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Pseudomonas aeruginosa defends against phages through type IV pilus glycosylation

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Since phages present a major challenge to survival in most environments, bacteria express a battery of anti-phage defences including CRISPR–Cas, restriction-modification and abortive infection systems[1–4]. Such strategies are effective, but the phage genome—which encodes potentially inhibitory gene products—is still allowed to enter the cell. The safest way to preclude phage infection is to block initial phage adsorption to the cell. Here, we describe a cell-surface modification that blocks infection by certain phages. Strains of the opportunistic pathogen *Pseudomonas aeruginosa* express one of five different type IV pilins (T4P)[5], two of which are glycosylated with O-antigen units[6] or polymers of D-arabinofuranose[7–9]. We propose that predation by bacteriophages that use T4P as receptors selects for strains that mask potential phage binding sites using glycosylation. Here, we show that both modifications protect *P. aeruginosa* from certain pilus-specific phages. Alterations to pilin sequence can also block phage infection, but glycosylation is considered less likely to create disadvantageous phenotypes. Through construction of chimeric phages, we show that specific phage tail proteins allow for infection of strains with glycosylated pili. These studies provide insight into first-line bacterial defences against predation and ways in which phages circumvent them, and provide a rationale for the prevalence of pilus glycosylation in nature.

Diverse phages infecting *Pseudomonas aeruginosa* use two main cell surface receptors, lipopolysaccharides (LPS) and T4P[10,11]. The evolution of different LPS O-antigen serotypes in *P. aeruginosa* has likely been shaped by coevolution with phages, since individual phages generally infect only limited serotypes. However, it is unclear how targeting of T4P by phages has affected the evolution of their components.

A wide variety of bacteria and archaea use T4P for adherence, biofilm formation, DNA uptake and twitching motility[12,13]. T4P are virulence factors for many pathogens, including *P. aeruginosa*[14–17]. While many *P. aeruginosa* phages use T4P as receptors[11,18,19], the exact manner in which they interact with pili remains unknown. T4P contain thousands of copies of the major pilin, PilA, plus small amounts of minor (low-abundance) pilins that form an assembly initiation complex to which major pilins are subsequently added, extending the pilus from its base in the inner membrane[20]. Thus, minor pilins are most likely positioned at the tip of assembled pili[21,22]. Electron microscopy studies of *P. aeruginosa* pilus-specific phages suggested they bind along the pilus length and are pulled to the cell surface when the pilus retracts[11]. These data suggest that the phages bind the major pilin, although this assumption has not been formally tested.

Each *P. aeruginosa* strain encodes one of five different major pilin variants at a conserved locus located between the *pilB* gene, encoding the pilus extension ATPase, and a transfer RNA^{Thr} gene[5] (Supplementary Fig. 1). The variants differ with respect to their length (150 to 173 residues), the number of amino acids (12 to 29 residues) between the two C-terminal Cys residues that form a critical disulfide-bonded loop or 'D-region' and the presence of specific pilin accessory genes downstream of the *pilA* gene. A census of nearly 300 strains[5] revealed that the most common variant is group I, a pilin glycosylated at a C-terminal Ser by its associated glycosyltransferase, TfpO[23]. The pilin glycan is an O-antigen unit, sourced from the O-specific antigen LPS biosynthetic pathway[6]. Group I pilins were over-represented (close to 70%) in strains isolated from cystic fibrosis patients, suggesting they provide a significant advantage in certain environments[5]. A second, distinct glycosylation system in group IV strains such as PA7 was also identified[7–9]. Group IV pilins are glycosylated by their cognate glycosyltransferase TfpW on multiple Ser and Thr residues with homopolymers of α1,5-linked D-arabinofuranose (D-Ara*f*). The genes encoding the biosynthesis of D-Ara*f* polymers are unlinked to the pilin locus, but glycosylation is important for stable pilin expression[8].

A convincing reason for the prevalence of pilin glycosylation in nature has not yet been advanced, though this modification has a modest effect on pathogenicity in mice[24] and decreases twitching motility[25,26]. In *Neisseria meningitidis*, an obligate human commensal, and in the genus *Acinetobacter*, glycosylation was proposed to block binding of pilin-specific antibodies[27,28]. A previous study of *P. aeruginosa* strain 1244, which expresses glycosylated group I pilins, concluded that this post-translational modification had no effect on phage susceptibility[24]. However, the design of those experiments meant that the effect could not really be addressed, since they used a phage capable of infecting the wild type expressing glycosylated pilins.

