May 2022

Pili and Chpc the Two Adaptor Proteins in Pseudomonas Aeruginosa Chp Chemosensory System

Samuel Engel
University of Wisconsin-Milwaukee

PILI AND CHPC THE TWO ADAPTOR PROTEINS IN *PSEUDOMONAS AERUGINOSA* CHP CHEMOSENSORY SYSTEM

by

Samuel E. Engel

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biological Sciences

at

The University of Wisconsin-Milwaukee

May 2022

ABSTRACT

PILI AND CHPC THE TWO ADAPTOR PROTEINS IN *PSEUDOMONAS AERUGINOSA* CHP CHEMOSENSORY SYSTEM

by

Samuel E. Engel

The University of Wisconsin-Milwaukee, 2022
Under the Supervision of Professor Sonia L. Bardy

Pseudomonas aeruginosa's Chp system is an important signal transduction system for its virulence and controls many cellular processes through adenosine 3',5' cyclic monophosphate (cAMP) and its interactions with virulence factor regulator (Vfr). Twitching motility, the other output of the Chp system also plays a large role in virulence. This study was completed to better understand the roles the adaptor proteins, PilI and ChpC, fill in the Chp system. The function of the adaptor proteins was investigated through deletion strains via fluorescence microscopy, β-galactosidase assays, twitching motility assays, and surface piliation assays. By viewing these outputs PilI's status as the dominant adaptor protein of the system was reinforced. ChpC's role as a structural support adaptor was also reinforced.

To :

Those

Who have helped me

on

My way

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

While my time at the University of Wisconsin Milwaukee is coming to a close I want to express gratitude to those who have helped me in my time here.

First and foremost, Dr. Bardy, thank you for taking a chance on me and bringing me into the lab. You have helped me grow not only as a scientist but as an individual. I appreciate everything you did for me. Thank you for your unwavering belief in me throughout the ups and downs of these past two years.

Second, thank you to my lab mates Alyssa and Caitlin for making time spent in lab more enjoyable. I feel fortunate to have been able to bounce ideas off you both as we all worked collectively towards our goals. I also appreciated our banter as it was something I would look forward to everyday.

Third, thank you to all my friends and family who have supported me throughout my time here at Milwaukee I appreciate everything you have done for me.

Introduction

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium that is an opportunistic pathogen. Often a cause of nosocomial infections, *P. aeruginosa* can be a death sentence for many people, most notably cystic fibrosis patients who have deficient innate defenses in their airways due to their condition. *P. aeruginosa* infects cystic fibrosis patients within and on their airways to form hardy biofilms that render the infection exceedingly difficult to get rid of even with robust antibiotics. One of its key modes of motility, twitching motility, or the ability of a bacteria to move along a surface, is essential to the formation of biofilms (12,13,14).

To understand twitching motility, it is necessary to understand the cellular structures that enable *P. aeruginosa* to move along surfaces. One of the main components necessary for twitching motility is a type four pilus (T4P). This polarly localized structure mainly consists of the major pilin, PilA, and multiple other minor subunits that are anchored on the inner membrane of *P. aeruginosa.* To twitch, *P. aeruginosa* adds PilA monomers to the base of the T4P through the ATPase PilB, extending the T4P (15). Once the end of the pilus has reached a surface it adheres. At this point the retraction ATPase, PilT, removes pilin monomers from the base of the T4P decreasing its length and thereby moving *P. aeruginosa* along the surface. This movement is regulated by a two-component signal transduction system called the Chp system (16).

As the name suggests, bacterial two component signal transduction systems have two main parts: a sensor kinase and a response regulator that reacts to the signal. Together, these make up one signaling unit. Two component systems allow rapid responses to extracellular conditions, whether favorable or adverse. The sensor kinase is, as the name suggests, also a histidine kinase with an ATP binding domain. Upon receiving a signal, the histidine kinase will

auto-phosphorylate its histidine residue. The response regulator then receives the phosphate group from the histidine kinase and transfer it to the aspartate residue on the response regulator (17). The response regulator then has a downstream impact which varies from regulating transcription to phosphorylating another protein in the signaling cascade.

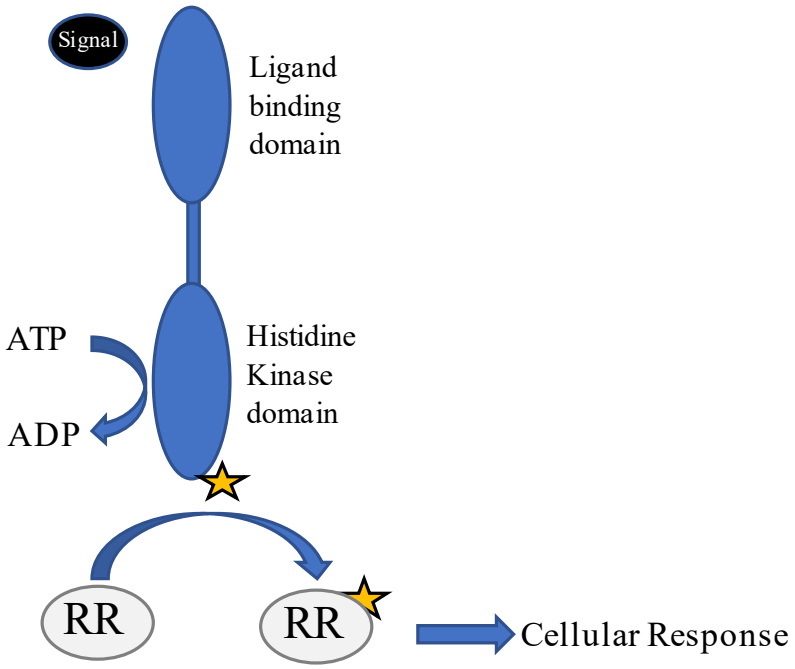


Figure 1: Simple model of a two-component system. Signal is received in the ligand binding domain of the protein. After the reception of the signal a conformational change allows the sensor kinase to take a phosphate group from intracellular ATP and auto phosphorylate its histidine residue. The response regulator then catalyzes the transfer of the phosphate from the histidine to its aspartate residue. The response regulator is now phosphorylated and will trigger downstream effects.

One common use of two component systems in bacterial cells is in chemotaxis, or the movement of a bacteria along a chemical gradient whether favorable or unfavorable. These systems are called chemosensory pathways. The best known and studied example of a chemosensory system exists in *Escherichia coli*. The basic structure is as follows; it receives a signal with its methyl accepting chemotaxis protein (MCP) which is passed through its adaptor protein CheW onto its histidine kinase CheA. The histidine kinase will phosphorylate the response regulator CheY which interacts with the flagellar motor of *E. coli* causing a change in the rotational direction of the motor. By changing rotation of the motor, the *E. coli* cell is able to alter its direction of travel before randomly starting off in a new direction. Multiple MCPs feed into this system through one adaptor protein, each new MCP is responsible for detecting different environmental cues to ensure proper adaptation to the environment (18).

In order to respond to the environment, *P. aeruginosa* employs four main chemosensory pathways with multiple chemoreceptors that have various inputs (10). The first is the F6 pathway which directly impacts swimming motility, as it is in control of the flagellar motor of *P. aeruginosa.* Depending on the signal received from outside of the cell, this pathway will either encourage a continuation of a run pattern or initiate a stop to change the course that hopefully will be more favorable but is randomly chosen (18). The second pathway is the F7 pathway, and while the true output of this pathway is unknown, it resembles a flagellar pathway from *E. coli*. It has been suggested that the F7 pathway could be the biological successor of the F6 pathway, as many species show intermediates between the two chemosensory systems with the F7 pathway seeming to be the final form (10). The third pathway is the Wsp pathway in *P. aeruginosa.* This pathway is heavily involved with the c-di-GMP regulation inside of the cell, which in turn helps turn the bacterium from its planktonic state to its biofilm creating state (10). Finally, the fourth

chemosensory system is the Chp system, which along with its components, will be the subject of this thesis. It is a surface sensing system that has two outputs (3, 10).

