



# Pf Bacteriophage and Their Impact on Pseudomonas Virulence, Mammalian Immunity, and Chronic Infections

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### Specialty section:

This article was submitted to  
Microbial Immunology,  
a section of the journal  
Frontiers in Immunology

**Received:** 12 November 2019

**Accepted:** 30 January 2020

**Published:** 21 February 2020

### Citation:

Secor PR, Burgener EB, Kinnersley M, Jennings LK, Roman-Cruz V, Popescu M, Van Belleghem JD, Haddock N, Copeland C, Michaels LA, de Vries CR, Chen Q, Pourtois J, Wheeler TJ, Milla CE and Bollyky PL (2020) Pf Bacteriophage and Their Impact on Pseudomonas Virulence, Mammalian Immunity, and Chronic Infections. *Front. Immunol.* 11:244. doi: 10.3389/fimmu.2020.00244

Pf bacteriophage are temperate phages that infect the bacterium *Pseudomonas aeruginosa*, a major cause of chronic lung infections in cystic fibrosis (CF) and other settings. Pf and other temperate phages have evolved complex, mutualistic relationships with their bacterial hosts that impact both bacterial phenotypes and chronic infection. We and others have reported that Pf phages are a virulence factor that promote the pathogenesis of *P. aeruginosa* infections in animal models and are associated with worse skin and lung infections in humans. Here we review the biology of Pf phage and what is known about its contributions to pathogenesis and clinical disease. First, we review the structure, genetics, and epidemiology of Pf phage. Next, we address the diverse and surprising ways that Pf phages contribute to *P. aeruginosa* phenotypes including effects on biofilm formation, antibiotic resistance, and motility. Then, we cover data indicating that Pf phages suppress mammalian immunity at sites of bacterial infection. Finally, we discuss recent literature implicating Pf in chronic *P. aeruginosa* infections in CF and other settings. Together, these reports suggest that Pf bacteriophage have direct effects on *P. aeruginosa* infections and that temperate phages are an exciting frontier in microbiology, immunology, and human health.

**Keywords:** bacteriophage, cystic fibrosis, wound, *Pseudomonas aeruginosa*, immunology, infection, Pf phage, Inovirus

## INTRODUCTION

*Pseudomonas aeruginosa* (*Pa*) is a major human pathogen associated with chronic infections in wounds, burns, and nosocomial settings (1–5). *Pa* is responsible for extensive mortality and billions of dollars in health care costs (6–8). In recognition of this, *Pa* was recently listed as a critical priority pathogen by the World Health Organization (WHO) (9).

*Pa* infections are highly problematic in cystic fibrosis (CF), an inherited disease associated with defective ion transport and the accumulation of thick, tenacious airway secretions. Individuals with CF are prone to chronic pulmonary infections that over time lead to poor lung function and increased mortality (10, 11).

*Pa* is particularly pathogenic in CF because of its ability to form robust biofilms (12–18). These are slimy conglomerates of polymers and microbial communities that allow *Pa* to colonize airways and other surfaces (19). *Pa* biofilms are especially tenacious and, once established, very difficult to clear.

Many antibiotics have limited penetration through biofilms (20, 21). Hence, bacteria encased within biofilms are able to tolerate antibiotic concentrations hundreds or thousands of times higher than planktonic bacteria (12, 22–24). Over time, this favors the emergence of antibiotic resistant bacteria (19, 25) and the predominance of multi-drug resistant (MDR) strains (15, 20, 26, 27). Individuals with CF are often infected with *Pa* strains that are resistant to whole classes of anti-pseudomonal antibiotics (28–30), including all oral antibiotic options (15, 31). *Pa* biofilms are also often composed of a heterogeneous bacterial population that includes individuals that are less susceptible to antibiotics, such as metabolically dormant persister cells (32).

*Pa* biofilms also contribute to immune evasion by defying phagocytic engulfment (33–38). Robust biofilm formation by *Pa* contributes to physical impedance of phagocytosis (39, 40) and biofilm polymers also have properties that contribute to immune evasion, including antagonism of complement-mediated opsonization [(35, 37, 41)]. *Pa* also resists efficient bacterial clearance by neutrophil extracellular traps (NETs) (42–44). These are networks of DNA and other polymers released from lysed neutrophils that entrap and destroy bacteria (45, 46). The ability to resist NET-mediated killing (NETosis) is highly strain dependent (42, 43). In the CF airway, ongoing robust immune activation and impaired bacterial clearance perpetuate a vicious cycle of inflammation (47–52).

In light of these effects on antibiotic tolerance and immune evasion, there is great interest in identifying novel biomarkers, virulence factors, and therapeutic targets associated with *Pa* biofilm infections.

Pf bacteriophages, filamentous Inoviruses produced by *Pa* (53, 54), have emerged as a new front in the fight against *Pa* biofilm infections. Here, we review the diverse and surprising ways that Pf phages contribute to the pathogenesis of chronic *Pa* infections in CF and other settings. First, we review the structure, genetics, and epidemiology of Pf phage. Next, we address how Pf phages contribute to *Pa* phenotypes including effects on biofilm formation, antibiotic resistance, and motility. Then, we examine data indicating that Pf modulates mammalian immunity at sites of bacterial infection. Finally, we discuss recent literature implicating Pf in chronic *Pa* infections in CF and other settings. The goal of this work is to provide a comprehensive and integrated resource for those interested in Pf biology and in phage contributions to bacterial pathogenesis.

## ***Pa* AND Pf BACTERIOPHAGES**

Pf phages are Inoviruses, a genus of temperate, non-enveloped filamentous viruses. Inoviruses are broadly distributed across

all biomes and infect both Gram-positive and Gram-negative bacterial species, and even some Archaea (54). While all lytic phage obligately lyse their bacterial hosts during propagation and most other lysogenic phage typically lyse their bacterial host at some point during their lifecycle, Inoviruses are unique in their ability to establish chronic infection cycles where virions are continuously extruded from the bacterial cell envelope without lysis (55).

Inoviruses like Pf are among the better characterized bacteriophages due to their extensive use in research and industry. Inoviruses are amenable to genetic manipulation and some species, such as M13 (*Escherichia coli*) are widely used in biotechnological applications, such as phage display (56) and as drug carriers (57). Pf phages are also a model system for studying the molecular mechanisms of nucleoprotein assembly and membrane transport (58–68).

In this section, we discuss Pf phage structure, genetics, and lifecycle. We then address the prevalence and diversity of Pf phages amongst *Pa* clinical isolates.

### **Pf Virion Structure**

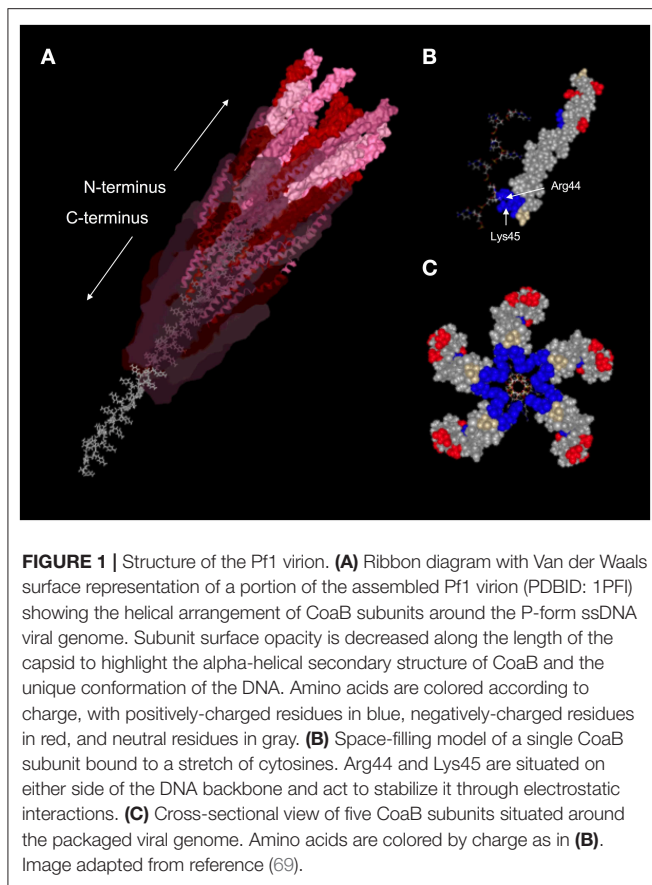
Pf virions are ~6–7 nm in diameter and vary in length from ~0.8–2 μm, depending on the strain and genome size in question. Like all Inoviruses, Pf virions are composed of thousands of copies of a single major coat protein (p8, CoaB in Pf) with minor coat proteins at either end involved in phage assembly and host recognition (**Figure 1**) (70–72). Structurally, Inoviruses can be subdivided into two classes based on helical symmetry. Class I Inoviruses are highly symmetrical and include species, such as Fd and M13, which infect *Escherichia coli*. Class II Inoviruses have a twisted, helical symmetry and include Pf phages (73).

