Bacteriophage moron JBD30-4 affects type IV pilus and flagellar function in Pseudomonas aeruginosa

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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Abstract

Bacteriophages can influence the virulence of Pseudomonas aeruginosa, an opportunistic pathogen causing significant morbidity and mortality in immunocompromised individuals. Morons, specific prophage genes not required for the phage life cycle, provide fitness advantages to bacteria under specific environmental conditions. JBD30-4 is a phage moron that inhibits P. aeruginosa twitching and swimming motility while remaining susceptible to phage infection. An interaction detected using the bacterial adenylate cyclase two-hybrid system suggests that JBD30-4 mediates this phenotype through an interaction with the type IV pilus response regulator PilH. Biochemical characterization of JBD30-4 revealed that the N-terminus of the protein is critical for function. Downregulation of flagella and type IV pili upon expression of JBD30-4 from a prophage, leading to a decrease in motility, may enable bacterial evasion from the host immune system during P. aeruginosa infection, promoting survival of the bacteria and the prophage genome contained within it.
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>ci-d-GMP</td>
<td>cyclic diguanosine monophosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EV</td>
<td>empty vector</td>
</tr>
<tr>
<td>gp</td>
<td>gene product</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
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<td>His$_6$</td>
<td>hexahistidine</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LES</td>
<td>Liverpool epidemic strain</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg</td>
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<td>millimolar</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>NaCl</td>
<td>sodium chloride</td>
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<td>amino-terminal</td>
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<tr>
<td>nm</td>
<td>nanometers</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pH</td>
<td>power of hydrogen</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>s</td>
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</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>T4P</td>
<td>type IV pilus/pili</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEV</td>
<td>tobacco etch virus</td>
</tr>
<tr>
<td>v/v</td>
<td>volume by volume</td>
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<tr>
<td>w/v</td>
<td>weight by volume</td>
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<tr>
<td>x g</td>
<td>times gravity</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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Chapter 1

1 Introduction

1.1 The bacteriophage life cycle

Bacteriophages, viruses that infect bacteria, are found in all environments, including water, soil, and the human body. They are the most abundant biological entity on the planet, with an estimated $10^{31}$ phages in the biosphere, outnumbering bacteria 10:1 (Whitman et al., 1998; Rohwer & Edwards, 2002). Upon infection of a bacterial cell, the phage may enter the lytic cycle, where it hijacks the cell machinery to produce new viral particles (Figure 1). Temperate phages are phages that can enter either the lytic or the lysogenic cycle upon infection. In the lysogenic cycle, the phage genome may be maintained as an extrachromosomal plasmid, or integrated into the bacterial chromosome, where the phage is referred to as a “prophage” (Lwoff, 1953). In this state, expression of most phage genes is silenced through the activity of a repressor protein to prevent virus particle production and maintain viability of the bacterial host (Waldor & Friedman, 2005). Lytic phages are incapable of entering the lysogenic cycle and thus cannot integrate their genomes into the bacterial chromosome.

Figure 1: Bacteriophages can initiate the lytic or lysogenic cycle upon infection of a bacterial cell. During the lytic cycle, the bacteriophage hijacks the cell machinery to create new phage particles. In the lysogenic cycle, the phage genome can be stably integrated into the bacterial chromosome as a prophage.
Temperate phages can cause the phenomenon of lysogenic conversion, which affects bacterial fitness, as the expression of genes from the prophage leads to the introduction of new fitness factors. Temperate phages can also influence bacterial fitness through gene disruption and protection from lytic infection (Brussow et al., 2004). In the lysogenic form, phages establish a long-term relationship with their host cell, and thus it is advantageous for them to encode genes that can provide fitness advantages to their host.

1.2 Bacteriophages and *Pseudomonas aeruginosa*

Prophages have been shown to be important in the virulence of the ubiquitous Gram-negative bacterium, *Pseudomonas aeruginosa*. *P. aeruginosa* is found in soil and water and infects plants and animals as an opportunistic pathogen. In the context of human infections, *P. aeruginosa* is a primary cause of nosocomial infections and is a particular concern for immunosuppressed individuals, severe burn wound victims, and those with chronic pulmonary diseases (Cross et al., 1983; Church et al., 2006; Yum et al., 2014). *P. aeruginosa* is a major cause of morbidity and mortality for Cystic Fibrosis (CF) patients, with about 80% of CF patients being chronically colonized by *P. aeruginosa* by the age of 20 (Tingpej et al., 2007). Factors contributing to the virulence of *P. aeruginosa* include the secretion of toxic compounds such as pyocyanin, toxin A, rhamnolipids, and alkaline protease (Liu, 1974; Klockgether & Tummler, 2017). Biofilm formation on implants, in chronic and burn wounds, and in the respiratory tract of CF patients make *P. aeruginosa* infections more difficult to treat due to reduced antimicrobial penetration and evasion of host immune defenses (Mulcahy et al., 2015; Wu et al., 2015; Bjarnsholt et al., 2009). The high level of intrinsic and acquired antibiotic resistance of *P. aeruginosa* makes it more challenging to eradicate and contributes to increased relapse and reoccurrence of infection (Pang et al., 2019).

The highly transmissible and virulent strain of *P. aeruginosa*, the Liverpool Epidemic Strain (LES), is the most abundant strain in CF patients in the United Kingdom (Scott & Pitt, 2004). The LES is associated with greater patient morbidity in CF patients compared to other *P. aeruginosa* strains and has even caused disease in healthy individuals (McCallum et al., 2002; Al-Aloul et al., 2004). LESB58, the earliest archived LES isolate, contains five prophages that contribute to the aggressiveness and success of this clone in disease states (Winstanley et al., 2008). Disruption of prophage genes in three of the five prophages of LESB58 decreased
competitiveness in a rat infection model, relative to wild type LESB58, demonstrating that the proteins expressed from those three prophages enhanced the growth and maintenance of the bacteria \textit{in vivo} (Winstanley \textit{et al.}, 2008). The presence of a prophage was also shown to be important in the hypertransmissibility of the Manchester epidemic strain of \textit{P. aeruginosa} (Lewis \textit{et al.}, 2005). The prophages of LESB58 and the Manchester epidemic strain exemplify the development of mutualistic relationships between \textit{P. aeruginosa} and its prophages. Therefore, we are using \textit{P. aeruginosa} as a model to study the effects of bacteriophage-encoded genes on the physiology and pathogenicity of this organism.

1.3 Phages influence bacterial phenotypes

Temperate phages have been shown to influence phenotypes in \textit{P. aeruginosa} through lysogenic conversion. Lysogenization of phage DMS3 in the \textit{P. aeruginosa} strain PA14 inhibits biofilm formation and swarming motility (Zegans \textit{et al.}, 2009). PA14 lysogens of phage MP22 also display impaired swarming motility (Chung \textit{et al.}, 2012). Phage D3112 infection in the \textit{P. aeruginosa} strain PAO1 causes complete loss of twitching motility (Chung \textit{et al.}, 2012; Chung \textit{et al.}, 2014). Lysogenization of phage D3 in PAO1 leads to serotype conversation (Kuzio & Kropinski, 1983), providing resistance to infection by other serotype-specific phages and promoting bacterial evasion from the host immune system. Lysogenic conversion of the FIZ15 bacteriophage in PAO1 leads to increased adhesion of the bacteria to human epithelial cells and increased bacterial resistance to killing by phagocytosis as a result of changes to the O-antigen receptor for phage infection, mediated by the FIZ15 prophage (Vaca-Pacheo \textit{et al.}, 1998). In a systematic study of the effect of prophages on phage resistance and bacterial behaviour in \textit{P. aeruginosa}, Bondy-Denomy \textit{et al.} found that lysogenization of the phages JBD26, MP22, and JBD1 in PA14 made the bacterial cells resistant to infection by pilus-specific phages but retained twitching motility, suggesting that pili were present and functional, but modified in some manner upon lysogenization, preventing further phage infection (Bondy-Denomy \textit{et al.}, 2016).

The specific prophage genes mediating the phenotypic changes caused by lysogenic conversion are referred to as open reading frames of unknown function (ORFans), or morons. The term “moron” was termed by Juhala \textit{et al.} (2000) to refer to the prophage expressed genes that are not essential for the phage life cycle and may increase host fitness. These genes are termed “morons” to indicate that “when one is present in the genome there is more DNA than when it is not
Expression of these genes is often controlled by independent transcriptional promoters and terminator elements, however, the use of the term moron has been expanded to include all of the non-conserved genes in phage genomes that do not have a phage function and may act as fitness factors (Brussow et al., 2004).

Figure 2: Phage-encoded genes can lead to a variety of changes in bacterial phenotypes. Genes expressed from a prophage can play important roles in bacterial physiology by affecting twitching motility through interactions with components of the type IV pilus, interfering with swarming motility, which requires flagella, through modifications to the O-antigen on lipopolysaccharides. Prophages can encode and mediate release of toxins and encode inhibitors of transcriptional regulators to prevent bacterial host silencing of phage DNA.

There are several reported examples of specific morons from *P. aeruginosa* phages impacting processes within their bacterial host that serve to protect both the bacterial host and the phage genome encoded within it. One such mechanism was elucidated by Wagemans et al., through the identification of a phage protein, LUZ24-gp4, the MvaT-inhibiting protein (Mip). MvaT is a transcriptional regulator that silences foreign, AT-rich DNA. Mip binds to MvaT and prevents binding of its target DNA, thus blocking MvaT from silencing the phage DNA from which Mip originated (Wagemans et al., 2015). Regulation of twitching motility, a key process in *P. aeruginosa*, by morons has been reported by Chung et al. D3112-gp05, a twitching inhibitory protein (Tip), binds to and prevents the localization of the type IV pilus extension ATPase PilB, inhibiting its function and preventing extension of the pilus. This leads to a complete loss of twitching motility and resistance to further infection by all pilus-specific phages (Chung et al., 2014). The phage ΦKZ encodes a degradosome-interacting protein (Dip) (ΦKZ gp37), which prevents activity of the RNA degradosome and protects newly synthesized viral RNA in infected
cells (Dendooven et al., 2017). The phage ΦKZ has also been found to encode its own tubulin/FtsZ-like cytoskeletal protein (TubZ) (ΦKZ gp39) to optimize production of phage progeny by localizing its own phage DNA in the center of the cell (Aylett et al., 2013). The phage ΦCTX carries a cytotoxin gene that, upon lysogenization of the phage, turns P. aeruginosa into a cytotoxin-producer that is more virulent than its isogenic non-producer (Hayashi et al., 1990; Baltch et al., 1994).

1.4 Twitching Motility in *P. aeruginosa*

Several phages have been identified that affect motility in *P. aeruginosa*, including the phages D3112, DMS3, and MP22 (Zegans et al., 2009; Chung et al., 2012; Chung et al., 2014). The Tip protein from phage D3112 prevents assembly of the type IV pili (T4P), which are long, thin hair-like appendages found on the surface of *P. aeruginosa* (Bradley, 1973; Pelicic, 2008), shown in Figure 3a. They are a key structure controlling a form of movement of *P. aeruginosa* on solid surfaces known as twitching motility, which is mediated by the extension and retraction of T4P (Figure 3b). T4P allow bacteria to crawl, walk, swarm, and skid as they explore surfaces (Burrows, 2012; Gibiansky et al., 2010).

