosa* biosurfactant rhamnolipid (*rhlA*) pathway was not negatively influenced by the Vfr function. Consequently, we demonstrated increased swarming activities in Δ*vfr* and *vfr* I44S compared to PA14-WT. PA14 twitching motility also proved that the loss of Vfr function leads to decreased type IV pili function. These phenotypic changes correlated to the function of Vfr once again confirmed that the *vfr* I44S mutations likely leads to loss-of-function similar to the Δ*vfr* genotype. The increase of motility and decrease of T3SS activity in *vfr* mutants leads to overall better fitness, enhanced biofilm formation, and long-term survival in facilitating colonization in the host.

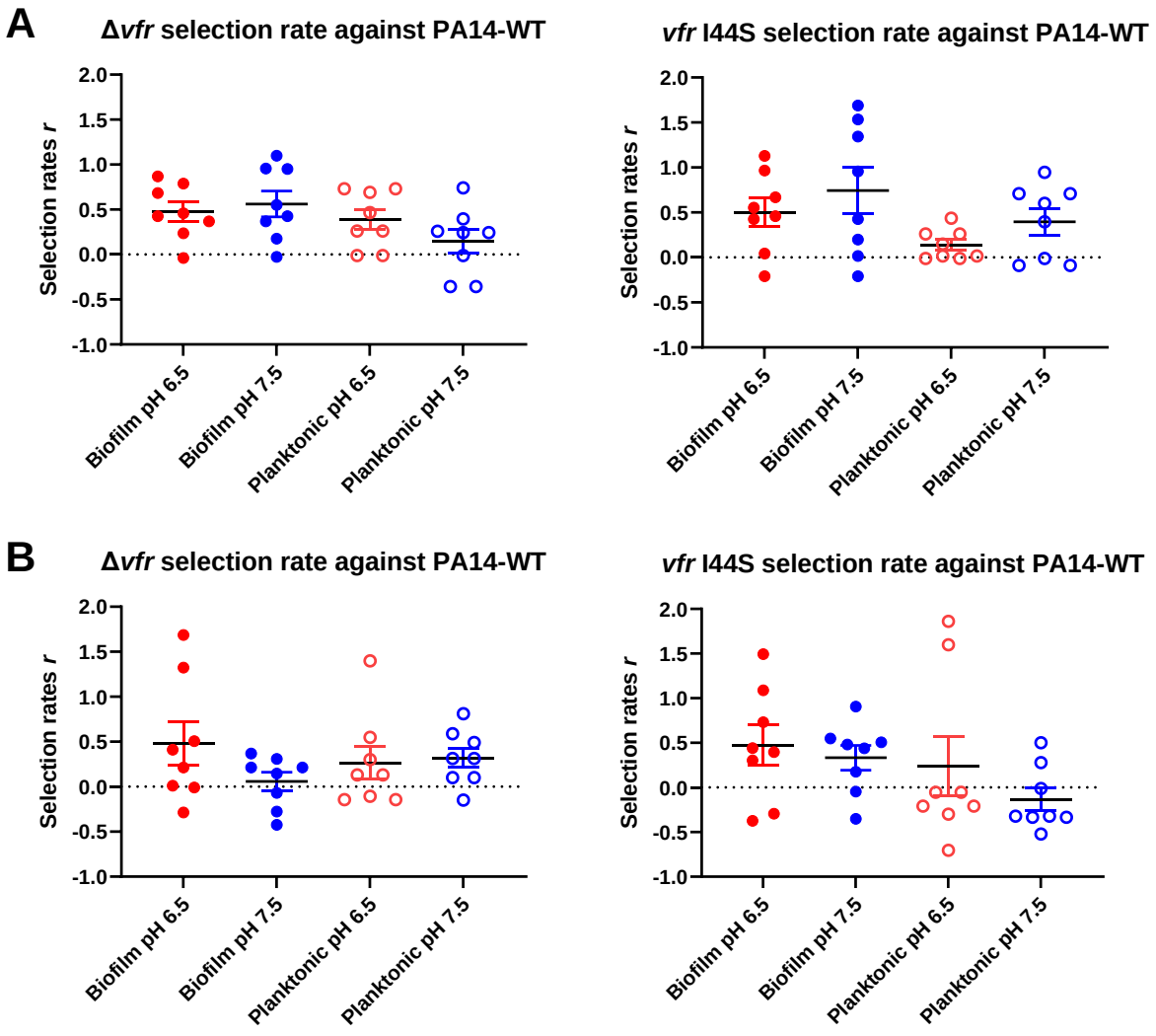


Figure 20. PA14 fitness.

