Microbial Biotechnology

WILEY

MICROBIAL BIOTECHNOLOGY Open Access

RESEARCH ARTICLE OPEN ACCESS

A Phage-Based Approach to Identify Antivirulence Inhibitors of Bacterial Type IV Pili

Tori M. Shimozono[1] | Nancy J. Vogelaar[2] | Megan T. O'Hara[1] | Zhaomin Yang[1,2,3]

[1]Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia, USA | [2]Virginia Tech Center for Drug Discovery, Virginia Tech, Blacksburg, Virginia, USA | [3]Center for Emerging, Zoonotic, and Arthropod-Borne Pathogens, Virginia Tech, Blacksburg, Virginia, USA

Correspondence: Zhaomin Yang (zmyang@vt.edu)

Received: 25 October 2024 | **Revised:** 18 December 2024 | **Accepted:** 26 December 2024

Funding: The research in the Yang laboratory is partially supported by the National Science Foundation grants MCB-1919455 and EFMA-2318093, as well as the CHRB award 208-07-23 to Z.Y. The Yang laboratory additionally received a pilot grant from the Virginia Tech Center for Emerging, Zoonotic and Arthropod-borne Pathogens. M.T.O. was the recipient of the Presidential Scholarship Initiative from Virginia Tech and the David Lyerly Undergraduate Microbiology Research Fellowship from the Department of Biological Sciences at Virginia Tech. The screening work was made possible by the support from the Virginia Tech Fralin Life Sciences Institute via FLSI Award 240065.

Keywords: antivirulence | high-throughput screen | phage | *Pseudomonas aeruginosa* | type 4 pilus (T4P)

ABSTRACT

The increasing threat of antibiotic resistance underscores the urgent need for innovative strategies to combat infectious diseases, including the development of antivirulants. Microbial pathogens rely on their virulence factors to initiate and sustain infections. Antivirulants are small molecules designed to target virulence factors, thereby attenuating the virulence of infectious microbes. The bacterial type IV pilus (T4P), an extracellular protein filament that depends on the T4P machinery (T4PM) for its biogenesis, dynamics and function, is a key virulence factor in many significant bacterial pathogens. While the T4PM presents a promising antivirulence target, the systematic identification of inhibitors for its multiple protein constituents remains a considerable challenge. Here we report a novel high-throughput screening (HTS) approach for discovering T4P inhibitors. It uses *Pseudomonas aeruginosa,* a high-priority pathogen, in combination with its T4P-targeting phage, φKMV. Screening of a library of 2168 compounds using an optimised protocol led to the identification of tuspetinib, based on its deterrence of the lysis of *P. aeruginosa* by φKMV. Our findings show that tuspetinib also inhibits two additional T4P-targeting phages, while having no effect on a phage that recognises lipopolysaccharides as its receptor. Additionally, tuspetinib impedes T4P-mediated motility in *P. aeruginosa* and *Acinetobacter* species without impacting growth or flagellar motility. This bacterium-phage pairing approach is applicable to a broad range of virulence factors that are required for phage infection, paving ways for the development of advanced chemotherapeutics against antibiotic-resistant infections.

1 | Introduction

Antimicrobial resistance (AMR) is a clear and present global threat to human health. It was estimated that 4.95 million and 1.27 million deaths in 2019 worldwide were associated with and directly attributed to bacterial AMR, respectively (Antimicrobial Resistance Collaborators 2022). A recent forecast (GBD Antimicrobial Resistance Collaborators 2024) projected that 39.1 million and 169 million of cumulative deaths in the next 25 years will be associated with and attributable to antimicrobial resistance, respectively. It is therefore imperative to develop new and innovative countermeasures against microbial infections. Antibiotics in both clinical and environmental settings select for and enrich resistance in all bacteria. This leads to the inevitable emergence and spread of bacterial AMR in both pathogenic and environmental microbes with horizontal gene transfer further

exacerbating the problem. Antivirulence is a promising strategy to combat the AMR crisis because it targets and disarms bacterial pathogens more selectively (Dickey, Cheung, and Otto 2017; Lau et al. 2023). Virulence factors are components or products of bacterial pathogens that contribute specifically to their ability to cause infections, but not their cell viability or general survival. Antivirulence therapeutics aim to inhibit or inactivate virulence factors, thereby attenuating or eliminating bacterial virulence. Antivirulants are small molecules that inhibit the function or production of virulence factors. Since virulence factors, in contrast to the targets of antibiotics, are not essential for bacterial viability, antivirulants are expected to have limited impacts on environmental and normal human microbiomes. The selectivity of antivirulants helps to mitigate a key driver for AMR development and spread, offering an encouraging alternative to traditional antibiotics.

The bacterial type IV pilus (T4P) is expressed as a virulence factor by many pathogens (Craig, Pique, and Tainer 2004; Piepenbrink and Sundberg 2016) that urgently need new countermeasures. These include the priority Gram-negative pathogens *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (WHO 2024). The extracellular pilus filament is composed of the major pilin PilA and minor pilins. The T4P machinery (T4PM), which is responsible for T4P biogenesis, dynamics and function, is composed of a set of core T4P or Pil proteins that organise into three interconnected complexes in Gram-negative bacteria (Hospenthal, Costa, and Waksman 2017; Craig, Forest, and Maier 2019; Ellison, Whitfield, and Brun 2022). These are the outer membrane (OM) secretin complex, the periplasmic alignment complex and the cytoplasmic membrane (CM)-associated motor complex. The OM complex contains a PilQ secretin multimeric channel that allows the pilus filament to pass through. The alignment complex includes multiple copies of PilM, PilN and PilO. The motor complex consists of the transmembrane protein PilC and one or more of the cytoplasmic ATPases PilB, PilT and PilU. PilB is the T4P assembly or extension motor, while PilT and PilU function as the main and the auxiliary disassembly or retraction motors, respectively. In bacterial pathogenesis, the T4P functions as an adhesin, facilitating bacterial attachment to biotic and abiotic surfaces (Craig, Pique, and Tainer 2004). The T4P also functions in twitching motility (Wall and Kaiser 1999; Wadhwa and Berg 2022) and natural competence (Dubnau and Blokesch 2019), in addition to its roles in bacterial pathogenesis.

While the T4PM is a validated virulence factor in many pathogens, the discovery of its inhibitors for the development of antivirulence chemotherapeutics remains challenging. A significant obstacle is the lack of assays that can be scaled up for the high-throughput screen (HTS) of compound libraries to identify T4P antagonists. As far as we are aware, there are two types of HTS assays that have been reported in the literature. One was to screen for molecules that disrupted the adhesion of *Neisseria menengitidis* to cultured human cells. This approach led to the discovery of P4MP4, an inhibitor of the PilB equivalent in *Neisseria* (Aubey et al. 2019; Duménil 2019), this approach, which is rigorous but difficult to scale up, targeted neisserial adhesins, only one of which is T4P. The other approach took advantage of *Chloracidobacterium thermophilum* PilB as a robust ATPase in vitro. Its use as a purified enzyme in HTS has led to the discovery of PilB inhibitors such as quercetin and benserazide that exhibited anti-T4P activity in multiple bacteria (Dye et al. 2021, 2022). While these PilB-based HTS assays are scalable and efficient, it targets only one of the many components of the T4PM and the inhibitors from such a screen may not be cell permeable. The limitations of these methods necessitate the development of innovative means for the experimental discovery of T4P inhibitors.