![Figure 3: *P. aeruginosa* uses T4P to engage in twitching motility and a single polar flagellum to perform swimming motility.](image)

T4P also play roles in surface attachment and adhesion, cell aggregation, and biofilm formation (Bradley, 1980; Mattick, 2002; Burrows, 2005). T4P are important for virulence, as mutants lacking T4P are impaired in mammalian host cell colonization and thus are less infectious (Farinha et al., 1994). Hyperpiliated *P. aeruginosa*, possessing T4P but lacking in twitching
motility, have reduced cytotoxicity towards epithelial cells in vitro, and were found to be defective in dissemination to the liver from a lung infection in vivo (Comolli et al., 1999). Nonpiliated and hyperpiliated P. aeruginosa exhibit reduced association and invasion of epithelial cells in vitro, attenuation of virulence, and a reduced ability to colonize cells in a murine corneal infection model (Zolfaghar et al., 2003). T4P are important in the initial stages of infection, during early epithelial interactions (Zolfaghar et al., 2003), but are often lost during chronic infection due to downregulation of surface pili; this is observed by the loss of surface piliation and twitching motility in P. aeruginosa isolates from chronically infected CF patients (Huus et al., 2016). T4P are also used as a receptor for infection by T4P-specific phages, such as the P. aeruginosa phages D3112 and DMS3 described earlier. T4P-specific phages are thought to bind to the tip of the pilus fiber, and brought into contact with or through the outer membrane of the bacterial cell upon T4P retraction, allowing for interaction with a secondary receptor and injection of genetic material into the cytoplasm of the cell (Bradley, 1973). Loss of surface piliation or T4P retraction leads to resistance to phage infection.

1.5 Components of the Type IV Pilus and Assembly System

![Diagram of T4P assembly and regulatory components](image)

Figure 4: Schematic representation of select P. aeruginosa T4P assembly and regulatory components. Proteins are labelled according to the Pil nomenclature. The minor pilins PilE, PilV, PilW, PilX, FimU, and PilY1 form an initiation complex for pilus formation and are incorporated into the growing fiber, made of the major pilin, PilA, which is processed by the peptidase PilD. A multimeric complex of PilQ and PilF form the secretin through which the T4P extends. PilM, PilN, PilO, PilP form an alignment complex surrounding the base of the pilus fibre to correctly position the growing pilus fiber through the secretin. A dimer of PilC with
hexameric PilB or PilT, depending on whether the pilus is extending or retracting, and PilU, make up the motor complex. PilZ and FimX interact with PilB to modulate its activity. PilG and PilH regulate the activity of PilB and PilT/PilU, respectively, in response to chemotactic signals. PilS and PilR are part of a two-component system regulating pilA expression.

The pilus is repeatedly assembled and disassembled during extension and retraction. The pilus fiber consists of hundreds of PilA subunits in a helical filament (Paranchych et al., 1979), along with the minor pilins that are assembled into the filament in low levels. The minor pilins, PilV, PilW, and PilX, form an inner membrane priming complex that then recruits PilE to form the initiation complex, shown in the schematic in Figure 4 (Alm & Mattick, 1995; Alm et al., 1996b; Alm & Mattick, 1996; Russell & Darzins, 1994, Nguyen et al., 2015a, 2015b). PilE links the priming complex to PilA for initiation of pilus fiber assembly (Nguyen et al., 2015b). The minor pilins are likely incorporated at the tip of the pilus (Giltner et al., 2010; Nguyen et al., 2015a). FimU is a connector that links the major pilin subunit, PilA, to the complex of minor pilins and PilY1 (Nguyen et al., 2015a). PilY1 is encoded within the minor pilin operon and is an essential calcium-dependent regulator of T4P assembly and twitching motility, and acts as a bacterial adhesin (Orans et al., 2010). The outer membrane secretin complex is responsible for creating the pore through which the T4P extends. It is composed of 14 subunits of PilQ (Koo et al., 2016) and the outer membrane lipoprotein PilF (Koo et al., 2008; Koo et al., 2013).

The inner membrane motor subcomplex that drives the extension and retraction of the pilus fiber is made of the dimeric integral membrane platform protein PilC, along with the cytoplasmic ATPases, PilB or PilT and PilU. PilC controls both pilus assembly and disassembly, through rotation by the extension ATPase PilB and the retraction ATPase PilT, which form hexamers in the motor complex (Takhar et al., 2013; McCallum et al., 2019). PilB is involved in extension of the T4P, converting chemical energy from ATP hydrolysis to mechanical energy required for pilus assembly (Mattick 2002; Savvides et al., 2003; Chiang et al., 2005), and PilT and PilU are involved in retraction and pilin depolymerization (Whitchurch et al., 1991; Whitchurch & Mattick, 1994). The exact function of PilU is not known, but it somehow modulates the activity of PilT (Whitchurch & Mattick, 1994). PilB and PilT are localized to both poles of the cell, but PilU is unipolar, suggesting that PilU may play a role in differentiating between the leading and lagging poles of the bacterial cell to control the direction of motility (Chiang et al., 2005; Leighton et al., 2015).
The alignment subcomplex connects the motor complex with the outer membrane complex and ensures that the growing pilus fiber is positioned correctly to extend through the secretin (Carter et al., 2017). This complex is composed of the proteins PilM, PilN, PilO, and PilP (Ayers et al., 2009; Tammam et al., 2011). PilM forms a ring in the inner membrane and binds the cytoplasmic N-terminus of PilN (Karuppiah, 2011). PilN forms heterodimers with the inner membrane protein PilO. This complex may influence which ATPase is bound to PilM, and also interacts with the inner membrane lipoprotein PilP, which interacts with PilQ to complete the transmembrane envelope (Tammam et al., 2013; McCallum et al., 2016).

Another important component of T4P biogenesis is PilD, a membrane-bound protease that is essential for T4P biogenesis, which processes the major and minor pilins prior to their assembly into the pilus fiber (Strom et al., 1994). The PilS and PilR two-component system regulates expression of the major pilin, PilA (Hobbs et al., 1993). PilS is a membrane-spanning sensor kinase and PilR is a cytoplasmic response regulator that activates pilA transcription (Boyd et al., 1994; Jin et al., 1994). PilG and PilH are part of the chemosensory system linking twitching motility to chemotaxis (the Chp system) (Darzins, 1993; Whitchurch et al., 2004). PilG regulates the activity of PilB and extension of T4P, while PilH is implicated in regulation of PilT and pilus retraction (Bertrand et al., 2010). FimX is a cyclic diguanosine monophosphate (c-di-GMP)-binding protein that interacts with PilB to promote assembly of T4P under low c-di-GMP levels in response to environmental signals (Huang et al., 2003; Jain et al., 2017). PilZ is another protein that regulates twitching motility and the activity of PilB in a manner that may be linked to FimX (Alm et al., 1996a; Jain et al., 2017).
The Pil-Chp system links chemotaxis to twitching motility

Figure 5: Schematic representation of the Chp chemosensory system in *P. aeruginosa*. The methyl-accepting chemotaxis protein PilJ receives mechanical and chemical signals from its environment and forms a complex with the adaptor proteins PilI and ChpC to activate ChpA. ChpA autophosphorylates and transfers a phosphate group to the response regulators PilH or PilG, both of which regulate the activity of the adenylate cyclase CyaB. Cyclic AMP produced by CyaB binds to the transcription factor Vfr, which then drives expression of virulence genes. PilH also interacts with PilT and/or PilU to regulate T4P retraction, while PilG regulates T4P extension through PilB. FimL serves as a scaffold to link FimV and PilG. Methylation of PilJ is regulated by ChpB and PilK. Dotted lines indicate the transfer of a functional group.

In *P. aeruginosa*, the Chp chemosensory system links environmental signals to twitching motility (Figure 5). PilJ is a methyl-accepting chemotaxis protein, which regulates twitching motility in response to levels of attractants or repellants in the environment by stimulating the histidine kinase ChpA through the adaptor proteins PilI and ChpC. Methylation of PilJ is regulated by the methyltransferase PilK and the methylesterase ChpB (Whitchurch et al., 2004; Bertrand et al., 2010). ChpA phosphorylates PilG and PilH, response regulators that mediate twitching motility in response to chemotactic signals through interactions with their respective T4P ATPases (Whitchurch et al., 2004). Upon phosphorylation, PilG regulates the activity of PilB, possible through PilZ or FimX to facilitate extension of T4P, while PilH interacts with PilT or PilU to regulate T4P retraction (Bertrand et al., 2010). FimV, which may also be part of the T4P alignment complex, binds peptidoglycan and is important for the formation of the outer membrane secretin (Wehbi et al. 2010; Inclan et al. 2016). FimV also has cyclic AMP (cAMP) -
dependent and -independent roles in the regulation of twitching motility and is involved in connecting twitching motility to the Chp chemosensory system (Buensuceso et al., 2016). FimL acts as a scaffold protein to link the Chp and T4P systems by directly connecting FimV to PilG (Inclan et al., 2016). Despite the importance of PilG and PilH in regulating twitching motility, some residual activity of PilB and PilT is retained in the absence of signaling from the Pil-Chp system (Bertrand et al., 2010). PilH is also hypothesized to act like a phosphate sink to attenuate Chp signaling in lieu of a phosphatase in the Pil-Chp system (Silversmith et al., 2016). PilG and PilH also regulate levels of intracellular cAMP through the regulation of CyaB, an adenyl cyclase that synthesizes cAMP (Fulcher et al., 2010). pilH mutants exhibit increased levels of intracellular cAMP, while pilG mutants exhibit decreased levels. In P. aeruginosa, cAMP modulates gene expression via binding to the cAMP receptor protein (Fulcher et al., 2010) Vfr (virulence factor regulator), a transcription factor that regulates more than 200 genes involved in virulence responses, including those for T4P and flagellum biosynthesis (Wolfgang et al., 2003).

1.7 Other key forms of motility in P. aeruginosa

Another key form of motility in P. aeruginosa is swimming motility, mediated by a single polar flagellum (Figure 3a, 3c). Swimming motility is used to move the bacteria through liquid environments and plays an important role in the virulence of P. aeruginosa. FliC is the flagellin monomer that makes up the long helical filament of the bacterial flagellum (Farinha et al. 1993). Flagella are important in the establishment of infection and may serve as a tether during initial interactions with epithelial cells (Feldman et al., 1998). The absence of surface flagellation was found to be associated with reduced virulence and invasion in a murine burn wound model (Drake & Montie, 1988). The flagellar gene fliF is required for flagellar assembly and contributes to bacterial binding to respiratory mucin, making the flagella important during initial colonization in infection (Arora et al., 1996). The flagellin monomer, FliC, is a proinflammatory molecule and activates the innate immune system through binding to Toll-like receptor 5 on the surface of epithelial and immune cells (Hayashi et al., 2001). Due to the ability of flagellin to provoke a strong innate immune response, it may be detrimental for P. aeruginosa to remain flagellated during bacterial infection, thus downregulation or even loss of surface flagellin would promote bacterial evasion from the host immune system. Similar to T4P, swimming motility and surface flagellation is found to be downregulated in chronic P. aeruginosa infection. In CF patients, particularly those with poor clinical condition, there is in vivo selection for mutants
lacking flagella. Luzar et al., demonstrated that isolates at the onset of infection were found to be motile but over the course of infection most of the bacteria no longer expressed functional flagella (Luzar et al., 1985).

Both flagella and T4P are required for a third form of motility known as swarming motility, which is induced on semisolid surfaces under nitrogen limitation and in response to certain amino acids (Köhler et al., 2000). Swarmer cells are usually elongated and hyperflagellated, possessing up to two flagella in *P. aeruginosa*. Loss of FliC or T4P leads to decreased or lost swarming motility (Köhler et al., 2000). Swarming motility is proposed to be important for colonization of natural environments and *in vivo* during infection (Köhler et al., 2000). Flagella may be required to overcome the adhesive interactions of T4P for swarming motility (Murray & Kazimierczak, 2008). T4P and flagella are important for detachment of bacteria from surfaces, and motility defects that affect the ability of bacteria to detach influence biofilm morphology in *P. aeruginosa* (Conrad et al., 2011). T4P are required for microcolony formation, while flagellin may serve as an adhesin, with the propulsive force generated by swimming motility initiating biofilm formation (O’Toole & Kolter, 1998; Toutain et al., 2007). In the absence of T4P and flagella, *P. aeruginosa* undergoes sliding motility, which takes place under the same conditions as swarming motility. Rhamnolipid, a biosurfactant produced by *P. aeruginosa* modulates swarming and sliding motility (Caiazza et al., 2005; Murray & Kazmierczak, 2008). Sliding motility is proposed to allow colonization of *P. aeruginosa* when flagellum and T4P expression is downregulated, such as during chronic infection (Murray & Kazimierczak, 2008).