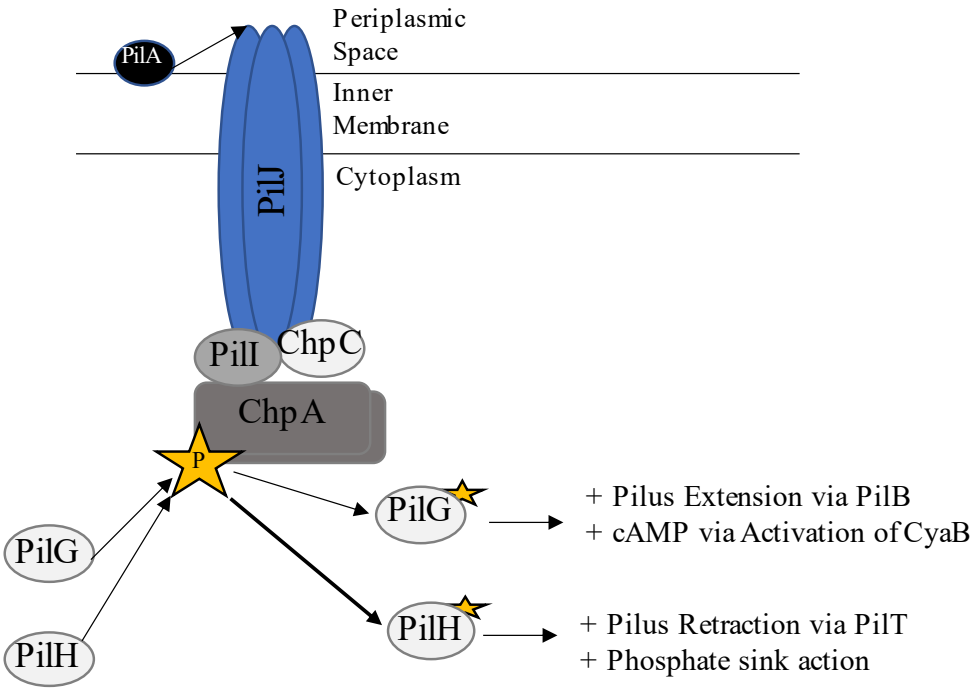


Figure 2: The Chp system. After receiving its signal, PilJ sends the signal downstream through PilI to ChpA, which has a histidine kinase domain that will auto phosphorylate and phosphorylate either PilG or PilH, the latter being the more favorable reaction. The downstream effects of the phosphorylation of PilG are increased pilus extension as well as increased cAMP that drives transcription of virulence genes when bound to Vfr.

The first output that it regulates is the function of T4P and thus one of the forms of motility in *P. aeruginosa.* The second output of this system is the regulation of the intracellular signaling molecule adenosine 3',5' cyclic monophosphate (cAMP). When bound to virulence factor regulator (Vfr), cAMP has drastic impacts on genetic regulation throughout the entire cell (3). Thus, the Chp system is essential for *P. aeruginosa* to be able to infect and then colonize its host.

The Chp system is a unique chemosensory system because it does not follow the canonical example of *E. coli* as it has two different outputs, neither of which control flagellar mediated motility. Even with this variation, the core signaling unit of the Chp system works much like the *E. coli* chemosensory systems core signaling unit, raising the question of why it has two different adaptor proteins with only one MCP. The Chp system works as follows: its MCP, PilJ, receives its signal, which triggers a conformational change in PilJ, and this signal is passed through the adaptor proteins PilI and ChpC that allow the histidine kinase ChpA to transfer a phosphate from ATP to either PilG or PilH, the response regulators of the Chp system. PilH has two roles, it is suggested to be a phosphate sink and is preferentially phosphorylated over PilG (19). It also functions as an activator for the ATPase, PilT which retracts extended pili and draws them into the cell. When PilG is phosphorylated two different outcomes happen: through the interactions of PilG and the ATPase, PilB, pili are extended from the cell. Additionally, intracellular cAMP levels are increased through a cascade starting with FimL and ending with CyaB, the main adenyl cyclase of *P. aeruginosa* (3, 24).

Once CyaB has been activated it will increase intracellular cAMP levels which then will bind to Vfr. Vfr is a regulatory protein that is very similar to CAP/CRP found within *E. coli*. Vfr and CAP/CRP form dimers when bound to cAMP and bind DNA to help drive the transcription of genes in the cell. When cAMP is bound to Vfr hundreds of downstream virulence factors are

upregulated, including the synthesis of T4P (3, 11). The activation of these virulence factors helps enable the successful colonization of the unfortunate host.

After the signal has been transduced PilJ still remains in the same post signal state. In order for the signaling unit of the MCP, HK, and response regulator to work again, the MCP must be reset. PilK, a predicted methyltransferase, then methylates PilJ to allow it to send a signal again. The methyl group cannot be allowed to sit on the MCP indefinitely though. ChpB, a methylesterase, is required to remove the methyl group allowing for the fine tuning of signaling through the setting and resetting of the MCP (3, 25)

The signaling unit of the Chp system does not work as a standalone unit. PilJ, the MCP, forms trimers of dimers which pair with adaptor proteins, PilI and ChpC to interact with dimers of ChpA. This signaling unit is then clustered into what is thought to be a hexagonal array that allows for the amplification of signals (20, 21). Multiple hexagonal signaling complexes bind together and form a larger array to sensitively react to signals. Several other species display this multi-adaptor approach as seen within the Chp system of *P. aeruginosa* so any additional information about these systems may have a broad impact, helping to understand how multiple adaptors can factor into a signaling array with one MCP. *Myxococcus xanthus* has two adaptors in its Frz system. One of these adaptors is a functional signal transducer, and the other is thought to be used for structural support (4). *Borrelia burgdorferi* has three adaptor proteins for one chemosensory system, two of which are key in signal transduction (23).

The Bardy Lab at University of Wisconsin Milwaukee has studied the two adaptor proteins and their role in signal transduction within the Chp chemosensory pathway to try and better understand why there are two adaptor proteins in one chemosensory system. Previous work used a Bacterial Two Hybrid Assay (BACTH) to study the protein-protein interactions seen

in the core signaling complex. The main takeaway from this set of experiments were that PilI and ChpC do not self-interact. Of the two adaptor proteins only PilI is able to interact with the histidine kinase of the system ChpA (5). With these new data a model of the Chp system hexagonal array was generated. In this model the removal of PilI leads to complete degradation of the array as ChpC is unable to bind ChpA to PilJ. However, as PilI can interact with ChpA the removal of ChpC only leads to a partial reduction in array size (5).

Once the protein interactions happening within the Chp system were understood this study observed the effect of the adaptor proteins on the phenotypic outputs of the Chp system as well as array formation. The output assays helped to better understand signaling through the Chp system with deletion strains highlighting the roles of the individual adaptors seen previously in the protein-protein interactions. Using a strain with genomic fluorescently labeled PilI, array formation was viewed by observing where the fluorescence was present in the cell. Through observing the size and brightness of the fluorescence with and without ChpC, fluorescent microscopy helped understand the structural role of ChpC in array formation.

These data our lab has gathered data suggests that of the two adaptor proteins, PilI is the main signal transducer. The other adaptor protein, ChpC, is mainly a structural protein that may not transmit signal but allows signal amplification by allowing many core signaling complexes to work together to efficiently transmit the signal from PilJ through ChpA to PilG and PilH. A better understanding of chemosensory systems with multiple adaptors will have implications beyond just the Chp system of *P. aeruginosa*. This study will help understand the roles multiple adaptor proteins can play in dual adaptor systems found in many different phyla. With this as a focus this study was undertaken to solidify the roles of the adaptor proteins of the Chp system.

Materials and Methods

Strains, Plasmids and Growth Conditions:

All *P. aeruginosa* and *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C unless otherwise stated. Liquid cultures were aerated using a rotational shaker and surface cultures were grown on 1.5% agar. Strains used in this study are listed in Table 1. Plasmids used in this study are listed in Table 2. Primers used in this study are listed in Table 3.

Twitching Motility Assays

Individual bacterial colonies were isolated and then stab inoculated into 1% agar LB plates and incubated at 37°C for 40 hours. Following incubation, the agar was removed and the diameter of the twitching zone on the plastic petri dish was measured.

Fluorescence Microscopy

Bacterial strains were streaked for isolation and incubated for 24 hours. Individual colonies were resuspended in 100uL of PBS in black microcentrifuge tubes and incubated on wet ice for 15 mins. After the ice bath incubation, 4uL of resuspended cell culture was pipetted directly onto a poly-lysine coverslip, (Cover slip: 25CIR-1D Lot: 042715-9) to promote adherence of bacterial cells. The cover slip was then placed on top of a glass slide. The coverslip and slide were incubated on ice and protected from light for 5 minutes to allow cells to lay flat onto coverslip. Cells were visualized using a Nikon Eclipse 90i upright microscope and images were captured via a Hamamatsu Orca -Flash 4.0 digital camera. Exposure was set for 600 ms and the Look up Tables (LUTs) were set at 0-1234 on NIS Elements software version 4.13

At least 300 cells were counted from images taken from 3 biological replicates for Δ*chpC* and Δ*pilJ*. At least 300 cells were counted from 2 biological replicates for the wildtype strain. Images were analyzed in ImageJ by measuring the intensity of fluorescence via its grayness from one pole to the other pole in a line after transferring the image into a 16bit format. Demographs were created according to the code cited in Guiseppi *et al* (4).

