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Genetic Analysis of the Chp Chemosensory System of Pseudomonas aeruginosa

by

Jacob Joseph Bertrand

DISSERTATION

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Dedication and Acknowledgments

For my family who offered their love and support, especially for my parents,
Dr. Ralph L. and Mrs. Dianne M. Bertrand, to whom I owe so much, and for my husband
Dr. C. Dale Young, my constant companion

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Abstract

Virulence of the opportunistic pathogen *Pseudomonas aeruginosa* involves the coordinate expression of a wide range of virulence factors including type IV pili, which are required for colonization of host tissues and for a type of flagella-independent surface translocation termed twitching motility. Twitching motility is controlled by a number of factors including a putative chemosensory system referred to as the Chp system. We conducted comprehensive genetic analysis of the function of Chp system genes by making in-frame deletions of all of the genes in the Chp cluster and assaying them for type IV pilus functions, including twitching motility, phage sensitivity, and pilin assembly. The majority of work in the current study focused on the core signaling components of the Chp system, which include a putative histidine kinase, ChpA, and two CheY-like response regulators, PilG and PilH. It remains unclear how components of the Chp system interface with the type IV pili motor proteins PilB, PilT, and PilU. Here we present genetic evidence confirming the role of ChpA, PilG, and PilB in the regulation of pilus extension and that of PilH and PilT in regulating pilus retraction. In addition, we present evidence that the diguanylate phosphodiesterase, FimX, is involved in regulating pilus extension, consistent with recent reports showing interactions with PilB. We demonstrate that ChpA, PilG, and PilB function upstream of PilH, PilT and PilU, that PilH functions upstream of PilT, and that PilT and PilB retain some activity in the absence of signaling input from components of the Chp system. We present evidence to suggest that despite its homology to PilT, PilU does not appear to play a role in the regulation of pilus retraction, and we show that it continues to localize to the cell pole in Chp system mutant backgrounds. We show that the histidine kinase domain of ChpA is

required for function as are the phospho-acceptor sites of both PilG and PilH. These findings suggest that ChpA–mediated control through PilG and PilH likely occurs through phosphorylation, and that this is important in the regulation of pilus extension and retraction. We present evidence suggesting that *pilA* transcription is regulated by intracellular PilA levels. We show that PilA is a negative regulator of *pilA* transcription in *P. aeruginosa* and that the Chp system functionally regulates *pilA* transcription by controlling PilA import and export. Finally we demonstrate that both ChpA and PilH play a role in the regulation of virulence factor production. ChpA regulates production of the type III secreted virulence factor, ExoT, and PilH regulates the production of a number of quorum sensing-regulated secreted virulence factors including pyocyanin and rhamnolipid.

Contents

List of Figures

List of Tables

Chapter 1

Type IV Pili and Twitching Motility

Introduction

Type IV pili (Tfp) are polarly localized filamentous appendages synthesized by a wide range of both pathogenic and environmental Gram-negative bacteria belonging to the β, γ, and δ subdivisions of the Proteobacteria (66). Tfp have a number of important biological functions. They act as adhesins to both biotic and abiotic surface, they serve as receptors for certain bacteriophages (11), they are required for DNA uptake in some naturally competent bacterial species, and they allow for a type of surface translocation termed twitching motility (TM, 66). Due both to their adhesive properties as well to the role the play in TM, Tfp are implicated in the colonization of both biotic and abiotic surfaces and have been shown to be involved in complex social behaviors such as fruiting body development in *Myxococcus xanthus* (47, 49, 114) and *P. aeruginosa* biofilm formation (5, 14, 54, 55, 81).

TM was originally described in *Acinetobacter calcoaceticus* (60). The name is derived from the observation that, when viewed in suspension, cells undergoing this type of motility appeared to move in a jerky or twitchy fashion. TM is distinguished from other forms of bacterial translocation, such as swimming and swarming, by the fact that it is flagella-independent. Instead TM is mediated by the coordinated extension, tethering, and retraction of Tfp (72, 88). TM has been most thoroughly characterized in *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and

Myxococcus xanthus, where it is also referred to as "social gliding motility". Fruiting body formation in *M. xanthus*, which requires Tfp, has served as a model for multicellular bacterial behavior and development (114). *N. gonorrhoeae*/*N. meningitidis* are important obligate human pathogens whose virulence is dependent upon Tfp (66).

P. aeruginosa has served as one of the principle models for the genetic and functional characterization of Tfp function, including TM, not only because it is easily cultured and genetically tractable, but also because it has been shown to infect a large number of diverse eukaryotes including amoebae, nematodes, insects, plants, mice and humans (17, 20, 44, 95). As an opportunistic pathogen, *P. aeruginosa* is the causative agent of both acute and chronic human infections ranging from minor skin infections to persistent, and often life-threatening, disease in hospitalized or immunocompromised patients such as those suffering from AIDS, extensive burns, who are undergoing chemotherapy, or who are recovering from major surgery (64). *P. aeruginosa* also chronically infects the cystic fibrosis lung and is the primary cause of respiratory morbidity and mortality in patients with this inherited disease (64). *P. aeruginosa* virulence involves the coordinate expression of a wide range of secreted and cell-associated virulence factors, including adhesins, exopolysaccharides, proteases, lipases, phospholipases, siderophores, biosurfactants, and exotoxins (29). Key among these virulence factors are the Tfp, which constitute the major adhesin of *P. aeruginosa* and have been shown to play a role in adherence to epithelial cells in culture and in virulence in several animal models of infection (29). Functional Tfp have also been shown to be involved in the formation of *P. aeruginosa* biofilms, which have been implicated in chronic infections (18).

Pilin Structure

Tfp are made up of a single proteinaceous subunit termed pilin and encoded by *pilA* .Tfp range from 5-7 nm is diameter and can be up to several μm in length. Pilins range from 145-160 amino acids in length and have a characteristic primary structure including a positively charged leader sequence and a highly conserved hydrophobic N-terminal domain, which makes up the core of the pilus (66). The leader sequence is cleaved and the N-terminal residue methylated by the prepilin peptidase PilD (62, 93). PilD is also involved in the cleavage of prepilin-like leader sequences of type II secreted proteins. A number of studies have shown that an invariant glycine residue at the -1 position is required for PilD-mediated cleavage and that a glutamate residue at the +5 position is required for pilus assembly and N-terminal methylation (58, 63, 91, 92). Other semiconserved regions include residues 30-55/56 and a C-terminal region containing a disulfide bridge that may be involved in receptor recognition (21, 34, 36, 96). The central and C-terminal two-thirds of pilin are relatively hydrophilic and contain the most prominent sites of structural and antigenic variation (96). Several species of bacteria have active mechanisms for varying the sequences of their pilins, presumably to evade host immune systems, pilus-specific bacteriophages, or to increase the repetoire of binding. In the case of *N. gonorrhoeae* and *N. meningitidis* this is accomplished by recombinational exchange of pilin sequences from silent loci into the expression locus (30, 70, 85).

The three-dimensional crystal structures of *N. gonorrhoeae* MS11 pilin and truncated versions of the pilins from *P. aeruginosa* strains PAK and K122-4 have been solved (36, 53, 82). The results of these studies point to a conserved general structure for

pilin which include: an asymmetrical α-β roll fold made up of an 85 Å α-helical spine formed by the hydrophobic N-terminal region, a 24 amino acid "sugar loop", a four-stranded anti-parallel β-sheet, and a C-terminal β-sheet and extended loop stabilized by a disulfide bridge. As mentioned above, in the pilus fiber, pilins are arranged in a helical structure with 5 subunits per turn, a 41 Å pitch, and an outer diameter of 60 Å. The hydrophobic core of the fiber is a parallel, overlapping, coiled-coil made up of the N-terminal α-helices. The outside of the fiber is a scaffold of β-sheets packed against the core. The outermost layer, which is not critical pilus structure is composed of a hypervariable region which is covalently linked to saccharide and a C-terminal tail. The similarities of the overall structures of pilins as well as the machineries required for their assembly/disassembly is underscored by the ability of a number of species to express functional heterologous pili (67, 101). Interestingly, however, when two types of pili are co-expressed in cells, two types of pili composed uniquely of one pilin or the other are produced, indicating some specificity in the interactions of homologous pilins (27).

Pilins can be modified post-translationally either by phosphorylation ofr glycosylation (66). *N. gonorrhoeae* pilin is phosphorylated on serine 68 though the modification does not appear to effect function. Likewise, *N. meningitidis* pilin is modified by an α-glycerophosphate likeage to serine 93/94, though, again, the effect of the modification is unclear. The pilins of *N. gonorrhoeae*, *N. meningitidis*, and some *P. aeruginosa* strains, though none of the well-characterized laboratory stains such as PAO1 or PAK, have also been shown to be glycosylated. In each instance the effect of the modification on Tfp function in unclear, although, at least in the case of *P. aeruginosa* strain 1244, glycosylation might confer some advantage during an infection (89).

Adhesion and Target Specificity of Type IV Pili

Tfp can bind to both biotic and abiotic surfaces. The ability of Tfp to bind to eukaryotic cells allows for not only colonization of host tissues but also for intimate contact with the host cells mediated by retraction (71, 94). Both of these activities are believed to be important virulence determinants. Binding of Tfp to abiotic surfaces appears to be nonspecific, although the interaction always occurs via an end of the pilus (88, 94). In the case of binding to eukaryotic cells, in general, and mammalian cells, in particular, pili appear to attach to specific receptors. For *P. aeruginosa* pili bind to the carbohydrate moiety of glycosphingolipids asialo-GM1 and asialo-GM2 on epithelial cells (34, 36). This interaction is mediated by a C-terminal disulfide-bonded region of 12-17 amino acids of *P. aeruginosa* pilin. This region is partly conserved in other Tfp expressing bacteria including *N. gonorrhoeae* and *N. meningitidis* (21, 36, 96). Binding of *N. gonorrhoeae* and *N. meningitidis* to epithelial and endothelial cells requires the bacterial PilC protein, which appears to act as a tip adhesin, although it is also found in the bacterial membrane (84, 107). *N. gonorrhoeae* encodes two variant copies of PilC (PilC1 and PilC2). Expression of at least one of these genes is required for the production of extracellular pili. It has recently been shown that expression of *pilC1* is negatively regulated by the pilus retraction machinery (112). *P. aeruginosa* encodes a *pilC* homologue, *pilY1*, which is found in the cell membrane and associated with extracellular pili and has been shown to be required for Tfp function (3). The exact role of PilY1 in regulating Tfp function remains to be elucidated.

The Physics of Twitching Motility

At the macroscopic level both *M. xanthus* and *P. aeruginosa* form flat spreading colonies with a hallmark rough edge comprised of a zone of cells twitching away from the colony (68, 87, 98). Mutants defective for TM form domes colonies with smooth edges. The twitching zones of *N. gonorrhoeae* and *N. meningitidis* can only be discerned microscopically (107). *P. aeruginosa* also exhibits TM at the interstitial surface between nutrient agar and plastic or glass (2, 22). The twitching zone radiates from the point of inoculation at a rate of approximately 1 mm/hr with overnight zones reaching a diameter of 2-3 cm under normal growth conditions (i.e., growth on rich media at 37 ˚C). These zones can be stained and offer a good semi-quantitative measure of TM activity.

At the microscopic level, TM can function both to aggregate and to disperse cells. In the first case, TM can bring cells together into complex structures such as fruiting bodies or biofilms. In the second case, TM can allow for expeditionary movement away from a colony over new surfaces. For both *M. xanthus* and *P. aeruginosa* either type of activity appears to be a communal process involving cell-cell contacts. Isolated cells of both species rarely move by TM unless they are within several micrometers of another cell corresponding to the length of a Tfp (72, 87, 98). Time-lapse video microscopy experiments have shown characteristic morphologies for *P. aeruginosa* and *M. xanthus* cells migrating via TM away from the center of a colony (66, 114). Spearhead-like aggregates of cells (typically 10-50 cells/aggregate in the case of *P. aeruginosa*) move radially outward from the center of the colony. Behind these aggregates, groups of cells stretch out and move into different directions and cross-connect with other groups, creating a lattice-like network of cells. Cells in the lattice appear to undergo a period of

dynamism, moving between groups and frequently reversing direction before finally settling into a more sedentary microcolony-like structure.

Type IV Pili and TM in Biofilm Formation

Early evidence for a role for Tfp in *P. aeruginosa* biofilm formation was provided by O'Toole and Kolter (81). Using time-lapse video microscopy, they showed that mutants defective in the synthesis of Tfp formed monolayers on a polyvinylchloride surface but failed to aggregate into microcolonies. They demonstrated further that flagella mutants failed to bind the surface altogether. These data suggested that initial binding to the substratum was not dependent upon Tfp but that subsequent aggregation into microcolonies was Tfp-dependent, leading to the hypothesis that TM was involved in the aggregation into microcolonies. In a series of elegant studies using fluorescent WT or strains defective for the production of Tfp or flagella in conjunction with confocal scanning laser microscopy, Klausen and colleagues demonstrated that initial attachment to the substratum does not absolutely require flagella or Tfp and that microcolony formation occurs by clonal growth (54, 55). Under growth favoring the formation of dynamic flat biofilms migration away from the microcolony to cover the surface is dependent upon functional Tfp (55). Under growth conditions favoring the formation of mushroom shaped mature biofilms, functional Tfp are required for colonization of the microcolony stalk by a motile cap population of cells (54). The same group went on to demonstrate that the motile subpopulation in these biofilms is more resistant to treatment with abtibiotics such as colistin and SDS (33). The mechanism for the resistance to colistin appears to be induction of 4-amino-4-deoxyarabinose biosynthesis in the motile

subpopulation; the binding of this compound to lipid A prevents colistin binding thereby conferring resistance (33). The regulatory circuitry underlying induction of 4-amino-4-deoxyarabinose biosynthesis specifically in the motile subpopulation is unknown. Consistent with the idea that Tfp function is important for biofilm development, mutation of components of the Chp chemosensory system (see below) results in biofilms with altered morphologies (chapter 3, 5).

Mechanics of Type IV Pilus Function

More that 40 genes involved in the biogenesis and function of Tfp have been identified in *P. aeruginosa*. Those involved in biogenesis and mechanical function include: *pilA*, *B, C, D, E, F, M, N, O, P, Q, T, U, V, W, X, Y1, Y2, Z, fimT, U, V,* and *X* (66). The mechanical aspect of Tfp function is tied to extension and retraction of Tfp. Extension and retraction of Tfp is regulated by two ATPases. The Tfp ATPases are members of the AAA+ family of motor proteins and function as ring-shaped oligomers (43, 48). *pilB* encodes the extension ATPase and *pilT* encodes the retraction ATPase. The role of a third ATPase in *P. aeruginosa*, encoded by *pilU*, remains unclear (16). A high degree of similarity with *pilT* (106) has led to the hypothesis that *pilU* may play some role in regulating pilus retraction. We present evidence in this study to suggest that while *pilU* is crucial to Tfp function, it does not appear to play a role in regulating retraction (see below and chapter 2). *pilU* homologues are also present in *Pseudomonas stutzeri* and *N. gonorrhoeae*. Mutations in *pilU* in these strain backgrounds lead to loss of visible TM and increased levels of surface pili, suggesting that PilU may play some

role in the regulation of pilus retraction, although this remains to be demonstrated explicitly (31, 108).

In addition to the extension ATPase, PilB, assembly, or extension of Tfp requires an a polytopic inner membrane protein, PilC (called PilF in *N. gonorrhoeae*), the prepilin peptidase, PilD, and a multimeric outer membrane protein, PilQ (1, 37, 59, 62, 65, 79, 83, 93). PilQ is a member of a family of proteins that form pores in the outer membrane through which the pili are believed to extrude (7, 53). PilQ forms a dodecameric doughnut-shaped complex. The diameter of the internal cavity closely matches that of the pilus. PilQ homologues are also involved in the extrusion of filamentous phage and in type II protein secretion.

pilQ is located within an operon encoding four other genes involved in Tfp function (*pilMNOPQ*) in *P. aeruginosa*, *M. xanthus*, *N. gonorrhoeae*m and other type IV piliated bacteria (66). *pilM* encodes a predicted 39 kDa protein with a signal sequence for localization to the cytoplasmic membrane. Both Walker A and Walker B boxes are encoded by *pilM*, indicating that the protein is likely to be an AAA+ ATPase (76). Domains of PilM also share homology with the *E. coli* rod-shape determining protein MreB and the cell division protein FtsA, which has led to the hypothesis that assembly of Tfp may be linked to cell shape or to the cell cycle (66). In support of this hypothesis are the observations that Tfp function in *P. aeruginosa* requires *fimV*, which encodes a putative peptidoglycan-binding domain (86), and *pilZ* expression of which is transcriptionally coupled to *holB*, which encodes the δ subunit of DNA polymerase III (66). The actual function of PilZ, however, is likely to be in the regulation of pilus assembly since it has been recently shown to bind PilB through FimX (see chapter 7, 32).

pilN and *pilO* encode proteins with putative N-terminal signal sequences, suggesting they are periplasmic proteins. Homologues of *pilN* are also required for type II secretion. PilO is involved in the glycosylation of *P. aeruginosa* strain 1244 pilin (89). *pilP* encodes a protein with a putative lipoprotein signal sequence, suggesting that it may be an outer membrane lipoprotein (76). In *N. gonorrhoeae*, *pilP* mutants fail to assemble PilQ (26). This has not been observed in *M. xanthus* or *P. aeruginosa* (66). However, in *M. xanthus*, PilM, PilN, PilO, PilP, and PilQ are all involved in the transfer of Tgl, presumably by maintaining Tgl in the outer membrane (78). Whether these proteins play a similar function for *P. aeruginosa* PilF remains to be determined.

Tfp function also requires a number of minor pilin-like proteins. In *P. aeruginosa*, the minor pilins are encoded by *pilE, V, W, X, fimT,* and *U* (*pilH, I, J, K,* and *comP* in *N. gonorrhoeae*, *N. meningitidis*), and all contain the signature hydrophobic N-terminal α-helical region (66). It has been hypothesized that these minor pilins may form the base structure for the pilus fiber similar to the pseudopilus structures that appear to be involved in type II protein secretion (66). The minor pilins may also be incorporated into the pilus structure itself. Indeed this appears to be the case for *N. meningitidis*, where incorporation of the minor pilin PilV into the pilus is crucial for colonization of endothelial cells and resistance to shear stresses in the bloodstream (19, 74).

Pilus retraction powers TM. Early evidence for the importance of retraction in Tfp function was provided by Bradley (10), who showed that hyperpiliated mutants of *P. aeruginosa* were not only defective in TM, but were also immune to infection by pilus-specific bacteriophages. Furthermore, electron micrographs showed that, in these hyperpiliated mutants, phages were not located on the cell surface at the junction between

the pili and the cell pole, as in WT, but rather bound at random points along the pili. From these experiments he concluded that hyperpiliation resulted from an inability of these mutants to retract their pili, resulting in increased levels on the cell surface and compromised phage infectivity and TM.

The role of retraction at the driving force behind TM was confirmed by three different studies in each of the major model systems. Using optical tweezers, Merz *et al* (72) measured the retractile force generated by *N. gonorrhoeae* pili on isolated cells positioned near a microcolony or tethered to latex beads positioned near smaller latex beads coated with anti-pilin antibodies. Retraction rates were determined to be approximately 1 μm/sec with up to 90 pN of force generated. Using time-lapse video microscopy of *P. aeruginosa* whose cells and pili had been fluorescently labeled, Skerker and Berg (88) determined that individual pili extend and retract at approximately 0.5 μm/sec. They also demonstrated that extension and retraction of multiple pili did not appear to be coordinately regulated, and showed that cell movement was associated uniquely with pilus retraction. Similar experiments in *M. xanthus* showed that WT cells tethered to a surface appeared to retract towards that surface via one pole, then to lie down parallel to the surface and to be pulled forward by extending, tethering, and retracting pili from the opposite pole (94). These experiments not only demonstrated that importance of pilus retraction in driving TM, but also provided evidence that pilus activity could alternate from one pole to the other, thus providing a mechanism for reversals in movement.

These experiments demonstrate the importance of pilus retraction in TM and also provide an indication of how cells could move in isolation to colonize new surfaces.

What is less clear, however, is how retraction functions to power motility when cells move in groups or aggregate to form fruiting bodies or microcolonies. During such processes, pili must, presumably, recognize and bind to specific receptors on neighboring cells. Pilus retraction would then bring the cells into close proximity allowing them to aggregate or to move as a group. No such receptors have been identified. However, TM in *M.* x*anthus* is dependent upon *tgl*, the product of which can stimulate pilus extension when delivered *in trans* (99). Specifically, *tgl*$^{-}$*pilA*$^{+}$ cells are deficient for TM but can be transiently stimulated to movement in the presence of *tgl*$^{+}$*pilA*$^{-}$ cells. It was though that Tgl, which is an outer membrane protein with a type II (lipoprotein) signal sequence, followed by six tandem degenerate 34-amino acid repeats involved in protein-protein interactions might serve as the Tfp receptor (99). *P. aeuginosa* encodes a similar protein, PilF, which has also be shown to be important for TM (87). However, it has been recently demonstrated that both Tgl and PilF are required for proper assembly of the outer membane secretin, PilQ (57, 78). In addition, at least in the case of Tgl, the protein can be transferred from one cell to another, explaining the temporary stimulation to movement of *tgl*$^{-}$*pilA*$^{+}$ cells in the presence of *tgl*$^{+}$*pilA*$^{-}$ cells (77). Further work will have to be done to identify Tfp receptors.

In *M. xanthus*, directed motion is achieved by regulating the rate of cellular reversals to bias the cell towards movement in a desired direction. This regulation is achieved, in part, by the Frz chemosensory system (see below). A component of this system, FrzS, has been shown to localize specifically to the piliated pole and to oscillate between poles during reversals in cell direction (73). This observation has led to the hypothesis that a subfraction of components involved in the regulation of Tfp function in

P. aeruginosa may be dedicated to determining which is the pilated pole and may switch from pole to pole during cell reversals. To date only three factors known to be involved in the regulation of Tfp function have been shown to have a monopolar localization. These include: the ATPase PilU, the phosphodiesterase FimX (38, 50), and the unique regulatory protein FimL (our unpublished data, 104). Other proteins involved in the regulation of Tfp function examined to date, including PilB and PilT (15), the putative Chp system methyl-accepting chemotaxis protein, PilJ (25), and the histidine kinase PilS (8), have been shown to have bi-polar localization. It is currently unknown: 1) if those proteins with monopolar localization, localize specifically to the piliated pole, 2) whether that localization is involved in determining which pole is piliated, 3) whether additional factors are involved, 3) whether some or all of these factors oscillate with reversals in cell direction, and 4) the mechanism by which oscillation is achieved. Further work will need to be done to answer these questions.

Regulation of Type IV Pilus Function

Of the more that 40 genes involved in the biogenesis and function of Tfp, a subset, including *pilG*, *H, I, J, K, R, S, chpA, B, C, D, E, algR, U, fimL, S, vfr,* and *crc* appear to play regulatory roles (66, 80, 102). These include both transcriptional regulation of biosynthetic components as well as chemosensory regulation of TM (66, 105). The exact function of some of these regulatory factors has yet to be determined.

A number of two-component regulatory systems are involved in the regulation of Tfp function. Transcription of *pilA* is regulated by the PilS/PilR two-component system (9, 41, 42, 45, 46). *pilS* encodes a membrane-bound, polarly-localized sensor kinase (8).

The signal to which PilS responds remains unknown. PilS phosphotransfers to the response regulator PilR, which, along with the sigma factor RpoN, binds to the *pilA* promoter to initiate transcription. *M. xanthus* encodes two sets of *pilS*/*pilR* homologues (pilS/pilR and pilS2/pilR2 110). Regulation of *pilA* transcription appears to differ somewhat in *M. xanthus* compared to *P. aeruginosa*. Whereas PilR is required for transcription of *pilA,* PilS appears to be a negative regulator. The functions of PilS2/PilR2 are currently unknown. We present evidence in chapter 2 suggesting that PilA is a negative regulator of *pilA* transcription in *P. aeruginosa*. PilA has been shown to play a negative regulatory role in *pilA* transcription in *M. xanthus*. It is intriguing to speculate that PilS may somehow be responding to intracellular PilA levels to regulate transcription on *pilA*.

The atypical sensor-regulator pair FimS/AlgR appears to be involved in the regulation of Tfp function in *P. aeruginosa*, although the mechanism remains unclear (103). Mutation of either *fimS* or *algR* results in decreased TM and levels of extracellular pili although *pilA* transcription is unaffected. AlgR appears to be a DNA binding protein that interacts with the sigma factor AlgU. AlgR, but not FimS, also regulates the production of the exopolysaccharide alginate in mucoid strains. FimS is unable to autophosphorylate suggesting that it either receives phosphate from an upstream donor or acts to dephosphorylate AlgR phosphorylated by another histidine kinase to influence AlgR function.

TM in *P. aeruginosa* and *M. xanthus*, though not in *N. gonorrhoeae* or *N. meningitidis*, is regulated by chemosensory signal transduction systems (Chp and Frz), which are similar to, but substantially more complex than, the Che chemosensory system

involved in the regulation of flagellar chemotaxis in *Escherichia coli* (for recent reviews see 4, 97). In the Che system, ligand binding to a methyl-accepting chemotaxis protein (MCP) receptor induces autophosphorylation of a histidine kinase (CheA), which phosphorylates a response regulator (CheY, Fig. 1). Phosphorylated CheY interacts with the flagellar motor to reverse the direction of rotation. Other components of the system include an adaptor protein (CheW), which facilitates interactions between CheA and the MCP, a methyltransferase (CheR) and a methylesterase (CheB), which modulate the methylation state of the MCP and are involved in the adaptive chemotactic response, and a phosphodiesterase (CheZ), which is involved in dephosphorylation of CheY.

In *M. xanthus* TM is regulated, in part, by the Frz chemosensory system (reviewed in 114). To date, chemosensory regulation of TM has been most thoroughly characterized in *M, xanthus*. Unlike the Chp chemosensory system in *P. aeruginosa*, which seems to be directly involved in signaling to ATPases driving pilus extension and retraction (see below), the Frz chemosensory system is believed to be involved primarily in the regulation of the frequency of cell reversals. Moreover, though they share some basic components common to chemotaxis two-component signaling systems, the two systems differ in important ways. The core components of the Frz system include a cytoplasmic chemoreceptor, FrzDC, two CheW homologues, FrzA and FrzB, a hybrid histidine kinase, FrzE, a methyltransferase and a methylesterase, FrzF and FrzG, and FrzZ, which contains two CheY-like response regulator domains (39, 40, 69, 100). In the current model for how the Frz system functions to regulate reversals, in the presence of an, as yet unidentified, attractant FrzCD induces autophosphorylation of FrzE, which is negatively regulated by its C-terminal CheY-like response regulator domain. FrzZ

accepts the phosphoryl group from FrzE and signals, via an unknown mechanism, to the master motility regulator MglA, which is a member of the Ras superfamily of small monomeric GTPases (35, 90). MglA, in turn, interacts with FrzS, which has been shown to localize to the pole at which Tfp are active and to oscillate between poles during changes in direction through a mechanism that is dependent upon the Frz chemosensory system (73). Thus, in the presence of an attractant, for example, the Frz system could function to inhibit changes in direction. Interestingly, like FrzS, PilU, which is a *P. aeruginosa* homologue of the retraction ATPase PilT, has a largely monopolar localization, one of only a handful of proteins involved in the regulation of *P. aeruginosa* TM to be localized to a single pole. We present evidence in chapter 2 suggesting that, despite the homology to PilT, PilU does not appear to play a role in the regulation of pilus retraction. However, like *frzS*, Δ*pilU* mutants are severely impaired for TM suggesting the apparent non-motile TM phenotype of Δ*pilU* may be due less to mechanical and more to spatial dysregulation of pilus extension and retraction, where pili are extending and retracting from either both poles or neither pole. If PilU does oscillate from pole to pole, the mechanism by which is does may be similar to that of FrzS, since like FrzS, PilU maintains a monopolar localization in both Chp system mutants (including Δ*chpA*, Δ*pilG*, Δ*pilH*, and Δ*pilG*Δ*pilH*, our unpublished data), as well as in other mutants implicated in the regulation of Tfp (15, 73). Whether or not PilU oscillates between poles and whether that oscillation affected in these mutant backgrounds remains to be determined.

In *P. aeruginosa* the putative chemosensory system involved in the regulation of TM is encoded by the *pilGHIJKchpABC* gene cluster (Fig. 1 22, 23, 24, 105). The core

signaling components of the Chp system, include the putative hybrid histidine kinase ChpA (105), and two CheY-like response regulators PilG and PilH (22, 23). ChpA is one of the most complex CheA homologs yet to be described. In addition to its histidine kinase domain, it possesses 8 potential sites of phosphorylation and a CheY-like receiver domain at its C-terminus. Previous studies (61) have demonstrated that the second and third phosphotransfer (Hpt) domains contribute to TM while the CheY domain is absolutely essential. Interestingly, the phosphorylation sites for two of these Hpt domains are predicted to be serine and threonine residues. In chapter 2 we present genetic evidence that phosphotransfer from ChpA to PilG, and PilH is likely important for TM and that PilG and PilH regulate TM by modulating the functions of the ATPases PilB and PilT, respectively. Given our results and those of others (61) several models for how ChpA might phosphorylate PilG and PilH emerge. These include simple phosphotransfer from autophosphorylated Hpt2 and/or Hpt3 to PilG and/or PilH, or a more elaborate phosphorelay scheme involving the C-terminal CheY domain of ChpA. A combination of biochemical and genetic analyses will be required to further elucidate this complex signaling pathway.

In addition to the putative hisidine kinase and the two CheY-like response regulators, the Chp system gene cluster encodes a putative methyl-accepting chemotaxis protein (MCP, PilJ, 23), two CheW-like adaptor proteins (PilI and ChpC, 23, 105), a putative methyltransferase (PilK, 24)), and a putative methylesterase (ChpB, 105)). Two additional genes, *chpD* and *chpE*, have no homology to Che system components. However, their proximity to other Chp system genes suggests that *chpD* and *chpE* may be co-transcribed and, therefore, may play some role in Chp system function. *chpD* encodes

a putative AraC-like transcriptional regulator and ChpE encodes a putative LysE family amino acid efflux protein (105). Insertion mutants in *pilI*, *pilJ*, and *pilK* have been shown previously to have defects in Tfp function, including TM and sensitivity to infection by pilus-specific phage (23, 24). Insertion mutants in *chpB*, *chpC*, *chpD*, and *chpE* had no defects in Tfp function (105). These phenotypes have been confirmed in in-frame deletion mutants (chapter 3). It remains to be determined if PilJ functions as an MCP, what chemical signal(s) it responds to, and whether it is the only MCP involved in the regulation of Tfp function. Previous studies have shown that both *P. aeruginosa* (51) and *M. xanthus* (52) demonstrate directed motility up phosphatidylethnolamine gradients. Whether PilJ is involved in this response is unclear. It also remains to be determined whether ChpA binds to PilJ and if either of the two CheW-like adaptor proteins (PilI and ChpC) are required for this interaction. Because a signal has yet to be identified for the Chp system, it remains unclear whether the system allows for an adaptive response to increasing concentrations of attractant or repellant. Once a signal is identified, however, the role that PilK and ChpB play in mediating an adaptive response can be investigated. Specifically, whether PilK is able to methylate PilJ or other MCP(s), whether ChpB is able to demethylate those receptors, and whether ChpA phosphorylation of ChpB is required for ChpB function. The roles of ChpD and ChpE, whether in the regulation of Tfp function or in another capacity remain to be determined. The majority of bacteria that possess Tfp do not encode *chpA* homologues (105). Those that do, however, appear maintain the Chp chemosensory system, in whole or in part (105). Interestingly, the bacteria that do have identifiable Chp systems are found in soil, water, and/or plants. These observations, taken together with the differences between the Frz and the Chp

systems, suggest that regulatory systems controlling Tfp function, in general, and the chemosensory regulation of TM, in particular, are likely to be quite diverse and may be tailored to an environmental niche or to bacterial lifestyle.

Swarming motility is a form of translocation that occurs on semisolid surfaces and results in complex motility patterns (13, 56). There is evidence to suggest that functional Tfp and flagella both play a role in swarming motility (56). In support of this notion, Caiazza and colleagues (12) have shown that mutation of either *pilJ* or *chpB* in the PA14 strain background results in altered swarming motility phenotypes. In addition, a recent study by Yeung *et al*. (113) demonstrated a swarming phenotype for PA14 *pilH* mutants. *pilJ* mutants have a hyperswarmer phenotype whereas *chpB* and *pilH* mutants show decreased swarming (12, 113). Interestingly, given the fact that deletion of *pilJ* in the PAO1 strain background results in decreased TM and presentation of surface pili, whereas deletion of *chpB* has no effect on TM or surface presentation (see chapter 3), these results suggest that Tfp may be playing a negative role in mediating swarming motility. However, Caiazza *et al*. (12) also show an inverse relationship between swarming motility and biofilm formation, wherein *pilJ* mutants fail to form robust biofilms and *chpB* mutants form biofilm with increased biomass relative to WT. Assays for biofilm formation with Δ*pilJ* and Δ*chpB* mutants in the PAO1 background suggest that *pilJ* mutants form mature biofilms with WT morphologies whereas *chpB* mutant biofilms have severely altered morphology (see chapter 3), suggesting that swarming motility and biofilm formation may be regulated differently in the two strain backgrounds. The differences in biofilm phenotypes could reflect different assay

conditions. Further work will need to be done to fully explore the role of the Chp system in both swarming motility and biofilm formation.

Regulation of Gene Expression by Chp System Components

Insertion mutants in *chpA* showed reduced cytotoxicity towards MCDK cells *in vitro* (105), and reduced virulence in both a *Drosophila* infection model (20) and a mouse model of acute pneumonia (105). These mutants were defective in the production of a number of quorum sensing regulated virulence factors (reviewed in 75), but the defect was not recapitulated in in-frame deletion mutants and could not be complemented in the insertion mutant by reintroduction of *chpA* onto the chromosome at the *chpA* locus. However, the N-terminal domain of ChpA is homologous to FimL, which has been shown to regulate not only TM but also the production of the type III secreted effector protein ExoT (102). The type III secretion system (T3SS) allows for the direct injection of bacterial effector proteins into the cytoplasm of a host cell, and has been shown to be involved in *P. aeruginosa* virulence in a number of *in vivo* infection models, including *Drosophila* and a mouse model of acute pneumonia (28). The TTSS is regulated in part by the cyclic-AMP (cAMP) binding transcription factor, Vfr, which also plays a role in the regulation of TM (6, 109), and the transcriptional regulator, ExsA (111). Vfr regulates transcription of *exsA* (109), although the mechanism is unknown. Our unpublished work has demonstrated a role for FimL in the production of cAMP (West, J., Inclan, Y., Bertrand, J., and Engel, J., manuscript in preparation). The defect in ExoT production in *fimL* mutants, therefore, is likely due to decreased production of cAMP and a consequent decrease in Vfr activity. The homology between FimL and the N-terminus

of ChpA raises the possibility that ChpA may also play a role in cAMP production. As such, ChpA might contribute to the regulation of T3SS by mediating Vfr activity. We present evidence in chapter 4 for a role for ChpA in the regulation of *exoT* and other T3SS gene expression. It remains to be determined if ChpA contributes to the regulation of T3SS gene expression primarily at the levels of cAMP production.

Similar to ChpA, The CheY-like response regulator PilH, in addition to the role it plays in the regulation of Tfp function, appears also to have a role in the regulation of *pilA* transcription and secreted virulence factor (VF) production. In chapter 2 we show that transcription of *pilA* is decreased in Δ*pilH* mutants under certain growth conditions, suggesting that PilH is a positive regulator of *pilA* transcription. We demonstrate further (chapter 5) that Δ *pilH* mutants have altered production of quorum sensing-regulated virulence factors (VFs). Specifically, we show that Δ *pilH* mutants have increased elastase and pyocyanin production, and decreased rhamnolipid production. Alterations in VF production correlated with changes in expression of biosynthetic genes. Expression of quorum sensing regulatory genes was not effected in Δ*pilH* mutants. The full range of genes regulated by PilH remains to be determined.

Conclusion

Tfp are involved in a number of important bacterial processes ranging from adhesion and phage adsorption, to DNA uptake, TM, and complex social behaviors such as fruiting body development and biofilm formation. All of these functions are based around the core process of extension and retraction of the Tfp. Particularly in the model species *P. aeruginosa*, *N. gonorrhoeae*, *N. meningitidis*, and *M. xanthus*, much has been

learned about the factors required for the biogenesis and mechanical functions of Tfp. We are beginning now to uncover and understand in greater depth those signaling systems involved in regulating the mechanical aspect of Tfp activity. A great deal more work remains to be done to fully appreciate the intricacies of these complex signaling systems, particularly with regard to how they interface with the extension and retraction machinery and whether and how they respond to changing environmental conditions to alter Tfp activity and influence bacterial behavior.

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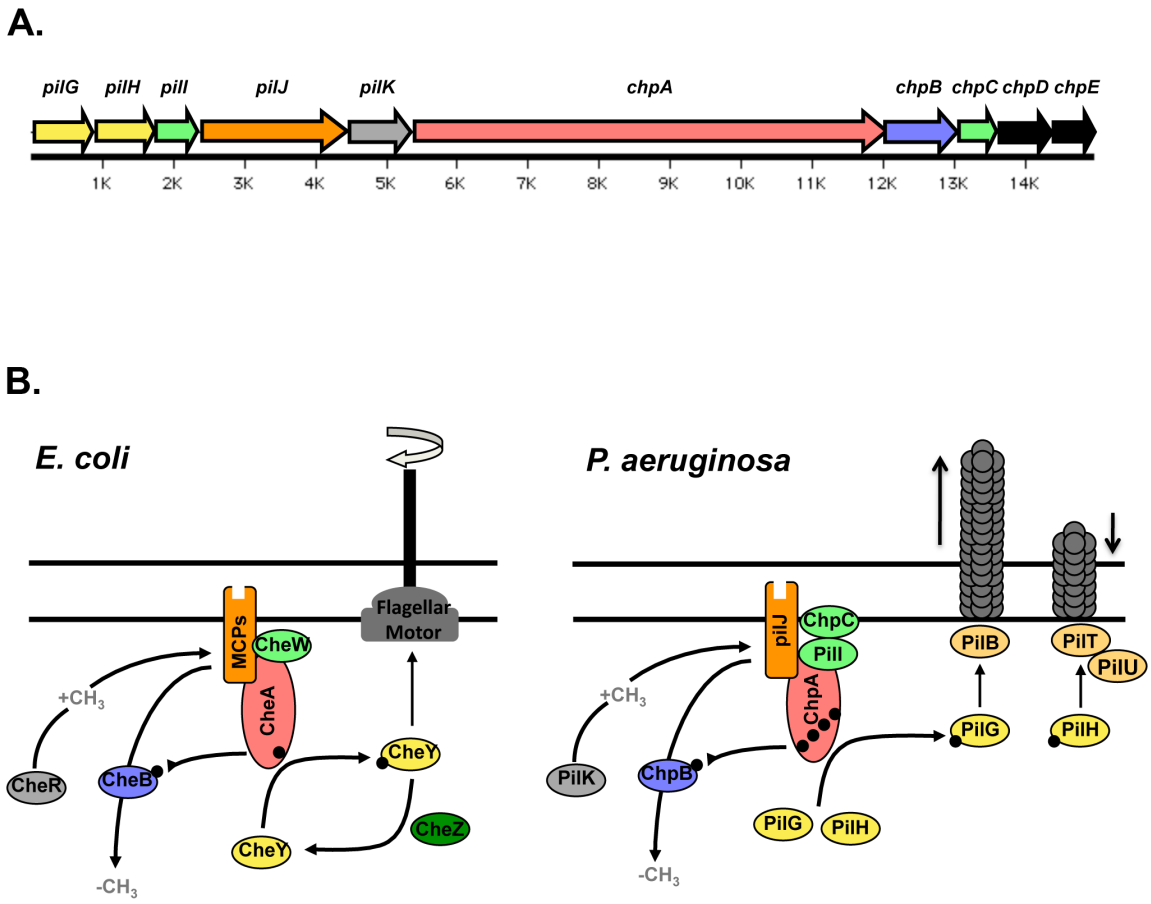

Figure 1. Model for the regulation of pilus extension and retraction by the Chp chemosensory system based on homology to the *E. coli* Che chemosensory system. (A) Organization of genes in the Chp cluster. (B) Hypothetical model for regulation of pilus extension and retraction by the Chp chemosensory system. The histidine kinase ChpA, couple to the MCP PilJ by one of two CheW homologues, PilI and/or ChpC, undergoes autophosphorylation and transfers phosphate groups to two CheY-like response regulator proteins, PilG and PilH. PilG-P interacts with the ATPase PilB to mediate pilus extension. PilH-P interacts with PilT and/or PilU to mediate pilus retraction. PilJ is methyated by the methyltransferase PilK and demethylated by the methylesterase ChpB.

Chapter 2

Extension and Retraction of Type IV Pili is Regulated by the Chp Chemosensory System

Introduction

Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacterium that is an opportunistic human pathogen and that has been shown to infect a large number of diverse eukaryotes including amoebae, nematodes, insects, plants, mice and humans (34). *P. aeruginosa* is the causative agent of both acute and chronic human infections ranging from minor skin infections to persistent, and often life-threatening, disease in hospitalized or immunocompromised patients such as those suffering from AIDS, extensive burns, who are (35) undergoing chemotherapy, or who are recovering from major surgery. *P. aeruginosa* also chronically infects the cystic fibrosis lung and is the primary cause of respiratory morbidity and mortality in patients with this inherited disease (35).

P. aeruginosa virulence involves the coordinate expression of a wide range of secreted and cell-associated virulence factors, including adhesins, exopolysaccharides, proteases, lipases, phospholipases, siderophores, biosurfactants, and exotoxins (15). Key among these virulence factors are the type IV pili (Tfp). Tfp are polarly localized filamentous appendages synthesized by a variety of Gram-negative bacteria including pathogens as well as environmental species such as *Myxococcus xanthus* (36). Tfp constitute the major adhesin of *P. aeruginosa* and have been shown to play a role in adherence to epithelial cells in culture and in virulence in several animal models of infection (15). In addition to the role that Tfp play in adhesion to host cells, they also

function as receptors for certain bacteriophages (6) and allow for a type of flagella-independent surface translocation termed twitching motility. TM is propelled by the coordinated extension, tethering, and retraction of Tfp (37, 44) and has been shown to be involved in complex social behaviors such as fruiting body development in *M. xanthus* (28, 29, 54) and biofilm formation in *P. aerugionsa* (3, 7, 30, 31, 40). *P. aeruginosa* biofilms have been implicated in chronic infections (10).