Pf phages package a single-stranded circular DNA genome. While most viruses package their genetic material into the smallest conformation possible, Inoviruses like Pf package their genome in the most extended conformation possible. The orientation of the packaged ssDNA Pf phage genome is also unusual; the genome is packaged in a *P* DNA confirmation where the DNA bases are inverted and pointing outwards with the C-terminus of CoaB capsid proteins reaching through the bases to stabilize the backbones of the anti-parallel DNA strands (**Figure 1**) (74).

Pf virions are relatively stiff filaments with a long persistence length and an overall negative charge density comparable to dsDNA (75). At sufficiently high concentrations, Pf phages, like other filamentous viruses, spontaneously align and assemble liquid crystalline structures (76). The uniform length, diameter, and charge density has made Pf and other Inoviruses a popular model system to study the soft matter physics of colloidal liquid crystals (77) and an effective tool to measure dipolar couplings in NMR structure determinations (78, 79). We discuss how the ability of Pf phages to assemble liquid crystalline structures in biofilms and airway secretions impacts *Pa* infection pathogenesis in a subsequent section below.

### **Pf Genome Structure**

Pf phages have a conserved core genome structure encoding genes necessary for DNA replication, virion assembly, and



morphogenesis. This is typically present in *Pa* as a prophage integrated into the bacterial chromosome (80). For this review, we refer to Pf gene names and numbers that correspond to the Pf4 prophage integrated into the chromosome of the reference strain *Pa* PAO1 (PA0715–PA0729) (**Figure 2**).

Characterized Pf genes in the core genome include *xisF4* (excisionase), *c* repressor gene (*pf4r*), PA0720 (single-stranded DNA binding protein), PA0723 (*coaB*, major coat protein), PA0724 (*coaA*, minor coat protein), PA0726 (morphogenesis protein), PA0727 (replication initiation protein), and PA0728 (*intF*, integrase). The functions of these genes are discussed in more detail below. The function of other genes in the Pf core genome remain to be characterized.

Accessory genes flank the core Pf genome (**Figure 2**) and are known as morons (they add more genes on) (81). While the functions of Pf phage morons are not well-characterized, other phage morons have been shown to reduce virulence factor production and inhibit *Pa* motility (82). Due to the presence of these accessory genes, the overall size of the Pf genome is variable amongst different Pf strains, ranging from ~7–12 kb.

## The Pf Life Cycle

The events around initial infection of bacteria with Inoviruses has been reasonably well-characterized. Pf virions initiate infection by adsorbing to the tip of type IV pili, an extracellular

appendage that mediates twitching motility in *Pa* (**Figure 3**) (83). Adsorption is mediated by the minor coat protein CoaA (PA0724), which is located on the end of the virion. The adsorbed virion is then drawn into the periplasm as the pili retracts where the phage coat protein CoaA contacts the secondary receptor TolA (72). TolA is part of the highly conserved Tol-Pal system that controls membrane integrity and invagination during cell division and is constitutively expressed and readily available for Pf phages to leverage to infect the host cell. CoaB capsid proteins are then drawn off the phage ssDNA genome and retained in the inner membrane while the ssDNA Pf genome is deposited into the cytoplasm. The ssDNA genome is then converted into a circular dsDNA replicative form (RF) by host enzymes and the RF serves as a template for transcription of phage genes.

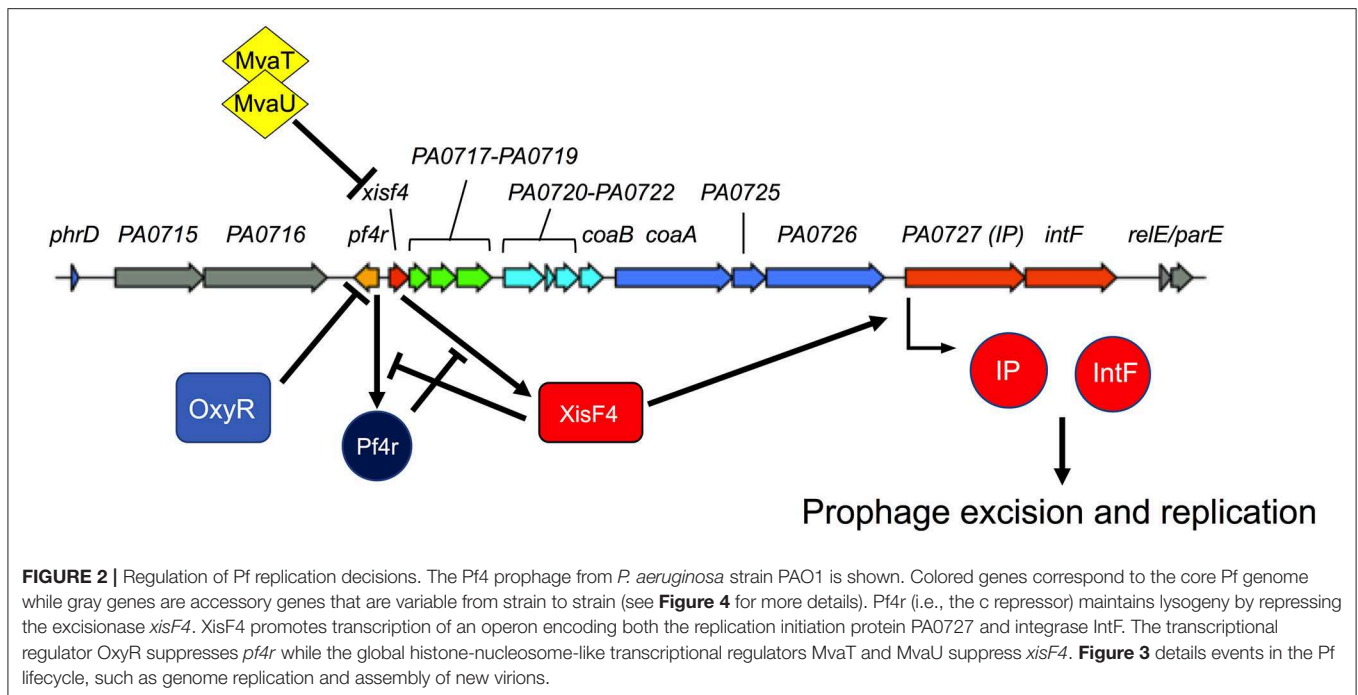
At this point, the phage must decide whether it will initiate a chronic infection and produce progeny virions or initiate a lysogenic lifecycle and integrate into the bacterial chromosome as a prophage (80). If conditions favor Pf entering a lysogenic lifecycle after infecting a naïve bacterial host, the RF can be integrated into the bacterial chromosome as a prophage by the Pf-encoded site-specific tyrosine recombinase IntF (84).

Recent work has shed light on the molecular details for how Pf phages maintain lysogeny or initiate a chronic infection cycle. Like other phages, Pf phages respond to host stress. For example, Pf5 replication is strongly induced when the substrate binding protein DppA1 (involved in peptide utilization) is inactivated (85). Although the mechanism is not known, it is thought that Pf5 senses nutrient limitation of its host through DppA1 and in response, initiates the chronic infection cycle. Given the importance of Pf phages for *Pa* biofilm formation and pathogenesis discussed below, the further characterization of factors that drive production of Pf phages is likely to be an area of active research.

Pf phages, like several other phages, also respond to oxidative stress. When *Pa* is exposed to H<sub>2</sub>O<sub>2</sub>, cysteine residues in the OxyR transcriptional regulator are oxidized (86). Oxidized OxyR then binds to the Pf4 prophage between PA0716 and PA0719 (86). The binding of OxyR to this site likely suppresses the *c* repressor gene *pf4r*, which maintains lysogeny and confers immunity to superinfection by exogenous Pf virions. Pf4r promotes lysogeny and superinfection exclusion by repressing transcription of the Pf excisionase gene *xisF4* (84) (**Figure 2**).