Surface Piliation Assay

Bacterial cells were streaked out for lawn growth and incubated at 37°C overnight with appropriate antibiotics. Cells were harvested by scraping lawn growth off plates and resuspending them in 500uL of 0.15M NaCl + 0.2% formaldehyde to generate a stock solution. The OD_{600} of a 1:100 dilution was measured, and the cultures were normalized to 800uL of 20 OD_{600} units in 0.15M NaCl + 0.2% formaldehyde. Surface proteins were then sheared off the cell via a 30-minute vortex followed by a 5-minute centrifugation at 12,000x*g* to pellet the intact bacterial cells. The resulting supernatant was precipitated overnight in 100mM $MgCl_2$ at 4°C. Precipitate was pelleted via a 25min centrifugation at 16873x*g* and then resuspended in 50uL of PBS. 10uL aliquots were mixed with 2x SDS loading dye and then run on a 15% acrylamide gel. Resulting gel was stained with G250 stain (7) and imaged via a Samsung Galaxy S20+. Resulting images were quantified via ImageJ using the "York method" (6).

Table 1: Strains used in this study		
Strain	Description	Source
E. coli Strains		
DH5α	Strain used for Cloning	New England Biolabs
BL21 DE3	Expression strain for T7 based promoters.	ThermoFisher
Lemo21 DE3	Expression strain for T7 based promoters with reducible activity of T7 RNA Polymerase	New England Biolabs
S17-1	Donor strain in conjugal mating in the generation Δ*pilI*+9 Δ*chpC* reporter strain	D. Saffarini
P. aeruginosa Strains		
PAO1	Igwelski wildtype	J. Harwood
Δ*pilI*::*lac*P1-*lacZ*	In frame deletion of *pilI* with 9 amino acids remaining introduced into PAO1::*lac*P1-*lacZ* reporter strain	(5)
*Δchp*C::*lac*P1-*lacZ*	In frame deletion of *chpC* with 9 amino acids remaining introduced into PAO1::*lac*P1-*lacZ* reporter strain	(5)
Δ*pilA*::*lac*P1-*lacZ*	In frame deletion of *pilA* with 9 amino acids remaining introduced into PAO1::*lac*P1-*lacZ* reporter strain	(5)
ΔcyaB::*lac*P1-*lacZ*	In frame deletion of *cyaB* with 9 amino acids remaining introduced to PAO1::l*ac*P1-*lacZ* reporter strain	(5)
ΔpilIΔchpC::*lac*P1-*lacZ*	In frame deletion of *pilI* with 9 amino acids remaining introduced to *ΔchpC*::*lac*P1-*lacZ* strain	This study
PilI-CFP	Chromosomal introduction of CFP fused PilI	(9)
PilI-CFP::Δ*pilJ*	PilI-CFP introduced to *ΔpilJ* strain	(9)
PilI-CFP::Δ*chpC*	PilI-CFP introduced to *ΔchpC* strain	This study

Table 2: Plasmids used in this study		
Plasmid	Description	Source
pEX18Tc	Gene replacement suicide vector conferring Tetracycline resistance	27
pEX18Tc:Δ*pilI*	pEX18Tc based plasmid designed for the deletion of *pilI*	5
pET-28a+	Expression vector conferring Kanamycin resistance with optional 6xHis tag addition at either N or C terminal ends	29
pET-28a+:*pilI*	*P. aeruginosa pilI* cloned into the HindIII and EcoRI restriction sites of pET28a+	This study
pET-28a+:*chpC*	*P. aeruginosa chpC* cloned into the HindIII and EcoRI restriction sites of pET28a+	This study
pJN105	Expression vector conferring Gentamicin resistance	28
pJN105:*cyaBR*$_{412H}$	Gene encoding constitutively active adenyl cyclase cloned into the EcoRI and XbaI restriction sites of pJN105	26

Table 3: Primers used in this study.	
Primer	Sequence (5' to 3') *
HindIIIPilJDNRev	CGAAGCTTGGCTCGAGCTCCGGAT
EcoRI-pilJFull-F	AGGAATTCCGAGAAGAACGACCGCAACCAG
FORpilI	**CAGCAAATGGGTCGC**GGATCCATGTCGGACGTTCAGACC
REVpilI	**CTCGAGTGCGGCCGC**AAGCTTTTATACGGCGACGTCGAG
FORchpC	**CAGCAAATGGGTCGC**GGATCCATGAACCAGGCCGTGATC
REVchpC	**CTCGAGTGCGGCCGC**AAGCTTTCAGATCAGGCCGGCGTC
*Bold indicates primer identity sequence to plasmid vector. Underline indicates identity sequence to gene	

β-galactosidase Assays

P. aeruginosa mutant strains with genomic addition of *lac*P1*lacZ* reporter construct were streaked out for lawn growth and incubated overnight at 37°C with the appropriate antibiotics (3). Lawn growth was then scraped from the plate and resuspended in 1mL of LB broth to form a stock culture. A 1:100 dilution of the stock culture was measured to determine the optical density (OD_{600}). A 1:20 dilution of the original stock culture was used in the β-galactosidase assays. 100uL of the 1:20 dilution was added to 400uL of Z-Buffer. Cells were lysed by adding 50uL chloroform and 25uL 0.1% SDS. Reaction tubes were inverted to mix and incubated at 30°C for 5 minutes. To start the reaction, 200uL of ONPG (4mg/mL in Z-buffer) was added to each tube. Once the reaction achieved a yellow color of similar intensity to LB media, the reaction was stopped by the addition of 500uL of 1M Na_2CO_3. Reaction tubes were then centrifuged at 16873x*g* for 3 minutes and the OD_{420} was measured taking care not to disturb the bottom layer of chloroform and cellular debris. Duration of the reaction was measured by recording the time when ONPG was added until the addition of Na_2CO_3. Miller units were calculated using the following formula:

$$\left\{\frac{OD420}{OD\ of\ used\ culture\ x\ volume\ in\ mL\ x\ time\ in\ minutes}\right\}\ x\ 1000$$

Generation of double deletion strain

The donor strain *E. coli* S17-1 (Δ*pilI*+9 pJN105) was grown up overnight at 37ºC. The recipient Δ*chpC* reporter strain was incubated overnight at 42 ºC. These cultures were mixed in a 3:1 ratio respectively and incubated on a LB plate overnight at 37ºC. After conjugal mating the

cells were diluted (10^{-1},10^{-2}, 10^{-3}) and plated on tetracycline 75ug/mL and chloramphenicol 5ug/mL LB agar and incubated overnight at 37ºC. Merodiploids were then replica plated onto LB plates with $Tet_{75}Chlor_5$ and 10% sucrose plates with $Tet_{75}Chlor_5$. The presence of the *pilI* deletion was verified via PCR.

Plasmid Cloning via RecA Independent Recombination

To express PilI and ChpC with N-terminal 6xHis tags, *pilI* and *chpC* were amplified via colony PCR. The primers for amplification encoded 15bp of similarity with the chosen expression vector pET-28a+. pET-28a+ was digested with HindIII and BamHI. Rather than ligating the gene inserts and plasmids *in vitro*, we took advantage of RecA Independent Recombination (RAIR) (1). The double digested pET-28a+ and *chpC* or *pilI* insert were added in a 1:1 molar ratio and transformed via heat shock into commercially competent DH5α (NEB). Initial verification of the constructs was done via mini prep and restriction enzyme digestion. Cloning was then verified via sequencing of the insert.

Transformations

Heat Shock: *E. coli* BL21-DE3 and DH5α were transformed by thawing cells on ice and then adding 1uL of the DNA into the reaction tube. The mixture was incubated on ice for 30 minutes and then placed into a 42°C water bath for 30 seconds to heat shock the cells. 1mL of Super Optimal broth with Catabolite repression (SOC) was added and the SOC competent cell mixture was incubated at 37°C with shaking for at least 1 hour. The transformation was then plated on LB agar plates with the correct antibiotic to select for resistant cells.

Electroporation: *E. coli* DH5α were transformed by thawing competent cells on ice and then adding 1uL of the DNA of choice into the reaction tube. The mixture was allowed to incubate for 30 minutes on ice. The DNA-cell suspension was added to a chilled 2mm electroporation cuvette. Electroporation was done via an Eppendorf Electroporator 2510 at 1600V. Directly after electroporation, 1mL of SOC was added and the transformants were allowed to recover at 37°C with shaking for at least 1 hour. Successful transformants were selected on LB agar with the appropriate antibiotics.

Induction of pET-28a+ expression vector

BL21 (DE3)

Bacterial cultures containing pET-28a+ based plasmids were grown overnight in LB broth containing 30ug/ml of kanamycin. The overnight culture was sub-cultured with a 1:100 dilution and incubated at 37ºC or 30ºC with aeration until exponential phase was reached (0.4 - 0.6 OD_{600}). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the indicated concentration. Strains were then induced at 37ºC for 1 hour. After induction OD_{600} was measured and 1mL of induced culture was pelleted and frozen down for protein purification.

Lemo21 DE3

Bacterial cultures containing pET-28a+ were grown overnight in LB broth containing 30ug/ml each of kanamycin and chloramphenicol. The overnight culture was sub-cultured at a 1:100 dilution. Rhamnose was added to the indicated concentration and incubated at 30ºC with aeration until exponential phase was reached (0.4 - 0.6 OD_{600}). At this point IPTG was added to a final concentration of 400mM. Strains were then induced at 30ºC for 1 hour. After induction

OD_{600} was measured and 1mL of induced culture was pelleted and frozen down for protein purification.