Bacterial fitness was tested after 24 hours of incubation in biofilm and planktonic lifestyles. The m63 (A) and SCFM (B) media were adjusted to pH 6.5 and 7.5. Results are SEM from five independent experiments.

Given the direct evidence of acidic pH stimulated cAMP production (Fig. 15B), acidic pH promotes the PA14-WT Vfr pathway activity, which leads to the production of acute virulence factors, such as T3SS. A nonsynonymous mutation of the *vfr* gene (*vfr* I44S) likely impairs the functionality of this pathway and converts PA14 to a mucoid biofilm producer. However, Fuchs *et al.* reported that Vfr activity is dependent on intracellular cAMP concentrations when activating

downstream virulence factors with one exception, the LasR pathway (Fuchs et al., 2010). Vfr can bind to the LasR promoter without cAMP. Therefore, we suspect that if the SNP in *vfr* I44S only affects cAMP binding affinity, the activities of the LasR pathway may not be dramatically affected. This notion was also reported before in a *vfr* functional study. Beatson *et al.* introduced a plasmid carrying $Vfr_{\Delta EQERS}$ (deletion of a putative cAMP binding domain) into a *vfr* mutant. The expression of this plasmid led to elastase production that is likely a result of LasR activation (Beatson et al., 2002). Since the cyclic diguanylate (c-di-GMP) pathway is left intact, the PA14 *vfr* I44S mutant eliminates its acute virulence and potentially starts distributing more cell resources to biofilm production. Our study suggests that the acidic CF-microenvironment is likely the driving force of this vicious bacterial evolution pathway to facilitate *P. aeruginosa* colonization.

The clinical association of *vfr* mutations has been demonstrated in previous longitudinal studies of CF respiratory *P. aeruginosa.* Smith *et al.* (E. E. Smith et al., 2006) reported that 7 of the 29 CF patients were confirmed to have nonsynonymous/indels mutations directly on the *vfr* gene (24%). The gene mutations in *cyaB*, a Vfr activator, were also identified in 3 patients (10%). In addition, genetic mutations in the Vfr-regulated T3SS and *las* quorum-sensing system accounted for 21% and 62% of the studied CF population, respectively. In another human longitudinal study, Markussen *et al.* reported a *vfr* missense mutation (T163P) from a single CF patient during a 32-year longitudinal follow-up (Markussen et al., 2014). Our study is the first to directly link the functional significance of *vfr* mutations to the *P. aeruginosa* adaptation to an acidic pH environment. The bead-based biofilm evolution model is a powerful tool to mimic natural bacterial evolutions in highly controlled conditions. Although it remains possible that some environmental or opportunistic *P. aeruginosa* strains might have already contained *vfr* mutations when they first contact CF patients, our results suggest it is more likely that *vfr* mutations were evolved as a result

of the acidic CF microenvironment. Nonetheless, it will still be interesting to examine in future studies how initially colonized *P. aeruginosa* with existing *vfr* mutations adapts to either acidic CF conditions or the neutral environment to simulate the CF lungs that are no longer acidic after successful CFTR-potentiator drug treatments (Abou Alaiwa et al., 2018).

The main goal of this study is to determine if the pathological changes of CF ASL pH promote the evolution of *P. aeruginosa* to chronic colonization. We found that *P. aeruginosa vfr* mutants adapted to evade host immune response but maintained their biofilm production abilities in acidic environments. This is a clear way for *P. aeruginosa* to survive in the CF lungs without frequently causing life-threatening respiratory stress, which is probably one reason why *P. aeruginosa* can colonize in the CF lungs for decades. One practical treatment strategy to eradicate *P. aeruginosa* from the CF airways would be to closely monitor and modulate the acidic pH in CF airway surface liquid (ASL). By increasing the acidic ASL pH to neutral, *P. aeruginosa* would form less biofilm and become less antibiotic-resistant (Lin et al., 2021), which would be less motivated for *P. aeruginosa* to evolve into the mutated *vfr* phenotypes. Another potential treatment is to prevent *P. aeruginosa* from evading the host immune response. Δ*vfr* represents one of the bacterial adaption steps to alter the host-pathogen interactions. A strategy to target Δ*vfr* mutations or to bypass the cAMP-Vfr signaling pathway in mildly activating T3SS could be used to alert the host and its immune response of colonizing *P. aeruginosa*.

There are limitations to this study. Our research focused on the effects of acidic pH on *P. aeruginosa* biofilm evolution to explain why *P. aeruginosa* is the most prevalent microbial species in the CF respiratory tract. Besides, different microbes co-exist in the CF lungs, and microbial interactions likely also play a role in *P. aeruginosa* biofilm formation (Alves et al., 2018; Hendricks et al., 2016), which was not specifically investigated in this study. Any microbial

growth that potentially acidifies the CF lung microenvironment could also be taken into consideration.