When *pf4r* is suppressed, as it is by OxyR, or when sufficiently high titers of infecting Pf virions overwhelm Pf4r, as may happen during superinfection, XisF4 is free to promote the transcription of the operon encoding the replication initiator protein (PA0727) and IntF (84). The initiator protein binds to RF DNA at the origin of replication, recruits the host helicase UvrD and DNA polymerase III, and initiates rolling circle replication (87). Rolling circle replication not only produces new copies of the dsDNA RF, but also new ssDNA Pf genomes. The newly synthesized ssDNA genomes are quickly coated by numerous copies of the single stranded-binding protein PA0720, stabilizing and protecting the ssDNA species until it can be packaged into a new virion.

While the RF and ssDNA pools expand in the bacterial cytoplasm, structural Pf proteins, such as CoaB are produced. CoaB is translated with an N-terminal leader peptide that



targets the CoaB preprotein to the inner membrane. After CoaB is inserted in the inner membrane in the correct orientation, it is processed into its mature form by host Sec/YidC enzymes (88). CoaB monomers interact with membrane-associated morphogenesis machinery (which likely includes the morphogenesis protein PA0726) to package the ssDNA Pf genome. As CoaB is incorporated into the growing virion, PA0720 is displaced and the nascent phage particle is extruded through the cell envelope (**Figure 3**).

### Superinfection of *Pa* by Pf Phages

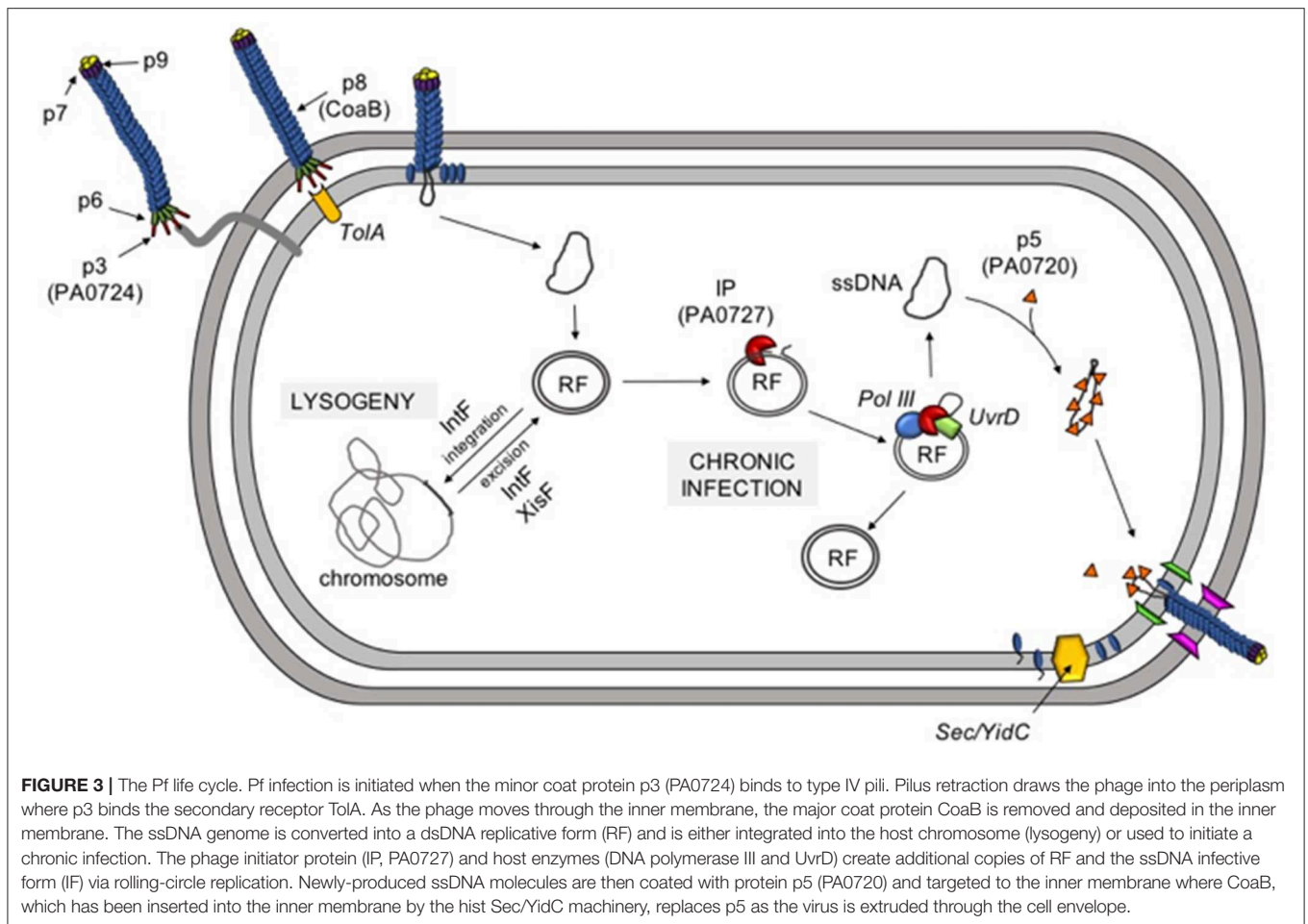
Once extruded from the host cell, Pf virions likely encounter a host that is already lysogenized by a Pf prophage. This is especially true when bacterial densities are high, as they are in biofilms. Superinfection occurs when exogenous Pf virions successfully infect a bacterial cell already lysogenized by Pf. Even though Pf (and other Inoviruses) can replicate without lysing their bacterial host, Pf superinfection can result in bacterial lysis. At present it is unclear how Pf superinfection causes bacterial lysis. It is possible that during superinfection more Pf virions are produced than an individual host cell can handle. For example, CoaB monomers or other Pf proteins could accumulate in the inner membrane to sufficiently high levels such that the cytoplasmic membrane is destabilized.

Superinfection by Pf phages plays important roles in *Pa* biofilm development. Evaluation of the within-population genetic diversity of *Pa* PAO1 during biofilm formation revealed that the Pf4 population diversified at an evolutionary rate comparable to RNA viruses, far faster than the rest of the genome (89). In conjunction with the diversification of the Pf4 population, titers of superinfective Pf4 virions as high as  $10^{11}$  plaque forming units per milliliter of biofilm effluent

were observed. Interestingly, all mutations in Pf4 were within or upstream of the c repressor gene *pf4r*, suggesting that superinfective Pf phages lose the ability to self-regulate their replication. Lastly, the study by McElroy *et al.* also found that mutations in type IV pili genes also accumulated in *Pa* biofilm populations, most likely in response to selective pressures exerted by the expansion of the Pf phage population (89).

*Pa* can suppress Pf phage through the histone-like nucleoid-structuring (H-NS) proteins MvaT and MvaU (83). MvaT and MvaU are global regulators of gene expression and coordinately suppress AT-rich elements in the *Pa* genome (90). The *Pa* genome is G-C-rich (~66% GC content) and MvaT and MvaU offer a mechanism for *Pa* to incorporate xenogenic DNA (such as phages and other horizontally acquired genetic material with a relatively high AT-content) into its genome in a regulated way. Because MvaT and MvaU regulate the same target genes, *Pa* can tolerate the loss of either *mvaT* or *mvaU*; however, inactivation of both *mvaT* and *mvaU* is lethal to *Pa* (90). A transposon mutagenesis study discovered that the conditional depletion of MvaT in a  $\Delta mvaU$  background induced Pf4 replication that resulted in the death of *Pa* (83). That study also demonstrated that Pf4 uses type IV pili as a cell surface receptor to initiate infection. Interestingly, when type IV pili were inactivated by deleting the *pilY* gene, depletion of MvaT in a  $\Delta mvaU$  background was tolerated, even though the Pf4 prophage was induced and infectious Pf4 virions were produced (83). This observation indicates that superinfection was required to cause cell death.

These observations are consistent with a model where within *Pa* biofilms, populations of Pf phages with inactivated c repressor genes expand and Pf virions are produced in abundance. These virions could then superinfect *Pa* at a sufficiently high multiplicity of infection to overwhelm the superinfection



exclusion mechanism mediated by Pf4r proteins produced by the resident Pf prophage. Under these conditions, individuals with type IV pili mutations would be selected for as they would be resistant to Pf superinfection.