Protein Purification

To purify the 6xHis tagged proteins, Bacterial Protein Extraction Reagent (BPER) with lysozyme and DNAse was added to the pelleted bacterial cells at a 4 mL:1 gram ratio in a microcentrifuge tube. The pellet was resuspended and incubated at 30°C for 15 minutes to lyse the cells. After incubation the mixture was centrifuged to separate the soluble proteins from the insoluble proteins. The soluble and insoluble fractions were resuspended in equal volumes, and SDS loading dye was added to a 1x final concentration.

SDS-PAGE and Immunoblotting

Proteins were separated on a 15% poly-acrylamide gel (7). Total proteins were stained via G250 Coomassie stain. To detect His-tagged proteins by immunoblotting, proteins were separated by SDS-PAGE. The poly-acrylamide gel was placed into a semi-dry transfer apparatus and the proteins were transferred to PVDF membrane for immunoblotting. After transfer the membrane was blocked using 5% skim milk in Tryptone Buffered Saline with Tween 20. Depending on the protein of interest, different primary and secondary antibodies were used. For PilI-CFP immunoblot the primary antibody was Rabbit anti-GFP (1:1000 dilution) and the secondary antibody was Goat anti-Rabbit Horseradish peroxidase (1:10,000 dilution). For the anti-His immunoblot the primary antibody was mouse monoclonal anti-His antibody (1:3000 dilution) with a Sheep anti-Mouse Horseradish peroxidase antibody (1:10,000 dilution). Immunoblots were developed using SuperSignal West Femto maximum sensitivity substrate kit and imaged on a Fotodyne Luminary system.

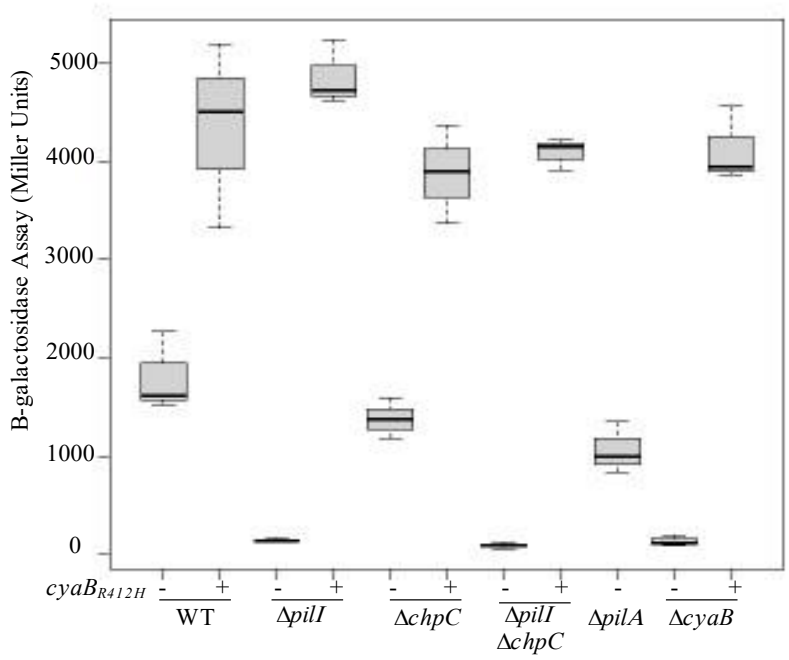
Results

PilI is the dominant signal transducer in the Chp System

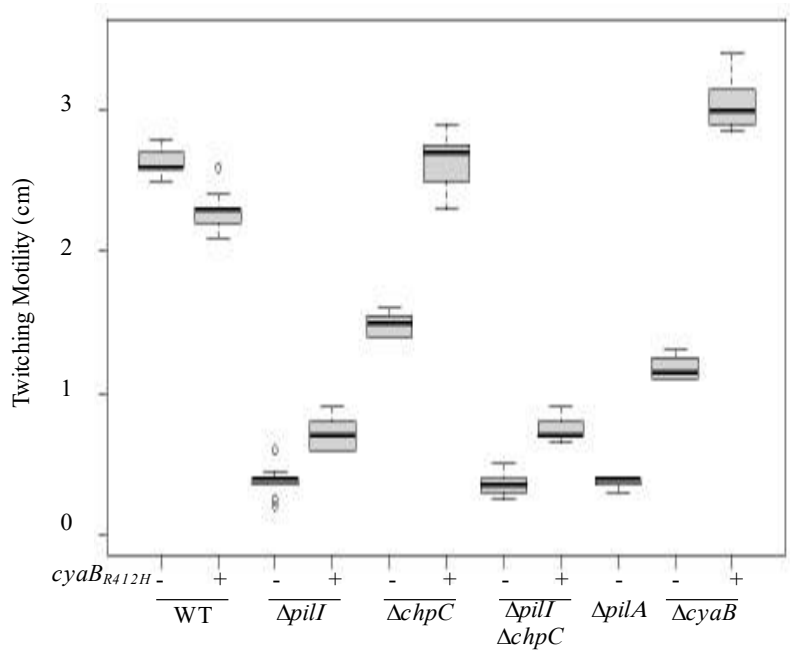
The Chp system has two main outputs: intracellular adenosine cAMP and twitching motility. Intracellular cAMP is positively regulated through the phosphorylation of response regulator, PilG and negatively regulated phosphorylation of response regulator, PilH. When PilG is phosphorylated, the adenylate cyclase, CyaB, is activated resulting in the production of ~90% of the cell's cAMP (7). Twitching motility is also regulated through phosphorylation of these two response regulators. PilG interacts with the ATPase PilB to extend the T4P and PilH works to retract the T4P by interacting with the ATPase, PilT (3,8).

It was previously shown that the *pilI* deletion strain had similar intracellular cAMP levels to a *cyaB* deletion strain (5). Upon restoration with a constitutively active CyaB ($CyaB_{R412H}$) intracellular levels of cAMP were no longer deficient. In contrast, when the *chpC* adaptor was deleted, there was only a partial reduction of intracellular levels of cAMP. This was once again able to be restored by the expression of $CyaB_{R412H}$ (Figure 3A). Similar patterns were seen in twitching motility when *pilI* and *chpC* were deleted individually. The Δ*pilI* strain lacked twitching motility much like the Δ*pilA* mutant which is missing the main component of T4P and is thus non-motile via twitching. Interestingly, even with restoration of intracellular cAMP through $CyaB_{R412H}$ expression, twitching motility was not restored to wildtype levels in the Δ*pilI* mutant. This contrasts with the twitching motility assay performed with the Δ*chpC* mutant, which had only a partial defect. Δ*chpC*'s defect in twitching motility could be fully restored back to wildtype levels via the addition of the constitutively active CyaB (Figure 3B). To see if the twitching motility results were due to a defect in T4P formation, a surface piliation assay was

A


B-galactosidase Assay (Miller Units)
5000
4000
3000
2000
1000
0
cyaB$_{R412H}$ - + - + - + - + - - + -
WT
ΔpilI
ΔchpC
ΔpilI ΔchpC
ΔpilA
ΔcyaB

B


Twitching Motility (cm)
3
2
1
0
cyaB$_{R412H}$ - + - + - + - + - - + -
WT
ΔpilI
ΔchpC
ΔpilI ΔchpC
ΔpilA
ΔcyaB

C

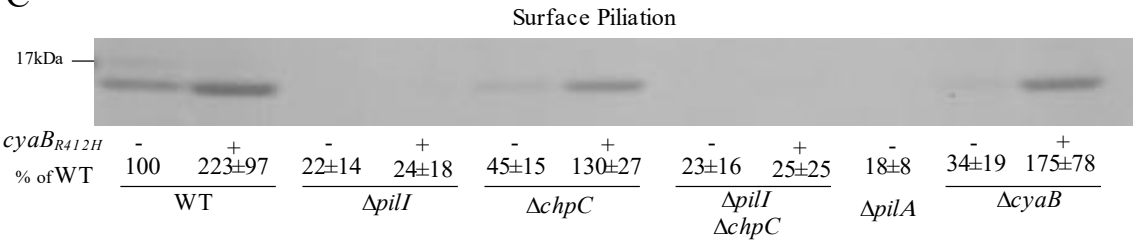

Surface Piliation
17kDa
cyaB$_{R412H}$ - + - + - + - + - - + -
% of WT 100 223±97 22±14 24±18 45±15 130±27 23±16 25±25 18±8 34±19 175±78
WT
ΔpilI
ΔchpC
ΔpilI ΔchpC
ΔpilA
ΔcyaB

