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Understanding the Link Between Chemotaxis and Biofilm Dispersion Systems in Pseudomonas Aeruginosa

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UNDERSTANDING THE LINK BETWEEN CHEMOTAXIS AND BIOFILM DISPERSION SYSTEMS IN *PSEUDOMONAS AERUGINOSA*

by

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ABSTRACT

UNDERSTANDING THE LINK BETWEEN CHEMOTAXIS AND BIOFILM DISPERSION SYSTEMS IN *PSEUDOMONAS AERUGINOSA*

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The opportunistic pathogen *P. aeruginosa* forms biofilms during chronic lung infections in cystic fibrosis patients, contributing to morbidity and mortality. Not only are biofilms antibiotic resistant but dispersal may release pathogenic bacteria throughout the body. Previous research discovered a novel interaction between DipA, a phosphodiesterase that promotes biofilm dispersal, and ParP, which localizes chemosensory clusters and has AIF (array integration and formation) domain homology to CheA and CheW of the chemotaxis system. This research was focused on further deciphering the role of ParP's AIF domain in mediating the interactions between DipA and CheA by using a bacterial two hybrid system. We also explored the functional redundancy of ParP and CheW AIF domains with swimming assays and found that ParP's AIF domain was unable to restore swimming motility in a Δ*cheW* mutant strain. This research may provide new targets for safe biofilm dispersal within the lungs of cystic fibrosis patients.

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Introduction

Chemotaxis System in *P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium with a unipolar flagellum. It is a model organism for investigation of biofilm formation and pathogenesis due to its natural ability to form biofilms, resistance to antimicrobials, numerous virulence factors, and network of genes that facilitate its ability to adapt to its environment called chemosensory systems [1]. These chemosensory systems are encoded in discrete gene clusters: *che I* and *V* (swimming motility), *chp/pil* (twitching motility and cAMP levels) and *wsp* (c-di-GMP levels and biofilm formation) [2]. The fifth chemosensory gene cluster (*cheII*) has unknown function but encodes chemotaxis protein homologs.

Swimming motility is a key to *P. aeruginosa* pathogenesis; without flagella the pathogen fails to establish infection [3]. Control of swimming motility occurs through the chemotaxis system, which in *P. aeruginosa* is thought to function similarly to that of *Escherichia coli*. Chemotaxis (Fig.1) has been studied extensively in *E. coli*: it starts with reception of an environmental signal by a methyl-accepting chemotaxis protein (MCP) trimer of dimers, which is relayed to a histidine kinase dimer (CheA) via the adaptor protein CheW. This initiates a two-component signal transduction system in which the first component, CheA, trans-autophosphorylates and then transfers its phosphate group to the second component the response regulator CheY [4]. CheA also phosphorylates CheB, a methyltransferase/response regulator that competes with constitutive methyltransferase CheR to adapt MCP sensing to signal concentration. Upon phosphorylation, CheY interacts with the flagellar motor by binding the rotational switch

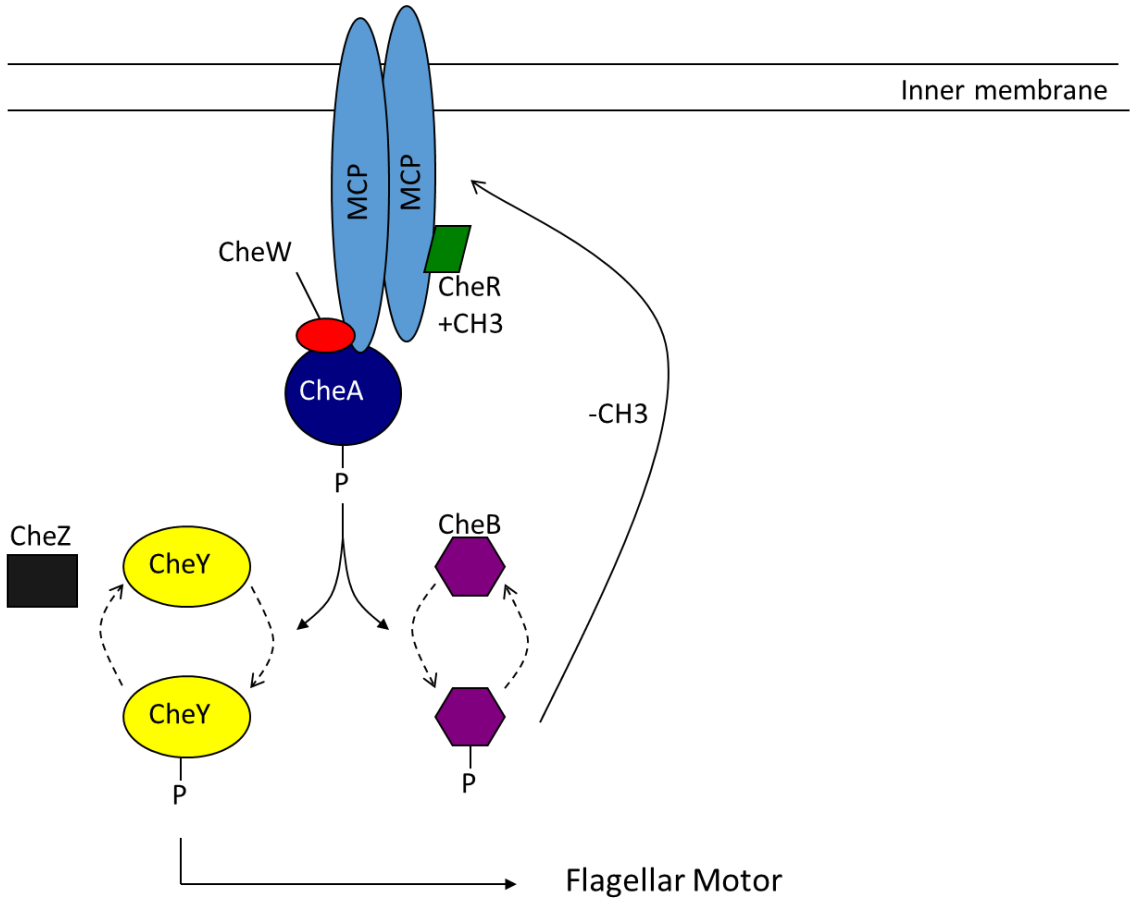


Figure 1 The chemotaxis system of *E. coli*. The methyl-accepting chemotaxis protein (MCP) dimer (light blue) respond to an environmental cue and activate CheA (royal blue) of the two-component transduction system. Phosphorylated CheY (yellow) diffuses to the flagellar motor resulting in a switch to clockwise rotation, bacterial tumble and directional change.

complex protein FliM to induce a change from counterclockwise rotation to clockwise. The change in rotation results in a tumble and allows the bacteria to randomly change swimming direction [4]. Ultimately, this system allows the bacteria to respond to its environment by swimming toward or away from different chemical stimuli. *P. aeruginosa* uses chemotaxis and swimming motility to colonize the lungs of CF patients, subsequently forming biofilms [3].

Differences between chemosensory systems of *E. coli* and *P. aeruginosa* include the number of gene clusters (*E. coli* has one while *P. aeruginosa* has five) and the spatial localization of the flagella. *E. coli* has both large polar chemotaxis arrays and nonuniform, lateral distribution of smaller chemotaxis arrays which coincides with peritrichous surface flagella [5]. Unipolar localization of chemosensory arrays and flagellum in *P. aeruginosa* is highly regulated [6]. It is proposed that localization of *P. aeruginosa* flagellum-associated chemosensory arrays is through a partitioning system that acts to divide cellular components upon division like the well-studied mechanism in *Vibrio cholerae* [2].