As we opened up new possibilities to chronic *P. aeruginosa* biofilm treatments, there are still debates on whether or not the CF lung microenvironment is significantly more acidic than non-CF subjects. Schultz *et al.* suggested that the in vivo measurement of ASL pH is similar to non-CF in CF children (André Schultz et al., 2017). However, many other studies support the notion of an acidic microenvironment in CF airways (Okshevsky & Meyer, 2015; Pezzulo et al., 2012; Shah et al., 2016; Tate et al., 2002; Wilton et al., 2016). The acidic CF microenvironment is much more complex than only the ASL pH changes caused by the CFTR malfunction. When microorganisms colonize the CF lungs, both the dead bacterial cells and dead host immune cells frequently release the acidifying eDNA that contributes to the acidification of the CF microenvironment, as evidenced by acidic exhaled breath condensate (Ojoo et al., 2005; Tate et al., 2002). Cowley *et al.* reported a low range of sputum pH (2.9-6.5) in CF children (Cowley et al., 2015). The importance of the acidic CF lung microenvironment should not be overlooked. Our study provided experimental evidence to demonstrate that the previously observed *vfr* mutations in CF lungs are likely an adapted result of *P. aeruginosa* to an acidic biofilm lifestyle selection. It is important to acknowledge that the acidity of CF lung microenvironment is likely not the only factor that contribute to the high prevalence of *P. aeruginosa* in the CF lungs. Previous research that looked into *P. aeruginosa* behavior in CF environments did not take acidic pH into consideration but still elucidated important mechanisms that could help better manage *P. aeruginosa* infections. For example, the quorum sensing system in *P. aeruginosa* utilizes special biomolecules for cell-to-cell communication during biofilm formation. The blocking of *P. aeruginosa* quorum sensing system activity reduces *P. aeruginosa* biofilm formation (Christiaen

et al., 2014; O'Loughlin et al., 2013). These studies are the foundation for bacterial biofilm research. Our data did not only agree with previous results, but also provided additional evidence that acidic pH could promote even stronger biofilm formation.

4.0 Final Conclusions

Acidic pH is one of the most important pathological factors in the lung microenvironment of CF. We investigated the correlation between acidic pH and the high prevalence of *P. aeruginosa* lung infections.

In this study, we first demonstrated that the majority of the tested clinical CF *P. aeruginosa* isolates formed more biofilm in acidic pH than in non-CF physiological pH. Acidic pH also induces immediate *P. aeruginosa* antibiotic tolerance. A rapidly increased antibiotic treatment in acidic pH significantly promotes *P. aeruginosa* antibiotic resistance regardless of killing mechanisms. Gene expression studies suggested that acidic pH directly modulates biofilm formation and expression of acute virulence factors. These data could potentially explain the mechanisms of how environmental *P. aeruginosa* quickly and preferably colonizes the CF lung, which is acidic compared to non-CF. Acidic pH in the CF-lung microenvironment is detrimental to the clinical prevention of *P. aeruginosa* infections. By neutralizing the acidic pH, *P. aeruginosa* colonization and acute virulence could be better managed. However, short-term observations of *P. aeruginosa* response and adaptation to the CF-specific pH and antibiotic treatments do not translate directly into the mechanism of long-term *P. aeruginosa* evolution since *P. aeruginosa* usually colonizes the CF lung for decades. Therefore, we performed a laboratory evolution experiment without antibiotic treatment and aimed to identify the underlying mechanisms that help *P. aeruginosa* survive and thrive in an acidic environment.

Based on our data and the current literature, we proposed a *P. aeruginosa*-acidic biofilm response pathway (Fig. 21).

