

ORGANIZATION OF PROTEINS FROM MULTIPLE CHEMOTAXIS SYSTEMS IN
RHIZOBIUM LEGUMINOSARUM AND IMPACT ON SHORT-TERM ROOT
COLONIZATION

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ABSTRACT

Bacteria must sense and adapt to a variety of environmental signals in order to survive. Motile bacteria utilize chemotaxis to orient their movement within a chemical gradient. Chemotaxis receptors (chemoreceptors) located in the membrane detect environmental signals which are then propagated through a signal transduction cascade to alter the motility pattern. In the model organism *Escherichia coli*, chemoreceptors sense changes in concentration gradients and relay these changes to chemotaxis cytoplasmic signaling proteins, including the chemotaxis histidine kinase CheA. In *E.coli*, five chemoreceptors signal to a single set of chemotaxis signaling proteins. Chemoreceptors assemble with CheA and other chemotaxis proteins into large patches called receptor arrays at the cell poles. This architecture is important for signal amplification and propagation; therefore, it is universally conserved across many bacterial species. In contrast to *E. coli*, *Rhizobium leguminosarum* biovar *viciae*, a nitrogen-fixing soil bacterium, encodes two chemotaxis operons (*Che1* and *Che2*) and 27 chemoreceptors. Previous work shows that Che1 is the major controller of chemotaxis while Che2 plays a minor role under laboratory conditions. However, it is not known how paralogs of chemotaxis proteins from both operons are organized to integrate signals into motility output. In this study, fluorescent microscopy to determine subcellular localization and protein-protein interactions were assessed between the histidine kinases from each operon (CheA1 and CheA2) as well as four chemoreceptors (HemAT, Mcp2A, McpC, McpD). We show that chemoreceptors segregate into distinct clusters based on length class, as expected. In addition, CheA1 could interact with both receptor clusters while CheA2 could only interact with one cluster. We also provide evidence for potential competition between CheA1 and CheA2 for integration into the arrays. Additionally, a preliminary study explores the role of different chemoreceptors in plant root colonization and suggests differential contributions of chemoreceptors to the competitive colonization of distinct rhizospheres.

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CHAPTER I. INTRODUCTION

Bacteria must be able to sense cues around them to navigate heterogeneous environments with a variety of chemical effectors. The movement of bacteria through chemical gradients either toward chemical attractants or away from chemical repellants is referred to as chemotaxis. Taxis in a chemical gradient is one of the most common motile bacterial responses; however, many bacteria show various forms of taxis such as aerotaxis (taxis specifically in oxygen gradients), phototaxis (taxis in light gradients), and energy taxis (taxis based on the indirect affect the cue has on metabolism) (Alexandre 2010; Wadhams and Armitage 2004). Motile bacteria such as *Escherichia coli* in the absence of a gradient will move in a series of random “runs” of continuous swimming and frequent “tumbles” that use Brownian motion to reorient swimming in a new direction (Porter, S.L., Wadhams, G.H., and Armitage, J.P. 2011). In the presence of an attractant or repellant, motile bacteria will increase the duration of runs and frequency of tumbles in a “random biased walk” toward a beneficial environment and away from potentially hazardous conditions (Figure 1). In order to sense changes in these chemical effectors, bacteria rely on chemoreceptors and a downstream signaling cascade (Wadhams and Armitage 2004). The simplest of these systems is found in *E. coli* which uses a signal transduction system consisting of five types of chemoreceptors and six chemotaxis proteins encoded within a single chemotaxis operon (Falke, J.J. and Hazelbauer, G.L. 2001). Typically, soil and aquatic bacteria possess multiple chemotaxis operons and a large quantity of chemoreceptors, with *Magnetospirillum magneticum* holding the record with 64 chemoreceptors (Lacal, J. et al 2010; Ashby, M.K. 2004; Porter, S.L., Wadhams, G.H., and Armitage, J.P. 2011). While much is known about chemotaxis signaling in *E. coli*, there is much to learn regarding chemotactic signaling in bacteria with more complex signaling systems.