In the polarly-flagellated bacterium *Vibrio cholerae*, the partitioning proteins ParA and ParB are important for nuclear division, tethering the duplicated chromosomes to opposite poles of cells for proper inheritance [7]. Another pair of partitioning proteins, ParC (a ParA-like ATP-ase) and its partner ParP, together tether chemotaxis clusters at the cell pole by capturing and preventing dissociation of the histidine kinase CheA [6]. Sequestration of chemotaxis clusters through the tripartite interaction of ParC-ParP-CheA results in proper chemosensory array localization at the cell pole [6]. Unipolar localization of chemotaxis clusters via ParP & ParC is modeled as "diffusion and capture" in tandem with cellular division (Fig. 2). The partitioning proteins diffuse and accumulate at the new cell pole with the onset of cellular division to capture and localize chemotactic arrays. The Par proteins continue to fluctuate between the poles as the cell

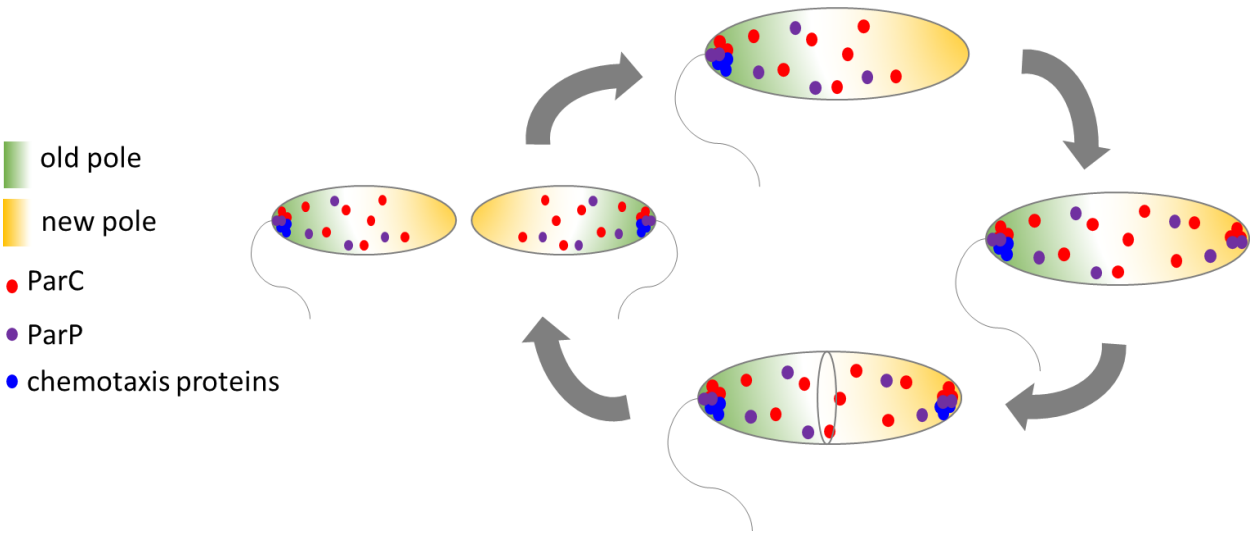


Figure 2 The partitioning system of *V. cholerae* (6). Partitioning proteins ParP and ParC diffuse from the "old" pole to the new and sequester chemotaxis arrays via interaction with CheA.

divides, resulting in flagellum-adjacent localization of the chemotaxis clusters in the new cells [6]. This mechanism facilitates proper inheritance of chemotaxis clusters and rapid chemosensory signaling to the flagellum.

ParC and ParP are encoded within the chemotaxis operon and predicted to be co-transcribed with chemotaxis genes [6]. Studies in *Vibrio* showed that Δ*parC* mutants are deficient in chemotaxis protein localization and ability to switch swimming direction [6]. Additionally, half of Δ*parP* mutants had laterally mislocalized chemotaxis proteins or lacked arrays altogether [7]. Previous studies in *P. aeruginosa* showed 45-50% loss of CheA foci formation in Δ*parC* and Δ*parP* strains, however the localization pattern of the foci was comparable to wild type [2].

The partitioning and chemotaxis systems together form chemosensory arrays at the cell pole. ParP, CheA, and CheW each have a homologous C-terminal nucleotide sequence called the Array Integration and Formation (AIF) domain [8]. It is known that CheA and CheW interact with the MCPs and each other via the AIF domains [8]. Additionally, studies in *Vibrio* revealed the ParP AIF domain interacts with the central LID domain of CheA while its N-terminus interacts with the sister partitioning protein, ParC [8].

Within the chemotaxis gene cluster, *cheW* is directly downstream of *parP* and is predicted to be co-transcribed [9]. Previous research in *P. aeruginosa* showed that CheW can partially complement swimming motility in a ParP knockout mutant, but ParP does not complement Δ*cheW.* Furthermore, double complementation with both ParP and CheW in Δ*parP* had greater restored swimming motility than either single complementation, suggesting that the expression and stability of these proteins are dependent on each other [2]. This prompted us to ask if full length ParP was unable to restore swimming motility in Δ*cheW* because of its N-terminal

extension. We hypothesized that a truncated ParP C-terminal construct would complement Δ*cheW*.

Biofilm Dispersal in *P. aeruginosa*

Biofilms are stratified colonies of sessile bacteria that increase collective resistance to host immune defenses and antimicrobials. This increased resistance contributes to chronic and persistent infection within a patient. Biofilm formation, or the transition from swimming motility to sessility, is another chemosensory-dependent virulence factor in *P. aeruginosa* and is regulated by intracellular cyclic dimeric guanosine monophosphate (c-di-GMP) levels.

Increased levels of this second messenger, synthesized by diguanylate cyclases (DGC) possessing a GGDEF domain, promote sessility and biofilm formation [10]. In *P. aeruginosa*, the Wsp chemosensory system regulates biofilm formation via modulation of c-di-GMP levels. The response regulator of this chemosensory system, WspR is homologous to CheY and also contains a GGDEF domain. Signal transduction through this system results in phosphorylation and clustering of WspR in the cytoplasm, and increased levels of c-di-GMP. Another chemosensory protein, WspF, is homologous to the methylesterase CheB which de-methylates the membrane-bound MCP trimer in the Che chemosensory system [11]. Deletion of *wspF* results in increased levels of c-di-GMP, cell aggregation and wrinkled colony formation [11].

Within a biofilm, degradation of c-di-GMP by phosphodiesterases results in dispersion [10]. The phosphodiesterase DipA is known to decrease c-di-GMP levels in response to environmental cues detected by the NicD complex (Fig. 3). NicD is a membrane-bound diguanylate cyclase which simultaneously increases c-di-GMP levels and phosphorylates the cytoplasmic protein BdlA in response to changes in extracellular glutamate levels [11]. BdlA is a homolog of the *E.*

coli MCP Aer, which is known to detect changes in reactive oxygen species via its Per-ARNT-Sim (PAS) sensory input domain. BdlA has two PAS domains which are thought to facilitate biofilm dispersion in a redox-dependent manner [12]. Additionally, PAS domains are known to form homodimers in protein interactions. Both known interaction partners of BdlA, DipA and RbdA, have PAS domains [12].

Although BdlA is an MCP homolog, it is not membrane-bound and is uncharacteristically phosphorylated [13]. Upon phosphorylation by NicD, BdlA requires proteolytic, non-processive cleavage between its PAS domains by protease ClpP and chaperone ClpD to become activated [14]. BdlA recruits phosphodiesterases DipA and RdbA to ultimately decrease cellular levels of c-di-GMP and promote biofilm dispersal [10]. Null *bdlA* mutants show a decrease in biofilm dispersion coupled with an increase in c-di-GMP levels [15]. Likewise, *dipA* and *rbdA* mutants showed impaired biofilm dispersal and virulence in vitro [14, 16]. In brief, the NicD DGC temporarily increases c-di-GMP levels to activate biofilm dispersal via BdlA. Dispersal is sustained by the subsequent recruitment of PDEs DipA and RbdA which decrease intracellular c-di-GMP [13].

Biofilm dispersal is considered a bacterial strategy to facilitate the shift from chronic infection to periodic, acute infection [10]. Biofilm dispersal is not simply a reversal of sessility but a distinct virulent phenotype [14]. Dispersed cells have decreased transcription of virulence genes, including cytotoxic and degradative proteins. Downregulation of virulence genes is thought to be a transient, protective measure to shield dispersed cells from the host immune system [14]. Ultimately, dispersion is key to chronic persistence and acute virulence of *P. aeruginosa* [14].