While investigating the host range of a group of pilus-specific phages we isolated previously[19], we noted that many were capable of infecting a 1244 *tfpO* mutant that produces unmodified pilins, but not its wild-type parent. These results hinted that pilin glycosylation might represent a mechanism of resistance to phages that use the pilus as a receptor. To test this hypothesis in the well characterized lab strain PAO1, we expressed *pilA* genes of interest from an arabinose-inducible plasmid in a PAO1 *pilA* null mutant[25]. Phage plaquing assays on these strains showed that all of the phages

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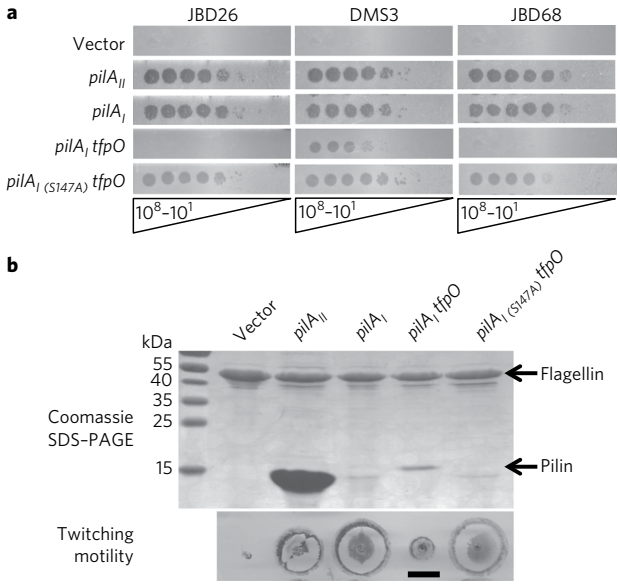

Fig. 1 | Infection of a *P. aeruginosa* PAO1 *pilA* mutant expressing non-glycosylated versus glycosylated pilins. a, Five microlitres each of serial tenfold dilutions (10^8–10^1) of phages JBD26, DMS3 and JBD68 were spotted onto a PAO1 *pilA* mutant expressing the genes indicated on the left, expressed from the pBADGr vector. Loss of pilin glycosylation in the S147A mutant restores phage susceptibility. Images are representative of six biological replicates. **b**, Surface piliation of the PAO1 *pilA* mutant complemented with its cognate pilin ($pilA_{II}$), or that of group I strain 1244 ($pilA_I$). Differences in the levels of recoverable surface pili do not correlate with phage susceptibility or twitching motility on polystyrene (bottom panel). Molecular weight standards in kDa are shown on the left. Scale bar, 1 cm. Gel image is representative of three biological replicates; twitching image is representative of three biological replicates, each with three technical replicates.

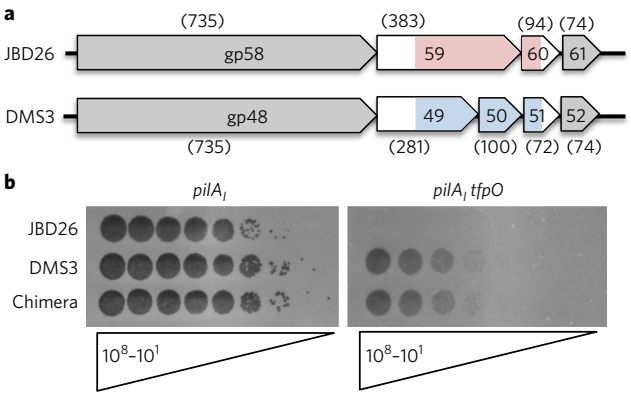

Fig. 2 | A chimeric phage expressing DMS3 genes in the JBD26 background gains the ability to infect a strain with glycosylated pilins. **a**, Map of JBD26 and DMS3 tail fibre genes and surrounding genomic conservation. The genomic organization of related genes from JBD26 and DMS3, and the predicted sizes of their products (amino acids, in brackets), are shown above each ORF. Regions where sequences from the two phages diverge are coloured pink and blue. The gp designations are omitted for most genes due to space limitations. Map not to scale. **b**, Plaque assay using 5 µl each of serial tenfold dilutions of phage (10^8–10^1). A chimeric JBD26 phage expressing the DMS3 gp49–52 instead of its own gp59–60 gains the ability to infect a PAO1 *pilA* mutant complemented with the pilin from group I strain 1244 ($pilA_I$), alone and with *tfpO*, encoding the cognate glycosyltransferase. Image is representative of six biological replicates.

replicated robustly on the strains expressing $PilA_{II}$ or unglycosylated $PilA_I$ (Supplementary Table 1). However, none of the phages except DMS3 detectably replicated on the strain expressing $PilA_I$ and its cognate glycosylation enzyme TfpO, suggesting that pilin glycosylation blocks phage replication.

To gain further insight into this phenomenon, we focused additional studies on three phages, JBD26, DMS3 and JBD68. JBD26 and DMS3 are similar in sequence and members of the dominant group in our collection (*P. aeruginosa* phage MP22-like)[19]. JBD68 is a completely distinct phage, and its putative tail proteins display no similarity to those of the other phages with only one exception (Supplementary Table 2). The plaquing ability of phages JBD26 and JBD68 was reduced > 10^5-fold when $PilA_I$ was coexpressed with TfpO, while the plaquing of DMS3 was reduced ~ 10^3-fold (Fig. 1a). To confirm that these effects were the result of pilin glycosylation, we mutated the C-terminal Ser of $PilA_I$ to Ala, which removes the glycan attachment site[23]. Coexpression of TfpO with the $PilA_I$ S147A mutant resulted in non-glycosylated pilins and failed to reduce the plaquing ability of the three phages (Fig. 1a). We showed previously that a PAO1 *pilA* mutant expressing group I pilins—regardless of glycosylation status—assembled substantially fewer surface pili compared to the strain expressing $PilA_{II}$ and, as previously reported, had reduced twitching[25] (Fig. 1b). However, this difference in surface piliation, assessed here by examining the amount of surface pili sheared from the recombinant strains, did not affect phage susceptibility (compare $pilA_{II}$ versus $pilA_I$ in Fig 1a). Since the $PilA_I$-TfpO expressing strain has more surface pili than the $PilA_I$ strain, the reduction in plaquing efficiency on the former was not due to reduced piliation (Fig. 1b).

