A Filamentous Bacteriophage Protein Inhibits Type IV Pili To Prevent Superinfection of *Pseudomonas aeruginosa*

Amelia K. Schmidt,[a] Alexa D. Fitzpatrick,[b] Caleb M. Schwartzkopf,[a] Dominick R. Faith,[a] Laura K. Jennings,[a] Alison Coluccio,[a] Devin J. Hunt,[a] Lia A. Michaels,[a] Aviv Hargil,[c] Qingquan Chen,[c] Paul L. Bollyky,[c] David W. Dorward,[d] Jenny Wachter,[e] Patricia A. Rosa,[e] Karen L. Maxwell,[b] Patrick R. Secor[a]

aDivision of Biological Sciences, University of Montana, Missoula, Montana, USA
bDepartment of Biochemistry, University of Toronto, Toronto, Ontario, Canada
cDivision of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University, Stanford, California, USA
dResearch Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA
eLaboratory of Bacteriology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

ABSTRACT *Pseudomonas aeruginosa* is an opportunistic pathogen that causes infections in a variety of settings. Many *P. aeruginosa* isolates are infected by filamentous Pf bacteriophage integrated into the bacterial chromosome as a prophage. Pf virions can be produced without lysing *P. aeruginosa*. However, cell lysis can occur during superinfection, which occurs when Pf virions successfully infect a host lysogenized by a Pf prophage. Temperate phages typically encode superinfection exclusion mechanisms to prevent host lysis by virions of the same or similar species. In this study, we sought to elucidate the superinfection exclusion mechanism of Pf phage. Initially, we observed that *P. aeruginosa* that survive Pf superinfection are transiently resistant to Pf-induced plaquing and are deficient in twitching motility, which is mediated by type IV pili (T4P). Pf utilize T4P as a cell surface receptor, suggesting that T4P are suppressed in bacteria that survive superinfection. We tested the hypothesis that a Pf-encoded protein suppresses T4P to mediate superinfection exclusion by expressing Pf proteins in *P. aeruginosa* and measuring plaquing and twitching motility. We found that the Pf protein PA0721, which we termed Pf superinfection exclusion (PfsE), promoted resistance to Pf infection and suppressed twitching motility by binding the T4P protein PilC. Because T4P play key roles in biofilm formation and virulence, the ability of Pf phage to modulate T4P via PfsE has implications in the ability of *P. aeruginosa* to persist at sites of infection.

IMPORTANCE Pf bacteriophage (phage) are filamentous viruses that infect *Pseudomonas aeruginosa* and enhance its virulence potential. Pf virions can lyse and kill *P. aeruginosa* through superinfection, which occurs when an already infected cell is infected by the same or similar phage. Here, we show that a small, highly conserved Pf phage protein (PA0721, PfsE) provides resistance to superinfection by phages that use the type IV pilus as a cell surface receptor. PfsE does this by inhibiting assembly of the type IV pilus via an interaction with PilC. As the type IV pilus plays important roles in virulence, the ability of Pf phage to modulate its assembly has implications for *P. aeruginosa* pathogenesis.

KEYWORDS Pf4, PilC, *Pseudomonas aeruginosa*, filamentous bacteriophage, superinfection exclusion, twitching motility, type IV pili

P seudomonas aeruginosa is an opportunistic pathogen that causes infection in wounds, on medical hardware, and in the airways of people with cystic fibrosis. *P. aeruginosa* itself can be infected by a variety of bacteriophages (phages). For example, many *P. aeruginosa* isolates are infected by temperate filamentous Pf phage, which can

integrate into the bacterial chromosome as a prophage (1, 2). During *P. aeruginosa* growth as a biofilm or at sites of infection, the Pf prophage is induced, and filamentous virions are produced (3–6). Like other filamentous phages, Pf can be extruded from the host cell without killing the host, allowing Pf virions to accumulate to high titers in biofilms (10^{11}/mL) (7) and infected tissues (10^7/gram) (8). However, cell lysis can occur when Pf superinfects *P. aeruginosa*, which occurs when multiple virions infect the same cell or when superinfective Pf variants emerge that contain mutations in the phage *c* repressor gene *Pf4r* (7, 9). Pf4-mediated cell lysis contributes to the maturation and dispersal stages of the *P. aeruginosa* biofilm life cycle (10–13).

Temperate phages typically encode superinfection exclusion mechanisms to stave off infection by competing phages in the environment. A common theme among superinfection exclusion mechanisms are proteins that inhibit or modify phage cell surface receptors such as type IV pili (T4P) (14), a common cell surface receptor for phages, including Pf4 (15). Many *P. aeruginosa* phages encode proteins that inhibit T4P to prevent superinfection (16). Specific examples include the Aqs1 protein encoded by phage DMS3 and the Tip protein encoded by phage D3112, which both inhibit T4P by binding to the T4P ATPase PilB (17, 18). Pf4 uses T4P as a cell surface receptor (15); however, a superinfection exclusion mechanism has not been characterized for the Pf phages that reside in *P. aeruginosa* genomes.

In this study, we show that the smallest protein encoded by Pf4, which we call PfsE (Pf superinfection exclusion), transiently inhibits T4P assembly through an interaction with the T4P platform protein PilC, providing resistance to further infection by T4P-dependent phages. By introducing point mutations to PfsE, we identified two aromatic residues (Y16 and W20) that may be required for PilC binding, T4P inhibition, and resistance to T4P-dependent phages. Furthermore, phage Pf4 engineered to lack the *pfsE* gene is able to kill *P. aeruginosa* more efficiently than the wild-type phage, demonstrating that this mechanism of superinfection reduces *P. aeruginosa* cell lysis. Filamentous inoviruses such as Pf are widespread among bacterial genomes, with even a few examples infecting Archaea (19). Thus, the superinfection exclusion mechanism described here may be relevant to many species of filamentous phage that infect diverse bacterial hosts.

RESULTS

Type IV pili are transiently suppressed in response to Pf4 superinfection. While working with phage Pf4 we noticed an interesting phenomenon where the surviving cells in a culture of PAO1 superinfected with Pf4 showed a decrease in twitching motility (Fig. 1A). As Pf4 uses T4P as a cell surface receptor (15), we tested the ability of these nontwitching cells to mediate resistance to Pf4-induced plaquing. We found that these cells were highly resistant to lysis by Pf4 virions (Fig. 1B) compared to uninfected PAO1, which retain the ability to twitch (Fig. 1C) and are sensitive to Pf4 superinfection (Fig. 1D). The resistance observed was similar to that seen for a PAO1 Δ*pilA* mutant, which completely lacks pilus on the cell surface (Fig. 1E and F). To determine if Pf4 superinfection selected for T4P-null mutants or transiently suppressed T4P expression, bacteria collected from Pf4-resistant lawns were subcultured in phage-free growth medium for 18 h, and then their ability to twitch and sensitivity to Pf4-induced plaquing was tested. Twitching motility and sensitivity to Pf4 superinfection was restored in subcultured bacteria (Fig. 1G and H), indicating that heritable mutations in T4P genes were not responsible for the twitch-deficient and Pf4-resistance phenotypes.