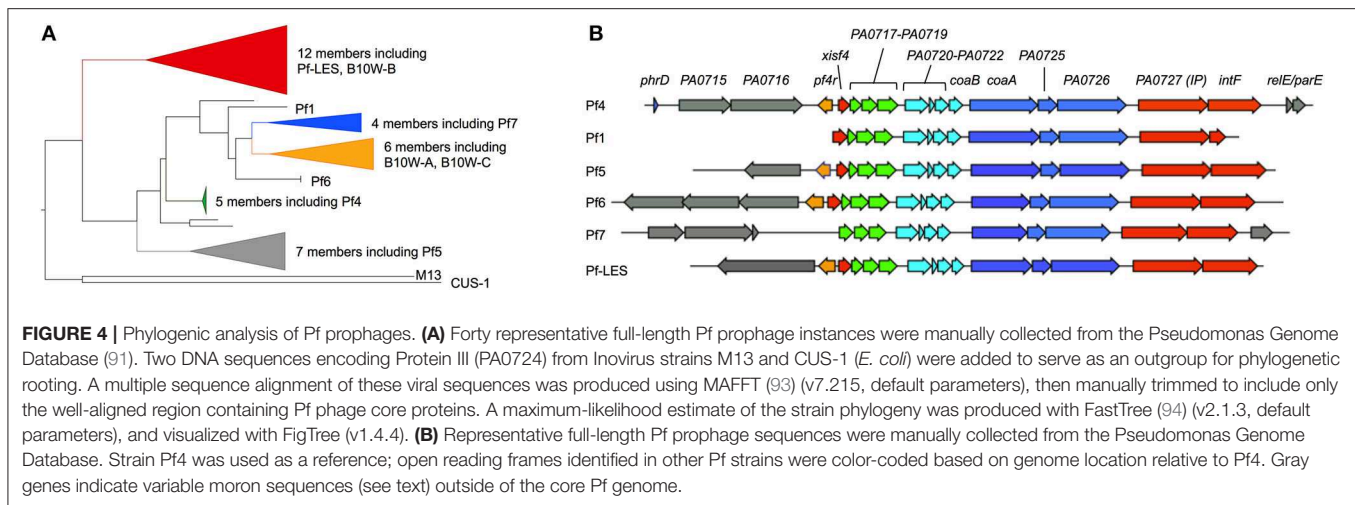
## Pf Diversity and Prevalence Amongst *Pa* Isolates

Routine whole genome sequencing of *Pa* isolates has revealed that Pf prophages are prevalent amongst *Pa* isolates. In one study, out of 241 *Pa* strains, ~60% were lysogenized by Pf (80). Of 2,226 genomes available through the Pseudomonas Genome Database (91), 52% encoded a Pf prophage (92). A phylogenetic analysis of Pf prophage sequences revealed that most known Pf prophages encode their own integrase *intF* (Figure 4). However, Pf1, the first Pf strain isolated in 1966 (95), does not encode an intact integrase and replicates episomally (it cannot enter a lysogenic lifecycle). Strains of Pf that do not integrate into the bacterial chromosome would not be detected by whole genome sequencing. Therefore, the prevalence of Pf phages amongst *Pa* isolates is likely underestimated.

Many Pf prophages integrate into bacterial tRNA genes, as is true with other temperate phages (96). For example, the Pf4 prophage infecting *Pa* strain PAO1 integrates into the tRNA-Gly

gene *PA0729.1*. It is worth noting that the subline strain MPAO1 harbors both Pf4 and a second similar Pf prophage designated Pf6 integrated into the tRNA-Met gene *PA4673.1* (97). Another example is Pf7, which is integrated into the tRNA-Met gene *PSPA7\_5361* in *Pa* strain PA7. Some Pf strains, however, integrate into other genomic locations. Strain Pf5, which infects *Pa* strain PA14, integrates between the C32 tRNA thiolase (*PA14\_48870*) and *dnaJ* (*PA14\_49040*). Other Pf prophages integrate into a homologous site in other strains of *Pa* including Pf-LES, which infects *Pa* epidemic strain LES58 (80) and an unclassified Pf strain infecting *Pa* strain 19660 (91).

We have observed that several strains of *Pa* are infected by multiple Pf prophages. This is in contrast to many species of phages (e.g., lambda) where, due to superinfection exclusion mechanisms, a preexisting phage prevents secondary infections by the same or closely related phage. As noted above, MPAO1 harbors both Pf4 and Pf6. Other strains, such as *Pa* B10W harbors three Pf prophages. Two of the three Pf prophages integrated into the B10W chromosome are genetically distinct from each other (Figure 4) indicating that the Pf prophages of BW10 were obtained from distinct infections by different strains of Pf phages along with potential duplications of the same Pf prophage. Given the roles Pf phages play in biofilm formation and infection



pathogenesis (see below), it would be interesting to compare the fitness of *Pa* strains lysogenized by a single Pf prophage compared to strains lysogenized with several Pf phages.

## Pf EFFECTS ON *Pa* PHENOTYPES AND FITNESS

Infection of the CF airway with *Pa* starts with planktonic (free living), variants often originating from the environment. Over time, as bacteria adapt to the CF airway, they environment, they form a biofilm (98). In this section we address the contribution of Pf phages to *Pa* biofilm formation and to other phenotypes involved in *Pa* pathogenicity.

### Pf Phages and Small Colony Variant (SCV) Morphology

In *Pa* PAO1 biofilms, bacterial cells shed from the biofilm display a small colony variant (SCV) morphology after they are grown on solid agar (99–101). The production of SCVs is correlated with the emergence of superinfective Pf4 phage. SCVs revert back to a “fuzzy” wild-type colony morphology after subculture in Pf phage-free lysogeny broth (101), indicating that the SCV morphology is a phenotypic response to Pf superinfection and not due to heritable mutations. Because Pf superinfection both suppresses type IV pili-mediated twitching motility and induces a severe growth lag in *Pa*, it is possible that the SCV morphology associated with chronic Pf infection is a result of reduced growth coupled to inhibited twitching motility. The emergence of SCVs from *Pa* biofilms appears to be variable amongst *Pa* strains—*Pa* PA14 biofilms do not shed cells that form SCVs even though Pf5 virions were present at high densities in PA14 biofilms (102). The underlying mechanism for this discrepancy is unknown, but it may relate to the different integration sites of Pf4 and Pf5 and/or the different moron genes encoded by Pf4 and Pf5 (Figure 4B).

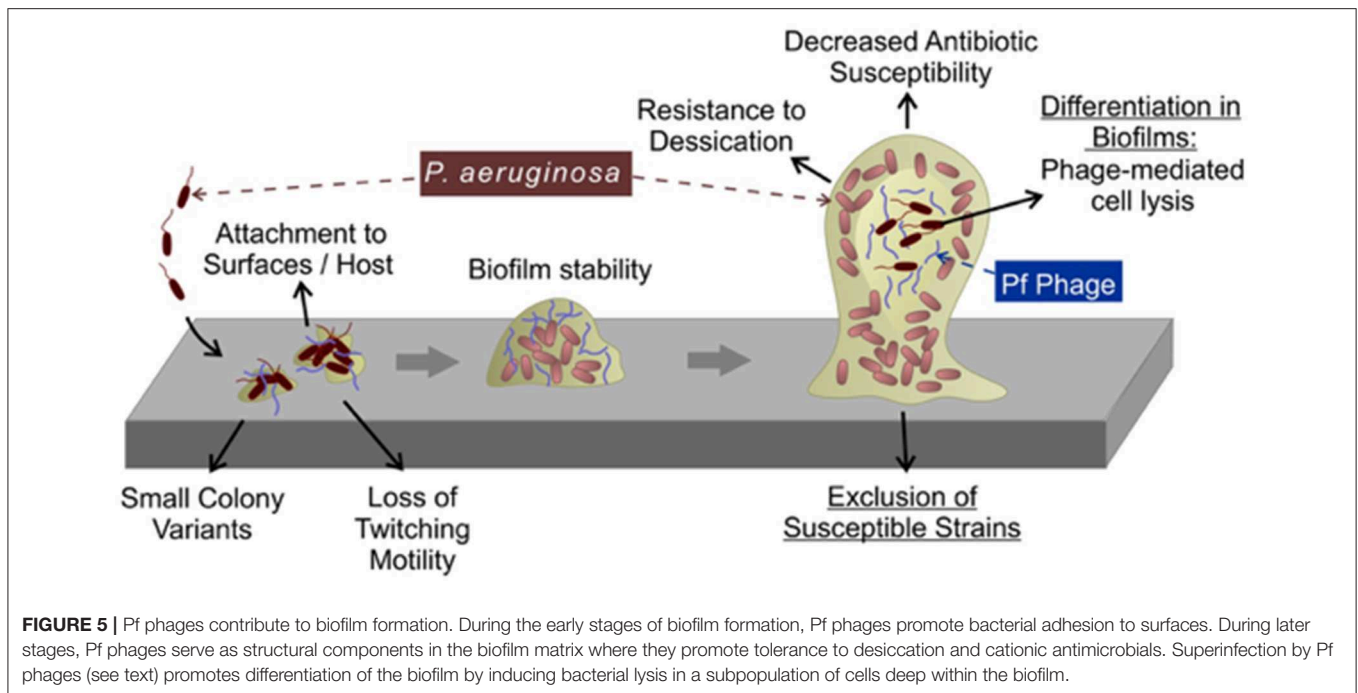
### Pf Phages and *Pa* Biofilm Formation