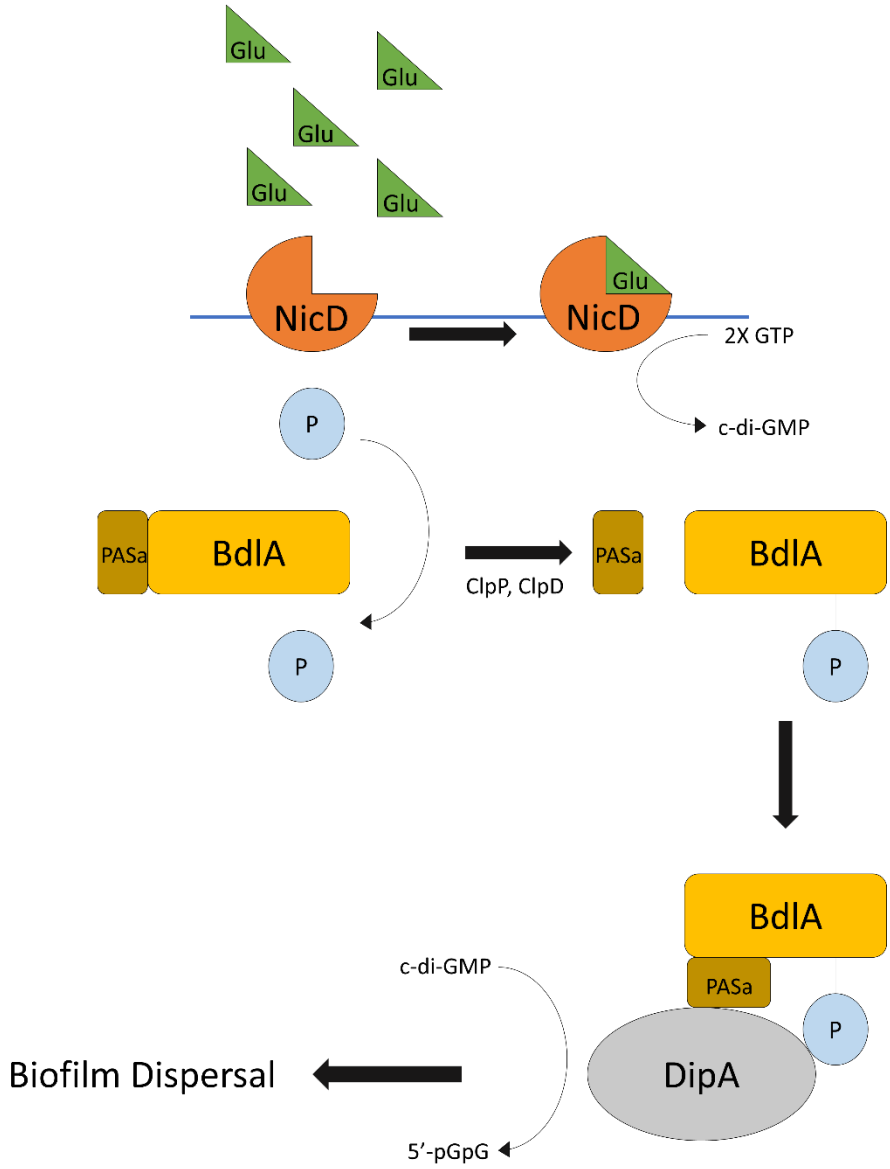


Figure 3 The NicD sensory complex (13). NicD phosphorylates BdlA in response to changes in extracellular glutamate levels. BdlA is then proteolytically cleaved and activates DipA to decrease intracellular c-di-GMP levels and promote biofilm dispersal.

DipA is a link between this biofilm dispersal system and the flagellar chemotaxis system in *P. aeruginosa* (Fig. 4). It is known that PDE activity of DipA is required for maintenance of intracellular c-di-GMP heterogeneity thereby regulating flagellar motility [17]. Previous research showed that DipA interacts with ParP, a novel interaction of this phosphodiesterase [2]. To further elucidate its interaction within the chemotaxis system, we asked if DipA interacts with the N-terminal domain of ParP or its C-terminal AIF domain. Additionally, DipA localization at the cell pole is dependent on CheA and phosphodiesterase activity of DipA is enhanced by phosphorylated CheA [17]. Furthermore, DipA and CheA were shown via coprecipitation assays to form a complex in *P. aeruginosa* but it is unknown if these proteins directly interact [17]. We therefore asked if DipA can directly interact with CheA.

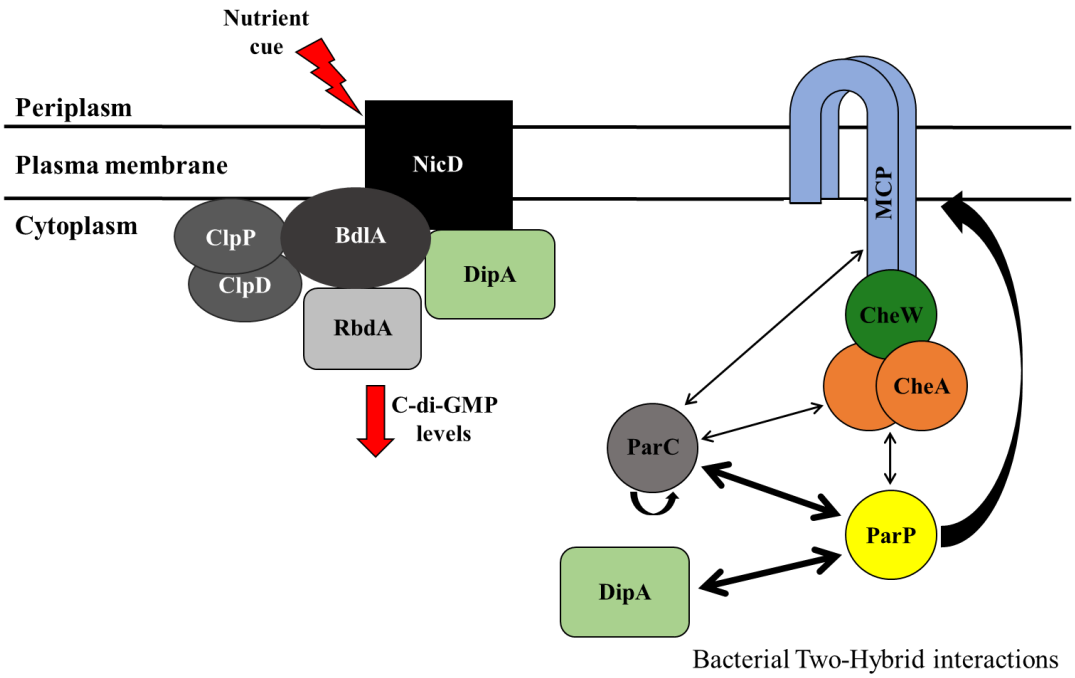


Figure 4 DipA interactions within NicD biofilm dispersal sensory complex and flagella-informing chemosensory array (2).

Materials & Methods

Bacterial Strains & Growth Conditions

E. coli and *P. aeruginosa* strains (Table 1) were grown at 37˚C over night in LB broth with aeration or on LB 1.5% agar plates. Antibiotics were used as appropriate: Ampicillin 100µg/mL, Kanamycin 50µg/mL, Gentamicin 10µg/mL, for *E. coli*, and Gentamicin 50µg/mL, Chloramphenicol 5µg/mL for *P. aeruginosa.*

BACTH Plasmid Gene Cloning

Bacterial genes of interest (Table 2) were first amplified using PCR (annealing at 62˚C for 30s, extension at 72˚C for 1.5min). PCR products for genes of interest were separated by gel electrophoresis, cut out of gel, and purified using Monarch Gel Extraction Kit or IBI Gel Extraction Kit. BACTH vectors (Table 3) were isolated from liquid culture using the IBI Mini Plasmid Kit. DNA was digested with appropriate restriction enzymes, separated by gel electrophoresis, and DNA was excised via gel extraction kit. Recovered DNA concentrations were measured using a Thermo Scientific NanoDrop 2000C Spectrophotometer. Ligations were incubated over night at 16˚C and DNA transformed the next day using DH5α *E. coli* competent cells: 2µl (or less than one-tenth cell volume) ligation added to 20µl competent cells, followed by incubation on ice for 30mins and heat shock at 42˚C for 30s. After recovery on ice for 5mins, SOC media was added and the cells were incubated for 1hr at 37˚C. Cultures were then plated on antibiotic-selective (Ampicillin or Kanamycin) LB media. Recombinant plasmids were confirmed first by gel electrophoresis and all genes of interest were sequenced to ensure no mutations were introduced.

Table 1 Strains used in this study

Strain	Description	Source
E. coli DHM1	*F-, cya-854, recA1, endA1, gyrA96 (Nal r), thi1, hsdR17, spoT1, rfbD1, glnV44(AS)*	Euromedex BACTH System Kit
E. coli S17-1	*TpR SmR recA thi pro hsdR- M+ RP4 2-Tc::Mu-Km::Tn7 λpir*	Daad Saffarini
E. coli NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
PAO1	*P. aeruginosa* PAO1 (Iglewski strain)	Carrie Harwood
PAO1 Δ*cheW*	deletion of PA1464 (*cheW*) in PAO1	[2]
PAO1 *fliC::tn*	Transposon (lacZhah) in PA1092 (*fliC*) in PAO1. Inserted at base 820 of 1467 Transposon mutant #45281	University of Washington PAO1 transposon mutant collection

Table 2 Genes of interest.

Genes	ORF* #	Truncation Coordinates (bp)
dipA	PA5017	
parP	PA1463	
parPc	PA1463	1593302-1594038
parPn	PA1463	1593256-1593301
parC	PA1462	
cheW	PA1464	
cheA	PA1458	

*****from *pseudomonas.com*, using the PAO1 reference genome

Table 3 Plasmids used in this study.

Plasmid	Description	Source
pKNT25	Empty vector with T25 CyaA fragment 5' end of multiple cloning site	Euromedex BACTH System Kit
pKT25	Empty vector with T25 CyaA fragment at 3' end of multiple cloning site	Euromedex BACTH System Kit
pUT18C	Empty vector with T18 CyaA fragment at 3' end of multiple cloning site	Euromedex BACTH System Kit
pUT18	Empty vector with T18 CyaA fragment at 5' end of multiple cloning site	Euromedex BACTH System Kit
pKT25-zip	Positive control with leucine zipper motif genetically fused to T25 CyaA fragment	Euromedex BACTH System Kit
pUT18C-zip	Positive control with leucine zipper motif genetically fused to T18 CyaA fragment	Euromedex BACTH System Kit
pJN105	Broad host range vector. pBBR-1 MCS5 AraC-pBAD derivative	[19]

Table 4 Primers used in this study.