PfsE suppresses twitching motility and protects *P. aeruginosa* from Pf4 superinfection. Many temperate phages possess superinfection exclusion mechanisms that prevent re-infection of an already infected cell (20). We hypothesized that a Pf4-encoded protein would suppress T4P as a mechanism to prevent Pf4 superinfection and lysis of the host cell. To test this hypothesis, we first deleted the Pf4 prophage from our in-house PAO1 strain (PAO1$^{\Delta Pf4}$). Pf4 proteins encoded by *PA0717-PA0728* in the core Pf4 genome (Fig. 2A) were then expressed individually from a plasmid in PAO1$^{\Delta Pf4}$, and twitching motility and sensitivity to Pf4-mediated lysis were then assessed. We identified
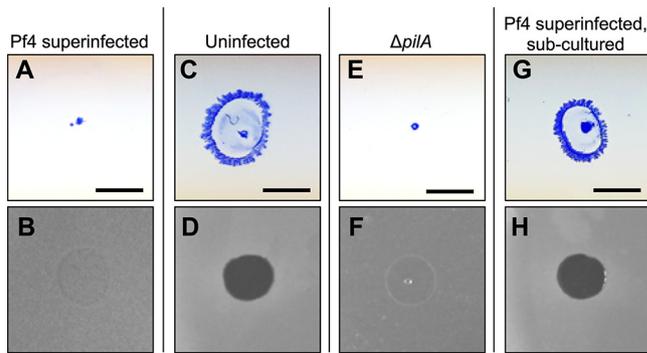
mBio®

FIG 1 Pf4 superinfection transiently suppresses twitching motility and promotes resistance to Pf4-induced plaquing. Twitch assays were performed by stabbing the indicated strain through the agar on a petri dish to the plastic surface below. After 24 h, the agar was removed and bacteria on the plastic dish were stained with Coomassie (upper panels A, C, E, and G). To measure sensitivity of *P. aeruginosa* to Pf4 superinfection, lawns of the indicated strains were spotted with 10^6 PFU of Pf4 in 3 μL (lower panels B, D, F, and H). Strains tested include (A and B) PAO1 superinfected with Pf4, (C and D) uninfected PAO1, (E and F) the twitch-deficient type IV pili mutant Δ*pilA*, and (G and H) Pf4 superinfected PAO1 that was subcultured in phage-free broth and replated. Scale bar 5 mm.

two proteins, PA0721 and PA0724, that suppressed twitching motility when overexpressed (Fig. 2B) and promoted resistance to Pf4 plaquing (Fig. 2C). PA0721 is a small 30 residue uncharacterized protein, and PA0724 is the Pf4 minor coat protein CoaA, which is involved in receptor binding during the initial steps of infection (2). These results were
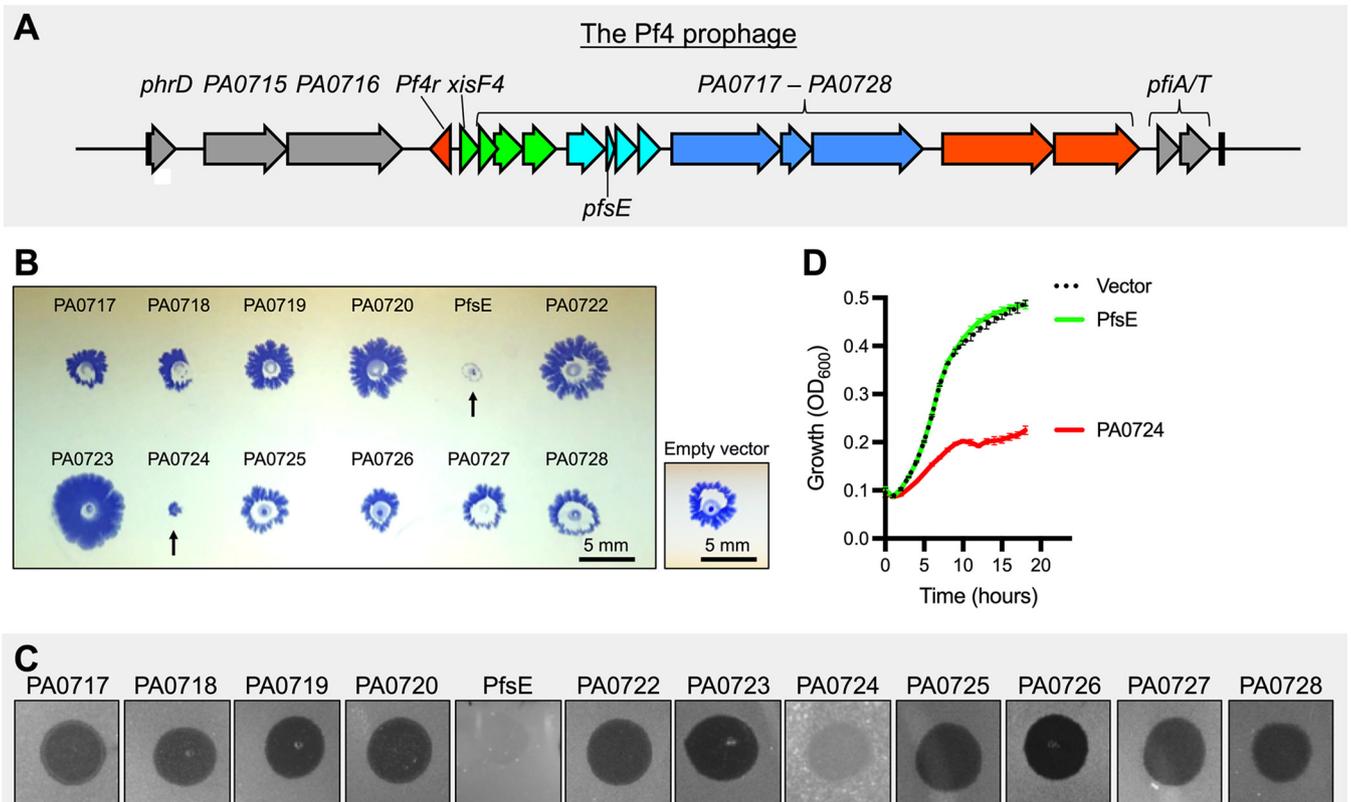

FIG 2 PfsE suppresses twitching motility and protects *P. aeruginosa* from Pf4 superinfection. (A) The Pf4 prophage is composed of core genes that are essential for the phage to complete its life cycle (*Pf4r* to *PA0728*) and flanking moron regions (gray) that add "more on" to the core genome (54). (B) Genes *PA0717–PA0728* in the core Pf4 genome were placed under the control of an arabinose-inducible promoter and expressed individually in *P. aeruginosa* PAO1^ΔPf4. Twitching was measured in the indicated strains after 24 h; arrows indicate strains with reduced twitching motility. Bar 5 mm. (C) 10^6 PFU of Pf4 were spotted onto lawns of PAO1^ΔPf4 expressing the indicated phage protein. (D) Growth curves in liquid culture for PAO1^ΔPf4 bacteria carrying the indicated expression vector. Cultures were grown in LB supplemented with 0.1% arabinose. Results are the mean ± SEM of three experiments.
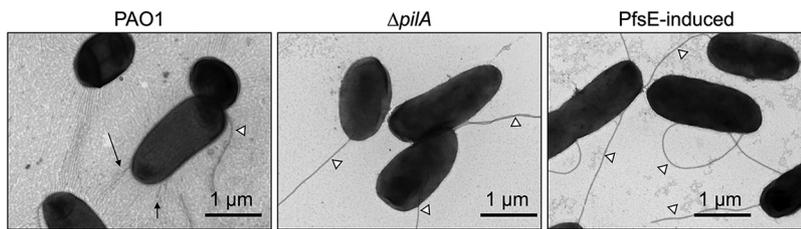
FIG 3 Type IV pili are not apparent on cells expressing PfsE. Transmission electron microscopy of the indicated strains of *P. aeruginosa* was performed. Midlogarithmic cells were washed, fixed, placed on a grid, and negatively stained with uranyl acetate. Arrows indicate potential pili on PAO1 cells. White triangles indicate flagella.

consistent with a previous study that found these proteins promote resistance to T4P-dependent long-tailed dsDNA phages DMS3m and JBD30 (19).

To determine if the observed twitching inhibition and phage resistance was a direct result of the biological function of these proteins or was due to toxicity of the overexpressed proteins, we examined the growth rates of cells expressing these proteins. We found that PAO1$^{\Delta Pf4}$ expressing PA0724 grew poorly compared to cells expressing PA0721 or PAO1$^{\Delta Pf4}$ carrying an empty expression vector (Fig. 2D). These observations suggest that PA0724 expression is toxic to *P. aeruginosa*, and it is possible that the twitch-deficient and phage resistance phenotypes associated with PA0724 expression are a result of this toxicity rather than a specific superinfection exclusion mechanism. Therefore, we turned our attention toward characterizing PA0721, which we refer to herein as PfsE (Pf superinfection exclusion).