Biogenesis, assembly, and function of Tfp requires more than 40 genes (36). Regulation of Tfp function is complex, and involves multiple signal transduction systems, including the two-component signaling systems *pilSR* and *algR*/*fimS*, the global carbon metabolism regulator *crc*, the virulence factor regulator *vfr*, and a putative chemosensory system encoded by the *pilGHIJKchpABC* gene cluster and referred to as the Chp system. The proteins encoded by these genes appear to comprise a chemosensory signal transduction pathway similar to the Che system involved in the regulation of flagellar chemotaxis in *Escherichia coli* (for recent reviews see 2, 47). As is the case with a growing number of bacterial chemotaxis systems, regulation of TM by the Chp system appears to be considerably more complex than its *E. coli* counterpart (28, 41).

The core signaling components of the Chp system include a putative histidine kinase, encoded by *chpA* (49), and two CheY-like response regulators, encoded by *pilG* and *pilH* (11, 12). Previous studies have suggested that *chpA* (49) and *pilG* (11) are involved in regulating pilus extension, whereas *pilH* (12) Is involved in regulating pilus retraction. Similarly, the ATPases encoded by *pilB* and *pilT* are postulated to be involved in mediating pilus extension and retraction, respectively (23). The role of the ATPase encoded by *pilU* is unclear. However, due to a high degree of similarity with

pilT, it has been hypothesized that *pilU* may play some role in regulating pilus retraction (9).

Here we use genetic approaches to test the hypothesis that phosphotransfer from ChpA to PilG, and PilH is important for TM and that PilG and PilH regulate TM through PilB and PilT, respectively (Fig. 2). Using double and triple mutants, we demonstrate that ChpA, PilG and PilB function upstream of PilH, PilT, and PilU and that PilH functions downstream of PilT. We provide evidence that PilB and PilT retain some activity in the absence of upstream signaling from imput from ChpA and PilG, and PilH, respectively. We show that the histidine kinase domain of ChpA is required for function as are the phospho-acceptor sites of both PilG and PilH. Finally, we present evidence suggesting that *pilA* transcription is regulated by intracellular PilA levels. We show that PilA is a negative regulator of *pilA* transcription in *P. aeruginosa* and that the Chp system functionally regulates *pilA* transcription by controlling PilA import and export.

Materials and Methods

Bacterial strains, plasmids and media. The *Escherichia coli* strains DH5α (*recA endA gyr96 hsd*R17 *thi-1 supE44 relA1* ϕ80 d*lacZ*Δ*M15*) was used in all genetic manipulations and in the preparation of DNA sequencing templates. *E. coli* strains S17-1 (*thi pro hsdR recA chr::RP4-2*) and SM10 (*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*) were used as donor strains in bacterial conjugation. The *P.s aeruginosa* strain used was PAO1 strain ATCC 15692 (American Type Culture Collection). Chemically competent *E. coli* cells (Invitrogen) were transformed according to the manufacture's instructions. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* and *P. aeruginosa* liquid cultures were maintained in Luria-

Bertani (LB) broth and solid media was prepared by adding 0.8-1.5% agar (Becton, Dickinson and Company Bacto™ Agar). *P. aeruginosa* strains were isolated from *E. coli* during the mating protocol on 1.5% Difco Pseudomonas Isolation Agar (PIA) (Becton, Dickinson and Company. The following antibiotic concentrations were used for the selection of *E. coli*: tetracycline 5 μg/mL, ampicillin 100 μg/mL, gentamicin 10 μg/mL, and kanamycin 50 μg/mL. The following antibiotic concentrations were used for the selection of *P. aeruginosa*: tetracycline 100 μg/mL, carbenicillin 250 μg/mL, and gentamicin 100 μg/mL.

Plasmid construction. All plasmids were purified using QIAprep spin miniprep columns (QIAGEN). Standard recombinant DNA manipulation techniques were used (42). Enzymes were purchased from New England Biolabs Inc. and used as recommended by the manufacturer. All oligonucleotides used in this study were designed based on the PAO1 genome sequence (http://www.pseudomonas.com) (45) and were synthesized by QIAGEN or Elim Biopharmaceuticals Inc. (Hayward, CA). The oligonucleotide primer sequences used for PCRs are listed in Table 2. PCR amplifications were carried out with Pfu Turbo DNA polymerase (Stratagene) or Herculase DNA polymerase (Stratagene) and products sequenced to ensure that no mutations were introduced during amplification. Intermediate cloning steps were performed using DH5α (Invitrogen). Plasmids were introduced into chemically competent *E. coli* strains.

Construction and complementation of in-frame deletion mutants. All matings were performed as described previously (49). All mutants were confirmed by Southern or PCR analysis.

ChpA. The *chpA* in-frame deletion mutant was constructed by allelic displacement and has been described previously (49). To complement Δ*chpA*, a 3′

fragment of *chpA* including 1 kb of 3′ flanking sequence was amplified using the chpA26.1/chpA29(Xba) PCR primer pair and cloned as an *Mlu*I/*Xba*I fragment into pJB219 (a modified version of pOK12 in which the *Kpn*I and *Cla*I sites have been removed from the MCS) to form pJB220. A 5′ fragment of *chpA* including 1 kb of 5′ flanking sequence was amplified using the chpA22(*Hind*III)/chpA25 PCR primer pair and cloned as an *Hind*III/*Mlu*I fragment into pJB220 to form pJB221. Full-length *chpA* and 1 kb of 5′ and 3′ flanking sequence were subcloned as a *Spe*I fragment from pJB221 to pJB100 to form pJB231. pJB231 was transformed into *E. coli* S17.1 and transformants were mated to Δ *chpA* to create a Δ *chpA* derivative complemented for *chpA* at its endogenous locus.

PilA. 5′ and 3′ *pilA* deletion construct fragments were amplified using the pilA1/pilA2 and pilA3/pilA4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T. The 5′ deletion construct was excised from pGEM-T as an *Nco*I/*Eco*RIII fragment. The 3′ deletion construct was excised from pGEM-T as an *Eco*RI/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Nco*I/*Xba*I fragment into pOK12. The deletion construct was excised from pOK12 as a *Spe*I fragment and ligated into the allelic exchange vector pJEN34 to form pJEN34PAO1KO3. pJEN34PAO1KO3 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilA* mutant. This procedure deleted amino acids 2–149 of the PilA protein. To complement Δ*pilA*, *pilA* and 1 kb of 5′ and 3′ flanking sequence were amplified using the pilA1(Spe)/pilA4(Spe) PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB241. pJB241 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilA* to create a Δ*pilA* derivative complemented for *pilA* at its endogenous locus.

PilB. 5′ and 3′ *pilB* deletion construct fragments were amplified using the

pilB1/pilB2 and pilB3/pilB4 PCR primer pairs. PCR products were used as templates in a sequence overlap extension (SOE) reaction with the pilB1/pilB4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB122. pJB122 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilB* mutant. This procedure deleted amino acids 4–564 of the PilB protein. To complement Δ*pilB*, *pilB* and 1 kb of 5′ and 3′ flanking sequence were amplified using the pilB1/pilB4 PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB246. pJB246 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilB* to create a Δ*pilB* derivative complemented for *pilB* at its endogenous locus.

PilG. 5′ and 3′ *pilG* deletion construct fragments were amplified using the pilG1/pilG2 and pilG3/pilG4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB43 (5′ deletion construct) and pJB44 (3′ deletion construct). The 5′ deletion construct was excised from pJB43 as an *Eco*RI/*Hind*III fragment. The 3′ deletion construct was excised from pJB44 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB60. The deletion construct was excised from pJB60 as a *Spe*I fragment and ligated into the allelic exchange vector pJEN34 to form pJB87. pJB87 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilG* mutant. This procedure deleted amino acids 5–87 of the PilG protein. To complement Δ*pilG*, *pilG* and 1 kb of 5′ and 3′ flanking sequence were amplified using the pilG1(Spe)/pilG4(Spe) PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB126. pJB126 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilG* to create a Δ*pilG* derivative complemented for *pilG* at its endogenous locus.

PilH. The construction and complementation of *pilH* has been described previously (3).

PilR. 5′ and 3′ *pilR* deletion construct fragments were amplified using the pilR1/pilR2 and pilR3/pilR4 PCR primer pairs. PCR products were used as templates in an SOE reaction with the pilR1/pilR4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB250. pJB250 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilR* mutant. This procedure deleted amino acids 4–443 of the PilR protein. To complement Δ*pilR*, *pilR* and 1 kb of 5′ and 3′ flanking sequence were amplified using the pilR1/pilR4 PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB251. pJB251 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilR* to create a Δ*pilR* derivative complemented for *pilR* at its endogenous locus.

PilT. 5′ and 3′ *pilT* deletion construct fragments were amplified using the pilT1/pilT5 and pilT6/pilT4 PCR primer pairs. PCR products were used as templates in a sequence overlap extension (SOE) reaction with the pilT1/pilT4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB109. pJB109 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilT* mutant. This procedure deleted amino acids 4-342 of the PilT protein. To complement Δ*pilT*, *pilT* and 1 kb of 5′ and 3′ flanking sequence were amplified using the pilT1/pilT4 PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB113. pJB113 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilT* to create a Δ*pilT* derivative complemented for *pilT* at its endogenous locus.

The *pilT* truncation mutant ($pilT^{Trunc}$) deletes 429 bp of the *pilT* coding sequence including the start codon (amino acids 1-143 of the PilT protein), and introduces stop

codons in all three reading frames. The mutant retains 783 bp of sequence upstream of *pilU*. The 5′ and 3′ truncation construct fragments were amplified using the pilT1(new)/pilT-Trunc2 PilT-Trunc3/pilT-Trunc4 PCR primer pairs. PCR products were used as templates in an SOE reaction with the pilT1(new)/pilT-Trunc4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB203. pJB203 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create the *pilT*-Trunc mutant.

PilU. 5′ and 3′ *pilU* deletion construct fragments were amplified using the pilU1/pilU5 and pilU6/pilU4 PCR primer pairs. PCR products were used as templates in an SOE reaction with the pilU1/pilU4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB111. pJB111 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilU* mutant. This procedure deleted amino acids 4–380 of the PilU protein. To complement Δ*pilU*, *pilU* and 1 kb of 5′ and 3′ flanking sequence were amplified using the pilT1/pilT4 PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB114. pJB114 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilU* to create a Δ*pilU* derivative complemented for *pilU* at its endogenous locus.

FliC. 5′ and 3′ *fliC* deletion construct fragments were amplified using the fliC1/fliC4 and fliC3/fliC4 PCR primer pairs. PCR products were used as templates in an SOE reaction with the fliC1/fliC4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB215. pJB215 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*fliC* mutant. This procedure deleted amino acids 4–486 of the FliC protein. To complement Δ *fliC*, *fliC* and 1 kb of 5′ and 3′ flanking sequence were amplified using the fliC1/fliC4 PCR primer pair. The product was cloned as a *Spe*I fragment into pJB100 to form pJB230. pJB230 was

transformed into *E. coli* S17.1 and transformants were mated to Δ*fliC* to create a Δ*fliC* derivative complemented for *fliC* at its endogenous locus.

A pEX100T-based *chpA* deletion construct was made by subcloning the *chpA* deletion construct as a *Spe*I fragment from pJB4 to pJB100 to form pJB116. pJB116 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilH*, Δ*pilT*, Δ*pilU*, and Δ*pilT*Δ*pilU* (see below) to create Δ *chpA*Δ*pilH*, Δ *chpA*Δ*pilT*, Δ *chpA*Δ*pilU*, and Δ*chpA*Δ*pilT*Δ*pilU*, respectively.

To construct Δ*pilG*Δ*pilH*, the 5′ deletion construct from pJB43 was excised as an *Xho*I/*Hind*III fragment, concatamerized with the 3′ deletion construct from pJB48 excised as a *Hind*III/*Xba*I fragment, and cloned as an *Xho*I/*Xba*I fragment into pOK12 to form pJB96. The deletion construct was excised from pJB96 as a *Spe*I fragment and ligated into the allelic exchange vector pJEN34 to form pJB97. pJB87 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an unmarked Δ*pilG*Δ*pilH* mutant. Gentamicin-resistant derivatives of the *pilT* and *pilU* deletion constructs were made by subcloning a gentamicin-resistance encoding cassette as a *Hind*III fragment from pX1918GT (43) into pJB109 and pJB111 to form pJB110 and pJB112, respectively. pJB110 and pJB112 were transformed into *E. coli* S17.1 and transformants were mated to Δ*pilG* to created Δ*pilGpilT*-gent and Δ*pilGpilU*-gent. Δ*pilGpilT*-gent and Δ*pilGpilU*-gent were then mated to S17.1 strains carrying pJB109 and pJB111 to create Δ*pilG*Δ*pilT* and Δ*pilG*Δ*pilU*. A pEX100T-based *pilG* deletion construct was made by subcloning the *pilG* deletion construct as a *Spe*I fragment from pJB59 to pJB100 to form pJB118. pJB118 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilT*Δ*pilU* (see below) to create Δ*pilG*Δ*pilT*Δ*pilU*.

Δ*pilB*Δ*pilH* and Δ *pilB*Δ*pilT*Δ*pilU* were constructed by mating Δ *pilH* and Δ*pilT*Δ*pilU*, respectively, to *E. coli* S17.1 carrying pJB122. Δ*pilB*Δ*pilT* and Δ*pilB*Δ*pilU*

were constructed by mating Δ*pilB* to *E. coli* S17.1 carrying pJB109 and pJB111, respectively.

5′ and 3′ *pilTpilU* deletion construct fragments were amplified using the pilU1/pilTpilU1 and pilTpilU2/pilU4 PCR primer pairs. PCR products were used as templates in an SOE reaction with the pilT1/pilU4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB163. pJB163 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create Δ*pilT*Δ*pilU*. A pEX100T-based *pilH* deletion construct was made by subcloning the *pilH* deletion construct as a *Spe*I fragment from pJB62 to pJB100 to form pJB119. pJB119 was transformed into *E. coli* S17.1 and transformants were mated to Δ*pilT*, Δ*pilU*, and Δ*pilT*Δ*pilU* to create Δ*pilH*Δ*pilT*, Δ*pilH*Δ*pilU*, and Δ*pilH*Δ*pilT*Δ*pilU*, respectively.

pilT(CTX-*pilU*) strains. *pilU* along with 1000 bp of upstream sequence was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system (20). The CTX-*pilU* construct was amplified using the pilU-CTX-Spe-F(1kb)/pilU-CTX-Hind-R PCR primer pair. The PCR product was cloned as a *Spe*I/*Hind*III fragment into mini-CTX2 to form pJB212. pJB212 was transformed into *E. coli* S17.1 and transformants were mated to all *pilT* mutant strains except *pilT*-Trunc and Δ*pilT*Δ*pilU* to create $pilT^{\text{CTX-}pilU}$ derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFlp2, as described previously (20).

chpA-FLAG and *chpA* point mutants. The region of *chpA* encoding the histidine kinase domain and the 2X-FLAG tag was subcloned as a *Cla*I/*Kpn*I fragment from pAL64 (32) into pOK12 to form pJB202. The *Kpn*I/*Cla*I fragment was subcloned from pJB202 into pJB221 from which the corresponding *Cla*I/*Kpn*I fragment had been removed to form pJB232. Full length *chpA*-FLAG including 1 kb 5′ and 3′ flanking

sequence was subcloned into pJB100 to form pJB234. pJB234 was transformed into *E. coli* S17.1 and transformants were mated to Δ*chpA* to create *chpA*-FLAG.

pJB202 was the substrate for a QuickChange (Stratagene) mutagenesis reaction using the chpA-HisKn-F/chpA-HisKn-R PCR primer pair. The mutation replaces each of the three residues in the signature DXG motif of the G1 box of ChpA with alanines (D2091A, D2092A, G2093A), *chpA*(AAA)-FLAG. The mutated histidine kinase domain, including the 2X-FLAG tag was subcloned as a *Kpn*I/*Cla*I fragment from pJB207 into pJB221 from which the corresponding *Cla*I/*Kpn*I fragment had been removed to form pJB233. Full length *chpA*(AAA)-FLAG including 1 kb 5′ and 3′ flanking sequence was subcloned into pJB100 to form pJB235. pJB235 was transformed into *E. coli* S17.1 and transformants were mated to Δ*chpA* to create *chpA*(AAA)-FLAG.

pilG-His, *pilH*-His, and *pilG* and *pilH* point mutants. 5′ and 3′ allelic exchange constructs to generate C-terminal 6-His tagged derivatives of *pilG* and *pilH* were amplified using the pilG1(Spe)/pilG5 and pilG6/pilG4(Spe), and pilH1/pilH15 and pilH16/pilH4 PCR primer pairs, respectively. PCR products were used as templates in SOE reactions with the pilG1(Spe)/pilG4(Spe) and pilH1/pilH4 primer pairs. The SOE products were cloned blunt into *Sma*I digested pEX100T to form pJB124 (*pilH*-His) and pJB127 (*pilG*-His). pJB124 and pJB127 were transformed into *E. coli* S17.1 and transformants were mated to Δ*pilH* and Δ *pilG* to create *pilH*-His and *pilG*-His respectively.

pilG-His and *pilH*-His allelic exchange constructs were amplified from pJB127 and pJB124, respectively, using pilG1(Spe)/pilG4(Spe) and pilH1(Spe/pilH4(Spe) PCR primer pairs cloned as Spe fragments into pOK12 to form pJB247 and pJB248. pJB247 and pJB248 were substrates for QuickChange (Stratagene) mutagenesis reactions using pilG-D58A-F/pilG-D58A-R and pilH-D52A-F/pilH-D52A-R PCR primer pairs to form

pJB252 and pJB254, respectively. The *pilG*(D58A)-His and *pilH*(D52A)-His allelic exchange constructs were subcloned as *Spe*I fragments from pJB252 and pJB254 into pJB100 to form pJB256 and pJB258. pJB256 and pJB258 were transformed into *E. coli* S17.1 and transformants were mated to Δ*pilG* and Δ*pilH* to create *pilG*(D58A)-His and *pilH*(D52A)-His.

P_{pilA}-*lacZ* reporter strains. A transcriptional fusion of the *pilA* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system (4). 500 bp of sequence upstream of the *pilA* start codon was amplified using the pilA-Reporter-F/pilA-Reporter-R PCR primer pair. The PCR product was cloned as an *Xho*I/*Bam*HI fragment into mCTX2-*lacZ* to form pJB249. pJB249 was transformed into *E. coli* S17.1 and transformants were mated to all single mutant strain (except Δ*pilT*) and $pilT^{Trunc}$ to create CTX-P_{pilA} derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFlp2, as described previously (4).

Twitching motility (TM) assay. Subsurface TM stab assays were performed as previously described. (1). Twitching motility was quantified by taking three separate measurements of twitching zone diameter from five independent stabs.

Generation of the α-PilA antibody. Polyclonal α-PilA antibodies were raised commercially in rabbits against the synthetic peptide WACKSTQDPMFTPKGCD (Invitrogen). The peptide represents amino acids 132-148 of PilA. For the current study, serum

Immunoblotting. Isolation of intracellular pilin for cells cultured on solid media was performed as follows. 100 µL of a 2 mL LB culture grown shaking overnight at 37 ˚C were plated to 1.5% LB agar and grown at 37 ˚C for 16 hrs. Following growth bacteria were scraped from the agar surface, and resuspended in 5 mL PBS. A volume of

cells equivalent to OD_{600} = 5.0 was brought up to 1 mL in PBS. Cells were vortexed for 30 min to remove surface pili and harvested by centrifugation (20,000 x g for 10 min). The supernatant was removed and cells were resuspended in 100 µL BugBuster Protein Extraction Reagent (Novagen). 1 µL of Benzonase nuclease (Novagen) was added, and the resuspended cells were incubated with gentle mixing at RT for 30 min. Cellular debris was removed by centrifugation (20,000 x g for 10 min) and 90 µL of supernatant was added to 90 µL 2X NuPage® LDS Protein Sample Buffer (Invitrogen) and 20 µL 10X NuPage® Sample Reducing Agent (Invitrogen). Samples were boiled for 5 min. Protein concentration was determined using the BioRad Protein Assay. To isolate surface pili, bacteria were grown as described above at 37 ˚C for 16 hrs on 1.5% LB agar, scraped from the agar surface, and resuspended in 5 mL PBS. A volume of cells equivalent to OD_{600} = 20.0 was brought up to 1 mL in PBS. Cells were vortexed at RT for 30 min to remove surface pili. The suspension was centrifuged at RT (20,000 x g for 10 min), the supernatant collected, and centrifuged a second time (20,000 x g for 10 min) to remove all cellular debris. The resulting supernatant was incubated overnight at 4 ˚C in 100 mM $MgCl_2$ to precipitate pili, as described previously (1). The precipitate was collected by centrifugation at 4˚C (20,000 x g for 20 min), the supernatant removed, and the pellet resuspended in 100 µL PBS and 100 µL 2X NuPage® LDS Protein Sample Buffer (Invitrogen) including 1X NuPage® Sample Reducing Agent (Invitrogen). Samples were boiled for 5 min.

Isolation of intracellular pilin for cells cultured in liquid media was performed as follows. Bacteria were grown shaking at 37 ˚C for 16 hrs in 10 mL LB. A volume of cells equivalent to OD_{600} = 5.0 was brought up to 1.5 mL in PBS and processed as described above. To isolate surface pili, bacteria were grown shaking at 37 ˚C for 16 hrs in 10 mL LB broth. A volume of cells equivalent to OD_{600} = 20.0 was brought up to 6

mL in PBS. Cells were harvested by centrifugation (6,000 X g for 10 min), resuspended in 1 mL PBS, and processed as described above.

To detect cell-associated and surface pilin for cells cultured on both solid and in liquid media, 15 μg of total cell-associated protein or 10 μL of surface pilin samples (1/20 of the total sample) were separated on NOVEX-NuPAGE 12% Bis-Tris SDS-PAGE gels (Invitrogen), and electroblotted onto Immun-Blot™ PVDF Membrane (BioRad) in a previously described Tris-glycine system (46). Membranes were blocked in 5% skim milk and probed with a 1:100,000 dilution of primary α-PilA antibody in 5% skim milk powder (0.05% Tween 20) in PBS. Membranes were then incubated with a 1:25,000 dilution of goat-anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories) in 5% skim milk powder (0.05% Tween 20) in PBS followed by detection by ECL using the Amersham™ ECL™ Western Blotting Detection Kit (GE Healthcare).

Cell-associated and surface flagellin samples for both plate and broth cultured cells were prepared as described for pilin samples samples. Detection of cell-associated and surface flagellin for both plate and broth cultured cells was carried out as for pilin samples, with the exception that membranes were probed with a 1:200,000 dilution of primary anti-FlaB antibody (a kind gift of Dr. Daniel Wozniak, Ohio State University 16).

To quantify levels of surface pili, samples were run in triplicate and immunoblotted for PilA and FliC as described above. Images of immunoblots were acquired on the ChemiDoc XRS (BioRad) and quantified using the Quantity One image analysis software. Surface pili levels were normalized to FliC.

Isolation and detection of *chpA*-FLAG and *chpA*(AAA)-FLAG was performed as described previously (32). Briefly, To prepare protein samples, cells were harvested

from 2 mL of culture grown shaking for 16 hrs at 37˚C, resuspended in 400 µL SDS-PAGE sample buffer, passaged through a 27½ gauge needle 3 times, and boiled for 5 min. 40 µ L (1/10 of total sample volumes) were separated by SDS-PAGE and immunoblotted with 2.5 µg/mL α-FLAG M2 monoclonal antibody.using 2.5 µg/mL α-FLAG M2 monoclonal antibody (Sigma).

Phage sensitivity assays. Phage sensitivity was assayed using the Tfp-specific *P. aeruginosa* bacteriophage PO4 (a kind gift of Dr. Lori Burrows, McMaster University), as previously described (48). Briefly, the phage stock was titred by adding 10 µL serial diluted phage to 100 µL PAO1 grown to stationary phase, incubating the mixture at room temperature for 30 min, adding the mixture to 3 mL 0.8% LB top agar, pouring onto 1.5% LB agar, and incubating overnight at 37 ˚C. To determine the phage sensitivity of mutant strains, cultures started from single colonies were grown in triplicate shaking for 16 hrs at 37 ˚C. 100 µL of culture were infected with 10 µL diluted phage and incubated at room temperature for 30 min. Following incubation, samples were added to 3 mL 0.8% LB top agar and incubated for 16 hrs at 37 ˚C. To quantify the data, plaques were counted and plaque forming units (Pfu) per milliliter of phage determined.

β-galactosidase assays. β-galactosidase assays were performed as previously described (48) with the following modifications. For assays performed on strains cultured in liquid media, cultures started from single colonies were grown in triplicate shaking for 16 hrs at 37 ˚C in 5 mL LB. For assays performed on strains cultured on solid media, cultures started from single colonies were grown in triplicate shaking overnight at 37 ˚C in 2 mL LB. 100 µL of overnight culture was plated to 1.5% LB agar and grown for 16 hrs. Following growth, cells were scraped from the agar surface, and resuspended in 5 mL PBS and diluted to OD_{600} = 4.0 before being assayed as described previously (48). Optical densities (OD_{600} and OD_{420}) were recorded using a

SPECTRAmax® microplate spectrophotometer (Molecular devices) and SoftMaxPro® 4.3.1. Specific activities were calculated relative to OD_{600}.

Statistical analysis. Statistical significance was determined by ANOVA using Instat software. Differences were considered to be significant at $P < 0.05$.

Results

ChpA, PilG, and PilB control TM by regulating pilus extension

We constructed in-frame deletion mutants in PAO1 for *chpA*, *pilG*, *pilH*, *pilB*, *pilT*, and *pilU* and assayed them for Tfp function including TM, intracellular and surface pilin levels, and phage sensitivity. The construction of the ChpA (49) and PilH mutants (3)has been described previously. This study represents the first in depth phenotypic analysis of in-frame deletions of these genes in a single strain background and is also the first instance in which all mutations have been complemented by reintroduction of the genes onto the chromosome at their endogenous loci. TM was measured by the subsurface stab assay. To determine surface- and intracellular pilin levels, plate-grown bacteria were harvested after 16 hrs. Surface pili were sheared by vigorous vortexing, the bacteria were removed by centrifugation, and the sheared pili were precipitated from the supernatant. Pilin in the pelleted bacteria constituted the intracellular pilin fraction. Infection with phage PO4 is a sensitive measure of surface pili. Tfp serve as phage receptor and functional Tfp are required for phage infection. Some mutants with no demonstrable surface piliation by western analysis remain sensitive to infection by phage PO4.

Consistent with previous results (11, 12, 39, 49-51), all mutants had defects in TM (Fig. 3A, B and Fig. 4A, B). The Δ*pilB* mutant lacked surface pili (Fig. 3C), and was

resistant to infection with phage PO4 (Table 3). The Δ*pilG* mutant also lacked detectable surface pili and Δ*chpA* had greatly reduced surface pili by western blot (Fig 1C). Both mutants, however, remained susceptible to phage infection (Table 3), suggesting that they assembled small amounts of functional surface pili. With the exception of *pilB* and *pilT* single double and triple mutants, infection of all strains with phage PO4 produced nearly as many plaques as when WT PAO1 was infected. However, in all cases plaques formed on mutant lawns were turbid when compared to plaques formed on WT lawns (data not shown). All mutants had wild type levels of flagellin on the cell surface, indicating that presentation of surface structures other than Tfp is not affected in these mutant backgrounds (Fig. 3C). Although Western analysis of cell lysates showed an approximate two-fold decrease in pilin production in the Δ*pilB*, Δ *pilG*, and Δ *chpA* mutants compared to wild type (Fig. 3C), this decrease is not sufficient to account for their profound defect in TM and surface presentation. TM, pilin production, surface piliation, and phage sensitivity could be complemented by re-introduction of each of the genes at their endogenous chromosomal loci (Fig. 3A, B, C). Taken together, and consistent with previous observations, these data suggest that *chpA*, *pilG*, and *pilB* are involved in the regulation of pilus extension.

PilH, PilT, and PilU control TM by regulating pilus retraction

We next examined Δ*pilH*, Δ*pilT*, and Δ*pilU* mutants to confirm their role in the regulation of pilus retraction. In contrast to Δ*chpA*, Δ*pilG*, and Δ*pilB*, all three mutants had WT levels of intracellular pilin (Fig. 4C). Δ*pilT* was completely defective for TM, having twitching zones comparable to those of a Δ *pilA* mutant (Fig. 4A, B). It had significantly increased levels of surface pili relative to WT ($p < 0.05$, Fig 4D), and was phage resistant (Fig 2C, Table 3). Δ*pilU* showed greatly decreased TM (Fig. 4A, B), but

was phage sensitive (Table 3) and had levels of surface pili statistically indistinguishable from WT (Fig. 4C, 4D). Δ*pilH* had a TM zone intermediate to wild type and Δ*pilA* (Fig. 4A, B). Similar to Δ*pilT*, it had significantly increased levels of surface pili relative to WT ($p < 0.05$, Fig. 4C, 4D), but in contrast was phage sensitive (Table 3). Interestingly, the morphology of plaques formed by PO4 phage on Δ*pilH* was distinguished by large zones of seemingly directional lysis and poorly defined plaques, even at low titers of phage (data not shown). This phenotype is unique among the Tfp regulatory factors addressed in the current study and does not appear to have been described for any other factor involved in the biogenesis or function of Tfp. TM, pilin production, surface piliation, and phage sensitivity could be complemented by re-introduction of each of the genes at their endogenous chromosomal loci (Fig. 4A, B, C).

Whereas all the other mutants (Δ*chpA,* Δ*pilG,* Δ*pilB*, Δ*pilH,* and Δ*pilU*) could be complemented for TM in *trans*, Δ*pilT* could only be complemented by re-introduction of *pilT* onto the chromosome at the *pilT* locus (data not shown). This finding suggests that the in-frame deletion of *pilT* might affect transcription of the adjacent *pilU* gene by disrupting *cis* regulatory sequences required for *pilU* transcription located within the *pilT* coding sequence. Consistent with this notion, a *pilT* truncation mutant ($pilT^{Trunc}$) in which the N-terminal 429 bp of *pilT* were deleted but which retains 783 bp of sequence upstream of *pilU*, is defective for TM, has increased surface pili, and could be complemented in by expression of *pilT* in *cis* or in *trans* (data not shown). For the remainder of our studies (except where indicated), we utilized a Δ*pilT* derivative strain ($\Delta pilT^{CTX\text{-}pilU}$) in which the *pilU* gene including 1000 bp of upstream sequence was introduced at an exogenous chromosomal locus using the mini-CTX system (20). This mutant was TM defective, had increased levels of surface pili, was phage resistant, and had wild type levels of intracellular pilin (Fig. 4A, B, C, and Table 3).

The phenotype of Δ*pilT*$^{\text{CTX-}pilU}$ differed from Δ*pilH* in several important ways. Δ*pilT*$^{\text{CTX-}pilU}$ is hyperpiliated relative to Δ*pilH* (Fig 2C, 4D) and was phage resistant whereas Δ*pilH* was phage sensitive. Although Δ*pilT*$^{\text{CTX-}pilU}$ exhibited increased surface pili relative to Δ*pilH* by western analysis, the difference was not statistically significant. Together, these data are consistent with the idea that PilT functions as a motor protein which serves to drive pilus retraction and whose activity is enhanced but is not completely dependent upon PilH. The function of PilU remains unclear. A high degree of homology to pilT (39% identical and 61% similar) suggests PilU may play a minor or accessory role in mediating pilus retraction (see below).

ChpA, PilG, and PilB function upstream of PilH, PilT, and PilU

We hypothesize that ChpA acts as a histidine kinase to phosphorylate PilG and/or PilH. Phosphorylated PilG, in turn mediates PilB dependent pilus extension, while phosphorylated PilH is required for PilT- and/or PilU-dependent pilus retraction. This model leads to the testable genetic prediction that double mutants in any factor involved in regulating pilus extension (*chpA*, *pilG*, or *pilB*) and any factor involved in regulating pilus retraction (*pilH*, *pilT*, and *pilU*) should have reduced surface pili. We constructed informative double mutants and assayed them for TM, intracellular and surface pilin levels, and phage sensitivity. All double mutants were defective for TM (Fig 3A, B). This is most notable in the case of *pilH* double mutants where the intermediate Δ*pilH* TM phenotype is decreased, indicating that ChpA, PilG, and PilB all function upstream of PilH.

chpA double mutants (Δ*chpA*Δ*pilH*, Δ *chpA*Δ*pilT*$^{\text{CTX-}pilU}$, and Δ *chpA*Δ*pilU*) retained some surface piliation (Fig. 5C), though less than seen in the single *pilH*, *pilT*, or *pilU* mutants (Fig 2C) but more than seen in the single *chpA* mutant (Fig 3C).

Furthermore, Δ*chpA*Δ*pilH* and Δ*chpA*Δ*pilT*$^{\text{CTX-}pilU}$ double mutants recapitulate Δ*pilH* and Δ*pilT*$^{\text{CTX-}pilU}$ phage sensitivity phenotypes (Table 3), respectively, indicating that PilH and PilT function downstream of ChpA. The observation that Δ *chpA*Δ*pilU* shows reduced surface piliation relative to WT (Fig. 5C) where Δ *pilU* has surface piliation levels similar to WT (Fig. 4C) indicates that, whatever role PilU plays in the regulation of pilus dynamics, it functions downstream of ChpA. Thus we conclude that ChpA functions upstream of PilH, PilT, and PilU.

We next compared various combinations of PilG or PilB double mutants. Δ*pilG*Δ*pilH*, Δ *pilG*Δ*pilT*$^{\text{CTX-}pilU}$, Δ *pilG*Δ*pilU*, Δ *pilB*Δ*pilH*, Δ *pilB*Δ*pilT*$^{\text{CTX-}pilU}$, and Δ*pilB*Δ*pilU* double mutants all lacked surface pili (Fig. 5C). Furthermore, Δ*pilG*Δ*pilH* and Δ *pilG*Δ*pilT*$^{\text{CTX-}pilU}$ had the same phage sensitivity as Δ*pilH* and Δ*pilT*$^{\text{CTX-}pilU}$, respectively (Table 3), indicating that PilH and PilT function downstream of PilG. Likewise, all Δ *pilB*Δ*pilH* and Δ *pilB*Δ*pilU* double mutants were resistant to phage infection, indicating that PilB functions upstream of PilH and PilU. These data are consistent with the model that both PilG and PilB function upstream of PilH, PilT, and PilU.

Our work (Table 3) and others (3, 11) have shown that *chpA* and *pilG* mutants remain susceptible to infection by PO4, while *pilB* mutants are immune to this pilus-specific phage (39). These observations suggest that some PilB-dependent pilus extension must occur in the absence of upstream signaling input from either ChpA or PilG. To test this hypothesis we constructed Δ*chpA*Δ*pilT*Δ*pilU*, Δ*pilG*Δ*pilT*Δ*pilU*, and Δ*pilB*Δ*pilT*Δ*pilU* triple mutants and assayed them for TM, pilin production, and surface presentation. We reasoned that in these sensitized genetic backgrounds, in which pilus retraction has been disabled, we would be able to detect any residual surface pili resulting from basal PilB activity. All triple mutants were defective for TM (Fig. 5A, B).

Detectable levels of surface pili were found for Δ*chpA*Δ*pilT*Δ*pilU* and Δ*pilG*Δ*pilT*Δ*pilU*, but not Δ*pilB*Δ*pilT*Δ*pilU* mutants (Fig. 5C). These data indicate that PilB-dependent extension occurs in the absence of signaling input from either ChpA or PilG and that PilB is absolutely required for pilus extension.

PilH functions downstream of PilT

Δ*pilT*$^{\text{CTX-}pilU}$ mutants are defective for TM and have increased levels of surface piliation relative to WT, Δ*pilH*, and Δ*pilU* strains (Fig. 4C). If PilH is involved in mediating PilT or PilU activity (i.e., is functionally downstream of PilT or PilU), we would predict that Δ*pilT* or Δ*pilU* would be epistatic to Δ*pilH*. Thus, Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$ and Δ *pilH*Δ*pilU* mutants should recapitulate Δ*pilT*$^{\text{CTX-}pilU}$ and Δ*pilU* phenotypes, respectively. We constructed Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$ and Δ*pilH*Δ*pilU* mutants and assayed them for TM, pilin production, surface presentation, and phage sensitivity.

The Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$ showed no TM, similar to Δ*pilT*$^{\text{CTX-}pilU}$ and was clearly distinguishable from Δ*pilH* (Fig. 6A, B). In addition, Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$ was resistant to phage infection (Table 3). Taken together, these data indicate that PilT functions upstream of PilH. Interestingly, the Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$ double mutant reproducibly showed significantly increased levels of surface pili relative to Δ*pilH* and Δ*pilT* ($p < 0.05$, Fig. 6C, D). Whereas Δ*pilT*$^{\text{CTX-}pilU}$ showed a 1.3-fold increase in surface pili relative to Δ*pilH* mutant, the double mutant exhibited 1.4-fold increase in surface pili relative to Δ*pilT*$^{\text{CTX-}pilU}$ and a 1.8-fold increase relative to Δ*pilH*. These data suggest that some additional retractile activity, enhanced perhaps by PilH, must be present.

Δ*pilH*Δ*pilU* had a TM phenotype and indistinguishable from Δ*pilU* (Fig 4A, B), a Δ*pilH* phage sensitivity phenotype (Table 3), and increased levels of surface pili relative to Δ*pilU* (Fig 4C, D). The simplest interpretation of these results is that both PilH and

PilU regulate TM, but that they do not operate in the same pathway to mediate retraction. The conclusion that PilU does not have retractile function is further supported by examination of Δ*pilT*Δ*pilU* and Δ*pilH*Δ*pilT*Δ*pilU*. Δ*pilT*Δ*pilU* had surface piliation levels similar to Δ*pilT*$^{\text{CTX-}pilU}$ (Fig 4C, D). Likewise, Δ*pilH*Δ*pilT*Δ*pilU* had surface piliation levels similar to Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$ (Fig 4C, D). Thus, loss of *pilU* did not further enhance the amount of surface pili, suggesting that the additional retractile activity is not encoded by *pilU*.

The phospho-acceptor sites of PilG and PilH are required for their function

Our results thus far are consistent with a model in which the CheY-like response regulator proteins PilG and/or PilH are targets of ChpA kinase activity, suggesting that their ability to be phosphorylated is required for their function. To test this hypothesis, we constructed strains in which we replaced the wild type allele with either WT His-tagged *pilG* and *pilH* (*pilG*-His, *pilH*-His) or His-tagged *pilG* and *pilH* derivatives in which the putative phospho-acceptor site aspartate residues were replaced with alanine residues (*pilG*(D58A)-His and *pilH*(D52A)-His). The various strains were assayed for TM, pilin production, and surface presentation. WT His-tagged PilG showed no defect in TM, intracellular pilin levels, or surface piliation (Fig. 7A, B, C), suggesting that the His tag did not interfere with function of PilG. In the case of PilH-His, the epitope tag slightly decreased its function, as the strain carrying the his-tagged protein showed slightly decreased TM relative to WT (Fig. 7A, B, C) and increased surface piliation, though not to the same extent as we observed in the Δ*pilH* mutant.

Consistent with the idea that signaling through PilG and PilH is required for their function, the phenotypes of *pilG*(D58A)-His or *pilH*(D52A)-His were indistinguishable from the in-frame deletion mutants of Δ*pilG* and Δ*pilH,* respectively. *pilG*(D58A)-His

was deficient for TM and lacked surface pili (Fig 5A, B, C). *pilH*(D52A)-His had an intermediate TM phenotype, increased surface pili, and formed aberrant plaques (Fig 5A, B, C, Table 3). We were unable to detect His-tagged wild type or mutant PilH or PilG by immunoblot analysis of either soluble or total cell lysates. Although we consider it unlikely, it is not possible to formally rule out that the defects observed in TM and surface piliation for *pilG*(D58A)-His and *pilH*(D52A)-His were secondary to reduced protein levels, though we consider this extremely unlikely as the equivalent point mutation has been made in many other CheY homologs without affecting protein levels or protein stability (e.g., 33).

The histidine kinase domain of ChpA is required for function

To test whether the histidine kinase activity of ChpA is required for its function, we mutated the histidine kinase domain. While histidine kinase mutants are typically constructed by altering the histidine residue that is the target of autophosphorylation (18), this approach is not possible for ChpA, as it encodes eight potential sites of phosphorylation six histidine-containing phosphotransfer domains and 2 novel serine- and threonine-containing phosphotransfer domains (49). Instead, we mutated the signature DXG motif of the G1 box of a chromosomal FLAG-tagged *chpA* derivative to AAA. The G1 box, along with the G2 box, is implicated in the binding and hydrolysis of ATP (19). *E. coli cheA* in which the DXG motif of the G1 box has been altered fails to autophosphorylate, shows decreased affinity for ATP *in vitro*, and is deficient for chemotaxis in vivo (13, 19). ChpA was C-terminally fused to FLAG so that levels of the wild type and mutant protein could be compared.

We assayed *chpA*-FLAG and *chpA*(AAA)-FLAG strains for TM, intracellular pilin levels, and surface piliation. The *chpA*-FLAG strain was indistinguishable from WT

in all assays (Fig. 8A, B, C), suggesting that the FLAG tag does not interfere with ChpA function. In contrast, the *chpA*(AAA)-FLAG histidine kinase mutant exhibited a phenotype similar to that of the Δ*chpA* mutant. It was defective for TM, had slightly reduced levels of intracellular pilin, and had greatly reduced surface pili (Fig. 8A, B, C). Immunoblots with α-FLAG showed equal production of ChpA-FLAG and ChpA(AAA)-FLAG, indicating that the defects in pilin function in the ChpA histidine kinase mutant was not due decreased protein levels (Fig. 8D). Interestingly, ChpA-FLAG and ChpA(AAA)-FLAG proteins were only detectable in insoluble cell lysate fraction (Fig 6D and data not shown), suggesting that ChpA may be membrane bound. In support of this idea, the TMpred program, which can be used to identify potential transmembrane regions and orientations, strongly predicts three transmembrane domains for ChpA (data not shown). These data provide genetic evidence that the histidine kinase activity of ChpA is required for its function and that Chp is membrane localized.