Upregulation of Pf phage genes are a common feature of *Pa* biofilms, as found in a metanalysis of transcriptional data from

*Pa* biofilms grown under various laboratory settings (103). In addition to gene expression data, *Pa* biofilms grown under different conditions (dripflow, chemostat, colony biofilms, etc.), are all associated with the production of abundant superinfective Pf phages at titers as high as  $10^{11}$  PFU/ml (89, 99, 100, 104). Furthermore, semiviscous (105) and anaerobic conditions (106) that mimic environments in biofilms and in late-stage CF-airways were found to induce Pf phages, although the mechanisms behind these induction cues are unknown. Collectively, these observations indicate that Pf phages are consistently induced in *Pa* biofilms, raising the possibility that Pf phages play important roles in biofilm formation. Indeed, Pf phages contribute to *Pa* biofilms in diverse and surprising ways.

The formation of a biofilm is initiated by bacterial attachment to a surface followed by the formation of microcolonies (107). As microcolonies differentiate and grow, voids form in the middle of these microcolonies that are created by cell lysis (100). Cell lysis is accompanied by the release of DNA into the extracellular space (eDNA), which adds structural integrity to the biofilm (108). The presence of superinfective Pf phages in the biofilm effluent correlates with cell lysis and eDNA release prior to the dispersion phase of the biofilm lifecycle (109, 110) (Figure 5). To this point, when Pf4 prophage was deleted from the *Pa* PAO1 chromosome, bacterial lysis and eDNA release were not observed (99). Bacterial lysis and eDNA release caused by superinfective Pf phages appears to be a regulated process; the two-component regulator BfmR was reported to suppress Pf phage-mediated cell lysis through PhdA (PA0691), an antitoxin homolog in the Phd (prevent-host-death) family of proteins (111). Deleting *bfmR* caused the premature induction of superinfective Pf phages, increased bacterial lysis and eDNA release, phenotypes opposite to the  $\Delta$ Pf4 prophage mutant. It is possible that PhdA induces Pf replication by interacting with the ParE plasmid stabilization system encoded by Pf4 (Figure 4B) (110). However, experimental evidence for this interaction is lacking.

The presence of Pf phages is also associated with phenotypes linked to the pathogenicity of *Pa* biofilms (Figure 5). Expecterated sputum from CF patients infected by *Pa* contain



$\sim 10^6$ - $10^9$  Pf virions per ml (92, 101). Filamentous Pf phages can dramatically increase the viscosity of CF airway polymers, such as mucin and DNA (92, 101). Furthermore, Pf phage-producing biofilms are more adherent to surfaces compared to *Pa* not producing Pf virions (92, 99–101). Thus, Pf virions could benefit *Pa* infecting CF airways by reducing bacterial clearance from the lung. Consistent with this idea, *Pa* superinfected by Pf established a non-invasive infection phenotype in a mouse model of pneumonia and did not disseminate from the lung whereas *Pa* not producing Pf virions disseminated to distal tissues, suggesting that Pf phages contribute to the establishment of conditions that promote localized chronic infection (112). Other filamentous phages, such as MDA $\Phi$  that infects *Neisseria meningitidis* likewise promotes adhesion to epithelial cells promoting host colonization (113). Thus, filamentous phages may play key roles in host colonization and infection initiation in various bacterial infections.

Rice *et al.* demonstrated that biofilms formed by the Pf phage-deficient mutant  $\Delta$ Pf4 were more sensitive to disruption by the detergent SDS relative to the parental strain *Pa* PAO1 lysogenized by Pf4 (99), suggesting that filamentous Pf phages contribute to the structural integrity of biofilms. As discussed above, Pf phages are stiff, negatively charged filaments that spontaneously assemble liquid crystalline structures. Our previous work revealed that Pf virions accumulate in the biofilm matrix where they spontaneously align and give the biofilm matrix liquid crystalline properties (101, 114). Bacteria living in a liquid crystalline biofilm matrix were resistant to desiccation, possibly because the highly ordered liquid crystalline matrix was better able to retain water compared to non-crystalline matrices. Furthermore, the negative charge carried by Pf phages facilitates the binding and sequestration of cationic

aminoglycoside antibiotics and cationic antimicrobial peptides (92, 101). This binding capacity was enhanced when Pf phages were in the liquid crystalline phase, allowing *Pa* to tolerate otherwise lethal doses of cationic antibiotics (101, 114). In clinical settings, tolerance to antimicrobials could promote the emergence of individuals with fixed antibiotic resistance, thus limiting treatment options and promoting chronic infection. Indeed, filamentous bacteriophages are associated with chronic *Pa* lung infections and antibiotic resistance in CF (92). Because all Inoviruses are composed of acidic coat proteins, filamentous bacteriophages may be an overlooked structural component of the biofilm matrix in other bacterial species.

Other ways that Pf phages could benefit *Pa* biofilms is by excluding susceptible strains (104). Consistent with this, recent work demonstrates that *Pa* isolates collected from older CF patients tend to be lysogenized by Pf compared to isolates collected earlier in disease (92). This observation raises the possibility that *Pa* strains not harboring Pf prophages may be replaced by strains lysogenized by Pf as the disease progresses to later stages. Future studies investigating the role of Pf phages in the establishment and maintenance of *Pa* lineages in chronic CF airway infections may provide insight into how *Pa* dominates CF airway infections.

## Pf EFFECTS ON MAMMALIAN IMMUNITY

There is increasing interest in the impact of phages on human immunology. This trend parallels the resurgence of efforts to develop phage therapies to treat chronic/multidrug-resistant bacterial infections (115, 116) as well as the rapid expansion of research into endogenous phages residing on and within the

human host (117–119). Much of this work has focused on CF and the viral pathogens of *Pa* (120, 121).

In this section, we discuss what is known about Pf and its impact on mammalian immunity. First, we discuss Pf and its impact on phagocytosis and bacterial clearance. Next, we address Pf effects on cytokine production and the inflammatory milieu. Finally, we discuss how Pf biology relates to what is known about mammalian immunity and other endogenous and exogenous phages and highlight the outstanding questions around these interactions.

## Pf Effects on Bacterial Clearance by Phagocytes

The ability of *Pa* to establish residency and persist in the human lung is dependent upon the bacterium's efficacy at evading innate immune attacks, particularly neutrophil- and alveolar macrophage-mediated anti-bactericidal defenses (122–126). *Pa* must also contend with multiple pattern-recognition receptors (PRRs) including Toll-like receptors 4 (TLR4), 5 (TLR5), and 9 (TLR9) that recognize bacterial pathogen-associated molecular patterns (PAMP) molecules including lipopolysaccharide (LPS) (127, 128), flagellin (128, 129), and DNA containing CpG motifs (130), respectively. PAMP-mediated stimulation of phagocytosis is the primary host defense against *Pa* infection, as evidenced by the fact that both animal models and people with defects in phagocytic cell function are highly susceptible to *Pa* infection (125, 131, 132).

*Pa* possess a diverse set of virulence factors that impede phagocytosis by both neutrophils and macrophages (38, 133). Many of these factors impact the machinery of phagocytosis, including inflammatory cytokine production, cellular recruitment, and bacterial engulfment (38, 134, 135). These effects on phagocytic clearance contribute to *Pa* immune evasion and persistence within infected tissues (136).

We recently reported that Pf directly inhibits phagocytosis and bacterial clearance (137). Pf causes a significant decrease in the number of *Pa* phagocytosed by murine-derived dendritic cells (DC), macrophages, and human U937-derived and primary macrophages (137). Additionally, murine phagocytes exposed to Pf4 phagocytosed fewer dead *E. coli* particles compared to saline controls, indicating that the inhibitory effects of Pf on phagocytosis are not limited to engulfment of *Pa* (137).