T4P are not apparent on cells expressing PfsE. Twitching motility requires bacteria to extend their pili outwards from the cell surface and then retract them to move along a solid surface. Thus, PfsE could inhibit twitching motility by preventing either pilus retraction or extension. If PfsE inhibits T4P retraction, cells are anticipated to have a piliated or hyperpiliated morphology. If PfsE inhibits T4P extension, then cells are expected to have few or no pili. To determine if PfsE interferes with extension or retraction, we used transmission electron microscopy to look for the presence of pili on the cell surface. We found that cells expressing PfsE showed no visible pili on the surface (Fig. 3), while wild-type PAO1 cells had structures consistent with T4P (Fig. 3). The lack of pili observed on the cells expressing PsfE was similar to a PAO1 Δ*pilA* mutant (Fig. 3), which is known to completely lack surface piliation (21). These data suggest that PfsE inhibits T4P extension rather than retraction.

PfsE protects *P. aeruginosa* from other T4P-dependent phage species. Many phages use the T4P as a cell surface receptor to infect bacteria (14). We hypothesized that the transient T4P suppression by PfsE that protected against Pf4 superinfection would also protect *P. aeruginosa* from nonfilamentous phages. To test this hypothesis, we examined the ability of phage JBD26, a temperate long-tailed dsDNA phage that uses the pilus as a cell surface receptor, to form plaques on lawns of PAO1, PAO1 Δ*pilA*, PAO1 superinfected with Pf4, PAO1 superinfected with Pf4 and then subcultured in phage-free media, or PAO1 expressing PfsE from a plasmid. Like Pf4, JBD26 was not able to infect cells that were superinfected by Pf4 or cells expressing PfsE (Fig. 4). We also tested the ability of phage CMS1, which does not depend on the pilus for infection, to form plaques on these strains. As expected, the plaquing ability of these phages was not affected by the absence of T4P (Δ*pilA*), expression of PsfE, or superinfection by Pf4 (Fig. 4). Furthermore, Pf4 superinfection and expression of PfsE were able to prevent infection by phage OMKO, a lytic pili-dependent phage, but not LPS-5, another pili-independent phage species (Fig. S1 in the supplemental material). These results demonstrate that PfsE protects *P. aeruginosa* from T4P-dependent phage species.

We also tested the ability of PfsE to inhibit flagellum-dependent swimming motility. PAO1$^{\Delta Pf4}$ expressing wild-type PfsE or PfsE point mutants did not affect swimming motility compared to PAO1$^{\Delta Pf4}$ carrying an empty expression vector (Fig. S2), indicating that PfsE does not affect flagellum-dependent swimming motility, which is consistent
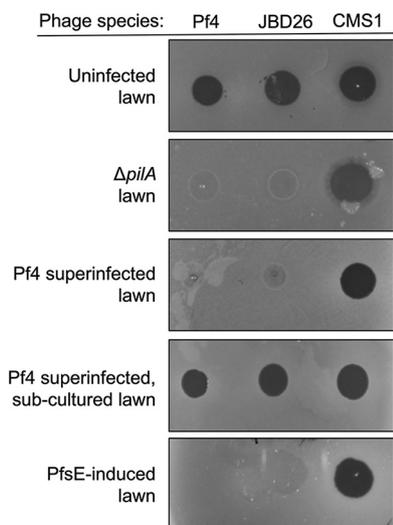
FIG 4 Pf4 superinfection and expression of PfsE promotes resistance to type IV pili (T4P)-dependent bacteriophages. Representative images of *P. aeruginosa* PAO1 lawns spotted with 10^6 PFU of Pf4, JBD26 (both T4P-dependent phages), or CMS1 (a T4P-independent phage). See also Fig. S1.

with our previous observation that Pf4 superinfection does not affect swimming motility in *P. aeruginosa* PAO1 (5).

Deletion of *pfsE* increases Pf4 virulence against *P. aeruginosa*. To definitively show that PsfE expression from Pf phage provides resistance to superinfection, we attempted to delete the *pfsE* gene from Pf4. All attempts to delete *pfsE* from the Pf4 prophage integrated into the PAO1 chromosome failed. We hypothesized that inactivating *pfsE* resulted in unregulated replication of Pf4, killing *pfsE* mutants, similar to how Pf4 kills *P. aeruginosa* PAO1 when the global repressors *mvaT* and MvaU are both disabled (15). To test this hypothesis, we attempted to delete *pfsE* from the PAO1 Δ*intF4* background. IntF4 (PA0728) is a Pf4-encoded site-specific tyrosine recombinase that catalyzes Pf4 prophage integration and excision (8, 9). In Δ*intF4*, the Pf4 prophage is trapped in the chromosome, preventing infectious virions from being produced (8, 15). We were successful in deleting *pfsE* from Δ*intF4*, creating the double mutant Δ*intF4*/*pfsE*, suggesting that when *pfsE* is inactivated, Pf4 replication kills *P. aeruginosa*.

We hypothesized that Δ*intF4*/*pfsE* Pf4 virions that lack the *pfsE* gene would not be able to regulate superinfection of the host bacterium, increasing host cell lysis. To test this, we induced and collected Pf4 virions from wild-type, Δ*intF4*, and Δ*intF4*/*pfsE* *P. aeruginosa*. To induce Pf4 virions from these strains, the Pf4 excisionase XisF4 was expressed in *trans* from a plasmid under the control of an arabinose-inducible promoter (9). To complement the Δ*intF4* mutation, IntF4 was also expressed from a plasmid in all strains tested. After overnight growth (18 h) in lysogeny broth (LB) supplemented with 0.1% arabinose, bacterial supernatants were filtered and DNase-treated, and Pf4 titers measured by qPCR, as previously described (22). Phage titers in each supernatant (wild type, Δ*intF4*, and Δ*intF4*/*pfsE*) were normalized to 6.95×10^7 virions per mL and were plated on a lawn of PAO1$^{\Delta Pf4}$ to determine how infective the mutant phages were. While the Δ*intF4* mutant phage titer was equal to wild-type Pf4, the Δ*intF4*/*pfsE* mutant phage was approximately 1,000-fold more infective (Fig. 5), indicating that *pfsE* restricts Pf4 infection and thereby protects the bacterial host from lysis.

PfsE binds to the T4P inner-membrane protein PilC. The T4P complex consists of four subcomplexes: the outer membrane complex, an alignment complex that spans the periplasm, and two inner membrane complexes (23). Because PfsE is predicted to localize in the inner membrane (24), we hypothesized that PfsE would interact with inner membrane proteins of the T4P complex or proteins that interact with the T4P inner membrane complex. To test this hypothesis, we used a bacterial adenylate cyclase two-hybrid (BACTH) assay (25) to detect interactions between PfsE and the T4P
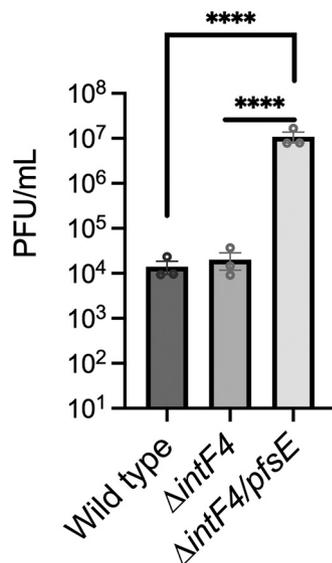
FIG 5 Pf4 virions not encoding *pfsE* are more virulent against *P. aeruginosa*. Wild-type and mutant Pf4 virions were induced by expressing both the Pf4 excisionase (*xisF4*) and integrase (*intF4*) in *trans* in wild-type, Δ*intF4*, or Δ*intF4/pfsE* backgrounds. Note that deletion of *pfsE* alone from the Pf4 prophage was not possible. Wild-type, Δ*intF4*, and Δ*intF4/pfsE* Pf4 virion titers were measured by qPCR and normalized to 6.95×10^7 virions per mL. Virions were then spotted as a 10× dilution series on lawns of ΔPf4 to enumerate PFU. Results are mean ± SEM of three experiments, unpaired Student's *t* test, ****, $P < 0.0001$.

proteins PilA, PilB, PilC, PilM, PilN, PilT, PilU, or PilW. In the BACTH assay, interactions between bait (PfsE) and prey (pilus proteins) are detected by β-galactosidase activity. High levels of β-galactosidase activity were observed only when PfsE was expressed with PilC (Fig. 6F), an inner membrane protein essential for T4P pilus biogenesis (26). Similar activity was observed when PilC was used as bait and PfsE as prey (Fig. 6F).