Figure 3: Outputs of the Chp system: The box and whisker plots were generated via Rstudio. *cyaB*$_{R412H}$ was expressed from pJN105 in the strains indicated with a "+". Empty vector (pJN105) is indicated with a " -". A) β-galactosidase assays were done as described in the materials and methods and are from 3 biological replicates, each performed in triplicate. B) Twitching motility data was gathered by Dr. Sonia Bardy and is from 9 distinct colonies. C) Coomassie stained SDS-PAGE showing surface PilA levels. Protein bands were analyzed via ImageJ "York Method" described in the materials and methods section. Percentages relative to wildtype were determined using 3 biological replicates and the cropped gel presented is the most representative of the pattern seen through the averages. All images were converted to 16bit to change them to grayscale. Representative image was chosen after relative band intensity was measured. Background noise measurement was taken above the protein band. Protein levels were compared as a percent of wildtype. +/- is the standard deviation given through =stdev in the excel workbook.

completed. This shears the surface proteins off the cell, so this assay can determine if the twitching motility defect was due to the cells not having functional pili. It was found that the Δ*pilI* strain levels of surface piliation were comparable to Δ*pilA*, a strain which does not encode the major pilin subunit of the T4P. These levels were not restored through the addition of intracellular cAMP using the CyaB$_{R412H}$. Once again, the deletion of *chpC* gave a different phenotypic result; its deletion did impact the levels of surface piliation, but they were restored via the addition of CyaB$_{R412H}$ (Figure3C).

To determine which of the two adaptors was the dominant adaptor, a double mutant, Δ*pilI*Δ*chpC* was created. The double deletion mutant was put through the same assays alongside the single mutants Δ*pilI* and Δ*chpC* and their data were compared to see which of the two

phenotypes the double deletion mimicked. In the three assays described above, the double deletion had near identical phenotypic results to the deletion of *pilI* (Figure 3).

Signaling array formation is reduced in Δ*chpC* strains

To better understand the localization of the adaptor protein PilI, and the role of its interacting partners on signaling array formation, fluorescence microscopy was used. Using a previously generated strain with a genomic PilI-CFP fusion it was possible to view PilI in the presence and absence of its interacting partners, thus giving insight into the formation of Chp signaling arrays. It was first necessary to determine the relative level of PilI-CFP in each of the deletion strains. PilI-CFP immunoblots were undertaken to better understand the whole cell level of the fluorescent protein. While the deletions of *pilJ* and *chpC* certainly had an impact on the whole cell levels of PilI-CFP as shown in Figure 4, PilI-CFP was still detectable in the cell so it would be detectable by fluorescence microscopy.

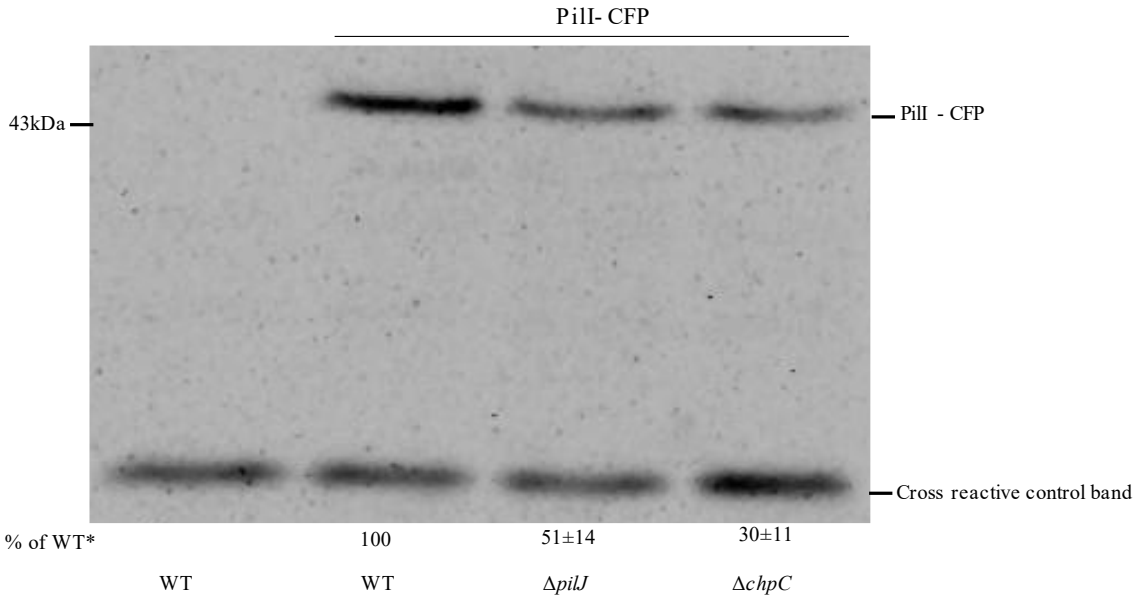


Figure 4: PilI-CFP Immunoblot. Immunoblots prepared as stated in the methods. Image of immunoblot was cropped to show relevant area only. Cross reactive band was seen in all strains including PAO1 without PilI-CFP. Three biological replicates were used to determine protein levels via ImageJ "York method". Image shown is representative image of the 3 biological replicates. +/- shown is the standard deviation as calculated through Excel's =st.dev function.

Table 4: Foci localization of PilI-CFP.

	PilI-CFP	PilI-CFP Δ*pilJ*	PilI-CFP Δ*chpC*
Number of Cells	315	304	315
Cells without Foci	164	249	206
Cells with Foci*	151	55	109
Polar Foci	110	41	79
Bipolar Foci**	18	6	15

*Indicates that the category of cells with foci includes polar, bipolar, and non-polar foci. **Bipolar foci were considered a subset of polar and included in the number of polar foci.

After whole cell levels of PilI-CFP were confirmed, fluorescent imaging took place. Demographs were constructed by measuring brightness from pole to pole as described in the materials and methods to better visualize the foci localization pattern. In the 47.9% of wildtype cells that displayed foci, PilI-CFP had 84.7% rate of foci localization to the poles of the cell as opposed to determination within the membrane throughout the cell. Wildtype PilI-CFP also showed consistent intensity of foci brightness as compared to the rest of the cell as displayed in the demographs by yellow color showing high level of fluorescence mainly at the poles (Figure 5A). Upon the deletion of the gene encoding *pilJ*, the MCP, fluorescence of PilI-CFP became diffuse throughout the cytoplasm and rarely formed foci, as only 18.1% of visualized cells were shown to have foci. The foci that did form were still polarly localized as 85.5% of visible foci were located at the poles (Figure 5B). When *chpC* was deleted the signaling array displayed a partial reduction, a pattern similar to those seen in the phenotypic outputs of the Chp system when compared to wildtype. There was a reduction in foci development as only 34.6% of visualized cells were shown to have foci. The foci in the Δ*chpC* deletion strain still retained polar localization though, as 86.2% of foci were located at the poles. However, fluorescence was also more diffuse than it was in the wildtype cell as higher intensity fluorescence was seen throughout the entire cell.