Pf phage also influence bacterial phenotypes in ways that are likely to prevent efficient phagocytosis. As discussed above, some strains of Pf phage contribute to the emergence of SCV strains (99, 110) which are known to drive exopolysaccharide-dependent resistance to macrophage phagocytosis (138). Furthermore, Pf contributions to biofilm formation, adhesiveness, and increased polymer viscosity could be expected to interfere with phagocytosis given that biofilms formed by *Pa* and other organisms are known to prevent opsonization and engulfment of the bacteria within (38, 139–141). Finally, the same structural attributes that promote liquid crystal assembly by Pf phages also drives bacterial aggregation (142) and loss of motility (112), features associated with diminished phagocytosis (135). We discuss reduced phagocytic uptake of bacteria through the lens of CF in later sections.

## Pf Effects on Cytokine Production and the Inflammatory Milieu

Inflammatory cytokines are critical to host immune clearance of *Pa*. It is well-established that TNF in particular plays a crucial role in stimulating phagocytosis (143, 144) and in polarizing macrophages toward an M1 phenotype associated with efficient bacterial clearance.

We recently reported that Pf phages are associated with decreased cellular production of TNF (137). These effects were associated with enhanced type 1 interferon levels. Consistent with a critical role for TNF in bacterial clearance, we observed that supplemental TNF could correct Pf effects on phagocytosis and bacterial clearance *in vivo* (137). Pf also suppressed the production of other cytokines and chemokines important for bacterial clearance, including CXCL1 and IL-17. Conversely, Pf increased production of IL-12 and type 1 interferon (112, 137). Fd phage, an *Inovirus* that infects *E. coli*, did not affect TNF $\alpha$  production or phagocytosis (137). The differences in the immunomodulatory properties of Pf and fd phages is unclear, but may relate to differences in coat protein/virion structure or amino acid composition.

Pf-dependent modulation of cytokine production by immune cells was associated with intracellular uptake of Pf phage. We reported that Pf is taken up by human and mouse macrophages, B cells, and dendritic cells within endosomal vesicles. These results are consistent with reports that other phages are internalized by mammalian cells, though those reports mostly implicated phagocytosis rather than endocytosis (145, 146). Further, there are indications that large numbers of phages are directionally transcytosed across epithelial layers into system circulation (147), raising the possibility that phages regularly modulate immune responses and impact human health and disease.

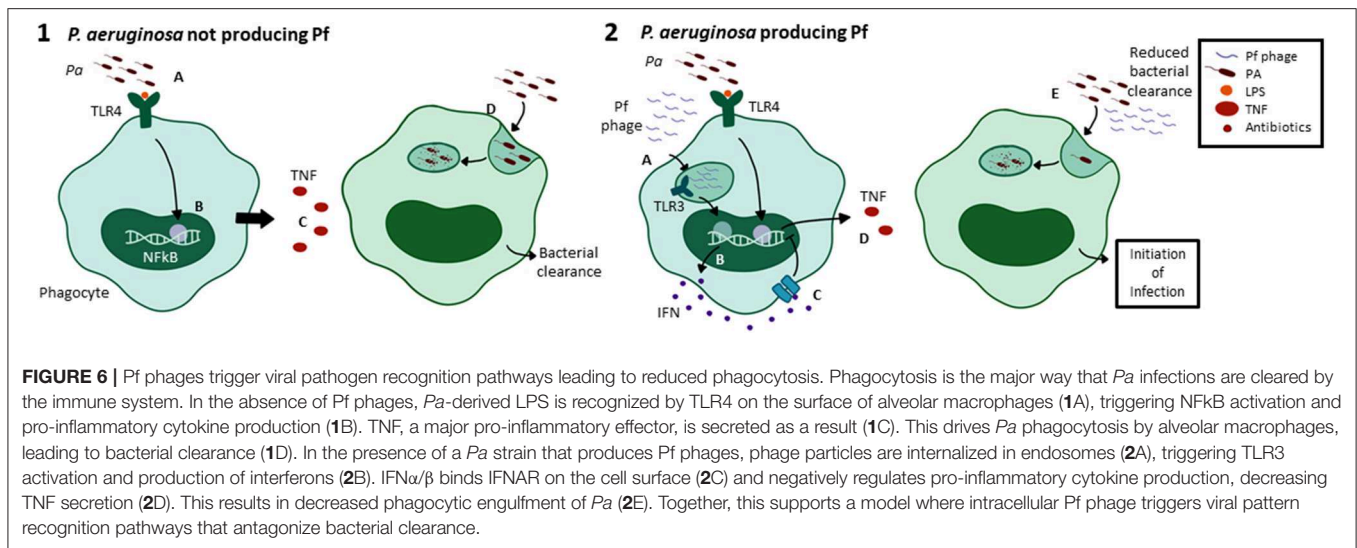
We observed that intracellular Pf phage reduce TNF production by triggering the viral pattern recognition receptor TLR3. In bone-marrow derived macrophages (BMDMs) from TLR2<sup>-/-</sup>, TLR9<sup>-/-</sup>, and MyD88<sup>-/-</sup> mice, Pf phage reduced TNF upon LPS stimulation, but in TRIF<sup>-/-</sup>, TLR3<sup>-/-</sup>, and IFNAR<sup>-/-</sup> BMDMs, Pf phage had no significant effect on TNF production (137).

Pf also impacts the polarization of macrophages in the inflammatory milieu. *Pa* PAO1 superinfected with Pf4 caused decreased expression of genes associated with pro-inflammatory M1 macrophage polarization, including Nos2, IL-12, and IFN- $\gamma$ ; along with increased expression of cytokines associated with anti-inflammatory M2 macrophage polarization, including IL-10, Nos1, and Arg1 (112, 148). Together with the aforementioned effects on cytokine production, these data indicate that internalization of Pf phages triggers anti-viral pattern recognition receptors that antagonize antibacterial immunity (Figure 6).

## The Emerging Picture of Phage/Immune Interactions

While our review here has focused on Pf phage, there is a growing body of literature on phage/immune interactions, primarily in the context of phage therapy. Much of that work is outside of the scope of this review and there are several recent, excellent summaries of that research (149–151). Nonetheless, it is worth





addressing the commonalities and differences of this larger body of work in relation to Pf phages.

The finding that Pf suppresses phagocytosis contrasts with reports that lytic phages promote phagocytosis and interact synergistically with phagocytes (152–156). There are also suggestions that some species of phage may promote the opsonization of bacteria by adhering to the bacterial surface and acting as a target for antibody binding (157). In addition, there are reports that phages may be induced when their bacterial host is phagocytosed, lysing the bacteria and promoting clearance (149, 158). Together, these data suggest that lytic phages may promote opposite effects on bacterial clearance compared to Pf. The mechanistic basis for the differences and similarities seen between lytic and lysogenic phages is unclear, particularly since lytic phages are also internalized by eukaryotic cells (159). However, there are recent examples; enteric Caudovirales phages (tailed phages that package linear dsDNA) initially purified for therapeutic use were found to stimulate increased production of pro-inflammatory cytokines (119). In the context of inflammatory bowel diseases, these phages directly exacerbated disease. While further, direct comparisons are needed, these distinctions could be important for the future prospects of lytic phage therapy.

While the impact on bacterial clearance may differ, the impact of cytokine production and the inflammatory milieu appears to be more similar. Studies with lytic phage have generally reported similar, anti-inflammatory effects *in vitro* (160–162) as well as *in vivo* (163, 164). In particular, lytic phage may also dampen the inflammatory effects of LPS and other bacterial products (165, 166). This stands in contrast to some data implicating other, endogenous eukaryotic viruses in chronic inflammation (167) and, it has been theorized, in protection against pathogenic viral infections (168).