In an attempt to map residues that are important for the PsfE/PilC interaction, we examined the PfsE protein sequence and identified a conserved cluster of aromatic residues, YAWGW (Fig. 6A and B). As clusters of aromatic residues often facilitate protein-protein binding interactions (27, 28), we hypothesized that these residues may be required for suppression of twitching motility and resistance to T4P-dependent phages. To test this hypothesis, we introduced into PfsE the following point mutations: PfsE^{Y16V}, PfsE^{W18A}, PfsE^{W20A}, and PfsE$^{Y16V/W18A/W20A}$. These variant proteins were expressed in PAO1$^{\Delta Pf4}$, and twitching motility and phage resistance were measured. PfsE^{Y16V}, PfsE^{W20A}, and PfsE$^{Y16V/W18A/W20A}$ all lost the ability to suppress twitching motility (Fig. 6C) and did not promote resistance to the T4P-dependent phages Pf4 or JBD26 (Fig. 6D). PfsE^{W18A} partially suppressed both twitch motility and infection by T4P-dependent phages. When the PfsE^{Y16V} or PfsE^{W20A} point mutants were used as bait with PilC as the prey, β-galactosidase activity was not detected. The PfsE^{W18A} mutant showed some β-galactosidase activity in the BACTH assay, consistent with its intermediate twitch and phage resistance phenotypes. To determine if the loss of twitching and phage resistance was due to loss of the protein interaction or to instability of the protein, we used a Western blot to examine the steady-state levels of 6×-His-tagged wild-type PfsE, PfsE^{Y16V}, and PfsE^{W20A} in *E. coli*. While we were able to detect wild-type PfsE using an anti-His antibody, we were unable to detect the PfsE^{Y16V} or PfsE^{W20A} point mutants (Fig. S3), suggesting that the loss of twitching motility, phage resistance, and the PilC interaction were due to low protein levels in the cell. These data suggest that Y16 and W20 play a role in stabilizing the PfsE protein fold or promoting its proper insertion into the membrane.

The binding of PfsE to the inner membrane protein PilC is consistent with the prediction that PfsE is itself an inner membrane protein. To determine the orientation that
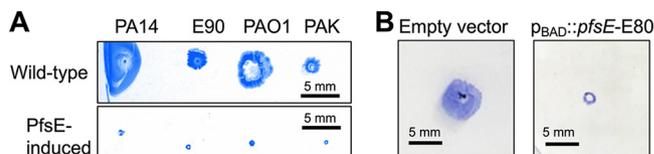
FIG 6 Conserved aromatic residues in PfsE are essential for the inhibition of type IV pili and resistance against Pf4 superinfection. (A) WebLogo (55) was used to construct a protein sequence logo for 312 PfsE sequences from Pf prophages infecting *P. aeruginosa* strains in the *Pseudomonas* genome database (24). Note that aromatic residues in the YXWGW motif (residues 16–20) are conserved (arrows). (B) There is 66% sequence identity (20/30) between two of the most highly diverged PfsE homologues found in Pf prophages residing in the *P. aeruginosa* PAO1 or E80 chromosomes. (C) Twitch motility was measured in PAO1$^{\Delta Pf4}$ expressing wild-type or modified PfsE. Results are mean ± SEM of 16 experiments, unpaired Student's *t* test, **, $P < 0.01$, ****, $P < 0.0001$. (D) Lawns of PAO1$^{\Delta Pf4}$ carrying the indicated expression vectors were induced with 0.01% arabinose and spotted with 3 μL of 10^9 PFU/mL stocks of Pf4, JBD26 (pili-dependent phages), or CMS1 (a pili-independent phage). (E) AlphaFold (52) was used to predict the structure of PfsE. (F) A bacterial adenylate cyclase two-hybrid (BACTH) assay (25) was used to detect interactions between PfsE and the indicated T4P proteins. Interactions between bait and prey proteins are detected by β-galactosidase activity, as indicated by the production of blue pigment. Representative colonies are shown. (G) A 6×-His tag was added to the N- or C-terminus of PfsE, expressed in PAO1$^{\Delta Pf4}$, and twitching was measured. Scale bar 5 mm.

PfsE inserts itself into the inner membrane, we tagged the N- or C-terminus of PfsE with a poly-histidine (His) tag, which is positively charged and unable to insert into lipid membranes. Tagged PfsE was expressed in PAO1$^{\Delta Pf4}$ and twitching motility measured. The N-terminal His tag had no impact on the ability of PfsE to inhibit twitching, while the C-terminal His tag prevented PfsE-mediated twitching inhibition (Fig. 6G). These results suggest that PfsE inserts into the inner membrane with the N-terminus oriented toward the cytoplasm.

As PilC is highly conserved across many different strains of *P. aeruginosa* (26), we tested the ability of PfsE to inhibit twitching motility in *P. aeruginosa* strains PA14, PAK, and E90. We found that the PfsE sequence from Pf4 inhibited twitching in each of these strains (Fig. 7A). When the divergent PfsE sequence from *P. aeruginosa* strain E80 (see Fig. 6B) was expressed in *P. aeruginosa* PAO1$^{\Delta Pf4}$, twitching motility was similarly inhibited (Fig. 7B). These results indicate a conserved mechanism of action.

DISCUSSION

Many *P. aeruginosa* isolates are Pf lysogens (i.e., they harbor one or more Pf prophages in their chromosome) (1, 2). Lysogenized bacteria defend against infection by the same or similar phage through a mechanism called superinfection exclusion. For example, previously characterized superinfection exclusion mechanisms employed by *P. aeruginosa* phages include proteins Aqs1 from phage DMS3 (17) and Tip from phage

FIG 7 Divergent PfsE sequences inhibit twitching motility in various *P. aeruginosa* strains. (A) PfsE was expressed in *trans* in the indicated strains of *P. aeruginosa* and twitching motility measured after 24 h. Representative images are shown. (B) Twitching motility was assessed in PAO1$^{\Delta Pf4}$ carrying either the empty vector pHERD20T or p$_{BAD}$-*pfsE*-E80 by standard twitch assay. Representative images are shown.

D3112 (18), both of which inhibit T4P by binding to the T4P assembly ATPase PilB, which energizes pilus extension (26). This is the first report, to our knowledge, of a phage protein binding PilC to suppress T4P and prevent superinfection. Our data support a model where the Pf-encoded protein PfsE mediates superinfection exclusion by transiently suppressing T4P by binding to PilC to prevent pilus extension, which inhibits twitching motility and prevents infection by T4P-dependent phages. PfsE interactions with PilC may inhibit T4P either by blocking PilC from rotating, PilA loading, or by interfering with interactions between PilC and other pili proteins such as PilB.