The Chp system may functionally regulate *pilA* transcription by controlling pilus extension and retraction

Immunoblot analysis of cell lysates prepared from Δ*chpA*, Δ *pilG*, and Δ *pilB* mutants shows an approximate two-fold decrease in intracellular pilin levels relative to WT (Fig. 3C). To determine if this defect occurred at the level of *pilA* transcription, a P_{pilA}-*lacZ* transcriptional fusion reporter was constructed, containing the 500 bp of sequence upstream of the *pilA* start codon, which are known to include all *cis* regulatory sequences required for *pilA* transcription (21, 22, 25, 27). This reporter fusion was integrated at the CTX site (4) in various mutant strain backgrounds. For bacteria cultured on solid media, *pilA* transcription is increased approximately two-fold in a Δ *pilA* background compared to WT (Fig. 9A; $p < 0.05$), suggesting that PilA may serve as a

negative regulator of *pilA* transcription. Unexpectedly, β-gatactosidase activity was reduced by approximately 90-95% relative to WT in Δ*chpA*, Δ*pilG*, and Δ*pilB*::CTX-P_{pilA}-*lacZ* reporter strains when assayed from bacteria cultured on solid media (Fig. 9A; $p < 0.05$). Although the defects in *pilA* transcription in the Δ*chpA*, Δ*pilG*, and Δ*pilB* mutant backgrounds do not appear as severe as in a Δ*pilR* mutant, where levels of transcription are similar to a promoterless *lacZ* gene incorporated at the CTX site (CTX-*lacZ*, Fig. 9A), they are not statistically different. PilR is the response regulator in the PilSR two-component regulatory system and has been shown, along with σ^{54}, to be required for *pilA* transcription (21, 22, 25, 27). *pilA* transcription was not affected in a Δ*fliC* background (Fig. 9A). Our finding that intracellular pilin levels are only decreased two fold in the Δ*chpA*, Δ *pilG*, and Δ *pilB* mutants whereas *pilA* transcription is decreased 90-95% suggests the possibility that intracellular levels of PilA may be regulated post-transcriptionally.

To further test this hypothesis, we measured *pilA* transcription in Δ*pilH*, Δ*pilT*, and Δ*pilU* mutants. As the CTX site of Δ$pilT^{\text{CTX-}pilU}$ already contained an exogenous gene (*pilU*), we introduced the P_{pilA}-*lacZ* reporter construct into the CTX site of $pilT^{\text{Trunc}}$. $pilT^{\text{Trunc}}$ reproducibly showed a 40% increase in β-galactosidase activity relative to WT when assayed from bacteria cultured on solid media (Fig. 9A; $P < 0.05$), consistent with the notion that pilin disassembly or intracellular pilin levels may regulate pilin transcription. *pilA* promoter activity was unchanged relative to WT in the Δ*pilU* mutant, further supporting the idea that PilU does not function in retraction. Interestingly, *pilA* transcription in the Δ*pilH* CTX-P_{pilA}-lacZ reporter strain, which is hyperpiliated, was reduced to 15% of WT, similar to the levels observed for Δ*chpA*, Δ*pilG*, and Δ*pilB* (Fig 8A). It is possible that *pilH* may have an additional, positive role in regulating *pilA* transcription.

We further investigated and compared *pilA* transcription in WT and mutant strains cultured in liquid media. For WT PAO1, levels of *pilA* transcription were decreased by approximately 95% for cells grown in liquid culture to stationary phase compared to cells cultured on solid media (Fig 8A; $p < 0.0001$). This finding suggests that *pilA* transcription is highly repressed in WT cells cultured in liquid media. Furthermore, given that the intracellular and surface pilin levels were similar under the two different growth conditions (Fig. 9C), these findings suggest that the turnover of pili is much slower in cells cultured in liquid compared to on solid media. Nonetheless, even in liquid culture, *pilA* transcription was increased 27-fold relative to WT in the Δ*pilA* background (Fig. 8B, $p < 0.05$). The absolute levels of *pilA* transcription for broth- and plate-cultured bacteria in the Δ*pilA* background were similar, suggesting that PilA functions as a negative regulator under both culture conditions and that *pilA* transcription was maximally derepressed in the absence of *pilA*.

Similar to bacteria cultured on solid media, $pilT^{Trunc}$ cultured in broth had increased levels of surface pili and showed a 3-fold increase in *pilA* transcription compared to WT (Fig 8A and B; $p < 0.05$). The observation that $pilT^{Trunc}$ shows a moderate increase in β-galactosidase activity suggests that pili remain functional under liquid culture conditions and, as such, PilT continues to act as a functional regulator of *pilA* transcription by mediating intracellular levels of PilA. Similarly, the hyperpiliated phenotype of Δ*pilH* suggests a role in regulating pilus retraction in liquid as on solid media (Fig. 9C).

We did not detect decreases in *pilA* transcription for mutants defective in pilin export (Δ*chpA*, Δ*pilG*, and Δ*pilB*) under liquid culture conditions (Fig. 9B). Of interest, levels of intracellular and surface pilin in Δ*chpA* and Δ*pilG* were similar to wild type, suggesting that the role they play in the regulation of pilus dynamics may be culture

condition specific (Fig. 9B). Δ*pilB*, however, lacks detectable surface pili when assayed from plates or out of broth, underscoring its essential role in controlling pilus extension (Fig. 9C). Unlike on solid media, the Δ*pilB* export defect does not correspond to a decrease in intracellular pilin levels or in *pilA* transcription (Fig. 9B). Similarly, an apparent increase in pilin production in Δ*pilH* does not correspond to increased *pilA* transcription (Fig. 9B). Some of these inconsistencies may be due, in part, to the global decrease in *pilA* transcription in cells cultured in liquid compared to on solid media which may be indicative of an overall decrease in pilus activity under liquid culture condition and which may make subtle changes in levels of *pilA* transcription difficult to detect.

Discussion

P. aeruginosa is a versatile pathogen that grows in diverse environments, in which it uses TM for locomotion, adherence, and biofilm formation. In previous work, we have described the initial characterization of the Chp signaling system, a chemosensory like system that regulates TM and biofilm formation (3, 49). In this work, we used genetic analysis to further understand the mechanism by which the Chp system controls TM. Our analysis of single, double, and triple mutants reveals that ChpA, PilG and PilB function upstream of PilH, PilT, and PilU. We also show that PilH functions downstream of PilT. Furthermore, we provide evidence that PilB and PilT retain some activity in the absence of upstream signaling from imput from ChpA and PilG, and PilH, respectively. We demonstrate that the histidine kinase domain of ChpA and the phospho-acceptor sites of PilG and PilH are required for function, supporting the hypothesis that ChpA acts as a histidine kinase to phosphorylate PilG and PilH, and that Chp system signaling is important for the regulation for TM. Finally, we present data consistent with the hypothesis that PilA is a negative regulator of *pilA* transcription and that the Chp

system, along with the ATPases PilB and PilT, serves to functionally regulate *pilA* transcription by modulating intracellular levels of PilA.

Through the construction and complementation of in-frame deletion mutants, we have confirmed the role of ChpA, PilG, and PilB in the regulation of pilus extension and the role of PilH and PilT in retraction. Although the *chpA* and *pilG* mutants show decreased levels of surface pilin, they demonstrate nearly wild-type sensitivity to infection by the Tfp-specific phage PO4, suggesting that small amounts of surface pili are sufficient to mediate infection with this phage. Interestingly, insertion mutants in *pilG* in the PAK strain background were reported to be resistant to other pilus-specific phages (11) suggesting, perhaps, that phage tropism may influence the degree to which certain strains are susceptible to infection.

Our extensive analysis of Tfp phenotypes of double and triple mutants in factors involved in extension (*chpA*, *pilG*, and *pilB*) and those involved in retraction (*pilH*, *pilT*, and possibly *pilU*) suggest that factors involved in extension function upstream of those involved in retraction. Furthermore, our data indicate that while PilB is absolutely required for pilus extension, it retains some extension activity in the absence of upstream signaling input from either ChpA or PilG. Tfp phenotypes of double and triple mutants in retraction factors suggest the presence of an additional retractile activity whose function may be enhanced by PilH, and which may or may not be encoded by *pilU* (see below). Our data also indicate that PilH acts functionally downstream of PilT, and that PilT retains some retractile activity in the absence of PilH.

PilU is a member of the family of hexameric secretion ATPases, and *pilU* is located adjacent to the *pilT* on the *P. aeruginos* chromosome, likely the result of a gene duplication event (51). Like $\Delta pilT^{\text{CTX-}pilU}$, $\Delta pilU$ is severely defective in TM. Unlike $\Delta pilT^{\text{CTX-}pilU}$, however, $\Delta pilU$ remains sensitive to phage PO4. Based on its similarity to

PilT and demonstrated ATPase activity *in vitro* (9), the most straightforward model is that PilU functions as an accessory retractile motor to PilT. Indeed, older published electron microscopy data shows hyperpiliation of a *pilU* insertion mutant (51), suggesting a role for PilU in retraction, though surface pili was not measured.

However, our data present conflicting roles for PilU. Some of our observations support its accessory role in pilus retraction. For example, the increased surface piliation observed in Δ*pilH*Δ*pilT* mutant relative to Δ*pilH* and Δ*pilT* suggests that surface pili can retract in the absence of PilH and PilT; PilU could be providing the energy for retraction in their absence. Furthermore, residual surface piliation due to basal PilB activity is only apparent in a Δ *pilG* background when both *pilT* and *pilU* are deleted (i.e., Δ*pilG*Δ*pilT*Δ*pilU*). Finally, levels of surface piliation in Δ *chpA*Δ*pilTpilU* are clearly increased relative to Δ *chpA* and Δ *chpA*Δ*pilT*$^{\text{CTX-}pilU}$. However, if PilU has retractile activity, even if minor, we would predict that Δ*pilT*Δ*pilU* would have increased surface piliation relative to Δ*pilT*$^{\text{CTX-}pilU}$. What we observed, though, is that the degree of surface piliation for Δ*pilT*Δ*pilU* is the same as that for Δ*pilT*$^{\text{CTX-}pilU}$ and that the surface piliation of Δ*pilH*Δ*pilT*Δ*pilU* is similar to Δ*pilH*Δ*pilT*. It is not known if the number or the length of the pili are different or if their spatial distribution is different in these mutants. Nonetheless, these observations suggest an additional PilH-dependent retraction factor that is not PilU.

Alternatively, or in addition to driving pilus retraction, PilU may play a role in determining the pole from which Tfp extend and retract. Indeed, recent subcellular localization studies shows that PilU has a largely monopolar distribution while PilT is found more frequently at both poles (8). A similar regulatory activity has been observed for Tfp-driven motility in *M. xanthus*, where changes in cell direction are mediated by disassembly of Tfp at one pole and reassembly at the other pole. FrzS has been shown to

localize to the pole at which Tfp are active and to oscillate between poles during changes in direction through a mechanism that is dependent upon the Frz chemosensory system (38). Like Δ*pilU*, *frzS* mutants are defective for Tfp-mediated motility. If PilU is functioning similarly to FrzS, the apparent non-motile TM phenotype of Δ*pilU* may be due less to mechanical and more to spatial dysregulation of pilus extension and retraction, where pili are extending and retracting from either both poles or neither pole. If PilU does oscillate from pole to pole, the mechanism by which is does may be similar to that of FrzS, since like FrzS, PilU maintains a monopolar localization in both Chp system mutants (including Δ*chpA*, Δ*pilG*, Δ*pilH*, and Δ*pilG*Δ*pilH*, our unpublished data), as well as in other mutants implicated in the regulation of Tfp (8). Whether or not the hypothetical oscillation of PilU is affected in these mutant backgrounds remains to be determined.

What is clear from all of these data is that, whether or not PilU has any retractile activity, its role in the regulation of Tfp function differs fundamentally from that of PilT. Interestingly, and consistent with potential different role for PilT and PilU, we provide genetic evidence suggesting that *pilT* and *pilU* are transcribed separately and that *cis* regulatory sequence required for *pilU* transcription are located within the *pilT* coding sequence. Specifically, we were able to complement the TM defect of a *pilT* truncation mutant or in ($\Delta pilT^{\text{CTX-}pilU}$), but not Δ*pilT*, by expression of *pilT* in *trans*. Our data are consistent with previous studies including the observations that: 1) insertion mutations in *pilT* are not polar on *pilU* , and 2) the *pilT* transcript was shown by Northern analysis to be 1.4 kb length, a size far to small to include both the *pilT* and *pilU* transcripts (51). Further work will need to be done to explicitly demonstrate the separate transcription of *pilT* and *pilU*, as well as to determine specific regulatory factors, and sequences required for *pilU* transcription.

We show for the first time that the histidine kinase domain of ChpA is essential for TM. ChpA is one of the most complex CheA homologs yet to be described. In addition to its histidine kinase domain, it possesses 8 potential sites of phosphorylation and a CheY-like receiver domain at its C-terminus. Previous studies have demonstrated that the second and third Hpt domains contribute to TM while the CheY domain is absolutely essential; a potential role in the regulation of swarming motility has also been shown for the fourth and fifth Hpt domains (32). It is possible that the ChpA histidine kinase domain catalyzes the autophosphorylation of Hpt2 and/or Hpt3, which then transfers phosphosphate to the C-terminal CheY domain. Alternatively, the phosphates may be transferred to PilG and/or PilH. A combination of biochemical and genetic analyses will be required to further elucidate this complex signaling pathway.

Our studies suggest a role for Tfp dynamics in regulating *pilA* transcription. This conclusion is based on three types of data. First, we found that *pilA* transcription is much lower in WT cells cultured in liquid compared to on solid media, despite the fact that the amount of intracellular and surface pili is the same. This finding suggests that pilin turnover is decreased in liquid grown cultures relative to plate grown cultures, and may correlate with a decreased need for pilus function (i.e., pilus retraction or extension) in liquid conditions. Second, in the absence of PilA or in the absence of retraction, *pilA* transcription is increased, suggesting that unassembled pilin subunits may function as a negative regulator of *pilA* transcription. Specifically, we observed a two-fold increase in *pilA* transcription in Δ*pilA* and a reproducible but smaller increase in transcription in $\Delta pilT^{\text{CTX-}pilU}$. When we attempted to overproduce PilA by expression from a plasmid borne copy under an inducible promoter, we were unable to increase pilin levels or to affect transcription from the native *pilA* promoter driving *lacZ* (our unpublished data). Our data is consistent with prior reports that showed negative feedback regulation of *pilA*

transcription by PilA in *P. aeruginosa* (14) and in *M. xanthus* (24, 53), where it is postulated that unassembled pilin subunits are sensed by the PilS/PilR two component regulatory system (24). Third, our data suggest that the Chp system, along with the ATPases PilB and PilT, serves to functionally regulate *pilA* transcription by modulating intracellular levels of PilA. Specifically, we show that mutants with defects in PilA export (Δ*chpA*, Δ*pilB*, and Δ*pilG*) have decreased *pilA* transcription whereas a mutant defective in pilin disassembly (Δ$pilT^{\text{CTX-}pilU}$) has increased *pilA* transcription. Consistent with our data, (26) published that *pilA* transcription by both Northern analysis and *lacZ*-assay was up-regulated in hyperpilated mutants and down-regulated in hypopiliated mutants. These data are similar to observations made in other organisms such as the cyanobacterium *Synechocystis* sp. PCC6803 where hyperpiliated *pilT1* mutants have been shown to accumulate higher than normal levels of *pilA1* transcript (5). Whitchurch and Mattick (51), in contrast, reported Northern blot analysis demonstrating decreased pilA mRNA in *pilT* or *pilU* insertion mutants. One attractive model is that as the pilus is dissembled, PilS senses pilin levels associated with the inner membrane and regulates PilR activity and *pilA* transcription.

We note that transposon insertion mutations in *pilG* and other Chp system components (*pilH*, *pilI*, and *pilJ*) have been reported to have wild type levels of *pilA* transcription in strain PAK (11, 12), although at least one *chpA* insertion mutant has been shown to have a defect in *pilA* transcription (49). Point mutations to an Hpt domain (H1088Q) and the CheY-domain (D2406N) of ChpA resulted in increased *pilA* transcription by Northern blot analysis (32). The reason for the discrepancy between the insertion mutant, Δ*chpA*, and the point mutants remains to be determined; it may reflect strain differences or differences in culture conditions. It is also unclear if the increase in *pilA* transcription in the *chpA* point mutants corresponds to increased surface piliation.

Mutations in components of other putative chemosensory systems proposed to regulate Tfp-driven motility have been shown to have defects in *pilA* transcription (52).

The phenotype of the *pilH* mutant was complex. Δ*pilH* has aberrant TM and increased surface pili. Based on its surface presentation phenotype, we would predict that the *pilH* mutant would show increased levels of *pilA* transcription; however, it is actually reduced to levels near those of Δ*chpA*, Δ*pilG*, and Δ*pilB*, suggesting that PilH may play some additional, positive role in regulating *pilA* transcription. The idea that PilH may have additional regulatory functions is supported by preliminary observations that, unique among the Chp system mutants, Δ*pilH* mutant shows altered pyocyanin and rhamnolipid production that correspond to change in *phzA1* and *rhlA* transcription and increased virulence in a *Drosophila melanogaster* infection model (our unpublished data). Furthermore, this phenotype may be tied to the ability of PilH to function as a signaling molecule as site-directed mutants of PilH, in which the aspartate residue in the putative phospho-acceptor site is replaced with alanine, recapitulate the virulence factor production phenotypes of the null mutant (our unpublished data). Further studies will need to be carried out to determine the full range of PilH function.

The data presented here provides genetic evidence consistent with a model in which ChpA acts as a histidine kinase to phosphorylate PilG and/or PilH. Phosphorylated PilG, in turn, modulates PilB to drive pilus extension, and phosphorylated PilH interacts with PilT and/or PilU to drive pilus retraction. Future studies will focus on complementing this genetic picture with biochemical support. It will be of interest to test whether (i) ChpA undergoes autophosphorylation, (ii) and if so, at which of its eight potential phosphorylation sites, and (iii) whether it can directly transfer phosphate to its own CheY domain or to PilG or PilH. Moreover, direct interactions between ChpA and PilG, ChpA and PilH, PilG and PilB, and PilH and

PilT/PilU remain to be shown. Interestingly, these interactions may be more complex than we previously imagined. A recent study demonstrated direct binding of PilZ and FimX, two proteins previously shown to have roles in the regulation of Tfp function, to PilB (17). Whether and how PilG interacts with this complex remains to be determined. Also unclear is if a similar or completely novel complex exists for PilT, and whether and how PilH might interact with those components of the retraction apparatus.

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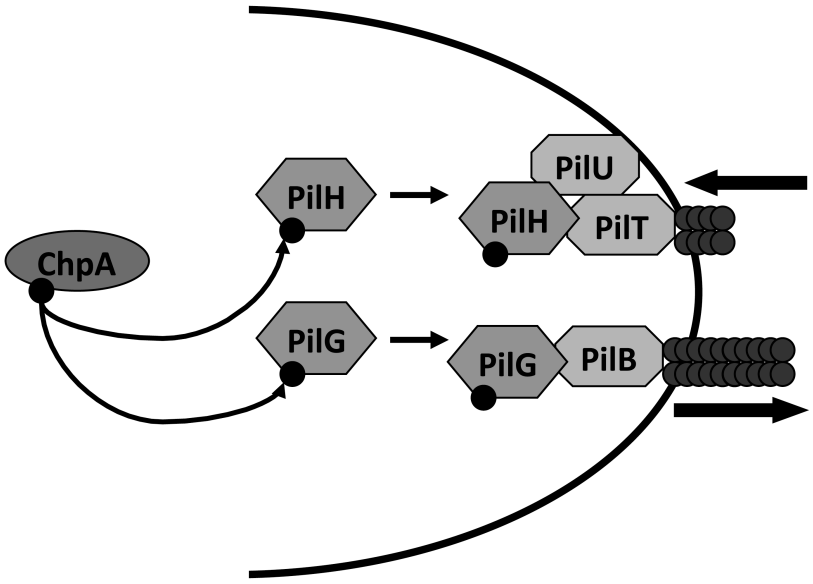

Figure 2. Model for Chp system signaling in the regulation of pilus extension and retraction. The histidine kinase ChpA undergoes autophosphorylation and transfers phosphate groups to two CheY-like response regulator proteins, PilG and PilH. PilG-P interacts with the ATPase PilB to mediate pilus extension. PilH-P interacts with PilT and/or PilU to mediate pilus retraction.

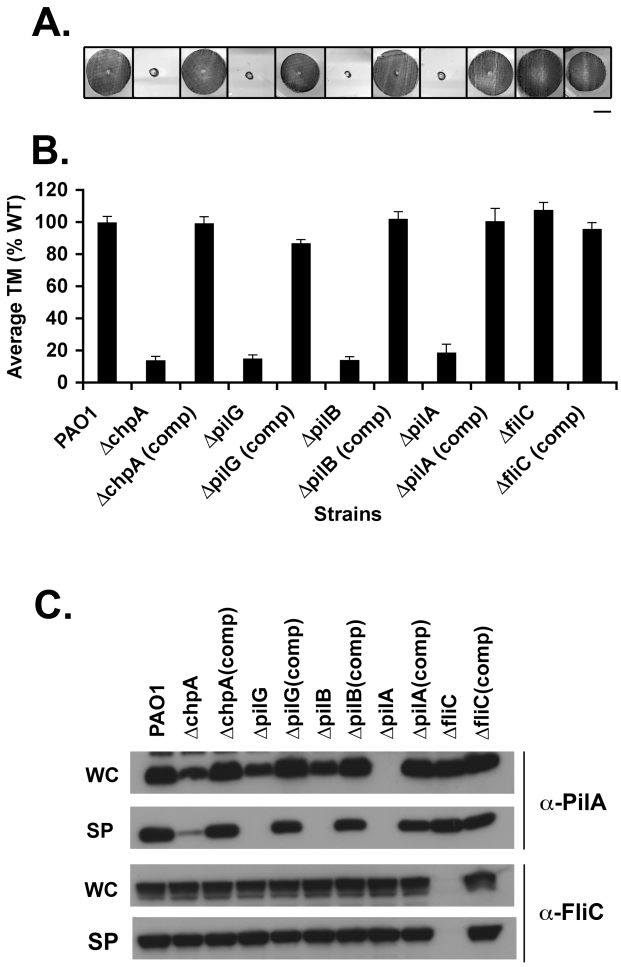

Figure 3. Assays of pilus function for mutants defective in pilus extension. (A) Subsurface TM assay of PAO1, the indicated in-frame deletion mutants, and complemented (comp) strains in which the wild type gene was reintroduced at its endogenous locus. TM assays were performed by the subsurface stab method followed by Coomassie blue staining, as described previously (REF). The bar represents 1 cm. (B) Graph depicting TM zone diameters for the indicated strains. The diameter is expressed as a percentage of PAO1. Shown are the means ± the standard deviation (SD, N=5) (C) Intracellular and surface pilin levels. For the indicated strains, surface pili (SP) were sheared by vigorous vortexing of bacteria cultured on solid media and separated

from cells (WC) by centrifugation. The sheared pili were precipitated. WC (15 µg total protein) and SP (5% of the total resuspended volume of precipitated pili) samples were separated by SDS-PAGE and immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC).

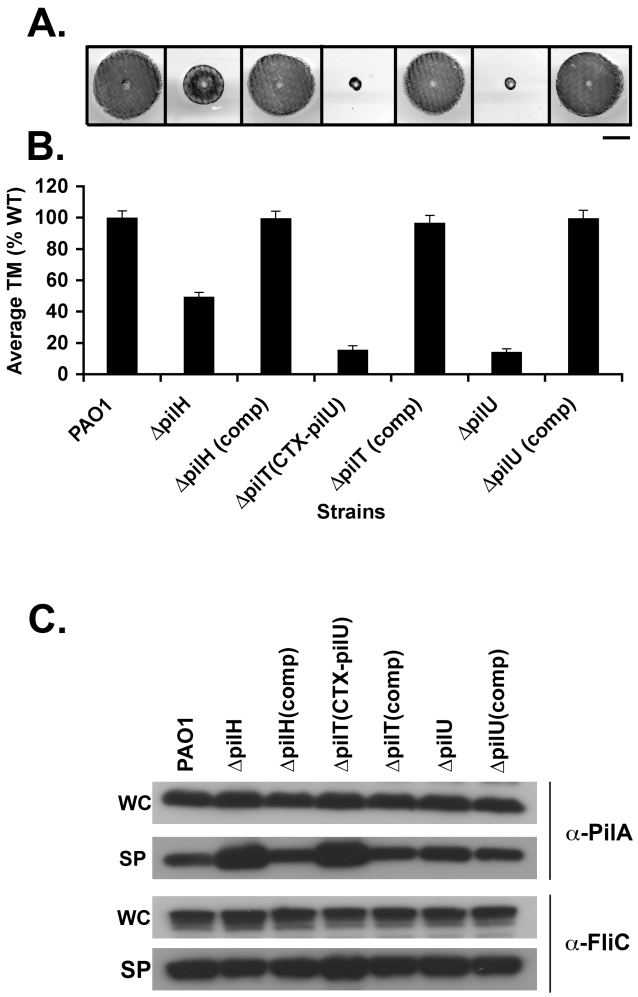

Figure 4. Assays of pilus function for mutants defective in pilus retraction. (A) Subsurface TM assays were performed as described in Fig. 2. The bar represents 1 cm. (B) Graph depicting average twitching zone diameters for the indicated strains. Shown are the means ± SD (N=5) (C) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC), as described in Fig. 2.

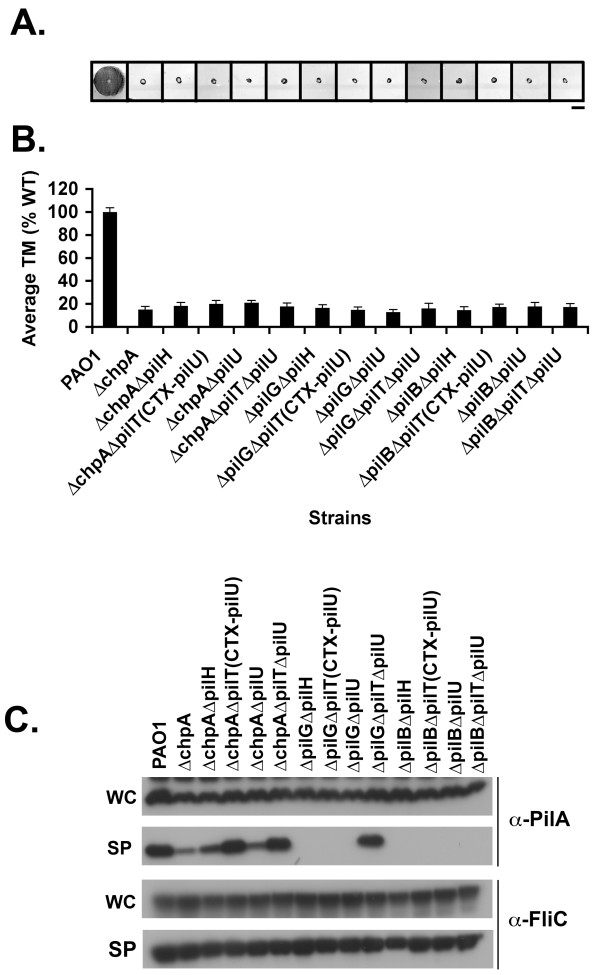

Figure 5. *chpA*, *pilG*, and *pilB* function upstream of *pilH*, *pilT*, and *pilU*. (A) Subsurface TM assays were performed as described in Fig. 2. The bar represents 1 cm. (B) Graph depicting average TM zone diameters for the indicated strains. Shown are the means ± SD (N=5) (C) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC), as described in Fig. 2.

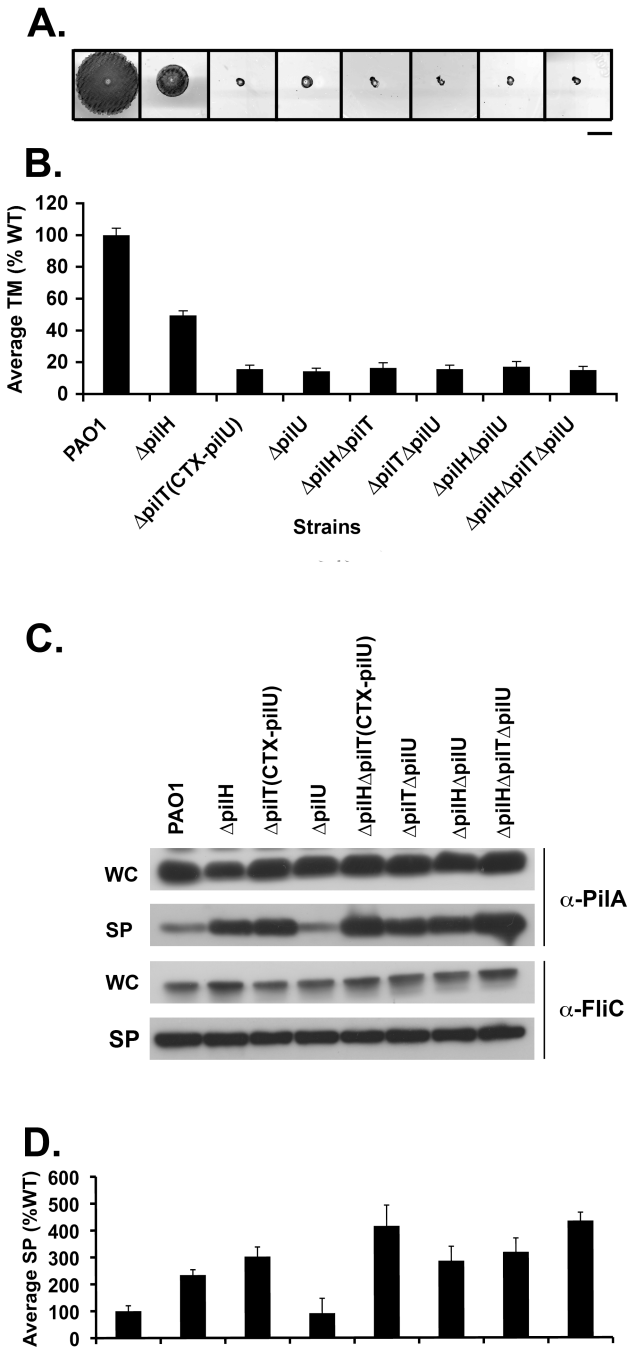
A.
B.
Average TM (% WT)
120
100
80
60
40
20
0
PAO1
ΔpilH
ΔpilT(CTX-pilU)
ΔpilU
ΔpilHΔpilT
ΔpilTΔpilU
ΔpilHΔpilU
ΔpilHΔpilTΔpilU
Strains
C.
PAO1
ΔpilH
ΔpilT(CTX-pilU)
ΔpilU
ΔpilHΔpilT(CTX-pilU)
ΔpilTΔpilU
ΔpilHΔpilU
ΔpilHΔpilTΔpilU
WC
SP
α-PilA
WC
SP
α-FliC
D.
Average SP (%WT)
600
500
400
300
200
100
0
PAO1
ΔpilH
ΔpilT(CTX-pilU)
ΔpilU
ΔpilHΔpilT(CTX-pilU)
ΔpilTΔpilU
ΔpilHΔpilU
ΔpilHΔpilTΔpilU
Strains

Figure 6. *pilH* functions downstream of *pilT*. (A) Subsurface TM assays were performed as described in Fig. 2. The bar represents 1 cm. (B) Graph depicting average TM zone diameters for the indicated strains. Shown are the means ± SD (N=5) (C) A 1:5 dilution of surface pili (SP) and cell associated (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC), as described in Fig. 2. (D) Quantification of surface piliation levels relative to a FliC loading control. Surface piliation levels are expressed as a percentage of PAO1. Shown are the means ± SD (N=3)

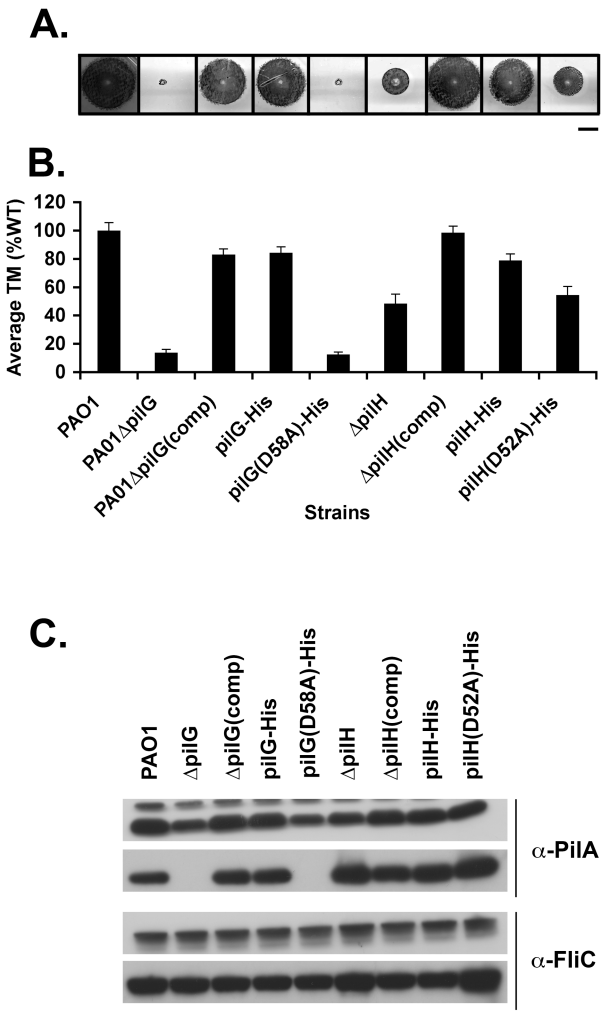

Figure 7. The phospho-acceptor sites of PilG and PilH are required for function. (A) Subsurface TM assays of PAO1, Δ*pilG*, Δ*pilG*(comp), Δ*pilH*, Δ*pilH*(comp), His-tagged *pilG* (*pilG*-His) and *pilH* (*pilH*-His), and His-tagged *pilG* and *pilH* point mutants, *pilG*(D58A)-His and *pilH*(D52A)-His. The bar represents 1 cm. (B) Graph depicting average TM zone diameters for the indicated strains. Shown are the means ± SD (N=5) (C) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC), as described in Fig. 2.

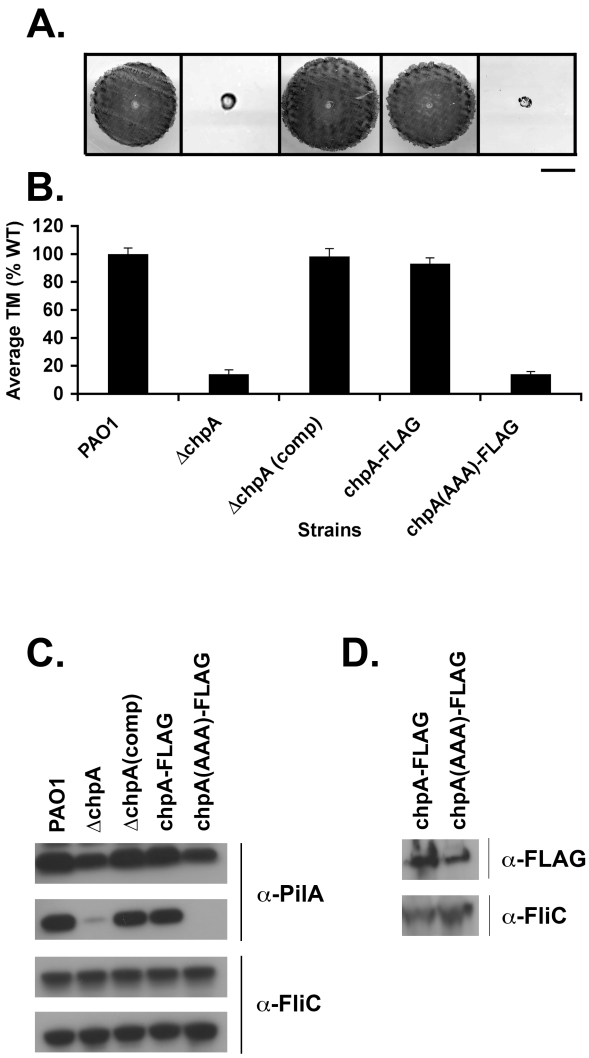

Figure 8. The histidine kinase domain of ChpA is required for function. (A) Subsurface TM assay of PAO1, Δ *chpA*, Δ *chpA*(comp), FLAG-tagged *chpA* (*chpA*-FLAG), and a FLAG-tagged *chpA* histidine kinase mutant (*chpA*(AAA)-FLAG). The bar represents 1 cm. (B) Graph depicting average TM zone diameters for the indicated strains. Shown are the means ± SD (N=5) (C) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC), as described in Fig. 2. (D) Levels of ChpA-FLAG and ChpA(AAA)-

FLAG. To prepare protein samples, cells were harvested from 2 mL of culture grown shaking for 16 hrs at 37˚C, resuspended in 400 µL SDS-PAGE sample buffer, passaged through a 27½ gauge needle 3 times, and boiled for 5 min. 10% of total sample volumes were separated by SDS-PAGE and immunoblotted with 2.5 µg/mL α-FLAG M2 monoclonal antibody.

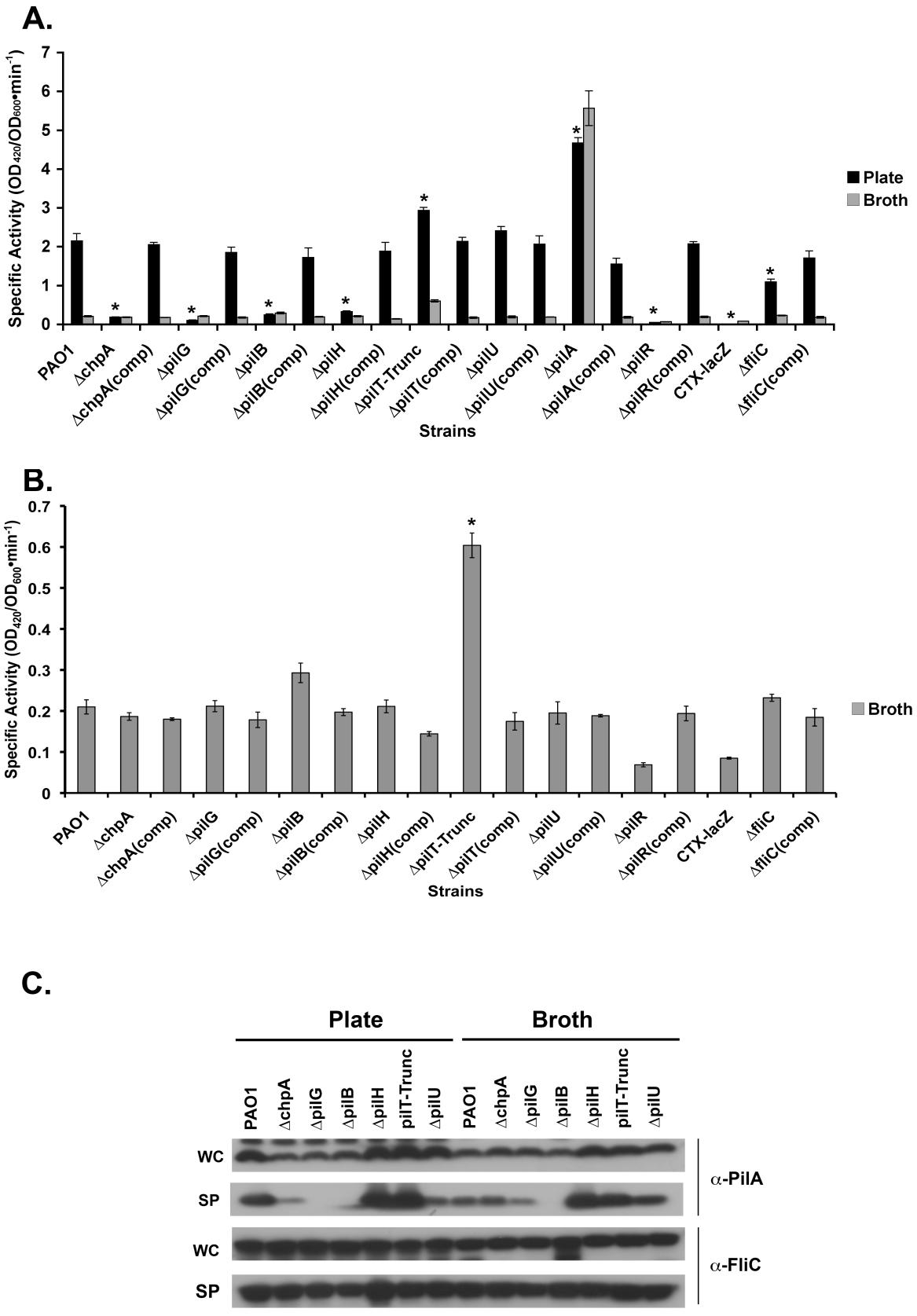
A.
Specific Activity (OD420/OD600•min-1)
Plate
Broth
PAO1
ΔchpA
ΔchpA(comp)
ΔpilG
ΔpilG(comp)
ΔpilB
ΔpilB(comp)
ΔpilH
ΔpilH(comp)
ΔpilT-Trunc
ΔpilT(comp)
ΔpilU
ΔpilU(comp)
ΔpilA
ΔpilA(comp)
ΔpilR
ΔpilR(comp)
CTX-lacZ
ΔfliC
ΔfliC(comp)
Strains
B.
Specific Activity (OD420/OD600•min-1)
Broth
Strains
C.
Plate
Broth
PAO1
ΔchpA
ΔpilG
ΔpilB
ΔpilH
pilT-Trunc
ΔpilU
WC
SP
α-PilA
α-FliC

Figure 9. The Chp system functionally regulates *pilA* transcription by mediating intracellular levels of PilA. (A) The indicated strains harboring the P_{pilA}-*lacZ* fusion and PAO1 harboring a promotorless *lacZ* gene (CTX-*lacZ*) integrated into the chromosome at the *attB* site were cultured in liquid (Panels A and B) or on solid media (Panel A) for 16 hrs. The β-galactosidase activity of samples was measured and specific activities relative to OD_{600} calculated. Shown are the means ± SD (N=3). Statistical significance was determined by ANOVA using Instat software. Differences from WT were considered to be significant at $P < 0.05$ and are indicated by *. (C) Surface pili (SP) and cell associated (WC) preparations of the indicated strains cultured in liquid (broth) or on solid media (plate) were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC), as described in Fig. 2.