## THE IMPACT OF Pf PHAGE ON *Pa* INFECTIONS IN CF AND OTHER SETTINGS

Recent reports have implicated Pf phage in clinical outcomes associated with *Pa* infections. Here, we discuss this literature and

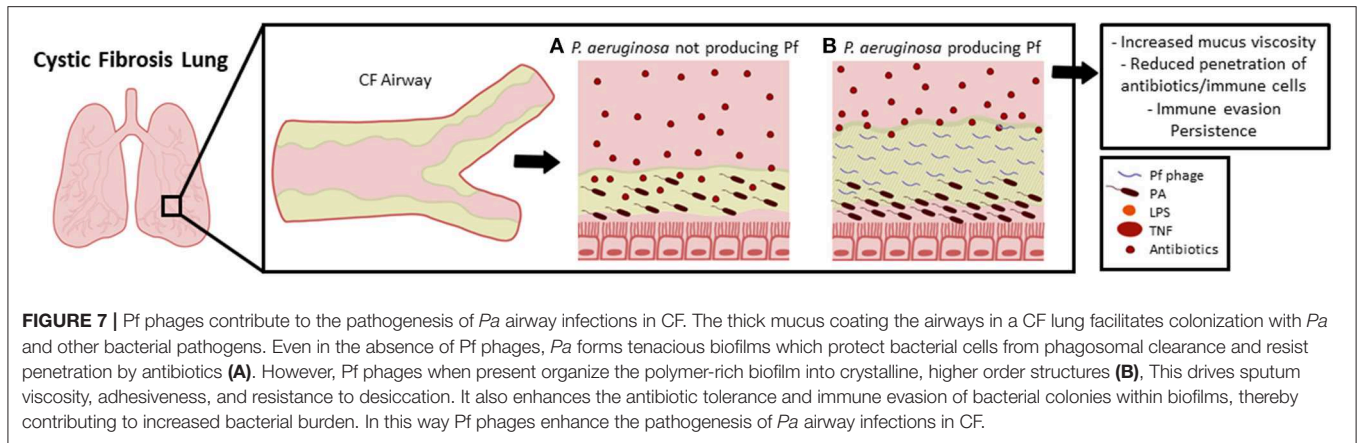
the potential role of Pf phage in CF lung infections. First, we discuss the links between Pf and clinical outcomes in CF. Next, we discuss the development of vaccines that target Pf to prevent infections with *Pa* and the implications of our work on lytic phage therapy. Finally, we address the relevance of our work on filamentous phage to studies on the lung microbiome.

## Pf Phage in CF Airway Infections

CF is a genetic disease where the cystic fibrosis transmembrane conductance regulator (CFTR) gene is mutated. In healthy individuals, the CFTR protein is expressed highly on the apical surface of epithelial cells. In people with CF, CFTR is absent or dysfunctional leading to an imbalance of chloride secretion and sodium absorption which causes dehydration of the airway surface (169–171). Without well-hydrated airway surface liquid, impaired ciliary beating and mucous transport lead to mucus stasis and buildup of viscous mucous in the airways. This in turn sets off a vicious cycle of bacterial infection and neutrophil dominated inflammation, resulting in severe and progressive obstructive pulmonary disease with significant morbidity and mortality (172, 173).

Lung disease in CF begins early in life with recurrent and chronic bacterial infections (172, 174). As the disease progresses, the airways of most patients with CF become colonized by *Pa* (175). In its early stages, *Pa* is intermittently recovered in respiratory cultures, before progressing to a chronic state (176, 177). By adulthood, nearly 60% of CF patients have chronic *Pa* infections (178, 179). Over time, *Pa* becomes a driving force in clinical outcomes in CF lung infections. Chronic endobronchial infection with *Pa* is associated with poor lung function, increased frequency of symptomatic episodes of pulmonary exacerbation, and increased mortality (180–183). While *Pa* eradication antibiotic protocols are now standard of care, success is unfortunately variable and not sustained (184, 185).

We recently identified an association between chronic *Pa* infections in CF patients and Pf phage. In 474 samples collected from a cohort of 34 Danish CF patients infected with *Pa*,



we found that 44.5% were positive for Pf phage (92). In a cross-sectional study of 58 *Pa* positive CF patients followed at the Stanford CF Clinic, 36% were Pf phage positive. In this cohort, detection of Pf phage was associated with older age, advanced lung disease, worse disease exacerbations, and antibiotic resistance (92). As additional evidence for the damaging effects of these altered responses, we also observed a correlation between the presence of Pf in sputum and the activity of the neutrophil enzyme Elastase (186), a known marker of airway destruction and bronchiectasis in CF. Further, we found that immune dysfunction correlates of the presence of Pf with sputum cytokine profiling revealing higher levels of IL12p70 and lower levels of ENA78, RESISTIN and TRAIL (186). These changes suggest disrupted immune function in response to infection (187–192). Broadly speaking, these data are consistent with the enhanced virulence associated with Pf production in a mouse model of *Pa* lung infection and with reports by ourselves and others describing contributions of Pf to chronic infection phenotypes (99, 101, 110, 112).

The effects of Pf on clinical outcomes were also associated with an increased rate of genetically-encoded antibiotic resistance to several anti-pseudomonal antibiotics—particularly aztreonam, amikacin, and meropenem. These same antibiotics were also sequestered by Pf phage *in vitro* whereas ciprofloxacin was not (92) (Figure 7). This raises the intriguing possibility that over time, treatment with these antibiotics favors the acquisition of Pf(+) strains of *Pa*. Consistent with this, we observed that only 1 of 11 pediatric patients in our cohort was Pf(+), whereas 20 of 46 adult patients were (92). Further, 10 of 10 CF patients who underwent lung transplantation were Pf(+) (101). In light of these data, we have proposed that *Pa* strains that produce Pf phage may have a selective advantage and be disproportionately represented in the lungs of older patients with CF. These data also suggest that it may be possible to use the presence of Pf phage to inform antibiotic choices.

These findings in *Pa*-infected CF patients echo the results of our recent investigations into the role of Pf phages in *Pa* chronic wound infections. In a prospective cohort study of 113 patients with wound infections we found that *Pa* was detected in 37

patients and that 25 of these (68%) were positive for Pf phage. Pf(+) strains of *Pa* were more likely than Pf(–) strains to be present in chronic wounds (137).

Together, these data suggest that Pf phages may contribute to clinical outcomes in *Pa* infections in CF as well as in other clinical contexts.

## The Development of Therapies That Target Pf Phages to Prevent *Pa* Infections

An effective vaccine against *Pa* would represent an important component in our armamentarium against this deadly bacterium. To date, there are no FDA approved vaccines for the prevention of *Pa* infections, though several are in various stages of development (193). Most of the vaccine candidates that have been evaluated have been against major components of *Pa* itself, including LPS, exopolysaccharides, extracellular and outer membrane proteins, flagella, or pili (193). Given the prevalence of Pf amongst *Pa* clinical isolates and its contribution to immune evasion and biofilm formation (92, 137), Pf represents an attractive and novel target for immunization against *Pa*.

The ability of antibodies to neutralize bacteriophages has been described and phage neutralizing antibodies have been detected in the sera of several species (151, 194, 195). Studies of sera from patients receiving phage therapy against *Staphylococcus aureus* showed the production of antibodies against the phages in question (196). In a different study, sera collected from healthy individuals contained neutralizing antibodies against naturally occurring bacteriophage (151, 197). On the other hand, other studies did not find evidence of phage targeted antibodies in phage therapy recipients (198). It may be that some phage are more antigenic than others in certain hosts, though the lack of standardized methodologies for these studies may also be a factor underlying these heterogeneous results.

Our recent work demonstrated that immunization against Pf provided protection against *Pa* infections in mice. Immunization was performed using a peptide derived from the CoaB coat protein of Pf phage conjugated to the carrier protein keyhole limpet hemocyanin (KLH). Mice vaccinated with this novel formulation were able to clear *Pa* infection challenges more often than unvaccinated mice. Similar rates of clearance were

obtained in the setting of passive immunization with monoclonal antibodies developed against the CoaB peptide. Further, anti-CoaB monoclonal antibodies (mAB) were effective in promoting opsonization of *Pa* (137). These data suggest that Pf phage is a potential candidate for a preventative vaccine against *Pa*.

Much work remains to be done before such vaccines could become a realistic possibility. In particular, the effectiveness of this strategy against clinical isolates, its specificity for *Pa*, and a detailed mechanism of action in regards to how it promotes bacterial clearance will all be necessary. Nonetheless, the prospect of such a vaccine is enticing. It is tempting to consider that this could be administered to individuals diagnosed with CF before they develop chronic *Pa* infections. Similarly, individuals with diabetes could be immunized before they develop their first *Pa* wound infection.

## The Relevance of Pf Studies for Lytic Phage Therapy

With the rising prevalence of antibiotic-resistant bacteria, novel approaches to deal with bacterial infections are an urgent need (199). Phage therapy is the application of bacteria-specific viruses to combat uncontrolled and undesired bacteria, such as those associated with infectious disease (200). Phage therapy for *Pa* infections has more than 50 years of history but there is a recent resurgence of interest in this approach (201–203). There are several potential advantages of phage therapy that make it an appealing alternative or adjunct to conventional antibiotics. These include the lytic activity of the phages, auto dosing, low inherent toxicity, minimal disruption of normal flora, narrower potential for inducing resistance, lack of cross-resistance with antibiotics, rapid discovery, formulation and application versatility, and effects on biofilm clearance (204). There nonetheless remain many potential issues that need to be addressed, including demonstrations of safety and efficacy in double-blinded, case controlled clinical trials.

