

A biofilm-tropic *Pseudomonas aeruginosa* bacteriophage uses the exopolysaccharide Psl as receptor

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This **valuable** study identifies a novel bacteriophage that can use the exopolysaccharide Psl of *Pseudomonas aeruginosa* to infect and disrupt biofilms. The work is **convincing** and suggests a novel approach to control biofilms that is relevant to researchers working on biofilms, specifically in *Pseudomonas*, on phage physiology and discovery, and on alternatives to controlling bacterial pathogens.

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Abstract

Bacteria in nature can exist in multicellular communities called biofilms. Biofilms also form in the course of many infections. *Pseudomonas aeruginosa* infections frequently involve biofilms, which contribute materially to the difficulty to treat these infections with antibiotic therapy. Many biofilm-related characteristics are controlled by the second messenger, cyclic-di-GMP, which is upregulated on surface contact. Among these factors is the exopolysaccharide Psl, which is a critically important component of the biofilm matrix. Here we describe the discovery of a *P. aeruginosa* bacteriophage, which we have called Clew-1, that directly binds to and uses Psl as a receptor. While this phage does not efficiently infect planktonically growing bacteria, it can disrupt *P. aeruginosa* biofilms and replicate in biofilm bacteria. We further demonstrate that the Clew-1 can reduce the bacterial burden in a mouse model of *P. aeruginosa* keratitis, which is characterized by the formation of a biofilm on the cornea. Due to its reliance on Psl for infection, Clew-1 does not actually form plaques on wild-type bacteria under standard *in vitro* conditions. This argues that our standard isolation

procedures likely exclude bacteriophage that are adapted to using biofilm markers for infection. Importantly, the manner in which we isolated Clew-1 can be easily extended to other strains of *P. aeruginosa* and indeed other bacterial species, which will fuel the discovery of other biofilm-tropic bacteriophage and expand their therapeutic use.

Introduction

Biofilms formed by bacteria at sites of infection significantly increase the difficulty of treatment with conventional antibiotic therapy. This increased resistance to antibiotic therapy has been attributed to a variety of factors, including reduced penetration of antibiotics (1, 2), as well as an increase in antibiotic-tolerant persister bacteria (3, 4). Formation of biofilms is a feature of many *P. aeruginosa* infections, including lung infections in cystic fibrosis patients (5, 6), wound, catheter, and device infections (7), as well as blinding corneal infections (8–10). In some instances, these biofilms have been found to be astonishingly antibiotic tolerant (11).

In addition to the antibiotic tolerance of bacteria in biofilms, there has been a significant increase in antibiotic resistant isolates (12). In fact, *P. aeruginosa* is one of the particularly worrisome ESKAPE group of pathogens (13). With the general rise of antibiotic-resistant isolates, phage therapy has garnered some interest as an alternative to treat these infections (14, 15). However, biofilm formation frequently interferes with phage infection (16), and even though a few bacteriophage that can target *P. aeruginosa* in a biofilm have been described (17, 18), the mechanism by which they infect these biofilm bacteria is unknown.

The extracellular matrix of *P. aeruginosa* biofilms is comprised of exopolysaccharides, including Psl, Pel, and alginate, as well as proteins and DNA (19, 20). Psl is of significant interest, since it is critical for biofilm formation, where it is needed for the initial surface attachment (21), as well as structural stability of the mature biofilm (22). Psl has been detected on the surface of individual *P. aeruginosa* bacteria in an apparent helical pattern (20). It is also deposited on surfaces by a subset of motile explorer bacteria during the early stages of aggregate formation (23). Psl production interferes with complement deposition and neutrophil functions, such as phagocytosis and ROS production (24). Moreover, Psl enhances the intracellular survival of phagocytosed *P. aeruginosa*, as well as survival in mouse models of lung and wound infection (24).

Here we describe the discovery of a bacteriophage that uses Psl, this crucial biofilm exopolysaccharide, as a receptor. Interestingly, this bacteriophage only infects a subpopulation of planktonically growing *P. aeruginosa*, but it can disrupt biofilms and replicates efficiently on biofilm-grown bacteria. Moreover, the phage can reduce the bacterial burden in a corneal infection model, which involves formation of a biofilm.

Results

Phage Clew-1 can form plaques on a Δ *fliF* mutant, but not wild type *P. aeruginosa*

We screened wastewater samples at the three Northeast Ohio Regional Sewer District water treatment plants in Cleveland for bacteriophage. The majority of phage in these samples used type IV pili as a receptor, and we wanted to exclude these from our screen. We had previously generated a Δ *fliF* Δ *pilA* double mutant strain in the lab and decided to use it to exclude both surface appendages as potential receptors. This turned out to be fortuitous, since, surprisingly, the screen identified four phage that could form plaques on the Δ *fliF* Δ *pilA* double mutant strain, but

not the parental wild type *P. aeruginosa* PAO1. We named these Cleveland wastewater-derived phage Clew-1, -3, -6, and -10. Subsequent tests determined that it was the *fliF* deletion that rendered *P. aeruginosa* permissive for infection by these phages. All four Clew phage can plaque on a *fliF* deletion mutant of *P. aeruginosa* PAO1, but not the corresponding wild-type strain or $\Delta pilA$ mutant strain. (Fig. 1A, S1A, S1B, S2). An unrelated Pbinavirus we isolated in the same screen, which uses O-antigen as receptor (Fig. S3), was used as a control in these experiments (Our control phage, Ocp-2). The Clew bacteriophages belong to the family of Bruynogheviruses (25) and are all highly related (Fig. 1B, S1C). Morphologically, like other members of the family, they are Podoviruses (Fig. 1C).

c-di-GMP levels control infection of *P. aeruginosa* by bacteriophage Clew-1

We next examined what part of the flagellum is involved in determining sensitivity to the Clew-1 phage. Mutations affecting the MS-ring ($\Delta fliF$) and associated proteins FliE or FliG (26) resulted in Clew-1 sensitivity. However, mutations in the ATPase complex only conferred partial sensitivity, and mutations affecting the hook or flagellar filament did not result in sensitivity, nor did a mutation that affects the type III secretion function of the flagellar basal body by impeding proton flux, *flhA*(R147A)(27), (Fig.S4). We therefore conclude that it is the presence of the MS-ring and not other aspects of the flagellum, such as assembly of the full flagellar structure or flagellar rotation, that control phage sensitivity.

Interestingly, we found that deletion of *fleQ*, which is required for transcription of flagellar genes (28), had a very minor effect on Clew-1 phage susceptibility of the wild-type bacteria, and actually decreased Clew-1 susceptibility of the $\Delta fliF$ mutant bacteria (Fig. S1E). FleQ is a c-di-GMP-responsive transcription factor that, among other things, reciprocally controls flagellar gene expression and production of biofilm-related characteristics, such as the production of the extracellular polysaccharides Psl and Pel, as well as the adhesin CdrA (28–30). We therefore examined whether manipulating c-di-GMP levels controls phage susceptibility. To this end we produced the c-di-GMP phosphodiesterase PA2133 from a plasmid (31) to artificially lower c-di-GMP levels in the $\Delta fliF2$ deletion mutant strain. Conversely, we artificially elevated c-di-GMP levels in the wild-type by deleting the *wspF* gene (31). Lowering c-di-GMP levels in the $\Delta fliF2$ mutant restored Clew-1 resistance (Fig. 1D), whereas deleting *wspF* rendered the parental PAO1 strain phage sensitive (Fig. 1E). Taken together, these data demonstrate that Clew-1 susceptibility is controlled by intracellular c-di-GMP levels and argue that absence of the MS-ring controls phage susceptibility through an increase in c-di-GMP.

Phage Clew-1 requires Psl for infection

To better understand the host factors that control susceptibility and resistance to Clew-1 infection, we carried out a pair of TnSeq experiments. In the first of these, we mutagenized the wild-type strain PAO1F with the mini-mariner transposon TnFAC (32), and the resultant mutant library was infected with phage Clew-1 at an MOI of 10 for 2 hours. The surviving bacteria were allowed to grow up after plating on an LB plate and the transposon insertion sites for the input and output pool were determined by Illumina sequencing. We identified insertion mutants that were depleted after infection (Fig. 2A). Two of the genes with the most significant depletion were *fliF* and *fliG*, consistent with our previous analysis indicating that these mutations sensitize PAO1 to Clew-1 infection. Interestingly we also noted depletion of *pch* and *bifA* insertions, both encoding phosphodiesterases that are involved in depleting c-di-GMP in the flagellated daughter cell after cell division (33–36). In fact, *pch* interacts with the chemotactic machinery (33), highlighting, here too, the importance of c-di-GMP in controlling Clew-1 sensitivity.

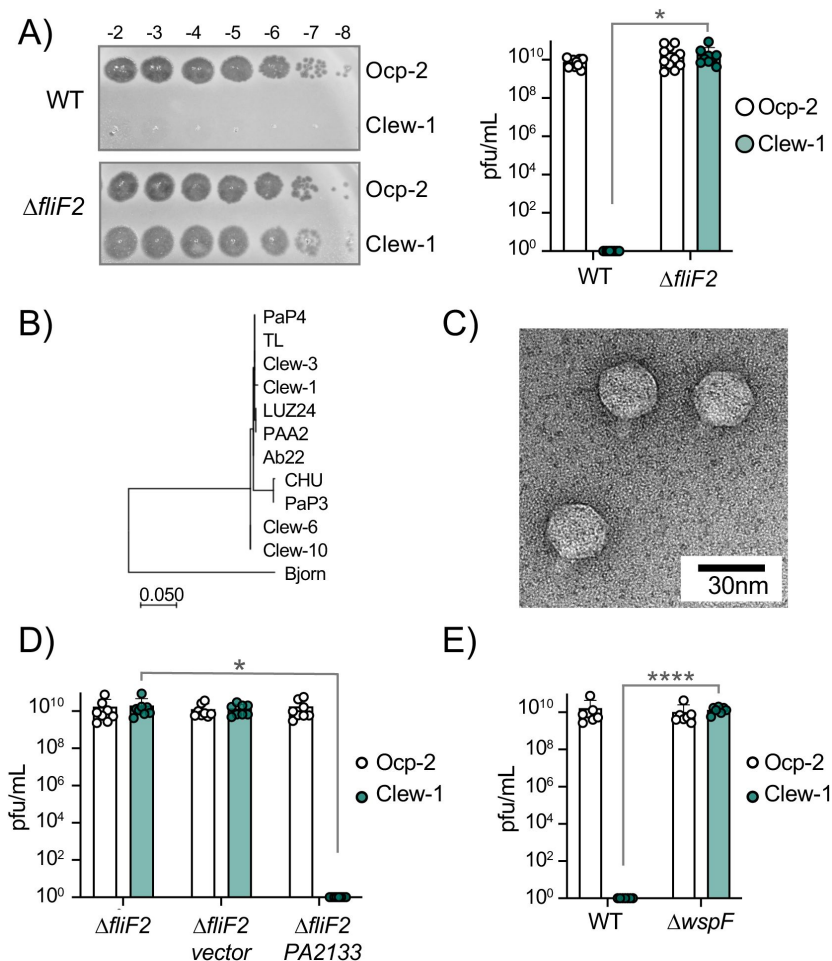


Fig. 1

c-di-GMP levels control infection of *P. aeruginosa* by bacteriophage Clew-1.