Here, we describe a novel approach to HTSs for the discovery of anti-T4P compounds using *P. aeruginosa* and its lytic phage φKMV as vehicles. We first confirmed and extended the previous report that φKMV uses the T4P as its receptor (Chibeu et al. 2009). An assay for a HTS based on this bacterium-phage pair was conceptualised and developed to screen for compounds that protect *P. aeruginosa* from φKMV lysis in liquid culture by monitoring optical density. Using an optimised protocol, tuspetinib was identified from a library of 2168 compounds, and it was confirmed to be a T4P inhibitor in *P. aeruginosa* and *Acinetobacter* species. Tuspetinib was found to protect *P. aeruginosa* from φKMV and two other T4P-targeting phages but not an LPS-targeting one. In addition, we demonstrate that tuspetinib inhibits T4P-mediated motility of *P. aeruginosa* and *Acinetobacter* species without observable effects on bacterial growth or the flagellated swimming motility of *P. aeruginosa*. This assay is powerful and scalable for discovering cell-permeable inhibitors against all possible T4PM constituents. The approach of using a bacterium-phage pair to screen for inhibitors is widely applicable to other bacterial structures that are used as phage receptors (de Melo, Morency, and Moineau 2024), providing a new and exciting venue to facilitate the research on antivirulence chemotherapeutics against bacterial AMR infections.

2 | Experimental Procedures

2.1 | Bacterial Strains and Phages

Table S1 lists *P. aeruginosa* strains used in this study. They were propagated on LB agar plates or in LB liquid media on an orbital shaker at 37°C. Other bacteria used in this study include *Acinetobacter nosocomialis* M2 (Niu et al. 2008) and its Δ*pilA* mutant (Harding et al. 2013) as well as *A. baumannii* strains AYE (Adams et al. 2008) and AB0057 (Hamidian et al. 2017). They were grown on MacConkey agar at 37°C.

P. aeruginosa phages used are the T4P-targeting φKMV (Lavigne et al. 2003), Luz19 (Lammens et al. 2009) and Ab05 (Essoh et al. 2015) as well as the LPS-targeting Luz7 (Ceyssens et al. 2010). Phage stocks were prepared (Budzik et al. 2004) and titered (Dreyer and Campbell-Renton 1933) similarly as previous described.

2.2 | Phage Infection in Liquid Culture

The infection of *P. aeruginosa* with φKMV at MOI = 10 was conducted with 96-well plates with a total volume of 100 μL per well. Here 5×10^7 cells from an overnight culture were mixed with 5×10^8 pfu of φKMV in LB with 10 mM $CaCl_2$ (LB + Ca).

Experiments at lower MOIs were conducted using a 384-well plate with each well containing 25 μL of log-phase *P. aeruginosa* cells at 2×10^8 cells/mL and an equal volume of φKMV at appropriate dilutions. The plate was incubated at 37°C in a Tecan Infinite F200 Pro Microplate reader with 250 rpm shaking which was paused during OD_{600} measurements.

2.3 | High-Throughput Screening

Used in this study were a Selleckchem (Boston, MA, USA) and a MedchemExpress (Monmouth Junction, NJ, USA) libraries with 273 and 2168 kinase inhibitors, respectively. Library screening was performed with 384-well plates at the Virginia Tech Center for Drug Discovery. Each well of a plate was first aliquoted 0.3 μL of a library compound in DMSO, followed by the addition of 30 μL of log-phase PAO1 cells at 2×10^8 cells/mL and 30 μL of φKMV at 4×10^4 pfu/mL, both in LB + Ca broth. OD_{600} was measured with a SpectraMax M5 plate reader pre- and post-incubation at 37°C with 400 rpm orbital shaking for 4 h. The difference between the two OD_{600} readings was used for data analysis and the identification of potential hits. Each screen plate included wells with DMSO and *P. aeruginosa* either without or with φKMV as positive and negative controls. Z′ factors were calculated from these controls as previously defined (Zhang, Chung, and Oldenburg 1999). In the trial screen with the smaller library, the effects of the compounds on *P. aeruginosa* growth were analysed in the absence of phages, and the lysis process was monitored every 30 min with a plate with wells containing *P. aeruginosa* cells with and without the φKMV phage.

2.4 | Validation of Tuspetinib as a T4P Inhibitor

The effect of tuspetinib on the lysis of PAO1 cells by phages was examined using 384-well plates as described for library screening except that sample wells were aliquoted 0.3 μL of tuspetinib at appropriate concentrations in DMSO. Plates were incubated at 37°C with 250 rpm shaking in a Tecan Infinite F200 Pro Microplate reader with OD_{600} measurements at regular time intervals.

The effect of tuspetinib on the twitching motility of *P. aeruginosa* and *Acinetobacter* species was examined using tissue culture-treated 6-well plates with LB media after 16 h of incubation as described previously (O'Hara et al. 2024). A *P. aeruginosa pilB* mutant (Table S1) and an *A. nosocomialis pilA* mutant (Harding et al. 2013) were used as controls for normalisation for data analysis. *P. aeruginosa* swimming motility was analysed as described (Samuel Chow, Jiang, and Nassour 2011) except that the assay was performed with 6-well plates with 4 mL of agar media in each. Swimming zones were measured after 16 h of incubation at 37°C with a Δ*fliC* mutant (Table S1) as the control. ImageJ was used for data acquisition and analysis of twitching and swimming motility.

3 | Results

3.1 | Bacteriophage φKMV Requires Functional T4P for *P. aeruginosa* Infection

φKMV was shown previously to infect the *P. aeruginosa* wild-type strain PAO1, but not its isogenic *pilA* mutant (Chibeu et al. 2009), suggesting that T4P is the φKMV receptor. We initially spotted serial dilutions of φKMV on the lawns of PAO1 and a *pilA* mutant. Clear zones or plaques formed on the wild type (Figure 1A), but not on the *pilA* mutant (Table S1), confirming the requirement of *pilA* by φKMV infection. PilB is the T4P assembly ATPase and it is required for T4P biogenesis. Similarly to the *pilA* mutant, the lawn of a *pilB* mutant produced no plaques or clearing zones in the presence of φKMV (Figure 1A). Also, we analysed mutants of additional *pil* genes essential for piliation (Ellison, Whitfield, and Brun 2022). These included *pilC*, *pilM, pilN, pilO and pilQ* as well as *pilE, pilY1* and *pilR* with the last three encoding a minor pilin, a pilus tip protein and a response regulator required for the transcription of *pilA*, respectively (Leighton et al. 2015). None of these mutants was able to form plaques upon φKMV infection. These observations collectively indicate that it is the T4P filament, not the mere presence of pilins or other T4PM components, that is essential for φKMV infection. These results are consistent with the conclusion that T4P functions as the receptor for the *P. aeruginosa* phage φKMV.