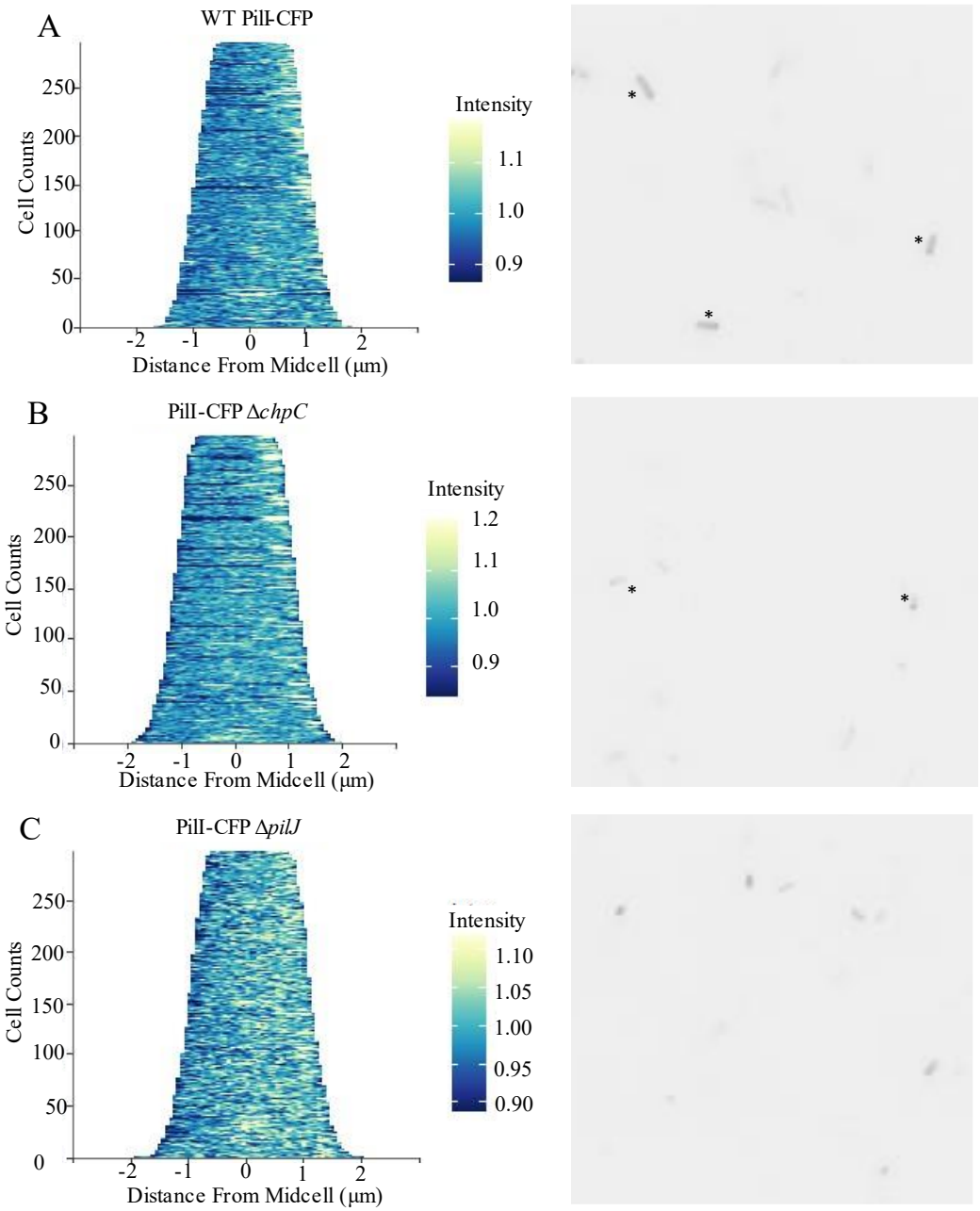


Figure 5: Fluorescence localization of PilI-CFP. PilI-CFP was imaged with and without interacting partners to determine localization patterns of foci. Measurements of fluorescence were taken as described in the materials and methods and assembled into a demograph by Alyssa Kline through the method laid out in (4). Astrisks indicate foci containing cells

Adaptor proteins have inducible expression in both BL21DE3 and Lemo21DE3

Previously our lab had undertaken BACTH assays to determine how the proteins in the Chp system interacted. It was found that PilI was able to interact with ChpA and ChpC. The other adaptor protein, ChpC, was only able to interact with PilI. ChpC and PilI did not show any self-interaction though. However due to the possibilities of BACTH causing false positives and negatives a protein pulldown experiment was initiated. I generated expression constructs of 6xHis-ChpC and 6xHis-PilI. This was accomplished via RAIR into DH5α as described in the materials and methods. After the generation of these constructs, it was necessary to determine if the 6xHis tagged proteins were able to be expressed in the quantity and quality that is necessary for pulldown assays. The first step was to determine if the expression of the proteins were inducible in a dose dependent manner as IPTG was increased. As displayed in Figure 6A, the plasmid-based transcription of the 6xHis tagged proteins were able to be induced with the addition of IPTG to the expression strains. While a dose dependent response is not directly seen that could be due to the large amount of protein present at the indicated induction levels. Since large amounts of protein were seen at the lowest level of induction, 0.1mM, this level of induction was used for fractionation experiments. Following induction, the cells were lysed using Bacterial Protein Extraction Reagent with lysozyme and DNase, and the insoluble and soluble fractions were isolated using centrifugation. The fractionated proteins were separated by SDS-PAGE. The majority of both PilI and ChpC were found in the insoluble fraction, even at the lowest level of IPTG induction used. 6xHis-PilI appeared more soluble than 6xHis-ChpC. Temperature of the induction was lowered to encourage the expression of soluble proteins, but the same fractionation pattern was seen at 30ºC as was seen at 37ºC (data not shown).

In a final attempt to solubilize the tagged proteins, the expression vectors were transformed into Lemo21-DE3. This expression strain is specifically designed to express 'hard to solubilize proteins' by decreasing the level of T7 RNA polymerase (T7 RNAp) in the cell. This is achieved by the pLemo plasmid included in this expression strain. pLemo encodes the lysozyme, LysY, the natural inhibitor of T7 RNAp. As pLemo is inducible via rhamnose (up to 2000mM) the more rhamnose present in the growth medium means that there will be less T7 RNAp and therefore less target protein will be transcribed and translated, ideally leading to a properly folded, soluble protein. These strains were also induced at 30ºC to encourage the formation of soluble proteins. However, the level of inhibition used did not allow enough tagged protein to be expressed and there were no visible protein bands of interest on the acrylamide gel. Due to time constraints the experiment was unable to be rerun with lower levels of rhamnose. This induction series did lead to the narrowing down of the induction level range needed for the generation of soluble ChpC and PilI to under 1000mM of rhamnose as no tagged protein is visible in the acrylamide protein gels in any of the strains containing rhamnose.

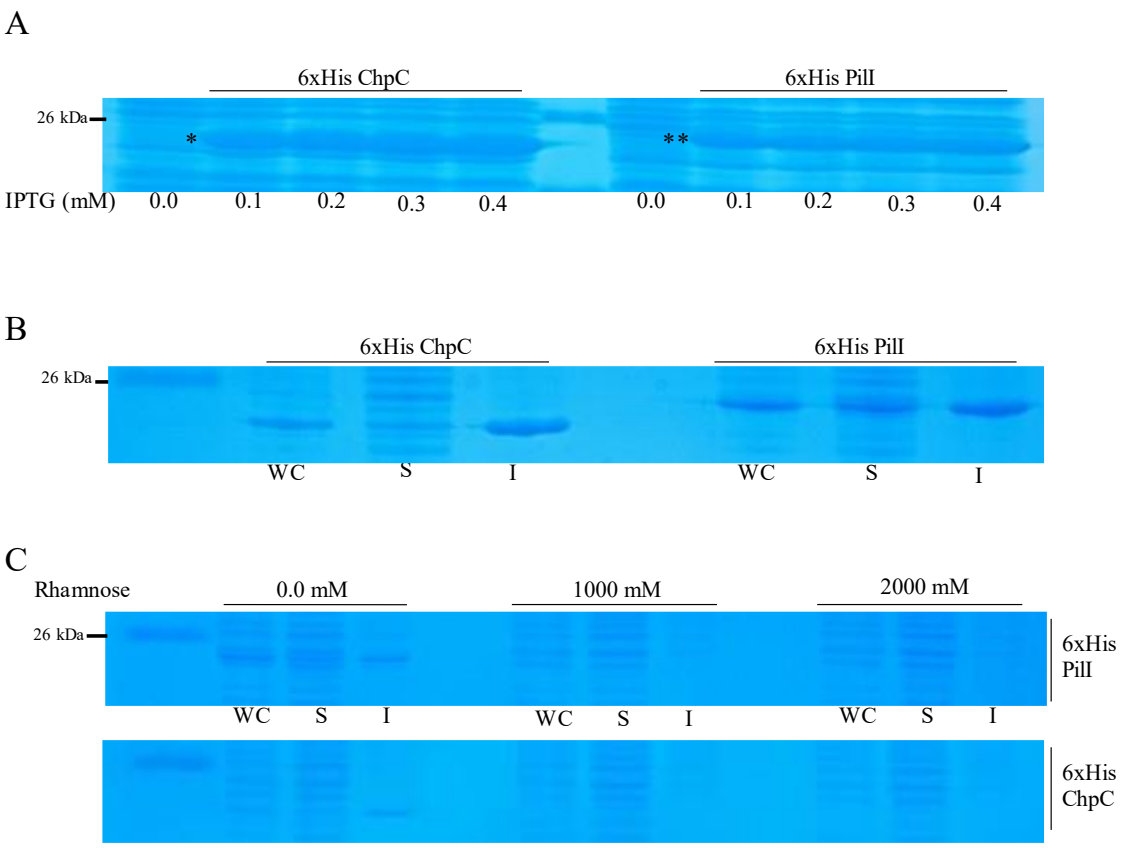


Figure 6: 6xHis Tagged proteins have inducible expression in BL21-DE3 and Lemo21-DE3. Poly-acrylamide gels were stained with G250 as described in the methods. Gels are cropped to show relevant portion of the gel only. WC indicates whole cell; S indicates soluble fraction and I indicates insoluble fraction. A) BL21 DE3 induction of tagged proteins. "*" indicates location of band of 6xHis ChpC and "**" indicated the location of 6xHis PilI. B) Fractionation of 0.1mM IPTG induction of BL21DE3 cells. C) Rhamnose induction of Lemo21-DE3 strain. IPTG was used at 400mM concentration in all samples.