Phage DMS3 was distinct from the others tested, in that it could replicate to some extent on cells with glycosylated pili. JBD26 and DMS3 are very similar non-contractile tailed phages, with 81% identity at the nucleotide level. However, one conspicuous difference between these phages occurs directly downstream of genes encoding their tail components. Here, the genome of JBD26 contains two open reading frames (ORFs)—*59* and *60*—between conserved ORFs *58* and *61*, while DMS3 has three ORFs—*49*–*51*—at the same position (Fig. 2a). The predicted gene products (gp) JBD26 gp59 and DMS3 gp49 are 86% identical over the first 153 amino acids (Fig 2a), but then their sequences diverge completely (Supplementary Fig. 2). Similarly, the last 47 residues of JBD26 gp60 and DMS3 gp51 are 96% identical, while the N termini of those proteins differ. DMS3 gp50 has no counterpart in JBD26. Given the positioning of the genes encoding these proteins at the 3'-end of the tail encoding regions within their respective genomes, we hypothesized that they may encode tail proteins involved in host range specificity. Thus, we replaced genes *59* and *60* of JBD26 with genes *49* to *51* of DMS3 by recombination within the conserved DNA regions flanking these genes (Fig. 2a). Strikingly, the JBD26/DMS3 chimeric phage infected strains expressing either non-glycosylated or glycosylated pilins to the same extent as DSM3 (Fig. 2b). These results suggest that DMS3 ORFS *49*–*51* encode the unique ability of DMS3 to use the glycosylated pilus for infection.

The C-terminal Ser that is glycosylated in group I pilins is adjacent to the D-region of PilA[23], suggesting that post-translational modification could mask this potential phage binding surface (Fig. 3a). Thus, we tested a set of previously engineered $PilA_{II}$ single-residue substitution mutants in the D-region for their ability to support phage infection[29]. Most substitutions had little effect on infection, but a $PilA_{II}$ P138R mutant was approximately 1,000-fold more resistant to JBD26 and JBD68 infection than wild type (Fig. 3b), while remaining susceptible to DMS3 and the chimeric phage (Fig. 3c). A P138A substitution had no effect on susceptibility, while a P138M mutant had slightly reduced susceptibility, suggesting a specific interaction between phages JBD26 and JBD68 and the