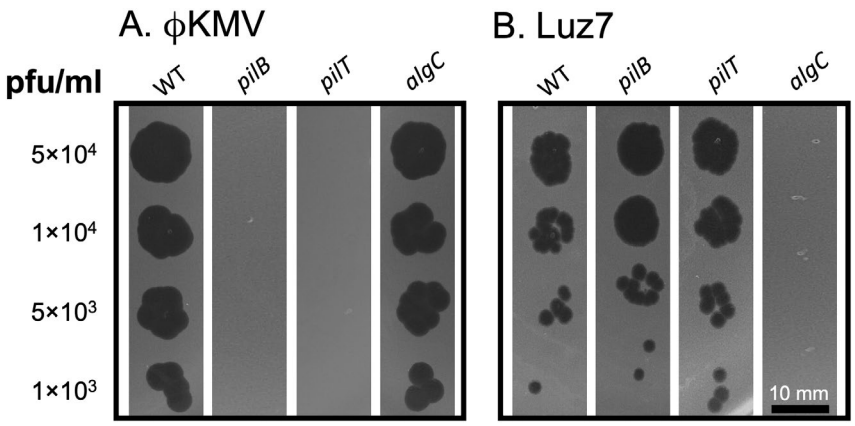

FIGURE 1 | Functional T4P is required for φKMV infection. Lawns of *P. aeruginosa* PAO1 (WT), PW8623 (*pilB*), PW1728 (*pilT*) and PAO1 *algC*::Tet mutant were infected by 2 μL of φKMV (A) or Luz7 (B) at indicated pfu/mL. Photographs were taken after incubation at 37°C for 12 h. Dark areas are clearing zones indicating successful phage infection. Three or more biological replicates were performed, each with technical quadruplicates. The 10 mm scale bar on the bottom right is applicable to both panels.

There are T4P or Pil proteins that are not required for piliation in *P. aeruginosa*. PilS is the cognate histidine sensor kinase of PilR. PilG, PilI, PilJ and PilK are components of a chemotaxis-like pathway with functions in the regulation of and by T4P (Bertrand, West, and Engel 2010; Geiger, Wong, and O'Toole 2024). Our results indicated that mutants of these regulatory genes are still susceptible to φKMV infection as analysed by the formation of plaques and clearing zones (Table S1). PilU and PilT are T4P retraction ATPases in *P. aeruginosa*. PilT is the main and essential one while PilU plays an accessory role to assist the function of PilT (Adams et al. 2019; Chlebek et al. 2019). Although both *pilT* and *pilU* mutants are hyperpiliated (Whitchurch and Mattick 1994). PilT, but not PilU, is absolutely required for T4P retraction (Talà et al. 2019). As shown in Figure 1A, on the lawn of a *pilT* mutant, φKMV did not produce plaques or any clearing. In contrast, a *pilU* mutant is still susceptible to φKMV infection in this assay (Table S1), which is reminiscent of PO4, another *P. aeruginosa* phage that infected a *pilU* mutant as well (Whitchurch and Mattick 1994). We additionally examined two other T4P-targeting phages Ab05 and Luz19 for their infections of the collection of *pil* mutants (Table S1; Figure S1) and observed the same patterns of infection as φKMV. These results here demonstrate that a functionally retractable T4P, not its mere presence, is required for φKMV and other T4P-targeting phages to infect *P. aeruginosa*.

It was observed previously (Chibeu et al. 2009) that while a *pilA* mutant is resistant to φKMV, it still displayed substantial phage absorption, albeit at a lower level than the wild type, suggesting the existence of a possible co-receptor. LPS is frequently used as a phage receptor in many bacteria (Silva, Storms, and Sauvageau 2016) and we examined if LPS is required for φKMV infection of *P. aeruginosa* with an *algC* mutant, which is defective in LPS O-antigen (Coyne Jr. et al. 1994). The results demonstrated that φKMV (Figure 1A), as well as Ab05 and Luz19 (Table S1), generated clearing zones and plaques on the lawn of the *algC* mutant. As a control, the LPS-targeting phage Luz7 (Ceyssens et al. 2009) was observed to infect the WT as well as the *pilB* and the *pilT* mutants, but not the *algC* mutant (Figure 1B). Together, these results indicate that φKMV, Ab05 and Luz19, while requiring functional T4P, do not use LPS as a receptor as does Luz7.

3.2 | *P. aeruginosa* In Liquid Culture Is Susceptible to φKMV Infection

We examined if the phage infections observable on agar plates (Figure 1 and S1) could occur in liquid media. *P. aeruginosa* cultures in 96-well plates were incubated without or with φKMV at a multiplicity of infection (MOI) of 10 with orbital shaking in a plate reader. At regular time intervals, shaking was paused and OD_{600} was measured. As shown in Figure 2A, cultures not infected with φKMV grew steadily as indicated by the increasing OD_{600} readings over time. In comparison, the OD_{600} of those infected with the phage remained relatively constant for the first 40 min, but started to decrease thereafter. By 130 min, their OD_{600} readings reached a low basal level and stabilised. Similar observations were made with the LPS-targeting phage Luz7 (Figure 2B) where the optical density of the cultures with phage decreased and stabilised in a similar time frame. Together, these results indicate that *P. aeruginosa* cells can be infected by φKMV and Luz7 in liquid culture in multiwell plates and that cell lysis can be monitored by optical density using plate readers. The estimated length of the infection cycle from these experiments is about 40 min for both phages, which is consistent with previous observations (Ceyssens 2009; Chibeu 2009) and coincides with the generation time for *P. aeruginosa* under our experiment conditions.

3.3 | Development of a High-Throughput Screen for Inhibitors of φKMV Infection

T4P inhibitors have potentials for the development of antivirulants against many bacterial pathogens. We considered utilising the protection of *P. aeruginosa* from φKMV lysis as a means to identify T4P inhibitors from compound libraries by HTS. To allow a potential T4P inhibitor to take effect, *P. aeruginosa* cells should be allowed to grow and multiply in the presence of a library compound before phage infection. One way to accomplish this in a HTS is to expose a log-phase bacterial culture simultaneously to a library compound and a phage at low MOI such that bacterial cells overwhelmingly outnumber phage particles

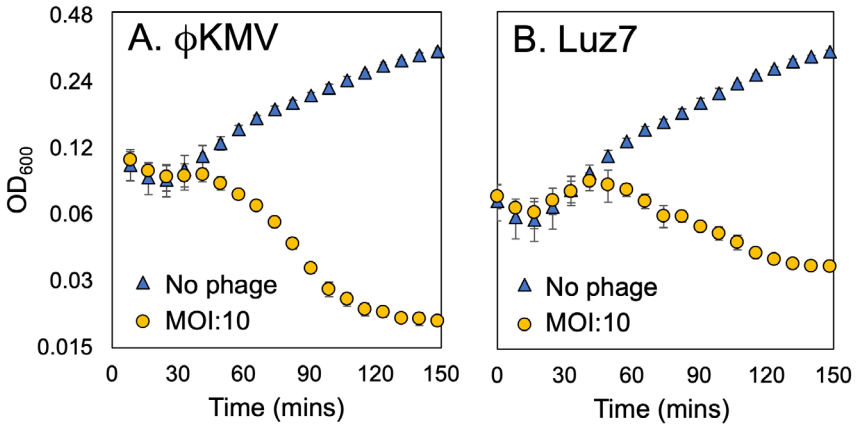

FIGURE 2 | Phage infection of *P. aeruginosa* occurs liquid culture. *P. aeruginosa* cultures in a 96-well plate were incubated without or with φKMV (A) or Luz7 (B) at an MOI of 10 in a microplate reader at 37°C with orbital shaking which was paused at regular time intervals for OD_{600} readings. Three or more biological replicates were performed, each with technical triplicates. Data presented are from one representative experiment with error bars indicating standard deviation.

at the onset. While a small number of cells would be infected and lysed early on, most cells would not encounter a live phage and would be able to proliferate before encountering newly produced phages at a later time. Depending on the initial MOI and the burst size of the phage, many cells could continue to grow and divide before phage particles eventually outnumber cells, leading to cell lysis and the collapse of a culture as monitored by OD_{600}. If a compound were protective against φKMV, the observed OD_{600} would be higher than control wells without any compound. This was the guiding principle for the development of the HTS assay using *P. aeruginosa* and φKMV in this study.