Table 1. Strains and plasmids used in chapter 2

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	
PAO1Δ*chpA*	In frame deletion of *chpA*	(55)
PAO1Δ*chpA*(comp)	PAO1Δ*chpA* complemented at the *chpA* locus	This study
PAO1Δ*fliC*	In frame deletion of *fliC*	This study
PAO1Δ*fliC*(comp)	PAO1Δ*fliC* complemented at the *fliC* locus	This study
PAO1Δ*pilA*	In frame deletion of *pilA*	This study
PAO1Δ*pilA*(comp)	PAO1Δ*pilA* complemented at the *pilA* locus	This study
PAO1Δ*pilB*	In frame deletion of *pilB*	This study
PAO1Δ*pilA*(comp)	PAO1Δ*pilB* complemented at the *pilB* locus	This study
PAO1Δ*pilG*	In frame deletion of *pilG*	This study
PAO1Δ*pilG*(comp)	PAO1Δ*pilG* complemented at the *pilG* locus	This study
PAO1Δ*pilH*	In frame deletion of *pilH*	(3)
PAO1Δ*pilH*(comp)	PAO1Δ*pilH* complemented at the *pilH* locus	This study
PAO1Δ*pilR*	In frame deletion of *pilR*	This study
PAO1Δ*pilR*(comp)	PAO1Δ*pilR* complemented at the *pilR* locus	This study
PAO1Δ*pilT*$^{\text{CTX-}pilU}$	In frame deletion of *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAO1*pilT*$^{\text{Trunc}}$	*pilT* truncation mutant, deletes 429 bp of *pilT* including start codon; stop codons in all three reading frames.	This study
PAO1Δ*pilT*(comp)	PAO1Δ*pilT* complemented at the *pilT* locus	This study
PAO1Δ*pilU*	In frame deletion of *pilU*	This study
PAO1Δ*pilU*(comp)	PAO1Δ*pilU* complemented at the *pilU* locus	This study
PAO1Δ*chpA*Δ*pilH*	In frame deletion of *chpA* and *pilH*	This study
PAO1Δ*chp*Δ*pilT*$^{\text{CTX-}pilU}$	In frame deletion of *chpA* and *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAO1Δ*chpA*Δ*pilU*	In frame deletion of *chpA* and *pilU*	This study
PAO1Δ*chpA*Δ*pilT*Δ*pilU*	In frame deletion of *chpA*, *pilT*, and *pilU*	This study
PAO1Δ*pilB*Δ*pilH*	In frame deletion of *pilB* and *pilH*	This study
PAO1Δ*pilB*Δ*pilT*$^{\text{CTX-}pilU}$	In frame deletion of *pilB* and *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAO1Δ*pilB*Δ*pilU*	In frame deletion of *pilB* and *pilU*	This study
PAO1Δ*pilB*Δ*pilT*Δ*pilU*	In frame deletion of *pilB*, *pilT*, and *pilU*	This study

PAO1Δ*pilG*Δ*pilH*	In frame deletion of *pilG* and *pilH*	This study
PAO1Δ*pilGpilT*-gent	Δ*pilG* with a Gm^R-tagged insertion in *pilT*	This study
PAO1Δ*pilG*Δ*pilT*$^{CTX\text{-}pilU}$	In frame deletion of *pilG* and *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAO1 Δ*pilGpilU*-gent	Δ*pilG* with a Gm^R-tagged insertion in *pilU*	This study
PAO1Δ*pilG*Δ*pilU*	In frame deletion of *pilG* and *pilU*	This study
PAO1Δ*pilG*Δ*pilT*Δ*pilU*	In frame deletion of *pilG*, *pilT*, and *pilU*	This study
PAO1Δ*pilH*Δ*pilT*$^{CTX\text{-}pilU}$	In frame deletion of *pilH* and *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAO1Δ*pilT*Δ*pilU*	In frame deletion of *pilT* and *pilU*	This study
PAO1Δ*pilH*Δ*pilU*	In frame deletion of *pilH* and *pilU*	This study
PAO1Δ*pilH*Δ*pilT*Δ*pilU*	In frame deletion of *pilH*, *pilT*, and *pilU*	This study
PAO1*chpA*-FLAG	*chpA* with a double FLAG tag inserted into the *Not*I/*Kpn*I sites of *chpA* at the *chpA* locus	This study
PAO1*chpA*(AAA)-FLAG	*chpA*-FLAG with ChpA D2091A, D2092A, G2093A	This study
PAO1*pilG*-His	*pilG* with a C-terminal 6-His tag at the *pilG* locus	This study
PAO1*pilG*(D58A)-His	*pilG*-His with PilG D58A	This study
PAO1*pilH*-His	*pilH* with a C-terminal 6-His tag at the *pilH* locus	This study
PAO1*pilH*(D52A)-His	*pilH*-His with PilH D52A	This study
PAO1 CTX-P_{pilA}-*lacZ*	PAO1; *pilA* promoter fused to *lacZ* at *attB* site	
PAO1Δ*chpA* CTX-P_{pilA}-*lacZ*	Δ*chpA*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*chpA*(comp) CTX-P_{pilA}-*lacZ*	Δ*chpA*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*fliC* CTX-P_{pilA}-*lacZ*	Δ*fliC*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*fliC*(comp) CTX-P_{pilA}-*lacZ*	Δ*fliC*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilA* CTX-P_{pilA}-*lacZ*	Δ*pilA*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilA*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilA*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilB* CTX-P_{pilA}-*lacZ*	Δ*pilB*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilA*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilB*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilG* CTX-P_{pilA}-*lacZ*	Δ*pilG*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilG*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilG*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study

PAO1Δ*pilH* CTX-P_{pilA}-*lacZ*	Δ*pilH*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilH*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilR* CTX-P_{pilA}-*lacZ*	Δ*pilR*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilR*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilR*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1*pilT*^{Trunc} CTX-P_{pilA}-*lacZ*	*pilT*-Trunc; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilT*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilT*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilU* CTX-P_{pilA}-*lacZ*	Δ*pilU*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilU*(comp) CTX-P_{pilA}-*lacZ*	Δ*pilU*(comp); *pilA* promoter fused to *lacZ* at *attB* site	This study
		This study
E. coli		This study
DH5α	*hsdR rec lacZYA ϕ80 lacZM15*	Invitrogen
S17-1 λpir	*Thi pro hsdR recA RP4-2*(Tc::Mu)(Km::Tn7)	Stratagene
SM10	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*	(13)
Plasmids		
pOK12	*E. coli* cloning vector, Km^R	(49)
pUCPSK	*P. aeruginosa-E. coli* shuttle vector, Ap^R	(51)
pGEM-T	*E. coli* cloning vector, Ap^R	Promega
pEX100T	Allelic replacement suicide plasmid, $Ap^R(Cb^R)$	(45)
pX1918GT	Source of Gm^R cassette, Ap^R	(45)
mCTX-lacZ	Contains promoterless *lacZ* for constructing transcriptional fusions at the *attB* site, Tc^R	(4)
mCTX2	Construct for introduction of exogenous DNA fragments at the *attB* site	(22)
pFLP2	Source of Flp recombinase, Ap^R	(21)
pAL64	pUCPSK carrying *chpA* with an internal double FLAG tag inserted between the native *Not*I/*Nru*I sites	(34)
pEN34	Allelic replacement suicide plasmid, Tc^R	(55)
pJEN34PAO1KO3	pJEN34 carrying Δ*pilA* on *Spe*I fragment	This study
pJB4	pOK12 carrying Δ*chpA* on *Eco*RI/*Xba*I fragment	(55)
pJB43	pGEM-T containing 1 KB region 5′ of *pilG*	This study
pJB44	pGEM-T containing 1 KB region 3′	This study

	of *pilG*	
pJB48	pGEM-T containing 1 KB region 3´ of *pilH*	(3)
pJB60	*Xho*I/*Hind*III fragment from pJB43 and *Hind*III/*Xba*I from pJB44 concatamerized and cloned into *Xho*I/*Xba*I sites of pOK12	This study
pJB62	pOK12 carrying Δ*pilH* on *Kpn*I/*Xba*I fragment	(3)
pJB87	pJEN34 carrying Δ*pilG* on *Spe*I fragment	This study
pJB96	*Xho*I/*Hind*III fragment from pJB43 and *Hind*III/*Xba*I from pJB48 concatamerized and cloned into *Xho*I/*Xba*I sites of pOK12	This study
pJB97	pJEN34 carrying Δ*pilG*Δ*pilH* on *Spe*I fragment	This study
pJB100	pEX100T derivative replacing *Sma*I site with *Spe*I site	
pJB109	pJB100 carrying Δ*pilT* on *Spe*I fragment	This study
pJB110	Gm^R-tagged Δ*pilT* in pJB100; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB109	This study
pJB111	pJB100 carrying Δ*pilU* on *Spe*I fragment	This study
pJB112	Gm^R-tagged Δ*pilU* in pJB100; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB109	This study
pJB113	pJB100 carrying Δ*pilT* complementation construct on *Spe*I fragment	This study
pJB114	pJB100 carrying Δ*pilU* complementation construct on *Spe*I fragment	This study
pJB116	pJB100 carrying Δ*chpA* on *Spe*I fragment; subcloned from pJB4	This study
pJB118	pJB100 carrying Δ*pilG* on *Spe*I fragment; subcloned from pJB60	This study
pJB119	pJB100 carrying Δ*pilH* on *Spe*I fragment; subcloned from pJB62	This study
pJB122	pJB100 carrying Δ*pilB* on *Spe*I fragment	This study
pJB124	pJB100 carrying *pilH*-His allelic exchange construct on *Spe*I fragment	This study
pJB126	pJB100 carrying Δ*pilG* complementation construct on *Spe*I fragment	This study
pJB127	pJB100 carrying *pilG*-His allelic exchange construct on *Spe*I fragment	This study
pJB163	pJB100 carrying Δ*pilT*Δ*pilU* on *Spe*I	This study

	fragment	
pJB202	FLAG-tagged *chpA* histidin kinase domain in pOK12; subcloned as *Kpn*I/*Cla*I fragment from pAL64	This study
pJB203	pJB100 carrying *pilT* truncation construct on *Spe*I fragment	This study
pJB207	*chpA*(AAA) histidine kinase domain in pOK12	This study
pJB212	*pilU* and 1 KB 5′ sequence cloned as *Spe*I/*Hind*III fragment into mCTX2	This study
pJB215	pJB100 carrying Δ*fliC* on *Spe*I fragment	This study
pJB219	Modified pOK12 lacking *Kpn*I and *Cla*I sites; *Bgl*II/*Sac*I fragment removed from pOK12 MCS	This study
pJB220	3′ *chpA* fragment including 1 KB 3′ flanking sequence in pJB219	This study
pJB221	*chpA* including 1 KB of 5′ and 3′ flanking sequence in pJB219	This study
pJB230	pJB100 carrying Δ*fliC* complementation construct on *Spe*I fragment	This study
pJB231	pJB100 carrying Δ*chpA* complementation construct on *Spe*I fragment; subcloned from pJB221	This study
pJB232	*chpA*-FLAG including 1 KB of 5′ and 3′ flanking sequence in pJB219; *Kpn*I/*Cla*I fragment from pJB202 subcloned into pJB221 from which the corresponding *Kpn*I/*Cla*I fragment had been removed	This study
pJB233	*chpA*(AAA)-FLAG including 1 KB of 5′ and 3′ flanking sequence in pJB219; *Kpn*I/*Cla*I fragment from pJB207 subcloned into pJB221 from which the corresponding *Kpn*I/*Cla*I fragment had been removed	This study
pJB234	pJB100 carrying *chpA*-FLAG allelic exchange on *Spe*I fragment; subcloned from pJB232	This study
pJB235	pJB100 carrying *chpA*-FLAG allelic exchange on *Spe*I fragment; subcloned from pJB233	This study
pJB241	pJB100 carrying Δ*pilA* complementation construct on *Spe*I fragment	This study
pJB246	pJB100 carrying Δ*pilB* complementation construct on *Spe*I fragment	This study
pJB247	pOK12 carrying *pilG*-His allelic exchange construct on *Spe*I fragment	This study

pJB248	pOK12 carrying *pilH*-His allelic exchange construct on *Spe*I fragment	This study
pJB249	*pilA* reporter construct cloned as *Xho*I/*BamH*I fragment into mCTX-*lacZ*	This study
pJB250	pJB100 carrying Δ*pilR* on *Spe*I fragment	This study
pJB251	pJB100 carrying Δ*pilR* complementation construct on *Spe*I fragment	This study
pJB252	pOK12 carrying *pilG*(D58A)-His allelic exchange construct on *Spe*I fragment	This study
pJB254	pOK12 carrying *pilH*(D582)-His allelic exchange construct on *Spe*I fragment	This study
pJB256	pJB100 carrying *pilG*(D58A)-His allelic exchange construct on *Spe*I fragment	This study
pJB258	pJB100 carrying *pilH*(D582)-His allelic exchange construct on *Spe*I fragment	This study

Table 2. Oligonucleotides used in chapter 2

Primer	Oligonucleotide sequence (5′→3′)[1]
chpA22	GGACTAGTAGATGGCGAGCGAGATGC
chpA25	GAGCTCCTCGCGCAGCGC
chpA26.1	GGCGCGCAACGGCCATTG
chpA29	GGACTAGTCGCAGCGTGCGGCCAGAT
chpA-HisKn-F	CTCCTCACCCTCTCCGCCGCCGCCGCCGGCATCCGCCTC
chpA-HisKn-R	GAGGCGGATGCCGGCGGCGGCGGCGGAGAGGGTGAGGAG
fliC1	GG ACTAGT ACCGTTTCGTGGTCTCGA
fliC2	TTAGCGCAGCAGAAGCTTAAGGGCCATGGTGATTTCCTCCAAAGG
fliC3	ATGGCCCTTAAGCTTCTGCTGCGCTAAGCCCGGGAACGGTCACTC
fliC4	GGACTAGTATTGATGGCGTCACGCAT
pilA1	CCATGGATGCCTAACCTCACCCTTGC
pilA1(Spe)	ACTAGTATGCCTAACCTCACCCTTGC
pilA2	GAATTCTAAGGTGATCGAAGGTGGCTTGTTTAG
pilA3	GAATTCCATGAATATCTCCATTGATATG
pilA4	TCTAGATTGATCCGTCCGTCCTGCGG
pilA4(Spe)	ACTAGTTTGATCCGTCCGTCCTGCGG
pilA-Reporter-F	CGCGGATCCGAATCTCTCCGTTGATTA
pilA-Reporter-R	CCGCTCGAGGTGTTGGCGGACCAGCTT
pilB1	GCACTAGTATCAACGAGGGCACCCTGCGC
pilB2	TTAATCCTTGGTAAGCTTGTCGTTCATGGGGAAGGAATCGCAGAAG GG
pilB3	ATGAACGACAAGCTTACCAAGGATTAA TCCATGGCGGACAAAGCGTTA
pilB4	GCACTAGTTGGATCGCCATGTTGGGAAAG
pilG1	CGGGGTACCCTCAGCGGCTGGGCCACGCCGCG
pilG1(Spe)	GCACTAGTCTCAGCGGCTGGGCCACGCCGCG
pilG2	AAGCTTTTGCTGTTCCATGTTCGCCC
pilG3	AAGCTTAAGGGGCGCATCGTCGGCTC
pilG4	TCTAGAATGAAGGGTTGCAGTGCCGC
pilG4(Spe)	GCACTAGTATGAAGGGTTGCAGTGCCGC
pilG5	TCAGTGGTGGTGGTGGTGGTGGGAAACGGCGTCCACCGGGGT
pilG6	ACCCCGGTGGACGCCGTTTCCCACCACCACCACCACCACTGA
pilG-D58A-F	AACATCATTTTCGTCGCCATCATGATGCCGCGC
pilG-D58A-R	GCGCGGCATCATGATGGCGACGAAAATGATGTT
pilH1	GGTACC CGTTATCGAAGGGCGGGTCC
pilH1(Spe)	GCACTAGTCGTTATCGAAGGGCGGGTCC
pilH4	TCTAGAGCTTTCTCGAAGTCGTTGCG
pilH4(Spe)	GCACTAGTGCTTTCTCGAAGTCGTTGCG
pilH15	TCAGTGGTGGTGGTGGTGGTGGCCCGCCAGCACCGCATTG
pilH16	CAATGCGGTGCTGGCGGGCCACCACCACCACCACCACTGA
pilH-D52A-F	GACGTGGTCCTGATGGCCATCGTCATGCCCGGC
pilH-D52A-R	GCCGGGCATGACGATGGCCATCAGGACCACGTC
pilR1	GGACTAGTCTGCAGCGCATGCGCACC
pilR2	TCAGTCGATGCCAAGCTTTCGGCTCATGCGTGCGGCTTCCGTCAG
pilR3	ATGAGCCGAAAGCTTGGCATCGACTGAAAGTGAAAAGGCCTGTCC
pilR4	GGACTAGTATCGTCTCGCCGCTCTAT
pilT1	GGACTAGT TGGCACACGGCTTTCATGG

pilT1(new)	GGACTAGT AACTCGCCGGCGATCTTC
pilT4	GGACTAGT TGTAGAGCTGGTACAGCGC
pilT5	TCAGAAGTTTTCAAGCTTAATATCCATGGGACTCCCCAATTACAAG C
pilT6	ATGGATATTAAGCTTGAAAACTTCTGATCCTGGCGCCGATCCGC
pilT-Trunc2	CAGGTAATCGAGTCTAGTCTAGACTAGGGGACTCCCCAATTACAA GCAAGCAGG
pilT-Trunc3	GGGAGTCCCCTAGTCTAGACTAGACTCGATTACCTGAACAACACC AAGTACCAC
pilT-Trunc4	GGACTAGTGAAGTTGCACTCATGGTT
pilU1	GGACTAGTAACACAAGCAGGTGCATGC
pilU4	GGACTAGTAAGGTGAAGAACCAGATCG
pilU5	TCAGCGGAAGCGTCTAGAGAATTCCATGATGTTCTCGCTCACTCAG G
pilU6	ATGGAATTCTCTAGACGCTTCCGCTGAACCGTCTTCAGTAGGCCAG C
pilTpilU1	TCAGCGGAAGCGAATATCCATGGGACTCCCCAATTACAAGC
pilTpilU2	ATGGATATTCGCTTCCGCTGAACCGTCTTCAGTAGGCCAGC
pilU-CTX-Spe-F	GGACTAGTGACCGGGTGGTCGACGTGTTC
pilU-CTX-Hind-R	CCCAAGCTTTCAGCGGAAGCGCCGGCCGGC

[1]Restriction endonuclease sequences are underlined

Table 3. Sensitivity (expressed as the average ± SD)of various strains to infection by the pilus-specific phage PO4

Strain	10^9 Pfu/mL
PA01	3.96 ± 0.53
Δ*chpA*	3.30 ± 0.70
Δ*chpA*(comp)	3.75 ± 0.79
Δ*pilG*	2.85 ± 0.72
Δ*pilG*(comp)	4.52 ±0.66
Δ*pilB*	0.00 ± 0.00*
Δ*pilB*(comp)	3.92 ± 0.32
Δ*pilA*	0.00 ± 0.00*
Δ*pilA*(comp)	4.12 ± 0.56
Δ*fliC*	3.92 ± 0.56
Δ*fliC*(comp)	5.41 ± 0.14
Δ*pilH*	2.96 ± 0.58†
Δ*pilH*(comp)	3.48 ± 0.45
Δ*pilT*$^{\text{CTX-}pilU}$	0.00 ± 0.00*
Δ*pilT*(comp)	3.39 ± 0.17
Δ*pilU*	2.88 ± 0.33
Δ*pilU*(comp)	4.11 ± 0.48
Δ*chpA*Δ*pilH*	4.00 ± 0.45†
Δ*chpA*Δ*pilT*$^{\text{CTX-}pilU}$	0.00 ± 0.00*
Δ*chpA*Δ*pilU*	1.85 ± 0.3^0
Δ*chpA*Δ*pilT*Δ*pilU*	0.00 ± 0.00*
Δ*pilG*Δ*pilH*	3.93 ± 0.72†
Δ*pilG*Δ*pilT*$^{\text{CTX-}pilU}$	0.00 ± 0.00*
Δ*pilG*Δ*pilU*	3.07 ± 0.78
Δ*pilG*Δ*pilT*Δ*pilU*	0.00 ± 0.00*
Δ*pilB*Δ*pilH*	0.00 ± 0.00*
Δ*pilB*Δ*pilT*$^{\text{CTX-}pilU}$	0.00 ± 0.00*
Δ*pilB*Δ*pilU*	0.00 ± 0.00*
Δ*pilB*Δ*pilT*Δ*pilU*	0.00 ± 0.00*
Δ*pilH*Δ*pilT*$^{\text{CTX-}pilU}$	0.00 ± 0.00*
Δ*pilT*Δ*pilU*	0.00 ± 0.00*
Δ*pilH*Δ*pilU*	2.87 ± 0.83†
Δ*pilH*Δ*pilT*Δ*pilU*	0.00 ± 0.00*
chpA-FLAG	3.45 ± 0.63
Δ*chpA*(AAA)-FLAG	3.28 ± 0.63
pilG-His	3.17 ± 0.29
pilG(D58A)-His	2.99 ± 0.39
pilH-His	3.31 ± 0.34
pilH(D52A)-His	4.23 ± 0.23†

*No plaques detectable even when strains were infected with 10^9 phage.

[†]Δ*pilH* plaque morphology distinguished by large zones of seemingly directional lysis and poorly defined plaques.

Chapter 3

Phenotypic Analysis of In-Frame Deletion Mutants of Chp System Genes

Introduction

The *pilGHIJKchpABC* gene cluster encodes a putative chemosensory system (the Chp system) similar to the Che system involved in the regulation of flagellar chemotaxis in *Escherichia coli* that has been previously implicated in the regulation of TM (1, 3-5, 13, 15). The functions of the core signaling components of the Chp system, including the putative histidine kinase ChpA and the two CheY-like response regulators PilG and PilH in the regulation of TM are discussed in Chapter 2. In addition to the putative histidine kinase and the two CheY-like response regulators, the Chp system gene cluster encodes a putative methyl-accepting chemotaxis protein (MCP and PilJ) (4), two CheW-like adaptor proteins (PilI and ChpC) (4, 15), a putative methyltransferase (PilK) (5), and a putative methylesterase (ChpB) (15). Two additional genes, *chpD* and *chpE*, have no homology to Che system components. However, their proximity to other Chp system genes suggests that *chpD* and *chpE* may play some role in Chp system function. *chpD* encodes a putative AraC-like transcriptional regulator and ChpE encodes a putative LysE family amino acid efflux protein (15). Insertion mutants in *pilI*, *pilJ*, and *pilK* have been shown previously to have defects in Tfp function, including decreased TM and sensitivity to infection by pilus-specific phage (4, 5). Insertion mutants in *chpB*, *chpC*, *chpD*, and *chpE* were reported to have no defects in Tfp function (15). To confirm the roles that these genes play in the regulation of Tfp function we constructed in-frame deletion

mutants in each of these genes and assayed them for TM and levels of intracellular and surface associated pilin. Our collaborators Drs. Sünje Pamp and Tim Tolker-Nielsen (Centre for Biomedical Microbiology, Technical University of Denmark, DK-2800, Lyngby, Denmark) assayed these mutants for biofilm formation in flow chambers.

Materials and Methods

Construction of in-frame deletion mutants. All matings were performed as described previously (15). All mutants were confirmed by Southern blot analysis or by PCR. Construction of Δ*pilG* and Δ*pilG*Δ*pilH* mutants are described in Chapter 2.

pilI. 5′ and 3′ *pilI* deletion construct fragments were amplified using the pilI1/pilI2 and pilI3/pilI4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB45 and pJB46, respectively. The 5′ deletion construct was excised from pJB45 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB46 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB61. Gentamicin-resistant derivatives of the *pilI* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB61 to form pJB70. Gm^R and untagged *pilI* deletion constructs were subcloned as *Spe*I fragments from pJB61 and pJB70 to pJEN34 to form pJB88 and pJB79, respectively. pJB79 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *pilI* insertion mutant, *pilI*-gent. pJB88 was transformed into *E. coli* S17.1 and transformants were mated to *pilI*-gent to create an unmarked Δ*pilI* mutant.

pilJ. 5′ and 3′ *pilJ* deletion construct fragments were amplified using the pilJ1/pilJ2 and pilJ3/pilJ4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB41 and pJB42, respectively. The 5′ deletion construct was excised from pJB41 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB42 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB59. Gentamicin-resistant derivatives of the *pilJ* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB59 to form pJB68. Gm^R and untagged *pilJ* deletion constructs were subcloned as *Spe*I fragments from pJB59 and pJB68 to pJEN34 to form pJB86 and pJB77, respectively. pJB77 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *pilJ* insertion mutant, *pilJ*-gent. pJB86 was transformed into *E. coli* S17.1 and transformants were mated to *pilJ*-gent to create an unmarked Δ*pilJ* mutant.

pilK. 5′ and 3′ *pilK* deletion construct fragments were amplified using the pilK1/pilK2 and pilK3/pilK4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB49 and pJB50, respectively. The 5′ deletion construct was excised from pJB49 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB50 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB63. Gentamicin-resistant derivatives of the *pilK* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB63 to form pJB72. Gm^R and untagged *pilK* deletion constructs were subcloned as *Spe*I fragments from pJB63 and pJB72 to pJEN34 to form pJB90 and pJB81, respectively.

pJB81 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *pilK* insertion mutant, *pilK*-gent. pJB90 was transformed into *E. coli* S17.1 and transformants were mated to *pilK*-gent to create an unmarked Δ*pilK* mutant.

chpB. 5′ and 3′ *pilB* deletion construct fragments were amplified using the chpB1/chpB2.1 and chpB3/chpB4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB51 and pJB52, respectively. The 5′ deletion construct was excised from pJB51 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB52 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB64. Gentamicin-resistant derivatives of the *chpB* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB64 to form pJB73. Gm^R and untagged *chpB* deletion constructs were subcloned as *Spe*I fragments from pJB64 and pJB73 to pJEN34 to form pJB91 and pJB82, respectively. pJB82 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *chpB* insertion mutant, *chpB*-gent. pJB91 was transformed into *E. coli* S17.1 and transformants were mated to *chpB*-gent to create an unmarked Δ*chpB* mutant.

chpC. 5′ and 3′ *pilC* deletion construct fragments were amplified using the chpC1/chpC2and chpC3/chpC4.1 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB53 and pJB54, respectively. The 5′ deletion construct was excised from pJB53 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB54 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct

fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB65. Gentamicin-resistant derivatives of the *chpC* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB65 to form pJB74. Gm^R and untagged *chpC* deletion constructs were subcloned as *Spe*I fragments from pJB65 and pJB74 to pJEN34 to form pJB92 and pJB83, respectively. pJB83 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *chpC* insertion mutant, *chpC*-gent. pJB92 was transformed into *E. coli* S17.1 and transformants were mated to *chpC*-gent to create an unmarked Δ*chpC* mutant.

chpD. 5′ and 3′ *pilD* deletion construct fragments were amplified using the chpD1/chpD2and chpD3/chpD4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB55 and pJB56, respectively. The 5′ deletion construct was excised from pJB55 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB56 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB66. Gentamicin-resistant derivatives of the *chpD* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB66 to form pJB75. Gm^R and untagged *chpD* deletion constructs were subcloned as *Spe*I fragments from pJB66 and pJB75 to pJEN34 to form pJB93 and pJB84, respectively. pJB84 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *chpD* insertion mutant, *chpD*-gent. pJB93 was transformed into *E. coli* S17.1 and transformants were mated to *chpD*-gent to create an unmarked Δ*chpD* mutant.

chpE. 5′ and 3′ *pilE* deletion construct fragments were amplified using the chpE1/chpE2and chpE3/chpE4 PCR primer pairs. PCR products were A-tailed and ligated into pGEM-T to form pJB57 and pJB58, respectively. The 5′ deletion construct was excised from pJB57 as an *Eco*RIII/*Hind*III fragment. The 3′ deletion construct was excised from pJB58 as a *Hind*III/*Xba*I fragment. Excised 5′ and 3′ deletion construct fragments were concatamerized and cloned as an *Eco*RI/*Xba*I fragment into pOK12 to form pJB67. Gentamicin-resistant derivatives of the *chpE* deletion construct were made by subcloning a Gm^R-encoding cassette as a *Hind*III fragment from pX1918GT into pJB67 to form pJB76. Gm^R and untagged *chpD* deletion constructs were subcloned as *Spe*I fragments from pJB67 and pJB76 to pJEN34 to form pJB94 and pJB85, respectively. pJB85 was transformed into *E. coli* S17.1 and transformants were mated to PAO1 to create an Gm^R-tagged *chpE* insertion mutant, *chpE*-gent. pJB94 was transformed into *E. coli* S17.1 and transformants were mated to *chpE*-gent to create an unmarked Δ*chpE* mutant.

gfp-, *yfp*-, and *cfp*-tagged strains were constructed as described previously (9). Biofilms were cultivated and images acquired and analyzed as described previously (9).

Results and Discussion

TM and surface presentation phenotypes Chp system mutants

We constructed in-frame deletion mutants in PAO1 for *pilI*, *pilJ*, *pilK*, *chpB*, *chpC*, *chpD*, and *chpE* and assayed them for Tfp function including TM, and intracellular and surface pilin levels. Consistent with previous results (4), Δ*pilI* and Δ*pilJ* were dramatically impaired for TM and lacked detectable levels of surface pili by Western blot

analysis (Fig. 10A, B). It remains to be determined if these mutants are susceptible to infection by pilus-specific phage, which would indicate the presence of a small number of pili on the cell surface. The Tfp-phenotypes of PAO1 Δ*pilJ* differ slightly from those of a PAK insertion mutant. Whereas the PAO1 mutant has a TM phenotype indistinguishable from that of Δ*pilA* at the resolution of the subsurface stab assay, and lacked detectable levels of surface pili by Western blot analysis, the PAK *pilJ* insertion mutant is reduced but not completely defective for TM and retains a small but detectable amount of pili on the cell surface (15). These differences may be specific to the strain background and may reflect a general difference in the way in which the Chp system contributes to the regulation of Tfp function. In, support of this is our finding that in-frame deletion mutants of *chpA* in the PAK background retain some TM and detectable levels of surface pili (data not shown), whereas the corresponding *chpA* mutant in PAO1 is completely defective for TM and has greatly reduced surface piliation. Consistent with previous reports (5) the TM phenotype of Δ*pilK* was indistinguishable from WT (Fig. 10A). In contrast to previous finding, however, Δ*pilK* had reduced levels of surface pili relative to WT (Fig. 10B). As with PAK insertion mutants (15), Δ*chpB*, Δ*chpD*, and Δ*chpE* were indistinguishable from WT for TM and exhibited WT levels of pili on the cell surface (Fig. 10A, B). Δ*chpC* showed slightly reduced TM relative to WT and presented reduced levels of pili on the cell surface (Fig. 10A, B), suggesting that that its role may be largely redundant with PilI. Decreased TM and/or surface presentation in in-frame deletion mutant backgrounds suggest a role for that gene in the regulation of Tfp dynamics. In each case, the exact nature of that role remains to be determined. Additionally, it has yet to be shown that mutants that have defects in Tfp function can be

complemented either in *cis* or in *trans*, although it is most likely that their defect in Tfp function is a result of the in-frame deletion and not a secondary mutation elsewhere in the genome

Future studies will focus on determining how the components of this system interact and how that contributes to the regulation of Tfp dynamics. Some specific avenues of investigation include, determining if PilJ functions as an MCP, what chemical signal(s) it responds to, and whether it is the only MCP involved in the regulation of Tfp function. Previous studies have shown that both *P. aeruginosa* (6) and *M. xanthus* (7) demonstrate directed motility towards phosphatidylethnolamine gradients. Whether PilJ is involved in this response is unclear. It also remains to be determined whether ChpA binds to PilJ and if either of the two CheW-like adaptor proteins (PilI and ChpC) are required for this interaction. We presented evidence in Chapter 2 consistent with the idea that ChpA contributes to the regulation of Tfp function by selectively phosphorylating PilG or PilH to drive pilus extension or retraction, respectively. It is intriguing to speculate that ChpA binding to PilJ or other MCP via PilI or chpC may play a role in determining which CheY homologue is phosphorylated. An alternate hypothesis is that PilI and ChpC function to couple ChpA to different MCPs. Because a signal has yet to be identified for the Chp system, it remains unclear whether the system allows for an adaptive response to increasing concentrations of attractant or repellant. Once a signal is identified, however, the role that PilK and ChpB play in mediating an adaptive response can be investigated. Specifically, whether PilK is able to methylate PilJ or other MCP(s), whether ChpB is able to demethylate those receptors, and whether ChpA phosphorylation

of ChpB is required for ChpB function. The roles of ChpD and ChpE, whether in the regulation of Tfp function or in another capacity remain to be determined.

Biofilm formation phenotypes of Chp system mutants

A number of studies have shown that functional Tfp play a role in the process of biofilm development (2, 8-10). To determine if components of the Chp system play a role in biofilm development, GFP-tagged WT and single in-frame Chp system deletion mutants were grown as biofilms in flow chambers in glucose supplemented minimal media at 30 ˚C for three days. Biofilm morphology was monitored by confocal laser scanning microscopy. These experiments were performed by our collaborators, Drs. Tim Tolker-Nielson and Sünje Pamp The results indicate that, despite defects in TM and surface presentation of pili for a number of Chp system mutants (Fig. 3A, B, Fig. 4A, B, Fig 10A, B), all mutants form mature biofilms with morphologies similar to that that of WT and that are clearly distinguishable from that of a Δ*pilA* mutant (Fig. 11). These data suggest that Tfp remain partially functional in Chp system mutant backgrounds under biofilm growth conditions and are consistent with our observations that a number of Chp system mutants remain sensitive to infection by pilus-specific phage and that very few functional pili appear to be required for TM (Table 3, Fig. 24A, C, Fig. 25). Slight morphological defects in mutant biofilms may point to more subtle regulatory roles for the Chp system during early stages of the development process.

Defects in gross morphology are more apparent in 4-day old mixed culture biofilms (Fig. 12). In these experiments, biofilms were grown from mixed cultures containing CFP-tagged Δ *pilA* and YFP-Tagged WT, Δ *pilH*, Δ *pilG*Δ*pilH*, Δ *chpA*, or Δ*chpB* mutants in flow chambers in glucose supplemented minimal media at 30 ˚C for

four days. The cap subpopulation for all biofilms was made up of Chp system mutant strains with the stalk subpopulation consisting almost exclusively of Δ*pilA*. These data are consistent with those presented for 3-day biofilms (Fig. 11), and suggest that Chp system mutant strains remain at least partially motile under these assay conditions (Fig. 12, 8). In contrast to 3-day biofilms, several Chp system mutants have distinct morphological defects (Fig. 12), suggesting that the Chp system may contribute to later stages of biofilm development. Δ*pilH* mutants present two distinct morphologies (Fig. 12B, C, F, G). In the first case, the biofilm is mostly flat with only small cap like structures protruding from the surface (Fig. 12B, F). In the second case, the biofilm forms large irregularly shaped caps, which appear rough compared to WT (Fig. 12A, C, E, G). The reasons for these two different phenotypes remain unclear. The biofilms formed by Δ*pilG*Δ*pilH* mutants appear similar to the second Δ*pilH* biofilm morphology (Fig. 12C, D, G, H), despite the fact that these mutants, like ΔpilG, have reduced TM and reduced surface pili Δ*chpA* mutants form small, irregular caps similar in appearance, though not in size, to those formed by Δ*pilH* and Δ*pilG*Δ*pilH* mutants. Taken together, these data suggest that fully functional Tfp may be required to form the large smooth caps characteristic of WT biofilms, although it is possible that these mutants are defective additional functions required for biofilm formation. One of the most distinctive morphologies is formed by the Δ*chpB* mutant. In this case, Δ*chpB* completely covers the layer of Δ*pilA* cells growing on the surface, forming only small dome structures rather than well developed caps (Fig. 12J, L). *chpB* encodes and putative methylesterase, which, in the *E. coli* Che system, is involved in adaptation to chemoattractant signals (13). It is interesting to speculate that the Δ*chpB* biofilm phenotype may result form the

inability of the cells to respond and adapt to chemical signals allowing them to direct their TM towards a specific location that will develop into the cap. Further work will have to be done to confirm or refute this hypothesis. In addition, it remains to be seen if older biofilms formed by Chp system mutants have similar phenotypes to those few mutants examined here, whether these defects can be complemented, and whether phosphotransfer between ChpA and PilG, pilH, and/or PilB are required for biofilm development.

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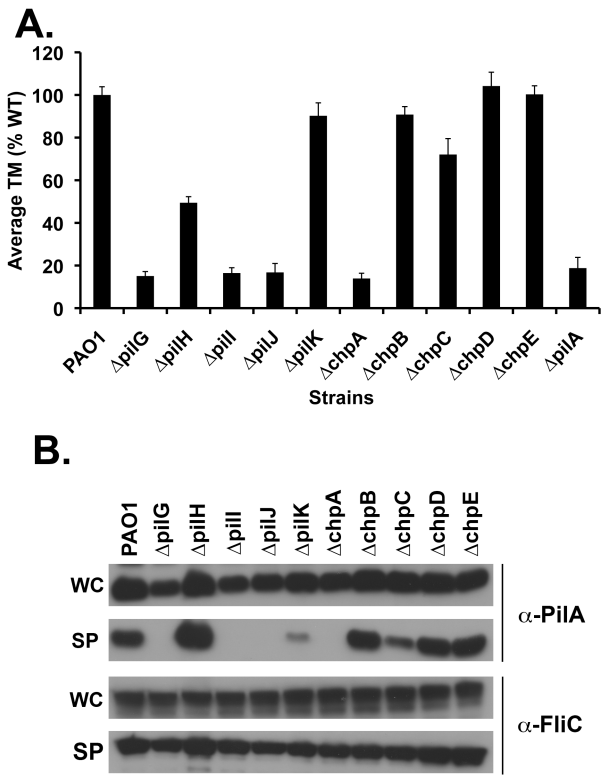

Figure 10. Assays of pilus function for Chp system mutants. (A) Graph depicting TM zone diameters for the indicated strains. The diameter is expressed as a percentage of PAO1. Shown are the means ± the standard deviation (SD, N=5) (B) Intracellular and surface pilin levels. For the indicated strains, surface pili (SP) were sheared by vigorous vortexing of bacteria cultured on solid media and separated from cells (WC) by centrifugation. The sheared pili were precipitated. WC (15 μg total protein) and SP (5% of the total resuspended volume of precipitated pili) samples were separated by SDS-PAGE and immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC).

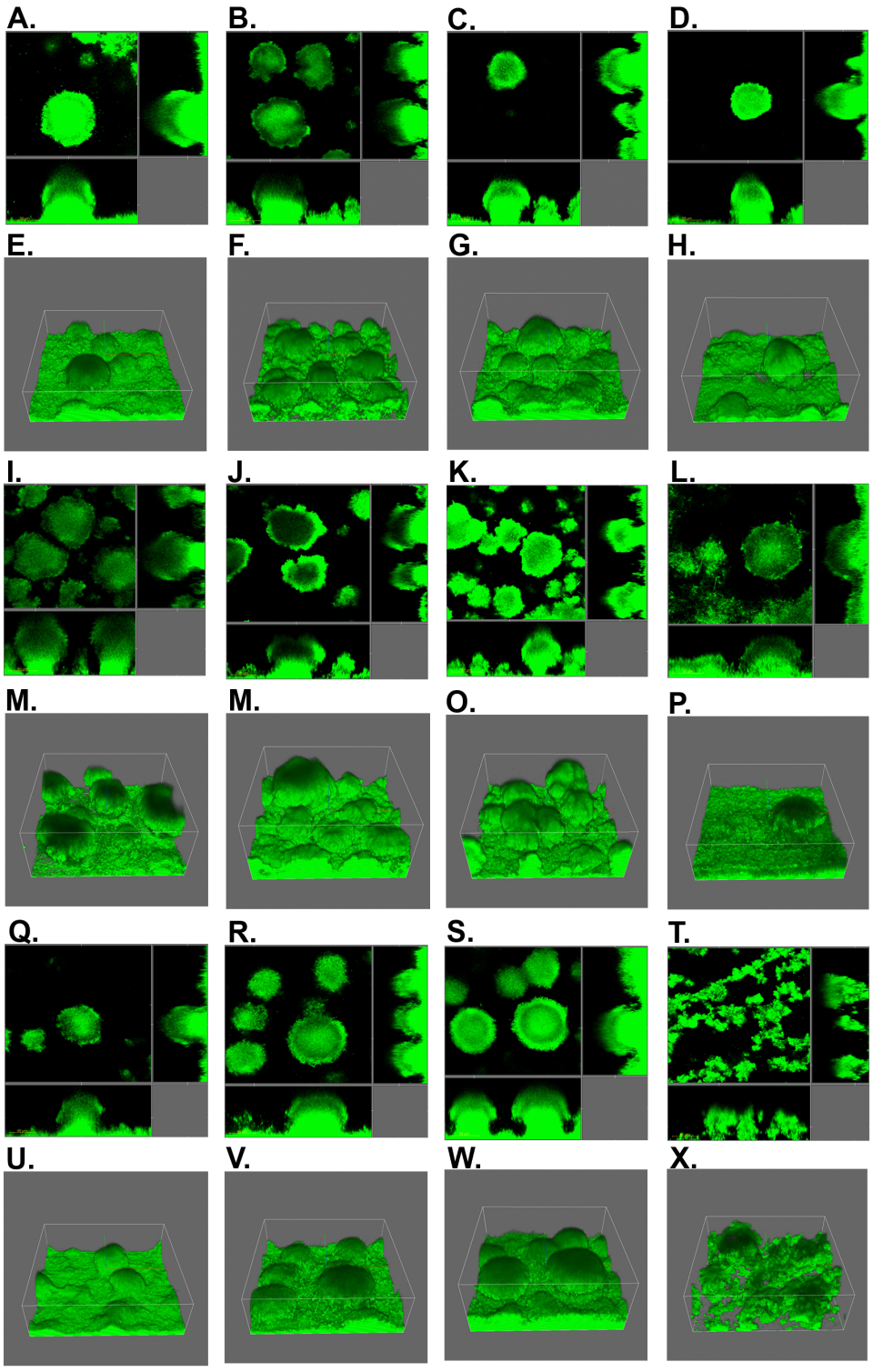

Figure 11. Three-Day biofilm formation phenotypes of Chp system mutants. Confocal laser scanning micrographs of 3-day old biofilms grown at 30 ˚C in the presence of

glucose minimal media formed by PAO1$_{gfp}$ (A and E), Δ*pilG*$_{gfp}$ (B and F), Δ*pilH*$_{gfp}$ (C and G), Δ*pilI*$_{gfp}$ (D and H), Δ*pilJ*$_{gfp}$ (I and M), Δ*pilK*$_{gfp}$ (J and N), Δ*chpA*$_{gfp}$ (K and O), Δ*chpB*$_{gfp}$ (L and P), Δ *chpC*$_{gfp}$ (Q and U), Δ *chpD*$_{gfp}$ (R and V), Δ *chpE*$_{gfp}$ (S and W), Δ*pilA*$_{gfp}$ (T and X). The central images (A – D , I – L, Q – T) show top-down views and the flanking images show vertical optical sections. 3-D confocal laser scanning microscopy images (E – H, M – P, U – X).

