AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Phage Morons Play an Important Role in *Pseudomonas aeruginosa* Phenotypes

Yu-Fan Tsao,[a] Véronique L. Taylor,[b] Smriti Kala,[b] Joseph Bondy-Denomy,[a]* Alima N. Khan,[b] Diane Bona,[b] Vincent Cattoir,[c]* Stephen Lory,[c] Alan R. Davidson,[a,b] Karen L. Maxwell[b]

[a]Department of Molecular Genetics, University of Toronto, Toronto, Canada
[b]Department of Biochemistry, University of Toronto, Toronto, Canada
[c]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

ABSTRACT The viruses that infect bacteria, known as phages, play a critical role in controlling bacterial populations in many diverse environments, including the human body. This control stems not only from phages killing bacteria but also from the formation of lysogens. In this state, the phage replication cycle is suppressed, and the phage genome is maintained in the bacterial cell in a form known as a prophage. Prophages often carry genes that benefit the host bacterial cell, since increasing the survival of the host cell by extension also increases the fitness of the prophage. These highly diverse and beneficial phage genes, which are not required for the life cycle of the phage itself, have been referred to as "morons," as their presence adds "more on" the phage genome in which they are found. While individual phage morons have been shown to contribute to bacterial virulence by a number of different mechanisms, there have been no systematic investigations of their activities. Using a library of phages that infect two different clinical isolates of *P. aeruginosa*, PAO1 and PA14, we compared the phenotypes imparted by the expression of individual phage morons. We identified morons that inhibit twitching and swimming motilities and observed an inhibition of the production of virulence factors such as rhamnolipids and elastase. This study demonstrates the scope of phage-mediated phenotypic changes and provides a framework for future studies of phage morons.

IMPORTANCE Environmental and clinical isolates of the bacterium *Pseudomonas aeruginosa* frequently contain viruses known as prophages. These prophages can alter the virulence of their bacterial hosts through the expression of nonessential genes known as "morons." In this study, we identified morons in a group of *Pseudomonas aeruginosa* phages and characterized the effects of their expression on bacterial behaviors. We found that many morons confer selective advantages for the bacterial host, some of which correlate with increased bacterial virulence. This work highlights the symbiotic relationship between bacteria and prophages and illustrates how phage morons can help bacteria adapt to different selective pressures and contribute to human diseases.

KEYWORDS *Pseudomonas aeruginosa*, bacteriophage resistance, bacteriophages, biofilms, motility

Although the viruses that infect bacteria (phages) are often regarded as parasites, a more accurate description for the majority of phages is that they are symbionts. This viewpoint arises from the realization that most bacterial genomes contain phage genomes, known as prophages, that are maintained in an inactive or "lysogenic" state. These temperate phages live a double life in which they sometimes behave as predators, infecting and lysing their host bacteria, and at other times integrate their

The "Since, If, Then" statements are in the right margins throughout the article.

Received 28 March 2018 **Accepted** 16 August 2018

Accepted manuscript posted online 27 August 2018

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

Editor George O'Toole, Geisel School of Medicine at Dartmouth

Address correspondence to Karen L. Maxwell, karen.maxwell@utoronto.ca.

* Present address: Joseph Bondy-Denomy, Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, California, USA; Vincent Cattoir, U1230 Inserm Unit, University of Rennes 1, and Department of Clinical Microbiology, Rennes University Hospital, Rennes, France.

genomes into that of the host, replicating passively with the bacterial genome. In the lysogenic state, phage replication is suppressed and the expression of the majority of the phage genes is repressed. However, a subset of prophage genes is expressed, and it is evolutionarily adaptive for the phage if these genes can confer a fitness advantage for the bacterial host. Thus, these phages are simultaneously both friend and enemy to their host.

Genes expressed from prophages may enhance bacterial survival by a number of mechanisms. For example, many prophages encode superinfection exclusion proteins that inhibit further phage infection by altering the bacterial cell envelope (1, 2). This is highly adaptive, as phage predation is a major challenge for bacteria in all environments. In other cases, the fitness advantages conferred by prophages increase the pathogenicity of bacterial strains infecting humans; thus, genes expressed from prophages are often recognized as virulence factors. For example, the *Escherichia coli* phage λ prophage-expressed *lom* and *bor* genes encode proteins that increase bacterial adherence to human buccal epithelial cells (3) and improve the survival of cells in animal serum (4), respectively. Similarly, *Pseudomonas aeruginosa* FIZ15 prophage expresses genes that increase adherence to epithelial cells and provide resistance to phagocytosis and human serum (5). Prophage genes also contribute significantly to bacterial pathogenesis in *Pseudomonas* lung infections in cystic fibrosis patients. For example, in the highly transmissible Liverpool epidemic strain, LESB58, three of its five prophages were shown to strongly enhance *in vivo* competitiveness in a chronic lung infection model in rats (6). The presence of a prophage was also shown to be important in the high transmissibility of the Manchester epidemic strain (7). The genomic sequencing of large numbers of pathogenic bacterial strains over the past decade has made it abundantly clear that the acquisition of prophages is a major evolutionary route by which free-living bacteria convert from being benign to being pathogenic (8).

Many prophage genes that have been shown to enhance bacterial fitness (9) are carried as genes known as "morons," a term coined by Juhala et al. in a comparative study of *E. coli* phages (10). Families of related phages have conserved gene orders that enable the identification of gene functions on the basis of both protein sequence similarity and genomic position. These conserved gene clusters frequently contain inserted genes found in only a subset of the phage family members. Since their presence adds "more on" the DNA compared to related phages that do not encode them, they were designated morons. Morons frequently have GC contents that differ from those of the surrounding genes, implying an acquisition by horizontal gene transfer, and often contain their own promoter and terminator sequences to enable expression from the prophage. The presence of morons in only a subset of phages within a given family implies that these genes are not essential for the life cycle of the phage but play an accessory role, possibly providing an evolutionary advantage only under certain conditions.

The demonstrated importance of prophages in *P. aeruginosa* virulence in cystic fibrosis epidemic strains motivated us to initiate a systematic investigation of the phenotypic alterations mediated by a group of closely related prophages introduced into a single *P. aeruginosa* strain background (11). We discovered that these prophages mediate diverse effects impacting phage resistance and bacterial motility. While the majority of genes in these phages were highly conserved among all members and could be ascribed functions required for the lytic or lysogenic life cycle of the phage, each phage also harbored a unique assortment of approximately 10 morons. To gain further insight into the mechanisms by which prophages alter bacterial behavior, here we characterized the effects of expressing 14 different moron genes in two different *P. aeruginosa* clinical strain backgrounds. The only criterion used for selecting the genes investigated in this study was that they were not conserved among phages in our collection. Nevertheless, we found that the expression of 85% of these genes markedly altered at least one measurable bacterial phenotype. Our results demonstrate the many ways by which morons can modulate bacterial phenotypes and highlight the complex impacts that prophages may exert on bacterial behavior and human disease.