Primer Name	Sequence (5' to 3')*
ParP N-terminus forward	agaggatcccatgagcgccgccaccgccac
ParP N-terminus reverse	ctcggtacccggcgcaactcgatgacttc
ParP C-terminus forward (BACTH)	agaggatcccgtgccgagcgccccggcg
ParP C-terminus reverse (BACTH)	ctcggtacccgatggtcgccgtgcaggcg
DipA forward	agaggatcccatgaaaagtcatcccgat
DipA reverse	ctcggtacccggtgcagggtgcggcaggg
ParC forward	gaggatcccatgaaagtctgggcagtc
ParC reverse	tgaattcgaggccacccgggtggccgg
CheW forward	gaggatcccatgagcaaagccaccgcg
CheW reverse	tgaattcgagatgctgcccagctccga
CheA forward	gaggatcccatgagcttcgacgccgat
CheA reverse	cggtacccggatgcgccgtgcgtaacg
ParP C-terminus forward (pJN105)	cttaagaattcatgagcgccccggcg
ParP C-terminus reverse (pJN105)	ctagagctctcaagtcgccgtgcagg

*Restriction enzyme sites are underlined.

Table 5 DHM1 Constructs

DHM1 Constructs	Plasmids
DipA-T18/ParP-T25	pUT18, pKNT25
T18-DipA/ParP-T25	pUT18C, pKNT25
DipA-T18/T25-ParPn	pUT18, pKT25
T18-DipA/T25-ParPn	pUT18C, pKT25
ParPc-T18/T25-DipA	pUT18, pKT25
T18-ParPc/T25-DipA	pUT18C, pKT25
ParPc-T18/DipA-T25	pUT18, pKNT25
T18-ParPc/DipA-T25	pUT18C, pKNT25
DipA-T18/CheA-T25	pUT18, pKNT25
T18-DipA/CheA-T25	pUT18C, pKNT25

BACTH DHM1 Electroporation Co-Transformations

E. coli DHM1 (Table 1) was made competent via glycerol method: over night cultures of DHM1 cells were washed twice with ice-cold 10% glycerol and concentrated. Aliquots of electrocompetent cells (50µl) were pipetted and 100ng of plasmid DNA added for both the T18 and T25 recombinant constructs. Following incubation on ice (30mins), cells were electroporated at 1600V. Electroporated cells were recovered in SOC at 37˚C for 1hr. Transformants were plated on antibiotic dual-selective (Ampicillin and Kanamycin) LB media.

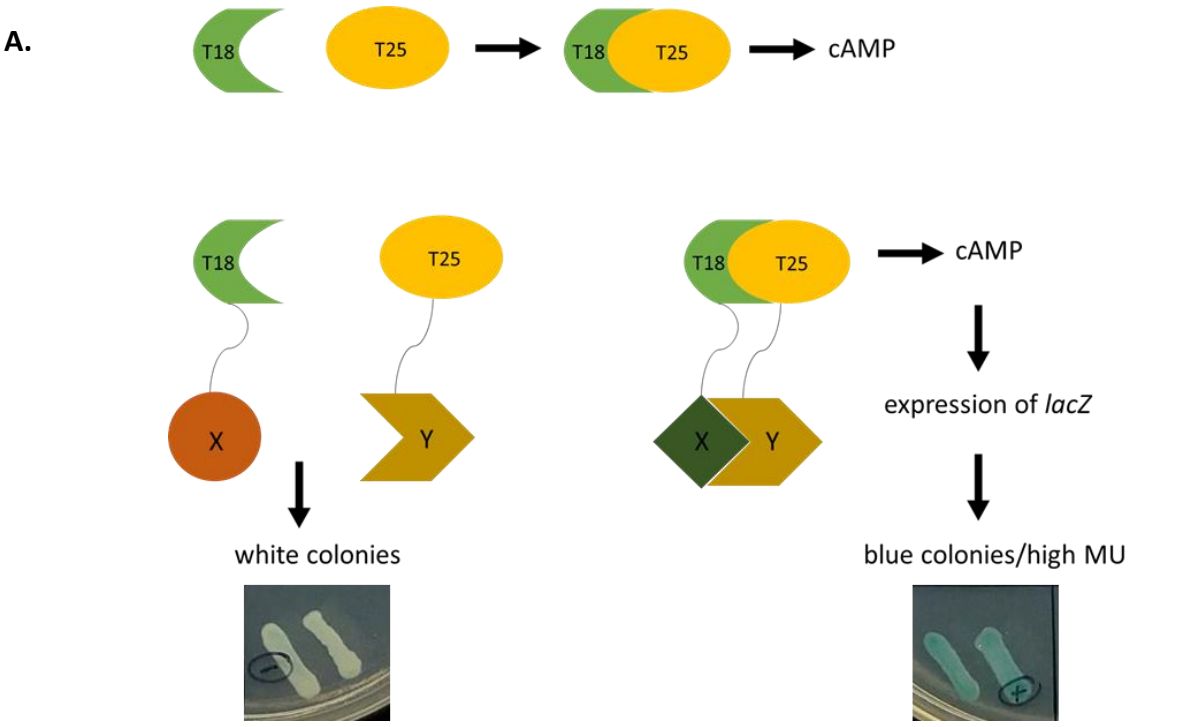
BACTH Protein Interaction Assays

Blue/White Screening

Strains were constructed and protein interactions tested using Bacterial Adenylate Cyclase Two-Hybrid System Kit (Euromoedex) (Table 5, Figure 5). Genes of interest (Table 3) were cloned into the BACTH plasmids (Table 2) and transformed into *E. coli* DHM1 strain (Table 1). Two individual colonies for each interaction were patched on antibiotic-selective, blue/white screening plates (Ampicillin 100µg/mL, Kanamycin 50µg/mL, 40µg/mL X-gal, 0.5mM IPTG) with appropriate empty vector controls and zip positive controls. Plates were incubated at 30˚C over night.

β-Galactosidase Assays

Liquid cultures of DHM1 transformants patched on blue/white screening plates were incubated over night at 30˚C with shaking then diluted to an OD_{600} of 0.1 in LB with appropriate antibiotics. After incubation with aeration at 30˚C for 2hrs, the OD_{600} was measured. At mid-late log growth (0.4 – 0.9) samples were used for β-galactosidase assay [2]. Aliquots of 200µL subculture and 300µL Z-Buffer were made in triplicate. To lyse the cells, 50µl chloroform and 25µl 0.1% SDS were added to aliquots with incubation at 30˚C for 5mins. Reactions were started with addition of ONPG (20mg/mL) and start times recorded. Reactions were stopped with



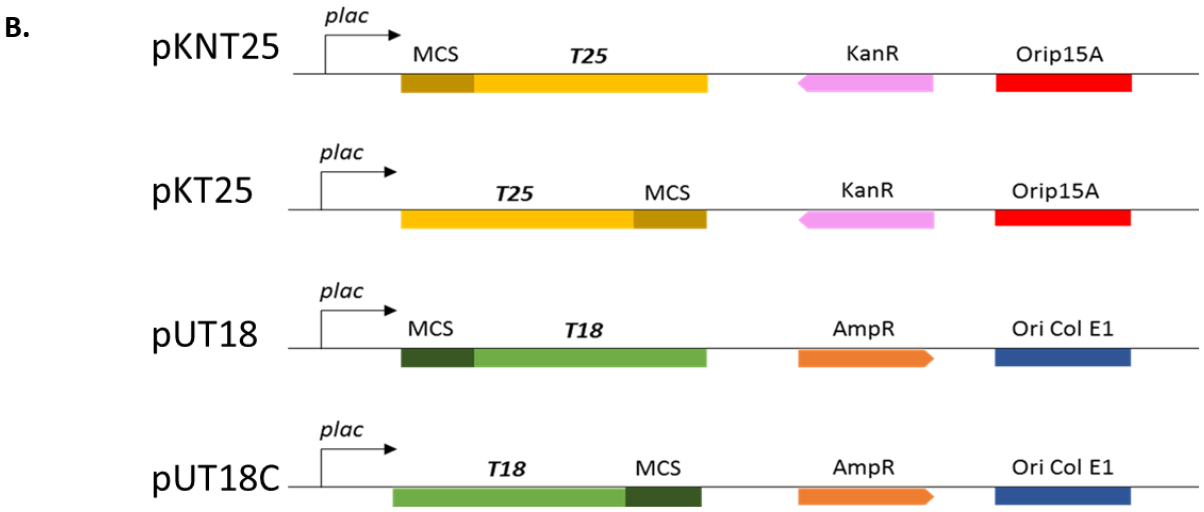


Figure 5 Schematic of the bacterial adenylate cyclase two-hybrid (BACTH) system blue/white screening assays (A) and gene organization of vectors (B).