A) Efficiency of plating experiment in which 3 μ L of a 10x dilution series of bacteriophage Ocp-2 or Clew-1 were spotted on wild-type PAO1F, or PAO1F $\Delta fliF2$. The adjacent graph shows the compiled results from 11 experiments. B) Maximum likelihood phylogenetic tree of Clew-1 relative to other Bruynogheviruses (including the type phage, LUZ24) and phage Bjorn as an outgroup. Branch lengths are measured in number of substitutions per site in the terminase large subunit. C) transmission electron micrograph of the Clew-1 phage. D) Efficiency of plating experiment as in (A) assaying the effect of expressing the phosphodiesterase PA2133 from a plasmid. E) Efficiency of plating experiment assaying the effect of deleting *wspF* on Clew-1 resistance. (* $p < 0.05$, **** $p < 0.0001$ by Student's T-test (A, E) or 1-way ANOVA with Šídák's multiple comparisons test (D))

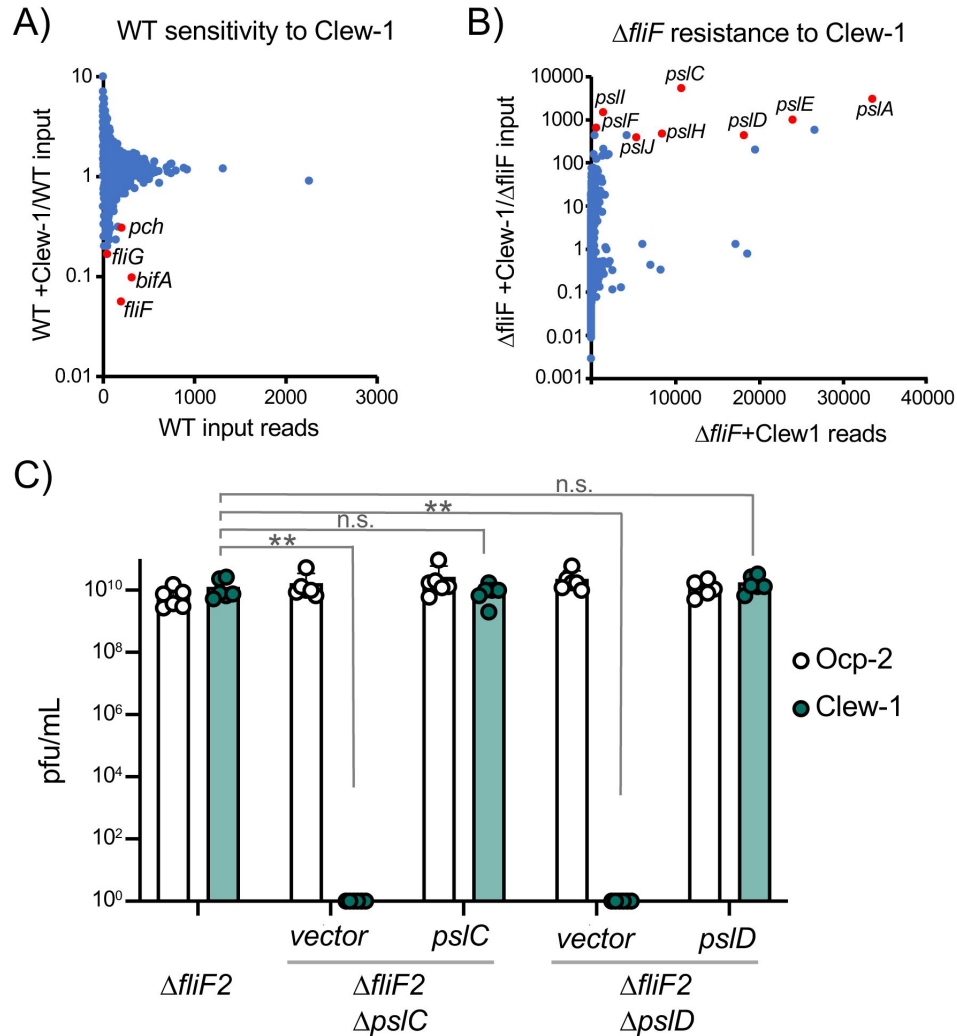


Fig. 2

Bacteriophage Clew-1 uses Psl as a receptor to infect *P. aeruginosa*.

A) TnSeq experiment in which a pool of mariner transposon mutants of strain PAO1F were infected with phage Clew-1 for 2h. The number of insertions in the output pool were plotted against the ratio of the output and input pool. B) Similar TnSeq analysis as in A) but using PAO1F $\Delta fliF2$. C) Efficiency of plating analysis on $\Delta fliF2 \Delta pslC$ and $\Delta fliF2 \Delta pslD$, Psl biosynthesis mutants, either harboring an empty vector or a complementing plasmid (n=6). Clew-1 values were compared by 1-way ANOVA with Šídák's multiple comparisons test (** $p < 0.01$, n.s. ... not significant).

In a reciprocal experiment, we carried out the TnSeq analysis in a *fliF* mutant strain. This analysis identified insertions in the *psl* operon as the most highly enriched group of mutants after Clew-1 selection, suggesting that Psl is required for phage infection (**Fig. 2B** [↗](#)). We examined the requirement for Psl explicitly by generating *pslC* and *pslD* mutants in the PAO1F Δ *fliF2* strain background. PslC is a glycosyltransferase required for Psl biosynthesis, while PslD is required for Psl export from the cell ([37](#) [↗](#), [38](#) [↗](#)). Deletion of either *pslC* or *pslD* rendered the *fliF* mutant bacteria Clew-1 resistant and sensitivity could be restored through complementation using a plasmid-borne copy of the deleted open reading frame (**Fig. 2C** [↗](#)). These data demonstrate that Psl production is required for infection of *P. aeruginosa* by phage Clew-1.

Phage Clew-1 attachment is Psl-dependent

We next examined whether attachment of Clew-1 to *P. aeruginosa* is Psl-dependent. We first used efficiency of center of infection (ECOI) analysis to examine attachment. In this analysis, the phage is allowed to adhere to the bacteria for 5 minutes, before washing the bacteria to remove unattached phage. The bacteria are then diluted, mixed with top agar and a sensitive indicator bacterium (Δ *fliF2*), and then plated to allow for plaque formation as a biological readout of attached bacteriophage. Attachment of phage Clew-1 is Psl-dependent. Interestingly, we were able to detect Psl-dependent attachment both with wild-type and Δ *fliF2* mutant bacteria (**Fig. 3A** [↗](#)), which contradicted our initial efficiency of plating experiments. We therefore reexamined phage susceptibility by monitoring phage infection in liquid media and generating lysis curves for wild-type and Δ *fliF2* mutant bacteria, as well as their Δ *pslC* mutant derivatives (**Fig. S5**). The Δ *fliF2* mutant strain was lysed after ~40 minutes of infection. The wild-type bacteria displayed a significant slowing of growth upon Clew-1 infection when compared to the uninfected culture, but not clear lysis as was observed with the Δ *fliF2* mutant. In both instances, deleting *pslC* abolished any phage-dependent effect on growth.

We hypothesized that perhaps, the difference between wild-type and Δ *fliF2* mutant bacteria is due to the fraction of cells that are producing Psl and therefore permissive for phage attachment. To test this hypothesis, we labeled phage Clew-1 with the DyLight594 fluorescent dye and examined attachment directly by microscopy (**Fig. 3B** [↗](#)). We observed a statistically significant increase in the percentage of bacteria with attached bacteriophage in the Δ *fliF2* mutant bacteria compared to the wild-type, arguing that the increase in c-di-GMP in the Δ *fliF2* mutant increases the fraction of Clew-1 susceptible cells in the population. As anticipated, no phage was observed attached to the corresponding Δ *pslC* mutant (**Fig. 3C** [↗](#)).

Phage Clew-1 binds to Psl directly

We next examined whether phage Clew-1 can bind to Psl directly. We first determined whether we could precipitate phage Clew-1 from filter sterilized culture supernatants of a Δ *fliF2* mutant using an antibody directed against Psl. The presence of the phage was determined by quantitative PCR. We were able to pull down phage Clew-1 in a Psl and antibody-dependent manner with Δ *fliF2* Δ *pslC* culture supernatants serving as a control (**Fig. 4A** [↗](#)). Notably, we observed some Psl-dependent attachment in the absence of antibody, arguing that Psl binds non-specifically to the magnetic beads we used in our experiments. Including the anti-Psl antibody resulted in a statistically significant increase compared to this background level of attachment (**Fig. 4A** [↗](#)). We next repeated the pulldown using a partially purified fraction of cell-associated Psl to repeat the pulldown and again found Psl and antibody dependent precipitation of phage Clew-1 (**Fig. S7**). Finally, we examined phage binding using a biotinylated, affinity purified preparation of Psl and found that we could pull down the phage using this Psl fraction as well, arguing that Clew-1 binds Psl directly (**Fig. 4B** [↗](#)).