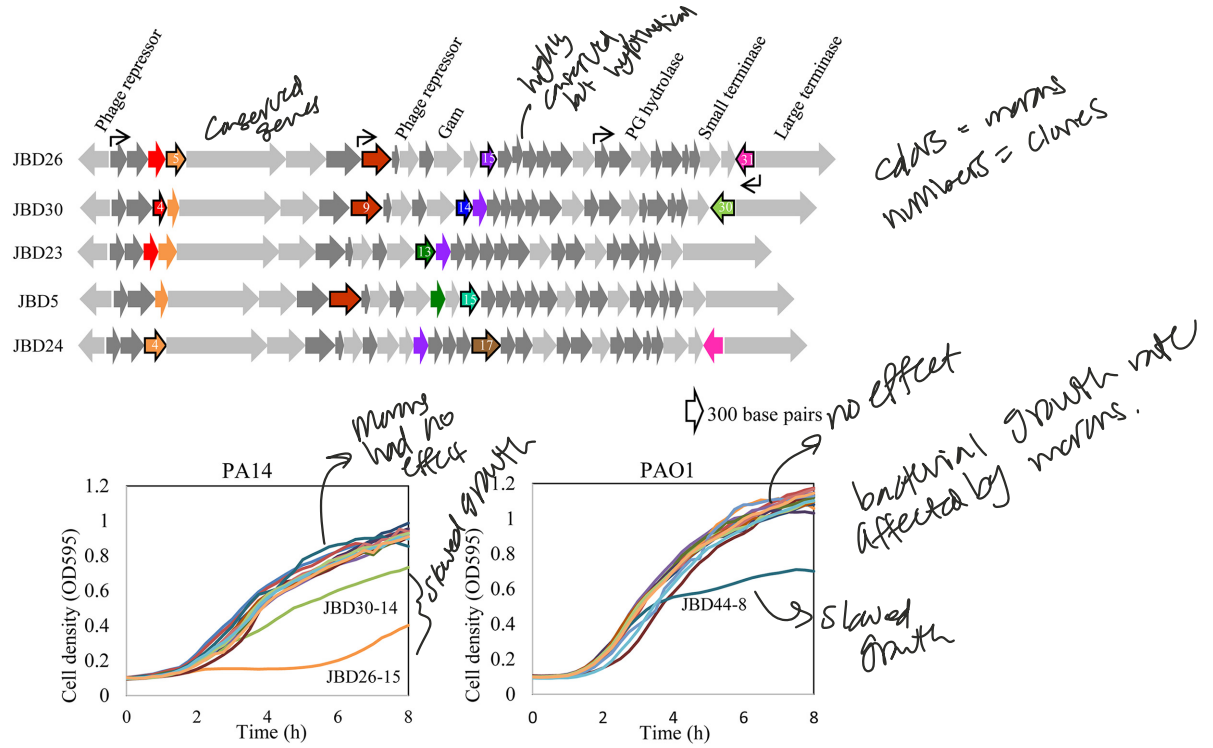

FIG 1 Phage morons are dispersed among phage genomes. (Top) A representative genome alignment of Mu-like phages JBD26, JBD30, JBD23, JBD5, and JBD24 illustrates the sporadic nature of moron gene insertion. The genomes of these related *P. aeruginosa* phages were aligned on the basis of sequence similarity and known gene functions. The light gray arrows represent conserved genes of known functions, and the dark gray arrows represent highly conserved hypothetical genes. Each moron gene sequence family is colored differently, with the numbers indicating the cloned genes examined in this study. The arrows indicate the gene orientation and direction of transcription, and the bent arrows denote predicted promoter sequences. (Bottom) Moron expression can affect bacterial growth rates. *P. aeruginosa* PA14 or PAO1 strains expressing individual morons from a plasmid were grown in liquid culture at 37°C, and the cell densities were monitored over 8 h. While most morons had no effect on bacterial growth rates, JBD26-15 significantly slowed the growth of PA14, while JBD30-14 slowed growth slightly. The expression of JBD44-8 significantly decreased the growth of PAO1.

RESULTS

Prophages harbor unique complements of morons. Our previous study of the effects of the integration of a group of prophages on *P. aeruginosa* behaviors suggested that the nonconserved phage genes led to phenotypic diversity in bacterial lysogens (11). However, no specific correlations between prophage gene complement and bacterial phenotypes was identified. To define the specific contributions of the nonconserved genes to *Pseudomonas* phenotypes, we selected 14 morons harbored by this group of phages for further studies (Fig. 1, top; Table 1). These genes encoded proteins of unknown function that ranged in size from 69 to 224 amino acids. While most are predicted to be cytoplasmic, a few are predicted to be localized to the periplasm and the inner membrane by the Consensus Constrained TOPology (CCTOP) prediction web server (12). We performed BLAST searches using these protein sequences and discovered that most had greater than 100 homologues in the same genomic locations in *P. aeruginosa* phages and prophages. Two morons were found to have homologues in diverse genomic contexts (e.g., near excisionase, terminase, and replicase genes) distributed across a wide variety of Gram-negative bacteria (Table 1).

Morons increase bacterial survival by protecting against further phage infection. To determine if the expression of these moron genes led to detectable phenotypes in the bacterial host cell, we cloned them into expression plasmids. We transformed *Pseudomonas aeruginosa* strains PA14 and PAO1 with each plasmid so that we could examine the effect of moron gene expression in two distinct strain backgrounds.

TABLE 1 Summary of morons included in this study

Moron	GenBank no.	Size (kDa)	% identity of homologs	No. of BLAST homologs[a]	Predicted subcellular localization	Other genera or orders containing homologs	Genomic location
JBD30-4	AFQ21918.1	12.5	50–100	>100	Cytoplasm	NA[b]	Conserved
JBD26-5	AGC24045.1	14.4	49–100	>100	Cytoplasm	NA	Conserved
JBD24-4	AFQ21860.1	15.1	47–94	>100	Cytoplasm	*Acinetobacter*	Conserved
JBD30-9	AFQ21923.1	23.6	35–100	>100	Inner membrane	*Azotobacter*	Conserved
JBD23-13	NA	13.6	33–100	>100	Cytoplasm	*Flavobacterium*, *Burkholderia*, *Pseudacidovorax*, *Rhizobium*, *Cupriavidus*	Conserved
JBD30-14	AFQ21928.1	7.5	32–100	96	Cytoplasm	*Chromobacterium*	Conserved
JBD26-15	AEY99428.1	11.2	29–100	>100	Cytoplasm	*Salmonella*, *Candidatus*	Conserved
JBD5-15	AFQ21812.1	8.1	29–100	>100	Cytoplasm	*Variovorax*, *Bradyrhizobium*, *Xanthomonas*	Conserved
JBD24-17	AFQ21873.1	23.9	30–100	>100	Inner membrane	*Laribacter*, *Hydrogenophilales*, *Rhodocyclales*, *Bordetella*, *Acinetobacter*, *Serratia*, *Vibrio*, *Chromobacterium*, *Burkholderia*, *Ralstonia*, *Candidatus*, *Curvibacter*[c]	Diverse
JBD26-31	AEY99485.1	9.3	87–100	78	Cytoplasm	NA	Conserved
JBD30-30	AFQ21944.1	18.3	22–100	>100	Periplasm	*Salmonella*, *Shigella*, *Escherichia*, *Neisseria*, *Achromobacter*, *Xanthomonas*, *Methylobacter*, *Ochrobactrum*, *Klebsiella*, *Yersinia*[c]	Diverse
JBD26-61	AEY99477.1	8.5	50–100	>100	Cytoplasm	NA	Conserved
JBD44-8	YP_009275495.1	5.7	63–100	5	Inner membrane	NA	Conserved
JBD44-9	YP_009275496.1	8.6	81–100	87	Periplasm	NA	Conserved

[a]Proteins with BLAST E-values $<10^{-4}$ were considered homologs.
[b]NA, not available.
[c]Representative genera or orders from the >15 total from BLAST.

PA14 is a highly virulent clinical isolate representing the most common clonal group worldwide (13), while PAO1 is a moderately virulent strain that belongs to a relatively rare clonal group (14). To ensure that there were no major growth defects resulting from the gene expression, we performed growth assays in rich liquid medium at 37°C using a Tecan microplate reader to monitor cell density. The expression of the morons in PA14 had no effect on the growth rates of the bacteria (Fig. 1, bottom) with the exception of JBD30-14, whose expression resulted in a slight but reproducibly slowed growth, and JBD26-15, which caused a very striking growth defect with little growth noted even after 8 h when it was expressed in strain PA14. Strikingly, the growth rates were unaffected when these genes were expressed in strain PAO1 (Fig. 1, bottom), highlighting the potential strain dependence of moron activity. This strain dependence was also observed for the PAO1 culture expressing JBD44-8, which initially grew at rates comparable to those of other cultures but reached stationary phase at a much lower cell density (Fig. 1, bottom). These results may stem from the differential expression of the genes in different host backgrounds or the interaction of the morons with a bacterial host cell component(s) that is unique to one of these strains.

As prophages and their bacterial hosts exist in a symbiotic relationship, anything that increases host cell survival by extension is also beneficial to the phage. Thus, prophages frequently encode proteins that block the ability of other phages to infect the bacterial lysogen (1, 15, 16). Prophages that contain the morons examined in this work were previously shown to block superinfection through a repressor-independent mechanism (11). However, the specific genes responsible for this phenotype were not determined. To determine if the morons in our collection conferred phage resistance on the bacterial cell, we examined the resistance of each of the strains to the killing

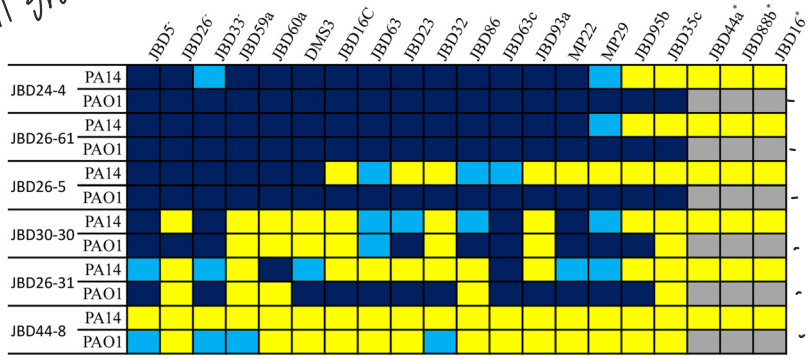

FIG 2 Phage morons increase resistance to further phage infection. The expression of six different morons inhibited the ability of phages to infect PA14 and PAO1. The genes expressed in each strain (PA14 and PAO1) are noted in the left-hand column. The phages tested for plaquing ability are listed along the top of the chart. Phages designated with an "*" rely on lipopolysaccharide as a receptor, phages designated with a "−" require both lipopolysaccharide and the type IV pilus as coreceptors, and the remaining phages rely on the type IV pilus as a receptor. Dark blue squares indicate resistance (>10^3-fold reduction in plaquing efficiency), light blue squares indicate partial resistance (10- to 100-fold reduction), and yellow squares indicate no effect on plaquing efficiency. The gray squares indicate phages that were unable to infect wild-type PAO1.