1M sodium carbonate after development of yellow coloration. The OD_{420} was measured and Miller Units were calculated. Assay was completed in triplicate with three colonies. The three biological replicates were plotted in box & whisker plots.

Construction of *parPc* Expression Strain

The 3' end of *parP* (Table 3) was cloned into pJN105 expression vector (Table 2). The C-terminal domain of ParP was PCR amplified (Table 2) and digested with EcoRI (5') and SacI (3') restriction enzymes. Vector was harvested from liquid culture, digested, and separated via gel electrophoresis. DNA was cut from the gel and recovered with a gel extraction kit. Vector and insert were ligated (1:7) and incubated at 16˚C over night. Ligations were transformed with *E.coli* DH5α (Table 1) competent cells as described above. Cultures were then plated on antibiotic-selective LB media. Recombinant plasmids were confirmed by gel electrophoresis and all genes of interest were sequenced to ensure no mutations were introduced. Recombinant plasmids were then transformed into PAO1Δ*cheW* strain (Table 1) by electroporation and transformants selected on LB Gentamycin (50µg/mL) plates.

Swimming Assays

P. aeruginosa strains (Table 1) were streaked on LB antibiotic-selective media and incubated over night at 37˚C. Isolated colonies were stab-inoculated into swimming media (1% tryptone, 0.5% NaCl, 0.3% agar, 50µg/mL Gentamicin) and incubated over night at 37˚C for 18hrs. Swimming zone diameters were measured and recorded (n=8). Media supplementation with arabinose (0%, 0.01%, 0.05%, 0.1%.) was used to analyze swimming with protein induction.

Results

DipA interacts with full length ParP

Previous research showed that the phosphodiesterase DipA of the biofilm dispersal system and the partitioning protein ParP of the flagellar chemosensory system directly interact [2]. This study used a different bacterial two-hybrid assay system (BACTH) compared to the previous results, and it was therefore necessary to confirm this interaction. The BACTH system used in this study is dependent on the reconstitution of two halves (T18 and T25) of the CyaA adenylate cyclase from *Bordetella pertussis*. Genes of interest are cloned into separate plasmids encoding either the T18 or T25 fragment of CyaA, and when expressed, proteins are fused to a non-functional half of the adenylate cyclase catalytic domain (Figure 5). If proteins of interest interact, the complementary CyaA halves are reconstituted, cyclic AMP is produced, and cAMP-CAP complex binds the *lac* promoter in *E. coli* to upregulate expression of β-galactosidase. High β-galactosidase activity is correlated with blue colonies (cleavage of X-gal substrate) and protein interaction. White colonies lack β-galactosidase activity and are negative for protein interaction. The direct interaction between DipA and ParP was confirmed via blue/white screening (Figure 6B) and quantitative β-galactosidase assay (Figure 6C). The T18-DipA/ParP-T25 configuration showed an interaction on both the blue/white patch plate and β-galactosidase assay. While the DipA-T18/ParP-T25 configuration showed interaction on the blue/white patch plate, it did not have a significant number of Miller Units in the β-galactosidase assay (Figure 6B&C).

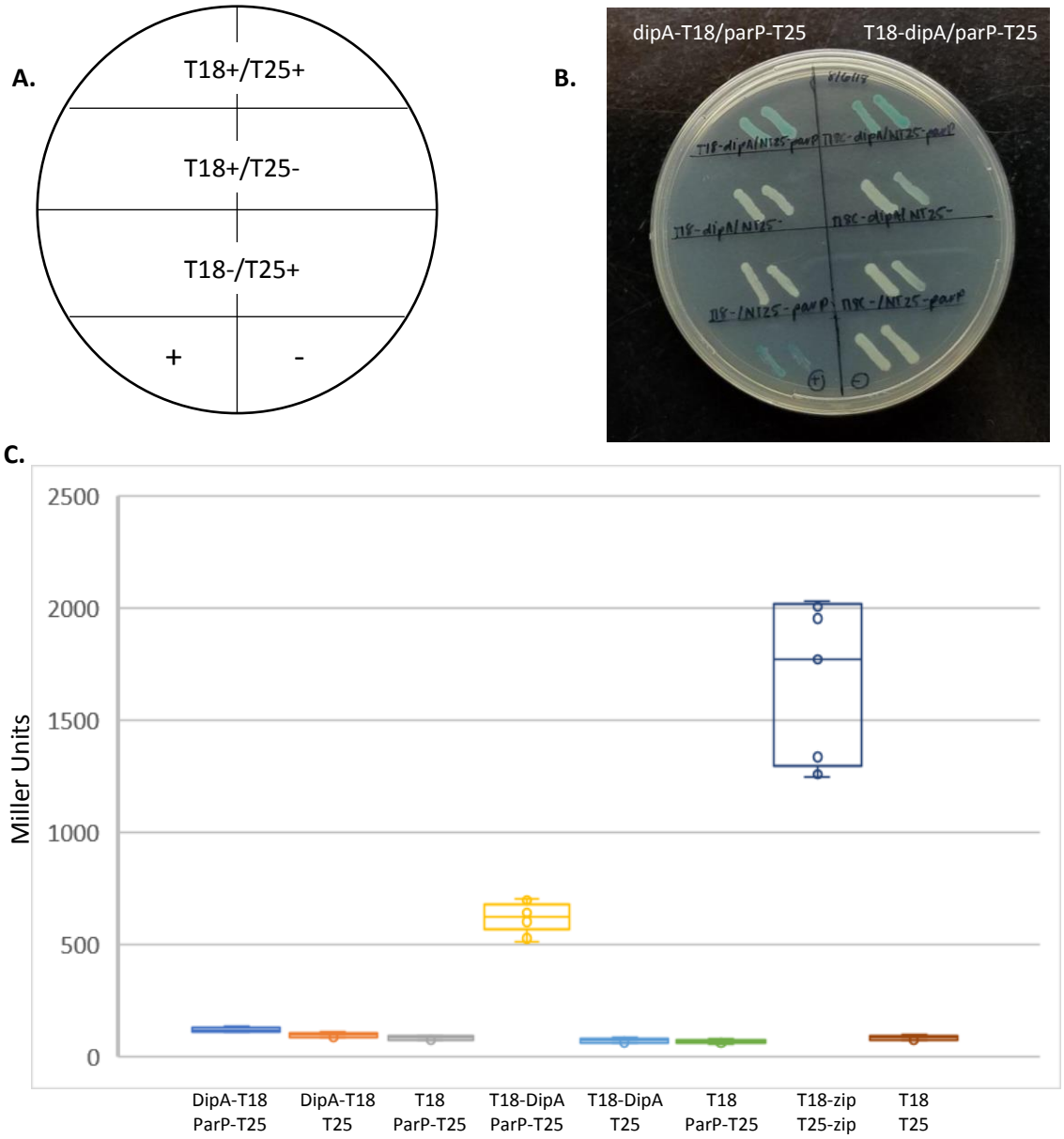


Figure 6 DipA interacts with ParP in the BACTH System. **A)** Schematic of blue/white screening plate layout. Interaction strains are at the top, below are appropriate empty vector controls, and at bottom are positive and negative controls. (+) indicates presence of protein and (-) indicates empty vector. **B)** Blue/white screening in *E. coli* DHM1 showed DipA interaction with ParP (blue) and no interaction in empty vector controls (white). **C)** β-galactosidase assay confirming DipA/ParP protein interaction. T18-DipA/ParP-T25 configuration had significant (>500 MU) activity indicating a positive interaction. The horizontal line within the box & whisker plots indicates the median of data set (n=3 assayed in triplicate). Whiskers of the box indicate variability of upper and lower quartiles.

DipA interacts with the C-terminal AIF domain of ParP

After confirming interaction between DipA and ParP within the BACTH system, the question of how DipA interacts with ParP was addressed using truncated ParP constructs. ParP has a C-terminal AIF domain ($ParP_{AIF}$) that is homologous to the C-termini of both CheA and CheW. Because the AIF domains are occupied in the core chemotactic unit, it was hypothesized that DipA would interact with ParP's free-hanging N-terminus (which interacts with sister partitioning protein ParC) [8]. The AIF domain and N-terminal truncations were expressed separately and tested for interaction with DipA. Surprisingly, there was no evidence of ParP's N-terminus interacting with DipA in any of the four BACTH configurations tested (Figure 7B). This was confirmed by β-galactosidase assay data (7C). However, $ParP_{AIF}$ did interact with DipA in two of the BACTH configurations tested (in which $ParP_{AIF}$ was fused to the T18 fragment of CyaA at either the N-terminus or C-terminus) (Figure 8).