To our knowledge, a transient superinfection exclusion phenotype has not previously been described for a phage. However, transient superinfection exclusion has been observed in animal viruses such as the RNA Pestivirus that causes bovine viral diarrhea (29). The transient nature of PfsE-mediated T4P inhibition may be related to highly stable PfsE-PilC binding interactions. If true, then if PfsE is downregulated as Pf re-enters the lysogenic replication life cycle, tight binding interactions between PfsE and PilC may titrate out PfsE as the cells divide, restoring T4P function. Alternatively, the conserved acidic residue D22 (see Fig. 6A) may pull the C-terminus of PfsE into the periplasmic space where PfsE could interact with periplasmic proteins that could disrupt PfsE-PilC binding (e.g., a periplasmic protease could degrade PfsE).

Our data suggest that PfsE inhibits pili extension. By inhibiting extension of the T4P cell surface receptor, PfsE may reduce the number of Pf virions that are "wasted" on nonproductive infections of an already infected host. This would allow Pf virions to accumulate in the environment, allowing Pf phage to spread and infect naive *P. aeruginosa* hosts. *P. aeruginosa* also benefits from the accumulation of filamentous Pf virions in the environment; as Pf virions accumulate in polymer-rich environments such as the biofilm matrix or host secretions (e.g., mucus), they spontaneously align, creating a large liquid crystalline lattice that protects *P. aeruginosa* from desiccation and some antibiotics (8, 30, 31). When encountered by immune cells, Pf virions induce a type I interferon antiviral response, which reduces the phagocytic uptake of bacteria by macrophages (4). Collectively, these phenotypes help explain why in *P. aeruginosa* PAO1 deleting the Pf4 prophage from the chromosome reduces bacterial virulence in murine lung (10) and wound (4) infection models.

PfsE inhibition of T4P may affect other bacterial behaviors. For example, in *P. aeruginosa*, T4P play important roles in virulence and biofilm formation (32, 33). T4P are critical virulence determinants in *P. aeruginosa* (33), and inhibition of T4P by PfsE could affect *P. aeruginosa* virulence potential. This possibility is consistent with our previous work demonstrating that Pf4 superinfection promotes a noninvasive infection phenotype *in vivo* (5). Under some conditions such as nutrient limitation, suppression of T4P is thought to contribute to biofilm dispersion (34). Pf4 superinfection contributes to the biofilm life cycle as well by inducing cell death and lysis, which produces the characteristic voids in the center of mature microcolonies of biofilms grown in flow cells (10–12). In dispersed cell populations, Pf4 gene expression is upregulated while T4P genes are downregulated (34, 35). Thus, it is possible that in response to Pf4 superinfection, PfsE expression contributes to biofilm dispersal by suppressing T4P.

Phage therapy holds great potential in combating multidrug-resistant bacterial

infections in several settings (36–41). Unfortunately, bacteria can develop resistance to therapeutic phages, causing treatment failure ((42) and references therein). In some cases, heritable phage resistance mutations cannot account for phage therapy failure as bacteria remain sensitive to phage infection *ex vivo* (42–45). Because Pf prophages are prevalent among *P. aeruginosa* clinical isolates and PfsE is encoded by all Pf lysogens, it is possible that PfsE could cause some phage therapies to fail. Conversely, PfsE could be leveraged as a therapeutic. Recent work demonstrates that the Tip protein from phage D3112 inhibits T4P by blocking the activity of PilB (18). A peptide mimic of the Tip protein inhibits T4P *in vitro*, and when given topically to *P. aeruginosa*, the peptide reduced virulence in a *Drosophila* infection model (46). This approach could potentially be adapted to PfsE by synthesizing a peptide that contains the aromatic amino acid motif YAWGW.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains, plasmids, and their sources are listed in Table 1, and primers are listed in Table 2. Unless indicated otherwise, bacteria were grown in lysogeny broth (LB) at 37°C with shaking and supplemented with antibiotics (Sigma) or 0.1% IPTG when appropriate. Unless otherwise noted, antibiotics were used at the following concentrations: gentamicin (10 or 30 μg mL^{-1}), ampicillin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}), carbenicillin (50 μg mL^{-1}).

Construction of strain ΔPf4. The Pf4 prophage was deleted from the PAO1 chromosome by allelic exchange (47), producing a clean and unmarked ΔPf4 deletion with the Pf4 att site intact. All primers used for strain construction are given in Table 2. The Pf4 prophage contains a toxin-antitoxin (TA) pair (48). The presence of the Pf4-encoded PfiTA system likely explains the low efficiency at which the Pf4 prophage can be deleted from the PAO1 chromosome (10); deletion of the Pf4 prophage results in loss of the antitoxin gene *pfiA*, and cells without the antitoxin are killed by the longer-lived toxin PfiT (48). Thus, the *pfiT* toxin gene was first deleted from PAO1 by allelic exchange (47). Briefly, the upstream region of *pfiT* (*pfiT'*) and the downstream region of *pfiT* (*'pfiT*) were amplified using the primer pairs attB1-pfiT-UpF/pfiT-UpR and PfiT-DownF/attB2-PfiT-DownR, respectively (Table 2). These were then assembled using SOE-PCR. The resulting fragment was cloned into pENTRpEX18-Gm, transformed into *Escherichia coli* S17λpir, and subsequently mobilized into *P. aeruginosa* PAO1 via biparental mating. Merodiploid *P. aeruginosa* was selected on Vogel-Bonner minimal medium (VBMM) agar containing 60 μg ml^{-1} gentamicin, followed by recovery of deletion mutants on no-salt LB (NSLB) medium containing 10% sucrose. Candidate mutants were confirmed by PCR and sequencing using primer pair PfiT seq F/PfiT seq R. The remaining Pf4 prophage was then deleted from Δ*pfiT* using the same allelic exchange strategy described for Δ*pfiT* using primers Pf4-UpF-GWL, Pf4-UpR-GM, Pf4-DnF-GM, and Pf4-DnR-GWR (Table 2), producing an unmarked clean deletion of Pf4 with an intact att site. Candidate mutants were confirmed by PCR using primer pair pf4-out F/pf4-out R and sequence confirmed. Supernatants collected from overnight cultures of this ΔPf4 strain did not produce detectable plaques on lawns of PAO1 or ΔPf4, and the ΔPf4 genotype was routinely PCR confirmed prior to experiments using this strain to avoid reinfection by exogenous Pf4 virions in the laboratory environment.

Phage expression constructs. The indicated Pf4 genes or *pfsE* point mutant genes were cloned into the arabinose-inducible expression constructs pHERD20T or pHERD30T (49), were obtained from reference (19), or were made by Genewiz (Table 1). Final constructs were all sequence verified.

Twitch motility assays. Twitching motility was assessed by stab inoculating the indicated strains through a 1.5% LB agar plate to the underlying plastic dish. Agar was supplemented with 0.1% arabinose or antibiotics when appropriate. After incubation for 24 h, the agar was carefully removed, and the zone of motility on the plastic dish was visualized and measured after staining with 0.05% Coomassie brilliant blue, as previously described (50). Twitch zones were measured by placing the plastic dish onto a ruler and imaging with a Bio-Rad GelDoc GO imaging system using preset parameters for Coomassie-stained gels.

Plaque assays. Plaque assays were performed using ΔPf4 or isogenic PAO1 as indicator strains grown on LB plates. Phages in filtered supernatants were serially diluted 10× in PBS and spotted onto lawns of the indicated indicator strain. Plaques were imaged after 18 h of growth at 37°C.

Pf4 phage virion quantitation by qPCR. Pf4 virion copy number was measured using qPCR as previously described (22). Briefly, filtered supernatants were treated with DNase I (10 μL of a 10 mg/mL stock per mL supernatant) followed by incubation at 70°C for 10 min to inactivate the DNase. Ten μL reaction volumes contained 5 μL SYBR Select Master Mix (Life Technologies, Grand Island, NY), 100 nM primer attR-F and attL-R (Table 2), and 2 μL supernatant. Primers attR-F and attL-R amplify the recircularization sequence of the Pf4 replicative form and thus do not amplify linear Pf4 prophage sequences that may be present in contaminating chromosomal DNA. Cycling conditions were as follows: 50°C 2 min, 95°C 2 min, (95°C, 15 sec, 60°C 1 min) × 40 cycles. A standard curve was constructed using plasmids containing the template sequence at a known copy number per milliliter. Pf4 copy numbers were then calculated by fitting Ct values of the unknown samples to the standard curve.