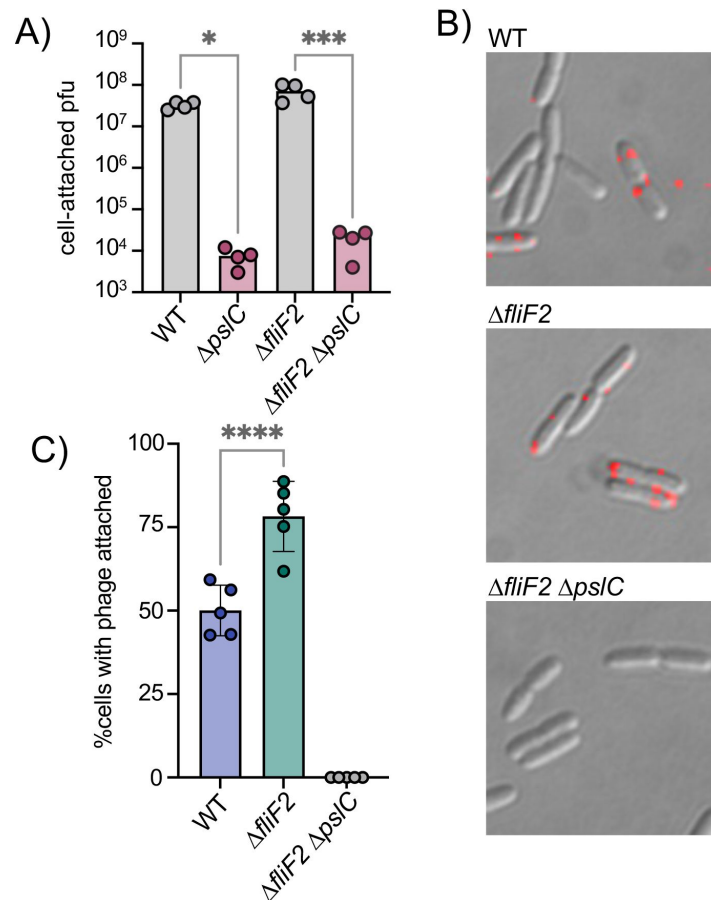


Fig. 3.

The $\Delta fliF2$ mutation changes the fraction of cells that phage Clew-1 binds to.

A) Efficiency of center of infection analysis. The indicated strain was infected for 5 minutes at an MOI of 0.01, the bacteria were pelleted, washed 3x with PBS, then diluted and mixed with an excess of the $\Delta fliF2$ mutant strain, top agar and plated on an LB agar plate. The number of plaques was used to calculate the number of phage that attached and productively infected the initial strain. B) Phage Clew-1 was labeled with DyLight594 fluorophores, bound to the indicated wild-type or mutant bacteria (15 minutes in LB), washed and fixed with paraformaldehyde. Phage attached to bacteria were imaged by fluorescence microscopy and attachment was quantified over 5 biological replicates, shown in C). Attachment was compared by 1-way ANOVA with Šídák's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

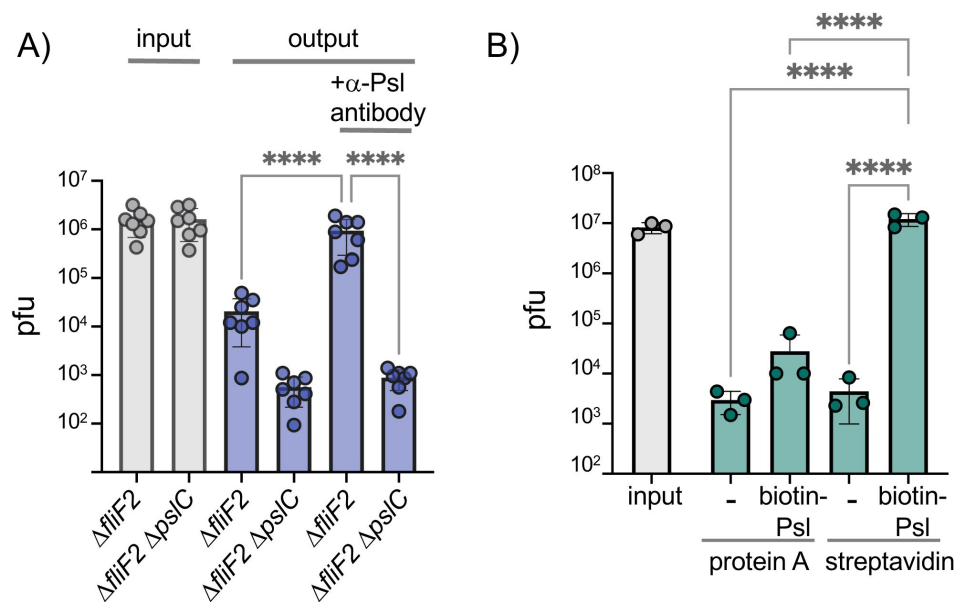


Fig. 4.

Phage Clew-1 binds to Psl.

A) Sterile filtered mid-log culture supernatants of PAO1F Δ fliF2 or PAO1F Δ fliF2 Δ pslC were incubated with phage Clew-1, as well as magnetic protein-A beads and, where indicated, a rabbit, anti-Psl antiserum. Beads were collected, washed 3x, and phage in the input and output samples were quantified by qPCR (7 independent replicates.) B) Phage Clew-1 was incubated for 1h in SM buffer with affinity purified, biotinylated Psl (biotin-Psl) and magnetic protein A beads, or magnetic streptavidin beads (SA), where indicated. Beads were collected and washed 3x, and phage in the input and output samples were quantified by qPCR (3 independent replicates). Statistical significance was determined by ANOVA with Sidák post-hoc test (**** $p < 0.0001$).

Phage Clew-1 infects wild-type *P. aeruginosa* in biofilms

Since Clew-1 exploits Psl for infection and Psl is a key component of the biofilm matrix of most strains of *P. aeruginosa* (39, 40), we hypothesized that, perhaps, Clew-1 can infect biofilm bacteria. We used a static-biofilm model to test this hypothesis. Biofilms were established overnight in a 96-well plate. One plate was washed and fixed with ethanol to quantify the day one biofilm mass using crystal violet staining. In a second plate, established in parallel, the biofilms were washed with PBS and LB was added back, either without addition, or with 10^9 pfu of phage Clew-1 or phage Ocp-2. The plates were incubated overnight and the next day, the day 2 biofilm mass was quantified using crystal violet. A similar experiment was carried out in 5-mL culture tubes to illustrate the result is shown in Fig. 5A. The averages of 5 biological replicates in the 96-well experiment are shown in Fig. 5B. Treatment of the day one biofilm with phage Clew-1 resulted in a statistically significant decrease in biofilm mass compared to the biomass present at day 1. Phage Ocp-2 infection, on the other hand, did not result in a reduction in biofilm (Fig. 5B). Notably, phage Clew-1 was not able to reduce a biofilm formed by *P. aeruginosa* strain PA14, a natural *psl* mutant (Fig. S8).

To corroborate this result, we also conducted a converse experiment where we monitored the ability of phage Clew-1 or Ocp-2 to replicate on biofilm bacteria over a two-hour period. Here, biofilms were generated overnight in 5-mL culture tubes, the biofilms were washed with PBS and exposed to 10^5 pfu/mL phage Clew-1 or phage Ocp-2 for 2 hours. At the end of the experiment, the culture supernatants were filter sterilized and the phage were titered. Consistent with the reduction in biofilm mass seen in Fig. 5B, we found that phage Clew-1, but not Ocp-2, was able to replicate when grown on biofilm bacteria (Fig. 5C).

In order to determine distinguish whether Clew-1 infection results in decolonization of bacteria from the biofilm, or killing of biofilm bacteria, we carried out two complementary experiments. In the first experiment, we generated wild type *P. aeruginosa* biofilms on polystyrene microbeads grown overnight with agitation. The beads were then washed with PBS and incubated for 2 hours either in LB or LB with Clew-1. Bacteria were released from the biofilm by sonication and the input CFU (total CFU attached to beads) were compared to the output after 2h of incubation (sum of supernatant and bead-associated viable bacteria). Clew-1 resulted in a significant reduction in viable CFU, arguing that phage infection kills biofilm bacteria (Fig. 5D). In a related experiment, we used LIVE/DEAD staining of bacteria to assess bacterial viability by microscopy. We established biofilms in microwell slides overnight, then added Syto 9 and propidium iodide (PI) to each well along with phage Clew-1, or a buffer control, and incubated the slide overnight again before imaging the biofilms by confocal microscopy. Infection with Clew-1 resulted in a significant increase in the fraction of the biofilm volume that is PI-positive, indicating that the biofilm bacteria are dying (Fig. 5E).

Phage Clew-1 can clear *P. aeruginosa* in a mouse keratitis model

Given the ability of phage Clew-1 to infect *P. aeruginosa* biofilms, we next examined whether Clew-1 could be used to treat a *P. aeruginosa* infection. Corneal infections by *P. aeruginosa* involve formation of a biofilm (8, 40). In fact, a bivalent antibody directed against Psl and the type III secretion needle-tip protein, PcrV, was found to be effective in clearing such corneal infections (8). We therefore examined the ability of Clew-1 to reduce the *P. aeruginosa* bacterial burden in a corneal infection model. Mice were infected with 5×10^4 cfu of wild-type *P. aeruginosa* strain PAO1 and given a topical application of 5×10^9 pfu Clew-1 in 5 μ L of PBS, or PBS alone, at 24h and 48h post-infection (Fig. 6A). After 48 hours the infection we quantified corneal opacity, a measure that correlates with the infiltration of immune cells (41–43), and GFP fluorescence (produced by the *P. aeruginosa* strain used in the infection) by image analysis. We also assessed the bacterial burden (colony forming units). Mice infected with PAO1 developed severe corneal