Discussion

Bacteria must always be responding and adapting to the ever-changing environmental conditions as their survival depends on it. One of the many adaptations bacteria have developed are chemosensory systems. These two component systems that are sensitive to the environment or intracellular signals allow bacteria to alter gene expression, movement, and even change their life cycle as in sporulation (30). The main parts of a chemosensory system are the MCP, adaptor proteins and histidine kinase. The MCP receives a signal and passes it through adaptors proteins to the histidine kinase of the system. The best studied chemosensory system belongs to *E. coli* which encodes multiple MCPs, one adaptor protein, CheW, and one histidine kinase, CheA (18).

While there are many chemosensory systems that exist in the bacterial world some chemosensory systems employ the tactic of having two adaptors to connect one MCP to one histidine kinase. Multiple adaptor chemosensory systems are found in several different phyla and are responsible for flagellar motility, alternative cellular function, and T4P motility (17). *Pseudomonas aeruginosa'*s Chp system is one of these unique systems. This two-adaptor structure differs from the canonical example of *E. coli*'s chemosensory system, which only encodes for one adaptor protein for multiple MCPs (18). Another bacterial chemotaxis system that also strays from the model set by *E. coli* is the *Myxococcus xanthus* Frz chemosensory system which also has two adaptors. One adaptor, FrzA, is responsible for interacting with the respective histidine kinase of the system, FrzE, while the other, FrzB does not interact with FrzE at all. It was proposed that FrzB helps confer stability to multiple smaller arrays and that it could allow the addition of separate MCPs to link up to create a larger overall array (4). *Borrelia burgdorferi*, the causative agent of Lyme's disease, encodes for 3 different adaptor proteins, $CheW_1$, $CheW_2$, and $CheW_3$. Of these three adaptor proteins two ($CheW_1$ and $CheW_3$) are

necessary for interaction between its histidine kinase and MCP and thus chemotaxis (23). This differs from *M. xanthus* which only requires one adaptor to form the core signaling complex. Yet another species, *Azospirillum brasilense* encodes separate chemosensory pathways all together and yet is able to integrate these differing MCPs and their paralogs of CheA and CheW into one vast signaling array (22). This allows for additional variability when chemosensory arrays are assembled. As different bacterial species use their adaptor proteins in different ways, the dual adaptor Chp system of *P. aeruginosa* was chosen to better understand the role of multiple adaptor proteins existing in one chemosensory system.

Previous studies from the Bardy lab have researched protein-protein interactions seen within the Chp system (5, 7). Most important to this study were the interactions had by the adaptor proteins. PilI was shown to interact with ChpA, the histidine kinase of the Chp system. It was also shown to interact with ChpC, the other adaptor protein of the system. ChpC however, only displayed interaction with PilI in this system. Also of note is that neither ChpC nor PilI self-interacted. Since PilI was the only adaptor protein that interacted with the ChpA, PilI was hypothesized to be the adaptor protein that was able to pass on the signal from PilJ downstream to ChpA. Due to its lack of interaction with ChpA, ChpC was hypothesized to be responsible for enhancing the signaling array from a structural standpoint. As far as chemosensory systems using two adaptors, this theoretical layout is the most similar to the Frz system of *M. xanthus* (4). Only one adaptor is used to transmit the signal from the MCP to the histidine kinase unlike in *B. burgdorferi* where both adaptor proteins are necessary for chemotaxis and interact with their respective histidine kinase (23).

To study and understand the Chp system better deletion strains Δ*pilI*, Δ*chpC* and a strain lacking both genes, Δ*pilI*Δ*chpC*, were tested for the phenotypic outputs of the Chp system.

While deleting either of the genes had an impact on the twitching motility and intracellular cAMP, deleting *pilI* had a larger impact than the deletion of *chpC*. This pattern of a partial deletion of output for *chpC* and near complete loss of output after the deletion of *pilI* was seen in the twitching motility assays, β-galactosidase assays, as well as the surface piliation assays (Figure 1). We restored cAMP back into these deletion strains through expression of $CyaB_{R412H}$ and tested the Chp system outputs as well. Predictably the β-galactosidase assay showed a high level of intracellular cAMP when $CyaB_{R412H}$ was expressed in all strains. Interestingly, the increased level of intracellular cAMP was not able to restore wild type levels of either twitching or surface piliation in the *pilI* deletion strain. As it has been shown that while the major pilin subunit PilA is cAMP dependent for biogenesis, twitching motility remains cAMP independent needing a signal from the Chp system (3). The fact that twitching motility and surface piliation were not able to be restored to wildtype levels in the *pilI* deletion strain even when cAMP was present at elevated levels strengthens our hypothesis that PilI is needed to transmit signal through the Chp system. Only then can PilB and PilT, the ATPases that control the extension and retraction of the pilus respectively, function properly and move the cell along a surface.

The next question this study addressed is which of the two adaptor proteins is the dominant adaptor protein. The double deletion, Δ*pilI*Δ*chpC*, was tested through the same phenotypic assays as the individual deletions. In each assay the double deletion mimicked the phenotypic results that Δ*pilI* displayed solidifying its position as the dominant adaptor protein (Figure 1). Once again this is a similar system design as what is theorized to occur with the *M. xanthus* Frz system albeit with a few differences. FrzA is the signal transducer but FrzB helps stabilize smaller arrays that form on the nucleoid and possibly incorporates other MCPs into the signaling array (4). However, the localization pattern seen in the Frz system is different than

what was seen with the fluorescence microscopy completed as part of this study. Where typically only one array was seen, with polar localization. Fluorescence microscopy images were taken of PilI-CFP with and without its interacting partners, PilJ and ChpC, to learn more about the localization tendencies of PilI in the Chp system. In each of these strains the foci were typically found at the cell poles (~85%). This suggests that PilI does not require all of its interacting partners to localize to the poles. However, when PilJ, the MCP of the Chp system, was absent PilI-CFP foci formation was dramatically reduced. In wildtype PilI-CFP foci were found in 47.9% of cells while just 18.1% of Δ*pilJ* cells had detectible foci. This seems to indicate that while the foci that do form and continue to localize to the poles, PilJ is a critical building block of the Chp signaling array and causes array instability when absent. While the deletion of *chpC* did not have as dramatic of an effect as *pilJ* deletion, there was still a decrease in array formation as 34.6% of Δ*chpC* cells displayed foci, a reduction of 13.3% compared to WT. This partial decrease in array formation is much like the partial defect seen with the phenotypic assays giving support to ChpC having a structural support role. It is also important to note while the PilI-CFP immunoblot did also show decreased levels of PilI-CFP in the *chpC* and *pilJ* mutants this decrease was not proportional to the decrease in array formation seen in fluorescence microscopy. Bringing these valuable data together shows that ChpC is an important building block in the generation of the Chp signaling array.

In conclusion, the research done in this study gives increased robustness to the groundwork laid out previously in this lab. This study reinforces PilI as the dominant adaptor protein. Phenotypic assays demonstrate the deletion of ChpC only gives a partial reduction to the outputs of the Chp system whereas the deletion of PilI removes output of the Chp system. This remains true for β-galactosidase assays and pili associated assays as they report levels closely

resembling defective CyaB and PilA respectively. Also of note is that when both adaptor proteins are deleted the mutant strains phenocopy the deletion of *pilI*. Taken together with the protein interaction studies previously undertaken in the lab these data strongly support PilI as the dominant adaptor protein in the cell. Also with this understanding ChpC is able to play a structural support role in the Chp system increasing array size to improve signaling. These insights into one dual adaptor chemosensory system will help display the variability in which bacterial species can use multiple adaptors in one system to better adapt to their surroundings. Without a doubt there is still more to learn about these unique systems and the various ways bacteria can employ them.

References

1. Watson, Jake F., and Javier García-Nafría. "In Vivo DNA Assembly Using Common Laboratory Bacteria: A Re-Emerging Tool to Simplify Molecular Cloning." *Journal of Biological Chemistry* 294, no. 42 (October 18, 2019): 15271–81. https://doi.org/10.1074/jbc.REV119.009109.
2. Schneider, Caroline A., Wayne S. Rasband, and Kevin W. Eliceiri. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods* 9, no. 7 (July 2012): 671–75. https://doi.org/10.1038/nmeth.2089.
3. Fulcher, Nanette B., Phillip M. Holliday, Erich Klem, Martin J. Cann, and Matthew C. Wolfgang. "The Pseudomonas Aeruginosa Chp Chemosensory System Regulates Intracellular CAMP Levels by Modulating Adenylate Cyclase Activity." *Molecular Microbiology* 76, no. 4 (May 2010): 889–904. https://doi.org/10.1111/j.1365-2958.2010.07135.x.
4. Guiseppi, Annick, Juan Jesus Vicente, Julien Herrou, Deborah Byrne, Aurelie Barneoud, Audrey Moine, Leon Espinosa, et al. "A Divergent CheW Confers Plasticity to Nucleoid-Associated Chemosensory Arrays." *PLOS Genetics* 15, no. 12 (December 20, 2019): e1008533. https://doi.org/10.1371/journal.pgen.1008533.
5. Hying, Zachary, "A Tale of Two Adaptors: the Role of Two Adaptor Proteins in Pseudomonas Aeruginosa CHP Chemosensory System Signal Transduction and Implications for Chemosensory Array Formation" (2020). *Theses and Dissertations*. 2386.