DipA interacts with histidine kinase CheA

Because DipA interacts with the AIF domain of ParP, we were prompted to ask if DipA interacts with other chemosensory system proteins harboring an AIF domain. Previous research in *P. aeruginosa* showed that CheA and DipA form a complex, but it was previously unknown if these proteins directly interacted [17]. Additionally, DipA phosphodiesterase activity and localization at the cell pole is dependent on CheA [17]. We therefore tested for the direct interaction of DipA with the histidine kinase CheA via the BACTH system. Our results show that DipA weakly interacts with full length CheA via blue/white screening and β-galactosidase assays (Figure 9B&C). The discrepancy between the T18-DipA/CheA-T25 colonies was investigated further: an additional 48 colonies were patched on blue/white screening plates yielding 30 blue (62.5%) and 18 white colonies (37.5%).

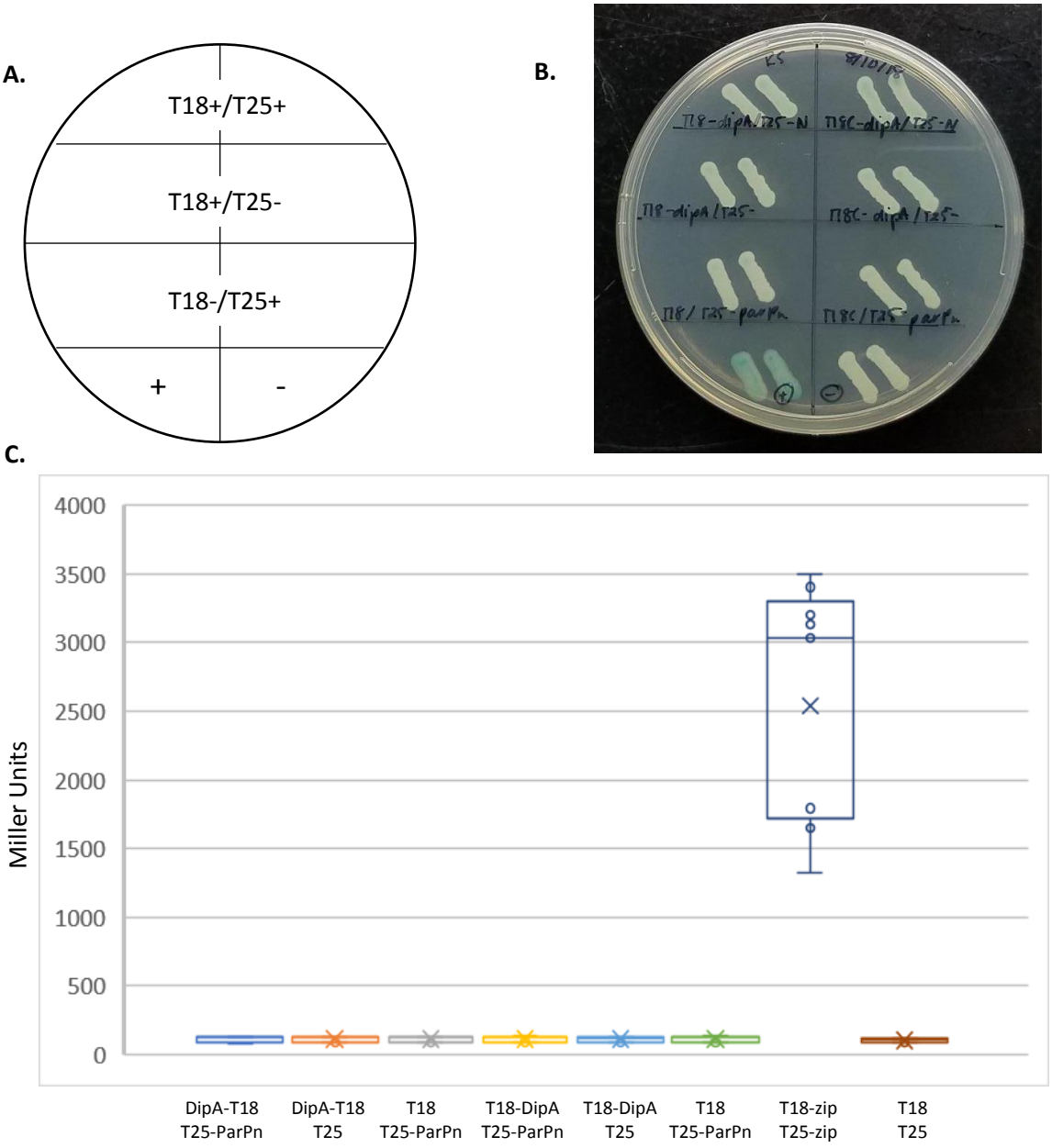


Figure 7 DipA does not interact with the N-terminus of ParP. **A)** Schematic of blue/white screening plate layout. **B)** Blue/white screening plate showing no interaction between DipA and N-terminus of ParP (white). **C)** β-galactosidase assay confirming no interaction between DipA and ParP N-terminal domain: both interaction strains had enzyme activity similar to that of the negative control.

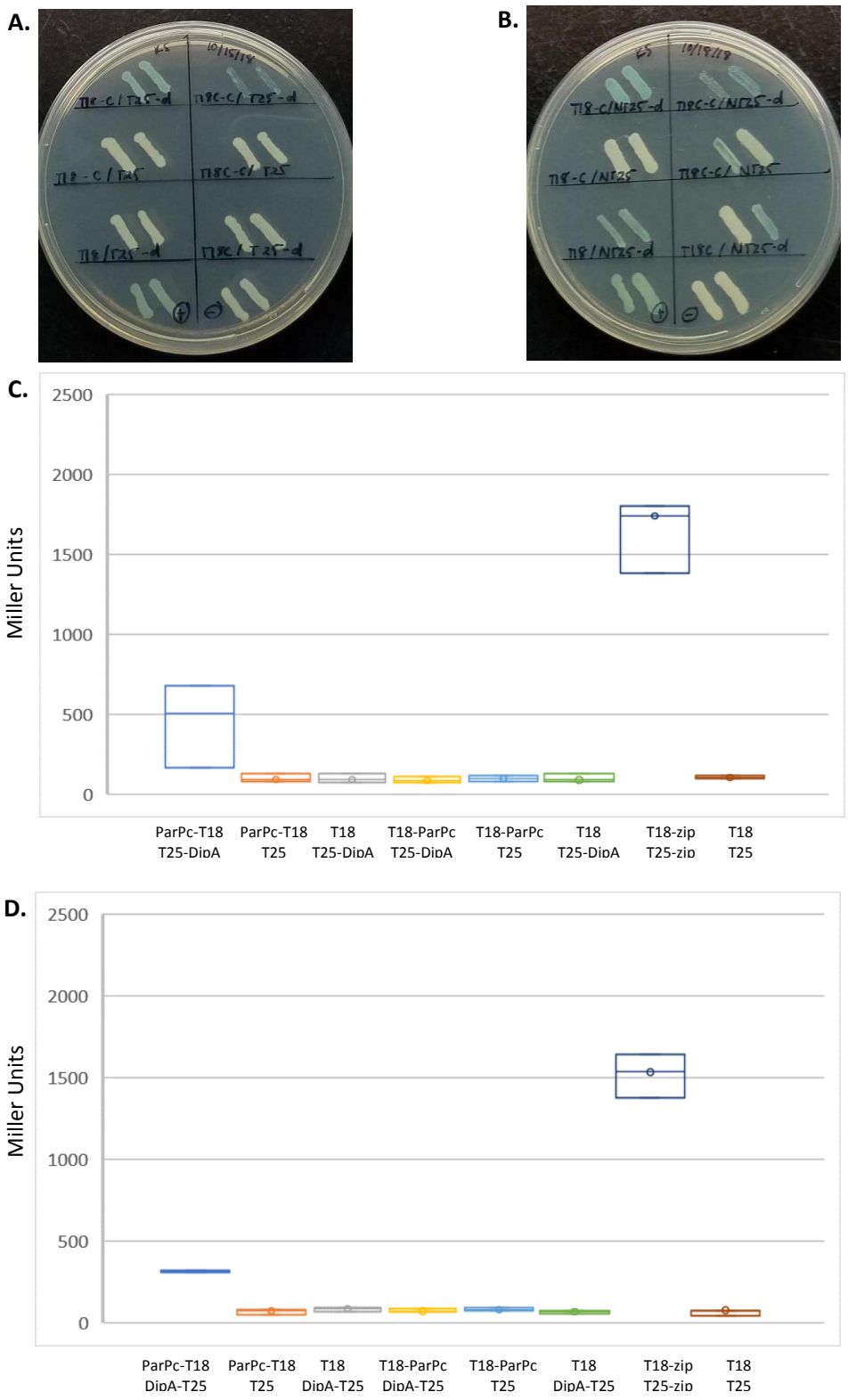


Figure 8 DipA interacts with the C-terminal AIF domain of ParP. **A)** Blue/white screening plate showing interaction of $ParP_{AIF}$-T18/T25-DipA configuration. **B)** Blue/white screening plate showing $ParP_{AIF}$-T18/DipA-T25 configuration interaction. **C)** β-galactosidase assay confirming $ParP_{AIF}$-T18/T25-DipA interaction (MU >500). **D)** β-galactosidase assay confirming $ParP_{AIF}$-T18/DipA-T25 interaction (MU >300).