Pf4 virion induction. *P. aeruginosa* strains PAO1, Δ*intF4*, and Δ*intF4/pfsE* were made competent by 300 mM sucrose washes (51) and electroporated with the arabinose-inducible expression vectors pHERD20T-*xisF4* and pHERD30T-*intF4*. Double transformants were grown in LB supplemented with

TABLE 1 Bacterial strains, phage, and plasmids used in this study

Strain	Description	Source
Escherichia coli		
DH5α	New England Biolabs	(15)
S17	λpir-positive strain	(15)
BTH101	BACTH reporter strain	(25)
P. aeruginosa		
PAO1	Wild type	(56)
PAO1 $\Delta pilA$	Clean deletion of *pilA* from PAO1	(57)
PAO1 $\Delta intF4$	Clean deletion of *intF4* from PAO1	(8)
PAO1 $\Delta intF4/pfsE$	Clean deletion of *pfsE* from PAO1 $\Delta intF4$	This study
PAO1 ΔPf4	Clean deletion of the Pf4 prophage from PAO1	This study
PA14	Wild type	(58)
PAK	Wild type	ATCC 25102
E90	Clinical CF *P. aeruginosa* isolate	(59)
Bacteriophage strains		
Pf4	Inoviridae	(8)
JBD26	Siphoviridae	(20)
CMS1	Podoviridae	This study
DMS3	Siphoviridae	(60)
OMK01	Myoviridae	(61)
LPS-5	Podoviridae	Felix Biotech
Plasmids		
pHERD20T	AmpR, expression vector with araC-P$_{BAD}$ promoter	(49)
pHERD30T	GmR, expression vector with araC-P$_{BAD}$ promoter	(49)
pHERD30T-*PA0717*	pBAD::*PA0717*	(19)
pHERD30T-*PA0718*	pBAD::*PA0718*	(19)
pHERD30T-*PA0719*	pBAD::*PA0719*	This study
pHERD30T-*PA0720*	pBAD::*PA0720*	(19)
pHERD30T-*pfsE*	pBAD::*pfsE*	(19)
pHERD30T-*PA0722*	pBAD::*PA0722*	This study
pHERD30T-*PA0723*	pBAD::*PA0723*	This study
pHERD30T-*PA0724*	pBAD::*PA0724*	This study
pHERD30T-*PA0725*	pBAD::*PA0725*	(19)
pHERD30T-*PA0726*	pBAD::*PA0726*	This study
pHERD30T-*PA0727*	pBAD::*PA0727*	This study
pHERD30T-*intF4*	pBAD::*intF4*	This study
pHERD20T-*xisF4*	pBAD::*xisF4*	(9)
pKT25	BACTH construct	(25)
pUT18C	BACTH construct	(25)
pHERD20T-*pfsE*	pBAD::*pfsE*	This study
pHERD20T-*pfsE*[Y16V]	pBAD:: *pfsE*[Y16V]	This study
pHERD20T-*pfsE*[W18A]	pBAD:: *pfsE*[W18A]	This study
pHERD20T-*pfsE*[W20A]	pBAD:: *pfsE*[W20A]	This study
pHERD20T-*pfsE*[Y16A/W18A/W20A]	pBAD:: *pfsE*[Y16A/W18A/W20A]	This study

gentamicin and carbenicillin to an OD$_{600}$ of 0.3 and induced with 0.1% arabinose. Bacteria were grown for 18 h, pelleted by centrifugation, and supernatants were filtered through a 0.22 μm filter (Millipore Millex GP) followed by DNase treatment. Pf4 virion titers were measured by qPCR, as described above. Pf4 copy numbers in each supernatant were normalized to the same titer by diluting with PBS.

Growth curves. Overnight cultures were diluted to an OD$_{600}$ of 0.05 in 96-well plates containing LB and, if necessary, the appropriate antibiotics. Over the course of 24 h, OD$_{600}$ was measured in a CLARIOstar (BMG Labtech) plate reader at 37C with shaking prior to each measurement.

Transmission electron microscopy. Cells were grown to midlog (OD$_{600}$ 0.4), washed with PBS, fixed with 4% formamide, and placed on a grid and negatively stained with uranyl acetate. Cells were imaged on a Hitachi H-7800 120 kV TEM.

Bacterial two-hybrid assays. Bacterial two-hybrid assays were performed as described previously (25). PfsE was cloned into plasmid constructs (pKT25, pUT18C) using relevant primers (Table 2). *Escherichia coli* BTH101 cells were cotransformed with plasmid constructs containing different sets of genes. Three independent colonies were grown overnight at 30°C in LB media containing the appropriate selection, 2 μL of each was plated onto X-gal, and MacConkey agar plates containing the appropriate selection and 1 mM IPTG and incubated at 30°C for 48 h. A color change on both plates indicates an interaction between the genes encoded in the plasmids.

TABLE 2 Primers used in this study

Purpose/name	Sequence (5′–3′)
Cloning	
PfsE_p18CFwd	TACGTCTAGAGCTCCGCTATCTCTCGCTGTTCGCGGTAGG
PfsE_p18CRev	TACGGGTACCTCAAACAGTCAGGGAGGCCGCTAGG
PfsE_Y16VFwd	CTGGCCACCGGCGTGGCCTGG
PfsE_Y16VRev	CCAGCCCCAGGCCACGCCGGT
PfsE_W18AFwd	ACCGGCTACGCCGCCGGCTGG
PfsE_W18ARev	TCGATCCAGCCGGCGGCGTAGC
PfsE_W20AFwd	TACGCCTGGGGCGCCATCGACG
PfsE_W20ARev	CTAGGCCGTCGATGGCGCCCCAGG
ΔpfiT primers	
attB1-pfiT-UpF	ggggataagtttgtacaaaaaagcaggcttcTTCAACCCGCTCATAGGTT
pfiT-UpR	TCAGGAGTAGAAAGCCATCACATTAAACCTCCTTATTCTGG
PfiT-DownF	TGATGGCTTTCTACTCCTGA
attB2-PfiT-DownR	ggggaccactttgtacaagaaagctgggtaAGCCGCTCAACCCGATCTA
PfiT seq F	CCACACGTTCGCCAGTCACTT
PfiT seq R	AATGCCGGCCACTTCATCGAC
ΔPf4 primers	
Pf4-UpF-GWL	tacaaaaaagcaggctTCTGGGAATACGACGGGGGC
Pf4-UpR-GM	tcagagcgcttttgaagctaattcgGATCCCAATGCAAAAGCCCC
Pf4-DnF-GM	aggaacttcaagatccccaattcgCGTCATGAGCTTGGGAAGCT
Pf4-DnR-GWR	tacaagaaagctgggtTGGCAGCAGACCCAGGACGC
pf4-out F	AGTGGCGGTTATCGGATGAC
pf4-out R	TCATTGGGAGGCGCTTTCAT

PfsE modeling. AlphaFold (52) was used to predict the secondary structure of PfsE. The .pdb file was downloaded for PfsE "model one" and visualized using UCSF ChimeraX (53).

Statistical analyses. Differences between data sets were evaluated by an unpaired Student's t test, using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). P values of < 0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.7 MB.
FIG S2, TIF file, 0.2 MB.
FIG S3, TIF file, 0.4 MB.