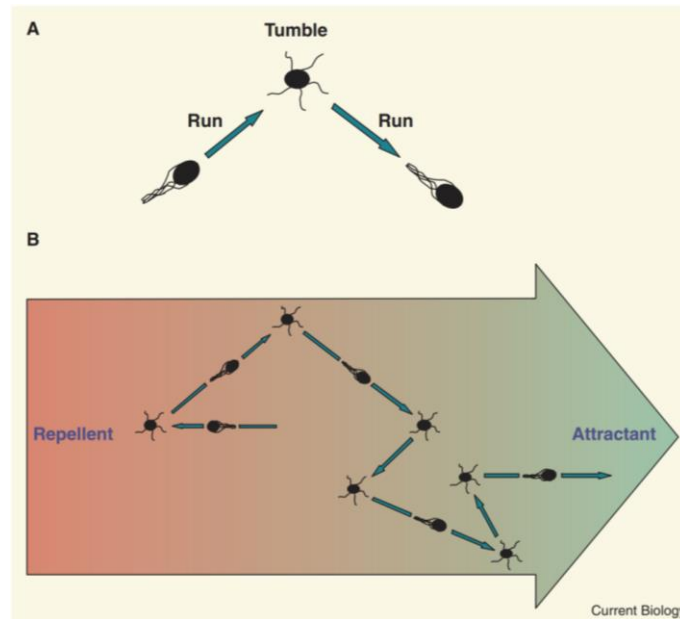


Figure 1: Differential movement in the presence or absence of a chemical gradient. A) Motile cell movement in the absence of a gradient. B) Random biased walk of motile bacteria toward an attractant and away from a repellent in a chemical gradient (Webre, D.J., Wolanin, P.M., and Stock, J.B. 2003).

Chemotaxis in *E. coli*

In order to sense chemical effectors in the environment surrounding the cell, motile bacteria rely on information received through chemoreceptors primarily localized to the cell pole. These receptors sense both attractants and repellants and pass the signal through a signaling cascade to the flagellar motor proteins. In the *E. coli* model system, binding of an attractant to a chemoreceptor does not alter the cell swimming bias. However, if an attractant binds to a chemoreceptor, it triggers a piston-like conformational shift in the transmembrane region of the receptor (Figure 2) (Hazelbauer, G.L., Falke, J.J., and Parkinson, J.S. 2007; Khursigara, C.M. et al 2008). This conformational change results in autophosphorylation from ATP of the histidine kinase CheA that is bound to the cytoplasmic portion of the chemoreceptor with the help of scaffolding protein CheW (Sankhe, G.D., Dixit, N.M., and Saini, D.K. 2018; Porter, S.L., Wadhams, G.H., and Armitage, J.P.). CheA is a dimeric protein made up of five structural domains (P1-P5). P1 is a histidine phosphotransfer domain which is phosphorylated by the P4 kinase domain. P2 binds the response regulator CheY and the methylesterase CheB. P3 controls dimerization and P5 is a CheW-like domain that binds both CheW and the cytoplasmic tips of chemoreceptors (Li, X. et al 2013). The phosphoryl group on CheA is transferred to CheY, a diffuse response regulator protein. CheY-P has reduced affinity for CheA and increased affinity to the flagellar motor (Bren, A. and Eisenbach, M. 2000). Binding of CheY-P to the flagellar motor switch protein FliM results in a switch in the direction of flagellar rotation, and thus a “tumble”. (Welch, M., Oosawa, K., Aizawa, S.-I., and Eisenbach, M. 1993). The probability of a shift in flagellar rotation increases as more CheY-P bind to FliM subunits (Porter, S.L., Wadhams, G.H., and Armitage, J.P. 2011). CheY-P has a short half-life of approximately 20 seconds (Hess, J.F., Oosawa, K., Kaplan, N., and Simon, M.I. 1988), allowing for rapid behavioral responses to stimuli. CheY-P is dephosphorylated by phosphatase CheZ which

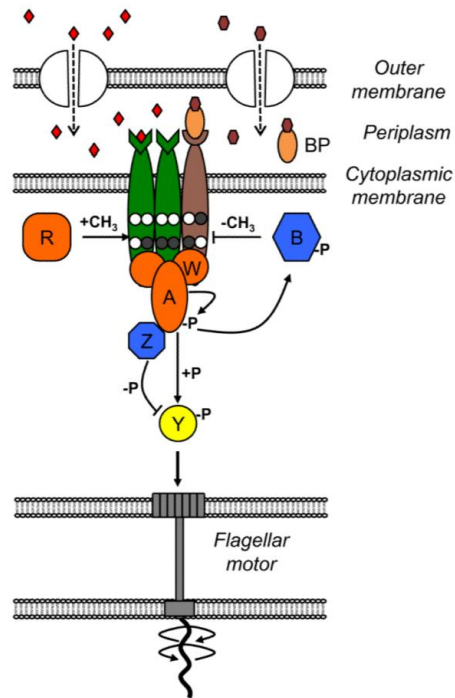


Figure 2: Chemotaxis signaling cascade in *E. coli* (Sourjik, V. and Wingreen, N.S. 2012).