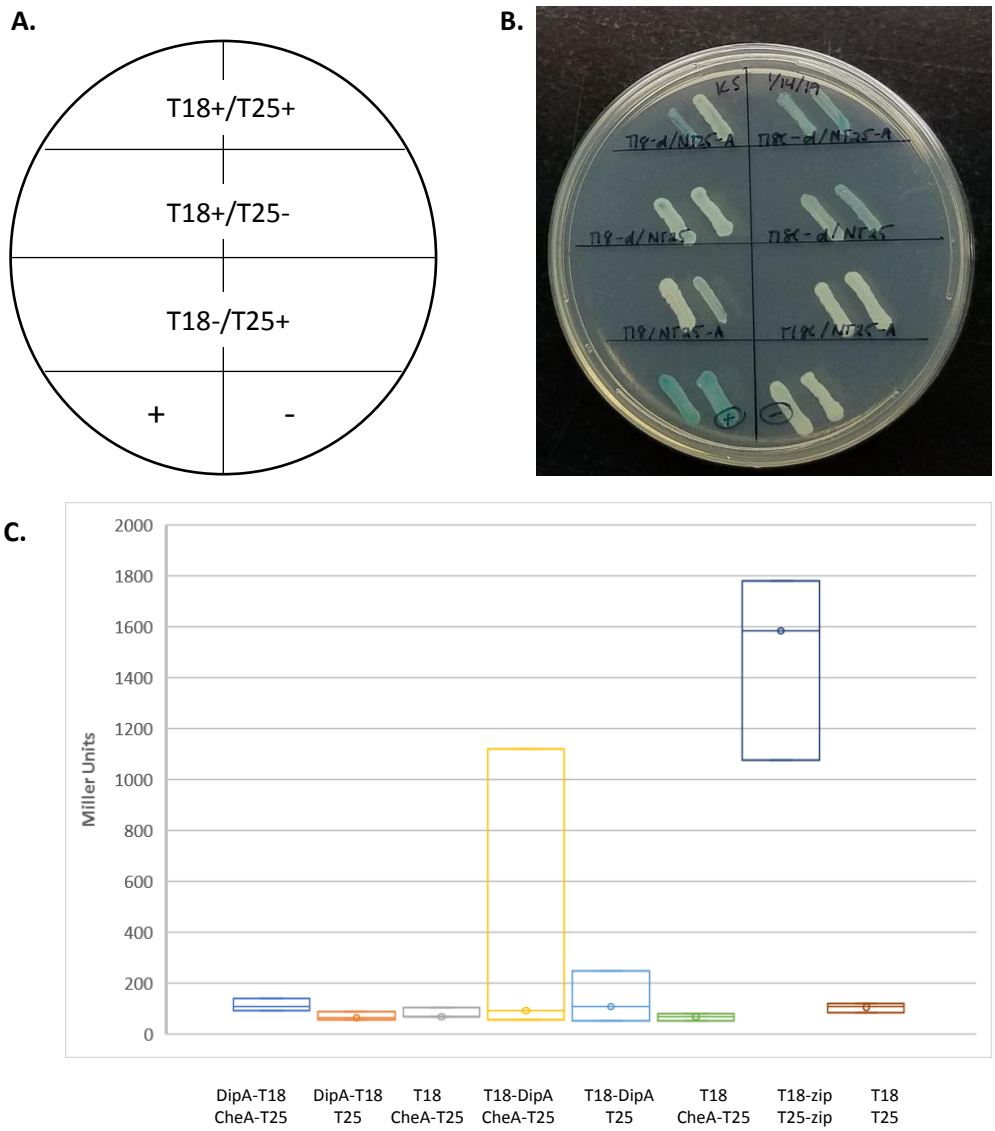


Figure 9 DipA interacts directly with CheA. **A)** Schematic of blue/white screening plate layout. **B)** Blue/white screening plate showing T18-DipA/CheA-T25 configuration interaction. **C)** β-galactosidase assay confirming interaction of T18-DipA/CheA-T25 (MU>1000).

The C-terminal AIF domain of ParP does not restore swimming motility in a Δ*cheW*

Within the chemotaxis gene cluster, *cheW* is directly downstream of *parP* and predicted to be co-transcribed [9]. $ParP_{AIF}$ is homologous to CheW and it was hypothesized that these AIF domains may be functionally redundant. Previous research showed that Δ*parP* had a 60% decrease in swimming motility while Δ*cheW* is null for swimming. Additionally, CheW complementation partially restores swimming motility in a Δ*parP* mutant (~67%). Furthermore, double complementation with both CheW and ParP resulted in a greater restoration of swimming motility (~89%) than either single complementation. However, full length ParP did not complement a Δ*cheW* mutant. We tested if the N-terminal extension of ParP prevented it from restoring swimming motility in the Δ*cheW* strain. Expression of $ParP_{AIF}$ from an arabinose inducible promoter failed to restore swimming motility in a Δ*cheW* mutant strain (Figure 10).

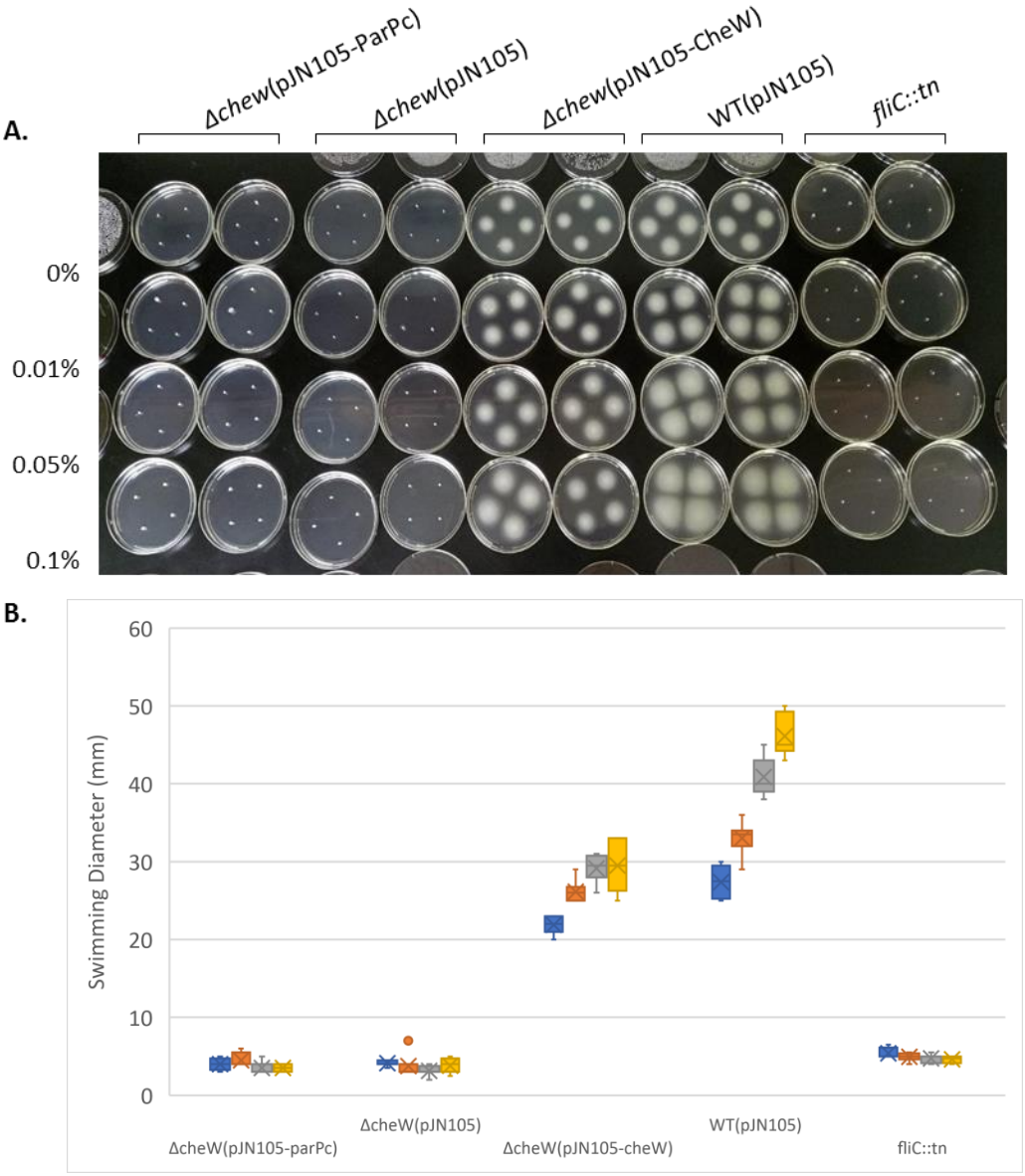


Figure 10 C-terminal AIF domain of ParP is not functionally equivalent to CheW. **A)** Arabinose induction series swimming assay plates. **B)** Quantification of average swimming diameter on arabinose induction swimming assay plates (n=8). Arabinose concentrations tested were 0% (blue), 0.01% (orange), 0.05% (grey), 0.1% (yellow).