activity of a panel of phages. We examined a collection of 20 *Pseudomonas* phages that use the type IV pilus, O antigen, or both as receptors for the infection of the host cell. Serial dilutions of these phages were spotted on lawns of PA14 and PAO1 expressing the individual morons, and plaque formation was assessed. The expression of six different morons was found to make the cells resistant (>10^3-fold reduction in plaquing efficiency, denoted by dark blue squares) or partially resistant (10- to 100-fold reduction, denoted by light blue squares) to phage infection (Fig. 2). JBD24-4 and JBD26-61 strongly inhibited plaque formation by almost all phages tested in both PAO1 and PA14. In contrast, JBD26-5 broadly inhibited plaque formation in PAO1, but was much less effective at blocking phage infection when expressed in PA14. A homologue of JBD26-5 from phage D3112 was previously shown to inhibit phage infection through an interaction with PilB, the type IV pilus assembly/extension ATPase (17). JBD30-30 and JBD26-31 were less efficient at inhibiting plaque formation, with fewer phages blocked by their activities. JBD44-8 provided partial resistance against four phages when expressed in PAO1 and showed no inhibitory activity in PA14. These results show that several morons function to increase the resistance of the bacteria against a variety of phages. As the patterns of resistance are altered for different morons and there are host-specific differences observed, they are likely acting through different mechanisms and are interacting with different host components in the bacterial cell.

Bacterial motility is influenced by moron expression. To gain insight into the mechanism of phage resistance imparted by moron expression, we next assessed bacterial motility. As many of the phages in our collection are dependent on the type IV pilus for infection (11), one way in which the bacterial lysogen could protect itself is through abrogating pilus assembly or modifying the pilus in some way to prevent superinfecting phages from attaching productively to it. We previously showed that prophages containing the morons examined in this work abrogated the twitching motility of their bacterial host, and this was strongly correlated with phage resistance (11). To determine the particular genes that were responsible for this phenotype, we measured the twitching motility of strains expressing each of the morons individually. Twitching motility assays, in which bacteria move along a solid medium via pilus activity, revealed that approximately half of the morons decreased bacterial twitching motility (Fig. 3a). Similar to the phage resistance patterns, twitching behaviors were found to differ between PA14 and PAO1. Five morons decreased twitching when expressed in both PA14 (black bars) and PAO1 (gray bars), while two (JBD30-4 and JBD24-17) caused twitching defects only in strain PAO1. In addition, within a single *P.*

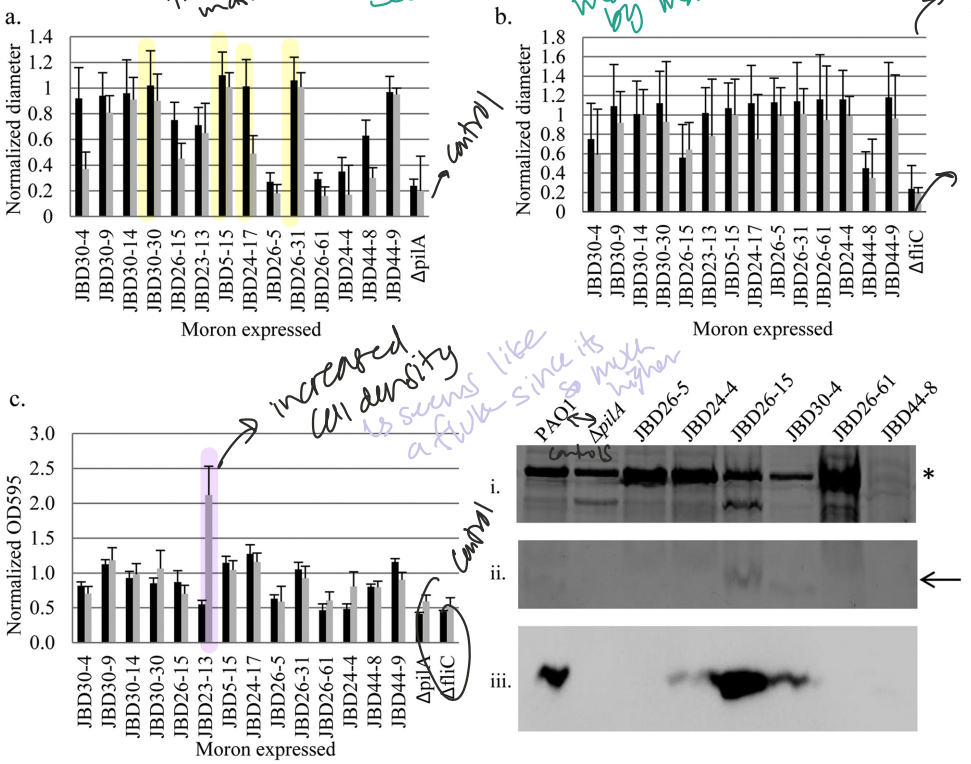

FIG 3 Moron expression can cause changes in bacterial twitching motility, swimming motility, and biofilm production. *P. aeruginosa* PA14 (black bars) and PAO1 (gray bars) strains overexpressing individual morons were assessed for their ability to twitch (a), swim (b), and produce biofilm (c). Type IV pilus (*pilA*) and flagellum (*fliC*) knockout controls were also included for comparison. The experiments were repeated on three different occasions, with each experiment containing three technical replicates. All measurements were normalized to the empty vector control in PAO1 or PA14. (c, right) Assessment of the pili and flagella present on the surfaces of cells expressing different morons. A shearing assay followed by analysis of the surface proteins using SDS-PAGE followed by Coomassie staining revealed that (i) flagella are reduced on the surfaces of cells expressing JBD44-8 (fliC protein is noted with an "*") and (ii) the expression of JBD26-15 leads to hyperpiliation (PilA is indicated by the arrow). (iii) Western blot of panel ii reveals that there are pili present on the surfaces of strains expressing JBD26-15 and JBD30-4, while the remaining nontwitching mutants have no surface pili. Wild-type POA1 is provided for comparison.

aeruginosa strain, different morons affected twitching motility to different degrees. For example, the expression of JBD26-5, JBD26-61, and JBD24-4 completely abrogated twitching motility, resulting in twitch zone sizes comparable to those of the pilus knockout, while others (e.g., JBD26-15 and JBD44-8) showed less severe twitching defects. To determine whether the observed twitching defects were due to a loss of pili on the surfaces of the bacteria, we performed a shearing assay in the PAO1 strain background. The strains harboring morons that completely abrogated twitching motility (JBD26-5, JBD26-61, and JBD24-4) had no pili present on the surfaces of the cells, while that with JBD26-15, which reduced but did not abrogate twitching, was hyperpiliated (Fig. 3c, right lower panel). These data suggest that these genes are acting through at least two different mechanisms, one that prevents the assembly of the pilus on the surface of the cell and one that prevents pilus retraction. Additional evidence that there are at least two mechanisms of activity at play is provided by the phage resistance profiles of these twitching mutant strains; those with morons that prevent the assembly of the pilus on the surface of the cell are completely resistant to phages that require the pilus for infection, while the nontwitching hyperpiliated strain is still susceptible (Fig. 2).

Phages have also been shown to use the flagellum for infection (18, 19). Thus, moron-expressing strains were assayed for altered swimming motility. The expression of three morons (JBD30-4, JBD26-15, and JBD44-8) caused swimming defects in both

PA14 and PAO1 (Fig. 3b). However, the effects we observed on swimming motility were moderate, with none leading to inhibition as great as that of the flagellar (*fliC*) knockout. We tested for the presence of flagella on the surfaces of the cells using a shearing assay and found that JBD44-8, which was most severely compromised for swimming motility, had little detectable flagellar protein (Fig. 3c, right upper panel). In summary, we found that the expression of several morons affected twitching and swimming motility, and in some cases, these alterations occurred in a strain-dependent manner.

Moron expression can alter bacterial virulence factor production. Since prophage acquisition has been associated with changes in bacterial virulence (20, 21), we examined the effects of moron expression on a number of bacterial phenotypes that are associated with increased virulence. For example, biofilms mediate attachment to mammalian cells, aid in immune system evasion, and increase antibiotic resistance (22–24), and so we examined the ability of the moron genes in our collection to mediate changes in biofilm formation. We grew bacteria expressing each of the morons in 96-well plates overnight and then quantified the amount of biofilm by staining the biomass adhered to the plate with crystal violet. As can be seen in Fig. 3c, JBD23-13 led to a large increase in biofilm when expressed in strain PAO1. Strikingly, it had the opposite effect and decreased biofilm formation when expressed in PA14. We found that the morons that inhibited twitching motility to the same degree as for the *pilA* mutant also showed decreased biofilm production (JBD26-5, JBD24-4, and JBD26-61). This was expected, as type IV pili have been shown to play important roles in the processes required for biofilm formation, including cell aggregation, microcolony formation, and surface attachment (25–27).