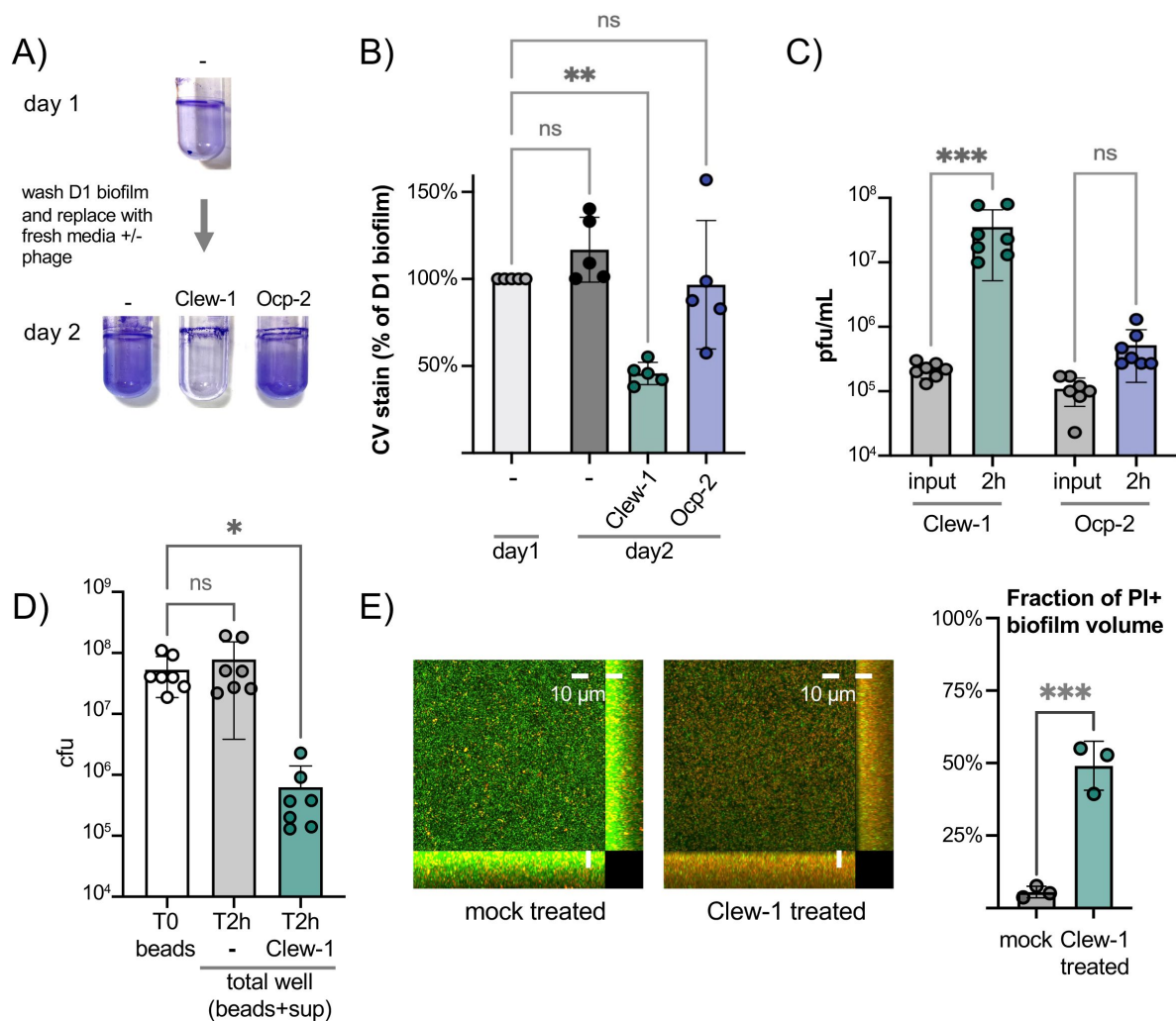


Fig. 5.

Phage Clew-1 can infect *P. aeruginosa* in biofilms.

A) Biofilms of wild type *P. aeruginosa* PAO1F were established overnight in 5 mL culture tubes (1 mL culture), the tubes were washed with PBS and 1.2 mL LB, LB with 10¹⁰ pfu phage Clew-1, or LB with 10¹⁰ pfu phage Ocp-2 were added back to each tube (1 was fixed with EtOH to represent the 1-day old biofilm). The following day all biofilms were washed with PBS and stained with crystal violet. B) PAO1F biofilms were established overnight in a 96-well plate (150 μL of culture, 6 technical replicates/condition), washed and incubated overnight with 200 μL of LB or LB with 10⁹ pfu bacteriophage Clew-1 or Ocp-2. The biofilms were then washed, fixed, and stained with crystal violet, which was then quantified spectrophotometrically. The day 1 controls were set to 100% (5 biological replicates). C) Growth of phage Clew-1 or Ocp-2 was assayed by establishing a static biofilm in 5 mL culture tubes overnight. The biofilms were washed with PBS, then LB with 10⁵ pfu/mL of phage Clew-1 or Ocp-2 was added back. Biofilms were incubated at 37°C for 2h 15 minutes, the culture supernatants were filter sterilized and input and output phage concentrations were tittered (6 biological replicates). D) Wild-type PAO1 biofilms were grown overnight, with agitation, on 6 mm polystyrene beads. Biofilms were rinsed with PBS and incubated with LB or LB with 10⁹ pfu Clew-1 for 2h. The sum of bead-associated and supernatant CFU were titered at the 2h mark and compared to the bead-associated biofilm input (T0) to assess total viable CFU (7 biological replicates). E) Biofilms were grown overnight in 8-well slides, at which point the LIVE/DEAD stain dyes, Syto 9 and propidium iodide were added, either on their own (mock) or in the presence of Clew-1. After another 24h of incubation, biofilms were imaged by confocal microscopy. Maximum intensity projections of the collected Z-stacks for one replicate are shown, along with YZ and XZ projections to the right and bottom of the image, respectively. The fraction of the total biofilm volume in the image stack that is propidium iodide positive was determined using BiofilmQ (3 biological replicates). Statistical significance was determined by ANOVA with Šídák's multiple comparisons test, except for panel E, where a two-tailed, unpaired T-test was applied (* p < 0.05, ** p < 0.01, *** p < 0.001).

disease manifest as corneal opacification in the region of bacterial growth indicated by GFP fluorescence (**Figure 6B-D**). Phage Clew-1 treatment was able to significantly reduce the bacterial burden, and in some instances completely eradicate the infection, as measured by GFP fluorescence and CFU (**Fig. 6D, E**). Corneal opacity, was similarly reduced by the phage treatment, indicating that inflammation was also starting to resolve (**Fig. 6B, C**).

Summary

We describe the isolation of four phage belonging to the family of Bruynogheviruses that use the *P. aeruginosa* exopolysaccharide Psl as a receptor. Psl is not a capsular polysaccharide, so this distinguishes the Clew phages from phages such as KP32 that infect *Klebsiella pneumoniae*. Moreover, these *Klebsiella* phages use a capsular depolymerase to break down the capsular polysaccharide (44, 45). Clew-1, on the other hand, has no such activity (Fig. S9) arguing that the role of Psl in infection is distinct from that seen in capsule-targeting bacteriophage.

Phage Clew-1 has the surprising quality that it fails to plaque on wild-type *P. aeruginosa* PAO1, but forms plaques on a *fliF* mutant. We determined that the *fliF* mutation generates a c-di-GMP dependent signal that up-regulates Psl production. Importantly, it increases the fraction of bacteria to which the phage can bind, resulting in efficient lysis in liquid cultures, and plaque formation in top agar. Plaque formation is likely masked in the wild-type bacteria by the fraction of cells that are not phage-susceptible. Notably, certain Bruynogheviruses are able to bind to *P. aeruginosa* PAO1, but not plaque (46). We now have an explanation for this observation.

The identification of Psl as phage receptor prompted us to examine the ability of phage Clew-1 to infect wild-type *P. aeruginosa* in a biofilm. We found that phage Clew-1, unlike the unrelated Ocp-2 phage, was able to disrupt biofilms formed by wild-type bacteria. Moreover, Clew-1 was able to actively replicate on biofilm bacteria, while phage Ocp-2 could not. Taken together, our data suggests that phage Clew-1 has specialized to replicate on *P. aeruginosa* growing in a biofilm. Given the prevalence of bacterial biofilms in nature, this specialization makes sense. Moreover, our observation suggests that we may have underestimated the prevalence of biofilm-tropic bacteriophage since standard isolation techniques using plaque formation of wild-type bacteria would miss phage akin to Clew-1. In fact, another bacteriophage was recently described that requires an intact *psl* operon for replication and can only plaque on PAO1 with elevated c-di-GMP levels (35). This bacteriophage, Knedl, belongs to the family of Iggyviruses (47), highlighting that more biofilm-tropic bacteriophage wait to be discovered. Our data also suggest that, for *P. aeruginosa*, using a $\Delta fliF \Delta pilA$ double mutant will allow us to enrich for biofilm-specific bacteriophage, by excluding dominant type IV pilus-dependent phage and up-regulating biofilm-specific surface structures such as Psl, Pel, and CdrA. Given the importance of biofilms in contributing to the antibiotic resistance of *P. aeruginosa* in infections such as the CF lung, catheters or wound infections, treatment modalities that are targeted towards biofilm bacteria are sorely needed. Indeed, phage Clew-1 shows some promise in this regard, since it was able to kill biofilm bacteria *in vitro*, and control *P. aeruginosa* infection in a mouse model of keratitis, which involves biofilm formation at the site of infection. While Clew-1 plaques can sometimes look turbid, this is likely a function of the fact that Clew-1 can only bind to a fraction of the bacterial population (80-90% in the case of PAO1 $\Delta fliF2$, **Fig. 3**). We have not been able to isolate Clew-1 lysogens, which is consistent with observations using other Bruynogheviruses (18, 48, 49) and argues that Clew-1 is lytic and not temperate. While many bacteriophages are not able to infect *P. aeruginosa* biofilms, some phage with the ability to target biofilms have been described, including the Bruynoghevirus Delta (18). We present here a way by which phage that target *P. aeruginosa* biofilms can be enriched during isolation.

Capsule-targeting bacteriophage tend to use LPS as a secondary receptor for tight binding to the target cell. While Clew-1 does not seem to require either A-or B-band LPS for infection (Fig. S3), we did hit several genes involved in the biosynthesis of outer core LPS in our TnSeq analysis (Table