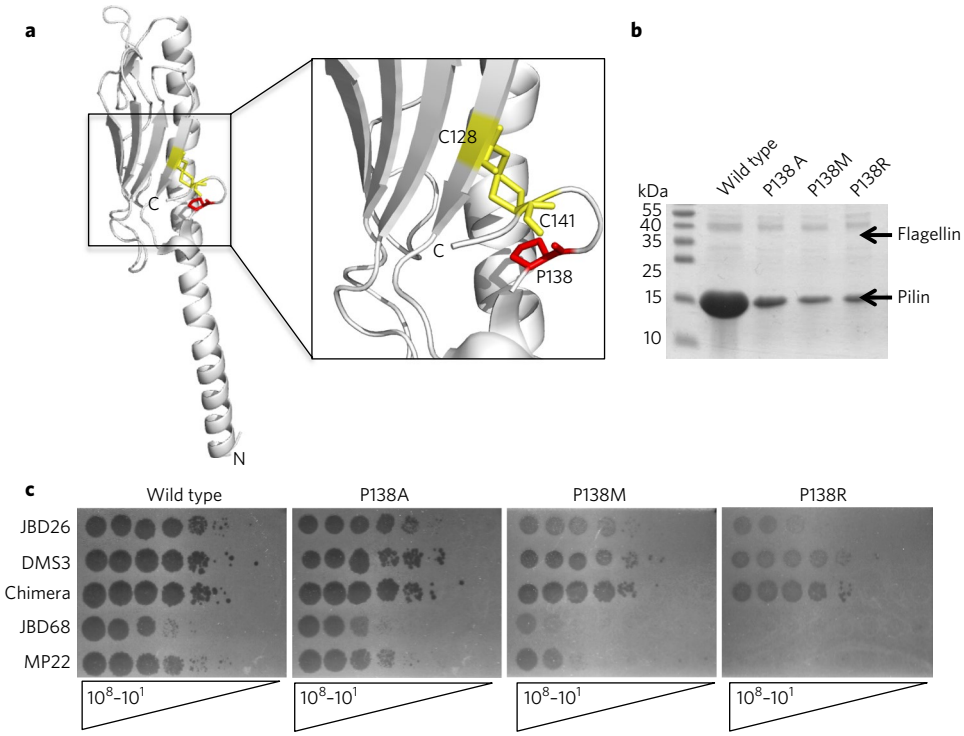

Fig. 3 | Pilin D-region sequence affects phage susceptibility. a, Model of a group II pilin showing the disulfide-bonded Cys residues (yellow) demarking the C terminal D-region and the Pro138 residue (red) targeted for site directed mutagenesis. **b**, Coomassie-stained SDS–PAGE of sheared surface proteins (flagellins and pilins), showing relative surface piliation of the $PilA_{II}$ point mutants. All three mutants have similar levels of piliation. The flagellins are used as a loading control. Molecular weight standards in kDa are shown on the left. Image is representative of four biological replicates. **c**, Plaque assays using 5 µl each of serial tenfold dilutions of the phage indicated on the left (10^8–10^1). Expression of the P138R pilin in the PAO1 *pilA* background reduces or blocks infection by JBD26, JBD68 and MP22, but not DMS3 or the JBD26 chimera expressing DMS3 tail proteins, implying that DSM3 binds elsewhere. These images are representative of three biological replicates. C, C terminus; N, N terminus.

D-region of $PilA_{II}$ that is disrupted by changes in side chain electrostatics. These results also suggest that DMS3 and the JBD26/DMS3 chimera interact with PilA in a different manner than JBD26 and JBD68, potentially explaining the ability of DMS3 to infect strains with glycosylated pilins.

P. aeruginosa strains expressing group IV pilins encode a distinct pilin glycosylation system that adds homooligomers of D-Ara*f* to multiple Ser and Thr residues on $PilA_{IV}$[7]. To determine whether this form of glycosylation also serves as a phage defence mechanism, we tested a set of PAO1 *pilA* mutant strains expressing unmodified or glycosylated $PilA_{IV}$. Since JBD26 poorly infects strains expressing $PilA_{IV}$, we used a closely related phage, MP22[30]. Unlike C-terminal glycosylation with O-antigen units, $PilA_{IV}$ glycosylation, which occurs in a different region of this pilin[7], blocked infection by DMS3 and the chimera, as well as MP22 (Fig. 4). Interestingly, JBD68 could not infect the strain producing unmodified $PilA_{IV}$, but could infect the strain expressing glycosylated $PilA_{IV}$ (Fig. 4), suggesting that it recognizes a new epitope on the modified pilin, or the pilin glycans themselves. These data show that both of *P. aeruginosa*'s pilin glycosylation systems have the capacity to protect against subsets of pilus-specific phages. In addition, phages like JBD68 appear to have evolved to exploit some forms of pilin glycosylation for cell surface binding.

Glycosylation of thousands of surface-exposed pilin subunits is energetically costly (each sugar requires the production of a nucleotide-activated precursor, and each glycan unit contains multiple sugars), so such modifications must provide a valuable benefit. Given the powerful impact of phage predation on bacterial evolution, our data provide a compelling rationale for post-translational modification of pilins in environmental and pathogenic bacteria. Glycosylation of *P. aeruginosa* pilins was suggested to protect against complement binding, opsonization by pulmonary surfactant protein A or proteolytic degradation by host or self-produced proteases[31,32]. Similar hypotheses have been advanced to explain the function of pilin glycosylation in *Neisseria* and *Acinetobacter*[27,28]. While these effects may have some impact within the context of infection, resistance to phage predation is likely of greater advantage to bacteria in a broader range of native environments and conditions. Although we do not yet know whether pilin glycosylation is a widespread anti-phage defence mechanism, we found that diverse Proteobacterial species encode homologues of TfpO adjacent to genes encoding PilA homologs (Supplementary Fig. 3, Supplementary Table 3), suggesting that this form of pilin glycosylation is common.

While T4P are well-established receptors for phages, the exact mode of phage binding has not been clarified. Electron micrographs show binding of phages along pilus filaments[11], presumably to the major pilin. Our data strongly support the conclusion that phages bind directly to PilA, rather than any of the minor pilins, for the following reasons: (1) Glycosylation of PilA blocks phage infection, and minor pilins are not glycosylated[20,22,33]. (2) The plaquing ability of the phages was affected by major pilin glycosylation status, and all assays were performed in PAO1 where minor pilins were invariant. (3) Specific amino acid substitutions in PilA changed the susceptibility of strains to phage infection (Fig. 3). Although we cannot rule out that some pilus-specific phages may bind to minor pilins, the phages studied here interact with the major pilin. As minor pilins are mainly localized to the pilus tip and present in minute quantities relative to the major pilin, binding of phages to PilA is considered a superior strategy for cell surface engagement.

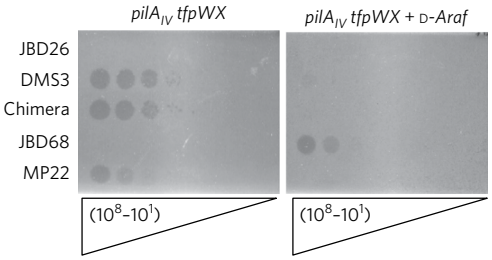

Fig. 4 | Pilin D-arabinofuranosylation blocks phage infection. Plaque assays using 5 µl each of serial tenfold dilutions of the phage indicated on the left (10^8–10^1). Expression of PilA$_{IV}$ and its accessory proteins TfpWX in the PAO1 *pilA* background results in expression of unmodified pilins (left). Addition of the genes encoding the synthesis of D-Araf polymers from pUCP20-6245-51 results in expression of glycosylated pilins[8] (right), and blocks infection by DMS3, the chimeric JBD26 phage and MP22. Image is representative of four biological replicates.