The ability of *P. aeruginosa* to infect and persist in the airway epithelial cells of cystic fibrosis patients is due in part to the overproduction of rhamnolipid and alginate virulence factors (28, 29). Using a simple assay for rhamnolipid production, we determined that while the expression of several moron genes led to a moderate decrease in rhamnolipid production, none led to an increase (Fig. 4a). In each case, the observed decrease was not as great as that observed for the *rmlC* mutant, which blocks the production of L-rhamnose. A single moron, JBD44-8, was shown to induce the formation of mucoid colonies when expressed in strain PAO1, suggesting that it led to alginate overproduction. The moron collection was also screened for changes in secreted virulence factors by protease, elastase, and lipase assays. We found that only elastase production was affected, and its production was decreased when two genes (JBD23-13 and JBD26-15) were expressed in the PAO1 background (Fig. 4c).

The innate immune response to an infection by *P. aeruginosa* requires the O-specific antigen component of lipopolysaccharide (LPS), and one way in which bacteria escape the immune response is by altering their O antigen. As *Pseudomonas* phages have been shown to encode enzymes that modify the O antigen (30), we examined the LPS profile of each moron-expressing strain. We observed a loss of the very-long-chain O-specific antigen when moron gene JBD30-9 was expressed in both PA14 and PAO1 (Fig. 4b). This protein, which is predicted to contain a single transmembrane domain (12), does not share detectable sequence similarity with the inhibitor of α-polymerase (*iap*) previously shown to disrupt Wzy/Wzz interaction (31). Thus, it represents a novel targeted inhibition of Wzz_2, the polysaccharide copolymerase responsible for imparting very-long-chain modality (32). Homologues of JBD30-9 occur in LPS-dependent phages JBD26, JBD5, ϕ297, and F1Z15, yet JBD30 does not require the O-specific antigen for infection (11). This altered O-specific antigen chain length may provide an advantage to the host by increasing phage resistance, as was recently shown in *P. aeruginosa* strain PAO1 (33).

As the ability to withstand antibiotic exposure increases the chance of successful infection and propagation in a human infection, and since *P. aeruginosa* infections are notoriously difficult to eradicate due to high levels of intrinsic antibiotic resistance (34), we also examined the ability of each phage moron to influence antibiotic susceptibility.

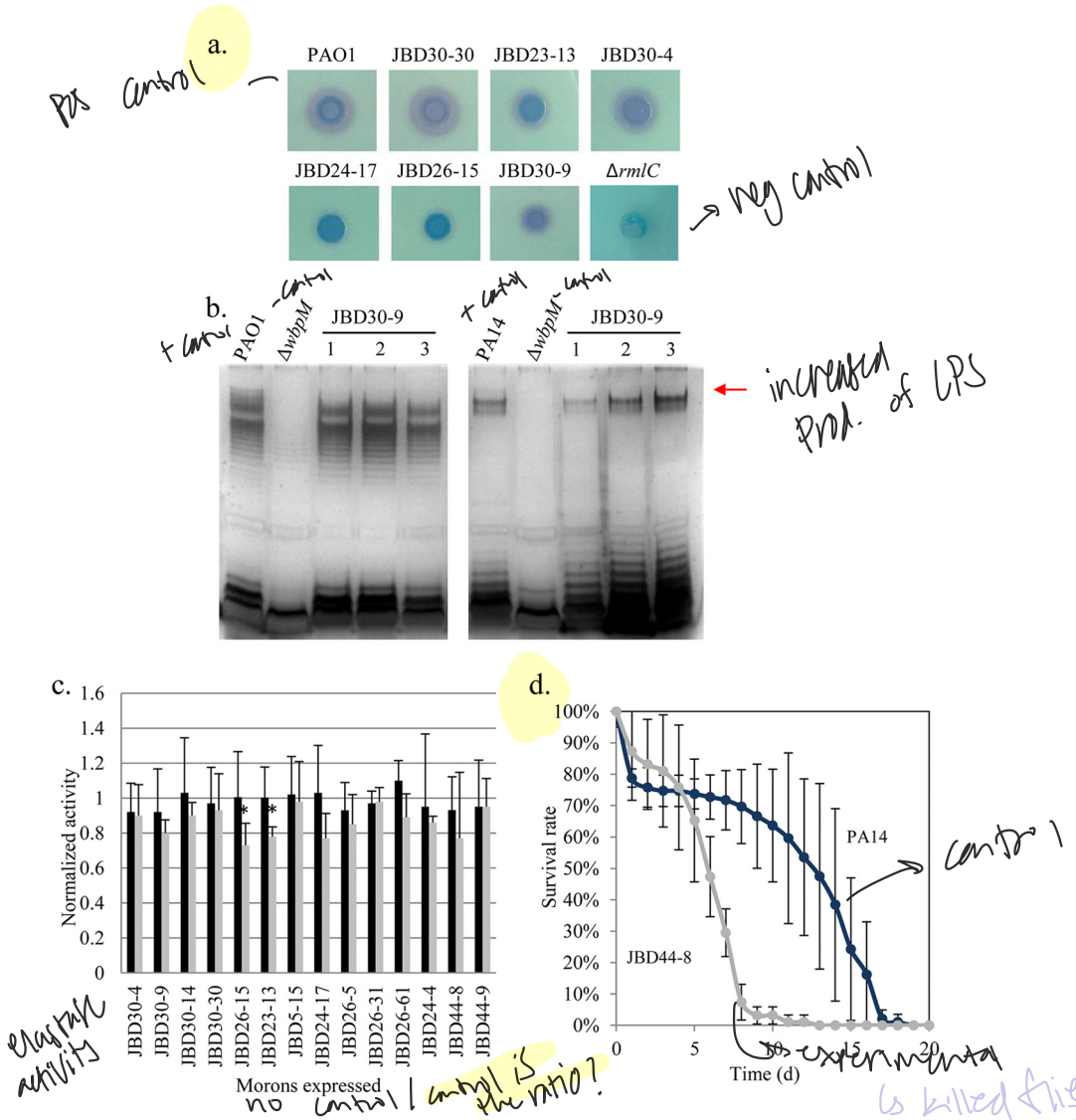

FIG 4 Phage morons lead to changes in phenotypes linked to virulence. (a) Rhamnolipid production was assessed for each moron using CTAB-methylene blue plates. The presence of a halo is indicative of rhamnolipid production. (b) Silver-stained SDS-polyacrylamide gels of lipopolysaccharides isolated from PAO1 (left) and PA14 (right) expressing JBD30-9. The arrow indicates the very-long O-specific antigen that disappears upon expression of JBD30-9. (c) Elastase production. The ratios between colony size and zone of clearing of PAO1 (gray bars) and PA14 (black bars) overexpressing individual morons. The "∗" designates significant decrease in elastase production. (d) JBD44-8 increases bacterial killing in *Drosophila melanogaster*. *P. aeruginosa* PA14 expressing JBD44-8 or an empty vector was fed to *D. melanogaster* and survival was monitored over 20 days.

We tested nine antibiotics (see Table 4) commonly used to treat bacterial infections using the disk diffusion method and found that no morons in our collection had an effect on antibiotic susceptibility of the tested strains.

To determine if the altered virulence phenotypes we observed *in vitro* had any effect on *in vivo* virulence, we tested strains expressing each moron gene in a chronic infection model in *Drosophila melanogaster*. *Drosophila* is an excellent model organism to study the pathogenesis of *P. aeruginosa* infections due to the striking similarities with the mammalian innate immune response, and it was previously used to identify virulence genes in *P. aeruginosa* (35–37). Using the fly oral model of infection, we monitored the survival of *Drosophila* fed PA14 expressing the different morons and compared them to those fed the PA14 wild type. The only moron that changed the killing kinetics in this assay was JBD44-8. Cells expressing JBD44-8 killed *Drosophila*

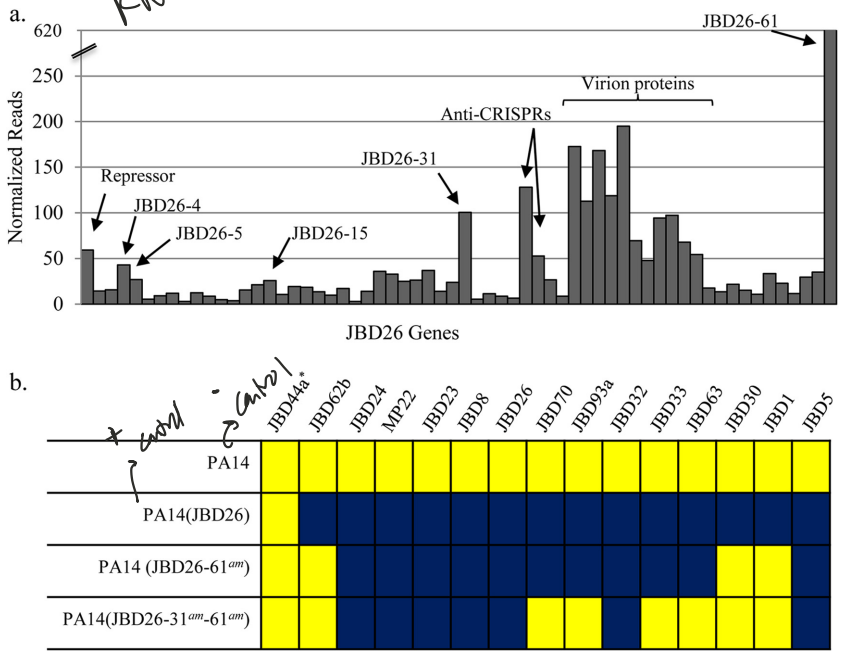

FIG 5 Phage morons are actively expressed from the lysogen. (a) RNA-seq analysis of a PA14(JBD26) lysogen reveals that a number of genes are expressed from the prophage during late exponential growth. (b) The deletion of genes JBD26-61 and JBD26-31 from the lysogen increases phage susceptibility. Yellow boxes denote phage sensitivity and blue boxes represent an increase in phage resistance of $>10^3$-fold compared to that of wild-type PA14.

faster, indicating an increase in virulence compared to that of the wild-type PA14 (Fig. 4d).