### 1.8 Identification of bacteriophage morons affecting motility in *P. aeruginosa*

Previously the Maxwell Lab conducted a systematic investigation of *P. aeruginosa* phage morons in the bacterial strains PAO1 and PA14 to characterize the effect of expression of specific phage morons on bacterial phenotypes. Tsao et al. found that expression of many morons from the collection led to changes in phenotypes that correlate with increased bacterial virulence and confer selective advantages for the bacterial host, highlighting the symbiotic relationship between bacteria and their prophages (Tsao et al., 2018). Eight unique morons were found to impair twitching motility, four of which are found together in the same phages: JBD26-5 (a homologue of the Tip protein) and JBD26-61, which completely abrogate twitching motility.
and provide resistance to T4P-specific phage infection, and JBD30-4 and JBD26-15, which decrease twitching motility. These four morons are found in the phages JBD23, JBD26, and JBD30, as shown in the partial genome alignment in Figure 6. JBD26-15 and JBD30-4, in addition to impairing twitching motility, also caused defects in swimming motility in the *P. aeruginosa* lab strains PAO1 and PA14. The study by Tsao *et al.*, was the first reported phage-mediated regulation of flagella and swimming motility in *P. aeruginosa*.

**Figure 6:** Schematic representation of partial genome alignments of *Pseudomonas aeruginosa* phages JBD30, JBD26, and JBD23. JBD30, JBD26, and JBD23 all have several morons that regulate twitching and swimming motility in *P. aeruginosa*. Genes required for phage replication and lysogeny are shown in light grey and labelled with their known or putative function. Phage genes encoding hypothetical proteins that are conserved across all phages in the family are shown in dark grey. Phage moron genes are shown in coloured arrows, with morons impacting twitching motility in the study by Tsao *et al.*, shown in the bordered arrows and labelled (JBD30-4 (red), JBD26-5, a homolog of the Tip protein (blue), JBD26-15 (purple), and JBD26-61 (orange) (Tsao *et al.*, 2018).

### 1.9 Thesis Objectives

Among two phage morons found in several JBD phages that altered both twitching and swimming motility in *P. aeruginosa*, one was selected to investigate, JBD30-4. In addition to decreasing twitching and swimming motility, JBD30-4 was found to decrease production of rhamnolipids. The decrease in twitching motility did not impact the sensitivity of bacteria expressing the moron to infection by T4P-specific phages (Tsao *et al.*, 2018). JBD30-4 presents a novel method of phage-mediated regulation of T4P and flagella that has not been explored in *P. aeruginosa*. This study aims to determine the mechanism of action of JBD30-4 by identifying an interacting partner and through structural characterization of this protein. The bacterial two-hybrid system and an *in vitro* protein pull-down assay were used to identify and validate the interaction between JBD30-4 and its putative interacting partner. To obtain insight into the function of JBD30-4 through its structure, the protein was purified and crystallization was attempted. Understanding the role of JBD30-4 in *P. aeruginosa* will provide insight into the
regulation of T4P and flagella and will reveal novel mechanisms of phage-mediated regulation of bacterial virulence.
2 Materials and Methods

2.1 Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in the study are listed in Tables 1 and 2, respectively. The list of oligonucleotides used to construct plasmids in this study are shown in Table 3. P. aeruginosa and Escherichia coli were cultured at 37 °C in lysogeny broth (LB) (Lennox) on LB agar, unless otherwise indicated, with antibiotics as needed. Antibiotics were used in the following concentrations: 100 μg mL⁻¹ ampicillin, 25 μg mL⁻¹ gentamicin, 50 μg mL⁻¹ streptomycin for E. coli and 50 μg mL⁻¹ gentamicin for P. aeruginosa. 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression in E. coli, and 0.05-0.5% (w/v) arabinose in P. aeruginosa.

Table 1: List of bacterial strains used in this study

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<tr>
<th>Bacterial Strain</th>
<th>Description</th>
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<td>Burrows Lab</td>
</tr>
<tr>
<td>P. aeruginosa PAO1ΔfliC</td>
<td>PAO1 with fliC deletion</td>
<td>Burrows Lab</td>
</tr>
<tr>
<td>P. aeruginosa PAO1-ΔpilA</td>
<td>Wild type PAO1 from</td>
<td>Howell Lab</td>
</tr>
<tr>
<td>P. aeruginosa PAO1ΔpilA</td>
<td>PAO1 with pilA deletion</td>
<td>Burrows Lab</td>
</tr>
<tr>
<td>E. Coli BTH101</td>
<td>F-, cya-99, araD139, galE15, galK16, rpsL1 (Str r), hsdR2, mcrA1, and mcrB1</td>
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<td>E. Coli BL21(ΔDE3)</td>
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<tr>
<td>E. coli DH5α</td>
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<td>New England Biolabs</td>
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Table 2: List of plasmids used in this study

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<td>Tsao et al., 2018</td>
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<tr>
<td>pHERD30T::jbd26-61</td>
<td>JBD26-61 in pHERD30T</td>
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<tr>
<td>Plasmid construct</td>
<td>Primer</td>
<td>Sequence</td>
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<td>Reverse</td>
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</tr>
</tbody>
</table>

**Table 3: List of oligonucleotides used to construct plasmids in this study**
2.2 Sequence analysis of JBD30-4 and protein structure prediction

Homologues of JBD30-4 were identified using NCBI BLAST (Johnson et al., 2008) with a search performed using the amino acid sequence of JBD30-4 (Genbank accession number YP_007392311.1) as the query, with an expect value threshold of 1. Further analysis of the genomic contexts of JBD30-4 homologues was performed using the annotated genomes available on Genbank. Secondary structure prediction was performed using JPred (Drozdetskiy et al., 2015). Disordered region prediction was performed by PredictProtein (Rost et al., 2004; Schlessinger et al., 2009). Tertiary structure prediction was performed using I-TASSER (Yang & Zhang, 2015; Zhang et al., 2017). Identification of paralogues of PilH in PAO1 was performed using the BLASTP search tool in the Pseudomonas Genome Database with an E-value cutoff of $10^{-12}$ (Winsor et al., 2016).

2.3 P. aeruginosa transformation and expression

PAO1 was cultured shaking overnight from a glycerol stock or a streak plate in 5 mL of lysogeny broth (LB) (Lennox) (1% (w/v) tryptone, 0.5% (w/v) sodium chloride, 0.5% (w/v) yeast extract) at 37 °C. 1 mL of overnight culture was centrifuged for 3 min at 5000 x g. Supernatant was removed and cell pellet was washed with 1 mL of 300 mM sucrose three times. After the final wash the pellet was resuspended in 100 μL of 300 mM sucrose and 75-100 ng of DNA was added. Cells were incubated with plasmid DNA for 15 min at room temperature and then transferred to BioRad 0.2 cm gap electroporation cuvettes, and electroporated at 2500 V. 1 mL of LB was added to the cells and the cells were transferred to culture tubes for recovery at 37 °C in a shaking incubator for 1 h. Cells were harvested by centrifugation at 6000 x g for 3 min, the supernatant was removed, and the cells were plated on the appropriate selection media.

2.4 Twitching motility assay

1% LB-agar plates were prepared for twitching assays with 1% (w/v) tryptone, 0.5% (w/v) sodium chloride, 0.5% (w/v) yeast extract, and 1% (w/v) agar. 25 mL of LB-agar was poured into each 10 cm petri plates and dried in the biosafety cabinet for 30 min with the lids off and used the same
day as prepared. To inoculate bacteria on the twitching plates, individual colonies from streak plate were selected with a toothpick and stabbed into the LB agar, touching the surface of the plastic petri dish. Plates were incubated at 37 °C for 24 h. Agar was removed from the petri dish and 1% (w/v) crystal violet (dissolved in water) was poured into the empty petri dish. After 1 min the crystal violet was removed and the plates were washed with double distilled water three times to remove residual crystal violet.

2.5 Phage plaquing assay

*P. aeruginosa* phages were freshly prepared by culturing lysogens in 3 mL of LB broth overnight at 37 °C. 10 drops of chloroform were added to each tube and culture tubes were transferred back into the shaking incubator at 37 °C for 10 min. 1 mL of each cell lysate was removed and centrifuged at 10000 x g for 5 min and the phage-containing supernatant was removed and stored at 4 °C until use. 150 μL of overnight cultures of PAO1 expressing empty vector or the gene of interest in pHERD30T was mixed with 3 mL of top agar (LB media with 0.7% (w/v) agar) with 10 mM magnesium sulfate. The appropriate selection antibiotics and inducing agents were added to the top agar mixture. Top agar was poured over pre-warmed thick plates (LB agar plates with 10 mM magnesium sulfate, 35 mL per 10 cm petri dish) in an even layer and allowed to solidify and dry under flame for 10 min. Phages were serially diluted 1:10 in SM buffer (100 mM sodium chloride, 8 mM magnesium sulfate, 50 mM Tris-HCl (pH 7.5)) and 2 μL of each dilution was spotted on the top agar. Spots were dried under flame for 10 min prior to incubating plates overnight at 30 °C.

2.6 Swimming motility assay

Swimming motility assays were performed with agar plates composed of 0.3% (w/v) agar, 1% (w/v) tryptone, and 0.5% (w/v) sodium chloride. The appropriate selection antibiotic and inducing agent was added to the media before pouring the plates (25 mL per 10 cm petri dish). Plates were dried for 30 min in the biosafety cabinet before use. Plates were inoculated by selecting a single, isolated colony from a streak plate using sterile toothpick or pipette tip and stabbing it into the agar layer of the plate, but not touching the plastic of the bottom of the plate. Plates were incubated upright for 24 h at 37 °C before imaging the plates and measuring the diameter of the swimming zone, or the area travelled.
2.7 Bacterial growth curves

Overnight cultures of PAO1 carrying either pHERD30T or pHERD30T::jbd30-4 were diluted to an OD$_{595}$ of 0.1 into LB with gentamicin (50 μg mL$^{-1}$) and 0.1% or 0.3% (w/v) arabinose. 200 μL of culture was grown in each well of a clear bottom 96-well plate, shaking at 37 °C in a TECAN Infinite 200 microplate reader, with the OD$_{595}$ measured every 15 min over 20 h of growth.

2.8 Isolation of sheared surface proteins and SDS-PAGE analysis

Single colonies were selected from the streak plate and were struck onto an LB agar plate (2% (w/v) agar) in a grid pattern, with one colony used for the vertical direction and another colony used for the horizontal direction. Plates were incubated at 37 °C for 24 h. Cells were scraped off the agar using a glass coverslip and added to a pre-weighed 15 mL conical tube. The mass of the cells was recorded. 1 mL of phosphate buffered saline (pH 7.5) was added to each tube and the cells were vortexed at maximum speed for 30 s. Cell suspension was centrifuged at 4000 x g for 10 min at 4 °C. Supernatant was removed and transferred to a fresh 1.5 mL microfuge tube and spun at 18000 x g for 15 min at 4 °C. Supernatant was then transferred to a fresh 1.5 mL microfuge tube and 1 M magnesium chloride was added to bring the solution to a final concentration of 0.1 M magnesium chloride. The tubes were inverted several times and incubated upright at 4 °C overnight. Samples were then centrifuged for 20 min at 18000 x g at 4 °C and the supernatant was removed. 1X sodium dodecyl sulfate loading dye (125 mM Tris, pH 6.8; 2% (w/v) 2-mercaptoethanol; 20% (v/v) glycerol; 0.001% (w/v) bromophenol blue; 4% (w/v) SDS), was added to each pellet proportionally to the mass of the cells recorded at the start of the experiment (10 μL loading dye per 30 mg cell mass). Pellets were resuspended and boiled at 95 °C for 10 min, then centrifuged at room temperature at 21000 x g for 30 s. 10 μL of sample was loaded onto an 18% SDS-PAGE gel, alongside BLUeye prestained protein ladder or PageRuler Unstained Protein Ladder. Gel was stained with Coomassie Brilliant Blue R-250 to visualize proteins.