Cultures of *P. aeruginosa* in 384-well plates were incubated without or with φKMV at MOIs from 2.5×10^{-1} to 2.5×10^{-4} with orbital shaking in a plate reader. As shown in Figure 3, OD_{600} of all cultures increased initially as was expected. Those without φKMV continued to increase their cell density over the course of the experiment. Depending on the initial MOI with φKMV, cultures reached peak cell densities at different times before OD_{600} started to decrease. The higher the MOI, the lower the maximum cell density and the sooner a culture collapsed. At the MOI of 2.5×10^{-1}, OD_{600} increased about three folds to reach its peak around 95 min, at which point, it started to decline to a basal level about 150 min post infection. At the MOI of 2.5×10^{-4}, cultures reached maximum cell densities in about 155 min with approximately an eight-fold increase in OD_{600}. Optical density decreased thereafter to stabilise at a basal level around 230 min. At this low initial MOI, it is reasonable to assume that a potential T4P inhibitor in a screening would have had sufficient time to exert its effect on the bacterial population before phages outnumber cells to collapse the bacterial culture.

3.4 | HTS Identified Inhibitors of φKMV Infection

As described in Experimental Procedures, a trial screen of a Selleckchem library with 273 kinase inhibitors was conducted at 15 μM and 50 μM, each in duplicate plates. The Z′ factors, calculated from control wells without and with phages from the four screening plates were 0.81, 0.78, 0.74 and 0.73, respectively (Figure S2A), indicating robustness of this phage-based HTS assay (Zhang, Chung, and Oldenburg 1999; Murray and Wigglesworth 2017). In addition, the inhibition of *P. aeruginosa* by the library compounds at 50 μM were examined in duplicate plates and no growth inhibition was observed for most compounds (Figure S2B). This trial screen, while not yielding any hit, showed the feasibility of HTS with *P. aeruginosa* and φKMV.

A MedChemExpresss library of 2168 kinase inhibitors was screened at 50 μM in duplicate plates as above. The resulting Z′ factors for the screening plates were close or above 0.7, comparable with the trial screen. As shown in Figure 4, two compounds, tuspetinib and doxorubicin, showed 71% and 88% protection of *P. aeruginosa* from φKMV lysis, respectively. Doxorubicin is a DNA-intercalating agent (Zhang et al. 2019) and an anthracycline-type chemotherapeutic drug for cancer treatment with severe cytotoxicity (Neefjes et al. 2024). This class of molecules such as acridine are known to have general antiphage activities (Hong and Kreuzer 2000) and antibiotic (Wainwright 2001; Tenconi and Rigali 2018). As such, doxorubicin was not pursued as a T4P inhibitor. The remainder of this study focused on tuspetinib, also known as HM43239, which is an inhibitor of the FMS-like tyrosine kinase 3 (FLT3) with potentials for the treatment of acute myeloid leukaemia (AML) (Daver et al. 2019, 2021; Wang et al. 2024). It is orally available and has shown favourable pharmacology and toxicology profiles (Sonowal et al. 2024). Interim results from a phase I/II clinical trial demonstrated tuspetinib to have promising clinical responses against relapsed AML in monotherapy and in combination with other chemotherapeutic agents (Daver et al. 2022, 2023, 2024).

3.5 | Tuspetinib Is Specific Against T4P-Targeting Phages

Tuspetinib was first examined for its effect on *P. aeruginosa* growth dynamics at different concentrations. As shown in Figure S3, no change in *P. aeruginosa* growth rate or yield was

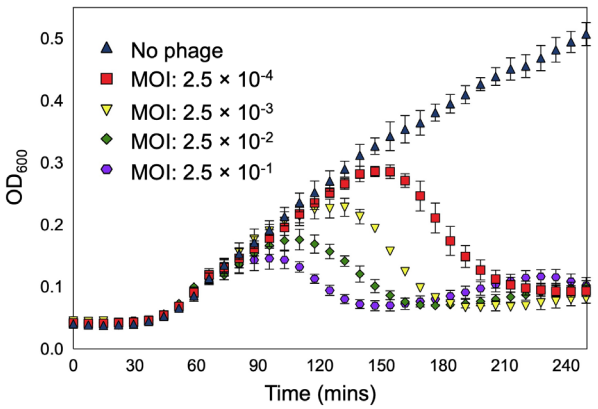

FIGURE 3 | φKMV infections at low MOI allows bacterial proliferation for multiple generations. *P. aeruginosa* PAO1 cultures in 384-well plates were incubated without or with φKMV at indicated MOIs. Plates were incubated at 37°C with orbital shaking with OD_{600} readings at regular time intervals. Three biological replicates were performed, each with technical quintuplicates. Data presented are from one representative experiment with error bars indicating standard deviation. Data for infections at three other MOIs are omitted for clarity.

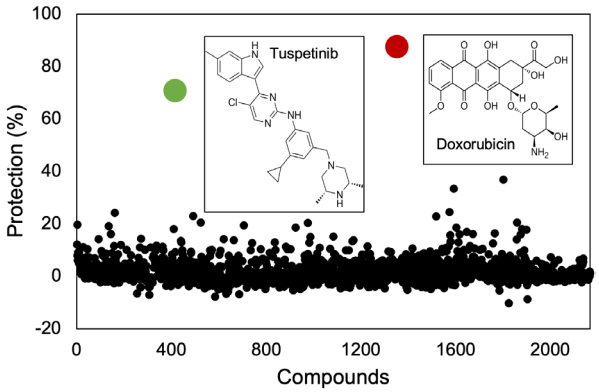

FIGURE 4 | HTS identified tuspetinib and doxorubicin. The MedChemExpress library of 2168 kinase inhibitor was screened at 50 μM as described in the main text. Each dot represents the average of two replicates. Error bars are omitted for clarity. Green and red dots indicate tuspetinib and doxorubicin, respectively.

observed at up to 200 μM of tuspetinib, four times of that in the HTS (Figure 4). Next, the dose response of φKMV infection to tuspetinib was examined in liquid cultures with continuous monitoring (Figure S4). At 25 μM, tuspetinib delayed the lysis of the *P. aeruginosa* culture for ~40 min in comparison with the solvent control. At 50 μM and 100 μM, it protected the *P. aeruginosa* culture for up to 250 min (Figures 5A and S4A). In addition, tuspetinib was observed to protect *P. aeruginosa* from the other two T4P-targeting phages Luz19 and Ab05 for up to 250 min (Figure 5C,D). In contrast, tuspetinib failed to protect *P. aeruginosa* from the LPS-targeting phage Luz7 in a similar assay (Figures 5B and S4B). Together, these results indicate that tuspetinib impairs the infection of *P. aeruginosa* by T4P-targeting but not LPS-targeting phages without affecting bacterial growth. These observations suggest that our novel HTS assay based on a bacterium-phage pair successfully identified tuspetinib as a likely anti-T4P compound effective in *P. aeruginosa*.