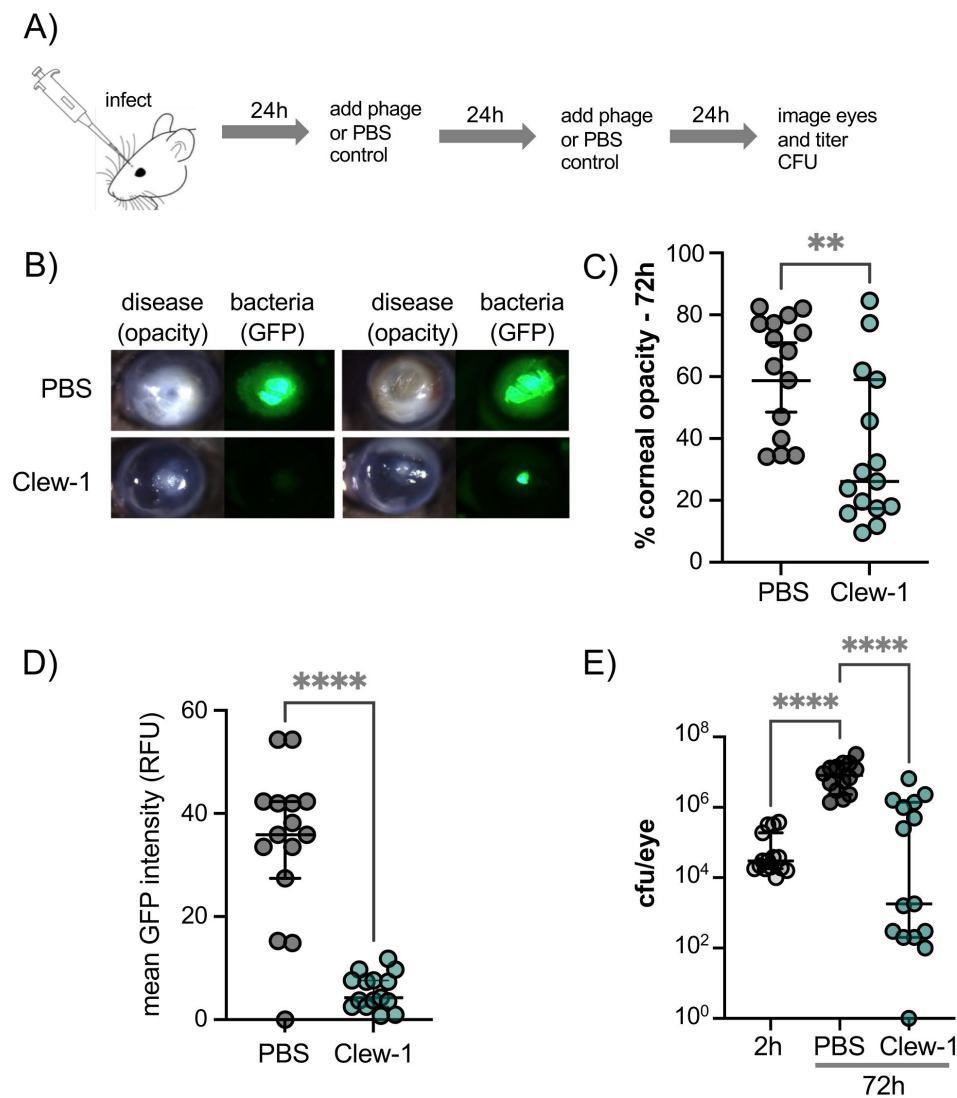


Fig. 6.

Phage Clew-1 reduces the bacterial burden in a mouse cornea model of infection.

A) Mice corneas were scratched and infected with 5×10^4 cfu strain PAO1F/pP25-GFPo, which produces GFP constitutively. Infected corneas were treated with 2×10^9 pfu phage Clew-1 or a PBS control at 24h and 48h post infection. B) At 72h post infection, the corneas were imaged by confocal microscopy to estimate the opacity (driven largely by the infiltration of neutrophils) and GFP fluorescence (produced by infecting *P. aeruginosa*). Representative images are shown in B). Opacity and GFP fluorescence were quantified by image analysis and are graphed in C) and D), respectively. E) Eyes were also homogenized and plated for CFU to determine the total bacterial burden at the end of the experiment. Significance was determined by Mann-Whitney test (C, D) or ANOVA with Kruskal Wallis post-hoc test (E) (** $p < 0.01$, **** $p < 0.0001$).

S3). We tested whether one of these genes, *ssg* (PA5001), is required for Clew-1 infection and found that this is the case (Fig. S3). How binding to Psl and LPS relate to the infection of *P. aeruginosa* by Clew-1 will be examined in a future study.

Another interesting aspect of the work described herein is the relationship between the presence of the MS-ring (FliF) and its associated proteins FliE and FliG with c-di-GMP levels. While it has been noted previously that flagellar mutations lead to increases in c-di-GMP levels and increased production of Psl upon surface contact (50), our results differ somewhat in that phage susceptibility was primarily the result of loss of the MS-ring and associated proteins (FliEFG), not, for example, the flagellar filament (FliC). This difference may be due to differences between planktonically grown (as in our study) and surface-attached bacteria. Surface contact leads to up-regulation of c-di-GMP through surface sensing by the Wsp chemosensory system (31, 51). Attached bacteria divide asymmetrically, with c-di-GMP levels decreasing in the flagellated daughter cell (33–35, 52). This asymmetry requires the phosphodiesterase Pch, which has been reported to bind to the chemosensory protein CheA (33, 34). A second phosphodiesterase, BifA, is also required for maintaining c-di-GMP homeostasis and developing an asymmetric program of cell division upon attachment to surfaces (35, 36). In our TnSeq experiment we found that insertions in flagellar genes, such as *fliF* and *fliG*, but also insertions in *pch* and *bifA* resulted in Clew-1 sensitivity. Whether the strong Clew-1 sensitivity associated with deletion of *fliF*, *fliE*, or *fliG* in our data, relative to deletions in other flagellar components, relates to a pivotal role of the MS-ring in controlling the activity of Pch and/or BifA is unclear, but worth further investigation. However, our work, along with the work of the Jenal group (35), suggests that phage such as Clew-1 or Knedl could be a useful tool for interrogating c-di-GMP signaling pathways in *P. aeruginosa*.

In summary, we have described here the isolation of a group of bacteriophages that target *P. aeruginosa* biofilms by using the exopolysaccharide Psl as a receptor. Consistent with the critical role of Psl as part of the *P. aeruginosa* biofilm matrix, we demonstrate that phage Clew-1 can replicate on biofilm bacteria and control *P. aeruginosa* in a mouse model of keratitis. Moreover, we have described a generalizable method that allows for the enrichment of biofilm-tropic bacteriophage, which is important due to their potential utility in combating biofilm infections that are notoriously recalcitrant to antibiotic therapy.

Methods

Strain construction and culture conditions

Bacterial strains were grown in LB (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) at 37°C unless indicated otherwise. Bacterial strains and plasmids used in this study are listed in table S1. Mutations were introduced into the genome of *P. aeruginosa* by allelic exchange. Briefly, flanks defining the mutation were amplified from the *P. aeruginosa* genome and cloned into plasmid pEXG2 by Gibson cloning. The primers used for the amplifications were designed using AmplifX 2.1.1 by Nicolas Jullien (Aix-Marseille Univ, CNRS, INP, Inst Neurophysiopathol, Marseille, France - <https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr>) and are noted in table S2. Plasmid pEXG2 harboring the mutation construct was transformed into *E. coli* strain SM10 and mated at 37°C into *P. aeruginosa* by mixing the donor and recipient strains on an LB plate. The mating mixture was then plated on an LB plate with 30µg/mL gentamicin and 5µg/mL triclosan and grown overnight at 37°C (selecting against the *E. coli* donor strain). Cointegrates were restructured and subsequently grown in LB lacking salt until the culture was barely turbid. The bacteria were then plated on a sucrose plate (5% sucrose, 10g/L tryptone, 5g/L yeast extract) and incubated overnight at 30°C. Sucrose resistant colonies were tested for gentamicin sensitivity and the presence of the mutation was tested by PCR.

Complementing plasmids were generated by amplifying the open reading frame and using Gibson assembly (53 [↗](#)) to clone it into pPSV37. The plasmids were then transformed into *P. aeruginosa* by electroporation.

For motility assays, individual bacterial colonies were used to inoculate motility agar plates (0.3% agar, LB plates) and incubated at 37°C for ~8h before imaging the plate.

Bacteriophage isolation and sequencing

Bacteriophage were isolated from filter sterilized waste-water samples from one of three Northeast Ohio Regional Sewer District wastewater treatment plants (Westerly, Southerly, or Easterly Sewage Treatment plant). Phage were isolated by picking individual plaques isolated on *P. aeruginosa* PAO1 Δ *fliF* Δ *pilA* and testing them for their ability to form plaques on the isolation strain or wild-type PAO1. Phage genome DNA was isolated as previously described (54 [↗](#)). Genomic DNA was sequenced by Illumina sequencing and assembled using Spades version 3.15.3 (55 [↗](#)). Genomes were annotated using PhageScope (56 [↗](#)). Genomes for Clew-1, Clew-3, Clew-6, and Clew-10, as well as Ocp-2, have been deposited in Genbank [accession# PQ790658.1, PQ790659.1, PQ790660.1, PQ790661.1, and PQ790662.1].

CsCl purification of bacteriophage

Bacteriophage were purified by CsCl gradient based on a published protocol (57 [↗](#)). Briefly, a 500mL culture of PAO1F Δ *fliF2* was grown in LB to an OD₆₀₀ of ~0.2 and inoculated with phage Clew-1 or Ocp-2 at an MOI of 0.025. After about 4h of culture, the bacteria were pelleted (12,000 x g, 15 min, 4 °C) and the supernatant was filtered through a 0.2µm filter. The supernatant was treated with DNase and RNase (1µg/mL each) overnight at 4°C. The following day, the phage were pelleted by centrifugation (overnight, 7,000 x g, 4 °C), the supernatant discarded and the pellets were resuspended in 1mL of SM buffer (50mM Tris.Cl (pH 7.5), 100mM NaCl, 8mM MgSO₄) without BSA each (~2h at 4°C). The concentrated phage prep was then spun at 12,000xg for 10 minutes to pellet remaining cell debris. At this point, 0.75g of CsCl/mL was added to the cleared supernatant and the mixture was spun for 20h at 4°C at 32000 rpm in Beckman Optima MAX-TL ultracentrifuge using an MLS-50 rotor to establish the gradient. The band with the phage was removed with a syringe and 20 gauge needle and transferred to a 3.5kDa cut-off dialysis cassette (Slide-A-Lyzer, Thermo). The phage prep was dialyzed overnight against SM, then 2x for 3h against SM, then overnight against PBS and 1x for 4h against PBS. The phage prep was tested for titer and, in the case of Clew-1, the ability to plaque on a Δ *fliF2* mutant, but not wild-type PAO1F.

Negative Stain Electron Microscopy

The negative stain experiment was done as described previously (58 [↗](#)). Briefly, a 3 µl phage Clew-1 sample (0.1-0.5 mg/ml) was loaded onto glow-discharge carbon coated grid for 60 s at room temperature and blotted with filter paper. The grid was touched with a water droplet and then blotted with filter paper. This process was repeated twice. The grid was then touched with a drop of 0.75% uranyl formate and blotted with filter paper. A second drop of 0.75% uranyl formate was applied to touch the grid for 30 s, blotted with filter paper and then air dried before data collection. The images were taken by Tecnai T20 (FEI Company) equipped with a Gatan 4K x 4K CCD camera at 80,000 x magnification.