2.9 Bacterial adenylate cyclase two-hybrid system

Interactions between proteins of interest and proteins involved in pilus biosynthesis in *P. aeruginosa* were tested using the bacterial adenylate cyclase two-hybrid (BACTH) assay. Full length pilus biosynthesis proteins were N- or C-terminally tagged with the T18 fragment of
adenylate cyclase. JBD30-4 was N-terminally tagged with the T25 fragment of adenylate cyclase by cloning it in frame into pKT25. pKT25::jbd30-4 was co-transformed into the cya mutant strain BTH101 with the pilus biosynthesis genes in the pUT18 or pUT18C vector. Transformations were plated on LB agar plates with 100 μg mL⁻¹ ampicillin and 50 μg mL⁻¹ kanamycin and incubated overnight at 37 °C. Single colonies were selected and cultured overnight at 30 °C in 1 mL of LB broth with ampicillin and kanamycin. 2 μL of each overnight culture was spotted onto LB agar plates containing 40 μg mL⁻¹ X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 1 mM IPTG and MacConkey agar plates with 1% (w/v) maltose and IPTG. Spots were allowed to dry under flame for 10 min prior to incubating at 30 °C overnight.

2.10 Protein expression

Plasmids were transformed into E. coli BL21(λDE3) cells and plated onto LB agar plates with 100 μg mL⁻¹ ampicillin. A single colony was selected to set up an overnight culture in 20 mL of LB broth with ampicillin. The starter culture was inoculated in 1.8 L of LB media with ampicillin and 500 μL of anti-foaming agent (Millipore Sigma) and grown at 37 °C until the culture reached an OD₆₀₀ of 0.8, then induced with 1 mM IPTG. The cultures were cooled to 16 °C and were grown overnight. Cells were harvested by spinning at 12000 x g for 20 min and resuspended in 37 mL of binding buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM imidazole) and sonicated for 30 s ten times, on ice. The cellular debris was collected by centrifugation at 34000 x g for 30 min. 750 μL nickel-NTA agarose slurry was added to each 40 mL of cleared lysate and incubated rotating at 4 °C for 1 h. The sample lysate was collected by gravity flow, and the resin was washed 10 times with 10 column volumes of wash buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 30 mM imidazole). The protein was eluted in 30 mL of elution buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 250 mM imidazole). Protein samples were dialyzed overnight in dialysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM DTT) with TEV protease (1 mg TEV protease per 100 mg target protein) at 4 °C. Nickel-NTA agarose was added to the dialyzed protein sample and the flow-through was collected and concentrated to 5 mL prior to loading onto the gel filtration column. Size exclusion chromatography was performed using the GE HiLoad® 16/600 Superdex® 75 preparation grade gel filtration column. Elution fractions containing the protein of interest were collected and concentrated prior to crystal trials. The MCSG-1 through MCSG-4 crystal screens (Anatrace) were used for crystal trials.
2.11 Denaturing protein preparation

*E. coli* BL21(λDE3) cells were grown overnight at 16 °C and harvested as described in section 2.10. Cell pellets were resuspended in 20 mL Buffer A (6 M GuHCl, 100 mM NaH₂PO₄, 114 mM Tris, 10 mM imidazole) and incubated for 1 h at room temperature. Cleared lysates were prepared by centrifuging the samples at 27000 x g for 15 min at room temperature. 1 mL of Ni-NTA agarose slurry was added to each supernatant fraction and incubated at room temperature for 15 min, then washed on a glass column with 20 mL of Buffer A four times. Protein was eluted with 15 mL of Buffer F (GuHCl 575 g/L, Glacial Acetic Acid (17.4 M) 11.5 ml/L) twice and dialyzed overnight at room temperature in refolding buffer (10 mM Tris, pH 7.5, 150 mM NaCl) with TEV protease.

2.12 Protein pull-downs with PilH-FLAG and JBD30-4-His₆

JBD30-4-His₆ was cloned into the p15TV-L vector and co-transformed into *E. coli* BL21(λDE3) with pCDF1b::pilH-FLAG and plated onto LB agar plates with 100 μg mL⁻¹ ampicillin and 50 μg mL⁻¹ streptomycin. 500 μL of an overnight culture was used to inoculate a 50 mL subculture that was grown to an OD₆₀₀ of 0.8 before the addition of 1 mM IPTG. The cultures were cooled to 16 °C and were grown overnight. Cells were harvested by centrifuging at 7000 x g for 10 min and resuspended in 1 mL binding buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM imidazole) and sonicated for 30 s 10 times, on ice. Cleared cell lysates were prepared by centrifuging the samples at 18000 x g for 10 min at 4 °C. 20 μL of nickel-NTA slurry was added to the supernatant and incubated at 4 °C for 1 h. Samples were centrifuged at 5000 x g for 1 minute and washed with 1 mL of wash buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 30 mM imidazole). Four washes were performed. The protein was eluted in 30 μL of elution buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 250 mM imidazole). Flow-through and elution fractions were collected, resuspended in 1X SDS loading dye, boiled at 95 °C for 10 min, resolved on a 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250.

2.13 Western blot analysis of protein samples

Protein samples were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membrane using a semi-dry transfer apparatus for 15 min at 10 V and blocked with 5% (w/v) bovine serum albumin in 1X Tris-buffered saline (TBS) at room temperature for 1 h. Blots were
incubated with rabbit monoclonal anti-FLAG or mouse anti-His\textsubscript{6} antibody at 1:10000 in 5% bovine serum albumin-TBST overnight at 4 °C. Blots were washed with 1X TBS with 0.1% (v/v) Tween 20 (TBST) 3 times for 5 min each and incubated with secondary goat anti-rabbit or anti-mouse IgG-HRP conjugated antibody at 1:10000 in 1X TBST for 1 h at room temperature. Blots were washed with 1X TBST five times for 5 min each and developed using the BioRad Clarity™ Western ECL Blotting Substrate.
Chapter 3

3 Results

3.1 Homologue identification and sequence analysis of JBD30-4

To determine how frequently phages and bacterial strains encode a homologue of JBD30-4, a BLAST search was performed using the amino acid sequence of JBD30-4 as the query. All hits, except for one, were identified in *P. aeruginosa* strains and phages and shared greater than 95% amino acid identity with JBD30-4. The most distantly related homologue was identified in *Pseudomonas oleovorans*, and shares 50% amino acid identity with JBD30-4. Homologues of JBD30-4 were identified in 11 different *Pseudomonas* phages. Secondary structure predictions of JBD30-4 using JPred (Drozdetskiy et al., 2015) predict that the protein is made of three to four alpha helices, separated by disordered regions. The first 15 residues in the N-terminal region and the last 15 residues at the C-terminus of JBD30-4 are predicted to constitute a disordered region in PredictProtein (Rost et al., 2004; Schlessinger et al., 2009). Though tertiary structure prediction by I-TASSER did not generate models with high confidence (Yang & Zhang, 2015; Zhang et al., 2017). The program PSORTb (Yu et al., 2010) predicts that JBD30-4 is localized in the cytoplasm of the cell.

3.2 JBD30-4 is found in the same genomic context in phages

Examination of the genomic context of JBD30-4 homologues in bacteriophages reveals that JBD30-4 is found as the fourth open reading frame in the phage genome (Figure 7), upstream of an 84-amino acid protein (JBD30-5) homologous (93% amino acid identity) to the C-terminal region of a previously characterized phage moron, Tip (D3112-gp05) (136-amino acids in length) known to prevent T4P formation (Chung et al., 2014). JBD30-4 is consistently found downstream of a hypothetical phage protein (JBD30-3) whose function is uncharacterized but is highly conserved (greater than 90% amino acid sequence similarity) across 50 *Pseudomonas* phages. Some phages in which JBD30-4 is not found, such as JBD5 and MP22 (Figure 7) possess a 69-amino acid protein that shares 56% amino acid identity with the C-terminus of the Tip protein (Chung et al., 2014). In *Pseudomonas* phages, JBD30-4 is only found upstream of Tip homologues that are equal to or greater than 84 amino acids in length. The only homologue of JBD30-4 not in *P. aeruginosa* strains or *Pseudomonas* phages is in *Pseudomonas oleovorans*. 
sharing 50% amino acid identity with JBD30-4. In *P. oleovorans*, the JBD30-4 homologue is likely contained within a prophage region, as it is in the same genomic context as the *Pseudomonas* phages shown in Figure 7, downstream of a phage repressor and upstream of a transposase. However, the homolog of JBD30-4 is not found adjacent to a Tip-like protein in *P. oleovorans* (Figure 7).

![Figure 7: JBD30-4 is found in the same genomic context across all phages in which it is present. A representative schematic of the genomic context in which JBD30-4 homologues are found in *Pseudomonas* phages and *P. oleovorans*. Phage names are shown on the left side of the figure, known or putative function of the genes are written above the arrows. Solid dark grey arrows indicate proteins genes with greater than 90% sequence identity with the corresponding gene in JBD30, light grey outlined arrows represent proteins with 60-89% sequence identity, and crosshatched arrows depict proteins with 35-59% sequence identity.]

### 3.3 Expression of JBD30-4 decreases twitching motility in a concentration-dependent manner

Previously the effect of expression of JBD30-4 in the *P. aeruginosa* strains PAO1 was characterized by expressing the phage moron from the arabinose-inducible expression vector pHERD30T (Qiu *et al.*, 2008). 0.1% w/v arabinose was used to induce protein expression in the studies performed by Tsao *et al.*, and it was found that expression of JBD30-4 under this condition decreased but did not abrogate motility, and sensitivity to infection by T4P-specific phages was unaffected (Tsao *et al.*, 2018). To determine whether the effect of JBD30-4 expression on motility and phage sensitivity phenotypes would be enhanced by increasing the
level of arabinose induction, and therefore the level of JBD30-4 protein expression, phage plaquing, swimming, and twitching motility assays were performed using higher levels of arabinose. The twitching motility assay was performed in the strain PAO1 expressing JBD30-4 with varying concentrations of the inducing agent arabinose. JBD26-61, a phage moron that inhibits twitching motility and causes loss of surface piliation, was used as a control in these experiments. Expression of JBD30-4 decreased twitching motility in PAO1 in a concentration-dependent manner, with a slight decrease at low levels of arabinose induction (0.05% (w/v) arabinose), a significant decrease at 0.1% arabinose, and the greatest decrease with 0.3% arabinose (Figure 8). At 0.3% arabinose induction, the level of twitching motility by PAO1-pHERD30T::jbd30-4 was similar to that of PAO1-pHERD30T::jbd26-61. Expression of JBD26-61 in PAO1 at all concentrations of arabinose tested led to complete abrogation of twitching motility.

**Figure 8: Expression of JBD30-4 decreases twitching motility in a concentration-dependent manner.** Twitching motility zones for PAO1 expressing the indicated morons from the expression vector pHERD30T at varying concentrations of the inducer, arabinose (w/v), were visualized with crystal violet staining of the Petri dish surface over 24 h on 1% LB agar plates.

3.4 Expression of JBD30-4 in PAO1 at high levels of arabinose does not impact sensitivity to phage infection

As there was a greater decrease in twitching motility when JBD30-4 was expressed in PAO1 at higher concentrations of arabinose than originally observed by Tsao et al., phage plaquing assays
were performed to determine whether this decrease in twitching motility correlated with an impact on sensitivity to T4P-specific phage infection. Previously it was observed that expression of JBD30-4 at 0.1% arabinose induction did not change the sensitivity of *P. aeruginosa* to phage infection. Lawns of PAO1 expressing JBD30-4 or JBD26-61 were spotted with several pilus-specific phages (JBD26, JBD23, JBD33, DMS3) and an LPS-specific phage (D3). PAO1-pHERD30T was sensitive to infection by all the phages tested (Figure 9), evidenced by the clearings formed where phages were spotted, with fewer plaques visible at lower concentrations of phage. PAO1-pHERD30T::jbd26-61, which lacks surface piliation, was resistant to infection by all the pilus-specific phages, while phage D3, which uses LPS as the cell surface receptor for infection, was unaffected. Lawns of PAO1-pHERD30T::jbd30-4 display a plaquing pattern identical to PAO1-pHERD30T (vector control) for all phages tested. This data is consistent with the results obtained by Chung *et al.*, where PAO1 expressing a homologue of JBD30-4 (D3112-gp04) was sensitive to infection by T4P-specific phages D3112, MP22, and DMS3 (Chung *et al.*, 2014). These data indicate that the impairment in T4P function observed does not change the sensitivity of PAO1-pHERD30T::jbd30-4 to phage infection, and suggests that some surface piliation is maintained, despite the loss of twitching motility.