REFERENCES

1. Knezevic P, Voet M, Lavigne R. 2015. Prevalence of Pf1-like (pro)phage genetic elements among *Pseudomonas aeruginosa* isolates. Virology 483: 64–71. https://doi.org/10.1016/j.virol.2015.04.008.
2. 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, Bollyky PL. 2020. Pf bacteriophage and their impact on pseudomonas virulence, mammalian immunity, and chronic infections. Front Immunol 11:244. https://doi.org/10.3389/fimmu.2020.00244.
3. Burgener EB, Sweere JM, Bach MS, Secor PR, Haddock N, Jennings LK, Marvig RL, Johansen HK, Rossi E, Cao X, Tian L, Nedelec L, Molin S, Bollyky PL, Milla CE. 2019. Filamentous bacteriophages are associated with chronic *Pseudomonas* lung infections and antibiotic resistance in cystic fibrosis. Sci Transl Med 11:eaau9748. https://doi.org/10.1126/scitranslmed.aau9748.
4. Sweere JM, Van Belleghem JD, Ishak H, Bach MS, Popescu M, Sunkari V, Kaber G, Manasherob R, Suh GA, Cao X, de Vries CR, Lam DN, Marshall PL, Birukova M, Katznelson E, Lazzareschi DV, Balaji S, Keswani SG, Hawn TR,

Secor PR, Bollyky PL. 2019. Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection. Science 363:6513. https://doi.org/10.1126/science.aat9691.

5. Secor PR, Michaels LA, Smigiel KS, Rohani MG, Jennings LK, Hisert KB, Arrigoni A, Braun KR, Birkland TP, Lai Y, Hallstrand TS, Bollyky PL, Singh PK, Parks WC. 2017. Filamentous bacteriophage produced by *Pseudomonas aeruginosa* alters the inflammatory response and promotes noninvasive infection *in vivo*. Infect Immun 85:e00648-16. https://doi.org/10.1128/IAI.00648-16.

6. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 413:860–864. https://doi.org/10.1038/35101627.

7. McElroy KE, Hui JG, Woo JK, Luk AW, Webb JS, Kjelleberg S, Rice SA, Thomas T. 2014. Strain-specific parallel evolution drives short-term diversification during *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci U S A 111:E1419–E1427. https://doi.org/10.1073/pnas.1314340111.

8. Secor PR, Sweere JM, Michaels LA, Malkovskiy AV, Lazzareschi D, Katznelson E, Rajadas J, Birnbaum ME, Arrigoni A, Braun KR, Evanko SP, Stevens DA, Kaminsky W, Singh PK, Parks WC, Bollyky PL. 2015. Filamentous bacteriophage promote biofilm assembly and function. Cell Host Microbe 18:549–559. https://doi.org/10.1016/j.chom.2015.10.013.

9. Li Y, Liu X, Tang K, Wang P, Zeng Z, Guo Y, Wang X. 2019. Excisionase in Pf filamentous prophage controls lysis-lysogeny decision-making in *Pseudomonas aeruginosa*. Mol Microbiol 111:495–513. https://doi.org/10.1111/mmi.14170.

10. Rice SA, Tan CH, Mikkelsen PJ, Kung V, Woo J, Tay M, Hauser A, McDougald D, Webb JS, Kjelleberg S. 2009. The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. ISME J 3:271–282. https://doi.org/10.1038/ismej.2008.109.

11. Webb JS, Lau M, Kjelleberg S. 2004. Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. J Bacteriol 186:8066–8073. https://doi.org/10.1128/JB.186.23.8066-8073.2004.

12. Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, Givskov M, Kjelleberg S. 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. J Bacteriol 185:4585–4592. https://doi.org/10.1128/JB.185.15.4585-4592.2003.

13. Petrova OE, Schurr JR, Schurr MJ, Sauer K. 2011. The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. Mol Microbiol 81:767–783. https://doi.org/10.1111/j.1365-2958.2011.07733.x.

14. Bertozzi Silva J, Storms Z, Sauvageau D. 2016. Host receptors for bacteriophage adsorption. FEMS Microbiol Lett 363:fnw002. https://doi.org/10.1093/femsle/fnw002.

15. Castang S, Dove SL. 2012. Basis for the essentiality of H-NS family members in *Pseudomonas aeruginosa*. J Bacteriol 194:5101–5109. https://doi.org/10.1128/JB.00932-12.

16. Tsao Y-F, Taylor VL, Kala S, Bondy-Denomy J, Khan AN, Bona D, Cattoir V, Lory S, Davidson AR, Maxwell KL. 2018. Phage morons play an important role in *Pseudomonas aeruginosa* phenotypes. J Bacteriol 200:e00189-18. https://doi.org/10.1128/JB.00189-18.

17. Shah M, Taylor VL, Bona D, Tsao Y, Stanley SY, Pimentel-Elardo SM, McCallum M, Bondy-Denomy J, Howell PL, Nodwell JR, Davidson AR, Moraes TF, Maxwell KL. 2021. A phage-encoded anti-activator inhibits quorum sensing in *Pseudomonas aeruginosa*. Mol Cell 81:571–583.e6. https://doi.org/10.1016/j.molcel.2020.12.011.

18. Chung IY, Jang HJ, Bae HW, Cho YH. 2014. A phage protein that inhibits the bacterial ATPase required for type IV pilus assembly. Proc Natl Acad Sci U S A 111:11503–11508. https://doi.org/10.1073/pnas.1403537111.

19. Roux S, Krupovic M, Daly RA, Borges AL, Nayfach S, Schulz F, Sharrar A, Matheus Carnevali PB, Cheng JF, Ivanova NN, Bondy-Denomy J, Wrighton KC, Woyke T, Visel A, Kyrpides NC, Eloe-Fadrosh EA. 2019. Cryptic inoviruses revealed as pervasive in bacteria and archaea across Earth's biomes. Nat Microbiol 4:1895–1906. https://doi.org/10.1038/s41564-019-0510-x.

20. Bondy-Denomy J, Qian J, Westra ER, Buckling A, Guttman DS, Davidson AR, Maxwell KL. 2016. Prophages mediate defense against phage infection through diverse mechanisms. ISME J 10:2854–2866. https://doi.org/10.1038/ismej.2016.79.

21. Murray TS, Kazmierczak BI. 2008. *Pseudomonas aeruginosa* exhibits sliding motility in the absence of type IV pili and flagella. J Bacteriol 190:2700–2708. https://doi.org/10.1128/JB.01620-07.

22. Burgener EB, Secor PR, Tracy MC, Sweere JM, Bik EM, Milla CE, Bollyky PL. 2020. Methods for extraction and detection of Pf bacteriophage DNA from the sputum of patients with cystic fibrosis. Phage (New Rochelle) 1:100–108. https://doi.org/10.1089/phage.2020.0003.

23. Burrows LL. 2012. Pseudomonas aeruginosa twitching motility: type IV pili in action. Annu Rev Microbiol 66:493–520. https://doi.org/10.1146/annurev-micro-092611-150055.

24. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS. 2016. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the Pseudomonas genome database. Nucleic Acids Res 44:D646–D653. https://doi.org/10.1093/nar/gkv1227.

25. McCallum M, Tammam S, Little DJ, Robinson H, Koo J, Shah M, Calmettes C, Moraes TF, Burrows LL, Howell PL. 2016. PilN binding modulates the structure and binding partners of the *Pseudomonas aeruginosa* type IVa pilus protein PilM. J Biol Chem 291:11003–11015. https://doi.org/10.1074/jbc.M116.718353.

26. Takhar HK, Kemp K, Kim M, Howell PL, Burrows LL. 2013. The platform protein is essential for type IV pilus biogenesis. J Biol Chem 288:9721–9728. https://doi.org/10.1074/jbc.M113.453506.

27. Lanzarotti E, Biekofsky RR, Estrin DA, Marti MA, Turjanski AG. 2011. Aromatic-aromatic interactions in proteins: beyond the dimer. J Chem Inf Model 51:1623–1633. https://doi.org/10.1021/ci200062e.

28. Stahelin RV, Cho W. 2001. Differential roles of ionic, aliphatic, and aromatic residues in membrane-protein interactions: a surface plasmon resonance study on phospholipases A2. Biochemistry 40:4672–4678. https://doi.org/10.1021/bi0020325.