3.6 | Tuspetinib Inhibits T4P-Mediated Twitching Motility in Multiple Bacteria

Besides its function as a phage receptor, T4P is a motility apparatus that powers bacterial twitching in *P. aeruginosa* and other bacteria. The effect of tuspetinib on *P. aeruginosa* twitching was examined with the twitching areas measured in the presence of tuspetinib at different concentrations. As shown in Figure 6A, *P. aeruginosa* twitching displayed a clear dose-dependent response to tuspetinib. At concentrations below or equal to 6.25 μM, tuspetinib showed no obvious effect on *P. aeruginosa* twitching. At 12.5 μM and above, it increasingly inhibited *P. aeruginosa* twitching with increasing concentrations. Its half maximal inhibitory concentration (IC_{50}) of *P. aeruginosa* twitching was determined to be 19.4 ± 1.9 μM. Besides twitching, *P. aeruginosa* possesses flagellated swimming motility which can be analysed with media containing low concentrations of agar (Samuel Chow, Jiang, and Nassour 2011). We quantified *P. aeruginosa* swimming in the presence of tuspetinib. As shown in Figure S5, there is no statistical difference in *P. aeruginosa* swimming between the absence and presence of tuspetinib at the various concentrations. These results demonstrate that tuspetinib is a specific inhibitor of T4P functions in *P. aeruginosa* without any observable effect on swimming motility (Figure S5) or *P. aeruginosa* growth (Figure S3).

Tuspetinib was examined to determine if it is effective as an anti-T4P compound beyond *P. aeruginosa*. *Acinetobacter nosocomialis* is a member of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex (Nemec et al. 2011), and the *A. nosocomialis* M2 strain has been used extensively for the studies of T4P-dependent twitching motility in this species (Ronish et al. 2019; Vo et al. 2023; O'Hara et al. 2024). As shown

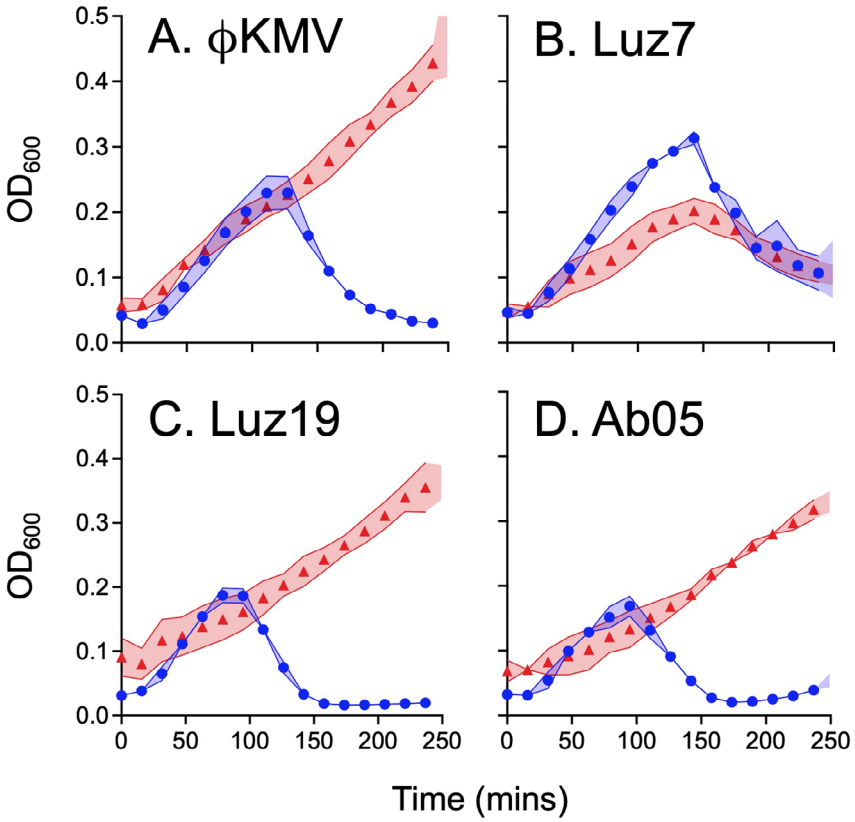

FIGURE 5 | Tuspetinib protects *P. aeruginosa* from phages. PAO1 cultures in 384-well plates were infected by phages φKMV (A), Luz7 (B), Luz19 (C) or Ab05 (D) at an MOI of 2.5×10^{-4} in the presence (red triangles) or the absence (blue circles) of 50 μM tuspetinib. Plates were incubated at 37°C with orbital shaking with OD_{600} readings at regular time intervals. Three biological replicates were performed, each with at least technical triplicates. Data presented are from one representative experiment with standard deviations indicated by shading. Data for controls without phage are not included for clarity. See Figure S4 for additional results.

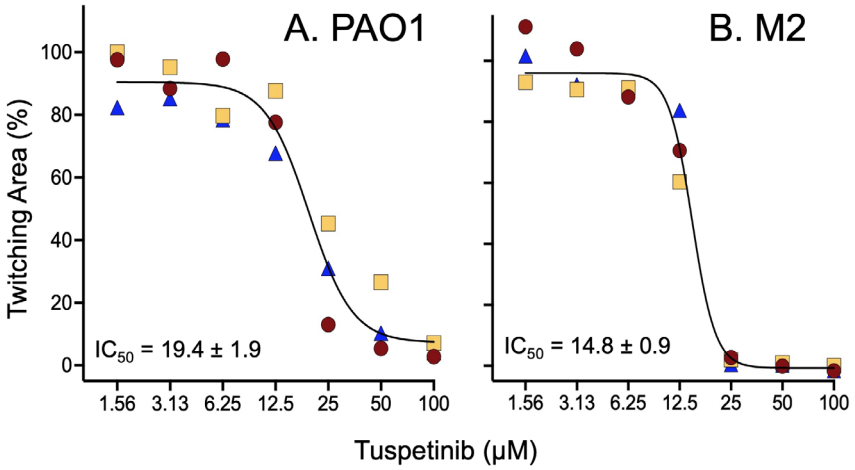

FIGURE 6 | Tuspetinib inhibits twitching motility in multiple pathogens. Twitching motility of *P. aeruginosa* PAO1 (A) and *A. nosocomialis* M2 (B) was examined and analysed as described in Experimental Procedures in the presence of tuspetinib at different concentrations. Twitching area is expressed as percentages (%) of the control without tuspetinib. Data presented here are averages from three biological experiments (represented by a different shape and colour) each performed in triplicate. Errors are omitted for clarity. IC_{50} was calculated using GraphPad Prism.

in Figure 6B, the twitching motility of *A. nosocomialis* is similarly inhibited by tuspetinib in a dose-dependent manner as that of *P. aeruginosa* (Figure 6A). The calculated IC_{50} of tuspetinib for this inhibition is $14.8 \pm 0.9\,\mu M$. Like the observations with *P. aeruginosa* (Figure S3), tuspetinib displayed no inhibition of *A. nosocomialis* growth (Figure S6). Additionally, we examined the twitching of two clinical isolates of *A. baumannii* strains, AYE and AB0057 (Adams et al. 2008; Hamidian et al. 2017), for their response to tuspetinib. As shown in Figure S7, the twitching motility of these two strains is similarly inhibited by tuspetinib in a concentration-dependent manner. The inhibition of twitching motility in *P. aeruginosa* and *Acinetobacter* species demonstrates that tuspetinib targets a conserved T4P component in these bacteria with promises for the development of a broad-spectrum antivirulant against T4P as a virulence factor.