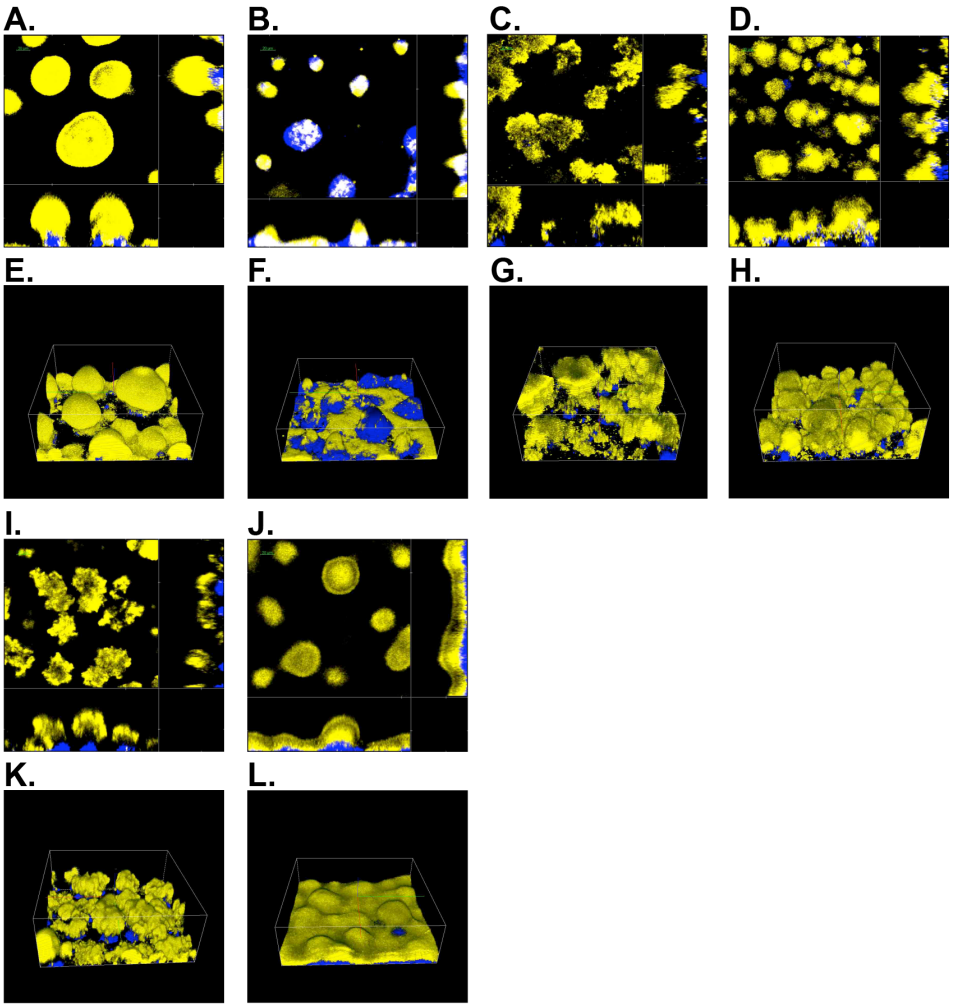

Figure 12. Four-Day biofilm formation phenotypes of select Chp system mutants. Confocal laser scanning micrographs of 4-day old biofilms grown at 30 ˚C in the presence of glucose minimal media formed by mixtures of PAO1$_{yfp}$ and Δ*pilA*$_{cfp}$ (A and E), Δ*pilH*$_{yfp}$ and Δ*pilA*$_{cfp}$ (B, C, F, and G), Δ*pilG*Δ*pilH*$_{yfp}$ and Δ*pilA*$_{cfp}$ (D and H), Δ*chpA*$_{yfp}$ and Δ*pilA*$_{cfp}$ (I and K), and Δ*chpB*$_{yfp}$ and Δ*pilA*$_{cfp}$ (J and L). The central images (A – D , I – J) show top-down views and the flanking images show vertical optical sections. 3-D confocal laser scanning microscopy images (E – H, K – L).

Table 4. Strains and plasmids used in chapter 3

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	
PAO1Δ*chpA*	In frame deletion of *chpA*	(15)
PAO1Δ*pilG*	In frame deletion of *pilG*	This study
PAO1Δ*pilH*	In frame deletion of *pilH*	(2)
PAO1Δ*pilG*Δ*pilH*	In frame deletion of *pilG* and *pilH*	This study
PAO1 *pilI*-gent	Gm^R-tagged *pilI* insertion mutant	This study
PAO1Δ*pilI*	In frame deletion of *pilI*	This study
PAO1 *pilJ*-gent	Gm^R-tagged *pilJ* insertion mutant	This study
PAO1Δ*pilJ*	In frame deletion of *pilJ*	This study
PAO1 *pilK*-gent	Gm^R-tagged *pilK* insertion mutant	This study
PAO1Δ*pilK*	In frame deletion of *pilK*	This study
PAO1 *chpB*-gent	Gm^R-tagged *chpB* insertion mutant	This study
PAO1Δ*chpB*	In frame deletion of *chpB*	This study
PAO1 *chpC*-gent	Gm^R-tagged *chpC* insertion mutant	This study
PAO1Δ*chpC*	In frame deletion of *pilC*	This study
PAO1 *chpD*-gent	Gm^R-tagged *chpD* insertion mutant	This study
PAO1Δ*chpD*	In frame deletion of *chpD*	This study
PAO1 *chpE*-gent	Gm^R-tagged *chpE* insertion mutant	This study
PAO1Δ*chpE*	In frame deletion of *chpE*	This study
PAO1Δ*pilA*	In frame deletion of *pilA*	This study
$PAO1_{gfp}$	PAO1 tagged with eGFP in a mini-Tn7 construct; Gm^R	(9)
$PAO1_{yfp}$	PAO1 tagged with eYFP in a mini-Tn7 construct; Gm^R	(9)
PAO1Δ$chpA_{gfp}$	Δ*chpA* tagged with eGFP in a mini-Tn7 construct; Gm^R	(2)
PAO1Δ$chpA_{yfp}$	Δ*chpA* tagged with eYFP in a mini-Tn7 construct; Gm^R	(2)
PAO1Δ$pilG_{gfp}$	Δ*pilG* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$pilH_{gfp}$	Δ*pilH* tagged with eGFP in a mini-Tn7 construct; Gm^R	(2)
PAO1Δ$pilH_{yfp}$	Δ*pilH* tagged with eYFP in a mini-Tn7 construct; Gm^R	(2)
PAO1Δ$pilI_{gfp}$	Δ*pilI* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$pilJ_{gfp}$	Δ*pilJ* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$pilK_{gfp}$	Δ*pilK* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$chpB_{gfp}$	Δ*chpB* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$chpB_{yfp}$	Δ*chpB* tagged with eYFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$chpC_{gfp}$	Δ*chpC* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$chpD_{gfp}$	Δ*chpD* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$chpE_{gfp}$	Δ*chpE* tagged with eGFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ*pilG*Δ$pilH_{yfp}$	Δ*pilG*Δ*pilH* tagged with eYFP in a mini-Tn7 construct; Gm^R	This study
PAO1Δ$pilA_{gfp}$	Δ*pilA* tagged with eGFP in a mini-Tn7 construct; Gm^R	(9)
PAO1Δ$pilA_{cfp}$	Δ*pilA* tagged with eCFP in a mini-Tn7 construct; Gm^R	(9)
E. coli		
DH5α	*hsdR rec lacZYA ϕ80 lacZM15*	Invitrogen
S17-1 λpir	*Thi pro hsdR recA RP4-2*(Tc::Mu)(Km::Tn7)	Stratagene
Plasmids		
pOK12	*E. coli* cloning vector, Km^R	(12)

pGEM-T	*E. coli* cloning vector, Ap^R	Promega
pX1918GT	Source of Gm^R cassette, Ap^R	(11)
pEN34	Allelic replacement suicide plasmid, Tc^R	(14)
pJB41	pGEM-T containing 1 KB region 5′ of *pilJ*	This study
pJB42	pGEM-T containing 1 KB region 3′ of *pilJ*	This study
pJB45	pGEM-T containing 1 KB region 5′ of *pilI*	This study
pJB46	pGEM-T containing 1 KB region 3′ of *pilI*	This study
pJB49	pGEM-T containing 1 KB region 5′ of *pilK*	This study
pJB50	pGEM-T containing 1 KB region 3′ of *pilK*	This study
pJB51	pGEM-T containing 1 KB region 5′ of *chpB*	This study
pJB52	pGEM-T containing 1 KB region 3′ of *chpB*	This study
pJB53	pGEM-T containing 1 KB region 5′ of *chpC*	This study
pJB54	pGEM-T containing 1 KB region 3′ of *chpC*	This study
pJB55	pGEM-T containing 1 KB region 5′ of *chpD*	This study
pJB56	pGEM-T containing 1 KB region 3′ of *chpD*	This study
pJB57	pGEM-T containing 1 KB region 5′ of *chpE*	This study
pJB58	pGEM-T containing 1 KB region 3′ of *chpE*	This study
pJB59	*Eco*RI/*Hind*III fragment from pJB41 and *Hind*III/*Xba*I from pJB42 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB61	*Eco*RI/*Hind*III fragment from pJB45 and *Hind*III/*Xba*I from pJB46 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB63	*Eco*RI/*Hind*III fragment from pJB49 and *Hind*III/*Xba*I from pJB50 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB64	*Eco*RI/*Hind*III fragment from pJB51 and *Hind*III/*Xba*I from pJB52 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB65	*Eco*RI/*Hind*III fragment from pJB53 and *Hind*III/*Xba*I from pJB54 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB66	*Eco*RI/*Hind*III fragment from pJB55 and *Hind*III/*Xba*I from pJB56 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB67	*Eco*RI/*Hind*III fragment from pJB57 and *Hind*III/*Xba*I from pJB58 concatamerized and cloned into *Eco*RI/*Xba*I sites of pOK12	This study
pJB68	Gm^R-tagged Δ*pilJ* in pOK12; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB59	This study
pJB70	Gm^R-tagged Δ*pilI* in pOK12; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB61	This study
pJB72	Gm^R-tagged Δ*pilK* in pOK12; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB63	This study
pJB73	Gm^R-tagged Δ*chpB* in pOK12; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB64	This study
pJB74	Gm^R-tagged Δ*chpC* in pOK12; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB65	This study
pJB75	Gm^R-tagged Δ*chpD* in pOK12; pX1918-derived Gm^R-cassette cloned as *Hind*III fragment into pJB67	This study
pJB76	Gm^R-tagged Δ*chpE* in pOK12; pX1918-derived Gm^R-	This study

	cassette cloned as *Hind*III fragment into pJB67	
pJB77	pJEN34 carrying Gm^R-tagged Δ*pilJ* on *Spe*I fragment	This study
pJB79	pJEN34 carrying Gm^R-tagged Δ*pilI* on *Spe*I fragment	This study
pJB81	pJEN34 carrying Gm^R-tagged Δ*pilK* on *Spe*I fragment	This study
pJB82	pJEN34 carrying Gm^R-tagged Δ*chpB* on *Spe*I fragment	This study
pJB83	pJEN34 carrying Gm^R-tagged Δ*chpC* on *Spe*I fragment	This study
pJB84	pJEN34 carrying Gm^R-tagged Δ*chpD* on *Spe*I fragment	This study
pJB85	pJEN34 carrying Gm^R-tagged Δ*chpE* on *Spe*I fragment	This study
pJB86	pJEN34 carrying pJB59-derived Δ*pilJ* on *Spe*I fragment	This study
pJB88	pJEN34 carrying pJB61-derived Δ*pilI* on *Spe*I fragment	This study
pJB90	pJEN34 carrying pJB63-derived Δ*pilK* on *Spe*I fragment	This study
pJB91	pJEN34 carrying pJB64-derived Δ*chpB* on *Spe*I fragment	This study
pJB92	pJEN34 carrying pJB65-derived Δ*chpC* on *Spe*I fragment	This study
pJB93	pJEN34 carrying pJB66-derived Δ*chpD* on *Spe*I fragment	This study
pJB94	pJEN34 carrying pJB67-derived Δ*chpE* on *Spe*I fragment	This study

Table 5. Oligonucleotides used in chapter 3

Primer	Oligonucleotide sequence (5′→3′)[1]
pilI1	GAATTCAAAGGCTCGCGATTGGGTCG
pilI2	AAGCTTCGAGGAGCTGGAAGGGGGTC
pilI3	AAGCTTTCGGCATGCAGCACTTCCCG
pilI4	TCTAGACCGAACCGGAGACGAACTCG
pilJ1	GAATTCAGATGTACAAGCTGACCGCC
pilJ2	AAGCTTCGAAAAGATTGCCTGCGTTG
pilJ3	AAGCTTTCGAGAAGGTATCCAAGACC
pilJ4	TCTAGACGACGGAAGTAGATCAGCAG
pilK1	GAATTCCCGCGACCGTGACCGAGGAC
pilK2	AAGCTTGCTGCAGTGACCAGACGCCG
pilK3	AAGCTTATTGCGAACGCCAGGCCGAC
pilK4	TCTAGATGACCACGCTACCGTTGGCC
chpB1	GAATTCTCTCCACCGCCGAAAAGG
chpB2.1	AAGCTTGTTGAGCACCACCTCGTAGCC
chpB3	AAGCTTGAACCCTACACCCCGTCCAT
chpB4	TCTAGATCTGCCCCGGGTTGTAGAGG
chpC1	GAATTCGGTCGGCCAGCATGAGTGAG
chpC2	AAGCTTTCATGCCATCCTGCTCGATC
chpC3	AAGCTTCGCGGGTGGTGGTCATCAA
chpC4.1	TCTAGAGCGAAACCGAACAGCAAAGC
chpD1	GAATTCCCTCTATGCCCAGCACATCG
chpD2	AAGCTTTGCTTCTCGAAGCGGTGCCC
chpD3	AAGCTTGTGGAGCATGCAACTGCGCA
chpD4	TCTAGAAGGGCGGACTACAGCACTGG
chpE1	GAATTCGCGCGGATTCCGGACCTGGC
chpE2	AAGCTTCGAACAGCAAAGCGGCGAGG
chpE3	AAGCTTTGTTCTTCGCCGGTTTCATG
chpE4	TCTAGAAAGAAACCCAGGGCGCGTTG

[1]Restriction endonuclease sequences are underlined

Chapter 4

ChpA Regulates Expression of the Type III Secreted Effector Protein ExoT

Introduction

Mutation of *chpA*, encoding the putative histidine kinase of the Chp chemosensory system, resulted in decreased virulence in both *in vitro* and *in vivo* infection models. Specifically, *chpA* mutants showed reduced cytotoxicity towards MCDK cells *in vitro* (13), and reduced virulence in both a *Drosophila* infection model (5) and a mouse model of acute pneumonia (13). Previous studies have shown a role for functional type IV pili (Tfp) in *P. aeruginosa* interactions with cultured cell lines as well as for virulence in animal models of disease (4, 9). Data presented here, along with previous studies (13), suggest that Tfp function is severely compromised in *chpA* mutants. The defect in Tfp function alone in *chpA* mutants could account for decreases in both cytotoxicity and virulence. However, the enormous signaling potential of ChpA, with nine potential phosphorylation sites (13), leaves open the possibility that it might be involved in the regulation of virulence factor production or more global gene expression, and that this might contribute to its virulence phenotype. Indeed, insertion mutants in *chpA* appeared to be defective in the production of quorum sensing regulated virulence factors (see Chapter 5), although the defect was not recapitulated in in-frame deletion mutants and could not be complemented in the insertion mutant by reintroduction of *chpA* onto the chromosome at the *chpA* locus. Nevertheless, the possibility remains that ChpA may play some role in the regulation of gene expression.

The N-terminal domain of ChpA is homologous to FimL, which has been shown to regulate the production of the type III secreted effector protein ExoT (11). The type III secretion system (T3SS) allows for the direct injection of bacterial effector proteins into the cytoplasm of a host cell, and has been shown to be involved in *P. aeruginosa* virulence in a number of *in vivo* infection models, including *Drosophila* and a mouse model of acute pneumonia (7). Using the T3SS, *P. aeruginosa* directly injects four known effectors into host cells. These include the cytotoxin ExoU, which has demonstrated phospholipase activity, the adenylate cyclase ExoY, whose function in host cells remains unclear, and two closely related bifunctional proteins ExoS and ExoT (7). ExoS and ExoT have N-terminal GTPase activating protein activity towards Rho family proteins, and C-terminal ADP ribosylase activity (7). The TTSS is regulated in part by the cyclic-AMP (cAMP) binding transcription factor, Vfr (14) and the transcriptional regulator, ExsA (15). Vfr regulates transcription of *exsA* (14), although the mechanism is unknown. ExsA, in addition to regulating its own transcription, regulates expression of the effector genes and genes involved in secretion and translocation.

Our unpublished work has demonstrated a role for FimL in the production of cAMP (West, J., Inclan, Y., Bertrand, J., and Engel, J., manuscript in preparation). The defect in ExoT production in *fimL* mutants, therefore, is likely due to decreased production of cAMP and a consequent decrease in Vfr activity. The homology between FimL and the N-terminus of ChpA raises the possibility that ChpA may also play a role in cAMP production. As such, ChpA might contribute to the regulation of T3SS by mediating Vfr activity.

To begin to address the questions of whether ChpA is involved in the regulation of T3SS virulence factor production we assayed *exoT* expression in three WT *P. aeruginosa* strain backgrounds and isogenic Δ *chpA* mutants. We also performed microarray experiments comparing Δ*chpA* mutants to wild type PAO1 cultured in both liquid and on solid media to determine if ChpA played a more global role in the regulation of gene expression. We show here that transcription and production of *exoT* is reduced in *chpA* mutants in PAO1, PAK, and PA103 strain backgrounds. Microarry data suggests that ChpA plays some role in the regulation of expression of the T3SS.

Materials and Methods

Construction of in-frame deletion mutants. All matings were performed as described previously (13). All mutants were confirmed by Southern or PCR analysis. In-frame deletion mutants of *chpA* (PAO1) and *vfr* (PAO1 and PA103) were constructed by allelic displacement as described previously (13) (11). pJB116 (see chapter 2) was transformed into *E. coli* S17.1 and transformants were mated to PAK to create PAKΔ*chpA*. 5′ and 3′ PA103 *chpA* deletion construct fragments were amplified from PA103 genomic DNA using the chpA-SOE-1/chpA-SOE-2 and chpA-SOE-3/chpA-SOE-4 PCR primer pairs. PCR products were used as templates in a sequence overlap extension (SOE) reaction with the chpA-SOE-1/chpA-SOE-4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB211. pJB211 was transformed into *E. coli* S17.1 and transformants were mated to PA103 to create PA103Δ*chpA*. The *vfr* deletion construct was subcloned as a *Spe*I fragment from pJEN51 (11) to pJB100 to form, pJB100(Δvfr).

P_{exoT}-*lacZ* reporter strains. A transcriptional fusion of the *exoT* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system (3). 500 bp of sequence upstream of the *exoT* start codon was amplified and cloned into mCTX2-*lacZ* to form mCTX-P_{exoT}-*lacZ*. mCTX-P_{exoT}-*lacZ* was transformed into *E. coli* S17.1 and transformants were mated to PAO1, PAK, PA103 and the isogenic Δ*chpA* and Δ*vfr* mutants (except PAK Δ*vfr*) to create CTX-P_{exoT} derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFlp2, as described previously (3). To creat PAKΔ*vfr* CTX-P_{exoT}-*lacZ*, PAK CTX-P_{exoT}-*lacZ* was mated to *E. coli* S17.1 carrying pJB100(Δvfr).

β-galactosidase assays. β-galactosidase assays were performed as previously described (12) with the following modifications. Cultures started from single colonies were grown in triplicate shaking for 16 hrs at 37 ˚C in 5 mL minimal media S (8) with or without the addition of 2.0 mM $CaCl_2$. Optical densities (OD_{600} and OD_{420}) were recorded using a SPECTRAmax® microplate spectrophotometer (Molecular devices) and SoftMaxPro® 4.3.1. Specific activities were calculated relative to OD_{600}.

Statistical analysis. Statistical significance was determined by ANOVA using Instat software. Differences were considered to be significant at $P < 0.05$.

Microarray analysis. For analysis of gene expression in cells grown in liquid culture, single colonies were used to start triplicate cultures in 25 mL LB broth. Cultures

were grown shaking overnight at 37 ˚C, diluted 1:100 in 50 mL fresh LB broth, and grown shaking for 16 hrs at 37 ˚C. Cultures were diluted using a minimum of 2x volume RNAprotect reagent (Qiagen) to OD_{600} = 1.0, and cells harvested from 1.5 mL diluted culture by centrifugation (20 000 x g for 10 min). For analysis of gene expression in cells cultured on solid media, single colonies were used to start triplicate cultures in 25 mL broth. Cultures were grown shaking overnight at 37 ˚C. Following overnight growth, 100 µL of culture were plated to LB agar and grown for 16 hrs at 37 ˚C. Cells were resuspended in 5 mL RNAprotect reagent (Qiagen) and diluted to OD_{600} = 1.0 in RNAprotect reagent (Qiagen). Cells were harvested from 1.5 mL of the resuspended samples. RNA isolation and processing for both borth and solid media cultured cells was performed identically. Cells from two pellets were lysed in 200 µL TE containing lysozyme (1 mg/mL) for 10 min at room temperature. Following lysozyme digestion, 700 µL buffer RLT (Qiagen) was added, and the samples were sonicated for 10 sec using a Branson Sonifier 150. 500 µL EtOH was added to sonicated samples. RNA was isolated using RNeasy mini columns (Qiagen), including on-column DNaseI digestion, according to the manufacture's specifications. The final elution volume was 30 µL. An additional *in vitro* DNaseI digestion was carried out using RQ DNaseI (Promega) for 1 hr at 37 ˚C according to the manufacture's specifications. DNase was removed over an RNeasy mini column (Qiagen) according to the manufacture's specifications. The absence of DNA in the sample was confirmed by PCR. The integrity of the RNA was assessed by visualization of the samples on a 1.7% formaldehyde-agarose gel.

cDNA was synthesized by pre-incubation of the RNA (12 µ g) with the nonspecific primer (NS)5 (750 ng) in a final volume of 30 µL for 10 min at 70 ˚C. Pre-

incubation was followed by addition of 1st Strand buffer (Invitrogen, 1X final concentration), DDT (10 mM final concentration), dNTPs (0.5 mM final concentration), RNase inhibitor (Ambion, 1.0 I/μL final concentration), and SuperScript II reverse transcriptase (Invitrogen, 25 U/μL final concentration) in a final volume of 30 μL. The reaction cycle for cDNA synthesis was: 25 ˚C for 10 min, 37 ˚C for 60 min, and 42 ˚C for 60 min. Reverse transcriptase was inactivated by incubation at 70 ˚C for 10 min. Residual RNA was removed by addition of 20 μL 1.0 N NaOH and incubation at 65 ˚C for 30 min, followed by addition of 20 μL 1.0 N HCl. cDNA was purified over a QIAquick PCR clean-up column (Qiagen) accordin to the manufacture's specifications. 7.0 μg cDNA was fragmented for 10 min at 37 ˚C in 1x One Phor-All buffer (Amersham) with DNaseI (Roche, 0.06 U/μg cDNA) followed by inactivation of the enzyme at 100 ˚C for 10 min. 4 μg fragmented cDNA was labeled using the Affymetrix terminal label protocol with terminal deoxynucleotidly transferase (Promega). Labeled cDNAs were hybridized to GeneChip *P. aeruginosa* Genome microarrays (Affymetrix). Hybridizations were carried out by the UCSF genomics core at the Gladstone Institute using an Affymetrix Fluidics Station according to the manufacturer's specifications.

Microarray data was analyzed using d Chip and Cyber-T (1, 2, 10). Results were considered significant for PPDE(< P) greater than 0.9.

Results and Discussion

ChpA regulates expression *exoT*

To determine if ChpA was involved in the regulation of the expression of the T3SS, we constructed in-frame deletions of *chpA* in the PAO1, PAK, and PA103 strain

backgrounds. Similar to PAO1Δ*chpA*, PA103Δ*chpA* mutants had no detectable TM by the subsurface stab assay or by colony morphology on the agar surface (data not shown). PAKΔ*chpA* showed greatly reduced TM relative to WT PAK (data not shown). All Δ*chpA* mutants had detectable levels of surface piliation by Western blot analysis, although less than that of their respective WT strains (data not shown).

To assess whether ChpA regulates the T3SS, we initially measured ExoT expression, as the effectors and the apparatus are coordinately regulated (15), and effects on *exoT* expression are likely to be generalizable to the entire T3SS regulon. A PAO1-derived P_{exoT}-*lacZ* reporter fusion was introduced onto the chromosome at the *attB* site of each of the WT and mutant strains. Isogenic Δ*vfr* CTX-P*exoT-lacZ* strains were included as a negative control. β-galactosidase activity was measured following 16 hrs of culture in MinS media in the presence or absence of Ca^{2+}. Under inducing conditions (i.e., in the absence of Ca^{2+}), *exoT* expression was reduced 2-3 fold in Δ*chpA* mutants for all strain backgrounds relative to the WT strain ($p < 0.05$), though not to the same levels as Δ*vfr* mutants (Fig. 13A, B). Interestingly, the levels of *exoT* expression under inducing conditions differed greatly for the three WT strains, with PA103 having the highest levels of expression and PAO1 the lowest. Indeed, *exoT* expression seems to be generally higher in PA103, with uninduced levels exceeding those of induced levels in PAO1 and similar to those in PAK. These data support the idea that ChpA is involved in the regulation of *exoT* expression, in multiple strain backgrounds, when grown in liquid media. Preliminary data suggests that decreased *exoT* expression in PAO1 Δ *chpA* mutants corresponds to decreased ExoT ptoduction and secretion (data not shown). Moreover, and consistent with the idea that ChpA plays a broader role in the regulation of

T3SS than just regulating expression of *exoT*, ExoS production appears to be decreased in PAO1 Δ*chpA* mutants. It remains to be determined whether the *exoT* expression defect in Δ*chpA* mutants can be complemented. In addition, the mechanism is by which ChpA regulates expression of *exoT* remains to be elucidated. Future work will focus on determining if cAMP production is altered in *chpA* mutant backgrounds.

Microarray analysis of ChpA mutants

In order to determine if genes other that *exoT* were regulated by ChpA, we carried out microarray experiments on WT and isogenic Δ*chpA* mutants cultured in rich media (LB) or on solid media (LB agar). cDNA, generated from RNA isolated from 16 hr broth or plate cultured, was fragmented, labeled, and hypridized to Affymetrix GeneChip arrays. Data were analyzed using the d Chip and cyber-T programs (1, 2, 10). Results were considered significant for PPDE(< P) greater than 0.9. The top 100 most significantly regulated genes for both cells cultured in broth and on solid media, sorted by PA number, are listed in Tables 3 and 4, respectively. Generally ChpA does not appear to be a global regulator of gene expression. Genes whose expression is significantly altered in Δ*chpA* are largely of unknown function, and do not, generally, include known transcription factors. However, several groups of genes known to be co-transcribed and several clusters of genes of unknown function do appear to be regulated by ChpA. These data suggest that ChpA does play some role in the regulation of gene expression. The full extent and biological significance of this regulation remains to be more fully explored.

Microarray analysis revealed that even under the non-inducing conditions used in these experiments, ExoT expression was reduced approximately 1.6 fold in Δ *chpA,* consistent with our P_{exoT}-*lacZ* reporter data. Expression of *exoS* and its putative chaperone *spcS* were also reduced approximately 1.5 and 1.9 fold, respectively. Interestingly, in addition to decreased expression of the effectors themselves, several additional *exsA* regulated genes also show decreased expression in Δ*chpA*, including the *pcrGVHpopBD* operon*,* involved in translocation, and *exsCEB* and *exsD*, involved in regulation of *exsA* expression. Interestingly, *exsA* expression does not appear to be altered in Δ*chpA*. These data suggest that ChpA may contribute to the regulation of T3SS by mediating ExsA production or activity at a post-transcriptional level. The mechanism, however, remains to be elucidated.

Interestingly, the microarray data from broth grown bacteria indicate that transcription of *pilA* is increased 1.3 fold in a Δ*chpA* mutant, an effect we did not see in experiments using the P_{pilA}-*lacZ* reporter (See Chapter 2). Moreover, microarray data for cells cultured on solid media did not show a significant decrease in *pilA* expression in Δ*chpA*, contrary to results gathered using the P_{pilA}-*lacZ* reporter (See Chapter 1). The reason for the apparent contradiction between the experiments is unclear.

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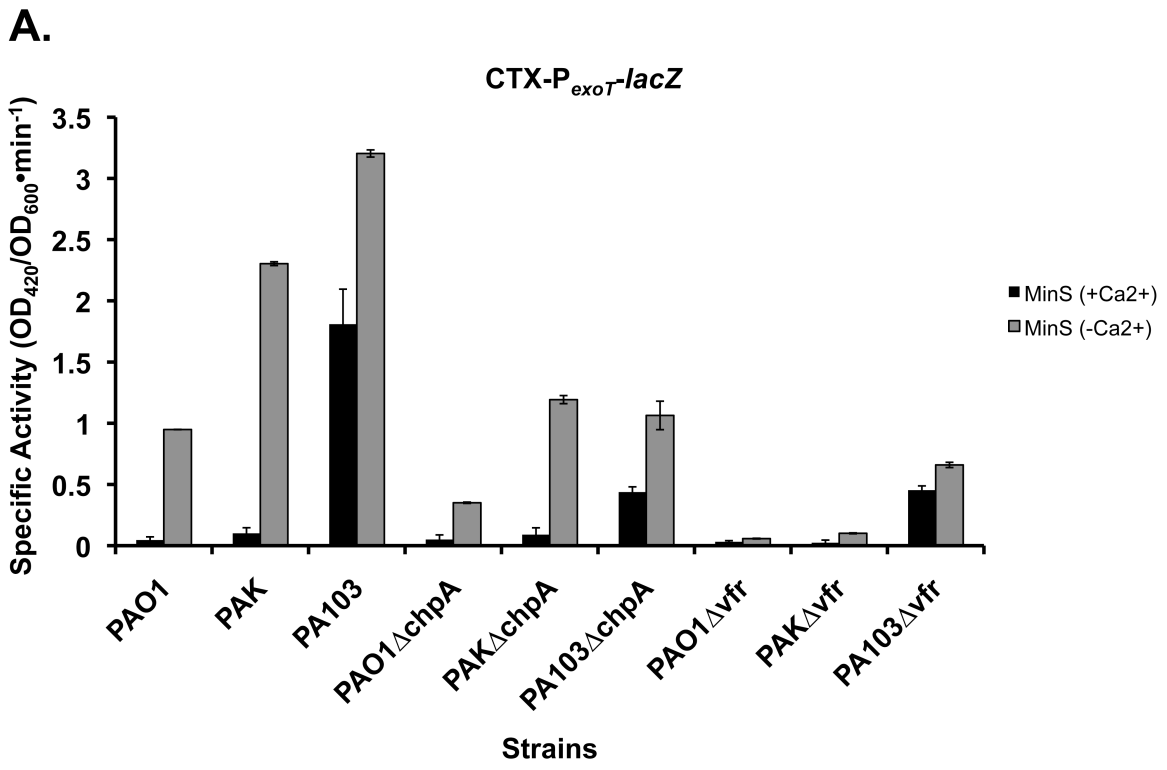
A.
CTX-P$_{exoT}$-lacZ
Specific Activity (OD$_{420}$/OD$_{600}$•min^{-1})
3.5
3
2.5
2
1.5
1
0.5
0
MinS (+Ca2+)
MinS (-Ca2+)
PAO1
PAK
PA103
PAO1ΔchpA
PAKΔchpA
PA103ΔchpA
PAO1Δvfr
PAKΔvfr
PA103Δvfr
Strains

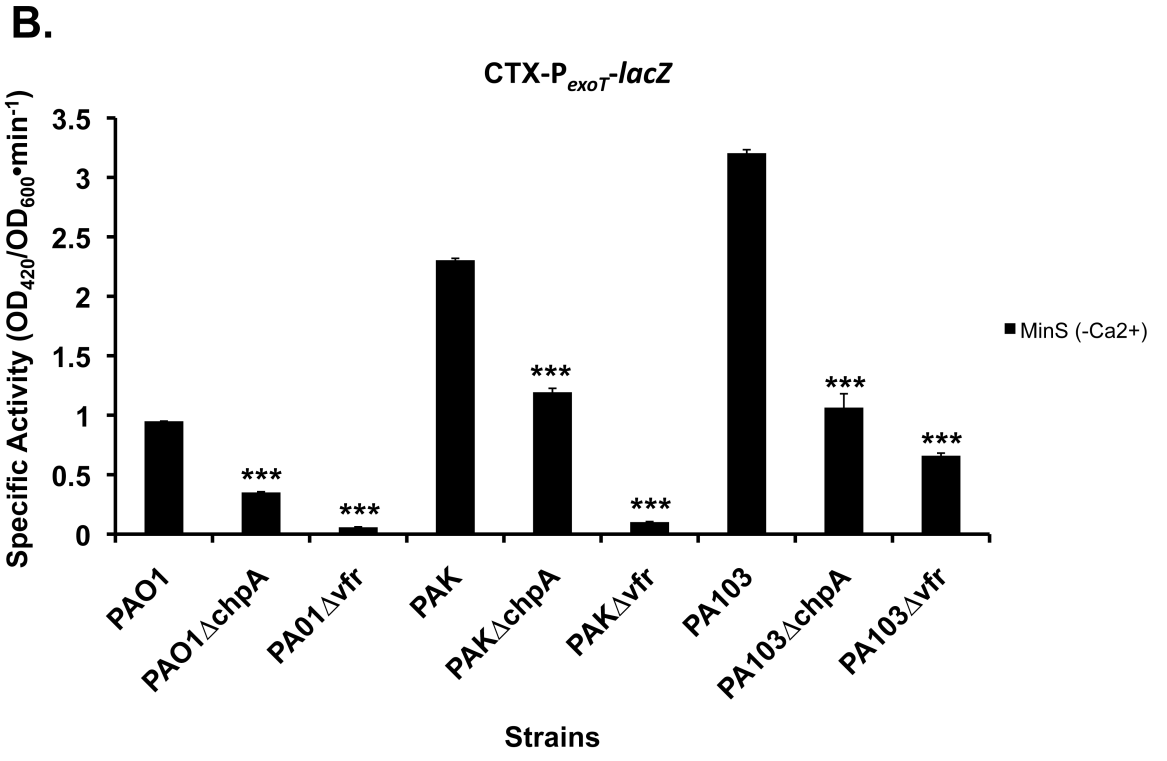
B.
CTX-P$_{exoT}$-lacZ
Specific Activity (OD$_{420}$/OD$_{600}$•min^{-1})
3.5
3
2.5
2
1.5
1
0.5
0
MinS (-Ca2+)
***
PAO1
PAO1ΔchpA
PA01Δvfr
PAK
PAKΔchpA
PAKΔvfr
PA103
PA103ΔchpA
PA103Δvfr
Strains

Figure 13. ChpA regulates *exoT* expression. (A) The indicated strains harboring a PAO1-derived P_{exoT}-*lacZ* fusion integrated into the chromosome at the *attB* site were cultured in MinS with (Panel A) or without Ca^{2+} (Panels A and B) for 16 hrs. The β-galactosidase activity of samples was measured and specific activities relative to OD_{600} calculated. Shown are the means ± SD (N=3). Statistical significance between WT and isogenic mutants was determined by ANOVA using Instat software (* $P < 0.001$).

Table 6. Strains and plasmids used in chapter 4

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	
PAO1Δ*chpA*	In frame deletion of *chpA*	(13)
PAO1Δ*vfr*	In frame deletion of *vfr*	(11)
PAK	Wild type	
PAKΔ*chpA*	In frame deletion of *chpA*	This study
PA103	Wild type	
PA103Δ*chpA*	In frame deletion of *chpA*	This study
PA103Δ*vfr*	In frame deletion of *vfr*	(11)
PAO1 CTX-P_{exoT}-*lacZ*	*pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*chpA* CTX-P_{exoT}-*lacZ*	Δ*chpA*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*vfr* CTX-P_{exoT}-*lacZ*	Δ*vfr*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAK CTX-P_{exoT}-*lacZ*	*pilA* promoter fused to *lacZ* at *attB* site	This study
PAKΔ*chpA* CTX-P_{exoT}-*lacZ*	Δ*chpA*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PAKΔ*vfr* CTX-P_{exoT}-*lacZ*	Δ*vfr*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PA103 CTX-P_{exoT}-*lacZ*	*pilA* promoter fused to *lacZ* at *attB* site	This study
PA103Δ*chpA* CTX-P_{exoT}-*lacZ*	Δ*chpA*; *pilA* promoter fused to *lacZ* at *attB* site	This study
PA103Δ*vfr* CTX-P_{exoT}-*lacZ*	Δ*vfr*; *pilA* promoter fused to *lacZ* at *attB* site	This study
E. coli		
DH5α	*hsdR rec lacZYA ϕ80 lacZM15*	Invitrogen
S17-1 λpir	*Thi pro hsdR recA RP4-2*(Tc::Mu)(Km::Tn7)	Stratagene
SM10	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*	(6)
Plasmids		
mCTX-P_{exoT}-*lacZ*	*exoT* reporter construct in mCTX-*lacZ*	This Study
pJEN51	pJEN34 carrying Δ*vfr* on SpeI fragment	(11)
pJB100	pEX100T derivative replacing *Sma*I site with *Spe*I site	This study
pJB100(Δvfr)	pJB100 carrying Δ*vfr* on SpeI fragment; subcloned from pJEN51	This Study
pJB116	pJB100 carrying Δ*chpA* on *Spe*I fragment	This study
pJB211	pJB100 carrying PA103 Δ*chpA* on SpeI fragment	This study

Table 7. Oligonucleotides used in chapter 4

Primer	Oligonucleotide sequence (5′→3′)[1]
chpA-SOE-1	GGACTAGTAAGATGGCGAGCGAGATG
chpA-SOE-2	TCACTCATGCTGTCCATTCATGTGCCTGAGTACCCCTTA
chpA-SOE-3	ATGAATGGACAGCATGAGTGAGCGCGTCACGCCACGGGT
chpA-SOE-4	GGACTAGTCTGGTTCATGTTTCGACT

[1]Restriction endonuclease sequences are underlined

Table 8. Top 100 gene differentially regulated in Δ*chpA* cultured in liquid media sorted by PA number

Gene Name or PA Number	Fold Change	PPDE (< P)	PPDE (P)
ig_1255042_1254309_at	-1.54	0.99	0.97
ig_1802453_1803626_at	1.66	0.97	0.93
ig_5242558_5243177_at	-1.65	0.96	0.91
ig_5820113_5820909_at	-1.59	0.95	0.90
ig_789144_788253_at	-2.05	0.97	0.95
ig_901046_901934_at	1.99	0.99	0.99
PA0044_exoT_at	-1.59	0.99	0.99
PA0080_at	-1.79	0.97	0.93
PA0200_i_at	1.55	0.96	0.93
PA0264_at	-1.50	0.99	0.98
PA0293_at	-1.59	0.98	0.95
PA0324_at	1.60	0.96	0.92
PA0380_i_at	-1.55	0.97	0.94
PA0413_at	-2.74	1.00	1.00
PA0415_at	-1.39	0.98	0.96
PA0551_epd_at	-1.38	0.95	0.90
PA0693_exbB2_at	1.74	0.96	0.91
PA1020_at	-1.45	0.97	0.94
PA1034_at	-1.74	0.99	0.98
PA1256_at	-1.44	0.95	0.90
PA1269_at	-1.53	0.96	0.92
PA1431_rsaL_at	-1.25	0.97	0.94
PA1490_at	2.50	0.99	0.97
PA1496_at	-1.45	0.96	0.91
PA1526_at	-1.54	0.97	0.95
PA1672_at	1.36	0.96	0.91
PA1695_pscP_at	-1.40	0.96	0.93
PA1705_pcrG_i_at	-1.50	0.96	0.93
PA1706_pcrV_at	-1.94	1.00	1.00
PA1707_pcrH_at	-1.96	1.00	1.00
PA1708_popB_at	-1.63	1.00	1.00
PA1709_popD_at	-1.86	1.00	1.00
PA1710_exsC_at	-1.55	1.00	0.99
PA1711_exsE_at	-1.75	1.00	1.00
PA1712_exsB_at	-1.54	0.96	0.91
PA1714_exsD_at	-1.48	0.99	0.99
PA1869_at	-1.31	0.95	0.90
PA1893_at	1.64	0.98	0.95
PA2004_at	-1.29	0.96	0.91
PA2076_at	1.80	0.97	0.94
PA2274_at	-1.31	0.96	0.92
PA2276_at	-1.58	0.97	0.93
PA2365_at	-1.48	0.99	0.98
PA2366_at	-1.39	0.96	0.92
PA2485_at	-1.69	0.96	0.92
PA2600_at	1.55	0.97	0.94

PA2619_infA_at	-1.71	0.98	0.96
PA2637_nuoA_at	-1.40	0.96	0.91
PA2727_at	-1.49	0.98	0.96
PA2772_at	-1.49	0.95	0.91
PA2861_ligT_at	-1.35	0.96	0.91
PA2946_at	-1.34	0.95	0.91
PA3002_mfd_at	-1.34	0.96	0.92
PA3009_at	-1.53	0.99	0.97
PA3126_ibpA_at	1.76	0.99	0.98
PA3148_wbpI_at	1.39	0.97	0.93
PA3195_gapA_at	-1.35	0.98	0.96
PA3243_minC_at	-1.49	1.00	0.99
PA3371_at	-1.44	0.97	0.94
PA3459_at	-1.36	0.96	0.93
PA3496_at	-1.42	1.00	0.99
PA3526_at	-1.37	0.97	0.93
PA3527_pyrC_at	-1.49	0.98	0.96
PA3560_fruA_at	-2.77	0.98	0.95
PA3621_fdxA_at	-1.58	0.99	0.97
PA3664_at	-1.38	0.96	0.92
PA3720_at	-1.54	0.99	0.99
PA3728_at	1.86	0.96	0.91
PA3798_at	-1.39	0.95	0.91
PA3841_exoS_at	-1.49	0.99	0.99
PA3842_at	-1.90	1.00	1.00
PA3843_at	-1.49	0.99	0.98
PA3861_rhlB_at	-1.36	0.97	0.94
PA3920_at	1.38	0.97	0.94
PA3976_thiE_at	-1.38	0.96	0.92
PA4013_at	1.98	0.99	0.97
PA4044_dxs_at	-1.42	0.97	0.93
PA4385_groEL_at	1.33	0.99	0.97
PA4431_at	-1.38	0.99	0.97
PA4454_at	-1.33	0.96	0.92
PA4461_at	-1.42	0.95	0.91
PA4525_pilA_at	1.32	0.95	0.90
PA4537_at	-1.51	1.00	0.99
PA4542_clpB_at	1.46	0.99	0.97
PA4563_rpsT_at	-1.59	0.97	0.94
PA4730_panC_at	-1.34	0.96	0.92
PA4746_at	-1.42	0.95	0.90
PA4791_at	1.33	0.96	0.91
PA4880_at	-1.53	0.99	0.97
PA5028_at	-1.33	0.96	0.91
PA5058_phaC2_at	-1.31	0.96	0.91
PA5110_fbp_at	-1.35	0.97	0.94
PA5133_at	-1.47	0.98	0.97
PA5216_at	-1.49	0.96	0.91
PA5276_lppL_i_at	-1.35	0.97	0.93