Chemoreceptors on the cytoplasmic membrane sense ligands from the environment. If a repellent is sensed, the signal is passed to CheA which becomes autophosphorylated. CheA is held to the chemoreceptors by the scaffolding protein CheW. That phosphoryl group is then passed to CheY which interacts with the flagellar motor. The phosphoryl group can also be passed to CheB which demethylates the chemoreceptors. CheR methylates the chemoreceptors. CheZ resets the system by dephosphorylating CheY.

enhances both speed and coordination of motor responses (Blat, Y. and Eisenbach, M. 1996). The sensitivity of chemoreceptors to chemical effectors can be modulated by addition and removal of methyl groups to conserved glutamate residues in the cytoplasmic region by CheR (methyltransferase) (Djordjevic, S. and Stock, A.M. 1998) and CheB (methylesterase) (Djordjevic, S. et al 1998). The combined activities of these two proteins adjusts movement bias to pre-stimulus levels and provides the cell a form of short-term memory, comparing current signals to those just previous.

Many bacteria diverge from the *E. coli* model in their basic chemotaxis proteins. While chemoreceptors, CheA, CheW, and CheY appear to be universal (Szurmant, H. and Ordal, G.W. 2004; Wuichet, K., Alexander, R.P., and Zhulin, I.B. 2007), many chemotactic archaea and bacteria have additional or modified proteins to assist the system in adaptation, signal transduction, and signal removal. Some archaea and gram-positive bacteria contain a CheD glutamine deamidase to mature chemoreceptors before they can be modified (Kristich, C.J. and Ordal, G.W. 2002). CheR and CheB proteins are almost universal across chemotactic bacteria and archaea but are noticeably absent in *H. pylori* (Szurmant, H. and Ordal, G.W. 2004; Terry, K., Go, A.C., and Ottemann, K.M. 2006). Many bacteria other than *E. coli* contain CheV, a CheW-REC fusion protein that is used for coupling at the baseplate and may play a role in adaptation as well (Fredrick, K.L. and Helmann, J.D. 1994; Alexander, R.P., Lowenthal, A.C., Harshey, R.M., and Ottemann, K.M. 2010). CheZ phosphatase is found in α , β/γ , δ , and ϵ - proteobacteria (Blat, Y. and Eisenbach, M. 1996; Wuichet, K., Alexander, R.P., and Zhulin, I.B. 2007). Bacteria lacking CheZ tend to have CheC, FliY, or the CheC homolog CheX as their phosphatase (Bischoff, D.S. and Ordal, G.W. 1992; Saulmon, M.M., Karatan, E., and Ordal, G.W. 2004; Silversmith, R.E. 2010). Only α -proteobacteria contain additional CheY proteins that act as phosphate sinks, notated CheY* (Scharf, B. and Schmitt, R. 2002). Thus, many diverging systems exist in addition to the base *E. coli* chemotaxis system.

Structure of bacterial chemoreceptors

Bacterial chemoreceptors, also referred to as methyl-accepting chemotaxis proteins (MCPs), are functional in a helical homodimer. Each monomer in the dimer typically contains an N-terminal ligand binding region (LBR) exposed to the periplasm and flanked by transmembrane domains as well as a C-terminal cytoplasmic signaling region (Figure 3). The LBR varies highly in sequence, indicating the diversity of compounds that are bound by MCPs (Alexander, R.P. and Zhulin, I.B. 2007). Currently, a majority of LBRs are unannotated in the SMART database (Lacal, J. et al 2010); therefore, the compound that binds any given LBR cannot be predicted from the sequence alone (Yang, W. and Briegel, A. 2019). Flanking the LBR in a typical MCP are transmembrane regions that anchor the receptor to the membrane. The HAMP domain is located between the transmembrane and signaling regions and serves to convert the signal received by the chemoreceptor (Salah Ud-Din, A.I.M. and Roujeinikova, A. 2017). The HAMP domain forms a stable four-helical parallel coiled-coil conformation in the presence of an attractant which results in loose packing of the downstream methylation domain and a kinase off state; the inverse is true in the presence of a repellent (Khursigara, C.M. et al 2008). The cytoplasmic signaling region forms a four-helix-bundle and is highly conserved among chemoreceptors as it functions to interact with the downstream components of the chemotaxis signaling cascade (Kim, K.K. et al 1999). The signaling region consists of a methylation domain, flexible bundle domain, and the signaling subdomain. The methylation domain is the location for CheB and CheR binding (Djordjevic, S. et al 1998; Levin, M.D., Shimizu, T.S., and Bray, D. 2002). The flexible bundle domain primarily serves to connect the methylation domain and signaling subdomain. The only conserved portion of this region is a glycine hinge that plays a role in the formation of trimers of dimers within signaling complexes (Parkinson, J.S., Hazelbauer, G.L., and Falke, J.J. 2015). The signaling subdomain is the location of CheW and