**Figure 9: Cells expressing JBD30-4 maintain sensitivity to infection in phage plaque assays in PAO1.** Five phage lysates were applied in 10-fold serial dilutions to lawns of PAO1 carrying pHERD30T (vector control), and PAO1 expressing either pHERD30T::jbd26-61 or pHERD30T::jbd30-4 at 0.5% arabinose (w/v) induction and plaque formation upon phage infection was assessed. JBD26, JBD23, JBD33, and DMS3 are T4P-specific phages and phage D3 is a LPS-specific phage.
3.5 Expression of JBD30-4 decreases swimming motility in a concentration-dependent manner

Noting that the effect of JBD30-4 expression on twitching motility was concentration-dependent, swimming motility assays were performed. Swimming motility was assayed by inoculating a bacterial colony through a layer of low-percentage agar medium, where the movement of the bacteria through the agar layer is observed. A PAO1ΔfliC strain of *P. aeruginosa* was used as a control for loss of swimming motility. This strain lacks FliC, the flagellin monomer, and functional flagella, thus swimming motility is abrogated. A concentration-dependent decrease in swimming motility was observed upon expression of JBD30-4 in PAO1 relative to the empty vector control (Figure 10). At 0.05% arabinose induction there was no change in swimming motility, while at 0.1% arabinose induction there was a substantial decrease in swimming motility. At 0.3% arabinose induction of JBD30-4 expression in PAO1, there was the greatest decrease in swimming motility, though it was not reduced to the level observed in the PAO1ΔfliC strain. Flares were visible in PAO1-pHERD30T::jbd30-4 at 0.3% arabinose induction, which may be indicative of revertants that have escaped the effect of JBD30-4 and have restored swimming motility. Flares were often, but not always observed in the swimming motility assay at 0.3% arabinose induction.

![Figure 10: Expression of JBD30-4 decreases swimming motility in a concentration-dependent manner.](image)

Swimming motility zones for PAO1ΔfliC or PAO1 with a pHERD30T empty vector control or pHERD30T::jbd30-4 at varying concentrations of the inducing agent, arabinose (w/v), were observed by the distance travelled by the bacteria over 24 h on 0.3% agar plates.
3.6 Expression of JBD30-4 in PAO1 at higher concentrations of arabinose causes growth defects

To determine whether the phenotypes of decreased swimming and twitching motility observed upon JBD30-4 expression in PAO1 were due to growth defects, growth curves were generated of PAO1 harbouring empty pHERD30T vector or pHERD30T::jbd30-4 (Figure 11). At 0.1% arabinose induction, there was no effect of JBD30-4 expression on bacterial growth in liquid culture. At 0.3% arabinose induction, a reduction in the growth rate was observed after 5 h, but at 20 h, the optical density of PAO1-pHERD30T::jbd30-4 nears that of PAO1-pHERD30T, suggesting that a defect in growth rate does not account for the motility phenotypes observed upon JBD30-4 expression. The increased growth after 15 h for PAO1 expressing JBD30-4 with 0.3% arabinose induction could be due to revertant cells that could escape the effect of JBD30-4 and selection for bacterial cells in liquid culture that persist and proliferate.

Figure 11: Expression of JBD30-4 in PAO1 causes slight growth defects at higher levels of protein expression. Growth curves of PAO1 carrying empty pHERD30T vector or pHERD30T::jbd30-4 grown in LB media in a 96-well plate at 0.1% or 0.3% arabinose induction. The optical density of the cells at 595 nm was measured every 15 min using the TECAN Infinite 200 plate reader.
3.7 Expression of JBD30-4 in PAO1 leads to decreased surface flagellation and piliation

The ability of pilus-specific phages to infect PAO1 expressing JBD30-4 indicates the presence of surface piliation despite the decrease in twitching motility. The presence of T4P and flagella can be assayed through a shearing assay, in which the surface appendages are isolated from the bacterial cell surface and separated and visualized via SDS-PAGE. Previously PAO1-BL (from the Burrows Lab) had been used for shearing assays with PAO1 expressing JBD30-4, but surface piliation in this wild type strain was undetectable by Coomassie stain on an SDS polyacrylamide gel of sheared proteins (Figure 12). As the level of basal surface piliation can differ between P. aeruginosa strains, several different PAO1 laboratory strains were tested in a shearing assay to identify one with higher levels of surface T4P than PAO1-BL (Figure 12). Three of nine different PAO1 lab strains had surface piliation detectable by Coomassie stain, and one of these strains, PAO1-HL (from the Howell Lab) was selected for use in a shearing assay.

![Figure 12: Different PAO1 laboratory strains have varying levels of surface piliation in surface shearing assays.](image)

Bacterial lawns of different PAO1 laboratory strains grown on 2% LB-agar were resuspended in phosphate-buffered saline and surface appendages were sheared by vigorous vortexing. Soluble proteins were precipitated from the supernatant with magnesium chloride. The resulting sheared proteins were separated by SDS-PAGE and proteins were visualized by staining with Coomassie Brilliant Blue. The flagellin monomer, FliC, and the pilin monomer, PilA, are indicated by the arrows.
pHERD30T empty vector, pHERD30T::jbd26-61, and pHERD30T::jbd30-4 were transformed into PAO1-RL to perform a shearing assay at 0.3% arabinose induction, where the greatest decrease in twitching and swimming motility was observed.

**Figure 13: PAO1-RL expressing JBD30-4 has decreased levels of flagellin and pilin monomers on the cell surface.** Bacterial lawns of PAO1ΔfliC, PAO1ΔpilA, or PAO1-RL with empty pHERD30T vector, pHERD30T::jbd30-4, pHERD30T::jbd26-61 grown on 2% LB-agar with 0.3% (w/v) arabinose were harvested and appendages were sheared by vigorous vortexing. Four biological replicates of the vector control and PAO1-RL-pHERD30T::jbd30-4 are shown. Soluble proteins were precipitated from the supernatant with magnesium chloride. The resulting sheared proteins were separated by SDS-PAGE and proteins were visualized by staining with Coomassie Brilliant Blue R-250. The flagellin monomer, FliC, and the pilin monomer, PilA, are indicated by the arrows.

In the Coomassie-stained SDS-PAGE gel of precipitated surface proteins (Figure 13), levels of the flagellin monomer, FliC, were significantly decreased upon expression of JBD30-4 relative to the vector control. A faint band is observed at approximately 50 kDa, the expected molecular weight of FliC, in samples from PAO1-RL-pHERD30T::jbd30-4, while there is no band observed in the PAO1ΔfliC lane at 50 kDa. This correlates with the observation that swimming motility is significantly decreased upon expression of JBD30-4 in PAO1, but not completely abrogated, as in the PAO1ΔfliC strain. Surface piliation upon expression of JBD30-4 is decreased relative to the empty vector control in PAO1, based on the decreased intensity of the band at 15 kDa, corresponding to the pilin monomer, PilA. Expression of JBD26-61 led to loss of surface piliation. Thus, the impaired swimming motility upon JBD30-4 expression in PAO1 is due to the significant decrease in surface FliC. The fact that PilA is still present on the surface of
the cell correlates with the finding that PAO1 expressing JBD30-4 is sensitive to infection by phages that use T4P for infection but does not explain the loss of twitching motility.

3.8 JBD30-4 interacts with the T4P response regulator, PilH

Given that the function of T4P is impacted by expression of JBD30-4 in PAO1, demonstrated by the decreased twitching motility and surface piliation, assays to determine whether JBD30-4 interacts with specific proteins involved T4P biogenesis and function, including regulatory proteins, were performed. To identify these interactions, the bacterial adenylate cyclase two-hybrid system (BACTH) was used, as it enables rapid screening of interactions between JBD30-4 and other proteins. 13 proteins involved in T4P function were tested using the BACTH system, shown in the schematic in Figure 14A. The selected proteins were fused to the T18 fragment of adenylate cyclase, while JBD30-4 was fused to the T25 fragment. The fusion proteins were co-transformed into the *E. coli* cya mutant strain BTH101, cultured, and spotted onto LB-X-gal or MacConkey-maltose agar indicator media (Figure 14B). Co-transformation of JBD30-4 with PilE, PilM, and PilH led to a colour change on X-gal plates, with PilH yielding the strongest colour change after 16 h of growth. On the MacConkey agar, the only interaction that yielded a colour change was that between T25-JBD30-4 and PilH-T18.
Figure 14: JBD30-4 interacts with the T4P biogenesis proteins PilH, PilM, and PilE using the BACTH system. (A) Select proteins involved with T4P assembly and regulation were tested for interactions with JBD30-4, shown in the schematic. Proteins tested are coloured, with proteins that yielded a positive interaction with JBD30-4 shown in blue. (B) Protein-protein interactions were tested using the bacterial adenylate cyclase two-hybrid (BACTH) system. JBD30-4 had an N-terminal T25 tag, and pilus biogenesis proteins had N- or C-terminal T18 tags. Interactions were tested in an *E. coli cya* mutant strain, BTH101, on LB agar with X-gal or MacConkey agar with maltose, resulting in blue or red colonies, respectively, upon reconstitution of the T18 and T25 fragments of adenylate cyclase. Empty vectors harbouring the T25 and T18 fragments of adenylate cyclase were used as a negative control (−) and proteins known to cause a colour change on indicator media were expressed in the positive control (+).

As the colour change on indicator plates appeared to be the most robust in the T25-JBD30-4 and PilH-T25 co-transformants, combinations of the constructs with the T18 and T25 tags were tested to determine whether interaction would persist if the adenylate cyclase domain fusions were reversed (Figure 15A), where JBD30-4 was always tagged at the N-terminus and PilH was tagged at the C-terminus. The co-transformants of JBD30-4 and PilH with the T25 and T18 tags turned blue on X-gal plates and none of the combinations of empty vectors transformed with a tagged construct changed colour on either indicator media (Figure 15B), demonstrating that the...
T18 and T25 fragments of adenylate cyclase were binding upon co-expression of JBD30-4 and PilH with both the T25 and T18 tags. These data suggest that a target binding partner of JBD30-4 is PilH.

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Figure 15: JBD30-4 and PilH interact using the BACTH system when the T18 and T25 tags are swapped. To confirm the interaction between JBD30-4 and PilH, different combinations of T18 and T25 tags on empty vectors, PilH and JBD30-4 were tested for interactions on X-gal and MacConkey agar. JBD30-4 was always tagged at the N-terminus and PilH was tagged at the C-terminus with the T18 or T25 fragments. Empty vectors (EV) harbouring the T25 and T18 fragments of adenylate cyclase were used as a negative control (-) and proteins known to cause a colour change on indicator media were expressed in the positive control (+). (A) The matrix of interactions and their results. (B) Interactions were tested in an E. coli cya mutant strain, BTH101, on indicator plates of LB agar with X-gal and MacConkey agar with maltose, which result in blue or red colonies, respectively, upon reconstitution of the T18 and T25 fragments of adenylate cyclase.