Phage moron genes are actively transcribed from a prophage. To determine the expression levels of the phage moron genes in their natural context, we used RNA sequencing (RNA-seq) to perform a transcriptomic analysis of a PA14 lysogen possessing the JBD26 prophage and observed gene expression across the whole prophage genome (Fig. 5). A total of 15 genes were expressed at levels equal to or greater than the expression level of the phage repressor protein (Table 2). The repressor is actively transcribed from the prophage as its activity is required to maintain the prophage in a quiescent state in the host chromosome. Ten of the genes for which transcription was observed are involved in the morphogenesis of the viral particle. This result can be explained by the natural high rate of spontaneous induction exhibited by this phage;

TABLE 2 Summary of genes actively transcribed in a JBD26 lysogen

Gene no.	No. of normalized reads	Function
1	59	Repressor protein
31	101	Unknown function
36	128	Anti-CRISPR
37	53	Anti-CRISPR
40	173	Head protease
41	113	Head decoration protein
42	168	Major head protein
43	119	Packaging chaperone
44	195	Conserved morphogenetic protein
45	70	Head-tail connector
47	94	Conserved morphogenetic protein
48	97	Tail tube
49	68	Tail chaperone
50	54	Tail chaperone
61	619	Unknown function

overnight growth of the PA14(JBD26) lysogen in the absence of any inducer molecules resulted in phage titers of ~10^9 phages/ml. The remaining four genes were morons; two encoded proteins that we previously determined inactivate the clustered regularly interspaced short palindromic repeat(s) (CRISPR)-Cas bacterial immune system (38, 39), and two (JBD26-31 and JBD26-61) encoded proteins of unknown function. Our results show that the expression of JBD26-31 led to increased phage resistance when expressed from a plasmid in PA14 and PAO1 (Fig. 2), while JBD26-61 provided complete resistance to phages that require the pilus for infection (Fig. 2), decreased twitching motility, and inhibited biofilm formation in both PAO1 and PA14 (Fig. 3). Previous work from our group showed that the PA14(JBD26) lysogen was highly resistant to infection by a variety of phages in a repressor-independent manner (11). To determine if the actively transcribed JBD26-31 and JBD26-61 genes prevent superinfection when they are expressed from the prophage, we created amber mutations and examined the ability of the prophage to resist infection by phages that use the pilus for infection. We found that a single amber mutation in gene 61 (JBD26^{61am}) caused a loss of resistance to several phages that require the pilus for infection (Fig. 5b). When this mutation was combined with a second amber mutation in gene 31 (JBD26^{31am61am}), the lysogen became sensitive to the activity of four additional phages (Fig. 5b). This indicates an active role for both JBD26-61 and JBD26-31 in protecting the lysogen from further phage infection and shows that they are expressed from the prophage at sufficient levels to provide resistance. It also illustrates that there are additional proteins encoded by phage JBD26 that contribute to phage resistance in the lysogen.

DISCUSSION

This study provides the first systematic investigation of the activities of a set of phage moron genes. Remarkably, even though the only criterion for the inclusion of genes in this study was their being nonconserved among *Pseudomonas* phages, 12 of 14 exerted a pronounced effect on at least one bacterial phenotype. The two genes that did not confer any detectable phenotype may be required for the infection process of the phage, which was not tested in this study, or they may only be expressed in specific strain backgrounds or under certain conditions. Further studies will be required to determine their roles. While phage resistance was the most common and dramatic phenotype conferred by moron genes (6 of 14 genes), we also observed effects on biofilm formation, virulence factor production, and both swimming and twitching motilities. The diversity of effects elicited by the expression of single moron genes highlights the scope and complexity of behavioral changes that may result from the presence of one or many prophages within a strain, each of which may contain many different moron genes.

A surprising result of our study was the high degree of strain-dependent variability that we observed by testing the moron genes in only two strain backgrounds (Table 3). For example, the expression of JBD26-5 in strain PAO1 prevented all phages tested from forming plaques, while the production of the same protein in PA14 resulted in resistance to only 9 of the 20 phages tested (Fig. 2). Even more striking, the expression of JBD23-13 produced opposite phenotypic effects in the two strains; it greatly increased biofilm production in PAO1 and decreased production in PA14. While PA14 and PAO1 possess very similar genome sequences, it is estimated that 4% to 8% of the genome is unique to each strain. These regions encode proteins known to be involved in the assembly of pili and fimbriae and O-specific antigen biosynthesis as well as encoding putative transcriptional regulators, ABC transporters, helicases, and many proteins of unknown function (40, 41). Genes within these regions must contribute to the strain-specific variations observed, either through direct protein-protein interactions or by modulating the expression of the moron genes.

Our observation that almost half of the moron genes tested increased phage resistance underscores the strong evolutionary pressure exerted by phages. The functional mechanisms of the moron proteins clearly differ, as some (e.g., JBD24-4 and JBD26-61) conferred resistance to a large number of phages in both PAO1 and PA14,

TABLE 3 Summary of host phenotype changes mediated by moron expression in *P. aeruginosa* strains PA14 and PAO1

		Effect of moron (JBD)[a]:													
Phenotype	**Strain**	**30-4**	**30-9**	**30-14**	**30-30**	**23-13**	**26-15**	**5-15**	**24-17**	**26-5**	**26-31**	**26-61**	**24-4**	**44-8**	**44-9**
Phage resistance	PAO1				↑					↑	↑	↑	↑	↑	
	PA14				↑					↑	↑	↑	↑		
Twitching	PAO1	↓					↓		↓	↓		↓	↓	↓	
	PA14					↓	↓			↓		↓	↓	↓	
Swimming	PAO1	↓				↓	↓							↓	
	PA14	↓					↓							↓	
Biofilm	PAO1					↑				↓		↓			
	PA14					↓						↓	↓		
LPS	PAO1		↓												
	PA14		↓												
Growth rate	PAO1													↓	
	PA14			↓			↓								
Rhamnolipid	PAO1	↓	↓			↓	↓		↓						
	PA14														
Elastase	PAO1					↓	↓								
	PA14														

[a] ↑, increase in observed phenotype; ↓, decrease in observed phenotype.

while others (e.g., JBD44-8) provided protection from only four phages. The expression of JBD26-5 and JBD26-61 caused decreased twitching motility, blocked pilus assembly on the cell surface, and increased phage resistance (Table 3). These phenotypes are linked, as the type IV pilus mediates twitching motility and serves as a cell surface receptor for all but three of the phages used in this study (42). Relevant to this point, a recent study revealed that a JBD26-5 homologue harbored by *Pseudomonas* phage D3112 binds to PilB, the type IV pilus assembly/extension ATPase, and prevents pilus assembly, thereby protecting against phage infection (17). Further illustrating the diversity of behaviors with respect to phage resistance, JBD26-15 decreased twitching motility but did not provide resistance to phage infection, while JBD30-30 and JBD26-31 conferred phage resistance but did not affect twitching motility. The strain expressing JBD26-15 was hyperpiliated, similar to *pilU* and *fimX* knockout strains that overexpress surface pili but are not motile on a solid surface (43, 44) and are susceptible to phages that use the pilus for infection. In contrast, JBD30-30 is predicted to localize to the inner membrane, similar to the gp15 superinfection exclusion protein from HK97 which functions by blocking phage genome injection (1). These data imply that the moron proteins tested inhibit both twitching motility and phage resistance by at least two different mechanisms. Phage resistance can also be mediated by the O-specific antigen present on the surface of the bacterial cell. While we identified seven different morons that affect the pilus, only a single moron (JBD30-9) (Fig. 4b) was shown to affect O antigen production. This may result from the collection of phages that were used in this study; 17 of 20 rely on the pilus for infection and it may be more adaptive for phages to block infection by other phages that are just like them.