6. Davarinejad H, 2015. “Quantifications of western blots with ImageJ.” University of York 3–6
7. Sharma, Swati, "Role of the Two Adaptor Proteins in the Chp Chemosensory System of Pseudomonas Aeruginosa" (2018). *Theses and Dissertations*. 1916. https://dc.uwm.edu/etd/1916
8. Bertrand, Jacob J., Joyce T. West, and Joanne N. Engel. “Genetic Analysis of the Regulation of Type IV Pilus Function by the Chp Chemosensory System of Pseudomonas Aeruginosa.” *Journal of Bacteriology* 192, no. 4 (February 15, 2010): 994–1010. https://doi.org/10.1128/JB.01390-09.
9. Jansari, Vibhuti Hemantkumar, "The Role of PILJ and Its Structural Domains in the Localization and Function of the CHP Chemosensory System in Pseudomonas Aeruginosa" (2017). Theses and Dissertations. 1646. https://dc.uwm.edu/etd/1646
10. Matilla, Miguel A., David Martín-Mora, Jose A. Gavira, and Tino Krell. “Pseudomonas Aeruginosa as a Model To Study Chemosensory Pathway Signaling.” *Microbiology and Molecular Biology Reviews : MMBR* 85, no. 1 (January 13, 2021): e00151-20. https://doi.org/10.1128/MMBR.00151-20.
11. Fuchs, Erin L., Evan D. Brutinel, Adriana K. Jones, Nanette B. Fulcher, Mark L. Urbanowski, Timothy L. Yahr, and Matthew C. Wolfgang. “The Pseudomonas Aeruginosa Vfr Regulator Controls Global Virulence Factor Expression through Cyclic AMP-Dependent and -Independent Mechanisms.” *Journal of Bacteriology* 192, no. 14 (July 2010): 3553–64. https://doi.org/10.1128/JB.00363-10.
12. Luo, Yun, Kun Zhao, Amy E. Baker, Sherry L. Kuchma, Kimberly A. Coggan, Matthew C. Wolfgang, Gerard C. L. Wong, and George A. O’Toole. “A Hierarchical Cascade of

Second Messengers Regulates Pseudomonas Aeruginosa Surface Behaviors." *MBio* 6, no. 1 (n.d.): e02456-14. https://doi.org/10.1128/mBio.02456-14.

13. Gloag, Erin S., Lynne Turnbull, Alan Huang, Pascal Vallotton, Huabin Wang, Laura M. Nolan, Lisa Mililli, et al. "Self-Organization of Bacterial Biofilms Is Facilitated by Extracellular DNA." *Proceedings of the National Academy of Sciences of the United States of America* 110, no. 28 (July 9, 2013): 11541–46. https://doi.org/10.1073/pnas.1218898110.
14. Semmler, A. B., C. B. Whitchurch, and J. S. Mattick. "A Re-Examination of Twitching Motility in Pseudomonas Aeruginosa." *Microbiology (Reading, England)* 145 (Pt 10) (October 1999): 2863–73. https://doi.org/10.1099/00221287-145-10-2863.
15. Craig, Lisa, Katrina T. Forest, and Berenike Maier. "Type IV Pili: Dynamics, Biophysics and Functional Consequences." *Nature Reviews. Microbiology* 17, no. 7 (July 2019): 429–40. https://doi.org/10.1038/s41579-019-0195-4.
16. Burrows, Lori L. "Pseudomonas Aeruginosa Twitching Motility: Type IV Pili in Action." *Annual Review of Microbiology* 66, no. 1 (2012): 493–520. https://doi.org/10.1146/annurev-micro-092611-150055.
17. Wuichet, Kristin, and Igor B. Zhulin. "Origins and Diversification of a Complex Signal Transduction System in Prokaryotes." *Science Signaling* 3, no. 128 (June 29, 2010): ra50. https://doi.org/10.1126/scisignal.2000724.
18. Wadhams, George H., and Judith P. Armitage. "Making Sense of It All: Bacterial Chemotaxis." *Nature Reviews. Molecular Cell Biology* 5, no. 12 (December 2004): 1024–37. https://doi.org/10.1038/nrm1524.

19. Silversmith, Ruth E., Boya Wang, Nanette B. Fulcher, Matthew C. Wolfgang, and Robert B. Bourret. "Phosphoryl Group Flow within the Pseudomonas Aeruginosa Pil-Chp Chemosensory System: DIFFERENTIAL FUNCTION OF THE EIGHT PHOSPHOTRANSFERASE AND THREE RECEIVER DOMAINS." *The Journal of Biological Chemistry* 291, no. 34 (August 19, 2016): 17677–91. https://doi.org/10.1074/jbc.M116.737528.

20. Piñas, Germán E., Vered Frank, Ady Vaknin, and John S. Parkinson. "The Source of High Signal Cooperativity in Bacterial Chemosensory Arrays." *Proceedings of the National Academy of Sciences* 113, no. 12 (March 22, 2016): 3335–40. https://doi.org/10.1073/pnas.1600216113.

21. Maddock, J. R., and L. Shapiro. "Polar Location of the Chemoreceptor Complex in the Escherichia Coli Cell." *Science (New York, N.Y.)* 259, no. 5102 (March 19, 1993): 1717–23. https://doi.org/10.1126/science.8456299.

22. O'Neal, Lindsey, Jessica M. Gullett, Anastasia Aksenova, Adam Hubler, Ariane Briegel, Davi Ortega, Andreas Kjær, Grant Jensen, and Gladys Alexandre. "Distinct Chemotaxis Protein Paralogs Assemble into Chemoreceptor Signaling Arrays To Coordinate Signaling Output." *MBio* 10, no. 5 (September 24, 2019): e01757-19. https://doi.org/10.1128/mBio.01757-19.

23. Zhang, Kai, Jun Liu, Youbin Tu, Hongbin Xu, Nyles W. Charon, and Chunhao Li. "Two CheW Coupling Proteins Are Essential in a Chemosensory Pathway of Borrelia Burgdorferi." *Molecular Microbiology* 85, no. 4 (August 2012): 782–94. https://doi.org/10.1111/j.1365-2958.2012.08139.x.

24. Buensuceso, Ryan N. C., Martin Daniel-Ivad, Sara L. N. Kilmury, Tiffany L. Leighton, Hanjeong Harvey, P. Lynne Howell, and Lori L. Burrows. "Cyclic AMP-Independent Control of Twitching Motility in Pseudomonas Aeruginosa." *Journal of Bacteriology* 199, no. 16 (July 25, 2017): e00188-17. https://doi.org/10.1128/JB.00188-17.

25. Whitchurch, Cynthia B., Andrew J. Leech, Michael D. Young, Derek Kennedy, Jennifer L. Sargent, Jacob J. Bertrand, Annalese B. T. Semmler, et al. "Characterization of a Complex Chemosensory Signal Transduction System Which Controls Twitching Motility in Pseudomonas Aeruginosa." *Molecular Microbiology* 52, no. 3 (May 2004): 873–93. https://doi.org/10.1111/j.1365-2958.2004.04026.x.

26. Jansari, Vibhuti H., Vishwakanth Y. Potharla, Geoff T. Riddell, and Sonia L. Bardy. "Twitching Motility and CAMP Levels: Signal Transduction through a Single Methyl-Accepting Chemotaxis Protein." *FEMS Microbiology Letters* 363, no. 12 (June 2016): fnw119. https://doi.org/10.1093/femsle/fnw119.

27. Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. "A Broad-Host-Range Flp-FRT Recombination System for Site-Specific Excision of Chromosomally-Located DNA Sequences: Application for Isolation of Unmarked Pseudomonas Aeruginosa Mutants." *Gene* 212, no. 1 (May 28, 1998): 77–86. https://doi.org/10.1016/s0378-1119(98)00130-9.

28. Newman, J. R., and C. Fuqua. "Broad-Host-Range Expression Vectors That Carry the L-Arabinose-Inducible Escherichia Coli AraBAD Promoter and the AraC Regulator." *Gene* 227, no. 2 (February 18, 1999): 197–203. https://doi.org/10.1016/s0378-1119(98)00601-5.

29. Rosenberg, A. H., B. N. Lade, D. S. Chui, S. W. Lin, J. J. Dunn, and F. W. Studier. "Vectors for Selective Expression of Cloned DNAs by T7 RNA Polymerase." *Gene* 56, no. 1 (1987): 125–35. https://doi.org/10.1016/0378-1119(87)90165-x.

30. Zschiedrich, Christopher P., Victoria Keidel, and Hendrik Szurmant. "Molecular Mechanisms of Two-Component Signal Transduction." *Journal of Molecular Biology* 428, no. 19 (September 25, 2016): 3752–75. https://doi.org/10.1016/j.jmb.2016.08.003.