4 | Discussion

Here we described the development and implementation of a novel approach for discovering T4P antagonists using *P. aeruginosa* and a T4P-targeting phage. For high-throughput screening, log-phase *P. aeruginosa* cells were mixed with φKMV at a low MOI in liquid growth media in multiwell plates such that bacteria cells outnumber phages by a ratio of 5000 to 1 at the onset of the experiment. From here on, it is a race between the phage and the bacterium in the increase of their numbers. Assuming a burst size of 100 pfu for φKMV (Lin et al. 2010; Sharma et al. 2021) and a synchronisation of φKMV infection cycle (Figure 2) with the doubling of *P. aeruginosa*, phage particles would surpass bacterial cells after two generations (Figure 3). After that, the demise of the bacterial culture will lead to drastic decreases in cell number which can be monitored by optical density. We reasoned that the time for two doublings of a culture would permit a T4P antagonist ample opportunity to influence the piliation levels of the surviving and proliferating cells, allowing the identification of a T4P inhibitor by its ability to protect cells against φKMV. The identification of hits from our screen validated the framework for the use of bacterium-phage pairs for HTS as was envisioned (Figure 4).

The HTS reported here with *P. aeruginosa* and φKMV overcomes a few limitations of previous approaches. Firstly, any hit identified by our screen is likely functional with the bacterium because it protects the whole cell from phage lysis. An inhibitor targeting a component of the motor or the periplasmic alignment complex is CM and/or OM permeable as was the case for P4MP4 (Aubey et al. 2019). Alternatively, a T4P antagonist identified with this screen may target the functions of the T4P filament (Poole et al. 2020) or its OM channel which presumably opens and closes during T4P assembly and disassembly (Hospenthal, Costa, and Waksman 2017; Craig, Forest, and Maier 2019; Ellison, Whitfield, and Brun 2022). This is in comparison with the HTS using purified proteins for the identification of PilB inhibitors (Dye et al. 2021, 2022), which may lead to inhibitors that are nonfunctional with intact cells. Secondly, this newly developed HTS assay identifies hits by optical density, which can be conveniently measured by a plate reader. It is similar in simplicity to the enzyme-based colorimetric or the fluorescence-based substrate binding assays with PilB (Dye et al. 2021, 2022). As such, it is cost effective and convenient to scale up for larger compound libraries. The microscope-based HTS measuring bacterial adhesion to cell cultures is more complex (Aubey et al. 2019). The procedures include, among other steps, preparation of primary endothelial cell culture, treatments with library compounds before and during bacterial adhesion, differential staining and fluorescent microscopic imaging with dual filter sets, followed by image analysis. While this protocol may capture the infection process the best in vitro, its complexity makes it challenging with larger compound libraries. Thirdly, our current screen, by virtue of antagonising the biogenesis or function of T4P as a phage receptor, is more specific to T4P instead of other molecules or proteins that function as adhesins (Aubey et al. 2019). Yet, unlike the PilB-based screen with one specific target, this bacterium-phage approach may identify inhibitors against any protein of the T4PM. The advantages of the current approach promise to accelerate the identification of T4P antagonists for the development of antivirulants against bacterial T4P.

An anticipated limitation of the current approach is the interference from compounds with antiphage activities such

as doxorubicin (Figure 4), which, like its related chemicals, may target mammalian, bacterial and phage topoisomerases (Kreuzer 1998). Other targets of interfering compounds include the diverse bacterial antiphage systems (Georjon and Bernheim 2023) because a potentiator of such systems may protect bacteria from phage lysis. Exclusion of such potentiators can be achieved by a counter screen using a phage that does not require T4P for infection. One candidate in this case can be the LPS-targeting phage Luz7 (Figures 2B and 5B). While not included in the current study, a counter screen with Luz7 will help to mitigate this limitation with larger compound libraries. Intriguingly, LPS is an endotoxin for many gram-negative bacterial pathogens. As such, Luz7, similarly as φKMV, may be used to screen for inhibitors of LPS biosynthesis and functions. Virulence factors including flagella, outer membrane porins and secretion systems are used as phage receptors (Silva, Storms, and Sauvageau 2016; Stone et al. 2019; de Melo, Morency, and Moineau 2024). The approach of using bacterium-phage pairs for the discovery of antivirulence compounds may be extended to these prominent virulence factors and their corresponding phages.

Multiple experiments demonstrated the specificity of tuspetinib as a genuine inhibitor of bacterial T4P and its functions. Tuspetinib is effective in protecting *P. aeruginosa* from lysis not only by φKMV, the phage used for the initial library screening, but also by two other T4P-targeting phages Luz19 and Ab05 (Figure 5). In addition, tuspetinib inhibits T4P-mediated twitching motility of *P. aeruginosa* as well as *A. nosocomialis* with IC_{50} of 19.4 μM and 14.8 μM, respectively (Figure 6). It also inhibited the twitching motility of *A. baumannii* (Figure S7), the top organism on the list of priority pathogens because of their antibiotic resistance (WHO 2024). In contrast, tuspetinib did not protect *P. aeruginosa* from lysis by the LPS-targeting phage Luz7 (Figure 5), and it inhibits neither *P. aeruginosa* flagellated swimming motility (Figure S5) nor bacterial growth (Figure S3). These results indicate that tuspetinib is a specific T4P inhibitor without antibiotic activity or non-specific inhibitory effects on bacterial motility. While the specific protein target of tuspetinib and its mode of action as a T4P inhibitor is yet to be established, its effectiveness in multiple pathogens shows promises for the development of broad-spectrum antivirulants against a conserved component of the T4P machinery.

Author Contributions

Tori M. Shimozono: conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **Nancy J. Vogelaar:** investigation, methodology, writing – review and editing, resources. **Megan T. O'Hara:** investigation, writing – review and editing, methodology. **Zhaomin Yang:** conceptualization, investigation, funding acquisition, writing – review and editing, visualization, formal analysis, project administration, supervision, resources.

Acknowledgements

We thank Dr. George O'Toole, Dr. Joanne Engel, Dr. Joanna Goldberg, Dr. Matthew Parsek, Dr. Joseph Bondy-Denomy and Dr. Robert Lavigne for providing experimental materials. We thank Dr. Keane Dye for his assistance at the early stages of this study.

Conflicts of Interest

An intellectual property disclosure has been filed on the approach of using bacterium-phage pairs for the identification of antivirulence compounds by Virginia Tech.

Data Availability Statement

Additional data that supports the findings of this study are available in Figures S1–S7 and Table S1 of this article.

References

Adams, D. W., J. M. Pereira, C. Stoudmann, S. Stutzmann, and M. Blokesch. 2019. "The Type IV Pilus Protein PilU Functions as a PilT-Dependent Retraction ATPase." *PLoS Genetics* 15: e1008393.

Adams, M. D., K. Goglin, N. Molyneaux, et al. 2008. "Comparative Genome Sequence Analysis of Multidrug-Resistant *Acinetobacter baumannii*." *Journal of Bacteriology* 190: 8053–8064.

Antimicrobial Resistance Collaborators. 2022. "Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis." *Lancet* 399: 629–655.

Aubey, F., J. P. Corre, Y. X. Kong, et al. 2019. "Inhibitors of the PilF ATPase Provoke Type IV Pilus Disassembly." *Proceedings of the National Academy of Sciences* 116: 8481–8486.

Bertrand, J. J., J. T. West, and J. N. Engel. 2010. "Genetic Analysis of the Regulation of Type IV Pilus Function by the Chp Chemosensory System of *Pseudomonas aeruginosa*." *Journal of Bacteriology* 192: 994–1010.

Budzik, J. M., W. A. Rosche, A. Rietsch, and G. A. O'Toole. 2004. "Isolation and Characterization of a Generalized Transducing Phage for *Pseudomonas aeruginosa* Strains PAO1 and PA14." *Journal of Bacteriology* 186: 3270–3273.