PA5285_at	-1.26	0.97	0.95
PA5286_at	-1.51	0.98	0.97
PA5300_cycB_at	-1.42	0.98	0.96
PA5351_at	-1.38	0.97	0.94
Pae_tRNA_Phe_f_at	1.88	0.99	0.97

Table 9. Top 100 gene differentially regulated in Δ*chpA* cultured on solid media sorted by PA number

Gene Name or PA Number	Fold Change	PPDE (< P)	PPDE (P)
ig_3206252_3206914_at	-5.86	1.00	1.00
PA0036_trpB_at	2.17	1.00	0.99
PA0093_at	-2.38	1.00	0.99
PA0521_at	2.30	1.00	0.99
PA0687_at	3.77	1.00	1.00
PA0707_toxR_at	3.12	1.00	1.00
PA0713_at	-4.22	1.00	1.00
PA0866_aroP2_at	2.62	1.00	1.00
PA0870_phhC_at	2.10	1.00	0.99
PA0881_at	2.53	1.00	1.00
PA0887_acsA_at	-8.94	1.00	0.99
PA0971_tolA_at	-2.38	1.00	0.99
PA1107_at	3.09	1.00	1.00
PA1134_at	2.96	1.00	1.00
PA1137_at	2.23	1.00	0.99
PA1178_oprH_at	2.03	1.00	0.99
PA1245_at	-2.72	1.00	1.00
PA1250_aprI_at	-2.45	1.00	0.99
PA1255_at	2.93	1.00	0.99
PA1256_at	2.56	1.00	0.99
PA1471_at	-2.89	1.00	1.00
PA1541_at	-3.68	1.00	1.00
PA1555_at	-5.17	1.00	1.00
PA1557_at	-4.01	1.00	1.00
PA1871_lasA_at	-2.57	1.00	1.00
PA1902_s_at	-4.40	1.00	0.99
PA1910_at	3.51	1.00	1.00
PA1911_at	2.38	1.00	0.99
PA2007_maiA_i_at	2.50	1.00	0.99
PA2033_at	2.61	1.00	0.99
PA2258_ptxR_at	3.07	1.00	1.00
PA2368_i_at	-2.13	1.00	0.99
PA2384_at	3.16	1.00	0.99
PA2385_at	3.08	1.00	0.99
PA2386_pvdA_at	4.15	1.00	1.00
PA2391_at	2.90	1.00	1.00
PA2392_at	2.51	1.00	0.99
PA2393_at	5.84	1.00	1.00
PA2394_at	4.12	1.00	1.00
PA2396_at	2.69	1.00	0.99
PA2397_pvdE_at	4.76	1.00	1.00
PA2398_fpvA_at	3.33	1.00	1.00
PA2399_pvdD_at	2.97	1.00	0.99
PA2402_at	4.90	1.00	1.00
PA2404_at	2.51	1.00	1.00
PA2405_at	3.49	1.00	1.00

PA2406_at	2.92	1.00	1.00
PA2407_at	2.44	1.00	0.99
PA2409_at	2.91	1.00	1.00
PA2412_at	4.02	1.00	1.00
PA2424_at	2.73	1.00	0.99
PA2425_at	3.25	1.00	0.99
PA2452_at	3.07	1.00	1.00
PA2478_at	2.68	1.00	0.99
PA2691_at	2.81	1.00	1.00
PA2803_at	3.26	1.00	1.00
PA2826_at	2.37	1.00	0.99
PA3234_at	-5.05	1.00	1.00
PA3235_at	-8.88	1.00	1.00
PA3329_at	-3.61	1.00	1.00
PA3392_nosZ_at	2.19	1.00	0.99
PA3516_at	-5.37	1.00	1.00
PA3584_glpD_at	-3.58	1.00	1.00
PA3600_at	7.75	1.00	1.00
PA3601_at	3.71	1.00	1.00
PA3753_at	2.55	1.00	1.00
PA3846_at	2.54	1.00	0.99
PA3847_at	2.93	1.00	0.99
PA3863_at	2.89	1.00	1.00
PA3870_moaA1_at	-4.16	1.00	1.00
PA3871_at	-3.72	1.00	1.00
PA3872_narI_at	-5.27	1.00	1.00
PA3873_narJ_at	-4.83	1.00	0.99
PA3874_narH_at	-5.73	1.00	1.00
PA3875_narG_at	-9.65	1.00	1.00
PA3876_narK2_at	-7.20	1.00	0.99
PA4113_at	-3.47	1.00	1.00
PA4168_at	2.97	1.00	1.00
PA4198_at	2.82	1.00	1.00
PA4205_at	-3.22	1.00	0.99
PA4206_at	-3.87	1.00	1.00
PA4211_g_at	-3.84	1.00	0.99
PA4290_at	-3.54	1.00	0.99
PA4295_at	-2.36	1.00	0.99
PA4352_at	-4.84	1.00	1.00
PA4380_at	2.50	1.00	0.99
PA4709_at	2.68	1.00	0.99
PA4759_dapB_at	2.30	1.00	0.99
PA4800_at	3.30	1.00	1.00
PA5043_pilN_at	-3.04	1.00	1.00
PA5054_hslU_at	1.83	1.00	0.99
PA5055_at	2.39	1.00	1.00
PA5119_glnA_at	2.21	1.00	0.99
PA5249_at	2.29	1.00	0.99
PA5361_phoR_at	2.32	1.00	0.99

PA5427_adhA_at	-4.54	1.00	1.00
PA5445_at	-5.57	1.00	0.99
PA5446_i_at	-3.26	1.00	1.00
PA5512_at	2.85	1.00	1.00

Chapter 5

PilH Regulates Production of Quorum Sensing-Dependent Virulence Factors and Mediates Virulence in a *Drosophila* Infection Model

Introduction

In-frame deletion mutants of *pilH* overproduce pyocyanin, which is a blue redox-active secondary metabolite (16) with antimicrobial activity that disrupts ciliary beating, inhibits lymphocyte proliferation, and alters phagocytic functions reviewed in (21). Due to its redox-active properties, pyocyanin generates reactive oxygen species that induce oxidative stress in both bacterial and mammalian cells (16, 21, 25). The production of pyocyanin is known to be regulated by the Rhl quorum sensing (QS) system (9), one of two classical QS systems encoded by *P. aeruginosa* (24). The other QS system is referred to as the Las system. Briefly, in these systems, the *lasI* or *rhlI* gene products direct the synthesis of unique homoserine lactone-derived signals (autoinducers), which freely diffuse into and out of cells. When autoinducer levels in a population reach a threshold concentration they bind to and activate the products of the *lasR* or *rhlR* genes. Autoinducer-bound LasR or RhlR serve as transcriptional activators for a number of downstream target genes including *lasI* and *rlhI*, establishing a positive feedback loop. They also directly regulate the transcription of genes involved in the production of secreted virulence factors including *lasB*, which encodes elastase, and *rlhAB*, which encodes a rhamnosyltransferase and is involved in the synthesis of the surfactant/hemolysin rhamnolipid (27). Regulation genes involved in the production of

other secreted VF, such as pyocyanin, and in the regulation of biofilm development appears to be indirect (8) (24). Quorum sensing in *P. aeruginosa* is hierarchical. Autoinducer-bound LasR activates transcription of *rhlR*. In addition the Las system autoinducer blocks the binding of the Rhl system autoinducer to RhlR. This second level of control, presumably, ensures that the two systems initiate their regulatory cascades sequentially and the in appropriate order. The system is further complicated by the presence of a third, non-homoserine lactone autoinducer, 2-heptyl-3-hydroxy-4-quinolone, designated the *Pseudomonas* quinolone signal (PQS) (28). Expression of PQS requires LasR and induces expression of *rhlI*, consistent with the idea that PQS functions as a link between the Las and Rlh systems allowing production of the Rlh autoinducer only after the establishment of the Las signaling cascade (15, 23, 28).

We investigated the mechanism by which PilH is involved in the regulation of QS-dependent VF production. We show here that Δ *pilH* mutants show increased production of elastase and pyocyanin, and decreased production of rhamnolipid. The increase in pyocyanin production correlates with increased transcription of one of the pyocyanin biosynthetic genes, *phzA1* (22). Likewise, decreased rhamnolipid production corresponded to decreased *rhlA* transcription (26, 27). Neither *lasR* nor *phnA*, a gene in the PQS biosynthesis pathway (11), showed altered transcription in Δ *pilH* mutant, suggesting that whatever its role, PilH functions downstream of these QS signaling regulators. We show that Δ*pilH* is hypervirulent in a *Drosophila* infection model (7, 14). Finally we show that Δ*pilH* mutants show decreased swarming relative to WT.

Materials and Methods

Construction of *lacZ* reporter strains. CTX-P$_{phzA1}$-*lacZ*. A transcriptional fusion of the *phzA1* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system (2). 500 bp of sequence upstream of the *phzA1* start codon was amplified using the phzA1-Reporter-F/phzA1-Reporter-R PCR primer pair. The PCR product was cloned as a *Hind*III/*Eco*RI fragment into mCTX2-*lacZ* to form pJB104. pJB104 was transformed into *E. coli* S17.1 and transformants were mated to PAO1, Δ*pilH*, and Δ*pilH*(comp) to create CTX-P$_{phzA1}$-*lacZ* derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFLP2, as described previously (2).

CTX-P$_{phzA2}$-*lacZ*. A transcriptional fusion of the *phzA2* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system (2). 500 bp of sequence upstream of the *phzA2* start codon was amplified using the phzA2-Reporter-F/phzA2-Reporter-R PCR primer pair. The PCR product was cloned as an *Eco*RI/*Bam*HI fragment into mCTX2-*lacZ* to form pJB105. pJB105 was transformed into *E. coli* S17.1 and transformants were mated to PAO1, Δ*pilH*, and Δ*pilH*(comp) to create CTX-P$_{phzA2}$-*lacZ* derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFLP2, as described previously (2).

CTX-P$_{phnA}$-*lacZ*. A transcriptional fusion of the *phnA* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system. 500 bp of sequence upstream of the *phnA* start codon was amplified using the phnA-Reporter-F/phnA-Reporter-R PCR primer pair. The

PCR product was cloned as a *Hind*III/*Eco*RI fragment into mCTX2-*lacZ* to form pJB106. pJB106 was transformed into *E. coli* S17.1 and transformants were mated to PAO1, Δ*pilH*, and Δ*pilH*(comp) to create CTX-P_{phnA}-*lacZ* derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFLP2.

CTX-P_{lasB}-*lacZ*. A transcriptional fusion of the *lasB* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system. 500 bp of sequence upstream of the *lasB* start codon was amplified using the lasB-Reporter-F/lasB-Reporter-R PCR primer pair. The PCR product was cloned as a *Hind*III/*Eco*RI fragment into mCTX2-*lacZ* to form pJB107. pJB107 was transformed into *E. coli* S17.1 and transformants were mated to PAO1, Δ*pilH*, and Δ*pilH*(comp) to create CTX-P_{lasB}-*lacZ* derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFLP2.

CTX-P_{rhlA}-*lacZ*. A transcriptional fusion of the *rhlA* promoter to *lacZ* was introduced in single copy onto the *P. aeruginosa* chromosome at the phage attachment site *attB* using the mini-CTX system. 500 bp of sequence upstream of the *phnA* start codon was amplified using the rhlA-Reporter-F/rhlA-Reporter-R PCR primer pair. The PCR product was cloned as a *Hind*III/*Eco*RI fragment into mCTX2-*lacZ* to form pJB108. pJB108 was transformed into *E. coli* S17.1 and transformants were mated to PAO1, Δ*pilH*, and Δ*pilH*(comp) to create CTX-P_{rhlA}-*lacZ* derivatives. The plasmid backbone was removed by mating strains to *E. coli* SM10 carrying pFLP2.

VF assays. Levels of specific secreted VF were measured from cleared culture supernatants. Cultures started from single colonies were grown in triplicate shaking in 5

mL LB for 16 hrs at 37 ˚C. Cells were removed by centrifugation (6,000 x g for 10 min) and the supernatant passed through a low protein binding 0.2 μm Acrodisc® Syringe Filter (Pall Corporation).

Elastase. Assays were preformed as described previously (27). Briefly, 10 mg of elastin congo red (ECR, Sigma) was resuspended in 500 μL ECR buffer (0.1 mM Tris-HCL, 1 mM $CaCl_2$, pH 7.2) and 500 μL filtered culture supernatant and incubated shaking at 37 ˚C for 4 hrs. Following incubation, the reaction was stopped by adding 100 μL 0.12 M EDTA. ECR was removed by centrifugation (14 000 g for 10 min) and the A_{495} of the supernatant determined.

Pyocyanin. Assays were performed as described previously (13). Briefly, 500 μL of filtered culture supernatant were extracted twice with 300 μL chloroform. The combined chloroform layers were back extracted with 150 μL 0.2 M HCl and the A_{520} determined.

Rhamnolipid. Assays were performed as described previously (18). Briefly, 500 μL of filtered culture supernatant was extracted twice with 1 mL diethyl ether. The ether fractions were back extracted with 200 μL 20 mM HCl to ensure that the entire aqueous phase was removed. Ether fractions were pooled and the ether allowed to evaporate. The residue was resuspended in 100 μL ddH_2O to which was added 900 μL 0.19% orcinol (w/v, Sigma) and 53% H_2SO_4 (v/v). Samples were incubated at 80 ˚C for 30 min, cooled for 15 min at room temperature, and the A_{421} determined.

β-galactosidase assays. β-galactosidase assays were performed as previously described (30) with the following modifications. Cultures started from single colonies

were grown in triplicate shaking for 16 hrs at 37 ˚C in 5 mL LB. Optical densities (OD_{600} and OD_{420}) were recorded using a SPECTRAmax® microplate spectrophotometer (Molecular devices) and SoftMaxPro® 4.3.1. Specific activities were calculated relative to OD_{600}.

Statistical analysis. Statistical significance was determined by ANOVA using Instat software. Differences were considered to be significant at $P < 0.05$.

***Drosophila* infection assays**. Bacteria were cultured in 2 mL M63 minimal media (29) lacking iron and supplemented with 10 mM succinate shaking at 37 ˚C for 16 hrs. Cultures were diluted to $OD_{600} = 0.001$ prior to injections into 20 one-week old male Oregon R flies. Injections were performed as described previously (4). To determine average colony forming units (CFUs) injected for each strain, cultures were serially diluted and plated for viable counts both before and after the injections. CFUs both before and after injections were determined and the overall average for the experiment determined by averaging both counts.

Swarming assays. Swarming assays were performed as described previously (5)

Results and Discussion

PilH regulates production of sensing-dependent virulence factors

In-frame deletion mutants of *pilH* overproduce the blue redox-active secondary metabolite pyocyanin, which has known antibiotic properties. Production of pyocyanin is

regulated by the Rhl QS system (9). To determine the degree to which pyocyanin was overproduced and to see if production of other QS-regulated secreted VFs, including those regulated by the Las system, were altered in Δ*pilH*, we assayed the supernatants from 16 hr LB grown WT PAO1 and Δ *pilH* cultures for the presence of elastase, pyocyanin, and rhamnolipid. Δ*pilH* mutants produced significantly more pyocyanin and elastase, and significantly less rhamnolipid relative to WT (Fig. 14A, B, C). It remains to be determined if these alterations in VF production in Δ*pilH* can be complemented by reintroduction of PilH. Curiously, pyocyanin and rhamnolipid production are both thought to be regulated primarily by the Rhl QS system (24). The reason for the inverse regulation of these two virulence factors in Δ*pilH* is unclear, though there is precedence for this. Mutations in the global transcriptional regulator of virulence gene expression, *mvaT*, also result in increased pyocyanin and decreased rhamnolipid production (12, 20). It is becoming increasingly clear that the regulation of virulence factor expression is complex. The role that PilH plays in that regulation, whether as part of the MvaT or another pathway will be the subject of future investigations. Interestingly, the ability of PilH to function as a signaling molecule may play some role in it regulation of VF production. A *pilH* point mutant, in which the putative phosphate accepting aspartate residue has been changed to alanine, appears to recapitulate the Δ *pilH* pyocyanin overproduction phenotype as judged by visual estimation.

PilH regulates expression of quorum sensing-dependent virulence factor biosynthesis genes

To investigate whether the changes we observed in the production of QS-dependent virulence were due to changes in the expression of VF biosynthesis genes, we constructed *lacZ* transcriptional fusions to the promoters of *lasB* (elastase), *rhlA* (rhamnolipid), *phzA1* and *phzA2* (pyocyanin). The reporter constructs contained 500 bp of sequence upstream of each gene. Reporter fusions were integrated at the *attB* site of WT PAO1, Δ*pilH*, and Δ*pilH*(comp). β-galactosidase activity was measured from 16 hr LB broth grown cultures. Transcription of *phzA1* was significantly increased in Δ*pilH* relative to WT (Fig. 15A, $p < 0.001$) and transcription of *rhlA* was significantly decreased (Fig. 15C, $p < 0.001$). Expression of both *phzA1* and *rhlA* were restored to WT levels in Δ*pilH*(comp) (Fig. 15A, C). Expression of *phzA2* was unchanged in Δ*pilH* (Fig. 15B) although the overall levels of expression were very low suggesting that *phzA2* may not be expressed under these conditions. Genes encoded by the *phzA1* and *phzA2* operons are both involved in the conversion of chorismic acid into phenazine-1-carboxylic acid, a precursor of pyocyanin (Fig. 16A, 22). The reason that expression of *phzA1* but not *phzA2* is increased in Δ*pilH* is not clear. However, mutations in *ptxR*, a regulator of expression of exotoxin A and other QS-dependent VFs, also results in overproduction of pycyanin, which correlates with increased *phzA1* but not *phzA2* expression (6). Although we detected increased elastase production, transcription of *lasB* was significantly decreased in Δ*pilH* (Fig. 15D, $p < 0.001$). The reason for this discrepancy is unclear, but the fact that we were unable to complement the *lasB* expression phenotype, indicates that the results should interpreted cautiously.

To determine if the changes in expression of VF biosynthetic genes corresponded to changes in QS signaling, we constructed *lacZ* transcriptional fusions to the promoters

of *lasR* and *phnA*. PhnA is required for the conversion of chorismic acid into anthranilic acid, a precursor of PQS (Fig. 16B, 11). The reporter constructs contained 500 bp of sequence upstream of each gene. Reporter fusions were integrated at the *attB* site (2) of WT PAO1 and Δ*pilH*. β-galactosidase activity was measured out of 16 hr broth grown culture. No changes in either *lasR* or *phnA* expression were detectable in Δ*pilH* (Fig. 15E, F). We also saw no changes in PQS levels by thin layer chromatography in Δ*pilH* relative to WT (data not shown). These results suggest that, whatever its role, PilH functions downstream of both the Las and the PQS QS systems. It remains to be determined if expression of *rlhI* or *rlhR* are altered in Δ*pilH*. Future work will focus on determining the exact role of PilH in the regulation of QS-dependent VF production.

Δ*pilH* mutants show increased virulence in a *Drosophila* infection model

We tested if Δ*pilH* showed altered virulence *in vivo*. The fruit fly, *Drosophila melanogaster*, is an established model for *P. aeruginosa* infection (7, 14). In fact, several Chp system mutants, including mutations in *chpA*, were previously shown to have decreased virulence in the *Drosophila* model (7). We infected *Drosophila* using a previously described subcutaneous injection model (4) with WT, Δ *pilH*, and Δ*pilH*(comp), and monitored the percentage of living flies over time (Fig. 17). Although preliminary, the data suggest that Δ*pilH* mutants are more virulence that WT PAO1 in a *Drosophila* infection model (Fig. 17). Δ *pilH*(comp) appeared to show decreased virulence relative to WT (Fig. 17), but this finding may reflect a lower average number of CFUs injected. It is unclear why Δ*pilH* shows increased virulence, although it is not due to increased surface piliation, as Δ*pilT* mutants show decreased virulence in the same

model (data not shown). One intriguing possibility is the overproduction of pyocyanin. This hypothesis could be tested by assaying the virulence phenotype of a Δ*pilH*Δ*phzA1* double mutant. Further work will need to be done to confirm the virulence phenotype of Δ*pilH* in the *Drosophila* model and to determine its causes.

Δ*pilH* mutants have an altered swarming phenotype

Swarming motility is a form of translocation that occurs on semisolid surfaces and requires functional type IV pili and flagella, and results in complex motility patterns (5, 19). Although their exact function is unclear, there is some evidence to suggest that rhamnolipid biosurfactants are important for swarming motility (5, 19). We were interested in determining if decreased rhamnolipid production in Δ*pilH* resulted in altered swarming motility. Swarming motility for PAO1 and Δ*pilH* was monitored as described previously (5). Although we failed to detect the well defined tendrils radiating out from the point of inoculation that have been observed for other strains (5), we did see a marked decrease in the size of the swarm zone for Δ*pilH* compared to WT (Fig. 18). These results are preliminary and further work will have to be done to determine: 1) if the result is repeatable, 2) if the Δ*pilH* swarming defect can be complemented, and 3) whether the Δ*pilH* defect in rhamnolipid production or type IV pilus function is primarily responsible for the swarming motility phenotype.

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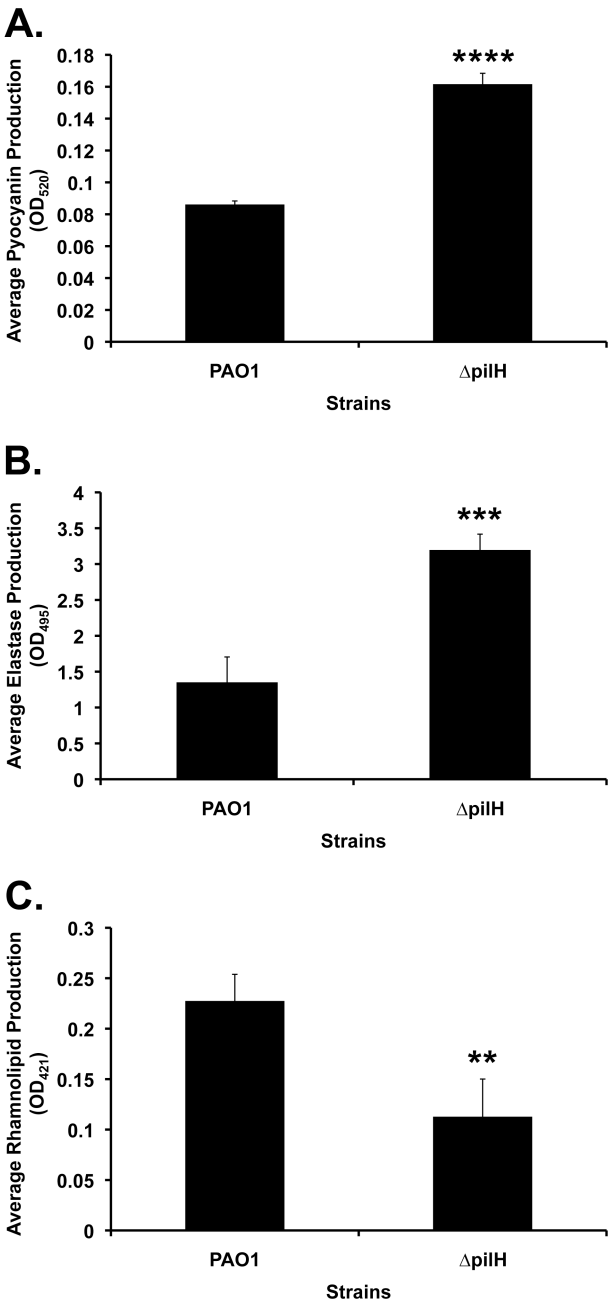

Figure 14. PilH regulates production of quorum sensing-dependent virulence factors. 16 hr LB PAO1 and Δ*pilH* broth culture supernatants were assayed for pyocyanin (A), elastase (B), and rhamnolipid (C). Shown are the means ± standard deviation (SD, N=3).

Statistical significance was determined by two-tailed t test using Instat software (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

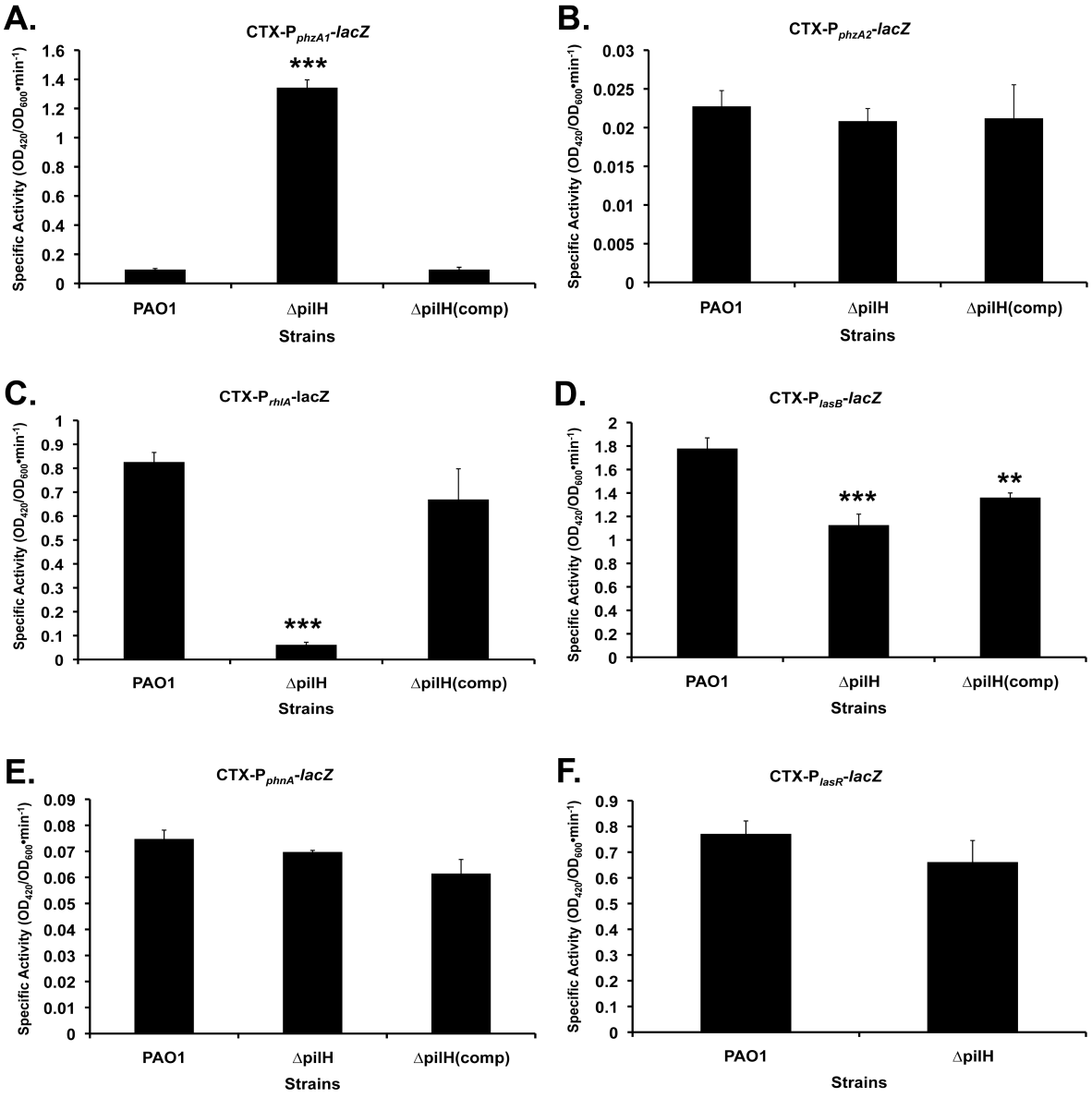

Figure 15. PilH regulates expression of quorum sensing-dependent virulence factor biosynthesis genes. PAO1, Δ*pilH*, and Δ*pilH*(comp) harboring P$_{phzA1}$-*lacZ* (A), P$_{phzA2}$-*lacZ* (B), P$_{rhlA}$-*lacZ* (C), P$_{lasB}$-*lacZ* (D), P$_{phnA}$-*lacZ* (E), or P$_{lasR}$-*lacZ* (F) integrated into the chromosome at the *attB* site were cultured in LB broth for 16 hrs. The β-galactosidase activity of samples was measured and specific activities relative to OD_{600} calculated.

Shown are the means ± SD (N=3). Statistical significance was determined by ANOVA using Instat software (** $P < 0.01$, *** $P < 0.001$).

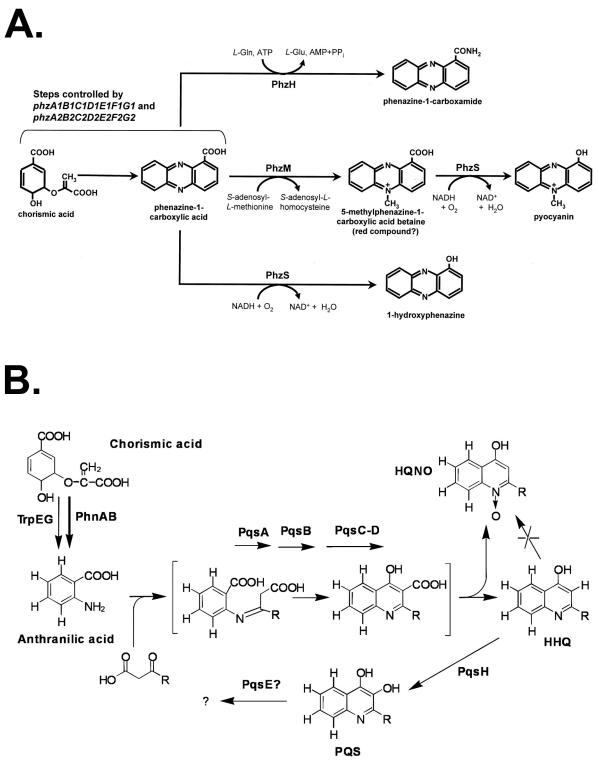

Figure 16. Pyocyanin and PQS biosynthesis pathways. (A) The pyocyanin biosynthesis pathway as characterized by Mavrodi *et al*. (22). (B) The PQS biosynthesis pathway as characterized by Déziel *et al*. (11).

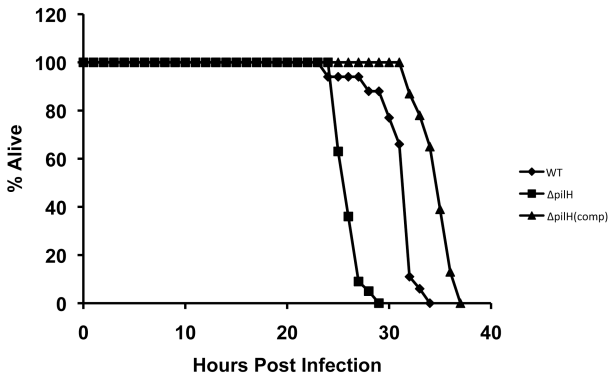

Figure 17. Δ*pilH* mutants show increased virulence in a *Drosophila* infection model. PAO1, Δ*pilH*, and Δ*pilH*(comp) were cultured in 2 mL M63 minimal media lacking iron and supplemented with 10 mM succinate. Cultures were diluted to OD_{600} = 0.001 and 50 nL injected into the anterior abdomen on the ventrolateral surface of 20 one-week old male flies (do we need to know the genotype of the flies?). The time to death was monitored and the percentage of flies alive as a function of time calculated. Average number of CFUs injected was determined to be 206 (PAO1), 200 (Δ*pilH*), and 98 (Δ*pilH*(comp)).

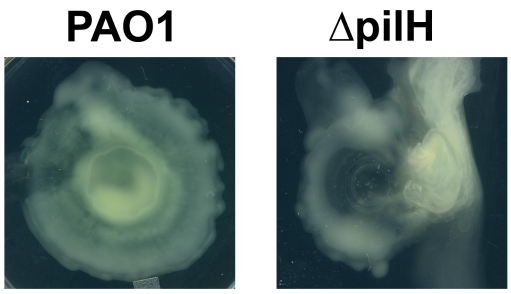

Figure 18. Δ*pilH* mutants have reduced swarming motility. PAO1 and Δ*pilH* were spot inoculated from 2 mL overnight LB broth cultures onto 0.5% LB agar and grown for 16 hrs at 37 ˚C. Shown are the swarm zones following incubation.

Table 10. Strains and plasmids used in chapter 5

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	This study
PAO1Δ*pilH*	In frame deletion of *pilH*	(1)
PAO1Δ*pilH*(comp)	PAO1Δ*pilH* complemented at the *pilH* locus	This study
PAO1 CTX-P$_{phzA1}$-*lacZ*	PAO1; *phzA1* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH* CTX-P$_{phzA1}$-*lacZ*	PAO1Δ*pilH*; *phzA1* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH*(comp) CTX-P$_{phzA1}$-*lacZ*	PAO1Δ*pilH*(comp); *phzA1* promoter fused to *lacZ* at *attB* site	This study
PAO1 CTX-P$_{phzA2}$-*lacZ*	PAO1; *phzA2* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH* CTX-P$_{phzA2}$-*lacZ*	PAO1Δ*pilH*; *phzA2* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH*(comp) CTX-P$_{phzA2}$-*lacZ*	PAO1Δ*pilH*(comp); *phzA2* promoter fused to *lacZ* at *attB* site	This study
PAO1 CTX-P$_{phnA}$-*lacZ*	PAO1; *phnA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH* CTX-P$_{phnA}$-*lacZ*	PAO1Δ*pilH*; *phnA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH*(comp) CTX-P$_{phnA}$-*lacZ*	PAO1Δ*pilH*(comp); *phnA* promoter fused to *lacZ* at *attB* site	This study
PAO1 CTX-P$_{lasB}$-*lacZ*	PAO1; *lasB* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH* CTX-P$_{lasB}$-*lacZ*	PAO1Δ*pilH*; *lasB* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH*(comp) CTX-P$_{lasB}$-*lacZ*	PAO1Δ*pilH*(comp); *lasB* promoter fused to *lacZ* at *attB* site	This study
PAO1 CTX-P$_{rhlA}$-*lacZ*	PAO1; *rhlA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH* CTX-P$_{rhlA}$-*lacZ*	PAO1Δ*pilH*; *rhlA* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH*(comp) CTX-P$_{rhlA}$-*lacZ*	PAO1Δ*pilH*(comp); *rhlA* promoter fused to *lacZ* at *attB* site	This study
PAO1 CTX-P$_{lasR}$-*lacZ*	PAO1; *lasR* promoter fused to *lacZ* at *attB* site	This study
PAO1Δ*pilH* CTX-P$_{lasR}$-*lacZ*	PAO1Δ*pilH*; *lasR* promoter fused to *lacZ* at *attB* site	This study
E. coli		
DH5α	*hsdR rec lacZYA ϕ80 lacZM15*	Invitrogen
S17-1 λpir	*Thi pro hsdR recA RP4-2*(Tc::Mu)(Km::Tn7)	Stratagene
SM10	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km lpir*	(10)

Plasmids		
mCTX-lacZ	Contains promoterless *lacZ* for constructing transcriptional fusions at the *attB* site, Tc^R	(2)
pFLP2	Source of Flp recombinase, Ap^R	(17)
pJB104	*phzA1* reporter construct cloned as *Hind*III/*Eco*RI fragment into mCTX-*lacZ*	This study
pJB105	*phzA2* reporter construct cloned as *Eco*RI/*BamH*I fragment into mCTX-*lacZ*	This study
pJB106	*phnA* reporter construct cloned as *Hind*III/*Eco*RI fragment into mCTX-*lacZ*	This study
pJB107	*lasB* reporter construct cloned as *Hind*III/*Eco*RI fragment into mCTX-*lacZ*	This study
pJB108	*rhlA* reporter construct cloned as *Hind*III/*Eco*RI fragment into mCTX-*lacZ*	This study

Table 11. Oligonucleotides used in chapter 5

Primer	Oligonucleotide sequence (5′→3′)[1]
phzA1-Reporter-F	CCCAAGCTTCCTGTTCCAGAGCCTTTTCC
phzA1-Reporter-R	CCGGAATTCGCGCCGCCTCCGAGAGGG
phzA2-Reporter-F	CCGGAATTCCATCGGCCTGCTCAACTG
phzA2-Reporter-R	CGCGGATCCGGTGCGAATCTCCGCCAG
phnA-Reporter-F	CCCAAGCTTTCTGCGGCGATGCCCTGG
phnA-Reporter-R	CCGGAATTCGGGCGACTGGCCTGCGCG
lasB-Reporter-F	CGCGGATCCCTTGTTCAGTTCTCCTGG
lasB-Reporter-R	CCCAAGCTTGCAGCAGCGGATCGTCGG
rhlA-Reporter-F	CCGGAATTCTTCACACCTCCCAAAAATTTTCG
rhlA-Reporter-R	CCCAAGCTTAGGCCTGCGAAGTGTCCTATAAG

[1]Restriction endonuclease sequences are underlined.

Chapter 6

Localization of PilU in Chp System Mutants

Introduction

A recent study by Chiang and colleagues (3) demonstrated that the ATPases PilB, PilT, and PilU have polar localization in the *P. aeruginosa* cell. Whereas PilB and PilT localize to both pole, PilU has a monopolar localization and appears to be present exclusively at the piliated pole (3). Only two other proteins implicated in the regulation of Tfp function have been shown to have monopolar localizations, the phosphodiesterase FimX (6, 7), and the unique regulatory protein FimL (our unpublished data 9). Other proteins involved in the regulation of Tfp function examined to date, including PilB and PilT (3), the putative Chp system methyl-accepting chemotaxis protein, PilJ (5), and the histidine kinase PilS (2), have been shown to have bi-polar localization. Furthermore, despite its homology to PilT (10) and demonstrated ATPase activity *in vitro* (4), data presented here (see Chapters 1 and 6) suggest that the function of PilU in the regulation of Tfp dynamics is something other than as a retraction ATPase. Taken together, these observation suggests that rather than contributing to pilus function primarily at the level of driving pilus retraction, PilU may play a role in determining the pole from which Tfp extend and retract. Indeed, a similar regulatory activity has been observed for Tfp-driven motility in *M. xanthus*, where changes in cell direction are mediated by disassembly of Tfp at one pole and reassembly at the other pole. FrzS has been shown to localize to the

pole at which Tfp are active and to oscillate between poles during changes in direction through a mechanism that is dependent upon the Frz chemosensory system (8).

We were interested in determining if PilU localization was dependent upon the Chp chemosensory system. It has previously been shown that PilB, PilT, and PilU localization is independent of various factors involved in the regulation of Tfp function, including, among others, FimX and PilJ, a putative MCP for the Chp system (3). We show here that PilU localization is unaffected by mutation of the core signaling components of the Chp system, *chpA*, *pilG*, and *pilH*. Therefore, if PilU does oscillate between poles to determine which is piliated, the mechanism by which is does so may be silimar to that of FrzS, which fails to oscillate in the absence of the Frz chemosensory system (8).

Materials and Methods

Fluorescent microscopy. To prepare strains, pUCP20Gm-*yfp-pilU* (3) was transformed into chemically competent PAO1 and mutant strain backgrounds, using previously published methods (1). Fluorescent microscopy was carried out as described previously (3). Briefly, single colonies growing on LB agar containing the appropriate antibiotics were resuspended in 10 μL phosphate-buffered saline on a glass slide, covered with a polylysine coated coverslip. Images were acquired at a magnification of x1000 under the oil immersion with a 1.45 NA objective on a Nikon Eclipse TE2000-E fluorescence microscope, using a CCD camera and processed by Simple PCI imaging software (Compix Inc). Images were processed with PhotoShop CS3 (Adobe).

Results and Discussion

To determine if PilU localization was affected by deletion of any of the core signaling components of the Chp chemosensory system, we transformed pUCP20Gm-*yfp-pilU* (3) into WT PAO1, Δ *chpA*, Δ *pilG*, Δ *pilH*, and Δ *pilG*Δ*pilH* mutants and monitored PilU localization by fluorescent microscopy. Monopolar localization of PilU was observed in all strain backgrounds (Fig. 19A-E). These results suggest that PilU localization is independent of Chp system components and signaling. They do not, however, rule out the possibility that the Chp system may be involved in regulating the, as yet hypothetical, oscillation of PilU from pole to pole. Indeed, FrzS, which determines the piliated pole in *M. xnthus*, continues to have a polar localization in the absence of the Frz chemosensory system, but fails to oscillate between poles (8). Further work will need to done to determine whether *P. aeruginosa* cells undergo reversals during TM, whether PilU oscillates between poles during those reversals, and what factors are required for both polar localization and oscillation of PilU.