When we examined the distribution of moron genes, we discovered each of the phages harbored an average of 11 different morons. Each phage has a unique combination, as if an assortment is drawn from a large pool that is available. Collectively, these genes would confer a particular set of capabilities on the prophage and thereby the bacterial lysogen that carries it. The morons may increase the fitness of the phage itself, for example, by the expression of anti-CRISPR proteins that inactivate the CRISPR-Cas bacterial immune system (38, 39). Alternatively, the morons may increase the fitness of the bacterial lysogen and thereby protect the prophage, for example, by increasing the resistance to further phage infection or adapting the lysogen to a unique ecological niche. The transcriptomic analyses of the JBD26 prophage revealed that morons are actively transcribed from the prophage and can account for some of the properties of lysogens. While not all morons were expressed, we only tested a single set of conditions in the laboratory. It is very likely that environmental signals also play a role in the expression and activity of phage morons. It was previously proposed that *P.*

aeruginosa shapes its accessory genome to favor survival in a wide variety of ecological niches (45). This concept can be extended to encompass the morons maintained within *Pseudomonas* prophages.

The work presented here emphasizes the complexity of determining how properties of individual moron genes will contribute to the overall behavior of bacterial lysogens. We uncovered a range of bacterial phenotypes that are influenced by the expression of a limited number of morons isolated from *P. aeruginosa* phages. Many of these genes modified phenotypes related to bacterial survivability and virulence and thus may play significant roles in the establishment and progression of bacterial infections. In addition to the combination of unique sets of morons within a single prophage, many bacterial strains contain multiple prophages, each of which might contribute a unique set of fitness factors. Thus, the addition of each new prophage might provide the strain with novel pathogenic properties. This study illustrates the importance of regarding prophages as active entities that express genes with diverse effects on the bacterial host and not simply as inert genetic elements waiting to kill them.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Each moron was amplified and cloned into the pHERD30T expression vector under the control of the pBAD promoter (46). The correct insertion into the plasmid was confirmed by DNA sequencing. The moron-expressing plasmids were then transformed into *P. aeruginosa* strains PA14 and PAO1 via electroporation (47), which were plated on lysogeny broth (LB) agar plates supplemented with gentamicin (50 μg/ml). Moron-expressing strains were grown overnight in LB medium or LB agar at 37°C in the presence of gentamicin (50 μg/ml) and 0.1% L-arabinose unless otherwise stated. All phenotypic analyses were performed in triplicates.

Bacterial growth curves. Overnight cultures were diluted 1:100 in fresh medium, and 200 μl of diluted cultures was grown at 37°C in 96-well microtiter plates in an Infinite F200 microplate reader (Tecan) for 8 h. Cell densities were measured every 15 min at an optical density of 595 nm (OD_{595}) to generate the growth curves.

Twitching motility assays. Bacteria were inoculated on freshly prepared 1% LB agar by stabbing isolated colonies to the bottoms of the plates. The plates were incubated for 24 h at 37°C. After incubating, the agar was carefully removed from the plates, and the biomass adhered to the bottom of the plates was stained with 1% crystal violet for 1 min followed by 3 washes with water (48). The plates were dried inverted overnight. To determine the distance traveled by the bacteria, the diameter of the stained area was measured.

Swimming motility assay. Bacteria were inoculated on freshly prepared semisolid medium containing 0.3% (wt/vol) agar, 1% (wt/vol) tryptone, and 0.5% (wt/vol) NaCl by stabbing isolated colonies to the bottoms of the plates. The plates were incubated for 24 h at 37°C, and the diameter of the area traveled by the bacteria was measured.

Lipopolysaccharide analysis. The lipopolysaccharide of overnight bacterial cultures grown in LB supplemented with 50 μg/ml gentamicin and 1% L-arabinose was harvested according to the protocol described by Hitchcock and Brown (49). Briefly, the cultures were equilibrated to an OD_{600} of 0.45, resuspended in Hitchcock and Brown lysis buffer, and treated overnight with proteinase K at 55°C. Isolated lipopolysaccharide samples were resolved using Tricine SDS-PAGE and visualized by the ultrafast silver stain method (50).

Preparation of *P. aeruginosa* phages and plaque assays. *P. aeruginosa* lysogens were grown overnight in LB medium at 37°C. Bacterial cells were collected by centrifugation, and a few drops of chloroform were added to the phage-containing supernatant to sterilize it; these phage lysates were stored at 4°C. For plaque assays, a lawn of bacteria was generated by adding 150 μl of overnight culture to 3 ml of 0.7% molten top agar and poured onto 1.5% LB agar plates. Two microliters of 10-fold serial dilutions of phages was spotted on the bacterial lawns, and the plates were incubated at 37°C overnight.

***Drosophila melanogaster* virulence assays.** The virulence assay protocol was modified from that of Shen et al. (51). In brief, 5 ml of molten 5% sucrose agar was poured into polystyrene *Drosophila* vials (VWR) and solidified overnight. The following day, bacterial cells from overnight cultures were collected by centrifugation, resuspended in 100 μl 5% sucrose solution, and spotted on filter paper (Whatman GF/A, 21 mm) placed on top of the solidified sucrose agar in fly vials. The vials were incubated at 37°C for 30 min, followed by 25°C for 30 min. Twenty prestarved 3- to 5-day-old male W1118 *Drosophila melanogaster* flies were transferred to each vial. The vials were kept in a 25°C humidity-controlled dark environment. The numbers of dead flies were recorded every 24 h for 21 days.

Secreted enzyme assay. *P. aeruginosa* moron-expressing strains were grown overnight in LB at 37°C and equilibrated to an OD_{600} of 0.6; 2 μl was spotted on each plate in the presence of 50 μg/ml gentamicin and 0.1% L-arabinose and grown for 48 h at 30°C. The diameter of each zone of clearing or observed precipitant was measured as a representation of enzyme activity (52). The protease assay protocol was adopted from that described by Sokol et al. using Trypticase soy agar (Bio-Basic) supplemented with 2% of skim milk powder (Bioshop) (53). The elastase assay was performed as described by Rust et al. (54) using reverse elastin plates. Briefly, tryptic soy agar plates (Difco) and 2% agar plates were cast and dried under laminar flow. An overlay of tryptone broth containing 2% agar supplemented with

TABLE 4 List of antibiotics tested in resistance assay

Drug name	Amount (μg)
Azithromycin	15
Cefoperazone	75
Ceftazidime	30
Imipenem	10
Ticarcillin	75
Piperacillin	100
Colistin	10
Amikacin	30
Aztreonam	30

0.5% elastin from bovine neck ligament (Sigma) was poured onto the 2% plates. To test for lipase activity, 1.5% agar plates were prepared as described by Lonon et al. (55). Briefly, peptone, sodium chloride, calcium chloride, and agar were sterilized, and after cooling, 1% Tween 80 was added to the solution before pouring.

Rhamnolipid production. The presence of rhamnolipid was qualitatively assessed by the drop-collapse method, and the presence of a halo when grown on agar plates supplemented with hexadecyltrimethylammonium bromide (CTAB) and methylene blue. Briefly, 5 μl of equilibrated overnight culture was spotted on the lid of a polystyrene 96-well plate equilibrated for 2 h with 2 μl of mineral oil. The collapse of the culture drop into the oil was assessed after 1 min. A lack of surfactant results in the beading of the culture on top of the oil (56). The CTAB plates were generated according to Siegmund and Wagner (57), wherein 1 liter of M8 medium salt agar (1.5%) was supplemented with 0.2 g of CTAB and 0.005 g of methylene blue, Casamino Acids (0.5%), glucose (0.2%), and 1 mM $MgSO_4$ in addition to 50 μg/ml of gentamicin and 0.1% L-arabinose. Another 2 μl of the aforementioned overnight culture was spotted on the plates and grown at 30°C for 48 h and an additional 24 h at 4°C to increase the contrast of the precipitant halo. The diameter of the halo produced is related to the total amount of rhamnolipid produced (57, 58).

Mucoid production. Single colonies were streaked out on pseudomonas isolation agar (PIA) supplemented with 0.1% L-arabinose and 50 μg/ml gentamicin and incubated at 37°C for 48 h (52).

Antimicrobial resistance assay. Two hundred microliters of moron-expressing *P. aeruginosa* strains was added to LB top agar and applied to an LB agar plate. Circular antibiotic-containing discs (Sensi-Disc; BD) were placed on top of the bacterial lawn, and the plates were incubated at 37°C overnight (Table 4). The diameters of clear zones around the discs were measured.

Quantification of biofilm production. The biofilm quantification assay protocol was adapted from that described in reference 59. In brief, an overnight bacterial culture was diluted 1:100 into M63 minimal medium (3 g monobasic potassium phosphate, 7 g dibasic potassium phosphate, 2 g ammonium sulfate dissolved in water for a 1-liter solution). One hundred microliters of diluted culture was distributed into 96-well vinyl round-bottom microtiter plates (Corning) and incubated at 37°C for 24 h. To stain the biomass in the wells, the bacterial culture was removed from the plates, and 100 μl of 0.1% crystal violet was added to the wells and incubated at room temperature for 15 min. The excess stain was removed, and the plates were dried inverted overnight. The residual dye was dissolved by adding 125 μl of 30% acetic acid to the wells and incubated at room temperature for 15 min. To quantify the stained biomass, the dye solution was transferred to flat-bottom microplates, and the OD_{595} of the solution was measured using the Infinite F200 microplate reader (Tecan).