Discussion

The localization of chemotaxis arrays near bacterial flagella is important for rapid signal transduction. Chemotaxis arrays within *V. cholerae* are localized to cell poles via partitioning proteins ParP and ParC. The tripartite interaction between ParC-ParP-CheA sequesters the chemotaxis histidine kinase CheA at the cell pole and prevents diffusion of chemotaxis clusters [6]. The C-termini of both ParP and CheA, along with adaptor protein CheW, are homologous array-integration-and-formation (AIF) domains [2]. This domain homology prompted investigation into whether these AIF domains are functionally redundant within the *P. aeruginosa* chemotaxis system.

Previous research showed that CheW can partially complement swimming motility within a Δ*parP* mutant but that overexpression of full length ParP did not restore swimming motility in a Δ*cheW* mutant, which is null for swimming in a soft-agar assay [2]. It was hypothesized that this failure to complement was because of ParP's N-terminal extension, and that the C-terminal AIF domain of ParP might restore swimming motility in Δ*cheW*. This research showed that $ParP_{AIF}$ was unable restore swimming motility in a Δ*cheW* strain, irrespective of arabinose concentration (Figure 10). $ParP_{AIF}$ is expressed from the inducible araC promoter in pJN105. Importantly, we were unable to confirm protein expression ($ParP_{AIF}$) through western blots due to lack of antibodies, which limits our ability to determine whether $ParP_{AIF}$ is not functionally equivalent to CheW or just not expressed. Earlier studies also suggested that the expression and/or stability of ParP and CheW are dependent on each other. Not only is *cheW* directly downstream of *parP* within the chemotaxis gene cluster, but expression of both ParP and CheW off a plasmid rescued swimming motility in a Δ*parP* mutant to a greater degree than either single complementation [2]. While unlikely, it is possible that in our swimming assay neither the full length or truncated ParP

protein restored swimming motility in Δ*cheW* because the co-expression of both proteins is necessary to elicit wild-type motility levels.

Previous research in *P. aeruginosa* demonstrated a novel interaction between ParP and DipA, a phosphodiesterase of the biofilm dispersal chemosensory system [2]. We asked how ParP interacts with DipA: via its N-terminus or C-terminus. Unexpectedly, this study showed that DipA interacts with $ParP_{AIF}$ but not ParP's N-terminal domain (Figure 7 & 8). In *V. cholerae*, the $ParP_{AIF}$ domain interacts with the LID domain of CheA and the protein interaction tip of the MCPs [8]. Together this data indicates the importance of the ParP AIF domain in mediating protein interactions within the chemosensory complex.

The interaction between DipA and $ParP_{AIF}$ prompted further investigation into DipA's ability to interact with homologous AIF domains within the chemotaxis array. Of the proteins with AIF domains, we focused our attention on the histidine kinase CheA. It is known that DipA's phosphodiesterase activity is promoted by, and its polar localization is dependent on, CheA [17]. Additionally, it was shown that DipA and CheA form a complex through co-immunoprecipitation, but it was previously unknown if these proteins directly interact [17]. Kulaskera et. al predicted that complex formation between DipA and CheA requires an adaptor protein, as CheA is known to only interact with CheW-like and response regulator receiver domains [17]. We propose that ParP facilitates proximity of DipA and CheA and enables CheA and DipA to directly interact, albeit weakly (Figure 9). Our research suggests formation of a DipA-ParP-CheA complex (Figure 11).

Given that DipA interacts with $ParP_{AIF}$, it can be hypothesized that DipA interacts with CheA via its AIF domain. To confirm this, a C-terminal CheA truncated construct should be made and

tested for interaction with DipA via BACTH assays. Finally, DipA's ability to interact with CheW should be tested given its AIF domain homology.

Although it does not possess an AIF domain, DipA has a degenerate GGDEF domain which may be sensory [17]. DipA is activated by BdlA in response to NicD glutamate sensing and degrades intracellular c-di-GMP to promote biofilm dispersal [17]. Decreased c-di-GMP levels downregulate expression of virulence and biofilm genes while simultaneously upregulating motility genes [18]. It is unknown if DipA diffuses to the cell pole after activation by BdlA or if there are separate intracellular pools acting independently [17]. It is possible that ParP mediates an interaction between DipA's GGDEF domain and CheA's AIF domain as part of an intracellular sensory mechanism to promote biofilm dispersal in response to extracellular cues sensed by the chemotaxis MCPs. This hypothesis is supported by our BACTH data: interaction between DipA and ParP was stronger than that with CheA (Figure 6 & 9). Further experimentation is required to confirm this hypothesis.

Additionally, a recent study outlines a Touch-Seed-and-Go virulence model in which DipA contributes to heterogeneity of bacterial progeny during the attachment process [20]. DipA asymmetrically associates with the bacterial flagellum during cellular division, maintaining low-levels of c-di-GMP in motile offspring, allowing dissemination and possible infection of new sites [20]. Therefore, it is also possible that DipA is sequestered at the cell pole by ParP and CheA, establishing c-di-GMP heterogeneity in surface-attached bacteria. This suggests that DipA and ParP not only have roles in biofilm dispersal but also initial surface adhesion for biofilm-fated cells.

This research on the relationship between swimming motility and biofilm dispersal may ultimately elucidate novel targets for safe biofilm dispersal in chronic infections of

immunocompromised individuals. *P. aeruginosa* is an opportunistic pathogen of patients with cystic fibrosis (CF), a human genetic disorder in which a chloride ion membrane channel mutation results in production of abnormally thick, sticky mucus. Although it colonizes CF patients at a young age it can become virulent in adulthood, contributing to the morbidity and mortality of the disease. *P. aeruginosa* forms biofilms within the lungs of CF patients, which are difficult to treat due to increased antibiotic resistance and heterogenous organization [1]. Sectional transcriptomic analysis showed that bacteria at the biofilm surface have upregulation of biofilm matrix factors and antimicrobial resistance genes, while centrally located bacteria have upregulated flagellar synthesis and ParP expression resulting in a "central hollowing phenotype" upon dispersal [1]. Therefore, although biofilm breakdown is an obvious treatment target, it may release pathogenic bacteria throughout the body and have dangerous health consequences for immunocompromised CF patients. Understanding the molecular mechanisms by which *P. aeruginosa* regulate biofilm dispersal will likely engender more effective clinical treatments for CF.

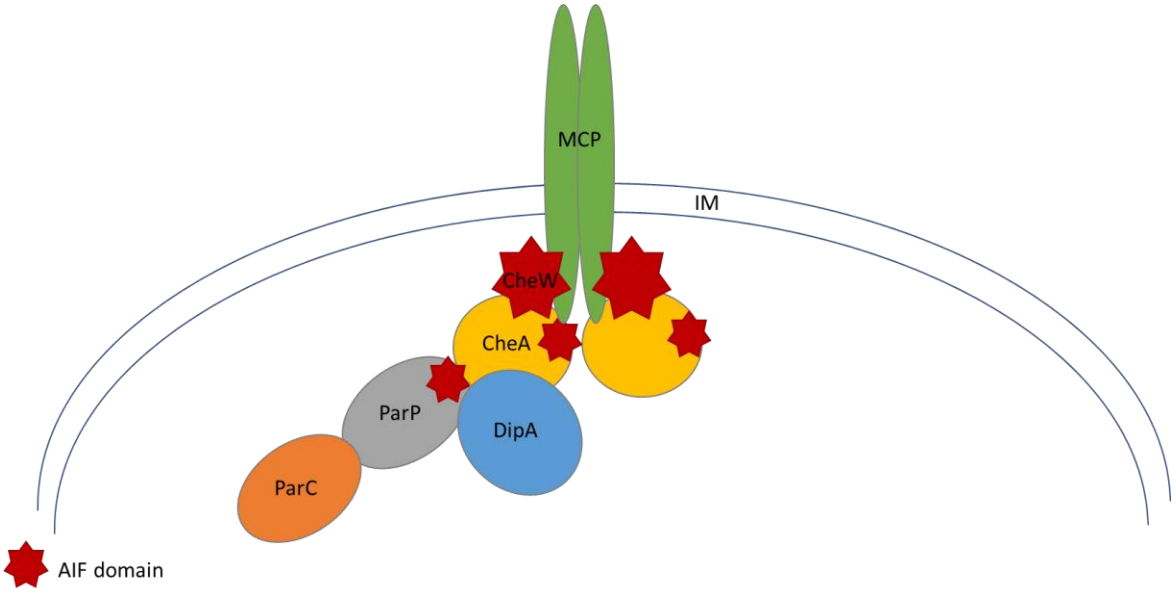


Figure 10 Updated model of polar chemotaxis array clusters. Our data suggest a ParP-DipA-CheA tripartite complex.

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