One important potential consideration is the nature of the immunological response induced by phages. As briefly summarized above, there are some suggestions that phage-mediated effects on the inflammatory milieu are generally well-tolerated without major side effects [(119, 137, 151, 155, 161, 205)]. However, this remains to be examined in a rigorous and systematic manner, ideally in the context of parallel evaluations of safety and efficacy. A better understanding of the interactions of phage—host—human interactions will provide valuable insight into predicting the outcome of phage therapy (151).

Data on filamentous, lysogenic phage are of unclear relevance for phage therapy studies. As noted above, diverse phage seem to have broadly non-inflammatory effects. However, to our knowledge lytic phage have not been reported to be directly immune suppressive in the way that Pf phages are reported to be. Indeed, there are reasons to think that the effects of lytic vs. temperate phage on local immunity may prove to be quite distinct. Not only are the viruses in question different genetically and phenotypically, but lytic phage are likely to be encountered in the context of bacterial detritus from lysed cells whereas Pf phage may not. It is also unclear to what extent different phage

are taken up intracellularly or trigger viral PRRs. Clearly the comparative effects of different phage is an important question for future research. In the meantime, in the absence of evidence, we favor a cautious approach to extrapolating results from work on Pf phages to other phages.

## Pf Phages in the Context of the Larger Lung Phageome

The virome is increasingly recognized as an integral part of the human microbiome, interacting with other members of the microbial community to shape host phenotypes (206). Bacteriophages make up a majority of this virome (207), but their impact on the larger microbial community within the lung and on lung health remain understudied. New sequencing methods have made metagenomics a more affordable and reliable approach to doing so (208) but substantial technical barriers remain (209, 210).

The lung phageome may be distinct in different disease states. One study suggested that in healthy individuals, the composition of the sputum phageome is highly heterogeneous and may reflect organisms in inhaled air or oral flora. In contrast, the sputum phageome of in CF individuals was highly similar (211, 212). Given recent findings regarding microbial heterogeneity within different lung regions of CF patients (213), it may be important to revisit the question of the lung phageome with more in depth sampling regimens. Nonetheless, this common footprint is remarkable. It may be that selective bacterial growth conditions lead to common, relatively stable microbial community compositions over time (212, 214) with metabolic activities that reflect host characteristics (211, 214, 215). Supporting this idea, Willner et al. found that the viral community in the lungs of a CF patient's non-CF spouse shared taxonomy with the CF virome but had the metabolic profile of a non-CF virome (211).

The contributions of Pf phages to the overall lung microbiome are undefined but potentially substantial. The impact of Pf phages on biofilm formation, antibiotic sequestration, virulence factors, and modulation of immunity described above are likely to influence not only *Pa* phenotypes but also the overall ecology of the lung.

Pf phages can also affect the flow of resources within the biofilm. We reported that Pf phages impact the growth of fungal organisms, specifically *Aspergillus* and *Candida*. As noted above, Pf phages bind to free iron through charge-based interactions, much in the same way that they sequester antibiotics, and this deprives fungi of this critical resource (216, 217). We also reported that Pf phages change the material properties of biofilms, making them far more viscous (101). In addition to increasing the tenacity and thickness of CF sputum, this is also likely to impact the movement and distribution of microbes within the biofilm. It seems probable that we are only scratching the surface of the ways in which Pf phages may influence microbial ecology.

Future studies of the lung microbiome in Pf(+) and Pf(−) subjects are likely to be highly informative. The integrated, simultaneous study of both bacterial and phage communities, including prophages and their induction (218) will be important.

## CONCLUDING REMARKS AND AREAS FOR FUTURE INVESTIGATION

Several themes emerge from this review. The relationship between Pf phages and *Pa* appears to be highly evolved and is clearly an important factor in *Pa* fitness. Despite the substantial energetic cost of producing so many phage particles, Pf phages are nonetheless common among *Pa* strains in multiple contexts, including in the CF lung.

Pf phage also contribute to *Pa* pathogenesis in multiple models, tissues, and human disease contexts. In the context of CF lung infections, Pf phages contribute to the impaired bacterial clearance and heightened inflammation that are characteristic of the disease. It seems likely that Pf contributions to antibiotic tolerance are a major driver in this setting. However, Pf effects on biofilm formation, antibiotic tolerance, microbial ecology, and immune modulation may be important as well.

It may be possible to exploit this biology for therapeutic gain. The anti-Pf vaccine discussed here is the clearest example of this. However, other applications are also possible. For example, the presence of Pf phages may also have potential to inform antibiotic treatment regimens. Manipulating the relationships between bacterial pathogens and their phages may also be an exciting therapeutic frontier.

Much about Pf phage biology remains unknown. While Pf phages are relatively well-characterized compared to most other phages, much remains unknown about their biology. In particular, the function of many Pf phage genes, the factors that govern Pf production, and the impact of Pf phages on *Pa* virulence factors are all promising areas of study that are likely to play important roles in *Pa* pathogenesis.

While the molecular details of the Pf lifecycle are reasonably well-defined, many questions remain regarding how the Pf lifecycle intersects with the functioning of their bacterial host. One obvious gap in our knowledge is the function of the uncharacterized genes in the core Pf genome. Another understudied aspect of Pf biology is their ability to bind not only cationic antimicrobials, but also multivalent cations like iron (75, 216, 217). Iron is a limiting nutrient at infection sites and the ability of Pf phages to bind and sequester this essential nutrient may have important implications in infection pathogenesis. Lastly, the ability of these filamentous phages to assemble liquid crystals in the extracellular environment likely has implications for the host-pathogen interactions.

The evolutionary origins of phage/mammalian host interactions are unclear. Recent work revealed that the (cGAS)-STING pathway, an innate antiviral defense mechanism in animals, has evolutionary roots with bacterial anti-phage defense mechanisms (219). Thus, phages may be triggering ancient highly conserved immune responses in animal cells. It seems unlikely that Pf phage evolved to manipulate mammalian immunity, given that mammals are not a natural host for *Pa*. Instead, it may be that these effects of Pf on animal immune systems enable *Pa* to likewise avoid predation by phagocytic predators, such as amoeba in the environment. Similar effects on

amoeba predation have been hypothesized for other *Pa* factors that impact mammalian phagocytosis, including exotoxins, alginate secretion, and the regulation of motility (136).

The relationship between Pf and *Pa* may also be dynamic and in flux. Bacteria-phage relationships can evolve from parasitic interactions to mutualistic relationships over time with the most extreme examples giving rise to new bacterial functions. Examples include the type VI secretion system (220) and pyocins (221) of *Pa*. The Pf prophage may likewise eventually evolve from an independent virus into a bacterial operon encoding a filamentous structural element of the extracellular biofilm matrix that also has immunomodulatory properties.

Many outstanding questions remain to be addressed in regards to the impact of phage on mammalian immunity. Inoviruses like Pf are widespread amongst bacteria (54) and it is unclear whether the immune effects outlined here for Pf are present in association with other filamentous phage. There are some hints that this may be the case. M13 filamentous phages produced by *E. coli* can be internalized *in vivo* (222) and *in vitro* (223). As mentioned above, a filamentous phage (MDA $\phi$ ) produced by *N. meningitidis* may likewise increase host-cell colonization, bacterial aggregation, and virulence (113). Given the extensive heterogeneity between and even within Inoviruses (54), it seems likely that here again further investigation will reveal substantial differences.

Relatively little is known about adaptive immune responses to Pf and other phages. Most studies suggest that humoral immunity (antibodies) against phage exists and is rapidly induced upon exposure to phage (194, 196, 197, 224, 225); the impact of these on phage therapy is an active area of investigation and may be particularly important for phage therapy (226).

In summary, Pf phages are an exciting frontier in microbiology, immunology, and human health. Further investigations are likely to yield additional insights into both *Pa* pathogenesis as well as novel therapeutic and prophylactic treatments.

## AUTHOR CONTRIBUTIONS

PS, EB, MK, LJ, VR-C, MP, JV, NH, CC, LM, CV, QC, JP, TW, CM, and PB all contributed to the writing, graphics, and editing of this work.

## FUNDING

PS was supported by NIH grants R01AI138981 and K22AI125282. PS and TW were supported by NIH grant P20GM103546. JV and CV were supported by grant T32 AI007502-22. PB was supported by grants R21AI133370, R21AI133240, R01AI12492093, grants from Stanford SPARK, the Falk Medical Research Trust, and the Cystic Fibrosis Foundation (CFF). MP was supported by a NSFGRF grant. EB was supported by grant T32 HL129970 and the Stanford Maternal Child Health Research Institute. CM was supported by grant R01 HL148184 and Ross Mosier CF Laboratories Research Fund.

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