Ceyssens, P. J. 2009. *Isolation and Characterization of Lytic Bacteriophages Infecting Pseudomonas aeruginosa*, 150. Leuven: *Department of Biosystems*: KU Leuven.

Ceyssens, P. J., A. Brabban, L. Rogge, et al. 2010. "Molecular and Physiological Analysis of Three Phages Belonging to the "N4-Like Viruses"." *Virology* 405: 26–30.

Ceyssens, P. J., J. P. Noben, H. W. Ackermann, et al. 2009. "Survey of *Pseudomonas aeruginosa* and Its Phages: de Novo Peptide Sequencing as a Novel Tool to Assess the Diversity of Worldwide Collected Viruses." *Environmental Microbiology* 11: 1303–1313.

Chibeu, A. 2009. *Pseudomonas aeruginosa Lytic Bacteriophage phiKMV Receptor Analysis*, 134. Leuven: *Department of Biosystems*: KU Leuven.

Chibeu, A., P. J. Ceyssens, K. Hertveldt, et al. 2009. "The Adsorption of *Pseudomonas aeruginosa* Bacteriophage Phi KMV Is Dependent on Expression Regulation of Type IV Pili Genes." *FEMS Microbiology Letters* 296: 210–218.

Chlebek, J. L., H. Q. Hughes, A. S. Ratkiewicz, et al. 2019. "PilT and PilU Are Homohexameric ATPases That Coordinate to Retract Type IVa Pili." *PLoS Genetics* 15: e1008448.

Coyne, M. J., Jr., K. S. Russell, C. L. Coyle, and J. B. Goldberg. 1994. "The *Pseudomonas aeruginosa algC* Gene Encodes Phosphoglucomutase, Required for the Synthesis of a Complete Lipopolysaccharide Core." *Journal of Bacteriology* 176: 3500–3507.

Craig, L., K. T. Forest, and B. Maier. 2019. "Type IV Pili: Dynamics, Biophysics and Functional Consequences." *Nature Reviews. Microbiology* 17: 429–440.

Craig, L., M. E. Pique, and J. A. Tainer. 2004. "Type IV Pilus Structure and Bacterial Pathogenicity." *Nature Reviews. Microbiology* 2: 363–378.

Daver, N., K. H. Lee, Y. Choi, et al. 2023. "Tuspetinib Myeloid Kinase Inhibitor Safety and Efficacy as Monotherapy and Combined With

Venetoclax in Phase 1/2 Trial of Patients With Relapsed or Refractory (R/R) Acute Myeloid Leukemia (AML)." *Blood* 142: 162.

Daver, N., K. H. Lee, Y. Choi, et al. 2024. "Phase 1 Safety and Efficacy of Tuspetinib Plus Venetoclax Combination Therapy in Study Participants With Relapsed or Refractory Acute Myeloid Leukemia (AML) Support Exploration of Triplet Combination Therapy of Tuspetinib Plus Venetoclax and Azacitidine for Newly Diagnosed AML." *Blood* 144: 4255.

Daver, N., K. H. Lee, B. A. Jonas, et al. 2022. "A Phase 1/2 Dose Escalation Study of the Myeloid Kinase Inhibitor HM43239 in Patients With Relapsed or Refractory Acute Myeloid Leukemia." *Blood* 140: 6197–6199.

Daver, N., K. H. Lee, C. W. Jung, et al. 2021. "First in Human (FIH) FLT3 and SYK Inhibitor HM43239 Shows Single Agent Activity in Patients (Pts) With Relapsed or Refractory (R/R) FLT3 Mutated and Wild-Type Acute Myeloid Leukemia (AML)." *Blood* 138: 702.

Daver, N. G., K. H. Lee, S. S. Yoon, et al. 2019. "HM43239, a Novel Potent Small Molecule FLT3 Inhibitor, in Acute Myeloid Leukemia (AML) With FMS-Like Tyrosine Kinase 3 (FLT3) Mutations: Phase 1/2 Study." *Blood* 134: 1331.

de Melo, A. G., C. Morency, and S. Moineau. 2024. "Virulence-Associated Factors as Targets for Phage Infection." *Current Opinion in Microbiology* 79: 102471.

Dickey, S. W., G. Y. C. Cheung, and M. Otto. 2017. "Different Drugs for Bad Bugs: Antivirulence Strategies in the Age of Antibiotic Resistance." *Nature Reviews. Drug Discovery* 16: 457–471.

Dreyer, G., and M. Campbell-Renton. 1933. "The Quantitative Determination of Bacteriophage Activity and Its Application to the Study of the Twort-d'Herelle Phenomenon." *Journal of Pathology and Bacteriology* 36: 399–423.

Dubnau, D., and M. Blokesch. 2019. "Mechanisms of DNA Uptake by Naturally Competent Bacteria." *Annual Review of Genetics* 53: 217–237.

Duménil, G. 2019. "Type IV Pili as a Therapeutic Target." *Trends in Microbiology* 27: 658–661.

Dye, K. J., N. J. Vogelaar, M. O'Hara, et al. 2022. "Discovery of Two Inhibitors of the Type IV Pilus Assembly ATPase PilB as Potential Antivirulence Compounds." *Microbiology Spectrum* 10: e03877-22.

Dye, K. J., N. J. Vogelaar, P. Sobrado, and Z. M. Yang. 2021. "High-Throughput Screen for Inhibitors of the Type IV Pilus Assembly ATPase PilB." *Msphere* 6: e00129-21.

Ellison, C. K., G. B. Whitfield, and Y. V. Brun. 2022. "Type IV Pili: Dynamic Bacterial Nanomachines." *FEMS Microbiology Reviews* 46: fuab053.

Essoh, C., L. Latino, C. Midoux, et al. 2015. "Investigation of a Large Collection of *Pseudomonas aeruginosa* Bacteriophages Collected From a Single Environmental Source in Abidjan, Cote d'Ivoire." *PLoS One* 10: e0130548.

GBD Antimicrobial Resistance Collaborators. 2024. "Global Burden of Bacterial Antimicrobial Resistance 1990-2021: A Systematic Analysis With Forecasts to 2050." *Lancet* 404: 1199–1226.

Geiger, C. J., G. C. L. Wong, and G. A. O'Toole. 2024. "A Bacterial Sense of Touch: T4P Retraction Motor as a Means of Surface Sensing by *Pseudomonas aeruginosa* PA14." *Journal of Bacteriology* 206: e0044223.

Georjon, H., and A. Bernheim. 2023. "The Highly Diverse Antiphage Defence Systems of Bacteria." *Nature Reviews. Microbiology* 21: 686–700.

Hamidian, M., P. Venepally, R. M. Hall, and M. D. Adams. 2017. "Corrected Genome Gequence of *Acinetobacter baumannii* Strain AB0057, an Sntibiotic-Resistant Isolate From Lineage 1 of Global Clone 1." *Genome Announcements* 5: e00836-17.

Harding, C. M., E. N. Tracy, M. D. Carruthers, P. N. Rather, L. A. Actis, and R. S. Munson. 2013. "*Acinetobacter baumannii* Strain M2 Produces Type IV Pili Which Play a Role in Natural Transformation and Twitching Motility but Not Surface-Associated Motility." *MBio* 4: e00360-13.

Hong, G., and K. N. Kreuzer. 2000. "An Antitumor Drug-Induced Topoisomerase Cleavage Complex Blocks a Bacteriophage T4 Replication Fork In Vivo." *Molecular and Cellular Biology* 20: 594–603.