The ability of the phages studied here to use both unmodified PilA$_I$ and PilA$_{II}$ as receptors is remarkable, as these proteins share only 25% identity outside of the highly conserved N-terminal 25 residues that are largely buried in the interior of assembled pilus filaments[34,35]. Binding to diverse PilA proteins likely reflects the results of the evolutionary drive for phages to use a wide variety of pili as receptors, and further emphasizes the difficulty of evading phage predation only through amino acid substitutions in PilA, which could have negative functional consequences. Pilus glycosylation may provide a more effective defence against phages, masking phage-binding sites through steric hindrance. Interestingly, some pilus-specific phages such as JBD68 appear to have evolved the capacity to recognize pilins with specific post-translational modifications (Fig. 4), potentially broadening their host range.

A role for DMS3 gp49, gp50 and/or gp51 in recognizing glycosylated pili was demonstrated using a chimeric phage (Fig. 2). DMS3 gp49 and JBD26 gp59 each have a conserved 100 residue N-terminal domain corresponding to Pfam protein family DUF2793 (PF10983). Proteins containing this domain are encoded in variety of tailed phage and prophage genomes. In non-contractile phage genomes, the DUF2793 protein is invariably encoded immediately downstream of the gene encoding the central fibre protein[36] as in JBD26 and DMS3 (Fig. 2a). Central fibre proteins and others encoded in the 3'-regions of tail operons are often involved in host cell receptor binding[36,37]. DUF2793 proteins are variable in length, with highly divergent C-terminal regions (Supplementary Fig. 2). The conserved N termini likely attach these proteins to the phage particle while the C termini may interact with variable host receptors. Mass spectrometry on purified phages closely related to JBD26 and DMS3 (Supplementary Table 4) confirmed that DUF2793 proteins are present in phage particles. We conclude that gp49 and gp59 are the components of DMS3 and JBD26 tails, respectively, responsible for binding to PilA. The difference in the effects of PilA mutations on infectivity of JBD26 versus DMS3 (Fig. 3c) implies that DMS3 gp49 and JBD26 gp59 interact with different regions of PilA, accounting for the distinct ability of DMS3 to bind glycosylated PilA$_I$. The identical behaviour of the JBD26/DMS3 chimeric phage and DMS3 implies that gp49 is responsible for conferring the unique host specificity of DMS3. The functions of DMS3 gp50 and gp51 are not known. However, the gp50 homologue encoded in JBD68, and the gp51 homologue encoded in JBD88a, were not detected in phage particles, implying that these proteins have no direct role in pilus binding. Since genes encoding homologs of these proteins in other phages are generally adjacent to genes encoding phage receptor proteins, we suspect that they may be chaperones that aid in the folding of receptor binding proteins.

The function of type IV pilin glycosylation has long remained a mystery. We showed that both of *P. aeruginosa*'s glycosylation systems block infection by many pilus-specific phages, and suggest that resistance to phage attack is a major evolutionary driver for these modifications. Since pili are targets for phage adsorption in many species, pilus glycosylation may be a widespread anti-phage defence system. The bulky nature of oligosaccharides and their typically negative charge can effectively block access to large portions of the pilus surface[27], conferring resistance to diverse phages. Glycosylation is likely a more broadly useful resistance mechanism than amino acid substitutions in PilA, a conclusion supported by the ability of the phages tested here to use PilA proteins of diverse sequence as receptors. Glycosylation could also block phage infection without compromising the important adaptive functions of the type IV pilus. In conclusion, pilus glycosylation is another fascinating manifestation of the evolutionary battle between bacteria and phages. The ability of DMS3 and JBD68 to each partially overcome specific forms of pilus glycosylation likely hints at a variety of mechanisms by which phages can circumvent this defence that await discovery.

Methods

Bacterial strains, phage and plasmids used in this work. Bacterial strains, phage and plasmids are listed in Supplementary Table 5. Unless noted otherwise, *Escherichia coli* and *P. aeruginosa* were grown at 37 °C in lysogeny broth (LB) or 1.5% LB agar plates supplemented with antibiotics at the following final concentrations when necessary (µg per ml): ampicillin, 100; kanamycin, 50; gentamicin (Gm), 15 for *E. coli* and 30 for *P. aeruginosa*. Plasmids were transformed by heat shock into chemically competent *E. coli* cells or by electroporation or biparental mating with *E. coli* SM10 into *P. aeruginosa* as previously described[8]. All constructs were verified by DNA sequencing.