3.9 Pull-down assay with JBD30-4-His$_6$ and PilH-FLAG does not recapitulate interaction between JBD30-4 and PilH

To confirm the BACTH result showing an interaction between JBD30-4 and PilH, a protein pull-down assay in E. coli with C-terminally His$_6$-tagged JBD30-4 and C-terminally FLAG-tagged PilH was performed as a complementary approach to the BACTH system to recapitulate the interaction of JBD30-4 and PilH observed in vitro. In the Coomassie-stained SDS polyacrylamide gel of fractions from the purification of either JBD30-4-His$_6$ alone, or JBD30-4-His$_6$ co-expressed with PilH-FLAG, an intense band corresponding to the size of PilH-FLAG is
visible in the flow-through fraction (Figure 16A). To determine whether any PilH-FLAG was present in the elution fraction of both JBD30-4 and PilH expressed together, a Western blot with anti-FLAG antibody was performed (Figure 16B). No PilH-FLAG was observed in the elution sample from the purification of co-expressed JBD30-4-His$_6$ and PilH-FLAG, and the interaction between JBD30-4 and PilH could not be recapitulated in vitro in this experiment. The presence of either an N- or C-terminal His$_6$-tag on JBD30-4 did not impair its functionality in this assay, as epitope-tagged JBD30-4 still inhibited twitching motility in vivo when expressed in PAO1 (Figure 17).

**Figure 16:** Pull-down assay with His$_6$-tagged JBD30-4 and FLAG-tagged PilH does not recapitulate the interaction between JBD30-4 and PilH. (A) E. coli BL21(λDE3) cells overexpressing JBD30-4-His$_6$ and PilH-FLAG were lysed and purified with nickel-NTA agarose and induced (I), flow-through (FT), and elution (E) fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. (B) Anti-FLAG Western blot of induced, flow-through, and elution samples.

**Figure 17:** N- and C-terminally His$_6$-tagged JBD30-4 retain functionality in twitching assays in PAO1. Twitching motility zones for PAO1 expressing the indicated morons from the expression vector pHERD30T at 0.5% arabinose were visualized with crystal violet staining of the Petri dish surface after growth for 24 h at 37 °C on 1% LB agar plates.
3.10 JBD30-4 is proteolytically cleaved during protein purification from *E. coli*

Though JBD30-4 has no sequence similarity to proteins of known function, it may have structural homology to previously characterized proteins, which could provide insight into the function and mechanism of action of JBD30-4. To perform structural characterization of JBD30-4, His6-JBD30-4 was expressed in *E. coli* BL21(λDE3) to purify the protein. In this system, JBD30-4 was largely found in inclusion bodies, and only a minimal amount of purified protein could be isolated from the soluble fraction (Figure 18A). To isolate His6-JBD30-4 from inclusion bodies, a denaturing protein purification was performed with guanidine hydrochloride, but the majority of the protein precipitated out of solution during removal of the denaturing agent through dialysis (Figure 18B).

![Figure 18: Nickel-NTA agarose-based purification of N-terminally His6-tagged JBD30-4.](image)

His6-JBD30-4 was expressed in *E. coli* BL21(λDE3) cells, protein expression was induced with 1 mM IPTG during log phase growth and cultured overnight at 16°C. (A) Induced cell lysates, insoluble fractions (inclusion bodies), flow-through, wash, and elution fractions were collected. (B) Soluble and precipitated protein fractions of dialyzed His6-JBD30-4 after isolation from inclusion bodies with guanidine hydrochloride were collected. Protein samples were resolved on 15% SDS-PAGE gels stained with Coomassie Brilliant Blue R-250.

Given that N-terminally His6-tagged JBD30-4 was insoluble, C-terminally His6-tagged JBD30-4 was purified to determine whether there would be an improvement in protein yield and solubility.
During purification of JBD30-4-His₆, there was one dominant band present in the induced fraction at about 15 kDa, corresponding to the expected molecular weight of JBD30-4-His₆, but in the elution fraction, there were several bands (Figure 19A). Notably, one band was consistent with the full-length form of JBD30-4 observed in the induced fraction, while another band between 10 kDa and 15 kDa was present as well. This lower molecular weight species may have been a more stable, proteolytically cleaved form of JBD30-4. To determine whether the higher and lower molecular weight species of JBD30-4 could be separated, and to identify whether the protein was being cleaved from the N- or C-terminus, the concentrated protein sample from Figure 19A was purified using size exclusion chromatography. Collected fractions were separated and visualized on a Coomassie-stained SDS-PAGE gel (Figure 19B). Though the input sample prior to size exclusion chromatography contained many contaminating proteins and may have included aggregated and degraded proteins (Figure 19B), full-length JBD30-4-His₆ and the proteolytically cleaved form could be separated. To determine if JBD30-4 was being proteolytically cleaved from its N- or C-terminus, select fractions were analyzed using SDS-PAGE and a Western blot was performed with anti-His₆ antibody (Figure 19C). A band was visible on the Western blot for the elution fractions at 45 mL and 79 mL, corresponding to the fractions where the full-length and truncated forms of JBD30-4-His₆ were eluting during size exclusion chromatography, suggesting that JBD30-4 cleaves from the N-terminus, while the His₆-tagged C-terminus remains intact. The 34 mL difference in elution volumes between full-length and truncated JBD30-4 does not reflect a difference between the molecular weights of the two proteins, as the elution fraction at 45 mL corresponds to the void volume of the column. Full-length JBD30-4 may have aggregated with the contaminating proteins, thus appearing in a lower elution volume than if the size exclusion chromatography input protein sample contained only full-length and truncated JBD30-4.
Figure 19: The N-terminus of JBD30-4 is proteolytically cleaved during purification. (A) JBD30-4-His6 was expressed in *E. coli* BL21(λDE3) cells, protein expression was induced with 1 mM IPTG during log phase growth and cultured overnight at 16°C. Induced cell lysates, insoluble fractions (inclusion bodies), flow-through, wash, elution, and dialyzed elution fractions were collected. (B) Samples of elution fractions from size exclusion chromatography (SEC) of JBD30-4-His6 purified with the GE HiLoad® 16/600 Superdex® 75 preparation grade gel filtration column were collected, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. (C) Western blot analysis of elution fractions collected from size exclusion chromatography with anti-His6 antibody.

As the exact difference in the molecular weight between truncated and full-length JBD30-4 was unknown, a sequence-based protein solubility prediction tool, PROSO II (Smialowski *et al.*, 2012), was used to estimate the minimum number of residues that needed to be removed from the N-terminus of JBD30-4 to generate a protein that is less susceptible to proteolysis. It was predicted that removal of the first 16 residues from JBD30-4 would yield a soluble protein product. Removal of the first 16 residues of JBD30-4 would yield a protein product of an estimated molecular weight of 11.3 kDa, compared to full-length JBD30-4-His6 with an approximate molecular weight of 13.3 kDa. A 2 kDa difference in the molecular weight between proteolytically cleaved and full-length JBD30-4 is plausible based on the migration patterns of the proteins during SDS-PAGE.

3.11 JBD30-4Δ1-16 is more soluble than JBD30-4 during purification from *E. coli*

To determine whether N-terminally cleaved form of JBD30-4 was more soluble during purification from *E. coli*, a construct of JBD30-4 lacking the first 16 amino acid residues
(JBD30-4\(^{Δ1-16}\)) was synthesized with a cleavable N-terminal His\(_6\)-tag. The solubility of His\(_6\)-JBD30-4\(^{Δ1-16}\) was significantly increased relative to His\(_6\)-JBD30-4, with a more intense band present in the elution lane of His\(_6\)-JBD30-4\(^{Δ1-16}\) than His\(_6\)-JBD30-4 (Figure 20A). After removal of the His\(_6\)-tag, JBD30-4\(^{Δ1-16}\) was purified further with size exclusion chromatography (Figure 20B), and select fractions were run on an SDS-PAGE gel (Figure 20C). JBD30-4\(^{Δ1-16}\) eluted between 79 to 86 mL, with the peak at 83 mL. Protein fractions from 88 mL to 90 mL appeared to contain degradation products, so elution fractions between 79 mL and 86 mL were pooled, concentrated and used to set up crystal trays, but no crystal hits have been identified to date.

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\text{Figure 20: Purification of N-terminally His}_6\text{-tagged JBD30-4 and JBD30-4}^{Δ1-16}. \ \text{(A) His}_6\text{-JBD30-4 and His}_6\text{-JBD30-4}^{Δ1-16} \ \text{were expressed in E. coli BL21(λDE3) cells, protein expression was induced with 1 mM IPTG during log phase growth and cultured overnight at 16 °C. Uninduced and induced cell lysates, insoluble fractions (inclusion bodies), flow-through, wash, elution, and elution fractions were collected. (B) Size exclusion chromatogram of JBD30-4}^{Δ1-16} \ \text{purified with the GE HiLoad® 16/600 Superdex® 75 preparation grade gel filtration column. (C) Samples from size exclusion chromatography elutions were collected, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue R-250.}
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3.12 Expression of JBD30-4\(^{Δ1-16}\) does not inhibit twitching or swimming motility in PAO1

JBD30-4\(^{Δ1-16}\) was more soluble than full-length JBD30-4\(^{Δ1-16}\) in vitro during purification from E. coli, but to determine whether the truncated form of JBD30-4 retained functionality in vivo in P. aeruginosa, swimming and twitching motility assays were performed with pHERD30T::jbd30-4\(^{Δ1-16}\) expressed in PAO1. PAO1 expressing pHERD30T::jbd30-4\(^{Δ1-16}\) twitched at the same level as the PAO1-pHERD30T empty vector control at 0.1% and 0.3% arabinose induction (Figure 21A), in contrast to PAO1-pHERD30T::jbd30-4, which is inhibited for twitching motility. Similarly, swimming motility was also unaffected by expression of JBD30-4\(^{Δ1-16}\) in PAO1.
These results show that the N-terminus is critical for the function of JBD30-4, as removal of the first 16 residues leads to loss of the inhibitory twitching and swimming motility phenotypes.

**Figure 21:** Expression of JBD30-4Δ1-16 does not impact twitching or swimming motility in PAO1. (A) Twitching motility zones for PAO1 expressing the indicated morons from the expression vector pHERD30T at varying concentrations of the inducer, arabinose, were visualized with crystal violet staining of the Petri dish surface over 24 h on 1% LB agar plates. (B) Swimming motility zones for the noted PAO1 strains at varying concentrations of the inducing agent, arabinose, was visualized as the distance travelled by the bacteria over 24 h on 0.3% LB agar plates.
Chapter 4

4 Discussion

4.1 The effect of JBD30-4 expression on motility in *P. aeruginosa*

Despite the prevalence of bacteriophages in all environments on Earth, little is known about the impact these phages can have on the physiology of the bacteria they infect. *P. aeruginosa*, an opportunistic pathogen causing serious morbidity and mortality in immunocompromised individuals, is found to harbor prophages that contribute to the virulence of this organism (Al-Aloul *et al.*, 2004; Winstanley *et al.*, 2008). Several examples of the effect of phage morons on *P. aeruginosa* physiology have been described (Tsao *et al.*, 2018), but only the function of a few morons has been characterized to date (Taylor *et al.*, 2013; Chung *et al.*, 2014; Wagemens *et al.*, 2015; Zhao *et al.*, 2016).

JBD30-4 is a phage moron shown to influence both twitching and swimming motility in *P. aeruginosa* and this study demonstrated that it may do so through an interaction with the T4P response regulator PilH. Loss of T4P and flagellum-mediated motility may be beneficial for *P. aeruginosa* in the context of bacterial infections. JBD30-4 may enable bacteria to avoid detection by the host immune system and persist within the host, resulting in chronic infection. JBD30-4 is an example of how phage morons may provide a fitness advantage to bacterial cells, and therefore the prophage, in the context of human infections.