RNA-seq of JBD26 lysogen. Prophage-free PA14 and the PA14(JBD26) lysogen were grown in LB to late exponential phase (OD_{600} of 3.5) at 37°C in shaking flasks, and total RNA was extracted using the hot phenol method. RNA samples were thoroughly treated by DNase I and assessed for quality using an Agilent 2100 bioanalyzer (RNA integrity number [RIN], >8). rRNA depletion was then performed using a MICROB Express kit (Ambion) according to the manufacturer's recommendations. cDNA was synthesized and prepared for Helicos sequencing as previously described (60). Raw reads were trimmed and mapped against the genomic sequence of *P. aeruginosa* UCBPP-PA14 (NC_008463.1) using CLC Genomics Workbench software v5.1 (CLC bio). The number of reads uniquely aligned to each coding sequence was determined using BEDTools software v2.16.2.

Constructing amber mutations in PA14. Site-directed mutagenesis was used to introduce amber mutations into pHERD30T plasmids harboring genes JBD26-31 and JBD26-61. These constructs were transformed into PA14, and wild-type JBD26 was spotted on a lawn of cells containing pHERD30T-JBD26^{61am}. Recombinant phages were isolated and spotted on lawns of PA14 containing CRISPR-RNA (cRNA) specific to the wild-type 5′ end of JBD26-61. Only phages that contained the amber mutation were able to plaque due to CRISPR targeting of the wild-type sequence. Another round of infection on pHERD30T-JBD26^{31am} and CRISPR selection was performed to obtain the double mutant. Lysogens containing the single or double mutant (JBD26^{61am} and JBD26^{31am61am}) were tested for phage resistance by spotting 10-fold serial dilutions of a panel of phages on lawns of each lysogen.

Shearing assays. Cell surface appendages (flagella and pili) were isolated using a method adapted from Castric (61, 62). The twitching-impaired strains with JBD constructs and PAO1 with the empty vector were streaked in a grid pattern on LB agar plates containing 50 μg/ml of gentamicin and 0.1% L-arabinose, and the Δ*pilA* strain was streaked on LB agar devoid of drugs and inducer. The plates were incubated for 24 h at 37°C. The bacteria were gently scraped from the agar surface by use of a sterile

coverslip and resuspended in 2 ml sterile phosphate-buffered saline (PBS; pH 7.4). To isolate the surface proteins, the samples were vortexed for 30 s to shear off surface appendages. The bacterial cells were removed by centrifuging the samples at 15,000 rpm for 5 min at room temperature. The supernatant was transferred to a fresh tube and centrifuged for an additional 25 min at 15,000 rpm to remove any remaining cells. To precipitate the sheared surface proteins, 1/10 volumes each of 5 M NaCl and 30% polyethylene glycol (PEG 8000) were added to the supernatant, and the samples were incubated on ice for 60 min. The samples were centrifuged at 15,000 rpm for 25 min at 4°C. After discarding the supernatants, the resulting pellets were resuspended in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye and analyzed by SDS-PAGE. Flagella were visualized using Coomassie stain, and the pilus was observed by Western blotting with primary antibodies specific to the PilA monomer (62) and secondary goat anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad).

ACKNOWLEDGMENTS

This work was supported by operating grants from the Canadian Institutes of Health Research to K.L.M. (MOP-136845) and A.R.D. (XNE-86943). J.B.-D. was supported by a Canadian Institutes of Health Research doctoral award and an Ontario Graduate Scholarship.

REFERENCES

1. Cumby N, Edwards AM, Davidson AR, Maxwell KL. 2012. The bacteriophage HK97 gp15 moron element encodes a novel superinfection exclusion protein. J Bacteriol 194:5012–5019. https://doi.org/10.1128/JB.00843-12.
2. Hofer B, Ruge M, Dreiseikelmann B. 1995. The superinfection exclusion gene (*sieA*) of bacteriophage P22: identification and overexpression of the gene and localization of the gene product. J Bacteriol 177:3080–3086. https://doi.org/10.1128/jb.177.11.3080-3086.1995.
3. Vica Pacheco S, Garcia Gonzalez O, Paniagua Contreras GL. 1997. The *lom* gene of bacteriophage lambda is involved in *Escherichia coli* K-12 adhesion to human buccal epithelial cells. FEMS Microbiol Lett 156:129–132. https://doi.org/10.1016/S0378-1097(97)00415-1.
4. Barondess JJ, Beckwith J. 1990. A bacterial virulence determinant encoded by lysogenic coliphage lambda. Nature 346:871–874. https://doi.org/10.1038/346871a0.
5. Vaca-Pacheco S, Paniagua-Contreras GL, Garcia-Gonzalez O, de la Garza M. 1999. The clinically isolated FIZ15 bacteriophage causes lysogenic conversion in *Pseudomonas aeruginosa* PAO1. Curr Microbiol 38:239–243. https://doi.org/10.1007/PL00006794.
6. Winstanley C, Langille MG, Fothergill JL, Kukavica-Ibrulj I, Paradis-Bleau C, Sanschagrin F, Thomson NR, Winsor GL, Quail MA, Lennard N, Bignell A, Clarke L, Seeger K, Saunders D, Harris D, Parkhill J, Hancock RE, Brinkman FS, Levesque RC. 2009. Newly introduced genomic prophage islands are critical determinants of *in vivo* competitiveness in the Liverpool epidemic strain of *Pseudomonas aeruginosa*. Genome Res 19:12–23. https://doi.org/10.1101/gr.086082.108.
7. Lewis DA, Jones A, Parkhill J, Speert DP, Govan JR, Lipuma JJ, Lory S, Webb AK, Mahenthiralingam E. 2005. Identification of DNA markers for a transmissible *Pseudomonas aeruginosa* cystic fibrosis strain. Am J Respir Cell Mol Biol 33:56–64. https://doi.org/10.1165/rcmb.2004-0352OC.
8. Busby B, Kristensen DM, Koonin EV. 2013. Contribution of phage-derived genomic islands to the virulence of facultative bacterial pathogens. Environ Microbiol 15:307–312. https://doi.org/10.1111/j.1462-2920.2012.02886.x.
9. Brüssow H, Canchaya C, Hardt W-D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol Mol Biol Rev 68:560–602. https://doi.org/10.1128/MMBR.68.3.560-602.2004.
10. Juhala RJ, Ford ME, Duda RL, Youlton A, Hatfull GF, Hendrix RW. 2000. Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. J Mol Biol 299:27–51. https://doi.org/10.1006/jmbi.2000.3729.
11. Bondy-Denomy J, Qian J, Westra ER, Buckling A, Guttman DS, Davidson AR, Maxwell KL. 2016. Prophages mediate defense against phage infection through diverse mechanisms. ISME J 10:2854–2866. https://doi.org/10.1038/ismej.2016.79.
12. Dobson L, Reményi I, Tusnády GE. 2015. CCTOP: a Consensus Constrained TOPology prediction web server. Nucleic Acids Res 43:W408–W412. https://doi.org/10.1093/nar/gkv451.
13. Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, Kohler T, van Delden C, Weinel C, Slickers P, Tummler B. 2007. Population structure of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 104:8101–8106. https://doi.org/10.1073/pnas.0609213104.
14. Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, Miyata S, Diggins LT, He J, Saucier M, Deziel E, Friedman L, Li L, Grills G, Montgomery K, Kucherlapati R, Rahme LG, Ausubel FM. 2006. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol 7:R90. https://doi.org/10.1186/gb-2006-7-10-r90.
15. Newton GJ, Daniels C, Burrows LL, Kropinski AM, Clarke AJ, Lam JS. 2001. Three-component-mediated serotype conversion in *Pseudomonas aeruginosa* by bacteriophage D3. Mol Microbiol 39:1237–1247. https://doi.org/10.1111/j.1365-2958.2001.02311.x.
16. Lehane Adele M, Korres H, Verma Naresh K. 2005. Bacteriophage-encoded glucosyltransferase GtrII of *Shigella flexneri*: membrane topology and identification of critical residues. Biochem J 389:137–143. https://doi.org/10.1042/BJ20050102.
17. Chung I-Y, Jang H-J, Bae H-W, Cho Y-H. 2014. A phage protein that inhibits the bacterial ATPase required for type IV pilus assembly. Proc Natl Acad Sci U S A 111:11503–11508. https://doi.org/10.1073/pnas.1403537111.
18. Choi Y, Shin H, Lee JH, Ryu S. 2013. Identification and characterization of a novel flagellum-dependent *Salmonella*-infecting bacteriophage, iEPS5. Appl Environ Microbiol 79:4829–4837. https://doi.org/10.1128/AEM.00706-13.
19. Shin H, Lee J-H, Kim H, Choi Y, Heu S, Ryu S. 2012. Receptor diversity and host interaction of bacteriophages infecting *Salmonella enterica* serovar Typhimurium. PLoS One 7:e43392. https://doi.org/10.1371/journal.pone.0043392.
20. Pallen MJ, Wren BW. 2007. Bacterial pathogenomics. Nature 449:835. https://doi.org/10.1038/nature06248.
21. De Smet J, Hendrix H, Blasdel BG, Danis-Wlodarczyk K, Lavigne R. 2017. *Pseudomonas* predators: understanding and exploiting phage-host interactions. Nat Rev Microbiol 15:517–530. https://doi.org/10.1038/nrmicro.2017.61.
22. Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322. https://doi.org/10.1126/science.284.5418.1318.
23. Mulcahy H, Charron-Mazenod L, Lewenza S. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog 4:e1000213. https://doi.org/10.1371/journal.ppat.1000213.
24. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. 2008. Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. Mol Microbiol 68:223–240. https://doi.org/10.1111/j.1365-2958.2008.06152.x.
25. Giltner CL, Nguyen Y, Burrows LL. 2012. Type IV pilin proteins: versatile molecular modules. Microbiol Mol Biol Rev 76:740–772. https://doi.org/10.1128/MMBR.00035-12.
26. Lassak K, Ghosh A, Albers S-V. 2012. Diversity, assembly and regulation of