Hospenthal, M. K., T. R. D. Costa, and G. Waksman. 2017. "A Comprehensive Guide to Pilus Biogenesis in Gram-Negative Bacteria." *Nature Reviews. Microbiology* 15: 365–379.

Kreuzer, K. N. 1998. "Bacteriophage T4, a Model System for Understanding the Mechanism of Type II Topoisomerase Inhibitors." *Biochimica et Biophysica Acta* 1400: 339–347.

Lammens, E., P. J. Ceyssens, M. Voet, K. Hertveldt, R. Lavigne, and G. Volckaert. 2009. "Representational Difference Analysis (RDA) of Bacteriophage Genomes." *Journal of Microbiological Methods* 77: 207–213.

Lau, W. Y. V., P. K. Taylor, F. S. L. Brinkman, and A. H. Y. Lee. 2023. "Pathogen-Associated Gene Discovery Workflows for Novel Antivirulence Therapeutic Development." *eBioMedicine* 88: 104429.

Lavigne, R., M. V. Burkal'tseva, J. Robben, et al. 2003. "The Genome of Bacteriophage φKMV, a T7-Like Virus Infecting *Pseudomonas aeruginosa*." *Virology* 312: 49–59.

Leighton, T. L., R. N. Buensuceso, P. L. Howell, and L. L. Burrows. 2015. "Biogenesis of *Pseudomonas aeruginosa* Type IV Pili and Regulation of Their Function." *Environmental Microbiology* 17: 4148–4163.

Lin, N. T., P. Y. Chiou, K. C. Chang, L. K. Chen, and M. J. Lai. 2010. "Isolation and Characterization of Phi AB2: A Novel Bacteriophage of *Acinetobacter baumannii*." *Research in Microbiology* 161: 308–314.

Murray, D., and M. Wigglesworth. 2017. *HTS Methods: Assay Design and Optimisation*. Vol. 1, 1–15. UK: Royal Society of Chemistry.

Neefjes, J., K. Gurova, J. Sarthy, G. Szabó, and S. Henikoff. 2024. "Chromatin as an Old and New Anticancer Target." *Trends in Cancer* 10: 696–707.

Nemec, A., L. Krizova, M. Maixnerova, et al. 2011. "Genotypic and Phenotypic Characterization of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* Complex With the Proposal of *Acinetobacter pittii* sp. nov. (Formerly Acinetobacter Genomic Species 3) and *Acinetobacter nosocomialis* sp. nov. (Formerly Acinetobacter Genomic Species 13TU)." *Research in Microbiology* 162: 393–404.

Niu, C., K. M. Clemmer, R. A. Bonomo, and P. N. Rather. 2008. "Isolation and Characterization of an Autoinducer Synthase From *Acinetobacter baumannii*." *Journal of Bacteriology* 190: 3386–3392.

O'Hara, M. T., T. M. Shimozono, K. J. Dye, D. Harris, and Z. Yang. 2024. "Surface Hydrophilicity Promotes Bacterial Twitching Motility." *Msphere* 9: e0039024.

Piepenbrink, K. H., and E. J. Sundberg. 2016. "Motility and Adhesion Through Type IV Pili in Gram-Positive Bacteria." *Biochemical Society Transactions* 44: 1659–1666.

Poole, J., C. J. Day, T. Haselhorst, et al. 2020. "Repurposed Drugs That Block the Gonococcus-Complement Receptor 3 Interaction Can Prevent and Cure Gonococcal Infection of Primary Human Cervical Epithelial Cells." *MBio* 11: e03046-19.

Ronish, L. A., E. Lillehoj, J. K. Fields, E. J. Sundberg, and K. H. Piepenbrink. 2019. "The Structure of PilA From *Acinetobacter baumannii* AB5075 Suggests a Mechanism for Functional Specialization in Acinetobacter Type IV Pili." *Journal of Biological Chemistry* 294: 218–230.

Samuel Chow, K. G., L. Jiang, and A. Nassour. 2011. "Salicylic Acid Affects Swimming, Twitching and Swarming Motility in *Pseudomonas aeruginosa*, Resulting in Decreased Biofilm Formation." *Journal of Experimental Microbiology and Immunology* 15: 22–29.

Sharma, S., S. Datta, S. Chatterjee, et al. 2021. "Isolation and Characterization of a Lytic Bacteriophage Against *Pseudomonas aeruginosa*." *Scientific Reports* 11: 19393.

Silva, J. B., Z. Storms, and D. Sauvageau. 2016. "Host Receptors for Bacteriophage Adsorption." *FEMS Microbiology Letters* 363: fnw002.

Sonowal, H., W. G. Rice, R. Bejar, et al. 2024. "Preclinical Development of Tuspetinib for the Treatment of Acute Myeloid Leukemia." *Cancer Research Communications*. https://doi.org/10.1158/2767-9764.CRC-24-0258.

Stone, E., K. Campbell, I. Grant, and O. McAuliffe. 2019. "Understanding and Exploiting Phage-Host Interactions." *Viruses* 11: 567.

Talà, L., A. Fineberg, P. Kukura, and A. Persat. 2019. "*Pseudomonas aeruginosa* Orchestrates Twitching Motility by Sequential Control of Type IV Pili Movements." *Nature Microbiology* 4: 774–780.

Tenconi, E., and S. Rigali. 2018. "Self-Resistance Mechanisms to DNA-Damaging Antitumor Antibiotics in Actinobacteria." *Current Opinion in Microbiology* 45: 100–108.

Vo, N., B. S. Sidner, Y. Yu, and K. H. Piepenbrink. 2023. "Type IV Pilus-Mediated Inhibition of *Acinetobacter baumannii* Biofilm Formation by Phenothiazine Compounds." *Microbiology Spectrum* 11: e0102323.

Wadhwa, N., and H. C. Berg. 2022. "Bacterial Motility: Machinery and Mechanisms." *Nature Reviews. Microbiology* 20: 161–173.

Wainwright, M. 2001. "Acridine—A Neglected Antibacterial Chromophore." *Journal of Antimicrobial Chemotherapy* 47: 1–13.

Wall, D., and D. Kaiser. 1999. "Type IV Pili and Cell Motility." *Molecular Microbiology* 32: 1–10.

Wang, Z. W., Y. An, J. Wang, and J. H. Lu. 2024. "Deciphering the Mechanism of HM43239 Inhibiting the Mutant F691L Resistant to Gilteritinib in FMS-Like Tyrosine Kinase 3." *Journal of Biomolecular Structure & Dynamics* 42: 5817–5826.

Whitchurch, C. B., and J. S. Mattick. 1994. "Characterization of a Gene, PilU, Required for Twitching Motility but Not Phage Sensitivity in *Pseudomonas aeruginosa*." *Molecular Microbiology* 13: 1079–1091.

WHO. 2024. *WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance*. Geneva, Switzerland: WHO.

Zhang, J. H., T. D. Y. Chung, and K. R. Oldenburg. 1999. "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays." *Journal of Biomolecular Screening* 4: 67–73.

Zhang, X., A. Poniewierski, K. Sozanski, Y. Zhou, A. Brzozowska-Elliott, and R. Holyst. 2019. "Fluorescence Correlation Spectroscopy for Multiple-Site Equilibrium Binding: A Case of Doxorubicin-DNA Interaction." *Physical Chemistry Chemical Physics* 21: 1572–1577.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.