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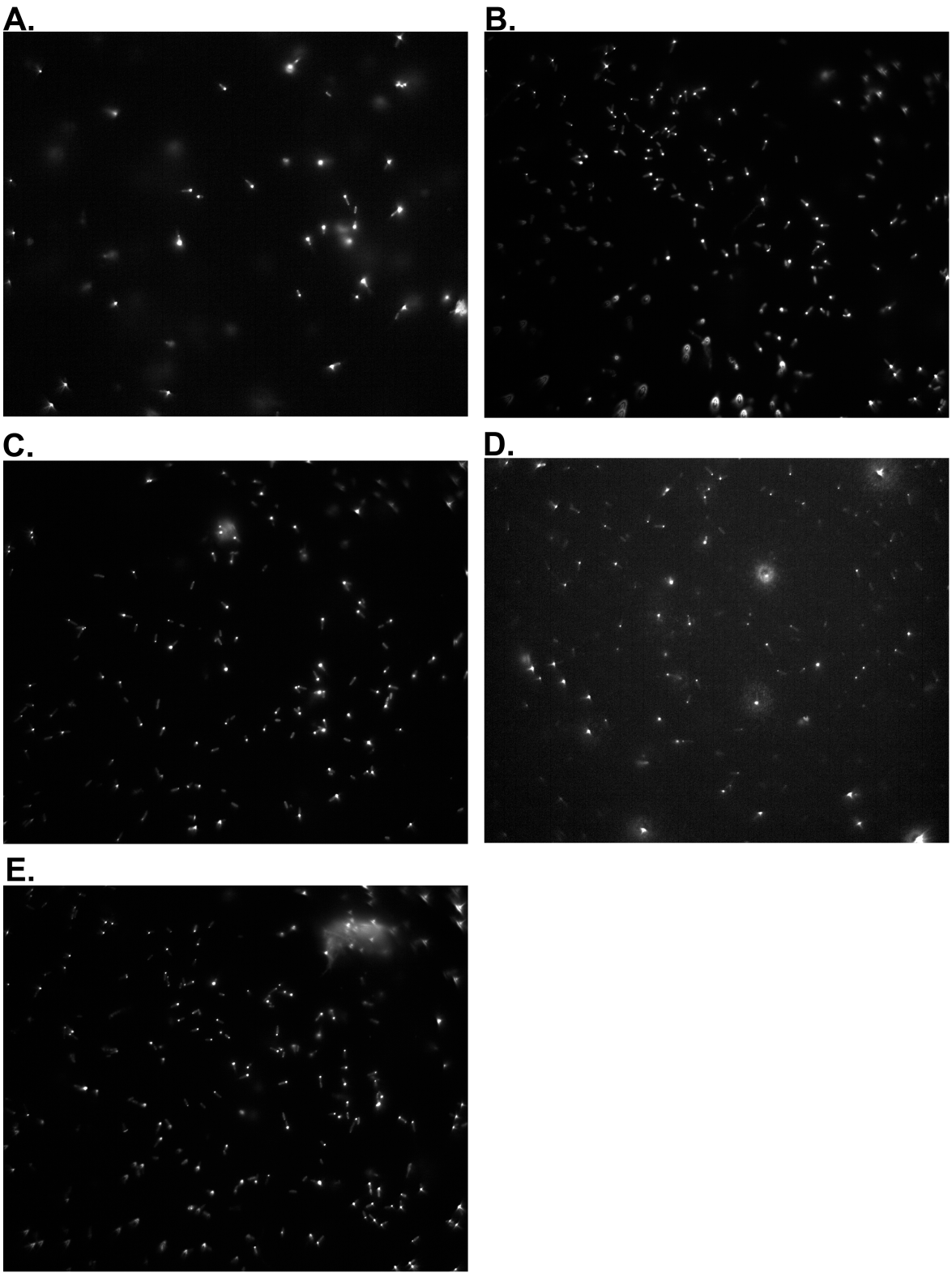
A.
B.
C.
D.
E.

Figure 19. PilU localization in Chp system mutant backgrounds. pUCP20Gm-*yfp-pilU* was transformed into WT PAO1 (A), Δ*chpA* (B), Δ*pilG* (C), Δ*pilH* (D), and Δ*pilG*Δ*pilH* (E). PilU localization was monitored by fluorescent microscopy.

Table 12. Strains and plasmids used in chapter 6

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	
PAO1Δ*chpA*	In frame deletion of *chpA*	This study
PAO1Δ*pilG*	In frame deletion of *pilG*	This study
PAO1Δ*pilH*	In frame deletion of *pilH*	This study
PAO1Δ*pilG*Δ*pilH*	In frame deletion of *pilG* and *pilH*	This study
Plasmids		
pUCP20Gm-*yfp-pilU*	YFP-PilU fusion construct	(3)

Chapter 7

FimX Is Involved in the Regulation of Type IV Pilus Extension

Introduction

FimX is a multidomain protein involved in the regulation of Type IV pilus (Tfp) function. *fimX* was originally identified in a transposon screen in the PAK strain background for mutants with impaired TM (4). Transposon insertions in *fimX* resulted in decreased TM and presentation of surface pili, suggesting that normal Tfp function was impaired. FimX has four predicted domains commonly found in proteins involved in signal transduction. These include: GGDEF and EAL domains characteristic of diguanylate cyclases and phosphodiesterases, respectively, a CheY-like response regulator domain, and a PAS domain, commonly found in signaling proteins and believed to be involved in sensing oxygen and redox potential (4, 5, 7). The CheY domain of FimX lacks a candidate catalytic aspartate residue, suggesting that FimX function is unlikely to be regulated through phosphorylation (5). However, C-terminal to the CheY domain is a putative membrane localization sequence. The initial characterization of FimX demonstrated a monopolar localization (4). Subsequent studies have shown that polar localization is dependent upon the putative membrane localization sequence (5). *fimX* mutants constructed in the PA103 strain background and in which the putative membrane localization sequence has been deleted show decreased TM and surface presentation of Tfp, and electron micrographs reveal that pili in these mutants can originate from non-polar sites (5). Furthermore, both the GGDEF and the EAL domains

have been shown to be required for FimX function, although only the EAL domain has demonstrable enzymatic activity *in vitro* (5). The exact mechanism by which FimX serves to regulate Tfp function remains ambiguous. A recent study, however, demonstrated direct binding of FimX and PilZ, a protein involved in Tfp biogenesis (1), to the PilB ATPase (3), suggesting that FimX may interface with components of the Chp system and/or the other Tfp motor ATPases, PilT and PilU. FimX is not required for PilU or PilT localization to the poles (2).

We carried out a preliminary genetic characterization of *fimX* to both confirm the Tfp phenotypes of Δ*fimX* in the PAO1 strain background and to begin to characterize its role in the regulation of Tfp function. Specifically, we constructed an unmarked in-frame deletion of *fimX* and assayed it for TM, intracellular pilin, and surface presentation. In addition, we constructed double mutants of *fimX* and the core Chp system signaling components (*chpA*, *pilG*, and *pilH*), as well as the Tfp motor ATPases (*pilB*, *pilT*, and *pilU*), and assayed then for Tfp function. We present data consistent with the idea that FimX is involved in the regulation of pilus extension, and that it functions downstream of ChpA and PilG and upstream of PilH and PilT. These data support the model that FimX functions as an accessory to PilB in the regulation of pilus extension.

Materials and Methods

Construction of in-frame deletion mutants. All matings were performed as described previously (6). All mutants were confirmed by PCR analysis. 5′ and 3′ *fimX* deletion construct fragments were amplified using the fimX1/fimX2 and fimX3/fimX4 PCR primer pairs. PCR products were used as templates in a sequence overlap extension

(SOE) reaction with the fimX1/fimX4 PCR primer pair. The SOE product was cloned as a *Spe*I fragment into pJB100 for form pJB123. pJB123 was transformed into *E. coli* S17.1 and transformants were mated to PAO1, Δ*chpA*, Δ*pilB* Δ*pilG*, Δ*pilH*, Δ*pilT*, and Δ*pilU* to create Δ*fimX*, Δ*fimX*Δ*chpA*, Δ *fimX*Δ*pilB*, Δ *fimX*Δ*pilG*, Δ *fimX*Δ*pilH*, Δ*fimX*Δ*pilT*, and Δ*fimX*Δ*pilU*.

Results and Discussion

To assess Tfp function of *fimX* mutans in the PAO1 strain background we constructed an unmarked in-frame deletion mutant and assayed it for TM, intracellular pilin, and surface pilin levels. Similar to the fimX mutation in the PAK strain background, in-frame deletion mutants of *fimX* in PAO1 have an intermediate TM phenotype, similar in magnitude to that seen for Δ*pilH* by the subsurface stab assay (Fig. 20A). Unlike PAK, but similar to PA103, no surface pilin was detectable in PAO1 Δ*fimX* by Western blot analysis (Fig. 20B). Intracellular pilin levels were unaffected in Δ*fimX*. The lack of detectable surface pili, as well as the recently described interaction of FimX with PilZ and PilB (3), led us to hypothesize that FimX is involved in the regulation of pilus extension or with PilB localization.

In light of the data presented in Chapter 2 and the recent report that FimX functions in concert with PilB to mediate pilus extension (3) makes the prediction that FimX should act downstream of ChpA and PilG and upstream of PilH, PilT, and, possibly, PilU. This model leads to the testable genetic predictions that: 1) Δ*fimX*Δ*chpA* and Δ *fimX*Δ*pilG* double mutants should recapitulate Δ *chpA* and Δ *pilG* phenotypes, respectively, and 2) Δ*fimX*Δ*pilH* and Δ*fimX*Δ*pilT* double mutants should show reduced

surface piliation relative to Δ*pilH* and Δ*pilT* but should recapitulate their TM phenotypes. To test these predictions we constructed Δ *fimX*Δ*chpA*, Δ *fimX*Δ*pilB*, Δ *fimX*Δ*pilG*, Δ*fimX*Δ*pilH*, Δ*fimX*Δ*pilT*, and Δ*fimX*Δ*pilU* double mutants and assayed them for TM, intracellular pilin, and surface presentation. Intracellular pilin levels were unaffected in all mutants. Consistent with our model, Δ*fimX*Δ*chpA* and Δ*fimX*Δ*pilG* mutants were defective for TM and lacked detectable levels of surface pili by western analysis (Fig. 20A, B). The loss of the intermediate Δ*fimX* TM phenotype indicates that ChpA and PilG both function upstream of FimX. The lack of TM and detectable surface pili in the Δ*fimX*Δ*pilB* double mutant is not surprising given the crucial role of PilB in regulating Tfp function through pilus extension (Fig. 20A, B). Similarly, our finding that Δ*fimX*Δ*pilT* recapitulated the TM phenotype of Δ*pilT* speaks to the indispensible role of PilT in mediating pilus retraction. Interestingly, Δ*fimX*Δ*pilH* double mutants showed TM that was decreased relative to both Δ*fimX* and Δ*pilH*, but still distinguishable from, and greater than, that of Δ*chpA*, Δ*pilG*, Δ*pilB*, Δ*pilT*, or Δ*pilU* (Fig. 20A). These data (along with those presented in Chapter 2) suggest that Tfp remain functional in the absence of both FimX and PilH, and support the notion that PilB and PilT retain some activity in the absence of FimX and PilH, respectively. Δ*fimX*Δ*pilH* and Δ*fimX*Δ*pilT* had detectable levels of surface pili, though less than those seen for Δ*pilH* and Δ*pilT,* respectively (Fig. 20B). This observation is consistent with the idea that FimX functions to regulate extension upstream of PilH and PilT, and further supports the idea that PilB retains some activity in the absence of FimX.

As with Δ*pilH*Δ*pilU* (see Chapter 2), Δ*fimX*Δ*pilU* appears to be a special case. Specifically, Δ*fimX*Δ*pilU* has a Δ*fimX* surface presentation phenotype and a Δ*pilU* TM

phenotype (Fig. 20A, B). The same is true for Δ*pilH*Δ*pilU* mutants. These data seem to suggest that FimX (and PilH, and, possibly, other Chp system components) and PilU function in separate pathways. The fact that Δ*pilU* and all *pilU* double mutants are severely deficient for TM, suggests that PilU is indispensible for TM. However, the observation that all *pilU* double mutants examined in this study, including Δ*fimX*Δ*pilU*, have levels of surface pili that recapitulate those of single deletion of the second gene, suggests that PilU does not directly regulate the mechanical aspect of Tfp function. The genetic argument against PilU having retractile activity and participating directly in mechanical aspects of regulation of Tfp function could be strengthened by examining the Tfp phenotypes of Δ *fimX*Δ*pilH*Δ*pilT*, Δ *fimX*Δ*pilH*Δ*pilU*, and Δ *fimX*Δ*pilH*Δ*pilT*Δ*pilU* mutants.

Taken together these data provide preliminary support for the notion that FimX functions to regulate pilus extension, possibly through its interactions with PilZ and PilB, downstream of ChpA and PilG and upstream of PilH and PilT.

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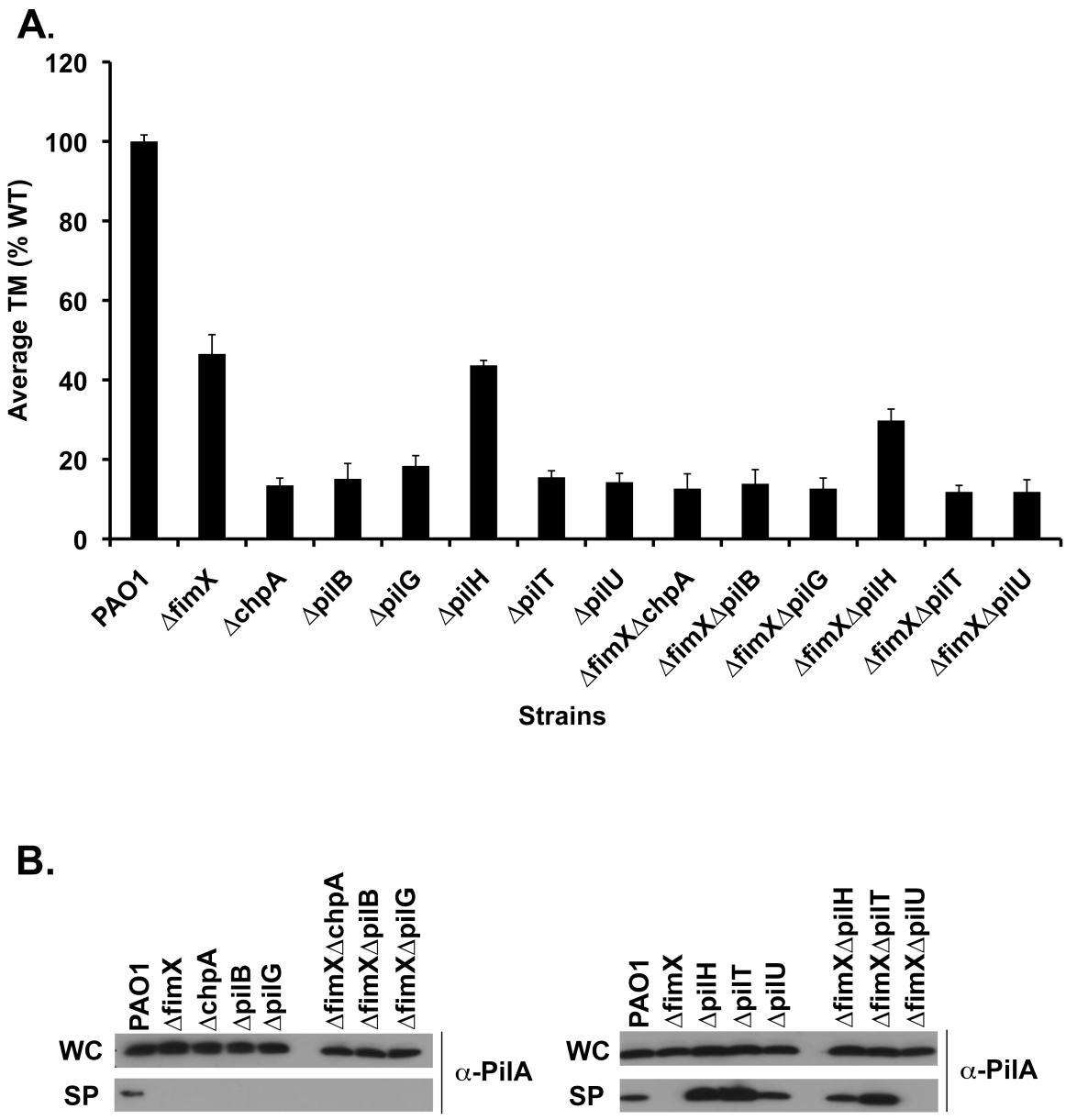

Figure 20. Assays of pilus function for *fimX* mutants. (A) Graph depicting TM zone diameters for the indicated strains. The diameter is expressed as a percentage of PAO1. Shown are the means ± the standard deviation (N=3) (B) Intracellular and surface pilin levels. For the indicated strains, surface pili (SP) were sheared by vigorous vortexing of bacteria cultured on solid media and separated from cells (WC) by centrifugation. The sheared pili were precipitated. WC (15 μ g total protein) and SP (5% of the total

resuspended volume of precipitated pili) samples were separated by SDS-PAGE and immunoblotted with a polyclonal antibody to PilA (α-pilA).

Table 13. Strains and plasmids used in chapter 7

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	
PAO1Δ*chpA*	In frame deletion of *chpA*	This study
PAO1Δ*pilB*	In frame deletion of *pilB*	This study
PAO1Δ*pilG*	In frame deletion of *pilG*	This study
PAO1Δ*pilH*	In frame deletion of *pilH*	This study
PAO1Δ*pilT*	In frame deletion of *pilT*	This study
PAO1Δ*chpU*	In frame deletion of *pilU*	This study
PAO1Δ*fimX*	In frame deletion of *fimX*	This study
PAO1Δ*fimX*Δ*chpA*	In frame deletion of *chpA* and *fimX*	This study
PAO1Δ*fimX*Δ*pilB*	In frame deletion of *pilB* and *fimX*	This study
PAO1Δ*fimX*Δ*pilG*	In frame deletion of *pilG* and *fimX*	This study
PAO1Δ*fimX*Δ*pilH*	In frame deletion of *pilH* and *fimX*	This study
PAO1Δ*fimX*Δ*pilT*	In frame deletion of *pilT* and *fimX*	This study
PAO1Δ*fimX*Δ*pilU*	In frame deletion of *pilU* and *fimX*	This study
E. coli		
DH5α	*hsdR rec lacZYA ϕ80 lacZM15*	Invitrogen
S17-1 λpir	*Thi pro hsdR recA RP4-2*(Tc::Mu)(Km::Tn7)	Stratagene
Plasmids		
pJB100	pEX100T derivative replacing *Sma*I site with *Spe*I site	This Study
pJB123	pJB100 carrying Δ*fimX* on *Spe*I fragment	This study

Table 14. Oligonucleotides used in chapter 7

Primer	Oligonucleotide sequence (5′→3′)[1]
fimX1	GCACTAGTGAAAGCACGCCTGCCGCTGAT
fimX2	TCATTCGTCTCCAAGCTTGATGGCCATGGAAAGGGCTCAGTCCGCGTC
fimX3	ATGGCCATCAAGCTTGGAGACGAATGAAGAACGGGCGCCCTGGGCGCC
fimX4	GCACTAGTCAAGGCGCGGCACATCGTGAC

[1]Restriction endonuclease sequences are underlined

Chapter 8

Phenotypic Analysis of In-Frame Deletion Mutants Complemented *in trans*

Introduction

To facilitate characterization of biochemical interactions between proteins involved in the regulation of pilus extension and retraction, we cloned genes of interest fused to epitopes recognized by commercially available antibodies. Antibodies to ChpA, PilG, PilH, PilB, PilT, PilU, and FimX either do not exist or had not been generated when we undertook the current investigations. Here we describe the preliminary phenotypic characterization of these constructs. We show that various tagged and untagged versions of *chpA*, *pilG*, *pilH*, *pilU*, and *fimX* are able to complement their respective in-frame deletion mutants for TM when expressed *in trans*. We show further that $pilT^{\mathrm{Tunc}}$ and $\Delta pilT^{\mathrm{CTX}\text{-}pilU}$, but not Δ*pilT* can be complemented by expression of tagged and untagged versions of *pilT in trans*, supporting the idea that *pilT* and *pilU* are transcribed separately and that deletion of *pilT* results in *pilU* silencing. We present evidence consistent with the idea that overexpression of *chpA* has a dominant negative effect on TM. We demonstrate that mutated versions of *chpA*, *pilG*, and *pilH* expressed *in trans* recapitulate the TM phenotypes of similar alleles integrated onto the *P. aeruginosa* chromosome, but that tagged versions of the WT proteins do not restore surface piliation to WT levels.

Materials and Methods

Construction of in-frame deletion mutants. In frame-deletion mutants, *pilT*$^{\text{Trunc}}$, and Δ*pilT*$^{\text{CTX-}pilU}$ were constructed as outlined in chapter 2. To create Δ*pilU*$^{\text{CTX-}pilU}$, Δ*pilU* was mated to *E. coli* S17.1 carrying pJB212. The plasmid backbone was removed by mating the resultant strain to *E. coli* SM10 carrying pFlp2.

Construction of plasmid based complementation and point mutant constructs. *chpA*. A 5′ fragment of *chpA* was amplified from PAO1 genomic DNA using the chpA24/chpA25 PCR primer pair and cloned as a *Sma*I/*Sac*I fragment into pOK12 to form pJB216. A 3′fragment of *chpA* was amplified from PAO1 enomic DNA using the chpA26.chpA27 PCR primer pair and cloned as a *Sac*I/*Hind*III fragment into pJB216 to form pJB217. Full-length *chpA* was subcloned as a *Spe*I fragment from pJB217 into pJB200 to form pJB226. A 3′ fragment of *chpA* was amplified using pJB232 (see chapter 2) as a template and cloned as a *Sac*I/*Hind*III fragment into pJB216 to form pJB242. Full-length *chpA*-FLAG was subcloned as a *Spe*I fragment into pJB200 to form pJB244. A 3′ fragment of *chpA* was amplified using pJB233 (see chapter 2) as a template and cloned as a *Sac*I/*Hind*III into pJB216 to form pJB243. Full-length *chpA*(AAA)-FLAG was subcloned as a *SpeI* fragment into pJB200 to form pJB245.

pilB. *pilB* was amplified from PAO1 genomic DNA using the pUC-pilB-XbaI-F/ pUC-pilB-SacI-R PCR primer pair. *pilB*-His was amplified from PAO1 genomic DNA using the pUC-pilB-XbaI-F/pUC-pilB-His-SacI-R PCR primer pair. *pilB*-HA was amplified from PAO1 genomic DNA using the pUC-pilB-XbaI-F/pUC-pilB-HA-SacI-R PCR primer pair. His-*pilB* was amplified from PAO1 genomic DNA using the pUC-pilB-XbaI-His-F/pUC-pilB-SacI-R PCR primer pair. HA-*pilB* was amplified from PAO1

genomic DNA using the pUC-pilB-XbaI-His-F/pUC-pilB-SacI-R PCR primer pair. All *pilB* constructs were cloned as *Xba*I/*Sac*I fragments into pUCP19Δ*lac* to form pJB154, pJB128, pJB129, pJB159, and pJB160. *pilB* was amplified from PAO1 genomic DNA using the pilB-Cherry-EcoRI-F/pilB-Cherry-XbaI-R PCR primer pair and cloned as an *EcoR*I/*Xba*I fragment into pCmCherry to form pJB149.

pilG. *pilG* was amplified from PAO1 genomic DNA using the pUC-pilG-HindIII-F/pUC-pilG-XbaI-R PCR primer pair. *pilG*-His was amplified from PAO1 genomic DNA using the pUC-pilG-HindIII-F/pUC-pilG-His-XbaI-R PCR primer pair. *pilG*-HA was amplified from PAO1 genomic DNA using the pUC-pilG-HindIII-F/pUC-pilG-HA-XbaI-R and pMBAD-pilG-XbaI-F/pMBAD-pilG-HA-HindIII-R PCR primer pairs. *pilG* constructs were cloned as *Hind*III/*Xba*I or *Xba*I/*Hind*III fragments (pMBAD18G) into pUCP19Δ*lac*, pMBAD18G, and pOK12 to form pJB155, pJB130, pJB131, pJB142, pJB164, and pJB165. pJB164 and pJB165 served as substrates for QuickChange (Stratagene) mutagenesis, as outlined in chapter 2, using the pilG-D58A-F/pilG-D58A-R primer pair to form pJB168 and pJB169. *pilG*(D58A)-His and *pilG*(D58A)-HA were subloned from pJB168 and pJB169, respectively, to pUCP19Δ*lac* as *Hind*III/*Xba*I fragments to form pJB176 and pJB177. *pilG* was amplified from PAO1 genomic DNA using the pilG-Cherry-BamHI-F/pilG-Cherry-XbaI-R PCR primer pair and cloned as a *BamH*I/*Xba*I fragment into pCmCherry to form pJB150.

pilH. *pilH* was amplified from PAO1 genomic DNA using the pUC-pilH-HindIII-F/pUC-pilH-XbaI-R PCR primer pair. *pilH*-His was amplified from PAO1 genomic DNA using the pUC-pilH-HindIII-F/pUC-pilH-His-XbaI-R and pMBAD-pilH-XbaI-F/pMBAD-pilH-His-HindIII-R PCR primer pairs. *pilH*-HA was amplified from

PAO1 genomic DNA using the pUC-pilH-HindIII-F/pUC-pilH-HA-XbaI-R and pMBAD-pilH-XbaI-F/pMBAD-pilH-HA-HindIII-R PCR primer pairs. *pilH* constructs were cloned as *Hind*III/*Xba*I or *Xba*I/*Hind*III (pMBAD18G) fragments into pUCP19Δ*lac*, pMBAD18G, and pOK12 to form pJB156, pJB132, pJB133, pJB143, pJB144, pJB166, and pJB167. pJB166 and pJB167 served as substrates for QuickChange (Stratagene) mutagenesis, as outlined in chapter 2, using the pilH-D52A-F/pilH-D52A-R primer pair to form pJB170 and pJB171. *pilH*(D52A)-His and *pilH*(D52A)-HA were subloned from pJB170 and pJB171, respectively, to pUCP19Δ*lac* as *Hind*III/*Xba*I fragments to form pJB180 and pJB181. *pilH* was amplified from PAO1 genomic DNA using the pilH-Cherry-BamHI-F/pilH-Cherry-XbaI-R PCR primer pair and cloned as a *BamH*I/*Xba*I fragment into pCmCherry to form pJB151.

pilT. *pilT* was amplified from PAO1 genomic DNA using the pUC-pilT-HindIII-F/pUC-pilT-XbaI-R PCR primer pair. *pilT*-His was amplified from PAO1 genomic DNA using the pUC-pilT-HindIII-F/pUC-pilT-His-XbaI-R and pMBAD-pilT-XbaI-F/pMBAD-pilT-His-HindIII-R PCR primer pairs. *pilT*-HA was amplified from PAO1 genomic DNA using the pUC-pilT-HindIII-F/pUC-pilT-HA-XbaI-R and pMBAD-pilT-XbaI-F/pMBAD-pilT-HA-HindIII-R PCR primer. His-*pilT* was amplified from PAO1 genomic DNA using the pUC-pilT-HindIII-His-F/pUC-pilT-XbaI-R PCR primer pair. HA-*pilT* was amplified from PAO1 genomic DNA using the pUC-pilT-HindIII-HA-F/pUC-pilT-XbaI-R PCR primer pair. *pilT* constructs were cloned as *Hind*III/*Xba*I or *Xba*I/*Hind*III (pMBAD18G) fragments into pUCP19Δ*lac* and pMBAD18G form pJB157, pJB135, pJB136, pJB145, pJB146, pJB161, and pJB162. *pilT* was amplified from PAO1 genomic

DNA using the pilT-Cherry-BamHI-F/pilT-Cherry-XbaI-R PCR primer pair and cloned as a *BamH*I/*Xba*I fragment into pCmCherry to form pJB152.

pilU. *pilU* was amplified from PAO1 genomic DNA using the pUC-pilU-HindIII-F/pUC-pilU-XbaI-R PCR primer pair. *pilU*-His was amplified from PAO1 genomic DNA using the pUC-pilU-HindIII-F/pUC-pilU-His-XbaI-R and pMBAD-pilU-XbaI-F/pMBAD-pilU-His-HindIII-R PCR primer pairs. *pilU*-HA was amplified from PAO1 genomic DNA using the pMBAD-pilU-XbaI-F/pMBAD-pilU-HA-HindIII-R PCR primer pair. *pilU* constructs were cloned as *Hind*III/*Xba*I or *Xba*I/*Hind*III (pMBAD18G) fragments into pUCP19Δ*lac* and pMBAD18G form pJB158, pJB137, pJB147, and pJB148. *pilU* was amplified from PAO1 genomic DNA using the pilU-Cherry-BamHI-F/pilU-Cherry-XbaI-R PCR primer pair and cloned as a *BamH*I/*Xba*I fragment into pCmCherry to form pJB153.

fimX. *fimX* was amplified from PAO1 genomic DNA using the pUC-fimX-HindIII-F/pUC-fimX-XbaI-R PCR primer pair. *fimX*-His was amplified from PAO1 genomic DNA using the pUC-fimX-HindIII-F/pUC-fimX-His-XbaI-R PCR primer pair. *fimX*-HA was amplified from PAO1 genomic DNA using the pUC-fimX-HindIII-F/pUC-fimX-HA-XbaI-R PCR primer pair. His-*fimX* was amplified from PAO1 genomic DNA using the pUC-fimX-HindIII-His-F/pUC-fimX-XbaI-R PCR primer pair. *fimX* constructs were cloned as *Hind*III/*Xba*I fragments into pUCP19Δ*lac* form pJB189, pJB187, pJB188, and pJB185.

Transformations into *P. aeruginosa* strains were carried out as described in chapter 2.

Phenotypic Assays. Phenotypic assays for twitching motility and intracellular production and surface presentation of PilA and FliC were carried out as described in chapter 2.

Results and Discussion

The TM phenotypes of in-frame deletion mutants can be complemented *in trans* by expression of tagged and untagged copies of their genes

In chapter 2, we describe analysis of the complemented in-frame deletion mutants. In those experiments, we re-introduced single copies of the wild type gene onto the chromosome at the endogenous locus of Δ*chpA*, Δ*pilG*, Δ*pilH*, Δp*ilT*, and Δ*pilU*. Here we have expressed, *in trans*, untagged and tagged versions of these genes cloned into high copy plasmids and introduced in their respective deletion mutant backgrounds to determine whether the TM phenotypes could be complemented. The results are summarized in tables 17 and 18. With the exception of Δ*pilT*, every in-frame deletion mutant could be complemented *in trans* by both tagged and untagged copies of its gene expressed from a high copy plasmid. $pilT^{\text{Trunc}}$ and Δ $pilT^{\text{CTX-}pilU}$ could both be complemented *in trans* by both tagged and untagged copies of *pilT* (Table 17, Fig. 23A, B). Δ*chpA* could also be complemented by expression of tagged and untagged version of *chpA in trans* but only when expressed from low copy plasmids (Table 19 and Fig. 24 and data not shown).

Deletion of *pilT* may affect transcription of *pilU*

Whereas all the other mutants could be complemented for TM in *trans*, Δ*pilT* could only be complemented by re-introduction of *pilT* onto the chromosome at the *pilT* locus (Table 3 and chapter 2). However, the TM defects of insertion mutants in PAK *pilT* and a PA103 *pilT* deletion could be complemented by expression of *pilT in trans* (Fig. 21). This finding suggests that the in-frame deletion of *pilT* might affect transcription of the adjacent *pilU* gene by disrupting *cis* regulatory sequences required for *pilU* transcription located within the *pilT* coding sequence. Consistent with this notion, a *pilT* truncation mutant ($pilT^{Trunc}$) in which the N-terminal 429 bp of *pilT* were deleted but which retains 783 bp of sequence upstream of *pilU*, is defective for TM, has increased surface pili, and could be complemented in by expression of tagged or untagged versions of *pilT in trans* (Fig. 22A, B, Fig. 23A). Likewise, a Δ*pilT* derivative strain ($\Delta pilT^{CTX\text{-}pilU}$) in which the *pilU* gene including 1000 bp of upstream sequence was introduced at the *attB* site also recapitulated Δ*pilT* TM and surface presentation phenotypes (Fig. 22A, B), but could be complemented by expression of tagged or untagged versions of *pilT in trans* (Fig 3B). The TM defect of Δ*pilU* was complemented by introduction of *pilU* at the *attB* site (Fig. 22C). These data provide genetic evidence suggesting that *pilT* and *pilU* are transcribed separately and are consistent with previous studies including the observations that: 1) insertion mutations in *pilT* are not polar on *pilU*, and 2) the *pilT* transcript was shown by northern analysis to be 1.4 kb length, a size far to small to include both the *pilT* and *pilU* transcripts (14). Further work will need to be done to explicitly demonstrate the separate transcription of *pilT* and *pilU*, as well as to determine specific regulatory factors, and sequences required for *pilU* transcription.

Surface piliation of Δ*chpA*, Δ*pilG*, and Δ*pilH* cannot be complemented by *in trans* expression of tagged or untagged copies of their genes

Overexpression of ChpA inhibits pilin function.

The Δ*chpA* TM defect can be complemented by expression of untagged or FLAG-tagged versions of *chpA* transcribed *in trans* from a low copy plasmid (Table 19, Fig. 24A). The Δ*chpA* TM defect could not be complemented by *in trans* expression of a *chpA* histidine kinase mutant (*chpA*(AAA)-FLAG), consistent with the idea that histidine kinase activity is required for ChpA function (Table 19, Fig. 24A). However, expression of ChpA from a high copy vector into a Δ*chpA* mutant fails to complement the TM defect and reduces TM in a WT background to levels resembling those of Δ *chpA* by the subsurface stab assay (data not shown). Likewise, TM is reduced slightly in WT in the presence of tagged or untagged *chpA* relative to the vector alone (Table 19, Fig. 24A). *chpA* is complemented to the reduced WT TM levels in the presence of tagged or untagged *chpA* (Table 19, Fig. 24A). Induction of *chpA* expression by the addition of IPTG reduces both WT and Δ*chpA* TM to levels seen in the Δ*chpA* mutant (Fig. 24B). These data all suggest that TM is extraordinarily sensitive to ChpA levels. It is possible that overexpression of ChpA titrates out or sequesters another protein that is required for TM. Consistent with a requirement for stoichiometric amounts of protein, this effect does not seem to depend on the histidine kinase actvity of ChpA. TM of PAO1 expressing *chpA*(AAA)-FLAG from a plasmid vector is reduced to levels similar to those seen in the presence of tagged or untagged WT *chpA* (Fig. 24A). However, because we have yet to determine levels of ChpA-FLAG and ChpA(AAA)-FLAG by immunoblot assays, we cannot rule out the possibility that the failure of ChpA(AAA)-FLAG to both

complement Δ *chpA* and to reduce TM in a WT background is due to decreased production or stability.

*Overexpression of PilG and PilH in Δ pilG and Δ pilH restores TM but fails to restore surface pilin levels***.**

We sought to test whether the phosphate-accepting aspartate residue of PilG or PilH was required for surface piliation and for TM. In chapter 2, we described experiments that show that introduction of *pilG*-His or *pilH*-His onto the chromosome at their endogenous loci complemented Δ*pilG* and Δ*pilH*, respectively, for TM and surface piliation. Introduction of His-tagged point mutant alleles of *pilG* or *pilH* in which the phosphate-accepting aspartate residue of phospho-transfer domain has been mutated to alanine recapitulated the null mutant phenotype. However, we were unable to detect either WT or point-mutated His-tagged protein. We therefore repeated these experiments but expressed HA-tagged versions of the proteins from a high copy number plasmid. The TM defects of both Δ*pilG* and Δ*pilH* can be complemented by expression of untagged and HA-tagged versions of PilG and PilH respectively (Table 3, Fig. 25A). TM defects cannot be complemented by *in trans* expression of HA-tagged point mutants of *pilG* or *pilH* in which the phosphate-accepting aspartate residue of phospho-transfer domain has been mutated to alanine (Table 17, Fig. 25A). These results are consistent with the idea that signaling through PilG and PilH are required for function. However, even with overproduction of the protein from a high copy number plasmid, we were not able to detect expression of the HA-tagged protein by immunoblot analysis (data not shown). Therefore, we cannot rule out the possibility that the expression level of these proteins

was too low to restore function, though we consider this extremely unlikely as the equivalent point mutation has been made in many other CheY homologs without affecting protein levels or protein stability (e.g., 7).

Measurement of surface pilin levels in the plasmid-containing mutants revealed some unexpected findings. Although TM was restored when tagged or untagged versions of the wild type gene was expressed in *trans* in Δ*chpA*, Δ*pilG*, and Δ*pilH* mutants, the amount of surface pili was not restored to wild type levels (Fig. 24C, Fig. 25B). The reasons for this are unclear, but they do not seem to be tied directly to a dysregulation of pilus function caused by an upset in the stoichiometry of regulatory signaling proteins. Indeed, introduction of a vector backbone alone into a WT background results in decreased surface piliation (Fig. 24C, Fig. 25B). This effect appears to be independent of vector copy number or antibiotic resistance encoded. These results indicate that restoration of TM does not necessarily correlate with restoration of surface piliation. Interestingly, the fact that TM can be restored to Δ*chpA* and Δ*pilG* mutants when no pili are detectable on the cell surface by western analysis, suggest that very few pili may be required for TM, and possibly other Tfp functions, although this remains to be demonstrated explicitly.

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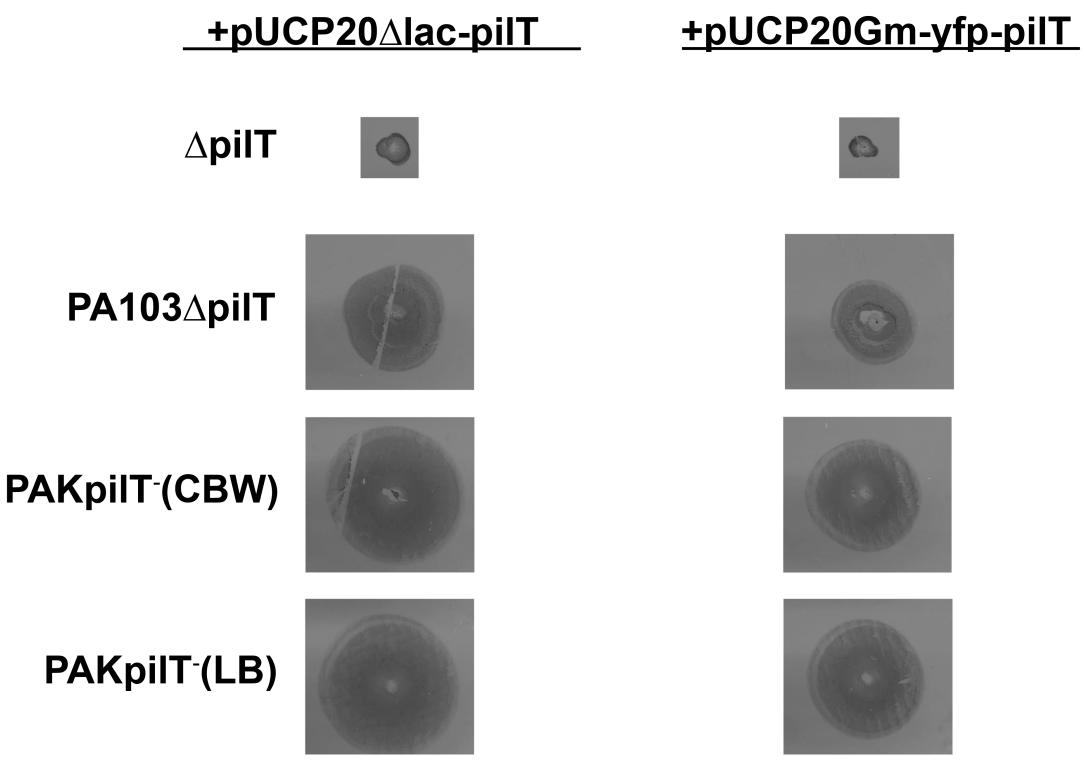


Figure 21. Complementation of *pilT* mutants *in trans*. Subsurface TM assay of the indicated strains transformed with either PAO1-derived *pilT* or PAK-derived *pilT* fused to *yfp*.

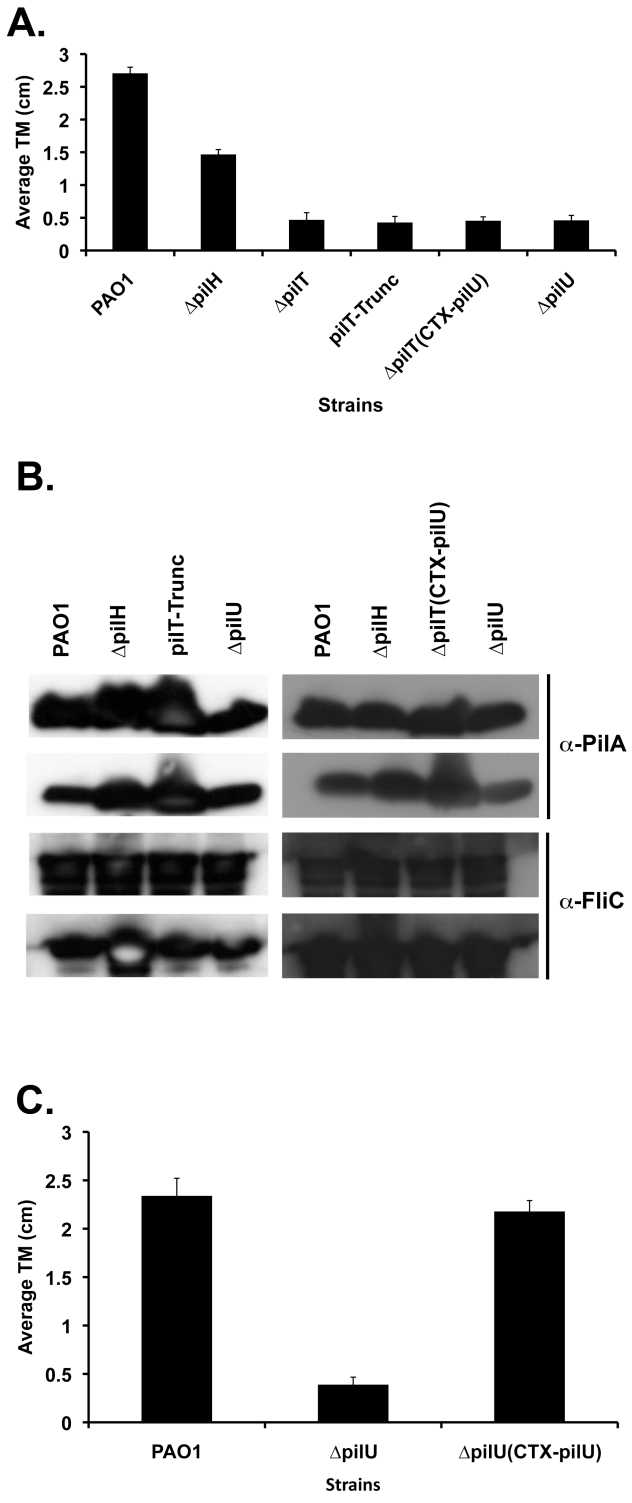


Figure 22. Assays of pilus function for *pilT*$^{\text{Trunc}}$ and *pilT*$^{\text{CTX-}pilU}$ mutants. (A) Graph depicting TM zone diameters for the indicated strains. Shown are the means ± the

standard deviation (SD, N=5). (B) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC). (C) Graph depicting TM zone diameters for the indicated strains. Shown are the means ± the standard deviation (SD, N=5).