Phage plaquing assays. Phage plaque assays were performed as described previously[38], with modifications. Briefly, bacteria were grown overnight at 37 °C with shaking, and then subcultured 1:100 in LB plus 8 mM MgSO4 and grown for 3 h at 37 °C with shaking. The subculture was then standardized to an optical density $(OD)_{600}$ of 0.3 in LB plus 8 mM MgSO4 and 100 µl was mixed with 8 ml top agar (LB plus 8 mM MgSO4, 0.6% agar), which was overlaid on a prepoured rectangular LB plus 8 mM MgSO4 1.5% agar plate containing antibiotics and L-arabinose where indicated. After allowing the top agar to solidify, it was air-dried with the lid off in a biosafety cabinet for 15 min. Phage stocks were standardized to a plaque-forming unit per ml of 10^8 and tenfold serially diluted in LB plus 8 mM MgSO4, and 5 µl of each dilution was spotted onto the prepared plates. After allowing the spots to air dry for 10 min with the lid on, the plates were incubated inverted for 18 h at 37 °C. The plates were then photographed and the titre estimated as the lowest dilution generating a complete zone of cell lysis. Each experiment was repeated a minimum of three times.

Twitching motility assays. Twitching assays were performed as described previously[25] with modifications. Assays were performed in Nunc OmniTrays (Nunc). Single colonies were stab inoculated to the bottom of a 1% LB agar plate which was incubated for 18 h at 30 °C. The agar was then carefully discarded, and the adherent bacteria stained with 1% (w/v) crystal violet dye, followed by washing with tap water to remove unbound dye. Twitching zone areas were measured using ImageJ software (NIH, Bethesda, MD, USA)[39]. All experiments were performed in triplicate with at least three independent replicates. Representative zones are shown.

Sheared surface protein preparation. The levels of surface-exposed pili and flagella were analysed as described previously[8]. Briefly, the strains of interest were streaked in a grid-like pattern on LB agar plates and incubated at 37 °C for ~16 h. The cells were scraped from the plates with glass coverslips and resuspended in 4.5 ml of phosphate buffered saline, pH 7.0. Surface proteins were sheared by vortexing the cell suspensions for 30 s. The suspensions were transferred to three separate 1.5 ml Eppendorf tubes and cells pelleted by centrifugation at 11,688 x *g* for 5 min. The supernatant was transferred to fresh tubes and centrifuged at 11,688 x *g* for 20 min to pellet remaining cells. After transfer of supernatants to new tubes, the surface proteins were precipitated by adding 1:10 volume of 5 M NaCl and 30% (w/v) polyethylene glycol (PEG 8000; Sigma Aldrich) to each tube and incubating on ice for 90 min. Precipitated proteins were collected by centrifugation at 11,688 x *g*, and resuspended in 150 µl of 1X sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris, pH 6.8, 2% β-mercaptoethanol, 20% glycerol, 4% SDS and 0.001% bromophenol blue). Samples were boiled for 10 min and separated on 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels. Proteins were visualized by staining with Coomassie brilliant blue.

Generation of a JBD26-DMS3 chimeric phage. To create a JBD26 chimeric phage expressing DMS3 tail protein, we first generated a JBD26 lysogen of strain PAO1.

The primers chimera1 5'-ACAAGAGAATTCCAGGATCAGTTCAATCTG-3' and chimera2 5'-ACAAGACTGCAGCTAGCCATTGTGCTGTAGCG-3', containing EcoRI and PstI restriction sites (underlined), were used to amplify from the middle of DMS3 gp48 to the end of gp52 (~3 kb). The resulting PCR product was gel-purified and ligated into the suicide vector, pEX18Gm[40]. After validation of the construct by DNA sequencing, it was introduced into *E. coli* SM10 by $CaCl_2$ transformation and transferred to the PAO1 JBD26 lysogen by biparental mating as described previously[8]. Following overnight incubation at 37 °C, mating mixtures were resuspended in 1 ml LB and 100 µl was plated onto *Pseudomonas* isolation agar containing 100 µg per ml of Gm to counterselect the *E. coli* donor. Colonies were picked from *Pseudomonas* isolation agar onto LB Gm30 agar and, from there, onto LB no salt, 5% sucrose agar to select against merodiploids. Colonies that grew on sucrose plates were streaked in parallel on LB Gm30 and LB agar, and Gm-sensitive colonies were tested by colony PCR using primers DMS3gp50p1 5'-GAACAGAATTCGAGGTGGTTCTGATGATGATCATC-3' and DMS3gp50p2 5'-GAACAGGATCCTCATTGCGGCAACTCCACAGG-3', designed to amplify DMS3 gp50. DNA from PCR-positive colonies was reamplified using primers DMS3gp49p1 5'-GAACAGAATTCAGGAGGCGTATCCGCATGAGC-3' and DMS3gp52p2 5'-ACAAGACTGCAGCTAGCCATTGTGCTGTAGCG-3', and the resulting amplicons sequenced to verify gene replacement.