Efficiency of plating experiments

To test phage plating efficiency, bacterial strains were back-diluted 1:200 from overnight cultures and grown to early log phase (OD₆₀₀ ~0.3). At this point, 50µL of culture were mixed with 3mL top agar (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 0.6% agar) and plated on an LB agar plate. Once solidified, 10-fold serial dilutions of the phage in SMB buffer (50mM Tris.Cl (pH 7.5), 100mM NaCl, 8mM MgSO₄, 0.1% bovine serum albumin). were spotted onto the agar using a multichannel pipette (3µL spots). The spots were allowed to dry and the plates incubated overnight at 37°.

Efficiency of Center of Infection (ECOI) experiments

P. aeruginosa strains were grown to mid-logarithmic phase in LB supplemented with 5mM MgCl₂ and 0.1mM MnCl₂ (LBMM) concentrated and resuspended at a concentration of 10⁹ cfu/mL in LBMM. 100μL bacterial suspensions were infected at an MOI of 1 with phage Clew-1 (2μL, 5*10¹⁰ pfu/mL) for 5 mins at 37°C, then pelleted (3' 10k RPM), washed 2x with 1mL LB, and resuspended in 100μL LB. The infected cells were serially diluted 10x, then 10μL of diluted, infected bacteria (10⁻⁴ for WT and *ΔfliF2*; 10⁻¹ for *ΔpslC* and *ΔfliF2 ΔpslC*) were mixed with 50μL of the mid-log PAO1F *ΔfliF2* culture and mixed with 2.5mL top agar, plated on an LB plate and incubated overnight at 37°C. The following day, plaques were counted to enumerate the cell-associated bacteria (59 [↗](#)).

TnSeq analysis

Strain PAO1F or PAO1F *ΔfliF* were mutagenized with transposon TnFac (32 [↗](#)), a mini-mariner transposon conferring gentamicin resistance. A pool 3*10⁶ (PAO1F) or 6*10⁶ (PAO1F *ΔfliF*) insertion mutants was grown overnight, then diluted 1:200 in fresh LB and grown to an OD₆₀₀ of 0.2. At this point the bacteria were infected at an MOI of 10 with phage Clew-1 and incubated for 2h to allow infection and killing of susceptible bacteria. Bacteria from 1mL culture were then pelleted, resuspended in 100μL LB with 5mM EGTA, and plated on a 3 LB plates with 30μg/mL gentamicin. The next day, surviving bacteria that had grown up were pooled and chromosomal DNA from the input and output pools were isolated using the GenElute™ Bacterial Genomic DNA Kit (Millipore-Sigma). Library preparation followed a published protocol (60 [↗](#)). Genomic DNA was sheared to ~300bp using a Covaris focused ultrasonicator. The sheared DNA was repaired using the NEBNext End Repair Module (New England Biolabs) and subsequently tailed with a polly-dC tail using Terminal Transferase (New England Biolabs). Tailed chromosomal DNA fragments were amplified in two consecutive steps, using primers Mar1x and olj376 for the first round and Mar2-InSeq paired with a TdT_Index primer for the second round, based on the published protocol (60 [↗](#)). The libraries were sequenced using an Illumina MiniSeq system using the transposon-specific primer MarSeq2. Reads with the correct Tn end sequence were mapped and tallied per site and per gene using previously described scripts ((60 [↗](#)) and <https://github.com/lq9/Tn-seq> [↗](#)). The data (hits and # of reads for each gene) are listed for each strain and condition in Table S3.

Clew-1 attachment by fluorescence microscopy

Bacteriophage Clew-1 was isolated from 500mL of culture and purified using a CsCl gradient, following a protocol published by the Center for Phage Technology at Texas A&M University. After dialysis overnight dialysis of the phage into SM buffer, the phage was dialyzed 3 more times against PBS (2x for 3h and once overnight). The purified phage was titered by efficiency of plating analysis and labeled with a Dylight594 NHS-ester (Invitrogen) at a concentration of 0.2mM, overnight in the dark. After labeling, the residual dye was removed by gel filtration using a Performa DTR gel filtration cartridge (EdgeBio) that had been equilibrated with PBS. The labeled phage preparation was titered to ensure that the phage concentration was unchanged and that the phage had not lost infectivity.

To assess phage attachment, wild type PAO1F, PAO1F *ΔfliF2*, or PAO1F *ΔfliF2 ΔpslC* harboring plasmid pP25-GFPo, which directs the constitutive production of GFP, were grown in LB to an OD₆₀₀ of ~0.3-0.4, normalized to an OD₆₀₀ of 0.3, and 0.5mL of the culture were infected for 10 minutes at 37°C with DyLight594-labeled Clew-1 phage at an MOI of 5. At this point, the infected bacteria were fixed with 1.6% paraformaldehyde [final concentration], incubated in the dark for 10 minutes, then the remaining paraformaldehyde was quenched through the addition of 200μL of 1M glycine (10 minutes at RT). The bacteria were washed 3x with 500μL of SM buffer and resuspended in 30μL SM buffer. 4μL were spotted onto an agarose pad, covered with a coverslip

and imaged using a Nikon Eclipse 90i fluorescence microscope. Images were adjusted for contrast and false-colored using the Acorn software package (Flying Meat Software), and cell-associated bacteriophage were counted in ImageJ.

Isolation and purification of Psl polysaccharide

Wild-type *P. aeruginosa* was grown for 18h in M63 minimal medium ($[\text{NH}_4]_2\text{SO}_4$, 2 g/l; KH_2PO_4 , 13.6 g/l; FeCl_3 , 0.5 mg/l, pH 7) supplemented with 0.5% Casamino acids (BD), 1 mM MgCl_2 , and 0.2% glucose. Bacterial cells were removed by centrifugation, the supernatant lyophilized, and Psl isolated by affinity chromatography.

The affinity column was prepared by resuspending 0.286 g of CNBr activated Sepharose (Purchased from GE Healthcare; cat#17-0430-01) in 1 M HCl (1 mL). It was subsequently filtered and washed with 1 M HCl (60 mL) and coupling buffer (1.5 mL; 0.1 M NaHCO_3 , 0.5 M NaCl, pH = 9). The activated Sepharose was added to a solution of Cam-003 ([61](#)) in coupling buffer (0.5 mL; 10 mg/mL) and was incubated for two hours at room temperature. The solvent was then removed by filtration, and the beads were washed with coupling buffer (3×1 mL). After removal of the solvent the sepharose was incubated with blocking buffer (2 mL; 0.1 M Tris, 0.5 M NaCl, pH = 8.5) for 2h at ambient temperature. The beads were washed with wash buffer (4 mL) and coupling buffer (4 mL) for four cycles until the OD280 of the wash was <0.01 . The derivatized beads were loaded onto a column and after washing with 5 column volumes of PBS-buffer (pH=7.4) the affinity column was ready to use.

Crude Psl (100 mg) was dialyzed (Thermo Scientific SnakeSkin™ Dialysis Tubing 3K MWCO) for three days and six exchanges of water and then concentrated to a final volume of 1 mL (10 mg/mL). It was loaded onto the affinity column and washed with PBS-buffer (4 mL) in order to remove all not-retained material. Next, the captured Psl was eluted with glycine buffer (4 mL; 100 mM glycine $\times$ HCl, pH=2.7). The glycine fraction was dialyzed (3K MWCO) for three days and six exchanges of water and after lyophilization, pure Psl (80 μg) was obtained.

The solution was lyophilized, and the residue was fractionated by gel permeation chromatography on a Bio-Gel P-2 column (90×1.5 cm), eluted with 10 mM NH_4HCO_3 . The collected fractions contained different size of Psl material: dimer (two repeating units), trimer (three repeating units) and high molecular weight polysaccharide. The high molecular weight polysaccharide fraction was used in our experiments.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry experiments were performed using Bruker ultrafleXtreme (Bruker Daltonics) mass spectrometer. All spectra were recorded in reflector positive-ion mode and the acquisition mass range was 200–6000 Da. Samples were prepared by mixing on the target 0.5 μL sample solutions with 0.5 μL aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

Precipitation of Clew-1 from culture supernatants and using purified Psl

For experiments in which binding of Clew-1 to Psl in culture supernatants was tested, PAO1F $\Delta\text{fliF}2$ or PAO1F $\Delta\text{fliF}2 \Delta\text{pslC}$ were grown to mid-logarithmic phase, then the bacteria were pelleted and the supernatants filter sterilized using a 0.2 μm filter. Culture supernatants were mixed with 1 μL of a rabbit, anti-Psl antibody ([37](#)) as well as 10^7 pfu of phage Clew-1. The mixture was incubated on ice for 1h, then 10 μL of magnetic protein A beads (BioRad), washed 2x with SMB + 0.05% Triton X-100 (SMBT) were added to the mixture and incubated for an additional 30 minutes on ice. The magnetic beads were collected, washed 3x with SMBT and resuspended in 100 μL of SMBT. Presence of Clew-1 in input and output samples was determined by quantitative PCR using primers designed to amplify the tail fiber gene, gp12.

Experiments using partially purified, cell-associated Psl were carried out in SM buffer. 100µL of SM-buffer were mixed with 10⁷ pfu of phage Clew-1, as well as 1µg of a partially purified, deproteinated fraction of cell-associated Psl (62 [↗](#)) and incubated for 1h on ice. All subsequent steps were the same as for the culture supernatants, above. Samples were resuspended in 100µL SMBT before quantifying Clew-1 levels.

Experiments using affinity purified, biotinylated Psl were carried out in SM. Here too, 10⁷ pfu Clew-1 were incubated with 1µg of affinity purified, biotinylated Psl. The samples were either incubated with streptavidin-coated Dynabeads (M280, Invitrogen) to precipitate the biotinylated Psl (or with magnetic protein A beads as a specificity control). Otherwise, the experiments were carried out as for the partially purified, cell-associated Psl fraction, above.

Static biofilm experiments

Static biofilm experiments were carried out based on a published protocol (63 [↗](#)). *P. aeruginosa* PAO1F was grown to mid-logarithmic phase in LB and then diluted to an OD₆₀₀ of 0.05 and used to inoculate either 5mL polystyrene tubes (1mL) or 6 wells in a polystyrene 96-well plate (150µL). The cultures were incubated overnight at 37°C in a humidified incubator with a 5% CO₂ atmosphere. The following day, 1 set of biofilm samples was washed three times with PBS, for 20 minutes fixed with 95% ethanol, and subsequently air dried after removing the ethanol. The remaining biofilm samples were washed 2x with PBS and reconstituted with pre-warmed LB (1.2mL in 5mL tube biofilms, 200µL in 96-well plates), or LB harboring either 10⁹ pfu of phage Clew-1 or phage Ocp-2. The biofilm samples were again incubated overnight at 37°C in a humidified incubator with a 5% CO₂ atmosphere, and subsequently washed and fixed as the control samples, above. The fixed and dried biofilms were stained with a 0.1% solution of crystal violet in water for 30 minutes, the staining solution was removed, and the biofilms were washed 2x with mili-Q water and rinsed twice with deionized water before drying the stained biofilm samples. The stained biofilms in the 5mL tubes were photographed against a white background. The stained biofilms in the 96-well plates were incubated for 20 minutes in 200µL 30% acetic acid to solubilize the crystal violet stain, which was subsequently quantified by spectrophotometry (absorbance at 590nm).