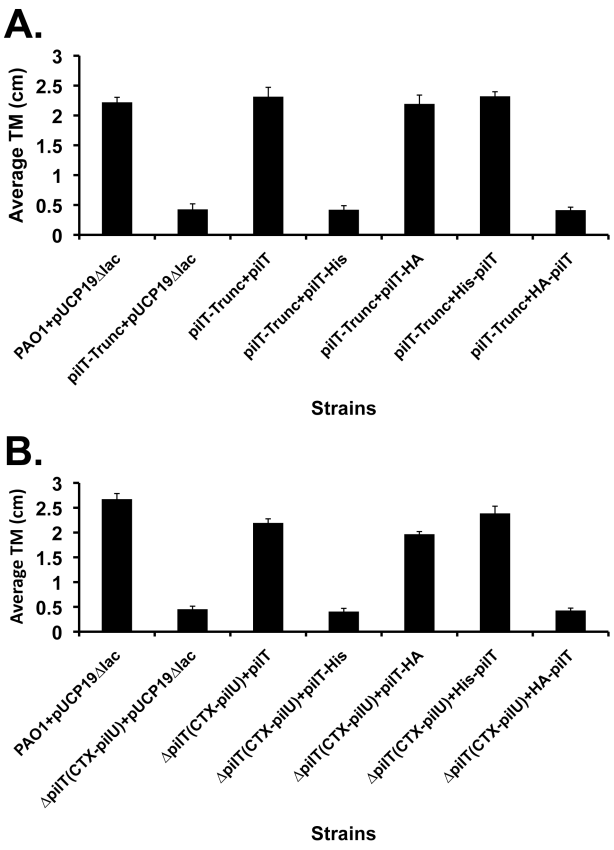
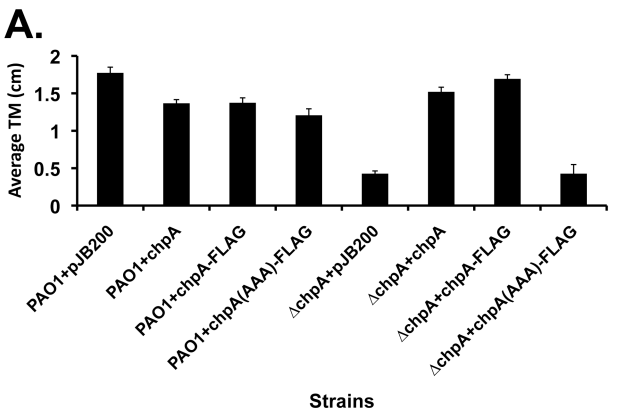
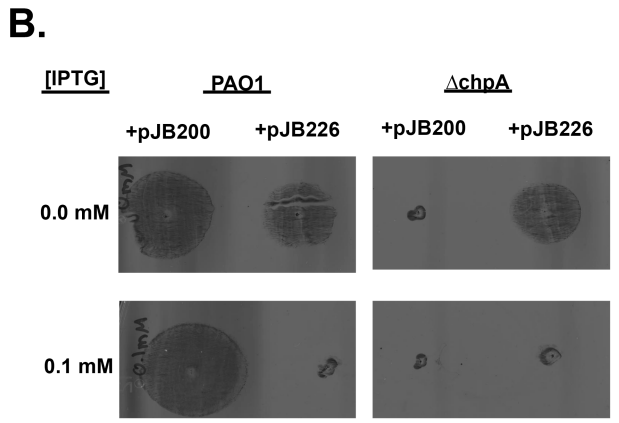


Figure 23. Complementation of *pilT*$^{\text{Trunc}}$ and *pilT*$^{\text{CTX-}pilU}$ mutants with tagged and untagged versions of *pilT in trans*. (A) Graph depicting TM zone diameters for *pilT*-Tunc mutants transformed with the indicated tagged and untagged versions of *pilT*. Shown are the means ± the standard deviation (SD, N=5). (B) Graph depicting TM zone diameters for *pilT*$^{\text{CTX-}pilU}$ mutants transformed with the indicated tagged and untagged versions of *pilT*. Shown are the means ± the standard deviation (SD, N=5).


A.
Average TM (cm)
2
1.5
1
0.5
0
PAO1+pJB200
PAO1+chpA
PAO1+chpA-FLAG
PAO1+chpA(AAA)-FLAG
ΔchpA+pJB200
ΔchpA+chpA
ΔchpA+chpA-FLAG
ΔchpA+chpA(AAA)-FLAG
Strains


B.
[IPTG]
PAO1
ΔchpA
+pJB200
+pJB226
+pJB200
+pJB226
0.0 mM
0.1 mM

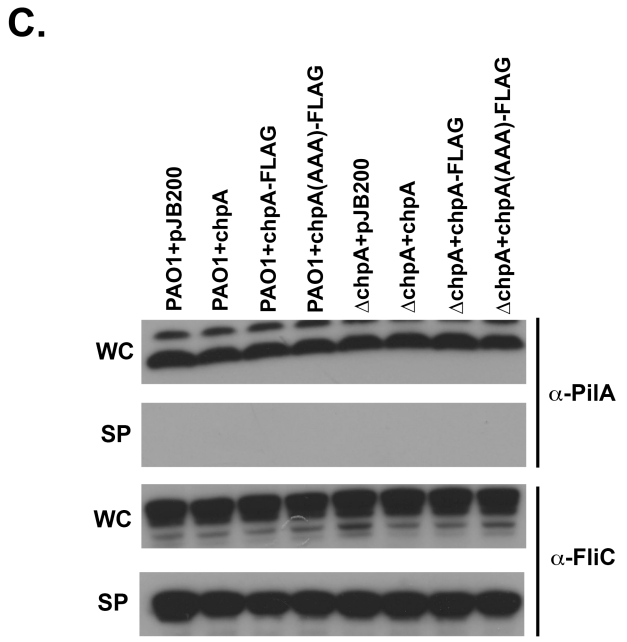

C.
PAO1+pJB200
PAO1+chpA
PAO1+chpA-FLAG
PAO1+chpA(AAA)-FLAG
ΔchpA+pJB200
ΔchpA+chpA
ΔchpA+chpA-FLAG
ΔchpA+chpA(AAA)-FLAG
WC
SP
α-PilA
WC
SP
α-FliC

Figure 24. Complementation of Δ*chpA* mutants with tagged and untagged versions of *chpA in trans*. (A) Graph depicting TM zone diameters for the indicated strains transformed with tagged and untagged versions of *chpA*. (B) Subsurface TM assay of the indicated strains transformed with either vector (pJB200) or untagged *chpA* (pJB226) in the presence or absence of IPTG. (C) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC).

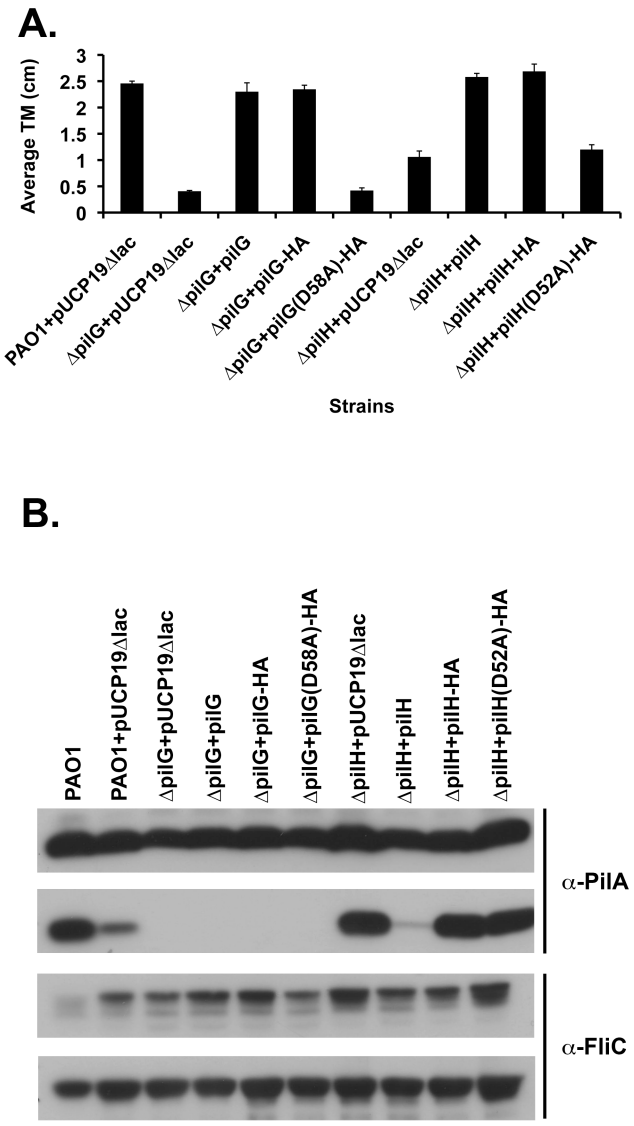


Figure 25. Complementation of Δ*pilG* and Δ*pilH* mutants with tagged and untagged versions of *pilG* and *pilH in trans*. (A) Graph depicting TM zone diameters for the indicated strains transformed with tagged and untagged versions of *pilG* or *pilH*. (B) Surface pili (SP) and intracellular (WC) preparations of the indicated strains were immunoblotted with a polyclonal antibody to PilA (α-pilA) or to FliC (α-FliC).

Table 15. Strains and plasmids used in chapter 8

Strain or plasmid	Genotype and relevant characteristics	Reference
P. aeruginosa		
PAO1	Wild type	
PAO1Δ*chpA*	In frame deletion of *chpA*	This study
PAO1Δ*pilB*	In frame deletion of *pilB*	This study
PAO1Δ*pilG*	In frame deletion of *pilG*	This study
PAO1Δ*pilH*	In frame deletion of *pilH*	This study
PAO1Δ*pilT*	In frame deletion of *pilT*	This study
PAO1Δ*pilU*	In frame deletion of *pilU*	This study
PAO1Δ*pilT*$^{\text{CTX-}pilU}$	In frame deletion of *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAO1*pilT*$^{\text{Trunc}}$	*pilT* truncation mutant, deletes 429 bp of *pilT* including start codon; stop codons in all three reading frames	This study
PAO1Δ*pilU*$^{\text{CTX-}pilU}$	In frame deletion of *pilU*; *pilU* and 1 kb upstream sequence at *attB* site	This study
PAK*pilT*$^{-}$ (CBW)	Insertion mutation *pilT*	(13)
PAK*pilT*$^{-}$ (LB)	Insertion mutation *pilT*	(1)
PA103Δ*pilT*	In frame deletion of *pilT*	(2)
PAO1+pUCPSK	PAO1 carrying pUCPSK	This study
PAO1+pUCP19Δ*lac*	PAO1 carrying pUCP19Δ*lac*	This study
PAO1+pMMB206	PAO1 carrying pMMB206	This study
PAO1+pAL64	PAO1 carrying *chpA*-FLAG on pUCPSK	This study
PAO1+pAL79	PAO1 carrying *chpA* on pMMB206	This study
PAO1+pJB200	PAO1 carrying pJB200	This study
PAO1+pJB226	PAO1 carrying *chpA* on pJB200	This study
PAO1+pJB244	PAO1 carrying *chpA*-FLAG on pJB200	This study
PAO1+pJB245	PAO1 carrying *chpA*(AAA)-FLAG on oJB200	This study
PAO1Δ*chpA*+pUCPSK	Δ*chpA* carrying pUCPSK	This study
PAO1Δ*chpA*+pMMB206	Δ*chpA* carrying pMMB206	This study
PAO1Δ*chpA*+pAL64	Δ*chpA* carrying *chpA*-FLAG on pUCPSK	This study
PAO1Δ*chpA*+pAL79	Δ*chpA* carrying *chpA* on pMMB206	This study
PAO1Δ*chpA*+pJB200	Δ*chpA* carrying pJB200	This study
PAO1Δ*chpA*+pJB226	Δ*chpA* carrying *chpA* on pJB200	This study
PAO1Δ*chpA*+pJB244	Δ*chpA* carrying *chpA*-FLAG on pJB200	This study
PAO1Δ*chpA*+pJB245	Δ*chpA* carrying *chpA*(AAA)-FLAG on oJB200	This study
PAO1Δ*pilB*+pUCP19Δ*lac*	Δ*pilB* carrying pUCP19Δ*lac*	This study
PAO1Δ*pilB*+pJB154	Δ*pilB* carrying *pilB* on pUCP19Δ*lac*	This study
PAO1Δ*pilB*+pJB129	Δ*pilB* carrying *pilB*-His on pUCP19Δ*lac*	This study
PAO1Δ*pilB*+pJB130	Δ*pilB* carrying *pilB*-HA on pUCP19Δ*lac*	This study
PAO1Δ*pilG*+pUCP19Δ*lac*	Δ*pilG* carrying pUCP19Δ*lac*	This study
PAO1Δ*pilG*+pJB155	Δ*pilG* carrying *pilG* on pUCP19Δ*lac*	This study
PAO1Δ*pilG*+pJB131	Δ*pilG* carrying *pilG*-His on pUCP19Δ*lac*	This study
PAO1Δ*pilG*+pJB132	Δ*pilG* carrying *pilG*-HA on pUCP19Δ*lac*	This study
PAO1Δ*pilH*+pUCP19Δ*lac*	Δ*pilH* carrying pUCP19Δ*lac*	This study
PAO1Δ*pilH*+pJB156	Δ*pilH* carrying *pilH* on pUCP19Δ*lac*	This study

PAO1Δ*pilH*+pJB133	Δ*pilH* carrying *pilH*-His on pUCP19Δ*lac*	This study
PAO1Δ*pilH*+pJB134	Δ*pilH* carrying *pilH*-HA on pUCP19Δ*lac*	This study
PAO1Δ*pilT*+pUCP19Δ*lac*	Δ*pilT* carrying pUCP19Δ*lac*	This study
PAO1Δ*pilT*+pJB157	Δ*pilT* carrying *pilT* on pUCP19Δ*lac*	This study
PAO1Δ*pilT*+pJB135	Δ*pilT* carrying *pilT*-His on pUCP19Δ*lac*	This study
PAO1Δ*pilT*+pJB136	Δ*pilT* carrying *pilT*-HA on pUCP19Δ*lac*	This study
PAO1Δ*pilU*+pUCP19Δ*lac*	Δ*pilU* carrying pUCP19Δ*lac*	This study
PAO1Δ*pilU*+pJB158	Δ*pilU* carrying *pilU* on pUCP19Δ*lac*	This study
PAO1Δ*pilU*+pJB137	Δ*pilU* carrying *pilU*-His on pUCP19Δ*lac*	This study
PAO1+pMBAD18G	PAO1 carrying pMBAD18G	This study
PAO1Δ*pilB*+pMBAD18G	Δ*pilB* carrying pMBD18G	This study
PAO1Δ*pilG*+pMBAD18G	Δ*pilG* carrying pMBD18G	This study
PAO1Δ*pilG*+pJB141	Δ*pilG* carrying *pilG*-His on pMBD18G	This study
PAO1Δ*pilG*+pJB142	Δ*pilG* carrying *pilG*-HA on pMBD18G	This study
PAO1Δ*pilH*+pMBAD18G	Δ*pilH* carrying pMBD18G	This study
PAO1Δ*pilH*+pJB143	Δ*pilH* carrying *pilH*-His on pMBD18G	This study
PAO1Δ*pilH*+pJB144	Δ*pilH* carrying *pilH*-HA on pMBD18G	This study
PAO1Δ*pilT*+pMBAD18G	Δ*pilT* carrying pMBD18G	This study
PAO1Δ*pilT*+pJB145	Δ*pilT* carrying *pilT*-His on pMBD18G	This study
PAO1Δ*pilT*+pJB146	Δ*pilT* carrying *pilT*-HA on pMBD18G	This study
PAO1Δ*pilU*+pMBAD18G	Δ*pilU* carrying pMBD18G	This study
PAO1Δ*pilU*+pJB147	Δ*pilU* carrying *pilU*-His on pMBD18G	This study
PAO1Δ*pilU*+pJB148	Δ*pilU* carrying *pilU*-HA on pMBD18G	This study
PAO1Δ$pilT^{\text{CTX-}pilU}$ +pUCP19Δ*lac*	Δ$pilT^{\text{CTX-}pilU}$ carrying pUCP19Δ*lac*	This study
PAO1Δ$pilT^{\text{CTX-}pilU}$+pJB157	Δ$pilT^{\text{CTX-}pilU}$ carrying *pilT* on pUCP19Δ*lac*	This study
PAO1Δ$pilT^{\text{CTX-}pilU}$+pJB135	Δ$pilT^{\text{CTX-}pilU}$ carrying *pilT*-His on pUCP19Δ*lac*	This study
PAO1Δ$pilT^{\text{CTX-}pilU}$+pJB136	Δ$pilT^{\text{CTX-}pilU}$ carrying *pilT*-HA on pUCP19Δ*lac*	This study
PAO1Δ$pilT^{\text{CTX-}pilU}$+pJB161	Δ$pilT^{\text{CTX-}pilU}$ carrying His-*pilT*-on pUCP19Δ*lac*	This study
PAO1Δ$pilT^{\text{CTX-}pilU}$+pJB162	Δ$pilT^{\text{CTX-}pilU}$ carrying His-*pilT*-on pUCP19Δ*lac*	This study
PAO1$pilT^{\text{Trunc}}$ +pUCP19Δ*lac*	$pilT^{\text{Trunc}}$ carrying *pilT* on pUCP19Δ*lac*	This study
PAO1$pilT^{\text{Trunc}}$+pJB157	$pilT^{\text{Trunc}}$ carrying *pilT*-His on pUCP19Δ*lac*	This study
PAO1$pilT^{\text{Trunc}}$ +pJB135	$pilT^{\text{Trunc}}$ carrying *pilT*-HA on pUCP19Δ*lac*	This study
PAO1$pilT^{\text{Trunc}}$ +pJB136	$pilT^{\text{Trunc}}$ carrying His-*pilT*-on pUCP19Δ*lac*	This study
PAO1$pilT^{\text{Trunc}}$ +pJB161	$pilT^{\text{Trunc}}$ carrying His-*pilT*-on pUCP19Δ*lac*	This study
PAO1$pilT^{\text{Trunc}}$ +pJB162	$pilT^{\text{Trunc}}$ carrying *pilT* on pUCP19Δ*lac*	This study
PA103Δ*pilT*+pJB157	PA103Δ*pilT* carrying *pilT* on pUCP19Δ*lac*	This study
PA103Δ*pilT*+pUCP20Gm *yfp-pilT*	PA103Δ*pilT* carrying *yfp-pilT* on pUCP20Gm	This study
PAK*pilT*˙(CBW)+pJB157	PAK*pilT*˙ carrying *pilT* on pUCP19Δ*lac*	This study
PAK*pilT*˙(CBW) +pUCP20Gm-*yfp-pilT*	PAK*pilT*˙ carrying *yfp-pilT* on pUCP20Gm	This study
PAK*pilT*˙(LB)+pJB157	PAK*pilT*˙ carrying *pilT* on pUCP19Δ*lac*	This study
PAK*pilT*˙(LB) +pUCP20Gm-*yfp-pilT*	PAK*pilT*˙ carrying *yfp-pilT* on pUCP20Gm	(1)
PAO1Δ*fimX*+pUCP19(Δ*lac*)	Δ*fimX* carrying pUCP19Δ*lac*	This study
PAO1Δ*fimX*+pJB185	Δ*fimX* carrying His-*fimX* on pUCP19Δ*lac*	This study
PAO1Δ*fimX*+pJB187	Δ*fimX* carrying *fimX*-His on pUCP19Δ*lac*	This study
PAO1Δ*fimX*+pJB188	Δ*fimX* carrying *fimX*-HA on pUCP19Δ*lac*	This study

PAO1Δ*fimX*+pJB189	Δ*fimX* carrying *fimX* on pUCP19Δ*lac*	This study
E. coli		
DH5α	*hsdR rec lacZYA ϕ80 lacZM15*	Invitrogen
S17-1 λpir	*Thi pro hsdR recA RP4-2*(Tc::Mu)(Km::Tn7)	Stratagene
SM10	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*	(3)
Plasmids		
pOK12	*E. coli* cloning vector, Km^R	(11)
pUCP19	*P. aeruginosa – E. coli* cloning vector, Ap^R	(9)
pUCPSK	*P. aeruginosa – E. coli* shuttle vector, Ap^R	(12)
pUCP19Δ*lac*	pUCP19 derivative in which *lacI* has been deleted	This study
pMBAD18G	pMBAD18 derivative, Gm^R	(4)
pCmCherry	Cherry red fusion construct, Cm^R	(10)
pUCP20Gm-*yfp-pilT*	YFP-PilT fusion construct	(1)
pMMB206	*P. aeruginosa* expression vector, Cm^R	(8)
pFlp2	Source of Flp recombinase, Ap^R	(5)
pAL64	*chpA*-FLAG carried on pUCPSK	(6)
pAL79	*chpA* carried on pMMB206	(6)
pJB129	*pilB*-His in pUCP19Δ*lac*; *Xba*I/*Sac*I fragment	This study
pJB130	*pilB*-HA in pUCP19Δ*lac*; *Xba*I/*Sac*I fragment	This study
pJB131	*pilG*-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB132	*pilG*-HA in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB133	*pilH*-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB134	*pilH*-HA in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB135	*pilT*-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB136	*pilT*-HA in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB137	*pilU*-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB142	*pilG*-HA in pMBAD18G; *Xba*I/*Hind*III fragment	This study
pJB143	*pilH*-His in pMBAD18G; *Xba*I/*Hind*III fragment	This study
pJB144	*pilH*-HA in pMBAD18G; *Xba*I/*Hind*III fragment	This study
pJB145	*pilT*-His in pMBAD18G; *Xba*I/*Hind*III fragment	This study
pJB146	*pilT*-HA in pMBAD18G; *Xba*I/*Hind*III fragment	This study
pJB147	*pilU*-His in pMBAD18G; *Xba*I/*Hind*III fragment	This study
pJB148	*pilU*-HA in pMBAD18G; *Xba*I/*Hind*III fragment	This study

pJB149	*pilB*-Cherry C-terminal fusion in pCmCherry; *Eco*RI/*Xba*I fragment	This study
pJB150	*pilG*-Cherry C-terminal fusion in pCmCherry; *Bam*HI/*Xba*I fragment	This study
pJB151	*pilH*-Cherry C-terminal fusion in pCmCherry; *Bam*HI/*Xba*I fragment	This study
pJB152	*pilT*-Cherry C-terminal fusion in pCmCherry; *Bam*HI/*Xba*I fragment	This study
pJB153	*pilU*-Cherry C-terminal fusion in pCmCherry; *Bam*HI/*Xba*I fragment	This study
pJB154	*pilB* in pUCP19Δ*lac*; *Xba*I/*Sac*I fragment	This study
pJB155	*pilG* in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB156	*pilH* in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB157	*pilT* in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB158	*pilU* in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB159	His-*pilB* in pUCP19Δ*lac*; *Xba*I/*Sac*I fragment	This study
pJB160	HA-*pilB* in pUCP19Δlac; *Xba*I/*Sac*I fragment	This study
pJB161	His-*pilT* in pUCP19Δlac; *Hind*III/*Xba*I fragment	This study
pJB162	HA-*pilT* in pUCP19Δlac; *Hind*III/*Xba*I fragment	This study
pJB164	*pilG*-His in pOK12	This study
pJB165	*pilG*-HA in pOK12	This study
pJB166	*pilH*-His in pOK12	This study
pJB167	*pilH*-HA in pOK12	This study
pJB168	*pilG*(D58A)-His in pOK12	This study
pJB169	*pilG*(D58A)-HA in pOK12	This study
pJB172	*pilH*(D52A)-His in pOK12	This study
pJB173	*pilH*(D58A)-HA in pOK12	This study
pJB176	*pilG*(D58A)-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB177	*pilG*(D58A)-HA in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB180	*pilH*(D52A)-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB181	*pilH*(D52A)-HA in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB185	His-*fimX* in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB187	*fimX*-His in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB188	*fimX*-HA in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB189	*fimX* in pUCP19Δ*lac*; *Hind*III/*Xba*I fragment	This study
pJB200	pMMB206 derivative replacing *Sma*I site with *Spe*I site	This study
pJB216	5′ *chpA* fragment in pOK12; *Sma*I/*Sac*I fragment	This study
pJB217	3′ *chpA* fragment in pOK12; *Sac*I/*Hind*III fragment	
pJB226	*chpA* in pJB200	

pJB232	*chpA*-FLAG in pJB219; see chapter 2	This study
pJB233	*chpA*(AAA)-FLAG in pJB219; see chapter 2	
pJB242	*chpA*-FLAG in pOK12	This study
pJB243	*chpA*(AAA)-FLAF in pOK12	This study
pJB244	*chpA*-FLAG in pJB200	This study
pJB245	*chpA*(AAA)-FLAF in pJB200	This study
pJB212	*pilU* and 1 KB 5′ sequence cloned as *Spe*I/*Hind*III fragment into mCTX2	This study

Table 16. Oligonucleotides used in chapter 8

Primer	Oligonucleotide sequence (5′→3′)[1]
chpA24	TGCATGCATATGAATGGAGTGGCTATG
chpA25	GAGCTCCTCGCGCAGCGC
chpA26	GAGCTCAGCACTCTGGAC
chpA27	CCCAAGCTTTCACTCATGCTGGCCGAC
pUC-pilB-XbaI-F	TGCTCTAGAATGAACGACAGCATCCAA
pUC-pilB-His-SacI-R	CCCGAGCT TTAGTGGTGGTGGTGGTGGTG ATCCTTGGTCACGCGGTTGAC
pUC-pilG-HindIII-F	CCCAAGCTTATGGAACAGCAATCCGAC
pUC-pilG-His-XbaI-R	TGC TCTAGATCAGTGGTGGTGGTGGTGG GGAAACGGCGTCCACCGGGGT
pUC-pilH-HindIII-F	CCCAAGCTTATGGCTCGTATTTTGATT
pUC-pilH-His-XbaI-R	TGCTCTAGATCAGTGGTGGTGGTGGTGGCCCGCCAGCA CCGCATTG
pUC-pilT-HindIII-F	CCCAAGCTTATGGATATTACCGAGCTG
pUC-pilT-His-XbaI-R	TGCTCTAGATCAGTGGTGGTGGTGGTGGAAGTTTTCCG GGATCTTCGC
pUC-pilU-HindIII-F	CCCAAGCTTATGGAATTCGAAAAGCTG
pUC-pilU-His-XbaI-R	TGCTCTAGATCAGTGGTGGTGGTGGTGGCGGAAGCGCC GGCCGGCAGG
pUC-pilB-HA-SacI-R	CCCGAGCTCTTAAGCGTAGTCTGGGACGTCGTATGGGTAAT CCTTGGTCACGCGGTTGAC
pUC-pilG-HA-XbaI-R	TGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAGG AAACGGCGTCCACCGGGGT
pUC-pilH-HA-XbaI-R	TGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAGC CCGCCAGCACCGCATTG
pUC-pilT-HA-XbaI-R	TGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAGA AGTTTTCCGGGATCTTCGC
pMBAD-pilG-XbaI-F	TGCTCTAGAATGGAACAGCAATCCGAC
pMBAD-pilH-XbaI-F	TGCTCTAGAATGGCTCGTATTTTGATT
pMBAD-pilH-His-HindIII-R	CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGGCCCGCCAGCA CCGCATTG
pMBAD-pilT-XbaI-F	TGCTCTAGAATGGATATTACCGAGCTG
pMBAD-pilT-His-HindIII-R	CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGGAAGTTTTCCG GGATCTTCGC
pMBAD-pilU-XbaI-F	TGCTCTAGAATGGAATTCGAAAAGCTG
pMBAD-pilU-His-HindIII-R	CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGGCGGAAGCGCC GGCCGGCAGG
pMBAD-pilG-HA-HindIII-R	CCCAAGCTTTCAAGCGTAGTCTGGGACGTCGTATGGGTAGG AAACGGCGTCCACCGGGGT
pMBAD-pilH-HA-HindIII-R	CCCAAGCTTTCAAGCGTAGTCTGGGACGTCGTATGGGTAGC CCGCCAGCACCGCATTG
pMBAD-pilT-HA-HindIII-R	CCCAAGCTTTCAAGCGTAGTCTGGGACGTCGTATGGGTAGA AGTTTTCCGGGATCTTCGC
pMBAD-pilU-HA-HindIII-R	CCCAAGCTTTCAAGCGTAGTCTGGGACGTCGTATGGGTAGC GGAAGCGCCGGCCGGCAGG
pUC-pilB-His-XbaI-F	TGCTCTAGAATGCACCACCACCACCACCACAACGACAGCAT CCAA

pUC-pilB-SacI-R	TGCGAGCTCTTAATCCTTGGTCACGCGGTT
pUC-pilB-HA-His-F	TGCTCTAGAATGTACCCATACGACGTCCCAGACTACGCTAA CGACAGCATCCAA
pUC-pilT-His-HindIII-F	CCCAAGCTTATGCACCACCACCACCACCACGATATTACCGA GCTG
pUC-pilT-XbaI-R	TGCTCTAGATCAGAAGTTTTCCGGGATCTT
pUC-pilT-HA-HindIII-F	CCCAAGCTTATGTACCCATACGACGTCCCAGACTACGCTGA TATTACCGAGCTG
pUC-pilG-XbaI-R	TGCTCTAGATCAGGAAACGGCGTCCACCGG
pUC-pilH-XbaI-R	TGCTCTAGATCAGCCCGCCAGCACCGCATT
pUC-pilU-XbaI-R	TGCTCTAGATCAGCGGAAGCGCCGGCCGGC
PilG-D58A-F	AACATCATTTTCGTCGCCATCATGATGCCGCGC
PilG-D58A-R	GCGCGGCATCATGATGGCGACGAAAATGATGTT
PilH-D52A-F	GACGTGGTCCTGATGGCCATCGTCATGCCCGGC
PilH-D52A-R	GCCGGGCATGACGATGGCCATCAGGACCACGTC
pUC-fimX-HindIII-F	CCCAAGCTTATGGCCATCGAAAAGAAAACC
pUC-fimX-XbaI-R	TGCTCTAGATCATTCGTCTCCCGAGGAGAA
pUC-fimX-His-HindIII-F	CCCAAGCTTATGCACCACCACCACCACCACGCCATCGAAAA GAAAACC
pUC-fimX-His-XbaI-R	TGCTCTAGATCAGTGGTGGTGGTGGTGGTGTTCGTCTCCCG AGGAGAA
pUC-fimX-HA-XbaI-R	TGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTATT CGTCTCCCGAGGAGAA
pilB-Cherry-EcoRI-F	CCGGAATTCATGAACGACAGCATCCAA
pilB-Cherry-XbaI-R	TGCTCTAGAATCCTTGGTCACGCGGTT
pilG-Cherry-BamHI-F	CGCGGATCCATGGAACAGCAATCCGAC
pilG-Cherry-XbaI-R	TGCTCTAGAGGAAACGGCGTCCACCGG
pilH-Cherry-BamHI-F	CGCGGATCCATGGCTCGTATTTTGATT
pilH-Cherry-XbaI-R	TGCTCTAGAGCCCGCCAGCACCGCATT
pilT-Cherry-BamHI-F	CGCGGATCCATGGATATTACCGAGCTG
pilT-Cherry-XbaI-R	TGCTCTAGAGAAGTTTTCCGGGATCTT
pilU-Cherry-BamHI-F	CGCGGATCCATGGAATTCGAAAAGCTG
pilU-Cherry-XbaI-R	TGCTCTAGAGCGGAAGCGCCGGCCGGC

[1]Restriction endonuclease sequences are underlined

Table 17. Complementation of TM phenotypes using pUCP19(Δ*lac*)-based constructs

Strain	Complementation of TM[1]					
	pUCP19(Δ*lac*)	*pilB*	*pilB*-His	*pilB*-HA	His-*pilB*	HA-*pilB*
Δ*pilB*	-	-[2]	-	-	+	+
	pUCP19(Δ*lac*)	*pilG*	*pilG*-His	*pilG*-HA	*pilG*(D58A)-His	*pilG*(D58A)-HA
Δ*pilG*	-	+	+/-	+	-	-
	pUCP19(Δ*lac*)	*pilH*	*pilH*-His	*pilH*-HA	*pilH*(D52A)-His	*pilH*(D52A)-HA
Δ*pilH*	-	+	+	+	-	-
	pUCP19(Δ*lac*)	*pilT*	*pilT*-His	*pilT*-HA	His-*pilT*	HA-*pilT*
Δ*pilT*	-	-	-	-	-	-
	pUCP19(Δ*lac*)	*pilT*	*pilT*-His	*pilT*-HA	His-*pilT*	HA-*pilT*
Δ*pilT*$^{\text{CTX-}pilU}$	-	+	-	+	+	-
	pUCP19(Δ*lac*)	*pilT*	*pilT*-His	*pilT*-HA	His-*pilT*	HA-*pilT*
pilT$^{\text{Trunc}}$	-	+	-	+	+	-
	pUCP19(Δ*lac*)	*pilU*	*pilU*-His	*pilU*-HA		
Δ*pilU*	-	+	+	N/D		
	pUCP19(Δ*lac*)	*fimX*	*fimX*-His	*fimX* -HA	His-*fimX*	HA-*fimX*
Δ*fimX*	-	+	+	+	+	N/D

[1]Complementation of the TM phenotype of the indicated strains by introduction of the indicated constructs (+, complementation to WT levels, -, no complementation, +/-, intermediate complementation, N/D, not done).
[2]Expression of *pilB* inhibits TM in WT PAO1.

Table 18. Complementation of TM Phenotypes using pMBAD18G- and pCmCherry-based constructs

Strain	Complementation of TM[1]				
	pMBAD18G	*pilB*-His	*pilB*-HA	pCmCherry	pCmCherry-*piB*
Δ*pilB*	-	N/D	N/D	-	+
	pMBAD18G	*pilG*-His	*pilG*-HA	pCmCherry	pCmCherry-*piG*
Δ*pilG*	-	N/D	+	-	-
	pMBAD18G	*pilH*-His	*pilH*-HA	pCmCherry	pCmCherry-*piH*
Δ*pilH*	-	+	-	-	+
	pMBAD18G	*pilT*-His	*pilT*-HA	pCmCherry	pCmCherry-*pilT*
Δ*pilT*	-	-	-	-	-
	pMBAD18G	*pilU*-His	*pilU*-HA	pCmCherry	pCmCherry-*piU*
Δ*pilU*	-	+	+	-	-

[1]Complementation of the TM phenotype of the indicated strains by introduction of the indicated constructs (+, complementation to WT levels, -, no complementation, +/-, intermediate complementation, N/D, not done)

Table 19. Complementation of Δ*chpA* TM Phenotypes using pUCPSK- and pJB200-based constructs

Strain	Complementation of TM[1]							
	pUCPSK	pAL64	pMMB206	pAL79	pJB200	chpA	chpA-FLAG	chpA(AAA)-FLAG
PAO1	+	-	+	+/-	+	+/-	+/-	+/-
Δ*chpA*	-	-	-	+/-	-	+/-	+/-	-

[1]TM phenotypes of the indicated strains transformed with the indicated constructs (+, WT TM, -, no TM, +/-, intermediate TM)

Chapter 9

Conclusions and Perspectives

Type IV pili (Tfp) are essential to the lifestyle of *Pseudomonas aeruginosa* both as an environmental bacterium as well as an opportunistic human pathogen. A great deal of work has been done to identify the genes involved in both the biosynthesis and function of Tfp, including those gene involved in the regulation of those functions. In the current study we add to this work through a preliminary characterization Chp chemosensory system including an analysis of the core signaling components and their genetic interactions with the ATPases that regulate pilus extension and retraction. Specifically we present genetic evidence confirming the role of ChpA, PilG, and PilB in the regulation of pilus extension and that of PilH and PilT in regulating pilus retraction. In addition, we present evidence that the diguanylate phosphodiesterase, FimX, is involved in regulating pilus extension, consistent with recent reports showing interactions with PilB. We demonstrate that ChpA, PilG, and PilB function upstream of PilH, PilT and PilU, that PilH functions upstream of PilT, and that PilT and PilB retain some activity in the absence of signaling input from components of the Chp system. We present evidence to suggest that despite its homology to PilT, PilU does not appear to play a role in the regulation of pilus retraction, and we show that it continues to localize to the cell pole in Chp system mutant backgrounds. We show that the histidine kinase domain of ChpA is required for function as are the phospho-acceptor sites of both PilG and PilH. These findings suggest that ChpA–mediated control through PilG and PilH likely occurs through phosphorylation, and that this is important in the regulation of pilus extension and retraction. We present evidence suggesting that *pilA* transcription is regulated by

intracellular PilA levels. We show that PilA is a negative regulator of *pilA* transcription in *P. aeruginosa* and that the Chp system functionally regulates *pilA* transcription by controlling PilA import and export. Finally we demonstrate that both ChpA and PilH play a role in the regulation of virulence factor production. ChpA regulates production of the type III secreted virulence factor, ExoT and the secretion system components, and PilH regulates the production of a number of quorum sensing-regulated secreted virulence factors including pyocyanin and rhamnolipid.

A great deal more work remains to be done to understand the full range of Tfp functions, how those functions are regulated, and how they contribute to the overall biology of the organism. Although in this regard there are many avenues open to future investigation, three general areas seem particularly relevant in light of the present study. First, the physical aspects of twitching motility must be examined in greater detail. Although a useful tool for the identification of genes involved in the biosynthesis and function of Tfp, the subsurface stab assay offers a limited view of TM. With only a handful of exceptions, most mutants identified to date appear completely defective for TM by this assay, having zones of TM similar to those of *pilA* mutants, and suggesting that subtle motility phenotypes are not readily detectable. Even in those cases where it exists, it is difficult to interpret the meaning of an intermediate TM phenotype. Therefore, the rates of motility under different environmental conditions must be quantified, as must the presence and frequency of cell reversals. This could be accomplished using a time-lapse video microscopy approach. Determining the migration rates and cell reversal frequencies for WT cells will allow us to interpret more broadly the motility defects of mutants with compromised Tfp function. As just one example: is

the TM defect of a *chpA* deletion mutant as determined by the subsurface stab assay caused by a complete lack of movement, slower kinetics, or by some alteration in reversal frequencies that results in no apparent net movement? The fact that *chpA* mutants form mature mushroom-capped biofilms, albeit with morphologies distinct from those of a WT biofilm, a process that has been shown to depend, at least in part, on TM, suggests that something more akin to one or both of the second two options may more likely be the case. Once these parameters have been established and the nature of the defects of mutants with altered TM determined, broader behavioral questions can begin to be addressed including, but not limited to, the following: 1) what kinds of chemical signals does *P. aeruginosa* respond to, 2) how do those signals influence the kinetics of TM in both WT cells and TM mutants, 3) how do TM mutants behave in mixed populations, and 4) how to defects in TM account for alterations in biofilm development and morphology. Alongside these investigations, complementary studies should begin to address the regulatory pathways involved in mediating cell reversals. As a preliminary step, the subcellular localizations of all components involved in the biogenesis and function of Tfp should be systematically determined. Of particular interest is whether or not components are localized to the cytoplasm or to one or both poles, and for those components that localize to a single pole whether or not it is the piliated pole. Candidates for modulators of reversals would comprise that subset of factors localized specifically to the piliated, or potentially, specifically to the non-piliated pole. Subsequent studies could focus on whether changes in direction correlated with oscillation of these components from pole to pole, and determining the mechanism through which this oscillation is achieved.

As with the subsurface stab assay, our current method for determining levels of surface piliation is low resolution and gives only a static picture of steady state levels of pilin on the cell surface. Using this approach it is impossible to say with certainty that similar levels of surface piliation reflect similarities in the actual pili on the surface both in terms of length as well as number. It is also impossible to determine whether pili on the surface are mislocalized. For these reasons, a comprehensive investigation should be undertaken to investigate the properties are localization of surface pili for mutants defective in both biosynthesis and function using a combination of both fluorescent and electron microscopy. Results from these experiments could be correlated with detailed TM data collected as described above to paint a more complete picture of how form and, potentially, location, effect function.

For a truly mechanistic appreciation of how Tfp function, detailed biochemical experiments will need to be undertaken. With respect to the Chp system, specifically, the results presented in this study would be strengthened by demonstrated physical interactions between ChpA and PilG and/or PilH, and between PilG and PilH and the ATPases PilB and PilT respectively. Likewise, the ability of ChpA to autophosphorylate and to phospho-transfer to PilG and/or PilH *in vitro* would provide support for our model. A detailed investigation of the kinetics of ChpA autophosphorylation and phosphotransfer might also reveal subtleties of Chp system regulation. Some questions include: 1) Which Hpt domains are phosphorylated, 2) do dedicated Hpt domains phospho-transfer to PilG and/or PilH 3) what is the role of N-terminal CheY domain, and 4) is signaling through ChpA to PilG and/or PilH a simple two-component system or is a more complicated phospho-relay cascade involving the CheY domain involved?

Moreover, is phosphorylation of PilG and/or PilH required for their interactions with PilB and PilT? Do PilG and PilH interact with the ATPases directly or indirectly? Looking outside the core signaling components themselves, hypothesis that the proteins encoded by Chp system genes function as a dedicated chemosensory would be supported through demonstration that ChpA interacts with PilJ via PilI and/or ChpC, and that PilJ is methyated by PilK and demethylated by PilB in a manner that depends on signaling impute from ChpA. Eventually similar lines of investigation could be expanded beyond the Chp system to include interactions between all know factors implicated in the biosynthesis and function of Tfp.

Preliminary observations that ChpA and PilH appear to play a role in the regulation of gene expression, raises additional questions about their biological functions outside regulation of TM. What is the complete range of genes whose expression is regulated by ChpA and PilH? How is this regulation accomplished, and what are the implications for *P. aeruginosa* biology for the coordinate regulation of gene expression and TM? Do other factors involved in the regulation of Tfp function also regulate gene expression? Through these studies and those described above, a clearer picture of how TM manifests behaviorally and how those behaviors are regulated will begin to emerge.

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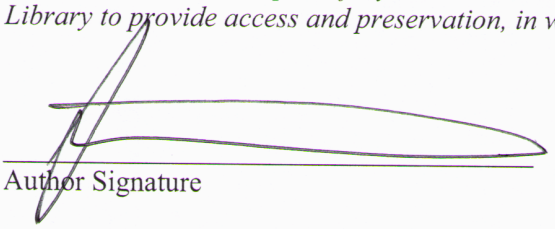

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