Using the BACTH system, PilH, along with PilE and PilM were identified as potential interacting partners for JBD30-4. The decrease in twitching motility and sensitivity to phage infection observed upon expression of JBD30-4 is consistent with the phenotype of *pilH* mutants (Bertrand *et al.*, 2010), supporting the hypothesis that JBD30-4 may be interacting with PilH. The interaction between JBD30-4 and PilH was maintained when the T18 and T25 tags on the proteins were swapped, while the location of the tags remained the same. *In vitro* pull-down assays with FLAG-tagged PilH failed to recapitulate the interaction. Proteolytic cleavage of JBD30-4 at the N-terminus during purification was observed, and a soluble truncation of JBD30-4 was generated. JBD30-4Δ1-16 which did not inhibit twitching or swimming motility in PAO1. Due to the observed loss of function, the N-terminal region of JBD30-4 could be necessary for the activity of the phage moron *in vivo* and responsible for the protein-protein interaction.
between JBD30-4 and PilH. However, it is also possible that JBD30-4Δ1-16 is also misfolding in a manner which renders it unable to interact with PilH. Whether this truncated form of JBD30-4 still interacts with PilH, PilE, or PilM in the BACTH system was not tested in this study.

4.2 JBD30-4 may regulate motility through an interaction with PilH

Though the decrease in twitching motility and sensitivity to phage infection observed upon JBD30-4 expression is similar to the phenotype of pilH mutants, hyperpiliation that is reported in pilH mutant strains of P. aeruginosa was not observed in this study (Bertrand et al., 2010; Fulcher et al., 2010). Instead, decreased levels of surface piliation were observed. PilH is not required for the activity of the retraction ATPase PilT (Bertrand et al., 2010), so T4P retraction would still be taking place even if JBD30-4 were inhibiting the activity of PilH. PilH is proposed to act as a phosphate sink upon phosphorylation by ChpA to limit phosphorylation of PilG. PilG activates CyaB to regulate cAMP levels and mediates the activity of PilB (Fulcher et al., 2010; Silversmith et al., 2016). Since cAMP levels are significantly elevated in pilH mutants (Fulcher et al., 2010), one way to further validate whether PilH is indeed the target of the activity of JBD30-4, is to quantify the level of intracellular cAMP upon JBD30-4 expression in PAO1 and compare it to a pilH mutant.

pilU mutants also exhibit sensitivity to phage infection and loss of twitching motility, and hyperpiliation was observed by electron microscopy (Whitchurch & Mattick 1991). However, when Bertrand et al., analyzed components of the Pil-Chp chemosensory system, they still observed sensitivity to phage infection and loss of twitching motility, but found that surface piliation in a shearing assay was equal to that of wild type PAO1 (Bertrand et al., 2010). The BACTH system was used to test for an interaction between JBD30-4 and PilU, but under the conditions tested, an interaction between the two was not observed. PilH was previously hypothesized to regulate the activity of PilU as well (Whitchurch et al., 2004). PilU may modulate the retractile activity of PilT, potentially by increasing the retraction force of PilT under high friction environments, rather than acting as an independent motor (Leighton et al., 2015; Tala et al., 2019). PilU is localized to only one pole of the bacterial cell during motility, while PilB and PilT are present at both poles (Chaing et al., 2005), so PilU may also function to differentiate between the leading and lagging pole during T4P-mediated movement. In pilU
mutants, the loss of motility may not be from loss of retraction, but the simultaneous extension and retraction from both poles of the cell (Leighton et al., 2015). If the interaction between JBD30-4 and PilH leads to decreased activity of PilU, but not PilT, this could lead to loss of twitching motility through bipolar T4P localization, and because T4P are still retracting, it could explain why hyperpiliation is not observed in PAO1 expressing JBD30-4.

4.3 The role of JBD30-4 in phage-mediated regulation of motility

The presence of JBD30-4 in a subset of phages within related families implies that it is not essential for the life cycle of the phage but may provide an evolutionary advantage under certain conditions (Tsao et al., 2018). In this study, the effect of JBD30-4 on motility was explored, but the initial study by Tsao et al., also observed decreased rhamnolipid production in PAO1 (Tsao et al., 2018). Rhamnolipid production, along with swimming and twitching motility, is required for effective swarming motility in _P. aeruginosa_ (Caiazza et al., 2005), so it would be expected that expression of JBD30-4 would also lead to loss of swarming motility. Non-motile _P. aeruginosa_ isolates from CF patients are frequently isolated (Mahenthiralingam et al., 1994; Gellatly et al., 2013), and more _P. aeruginosa_ isolates from chronic catheter urinary tract infections exhibit decreased motility (80% of strains) than from acute infections (20% of strains) (Tielen et al., 2011). Though environmental isolates are not typically reported as nonmotile (Mahenthiralingam et al., 1994), one group has identified decreased swimming and swarming motility in environmental isolates of _P. aeruginosa_ (Grosso-Becerra et al., 2014). Nonmotility may provide bacteria with a survival advantage in the context of chronic infections, allowing _P. aeruginosa_ to evade eukaryotic host defenses, maintain infection, and conserve energy (Luzar et al., 1985; Mahenthiralingam et al., 1994; Lau et al., 2005). FliC is a potent activator of Toll-like receptor 5, and stimulates the induction of proinflammatory cytokines, leading to bacterial clearance from the immune system (Hajam et al., 2017). Defects in swimming motility from loss of surface flagellin could promote survival of bacteria and protecting the prophage within it. Phage-mediated loss of motility and conservation of energy may also allow the phage to redirect metabolic resources towards virus production. JBD30-4 is flanked by a phage repressor and transposase in phage genomes, which are expressed in early during infection, suggesting that JBD30-4 may be expressed early during phage infection. During early infection, JBD30-4 could function to stall assembly of the flagellum and T4P to divert host resources towards phage
production. This could also keep bacteria adhered to a surface and prevent movement away from a community to ensure the propagation of nascent phage particles.

JBD30-4 homologues were identified in *P. aeruginosa* bacterial strains and phages, and in *P. oleovorans* (which has also been referred to as *P. pseudocaldigenes*). In *P. oleovorans*, the homologue of JBD30-4 is likely in the context of a prophage, as it is downstream of a phage repressor and upstream of a transposase, like that of the *Pseudomonas* phages in which JBD30-4 homologues are found. The presence of a JBD30-4 homologue in another *Pseudomonas* species demonstrates that this phage moron can undergo horizontal gene transfer. *P. oleovorans* also possesses homologues of components of the T4P and the Pil-Chp systems and can be isolated from clinical samples (Gautam *et al.*, 2015). JBD30-4 was found in both clinical and environmental isolates of *P. aeruginosa*, suggesting that expression of this phage moron may provide a fitness advantage to bacteria in various environments.

JBD30-4 was always found upstream of Tip homologues that were either the same length as full-length Tip (136 amino acids), or a truncated form of Tip lacking the extended N-terminal region (83 amino acids). Homologues of Tip from phages JBD5 and MP22 that are 69 amino acids in length still provide resistance to phage infection (Chung *et al.*, 2014), but JBD30-4 is only found upstream of Tip homologues that are equal to or greater than 83 amino acids in length. As some Tip and JBD30-4 homologues are found together in phages, JBD30-4 could play a role in regulating the activity of Tip. JBD30-4 is found in nearly 150 different *P. aeruginosa* strains; looking at the genomic contexts in which JBD30-4 is found in bacterial genomes, such as whether JBD30-4 is always found within a prophage and with Tip homologues, would provide further insight into the function of this phage moron. However, the presence of a JBD30-4 homologue in *P. oleovorans* without an adjacent Tip-like protein suggests that JBD30-4 does have its own function independent of Tip.

### 4.4 Validating interaction between JBD30-4 and PilH with pull-downs

Though the interaction between PilH and JBD30-4 was not validated through *in vitro* pull-downs under the conditions tested, this may have been due to the accumulation of JBD30-4 in inclusion bodies and decreased or lost functionality of PilH with a C-terminal FLAG-tag *in vivo*. It has been previously reported that PilH with a C-terminal His6-tag exhibits slightly reduced
functionality compared to untagged PilH in PAO1 (Bertrand et al., 2010). Given that a His6-tag affects functionality, the negatively charged nature of the FLAG-tag could have a deleterious effect on the function of PilH (Johnson et al., 2002), reducing functionality to the point that its activity or interaction with JBD30-4 is lost. In the BACTH system, PilH was tested with C-terminal tags, but the neutral charge of the T18 and T25 fragments may not have interfered with the function of PilH in the assay. Commercially available epitope tags, such as His6 and FLAG are highly charged, which can have an adverse effect on protein structure and function (Kim et al., 2016). Lost functionality due to the placement epitope tags could have caused other hits to be missed (false negatives) when screening with the BACTH system for interacting partners of JBD30-4 as well, by affecting protein stability or function. Expression tests can be performed with T18- and T25-tagged proteins to ensure the proteins are being stably expressed in the BACTH system. As the BACTH system is a two-hybrid system, any interactions with JBD30-4 that require a third protein would not be detected through this assay.

Given that tags may interfere with the functionality of PilH, in vitro pull-down assays in E. coli should use untagged PilH and tagged JBD30-4, as His6-tagged JBD30-4 does not exhibit a loss in functionality in vivo. Additionally, if the interaction between JBD30-4 and PilH is transient, it would be detected in the BACTH assay, but may not be apparent in in vitro pull-downs in E. coli. To overcome this, formaldehyde cross-linking could be used for stabilization of transient interactions. To avoid false-positives from co-overexpression of two recombinant proteins and two-hybrid systems, tagged JBD30-4 expressed in P. aeruginosa could be used in in vivo pull-downs. Instead of using an epitope tag to detect the presence of PilH, mass spectrometry or an antibody against PilH could be used instead to detect the interaction between the two proteins when JBD30-4 is expressed in P. aeruginosa. Since PilH may depend on phosphorylation by ChpA for its activity in vivo (Silversmith et al., 2016), pull-downs in E. coli may not reveal an interaction, as PilH would not be in its phosphorylated state, but pull-down assays in P. aeruginosa could overcome this issue.

4.5 Additional interacting partners for JBD30-4

In this study, the potential for PilH to serve as an interacting partner for JBD30-4 was explored. In the initial BACTH screen, co-expression of PilE or PilM with JBD30-4 exhibited a slight colour change on X-gal-containing media. While both X-gal and MacConkey indicator media
provide readouts of adenylate cyclase activity, LB-X-gal plates are more sensitive and susceptible to formation of pale blue colonies upon weak interaction of the adenylate cyclase domains due to the basal level of β-galactosidase activity in *E. coli* cells in the absence of cAMP. The residual β-galactosidase activity is insufficient to ferment maltose, as is required to observe a colour change on MacConkey agar (Karimova *et al.*, 2000). Thus, presence of a colour change on MacConkey agar upon coexpression of JBD30-4 and PilH with the BACTH system is likely indicative of a stable interaction, while the colour change on X-gal media upon coexpression of JBD30-4 and PilE or PilM may be indicative of a weak or nonspecific interaction. However, it is still possible that PilE and PilM could also be targets of JBD30-4. PilM is hypothesized to bind to the short, conserved N-terminal INLLPW motif (residues 4-9) of PilN (Sampaleanu *et al.*, 2009). JBD30-4 does not contain this motif, but the N-terminal regions of PilN and JBD30-4 share some similarities, with JBD30-4 possessing the sequence WVREEL (residues 15-20) while PilN contains the sequence WREEL (residues 9-13). It is possible that PilM recognized the WVREEL sequence in the disordered N-terminus of JBD30-4 and that led to the detection of an interaction in the BACTH system. Loss of individual components of the alignment complex, including PilM, leads to loss of surface piliation and twitching motility, except in retraction-deficient *P. aeruginosa*, indicating that there is some T4P assembly in the absence of the alignment complex (Ayers *et al.*, 2009; Takhar *et al.*, 2013; Leighton *et al.*, 2015). Thus, if JBD30-4 was interacting with and inhibiting the activity of PilM, surface piliation may still be taking place, which could explain the decreased levels of pilin on the cell surface upon expression of JBD30-4. Further analysis of the potential interaction between JBD30-4 and PilM is necessary.