Bead biofilm assay

For each technical replicate (3 per condition), one 6mm natural bead (Precision Plastic Ball Company) was placed in the well of a 24-well plate, and covered with 1mL of PAO1F in LB at an OD₆₀₀ of 0.05. The plate was sealed with parafilm and shaken overnight (150 rpm, 37°C). The following day all beads were washed by moving the bead to 1mL sterile PBS with a forceps. Three beads were placed individually into microcentrifuge tubes with 1mL PBS with 5mM MgCl₂, sonicated for 10 minutes in a water bath sonicator, and titered on LB plates to determine the input. The remaining beads were transferred into fresh wells of the 24-well plate with either 1mL LB or 1mL LB with 10⁹ pfu Clew-1 and incubated with shaking (37°C, 150rpm) for another 2 hours. At this point, the supernatant bacteria were titered, the beads were washed 1x with PBS and placed individually into microcentrifuge tubes with 1mL PBS with 5mM MgCl₂ and sonicated and titered, as above. The bacterial titers of the supernatant and bead-associated bacteria were added to obtain the total CFU in the well and compared to the input CFU (bead titer at T₀).

In some experiments, the phage treated samples were treated with a virucide before titering (64 [↗](#)). Here 250µL of 15% wt/vol, filter-sterilized black Tea was mixed with 730µL Milli-Q H₂O and mixed with 20µL of a freshly made, 200mM FeSO₄ solution just before use (4mM FeSO₄ final). The virucide was mixed with the sample, 1:1 and incubated for 10 minutes at room temperature before titering the bacteria. However, this did not significantly alter the outcome of the experiment, and the data presented are a combination of all experiments, with and without virucide.

LIVE/DEAD imaging of biofilms

Biofilms were generated in 8-well chamber slides (ibidi) by inoculating the well with 200 μ L of mid-log PAO1F (diluted to an OD₆₀₀ 0.05) and incubating the slide overnight in a humidified incubator (5% CO₂). The next day, the staining solution was added to each well (1 μ L of a 1:10 dilution of each component in DMSO + 8 μ L LB) as well as 40 μ L LB (no phage control) or 40 μ L LB with 10⁹ pfu Clew-1. The concentrations were based on a published protocol for using LIVE/DEAD staining to test antibiotic susceptibility of biofilms (65). The slides were incubated overnight and imaged using a 60x objective on a Nikon AX R laser scanning confocal microscope. The Z-stacks were analyzed and the propidium iodide positive fraction of the total biofilm volume was determined using the BiofilmQ software package (66).

Mouse keratitis model

C57BL/6 mice were purchased from Jackson Laboratories. The mice were housed in pathogen free conditions in microisolator cages and were treated according to institutional guidelines following approval by the University of California IACUC.

Overnight cultures of *P. aeruginosa* PAO1F/pP25-GFPo were grown to log phase (OD₆₀₀ of 0.2) in LB broth, then washed and resuspended in PBS at 2.5 × 10⁷ bacteria/mL. 7-12 weeks old C57BL/6 mice were anesthetized with ketamine/xylazine solution, the corneal epithelium was abraded with three parallel scratches using a sterile 26-gauge needle, and 2 μ L of a suspension of bacteria were added topically (approximately 5 × 10⁴ cfu per eye). At 24h and 48h, the mice were anesthetized and treated with 5 × 10⁹ pfu CsCl purified phage Clew-1 in PBS, or PBS alone. At 72h the mice were euthanized, and corneas were imaged by brightfield microscopy to detect opacification, or by fluorescence microscopy to detect GFP-expressing bacteria. Fluorescent intensity images were quantified using Image J software (NIH). To determine the bacterial load, whole eyes were homogenized in PBS using a TissueLyser II (Qiagen, 30 Hz for 3 minutes), and homogenates were serially diluted plated on LB agar plates for quantification of colony forming units (CFU) by manual counting. CFU were also determined at 2h to confirm the inoculum.

Growth curves

Strains PAO1F, PAO1F Δ *flhF*2, PAO1F Δ *psl*C, and PAO1F Δ *flhF*2 Δ *psl*C were grown to mid-logarithmic phase in LB, then diluted to a concentration of 10⁸ cfu/mL. For growth curve measurements (OD600), 3 technical replicates were set up in a 96-well plate for each strain/condition. 100 μ L of culture were mixed with 10 μ L PBS or 10 μ L with 10⁸ pfu Clew-1 and incubated at 37°C in an Agilent Cytation 5 Imaging Plate Reader with a heated chamber and orbital rotation between OD600 measurements. OD600 readings were taken every 5 minutes.

Culture Supernatant Psl blot

Strains PAO1F Δ *flhF*2 and PAO1F Δ *flhF*2 Δ *psl*C were grown to mid-logarithmic phase (OD600 ~0.5), the bacteria pelleted by centrifugation and the culture supernatant was sterilized using a 0.22 μ m syringe filter. 0.5mL supernatant samples were incubated for 1h at 37°C with or without 10⁷ pfu Clew-1 and subsequently diluted three times at a 1:3 ratio. 2 μ L of the undiluted culture supernatants and of each dilution were spotted onto a nitrocellulose filter and allowed to air-dry. The filter was then blocked with 5% non-fat milk in TBS-T (20mM Tris.Cl, 150mM NaCl, 0.1% Tween-20) for 30 minutes, washed 2x with TBS-T and incubated with the primary anti-Psl antibody (diluted 1:3000) in TBS-T overnight at 4°C. The following day, the blot was washed 3x with TBS-T, then incubated with secondary antibody (horse-radish peroxidase conjugated goat anti-rabbit antibody, Sigma) diluted 1:10000 in TBS-T for ~3h at room temperature. The blot was then washed 3x with TBS-T and developed using the Advanta WesternBright Sirius HRP substrate and imaged on a GE ImageQuant LAS4000 imager.

Analysis of Evolutionary Relatedness

The evolutionary relationship between Clew bacteriophage and other Bruynogheviruses was carried out using the Maximum Likelihood method and JTT matrix-based model (67 [↗](#)). The tree with the highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 12 amino acid sequences. There was a total of 485 positions in the final dataset. Evolutionary analyses were conducted in MEGA11(68 [↗](#), 69 [↗](#)). The genome comparison between Luz24 and the Clew phages was visualized using EasyFig (70 [↗](#)).

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Additional files

Supplementary Material [↗](#)

TnSeq data [↗](#)

Additional information

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Reviewer #1 (Public review):

Summary:

Walton et al. set out to isolate new phages targeting the opportunistic pathogen *Pseudomonas aeruginosa*. Using a double Δ fliF Δ pilA mutant strain, they were able to isolate 4 new phages, CLEW-1, -3, -6 and -10, that were unable to infect the parental PAO1F Wt strain. Further experiments showed that the 4 phages were only able to infect a Δ fliF strain, indicating a role of the MS-protein in the flagellum complex. Through further mutational analysis of the flagellum apparatus, the authors were able to identify the involvement of c-di-GMP in phage infection. Depletion of c-di-GMP levels by an inducible phosphodiesterase render the bacteria resistant to phage infection, while elevation of c-di-GMP through the Wsp system made the cells sensitive to infection by CLEW-1. Using TnSeq, the authors were able to not only reaffirm the involvement of c-di-GMP in phage infection but also able to identify the exopolysaccharide PSL as a downstream target for CLEW-1. C-di-GMP is a known regulator of PSL biosynthesis. The authors show that CLEW-1 binds directly to PSL on the cell surface and that deletion of the *pslC* gene resulted in complete phage resistance. The authors also provide evidence that the phage - PSL interaction happens during the biofilm mode of growth and that the addition of the CLEW-1 phage specifically resulted in a significant loss of biofilm biomass. Lastly, the authors set out to test if CLEW-1 could be used to resolve a biofilm infection using a mouse keratitis model. Unfortunately, while the authors noted a reduction in bacterial load assessed by GFP fluorescence, the keratitis did not resolve under the tested parameters.

Strengths:

The experiments carried out in this manuscript are thoughtful and rational, and sufficient explanation is provided for why the authors chose each specific set of experiments. The data presented strongly supports their conclusions and they give present compelling explanations for any deviation. The authors have not only developed a new technique for screening for phages targeting *P. aeruginosa*, but also highlights the importance of looking for phages during the biofilm mode of growth, as opposed to the more standard techniques involving planktonic cultures.

Weaknesses:

The authors did not include host-range testing or resistance development in this study, which would have strengthened the paper. Additionally, further characterisation of the CLEW-1 interaction with PSL at the molecular level would also have been welcomed. However, this will likely be the subject of future studies.

<https://doi.org/10.7554/eLife.102352.2.sa2>

Reviewer #2 (Public review):

This manuscript by Walton et al. suggests that they have identified a new bacteriophage that uses the exopolysaccharide Psl from *Pseudomonas aeruginosa* (PA) as a receptor. As Psl is an important component in biofilms, the authors suggest that this phage (and others similarly isolated) may be able to specifically target biofilm-growing bacteria.

Comments on revised version:

The authors have generally responded well to the reviewers' comments. This has served to improve this manuscript that has identified a new bacteriophage that uses the exopolysaccharide Psl from *Pseudomonas aeruginosa* as a receptor.