29. Lee YM, Tscherne DM, Yun SI, Frolov I, Rice CM. 2005. Dual mechanisms of pestiviral superinfection exclusion at entry and RNA replication. J Virol 79:3231–3242. https://doi.org/10.1128/JVI.79.6.3231-3242.2005.

30. Tarafder AK, von Kugelgen A, Mellul AJ, Schulze U, Aarts D, Bharat TAM. 2020. Phage liquid crystalline droplets form occlusive sheaths that encapsulate and protect infectious rod-shaped bacteria. Proc Natl Acad Sci U S A 117:4724–4731. https://doi.org/10.1073/pnas.1917726117.

31. Pourtois JD, Kratochvil MJ, Chen Q, Haddock NL, Burgener EB, De Leo GA, Bollyky PL. 2021. Filamentous bacteriophages and the competitive interaction between *Pseudomonas aeruginosa* strains under antibiotic treatment: a modeling study. mSystems 6:e0019321. https://doi.org/10.1128/mSystems.00193-21.

32. O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304. https://doi.org/10.1046/j.1365-2958.1998.01062.x.

33. Comolli JC, Hauser AR, Waite L, Whitchurch CB, Mattick JS, Engel JN. 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect Immun 67:3625–3630. https://doi.org/10.1128/IAI.67.7.3625-3630.1999.

34. Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P. 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol 186:7312–7326. https://doi.org/10.1128/JB.186.21.7312-7326.2004.

35. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol 184:1140–1154. https://doi.org/10.1128/jb.184.4.1140-1154.2002.

36. Wright A, Hawkins CH, Anggard EE, Harper DR. 2009. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. Clin Otolaryngol 34:349–357. https://doi.org/10.1111/j.1749-4486.2009.01973.x.

37. Bruttin A, Brussow H. 2005. Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. Antimicrob Agents Chemother 49:2874–2878. https://doi.org/10.1128/AAC.49.7.2874-2878.2005.

38. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. 2009. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care 18:237–243. https://doi.org/10.12968/jowc.2009.18.6.42801.

39. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, Barr JJ, Reed SL, Rohwer F, Benler S, Segall AM, Taplitz R, Smith DM, Kerr K, Kumaraswamy M, Nizet V, Lin L, McCauley MD, Strathdee SA, Benson CA, Pope RK, Leroux BM, Picel AC, Mateczun AJ, Cilwa KE, Regeimbal JM, Estrella LA, Wolfe DM, Henry MS, Quinones J, Salka S, Bishop-Lilly KA, Young R, Hamilton T. 2017. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. Antimicrob Agents Chemother 61:e00954-17. https://doi.org/10.1128/AAC.00954-17.

40. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, Gilmour KC, Soothill J, Jacobs-Sera D, Schooley RT, Hatfull GF, Spencer H. 2019. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. Nat Med 25: 730–733. https://doi.org/10.1038/s41591-019-0437-z.

41. Aslam S, Courtwright AM, Koval C, Lehman SM, Morales S, Furr CL, Rosas F, Brownstein MJ, Fackler JR, Sisson BM, Biswas B, Henry M, Luu T, Bivens BN, Hamilton T, Duplessis C, Logan C, Law N, Yung G, Turowski J, Anesi J, Strathdee SA, Schooley RT. 2019. Early clinical experience of bacterio- phage therapy in 3 lung transplant recipients. Am J Transplant 19: 2631–2639. https://doi.org/10.1111/ajt.15503.

42. Oechslin F. 2018. Resistance development to bacteriophages occurring during bacteriophage therapy. Viruses 10:351. https://doi.org/10.3390/v10070351.

43. Loc Carrillo C, Atterbury RJ, el-Shibiny A, Connerton PL, Dillon E, Scott A, Connerton IF. 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. Appl Environ Microbiol 71: 6554–6563. https://doi.org/10.1128/AEM.71.11.6554-6563.2005.

44. Scott AE, Timms AR, Connerton PL, Loc Carrillo C, Adzfa Radzum K, Connerton IF. 2007. Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. PLoS Pathog 3:e119. https://doi.org/10.1371/journal.ppat.0030119.

45. Carvalho CM, Gannon BW, Halfhide DE, Santos SB, Hayes CM, Roe JM, Azeredo J. 2010. The *in vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. BMC Microbiol 10:232. https://doi.org/10.1186/1471-2180-10-232.

46. Chung IY, Kim BO, Han JH, Park J, Kang HK, Park Y, Cho YH. 2021. A phage protein-derived antipathogenic peptide that targets type IV pilus assembly. Virulence 12:1377–1387. https://doi.org/10.1080/21505594.2021.1926411.

47. Hmelo LR, Borlee BR, Almblad H, Love ME, Randall TE, Tseng BS, Lin C, Irie Y, Storek KM, Yang JJ, Siehnel RJ, Howell PL, Singh PK, Tolker-Nielsen T, Parsek MR, Schweizer HP, Harrison JJ. 2015. Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. Nat Protoc 10:1820–1841. https://doi.org/10.1038/nprot.2015.115.

48. Li Y, Liu X, Tang K, Wang W, Guo Y, Wang X. 2020. Prophage encoding toxin/antitoxin system PfiT/PfiA inhibits Pf4 production in *Pseudomonas aeruginosa*. Microb Biotechnol 13:1132–1144. https://doi.org/10.1111/1751-7915.13570.

49. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. 2008. PBAD-based shut- tle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. Appl Environ Microbiol 74:7422–7426. https://doi.org/10.1128/AEM.01369-08.

50. Semmler AB, Whitchurch CB, Mattick JS. 1999. A re-examination of twitch- ing motility in *Pseudomonas aeruginosa*. Microbiology 145:2863–2873. https://doi.org/10.1099/00221287-145-10-2863.

51. Choi KH, Schweizer HP. 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc 1:153–161. https://doi.org/10.1038/nprot.2006.24.

52. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Zidek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596:583–589. https://doi.org/10.1038/s41586-021-03819-2.

53. Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH, Ferrin TE. 2021. UCSF ChimeraX: structure visualization for researchers, educators, and developers. Protein Sci 30:70–82. https://doi.org/10.1002/pro.3943.

54. Taylor VL, Fitzpatrick AD, Islam Z, Maxwell KL. 2019. The diverse impacts of phage morons on bacterial fitness and virulence. Adv Virus Res 103: 1–31. https://doi.org/10.1016/bs.aivir.2018.08.001.

55. Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: a sequence logo generator. Genome Res 14:1188–1190. https://doi.org/10.1101/gr.849004.

56. Holloway BW, Krishnapillai V, Morgan AF. 1979. Chromosomal genetics of *Pseudomonas*. Microbiol Rev 43:73–102. https://doi.org/10.1128/mr.43.1.73-102.1979.

57. Zhao K, Tseng BS, Beckerman B, Jin F, Gibiansky ML, Harrison JJ, Luijten E, Parsek MR, Wong GC. 2013. Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. Nature 497:388–391. https://doi.org/10.1038/nature12155.

58. Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, Secor PR, Tseng BS, Scian M, Filloux A, Wozniak DJ, Howell PL, Parsek MR. 2015. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. Proc Natl Acad Sci U S A 112:11353–11358. https://doi.org/10.1073/pnas.1503058112.

59. Cruz RL, Asfahl KL, Van den Bossche S, Coenye T, Crabbe A, Dandekar AA. 2020. RhlR-regulated acyl-homoserine lactone quorum sensing in a cystic fibrosis isolate of *Pseudomonas aeruginosa*. mBio 11. https://doi.org/10.1128/mBio.00532-20.

60. Budzik JM, Rosche WA, Rietsch A, O'Toole GA. 2004. Isolation and charac- terization of a generalized transducing phage for *Pseudomonas aerugi- nosa* strains PAO1 and PA14. J Bacteriol 186:3270–3273. https://doi.org/10.1128/JB.186.10.3270-3273.2004.

61. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. 2016. Phage selection restores antibiotic sensitivity in MDR *Pseudomonas aeru- ginosa*. Sci Rep 6:26717. https://doi.org/10.1038/srep26717.