PilE was also found to potentially interact with JBD30-4. Given that during extension of T4P, PilE is incorporated into the pilus and would be localized extracellularly, and that *pilE* mutants exhibit no surface piliation and resistance to phage infection (Nguyen *et al.*, 2015b), it is unlikely that PilE is the target of activity of JBD30-4, a cytoplasmic protein. Furthermore, when performing BACTH screens in our lab, we found PilE to be a frequently occurring hit, suggesting it may be a false positive, as two-hybrid system are prone to having (Legrain & Selig, 2000).
4.6 Further biochemical characterization of JBD30-4

During the initial expression and purification of JBD30-4 in *E. coli* cells, much of the protein was present in the insoluble fraction, with a minimal amount of protein present in the elution fraction. Overproduction of recombinant proteins in *E. coli* can result in the accumulation of the protein of interest in inclusion bodies as insoluble aggregates (Sørensen & Mortensen, 2004). A denaturing protein purification was performed to isolate JBD30-4 from inclusion bodies in *E. coli* did not yield soluble protein upon removal of the denaturing agent. Though additional denaturing preparation protocols could have been used to purify full length JBD30-4 from inclusion bodies, optimization of the refolding conditions for the protein are time-consuming and may not yield properly folded proteins (Sørensen & Mortensen, 2005). Future biochemical characterization of full-length JBD30-4 may be improved through purification in *P. aeruginosa* strains, as that is where JBD30-4 is naturally found and expressed. Cleavable tags that are designed to improve protein solubility, such as maltose-binding protein, N-utilization substance, and thioredoxin (LaVallie *et al.*, 1993; Kapust & Waugh, 1999; Davis *et al.*, 1999) could also be tested. Additionally, homologues of JBD30-4 from other *P. aeruginosa* strains or phages may exhibit greater solubility *in vitro*, particularly those with amino acid substitutions to charged residues (Chan *et al.*, 2013). Purification of JBD30-4-His$_6$ demonstrated that JBD30-4 was being proteolytically cleaved from the N-terminus, but the exact change in molecular weight could not be determined due to the impurity of the input sample during size exclusion chromatography. To accurately determine the molecular weight difference between full-length and proteolytically cleaved JBD30-4, a cleaner protein sample with a minimal amount of contaminating protein would need to be analyzed through size exclusion chromatography with multi-angle static light scattering. However, removal of the first 16 amino acids from the N-terminus of JBD30-4 did yield more soluble protein during purification. Though this N-terminally truncated form of JBD30-4 was nonfunctional *in vivo*, obtaining structural information on JBD30-4$^{Δ1-16}$ could be beneficial as it may uncover structural folds or identify surface-exposed residues to be targeted for mutagenesis.
Chapter 5

Future Directions

5.1 Homologues of PilH as interacting partners of JBD30-4

There are several paralogues of PilH in PAO1 that have 35% or more amino acid sequence similarity, with most of the similarity in the N-terminal region (Figure 22). To determine whether the interaction between JBD30-4 and PilH is specific, or if JBD30-4 is recognizing some motif in PilH that is similar to another protein, interactions with these different paralogues of PilH could be tested, especially because some of these proteins also play roles in controlling motility and virulence in *P. aeruginosa*.

![Figure 22: Partial amino acid sequence alignment of PilH homologues in P. aeruginosa.](image)

PilH has several paralogues in *P. aeruginosa* that share regions of sequence similarity that could account for an interaction observed between JBD30-4 and PilH in the BACTH system. Alignment shown with the clustal colour scheme and generated with Jalview (Waterhouse et al., 2009).

PilG, as described earlier, is involved in regulating the activity of PilB and controls cAMP levels (Bertrand et al., 2010; Fulcher et al., 2010). HsbR is a response regulator in the HtpB pathway that regulates twitching and swimming motility, biofilm formation, and chemotaxis (Valentini et al., 2016). ColR is part of the ColRS two-component regulatory system (Kivistik et al., 2006). When mutated in *Pseudomonas putida* it leads to downregulation of 22 motility proteins and induces the alginate regulator AmrZ, which is known to repress the activity of FleQ, the master regulator of flagellar motility (Tart et al., 2006; Mumm et al., 2016). AmrZ also regulates twitching motility and T4P biogenesis (Baynham et al., 2006). PhoB is a transcriptional regulator that, when phosphorylated, activates the Pho regulon, which regulates motility and rhamnolipid production, and under phosphate-depleted conditions, controls swarming motility (Blus-Kadosh et al., 2013; Peng et al., 2017). Given the number of paralogues of PilH that also affect T4P and
flagellar function in *P. aeruginosa*, it would be interesting to explore whether JBD30-4 also interacts with paralogues of PilH.

5.2 Effect of JBD30-4 expression on swimming motility

One aspect that remains unexplored in this study is the observation that expression of JBD30-4 in PAO1 leads to loss of swimming motility through a decrease in surface flagellin. It remains to be determined whether this is due to fewer cells in a mixed population having a flagellum, shorter flagella per cell, or no assembly of surface flagella but observation of flagellin in the shearing assay due to cell lysis. To determine whether cell lysis is taking place during the surface shearing assay, an antibody against an inner membrane protein, such as PilO, can be used (Nguyen et al., 2015b). Electron microscopy may be used to determine exactly what the status of surface flagellation is upon expression of JBD30-4 in PAO1. Though a direct interaction with PilH has not been confirmed, if JBD30-4 is indeed interacting with PilH, this could lead to a decrease in flagellar motility through the regulation of cAMP levels mediated by PilH. PilH is known to regulate the activity of the primary adenylate cyclase in *P. aeruginosa*, CyaB; *pilH* mutants have significantly increased levels of intracellular cAMP. cAMP is an allosteric activator of Vfr which regulates the expression of over 200 genes involved in global regulation of many *P. aeruginosa* virulence systems, including the type III secretion system, quorum sensing, exotoxin production, flagellar biosynthesis, and regulatory factors for T4P biogenesis (Wolfgang et al., 2003). Vfr overexpression specifically regulates flagellar biosynthesis by binding to the promoter of FleQ, the master regulator of flagellar biosynthesis, to repress flagellar gene expression (Dasgupta et al., 2002). To determine whether expression of JBD30-4 leads to a loss of swimming motility through this mechanism, an anti-Vfr antibody could be used to determine relative levels of Vfr in cells (Berry et al., 2018), and reverse transcription (RT) quantitative polymerase chain reaction (qPCR) (RT-qPCR) could be used to measure *fleQ* transcript production upon JBD30-4 expression in PAO1. However, Vfr does control many facets of *P. aeruginosa* physiology, so this is only one possible mechanism by which JBD30-4 could regulate swimming motility if there is an interaction with PilH.
5.3 Effect of JBD30-4 on intracellular c-di-GMP levels and motility

In this study a potential link between JBD30-4 and the second messenger cAMP was described, but c-di-GMP is another second messenger that plays an important role in regulating motility in *P. aeruginosa*. C-di-GMP is thought to trigger the switch from the planktonic, motile *P. aeruginosa* phenotype observed in isolates from acute infections, to the biofilm-forming, sessile lifestyle during chronic infection (Cole & Lee, 2016). Low c-di-GMP levels are associated decreased biofilm formation and increased motility (Cole & Lee, 2016). When c-di-GMP levels are high, *P. aeruginosa* exhibits decreased motility, as c-di-GMP binds FimX to repress twitching motility (Kazmierczak et al., 2006). FleQ, in the absence of c-di-GMP, positively regulates flagellar gene expression and represses expression of genes from the *psl* and *pel* exopolysaccharide operons, needed for biofilm formation. High c-di-GMP levels relieves the transcriptional repression by FleQ of exopolysaccharide genes promoting biofilm formation in *P. aeruginosa* (Hickman & Harwood, 2008; Cole & Lee, 2016). Since the loss of motility phenotypes caused by expression of JBD30-4 in PAO1 are similar to those observed during chronic *P. aeruginosa* infections, and c-di-GMP levels influence these phenotypes, future experiments could explore whether expression of JBD30-4 has an impact on c-di-GMP levels.

The initial systematic investigation performed by Tsao et al. (2018) did not identify an effect of JBD30-4 expression on biofilm formation under the conditions tested: a microtiter plate assay at 0.1% arabinose induction. If c-di-GMP levels are significantly altered upon JBD30-4 expression, alternative methods of assaying biofilm formation may need to be used, such as the use of flow chambers.

5.4 Additional approaches to determine the mechanism of action of JBD30-4 in *P. aeruginosa*

Given that JBD30-4 shuts down two key forms of motility in *P. aeruginosa*, it is possible that JBD30-4 interacts with some master transcriptional regulator that is linked to twitching and swimming motility in *P. aeruginosa*. In this study, the search for a possible interacting partner was biased to certain components of T4P assembly and function. An unbiased search could be performed in vivo to ensure that no hits are missed. As expression of JBD30-4 with a His6-tag in vivo does not affect functionality, in vivo pull-downs in *P. aeruginosa* strains with epitope-tagged JBD30-4 could be used to identify potential interacting partners for this phage moron.
This approach would also allow for identification of interacting partners under various conditions, such as nutrient-rich versus minimal media or plate growth versus liquid growth, to determine if there are different targets of the activity of JBD30-4 in different environmental contexts.

Since JBD30-4 is a phage protein, it is important to consider that when JBD30-4 is expressed from a lysogen, it is unlikely to be acting alone in regulating the motility of *P. aeruginosa*. In fact, Tsao *et al.*, showed with RT-qPCR that in the PA14(JBD26) lysogen, the homologue of JBD30-4 (JBD26-4) was expressed, but at lower levels than the repressor, while the phage moron JBD26-61, which prevents T4P assembly, was expressed 12-fold greater than the repressor, and another uncharacterized phage moron was expressed two-fold greater than the repressor (Tsao *et al.*, 2018). The difference in expression levels between the different morons in the same phage demonstrates that when expressed from the prophage, JBD30-4 may be working in concert with or dependent on the activity of other phage proteins and regulatory proteins in the cell. Thus, it is important to determine the activity of JBD30-4 in the context of a lysogen. RT-qPCR can be performed to determine when during phage infection JBD30-4 is most highly expressed, and to what levels. By determining the relative levels of expression of JBD30-4 from the lysogen, the concentration of the protein expression inducing agent can also be adjusted to more closely represent physiologically relevant levels of expression when performing assays in *P. aeruginosa*.

One approach to determining the mechanism of action of JBD30-4 in an unbiased manner would be to perform *in vivo* pull-downs with epitope-tagged JBD30-4 in *P. aeruginosa*. Potential interacting partners can be identified using mass spectrometry. Chemical crosslinking can be used to capture any transient interactions. It is possible that JBD30-4 interacts with multiple proteins, so an unbiased pull-down assay coupled with crosslinking at different timepoints, under different media and growth conditions, and at varying levels of protein expression may enable identification of a broader range of interacting partners for JBD30-4 and a provide a better idea of its mechanism of action. Furthermore, these pull-downs could be performed in the lysogen so that any other proteins from the prophage that JBD30-4 may interact with or are required for the stabilization or activity of JBD30-4 will also be present. This is particularly relevant for JBD30-4 as it is always found in phages upstream of Tip protein homologues, and downstream of an
uncharacterized, highly conserved phage protein, so these proteins could be important in the function of JBD30-4.

6 Conclusion

JBD30-4 is a phage moron that is involved in regulating twitching and swimming motility in *P. aeruginosa*, that could exert its activity through an interaction with the response regulator PilH. PilH links chemotaxis to twitching motility and regulates intracellular cAMP levels. In this study, it is demonstrated that swimming motility is lost through a decrease in surface flagellation, and T4P are still present on the cell surface despite the loss of motility. The importance of the N-terminal region of JBD30-4, which gets cleaved during protein purification in *E. coli*, is demonstrated through the inability of truncated JBD30-4 to shut down twitching and swimming motility. The loss in motility mediated by the phage moron may have implications for *P. aeruginosa* in the context of chronic infections, allowing for persistent infection through bacterial evasion from the host immune system, thus providing cells expressing JBD30-4 with a fitness advantage in certain contexts. JBD30-4 is carried with another phage moron, the Tip protein, in phage genomes, and these two morons may work in concert to elicit specific effects on bacterial physiology during phage infection. Understanding the role that JBD30-4 plays in regulating key motility processes in *P. aeruginosa* will provide insight into the mechanisms of phage regulation of bacterial physiology.
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