archaeal type IV pili-like and non-type-IV pili-like surface structures. Res Microbiol 163:630–644. https://doi.org/10.1016/j.resmic.2012.10.024.
27. Esquivel RN, Pohlschroder M. 2014. A conserved type IV pilin signal peptide H-domain is critical for the post-translational regulation of flagella-dependent motility. Mol Microbiol 93:494–504. https://doi.org/10.1111/mmi.12673.
28. Zulianello L, Canard C, Köhler T, Caille D, Lacroix J-S, Meda P. 2006. Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by *Pseudomonas aeruginosa*. Infect Immun 74:3134–3147. https://doi.org/10.1128/IAI.01772-05.
29. Ramphal R, Pier GB. 1985. Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. Infect Immun 47:1–4.
30. Kuzio J, Kropinski AM. 1983. O-antigen conversion in *Pseudomonas aeruginosa* PAO1 by bacteriophage D3. J Bacteriol 155:203–212.
31. Taylor VL, Udaskin ML, Islam ST, Lam JS. 2013. The D3 bacteriophage α-polymerase inhibitor (Iap) peptide disrupts O-antigen biosynthesis through mimicry of the chain length regulator Wzz in *Pseudomonas aeruginosa*. J Bacteriol 195:4735–4741. https://doi.org/10.1128/JB.00903-13.
32. Daniels C, Griffiths C, Cowles B, Lam JS. 2002. *Pseudomonas aeruginosa* O-antigen chain length is determined before ligation to lipid A core. Environ Microbiol 4:883–897. https://doi.org/10.1046/j.1462-2920.2002.00288.x.
33. Latino L, Caroff M, Pourcel C. 2017. Fine structure analysis of lipopolysaccharides in bacteriophage-resistant *Pseudomonas aeruginosa* PAO1 mutants. Microbiology 163:848–855. https://doi.org/10.1099/mic.0.000476.
34. Mesaros N, Nordmann P, Plesiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebecque P, Malfroot A, Tulkens PM, Van Bambeke F. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect 13:560–578. https://doi.org/10.1111/j.1469-0691.2007.01681.x.
35. Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg EP. 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 98:2752–2757. https://doi.org/10.1073/pnas.051624298.
36. D'Argenio DA, Gallagher LA, Berg CA, Manoil C. 2001. Drosophila as a model host for *Pseudomonas aeruginosa* infection. J Bacteriol 183:1466–1471. https://doi.org/10.1128/JB.183.4.1466-1471.2001.
37. Fauvarque MO, Bergeret E, Chabert J, Dacheux D, Satre M, Attree I. 2002. Role and activation of type III secretion system genes in *Pseudomonas aeruginosa*-induced *Drosophila* killing. Microb Pathog 32:287–295. https://doi.org/10.1006/mpat.2002.0504.
38. Bondy-Denomy J, Pawluk A, Maxwell KL, Davidson AR. 2013. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. Nature 493:429–432. https://doi.org/10.1038/nature11723.
39. Pawluk A, Bondy-Denomy J, Cheung VHW, Maxwell KL, Davidson AR. 2014. A new group of phage anti-CRISPR genes inhibits the type I-E CRISPR-Cas system of *Pseudomonas aeruginosa*. mBio 5:e00896. https://doi.org/10.1128/mBio.00896-14.
40. He J, Baldini RL, Deziel E, Saucier M, Zhang Q, Liberati NT, Lee D, Urbach J, Goodman HM, Rahme LG. 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc Natl Acad Sci U S A 101:2530–2535. https://doi.org/10.1073/pnas.0304622101.
41. Choi JY, Sifri CD, Goumnerov BC, Rahme LG, Ausubel FM, Calderwood SB. 2002. Identification of virulence genes in a pathogenic strain of *Pseudomonas aeruginosa* by representational difference analysis. J Bacteriol 184:952–961. https://doi.org/10.1128/jb.184.4.952-961.2002.
42. Craig L, Pique ME, Tainer JA. 2004. Type IV pilus structure and bacterial pathogenicity. Nat Rev Microbiol 2:363–378. https://doi.org/10.1038/nrmicro885.
43. Whitchurch Cynthia B, Mattick John S. 1994. Characterization of a gene, *pilU*, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa*. Mol Microbiol 13:1079–1091. https://doi.org/10.1111/j.1365-2958.1994.tb00499.x.
44. Jain R, Behrens A-J, Kaever V, Kazmierczak BI. 2012. Type IV pilus assembly in *Pseudomonas aeruginosa* over a broad range of cyclic di-GMP concentrations. J Bacteriol 194:4285–4294. https://doi.org/10.1128/JB.00803-12.
45. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, Rokas A, Yandava CN, Engels R, Zeng E, Olavarietta R, Doud M, Smith RS, Montgomery P, White JR, Godfrey PA, Kodira C, Birren B, Galagan JE, Lory S. 2008. Dynamics of *Pseudomonas aeruginosa* genome evolution. Proc Natl Acad Sci U S A 105:3100–3105. https://doi.org/10.1073/pnas.0711982105.
46. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. 2008. PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. Appl Environ Microbiol 74:7422–7426. https://doi.org/10.1128/AEM.01369-08.
47. Smith AW, Iglewski BH. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res 17:10509. https://doi.org/10.1093/nar/17.24.10509.
48. Semmler ABT, Whitchurch CB, Mattick JS. 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. Microbiology 145:2863–2873. https://doi.org/10.1099/00221287-145-10-2863.
49. Hitchcock PJ, Brown TM. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 154:269–277.
50. Fomsgaard A, Freudenberg MA, Galanos C. 1990. Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. J Clin Microbiol 28:2627–2631.
51. Shen L, Gao X, Wei J, Chen L, Zhao X, Li B, Duan K. 2012. PA2800 plays an important role in both antibiotic susceptibility and virulence in *Pseudomonas aeruginosa*. Curr Microbiol 65:601–609. https://doi.org/10.1007/s00284-012-0196-2.
52. Duong J, Booth SC, McCartney NK, Rabin HR, Parkins MD, Storey DG. 2015. Phenotypic and genotypic comparison of epidemic and non-epidemic strains of *Pseudomonas aeruginosa* from individuals with cystic fibrosis. PLoS One 10:e0143466. https://doi.org/10.1371/journal.pone.0143466.
53. Sokol PA, Ohman DE, Iglewski BH. 1979. A more sensitive plate assay for detection of protease production by *Pseudomonas aeruginosa*. J Clin Microbiol 9:538–540.
54. Rust L, Messing CR, Iglewski BH. 1994. Elastase assays. Methods Enzymol 235:554–562. https://doi.org/10.1016/0076-6879(94)35170-8.
55. Lonon MK, Woods DE, Straus DC. 1988. Production of lipase by clinical isolates of *Pseudomonas cepacia*. J Clin Microbiol 26:979–984.
56. Bodour AA, Miller-Maier RM. 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. J Microbiol Methods 32:273–280. https://doi.org/10.1016/S0167-7012(98)00031-1.
57. Siegmund I, Wagner F. 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnol Tech 5:265–268. https://doi.org/10.1007/BF02438660.
58. Pinzon NM, Ju L-K. 2009. Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. Biotechnol Lett 31:1583–1588. https://doi.org/10.1007/s10529-009-0049-7.
59. O'Toole GA. 2011. Microtiter dish biofilm formation assay. J Vis Exp 2011:2437. https://doi.org/10.3791/2437.
60. Cattoir V, Narasimhan G, Skurnik D, Aschard H, Roux D, Ramphal R, Jyot J, Lory S. 2013. Transcriptional response of mucoid *Pseudomonas aeruginosa* to human respiratory mucus. mBio 3:e00410-12. https://doi.org/10.1128/mBio.00410-12.
61. Castric P. 1995. *pilO*, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. Microbiology 141:1247–1254. https://doi.org/10.1099/13500872-141-5-1247.
62. Harvey H, Habash M, Aidoo F, Burrows LL. 2009. 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. https://doi.org/10.1128/JB.00943-09.