<https://doi.org/10.7554/eLife.102352.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public review):

Summary:

*Walton et al. set out to isolate new phages targeting the opportunistic pathogen *Pseudomonas aeruginosa*. Using a double Δ fliF Δ pilA mutant strain, they were able to isolate 4 new phages, CLEW-1, -3, -6, and -10, which were unable to infect the parental PAO1F Wt strain. Further experiments showed that the 4 phages were only able to infect a Δ fliF strain, indicating a role of the MS-protein in the flagellum complex. Through further mutational analysis of the flagellum apparatus, the authors were able to identify the involvement of c-di-GMP in phage infection. Depletion of c-di-GMP levels by an inducible phosphodiesterase renders the bacteria resistant to phage infection, while elevation of c-di-GMP through the Wsp system made the cells sensitive to infection by CLEW-1. Using TnSeq, the authors were able to not only reaffirm the involvement of c-di-GMP in phage infection but also able to identify the exopolysaccharide PSL as a downstream target for CLEW-1. C-di-GMP is a known regulator of PSL biosynthesis. The authors show that CLEW-1 binds directly to PSL on the cell surface and that deletion of the pslC gene resulted in complete phage resistance. The authors also provide evidence that the phage-PSL interaction happens during the biofilm mode of growth and that the addition of the CLEW-1 phage specifically resulted in a significant loss of biofilm biomass. Lastly, the authors set out to test if CLEW-1 could be used to resolve a biofilm infection using a mouse keratitis model. Unfortunately, while the authors noted a reduction in bacterial load assessed by GFP fluorescence, the keratitis did not resolve under the tested parameters.*

Strengths:

*The experiments carried out in this manuscript are thoughtful and rational and sufficient explanation is provided for why the authors chose each specific set of experiments. The data presented strongly supports their conclusions and they give present compelling explanations for any deviation. The authors have not only developed a new technique for screening for phages targeting *P. aeruginosa*, but also highlight the importance of looking for phages during the biofilm mode of growth, as opposed to the more standard techniques involving planktonic cultures.*

Weaknesses:

While the paper is strong, I do feel that further discussions could have gone into the decision to focus on CLEW-1 for the majority of the paper. The paper also doesn't provide any detailed information on the genetic composition of the phages. It is unclear if the phages isolated are temperate or virulent. Many temperate phages enter the lytic cycle in response to QS signalling, and while the data as it is doesn't suggest that is the case, perhaps the paper would be strengthened by further elimination of this possibility. At the very least it might be worth mentioning in the discussion section.

Thank you for your review. The genomes of all Clew phages and Ocp-2 have been uploaded [Genbank accession# PQ790658.1, PQ790659.1, PQ790660.1, PQ790661.1, and PQ790662.1]. It turns out that the Clew phage are highly related, which is highlighted by the genomic comparison in the supplementary figure S1. It therefore made sense to focus our in-depth analysis on one of the phage. We have included a supplementary figure (S1A), demonstrating that the other Clew phage also require an intact *psl* locus for infection, to make that logic clearer. The phage are virulent (there is apparently a bit of a debate about this with regard to Bruynogheviruses, but we have not been able to isolate lysogens). This is now mentioned in the discussion.

Reviewer #2 (Public review):

*This manuscript by Walton et al. suggests that they have identified a new bacteriophage that uses the exopolysaccharide Psl from *Pseudomonas aeruginosa* (PA) as a receptor. As Psl is an important component in biofilms, the authors suggest that this phage (and others similarly isolated) may be able to specifically target biofilm-growing bacteria. While an interesting suggestion, the manner in which this paper is written makes it difficult to draw this conclusion. Also, some of the results do not directly follow from the data as presented and some relevant controls seem to be missing.*

Thank you for your review. We would argue that the combination of demonstrating Psl-dependent binding of Clew-1 to *P. aeruginosa*, as well as demonstration of direct binding of Clew-1 to affinity-purified Psl, indicates that the phage binds directly to Psl and uses it as a receptor. In looking at the recommendations, it appears that the remark about controls refers to not using the Δ pslC mutant alone (as opposed to the Δ fliF2 Δ pslC double mutant) as a control for some of the binding experiments. However, since the Δ fliF2 mutant is more permissive for phage infection, analyzing the effect of deleting *pslC* in the context of the Δ fliF2 mutant background is the more stringent test.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

*First off, I would like to congratulate the authors on this study and manuscript. It is very well executed and the writing and flow of the paper are excellent. The findings are intriguing and I believe the paper will be very well received by both the phage, *Pseudomonas*, and biofilm communities.*

Thank you for your kind review of our work!

I have very little to critique about the paper but I have listed a few suggestions that I believe could strengthen the paper if corrected:

Comments and suggestions:

(1) The paper initially describes 4 isolated phages but no rationale is given for why they chose to continue with CLEW-1, as opposed to CLEW-3, -6, and -10. The paper would benefit from going into more detail with phage genomics and perhaps characterize the phage receptor binding to PSL.

Clew-1, -3, -6, and -10 are actually quite similar to one another. The genomes are now uploaded to Genbank [accession# PQ790658.1, PQ790659.1, PQ790660.1, and PQ790661.1]. They all require an intact Psl locus for infection, we have updated Fig. S1 to show this for the remaining Clew phage. In the end, it made sense to focus on one of these related phage and characterize it in depth.

(2) PA14 was used in some experiments but not listed in the strain table.

Thank you, this has been added in the resubmission.

(3) Would have been good to see more strains/isolates used.

We are currently characterizing the host range of Clew-1. It appears to be pretty limited, but this will likely be included in another paper that will focus on host range, not only of Clew-1, but other biofilm-tropic phage that we have isolated since then.

(4) Could purified PSL be added to make non-PSL strain (like PA14) susceptible?

We have tried adding purified Psl to a psl mutant strain, but this does not result phage sensitivity. Further characterization of the Psl receptor, is something we are currently working on, but will likely be a much bigger story than can be easily accommodated in a revised manuscript.

(5) No data on resistance development.

We have not done this as yet.

(6) Alternative biofilm models. Both in vitro and in vivo.

We agree that exploring the interaction of Clew-1 with biofilms in greater detail is a logical next step. The revised manuscript does have data on the viability of *P. aeruginosa* biofilm bacteria after Clew-1 infection using either a bead biofilm model or LIVE/DEAD staining of static biofilms. However, expanding on this further (setting up flow-cell biofilms, developing reporters to monitor phage infection, etc.) is beyond the scope of this initial report and characterization of Clew-1.

(7) There is a mistake in at least one reference. An unknown author is listed in reference 48. DA Garsin is not part of the paper. Might be worth looking into further mistakes in the reference list as I suspect this might be an issue related to the citation software.

Thank you. Yes, odd how that extra author got snuck in. This has been corrected.

(8) I don't seem to be able to locate a Genbank file or accession number. If it wasn't performed how was evolutionary relatedness data generated?

The genomes of all Clew phages and Ocp-2 have been uploaded [Genbank accession# PQ790658.1, PQ790659.1, PQ790660.1, PQ790661.1, and PQ790662.1]

(9) No genomic information about the isolated phages. Are they temperate or virulent? This would be important information as only strictly lytic phages are currently deemed appropriate for phage therapy.

These phage are virulent. We have only been able to isolate resistant bacteria from plaques, but they do not harbor the phage (as detected by PCR). This matches what other researchers have found for Bruynogheviruses.

Reviewer #2 (Recommendations for the authors):

Others have used different PA mutants lacking known phage receptors to pan for new phages. However, it is not totally clear how the screen here was selected for the Psl-specific phage. The authors used flagella and pili mutants and found Clew-1, -3, -6, and -10. These were all Bruynogheviruses. They also isolated a phage that uses the O antigen as a receptor. The family of this latter phage and how it is known to use this as a receptor is not described.

Phage Ocp-2 is a Pbunavirus. We added new supplementary figure S3, addressing the O-antigen receptor.

The authors focused on Clew-1, but the receptor for these other Clew phages is not presented. For Clew-1 the phage could plaque on the fliF deletion mutant but not the wild-type strain. The reason for this never appears to be addressed. The authors leap to consider the involvement of c-di-GMP, but how this relates to fliF appears to be lacking.

We have included a supplementary figure demonstrating that all the Clew phage require Psl for infection (Fig. S1A). As noted above, we have uploaded the genomic data that underpins the comparison in our supplementary figure. The phage are all closely related. It therefore made sense to focus on one of the phage for the analysis.

It is particularly unclear why this phage doesn't plaque on PAO1 as this strain does make Psl. Related to this, it actually looks like something is happening to PAO1 in Figure S4 (although what units are on the x-axis is not entirely clear).

We hypothesize that the fraction of susceptible cells in the population dictates whether the phage can make overt plaques. The supplementary figure S4 indicates that a subpopulation of the wild-type culture is susceptible and this is borne out by the fraction of wild type cells that the phage can bind to (~50%). The fliF mutation increases this frequency of susceptible cells to 80-90% (Fig. 3).

The Tnseq screen to identify receptors is clever and identifies additional phosphodiesterase genes, the deletion of which makes PAO1 susceptible. And the screen to find resistant fliF mutants identified genes involved in Psl. However, the link between the phosphodiesterase mutants and the amount of Psl produced never appears to be established. And the statement that Psl is required for infection (line 130) is never actually tested.

The link between c-di-GMP and Psl production is well-established in the literature. I think the requirement for Psl in infection is demonstrated multiple ways, including lack of plaque formation on psl mutant strains and lack of phage binding to strains that do not produce Psl, direct binding of the phage to affinity purified Psl.

Figure 2C describes using a Δ fliF2 strain but how this is different (or if it is different) from Δ fliF described in the text is never explained.

The difference in the deletions is explained in table S1, in the description for the deletion constructs used in their construction, pEXG2- Δ fliF and pEXG2- Δ fliF2 (Δ fliF2 is smaller than Δ fliF and can be complemented completely with our complementing plasmid, pP37-fliF, which is the reason why we used the Δ fliF2 mutation going forward, rather than the Δ fliF mutation on which the phage was originally isolated).

Similarly, there is a sentence (line 138) that "Attachment of Clew-1 is Psl-dependent" but this would appear to have no context.

The relevant figure, Fig. 3, is cited in the next sentence and is the subject of the remaining paragraphs in this section of the manuscript.

For Figure 3B, why wasn't the single Δ pslC mutant visualized in this analysis? Similar questions relate to the data in Figure 4.

Analyzing the effect of the pslC deletion in the context of the Δ fliF2 mutant background, which is more permissive for phage infection, is the more stringent test.

The efficacy of Clew-1 in the mouse keratitis model is intriguing but it is unclear why the CFU/eye are so variable. The description of how the experiment was actually carried out is not clear. Was only one eye scratched or both? Were controls included with a scratch and no bacteria ({plus minus} phage)?

One eye was infected. We did not conduct a no-bacteria control (just scratching the cornea is not sufficient to cause disease). The revised manuscript has an updated animal experiment in which we carried the infection forward to 72h with two phage treatments. Following this regiment, there is a significant decrease in CFU, as well as corneal opacity (disease). Variability of the data is a fairly common feature in animal experiments. There are a number of factors, such as does the mouse blink and remove some of the inoculum shortly after deposition of the bacteria or the phage after each treatment that could explain this variability.

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