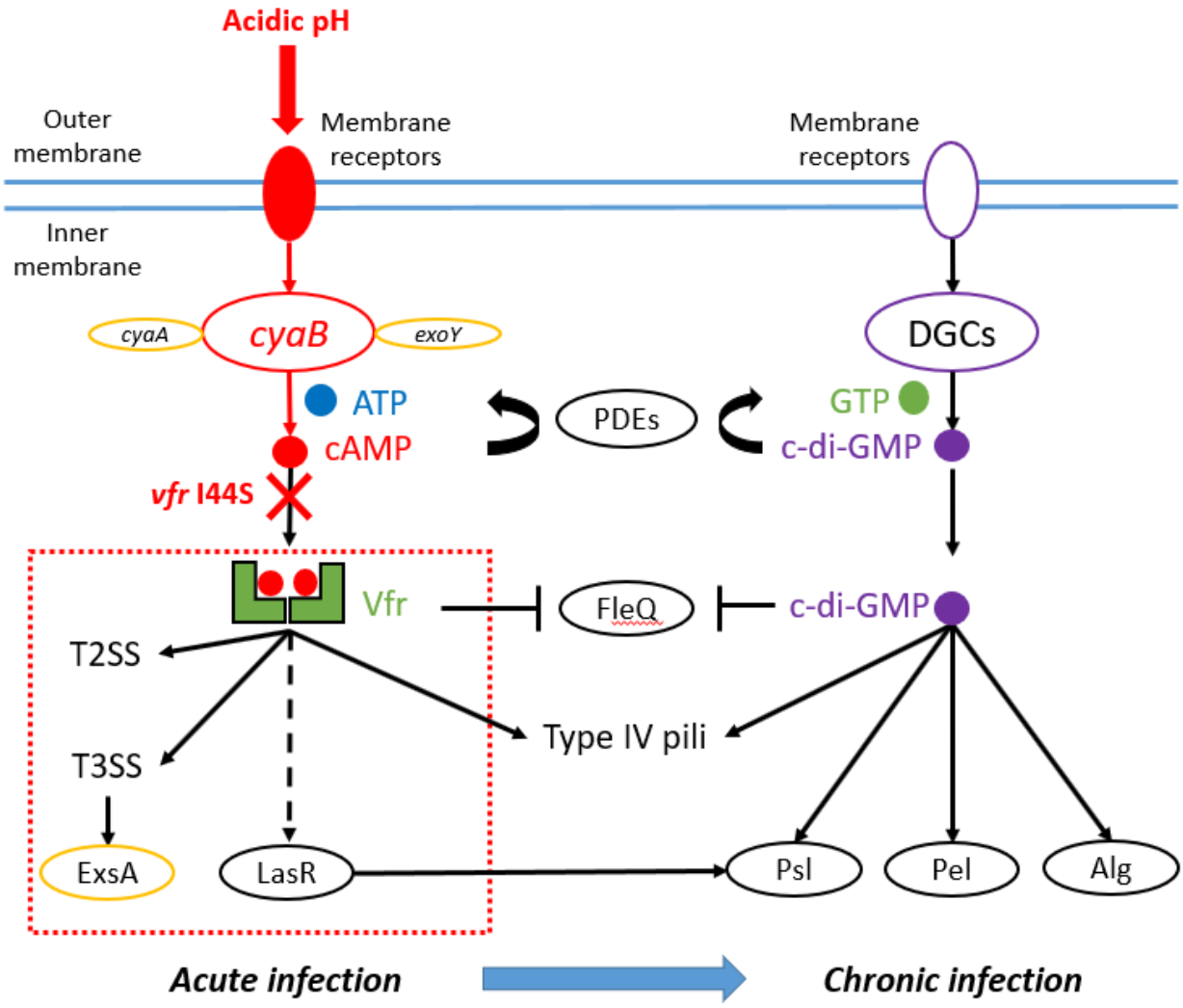


Figure 21. Schematic of the proposed mechanisms after the loss of Vfr function in P. aeruginosa.

Genes within the red dashed box are potentially affected by the *vfr* I44S mutation.

Given the direct evidence of acidic pH stimulated cAMP production (Fig. 12B), PA14-WT activates its Vfr pathway and starts producing acute virulence factors, such as T3SS. A nonsynonymous mutation of the *vfr* gene (*vfr* I44S) likely impairs the functionality of this pathway and converts PA14 to a mucoid biofilm producer. However, Fuchs et al. reported that Vfr activity is dependent on intracellular cAMP concentrations when activating downstream virulence factors

with one exception, the LasR pathway (Fuchs et al., 2010). Vfr can bind to the LasR promoter without available cAMP. Therefore, we suspect that if the SNP in *vfr* I44S only affects cAMP binding affinity, the activities of the LasR pathway may not be dramatically affected. This notion was also reported before in a *vfr* functional study (Beatson et al., 2002). Beatson et al. introduced a plasmid carrying $Vfr_{\Delta EQERS}$ (deletion of a putative cAMP binding domain) into a *vfr* mutant. The expression of this plasmid led to elastase production that is likely a result of LasR activation. Since the cyclic diguanylate (c-di-GMP) pathway is left intact, the PA14 *vfr* I44S mutant eliminates its acute virulence and potentially starts distributing more cell resources to biofilm production. Our study suggests that the acidic CF-microenvironment is likely the driving force of this vicious bacterial evolution pathway to facilitate *P. aeruginosa* colonization.

Our results strongly support the notion that acidic pH is one of the most important pathophysiological factors in the CF-lung microenvironment and its contribution to CF mortality should not be ignored. Developing new therapies that consider the acidic CF-lung pH could potentially contribute tremendously to the clinical management of *P. aeruginosa* infections and the extension of CF life expectancy.

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