To induce the excision of the chimeric phage, the lysogen was grown overnight in LB and subcultured 1:100 to OD_{600} 0.6 in 3 ml of the same medium, then treated with mitocycin C (final concentration 3 µg per ml) for 18 h. The remaining cells were lysed by adding ~250 µl chloroform and vortexing briefly to mix. The phages were titred by standard plaque assay. Tenfold serial dilutions of the lysate were prepared and 100 µl each mixed with 100 µl of PAO1 standardized to OD_{600} 0.3 in LB plus 10 mM $MgSO_4$ and added to 8 ml of top agar (LB plus MgSO4, 0.6% agar) that was overlaid onto a standard LB plus 10 mM $MgSO_4$ plate and allowed to solidify. After 18 h incubation at 37 °C, the dilution(s) giving countable plaques were used to calculate the plaque-forming units per ml of lysate.

Mass spectrometry analysis of phage particles. We subjected 3.8×10^9 phage particles from lysate purified twice by CsCl density gradient ultracentrifugation to tryptic digest as described previously[41]. Liquid chromatography tandem-mass spectrometry spectra were collected on a linear ion-trap instrument (LTQ; ThermoFisher) (*SPARC* BioCentre, The Hospital for Sick Children, Toronto, Canada). Proteins were identified using Mascot (Matrix Science, London, UK) and analysed in Scaffold version 3.0 (Proteome Software Inc., Portland, OR, USA). The protein identification cut off was set at a confidence level of 95% with a requirement for at least two peptides to match to a protein.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. Accession codes for protein and genome sequence data presented herein are provided in the Supplementary Information.

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References

1. Weinbauer, M. G. Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* **28**, 127–181 (2004).
2. Sampson, T. R. & Weiss, D. S. Alternative roles for CRISPR/Cas systems in bacterial pathogenesis. *PLoS Pathog.* **9**, e1003621 (2013).
3. Sorek, R., Lawrence, C. M. & Wiedenheft, B. CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu. Rev. Biochem.* **82**, 237–266 (2013).
4. Labrie, S. J., Samson, J. E. & Moineau, S. Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* **8**, 317–327 (2010).
5. Kus, J. V., Tullis, E., Cvitkovitch, D. G. & Burrows, L. L. Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology* **150**, 1315–1326 (2004).
6. DiGiandomenico, A. et al. Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: glycan substrate specificity. *Mol. Microbiol.* **46**, 519–530 (2002).
7. Kus, J. V. et al. Modification of *Pseudomonas aeruginosa* Pa5196 type IV pilins at multiple sites with D-Ara*f* by a novel GT-C family Arabinosyltransferase, TfpW. *J. Bacteriol.* **190**, 7464–7478 (2008).
8. Harvey, H., Kus, J. V., Tessier, L., Kelly, J. & Burrows, L. L. *Pseudomonas aeruginosa* D-arabinofuranose biosynthetic pathway and its role in type IV pilus assembly. *J. Biol. Chem.* **286**, 28128–28137 (2011).
9. Voisin, S. et al. Glycosylation of *Pseudomonas aeruginosa* strain Pa5196 type IV pilins with mycobacterium-like α-1,5-linked D-Ara*f* oligosaccharides. *J. Bacteriol.* **189**, 151–159 (2007).
10. Lam, J. S., Taylor, V. L., Islam, S. T., Hao, Y. & Kocincova, D. Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. *Front. Microbiol.* **2**, 118 (2011).
11. Bradley, D. E. & Pitt, T. L. Pilus-dependence of four *Pseudomonas aeruginosa* bacteriophages with non-contractile tails. *J. Gen. Virol.* **24**, 1–15 (1974).
12. Burrows, L. L. *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu. Rev. Microbiol.* **66**, 493–520 (2012).
13. Craig, L. & Li, J. Type IV pili: paradoxes in form and function. *Curr. Opin. Struct. Biol.* **18**, 267–277 (2008).
14. Heiniger, R. W., Winther-Larsen, H. C., Pickles, R. J., Koomey, M. & Wolfgang, M. C. Infection of human mucosal tissue by *Pseudomonas aeruginosa* requires sequential and mutually dependent virulence factors and a novel pilus-associated adhesin. *Cell. Microbiol.* **12**, 1158–1173 (2010).
15. Ohama, M. et al. Intratracheal immunization with pili protein protects against mortality associated with *Pseudomonas aeruginosa* pneumonia in mice. *FEMS Immunol. Med. Microbiol.* **47**, 107–115 (2006).
16. Hahn, H. P. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa* – a review. *Gene* **192**, 99–108 (1997).
17. Bucior, I., Pielage, J. F. & Engel, J. N. *Pseudomonas aeruginosa* pili and flagella mediate distinct binding and signaling events at the apical and basolateral surface of airway epithelium. *PLoS Pathog.* **8**, e1002616 (2012).
18. James, C. E. et al. Differential infection properties of three inducible prophages from an epidemic strain of *Pseudomonas aeruginosa*. *BMC Microbiol.* **12**, 216 (2012).
19. Bondy-Denomy, J. et al. Prophages mediate defense against phage infection through diverse mechanisms. *ISME J.* **10**, 2854–2866 (2016).
20. Nguyen, Y. et al. *Pseudomonas aeruginosa* minor pilins prime type IVa pilus assembly and promote surface display of the PilY1 adhesin. *J. Biol. Chem.* **290**, 601–611 (2015).
21. Winther-Larsen, H. C. et al. *Pseudomonas aeruginosa* type IV pilus expression in *Neisseria gonorrhoeae*: effects of pilin subunit composition on function and organelle dynamics. *J. Bacteriol.* **189**, 6676–6685 (2007).
22. Giltner, C. L., Habash, M. & Burrows, L. L. *Pseudomonas aeruginosa* minor pilins are incorporated into type IV pili. *J. Mol. Biol.* **398**, 444–461 (2010).
23. Comer, J. E., Marshall, M. A., Blanch, V. J., Deal, C. D. & Castric, P. Identification of the *Pseudomonas aeruginosa* 1244 pilin glycosylation site. *Infect. Immun.* **70**, 2837–2845 (2002).
24. Smedley, J. G. III et al. Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. *Infect. Immun.* **73**, 7922–7931 (2005).
25. Asikyan, M. L., Kus, J. V. & Burrows, L. L. Novel proteins that modulate type IV pilus retraction dynamics in *Pseudomonas aeruginosa*. *J. Bacteriol.* **190**, 7022–7034 (2008).
26. Allison, T. M., Conrad, S. & Castric, P. The group I pilin glycan affects type IVa pilus hydrophobicity and twitching motility in *Pseudomonas aeruginosa* 1244. *Microbiology* **161**, 1780–1789 (2015).
27. Gault, J. et al. *Neisseria meningitidis* type IV pili composed of sequence invariable pilins are masked by multisite glycosylation. *PLoS Pathog.* **11**, e1005162 (2015).
28. Piepenbrink, K. H. et al. Structural diversity in the type IV pili of multidrug-resistant *Acinetobacter*. *J. Biol. Chem.* **291**, 22924–22935 (2016).
29. Harvey, H., Habash, M., Aidoo, F. & Burrows, L. L. Single-residue changes in the C-terminal disulfide-bonded loop of the *Pseudomonas aeruginosa* type IV pilin influence pilus assembly and twitching motility. *J. Bacteriol.* **191**, 6513–6524 (2009).
30. Heo, Y. J., Chung, I. Y., Choi, K. B., Lau, G. W. & Cho, Y. H. Genome sequence comparison and superinfection between two related *Pseudomonas aeruginosa* phages, D3112 and MP22. *Microbiology* **153**, 2885–2895 (2007).
31. Castric, P., Cassels, F. J. & Carlson, R. W. Structural characterization of the *Pseudomonas aeruginosa* 1244 pilin glycan. *J. Biol. Chem.* **276**, 26479–26485 (2001).
32. Tan, R. M. et al. Type IV pilus glycosylation mediates resistance of *Pseudomonas aeruginosa* to opsonic activities of the pulmonary surfactant protein A. *Infect. Immun.* **83**, 1339–1346 (2015).
33. Nguyen, Y. et al. Structural and functional studies of the *Pseudomonas aeruginosa* minor pilin, PilE. *J. Biol. Chem.* **290**, 26856–26865 (2015).
34. Craig, L. et al. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol. Cell.* **11**, 1139–1150 (2003).
35. Kolappan, S. et al. Structure of the *Neisseria meningitidis* type IV pilus. *Nat. Commun.* **7**, 13015 (2016).
36. Davidson, A. R., Cardarelli, L., Pell, L. G., Radford, D. R. & Maxwell, K. L. Long noncontractile tail machines of bacteriophages. *Adv. Exp. Med. Biol.* **726**, 115–142 (2012).
37. Le, S. et al. Mapping the tail fiber as the receptor binding protein responsible for differential host specificity of *Pseudomonas aeruginosa* bacteriophages PaP1 and JG004. *PLoS ONE* **8**, e68562 (2013).
38. Bondy-Denomy, J., Pawluk, A., Maxwell, K. L. & Davidson, A. R. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature* **493**, 429–432 (2013).

39. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
40. Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86 (1998).
41. Lavigne, R., Ceyssens, P. J. & Robben, J. Phage proteomics: applications of mass spectrometry. *Methods Mol. Biol.* **502**, 239–251 (2009).

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Author contributions

H.H., A.R.D. and L.L.B. designed the study; H.H., J.B.-D., H.M. and K.M.S. performed experiments; H.H., A.R.D. and L.L.B. analysed the data; A.R.D. and L.L.B. wrote the manuscript with input from H.H., J.B.-D. and H.M. All authors approved the final version.

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Experimental design

1. Sample size

Describe how sample size was determined.

Bacteria: recombinant P. aeruginosa strains expressing 3 different pilins, with and without their associated accessory genes.
Phages: A previously characterized set of pilus-specific phages was used for this study (Bondy-Denomy, J. et al. Prophages mediate defense against phage infection through diverse mechanisms. ISME J 10, 2854-2866 (2016)).

2. Data exclusions

Describe any data exclusions.

No data were excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

The results were reproducible in multiple biological replicates - the specific number of replicates used for each experiment are indicated in the figure legends.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

All phages were initially tested against 3 bacterial strains. A subset of phages were selected for further study based on their infection patterns and genetic relatedness.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Blinding was not relevant to the study. The data are binary (lysis/no lysis).

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