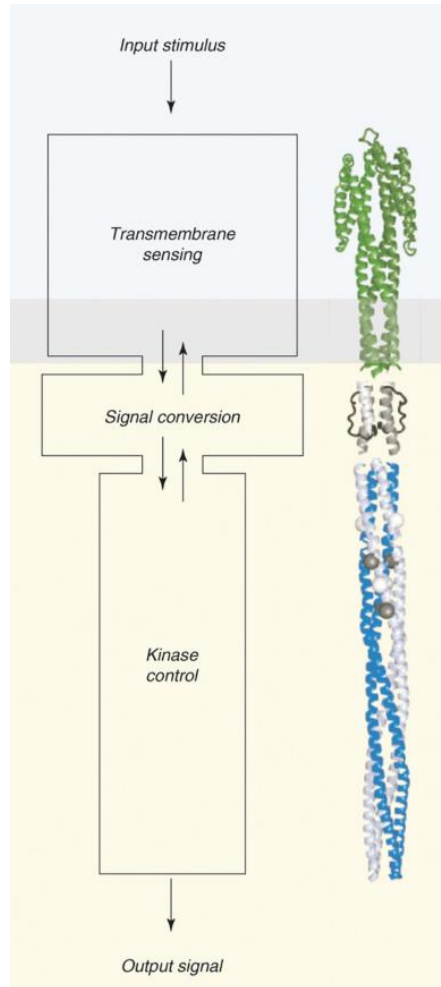


Figure 3: Ribbon diagram and schematic of a chemoreceptor dimer from *E. coli* (Hazelbauer, G.L., Falke, J.J., and Parkinson, J.S. 2007). The transmembrane sensing domain structure is variable as it depends on the ligand sensed. The remainder of the chemoreceptor makes up the conserved signaling domain. The signal conversion domain connects the sensing domain to the kinase control signaling domain. The cytoplasmic tip interacts with CheA and CheW.

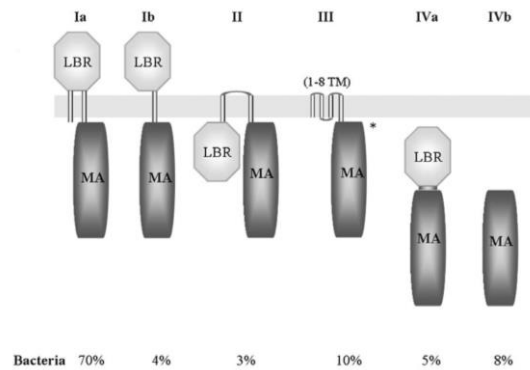


Figure 4: Classification of MCPs based on ligand binding region topology. Six categories exist to categorize the chemoreceptor based on the presence and placement of the ligand binding domain. Most chemoreceptors found in bacteria are class Ia. MA: methyl-accepting domain, LBR: ligand binding region (Lacal, J. et al 2010).

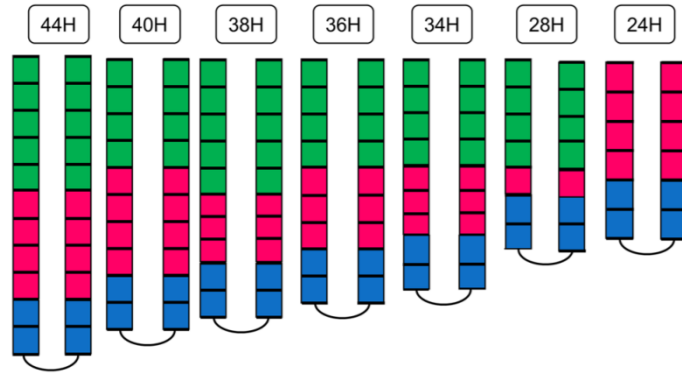


Figure 5: Classification of chemoreceptors based on signaling domain heptad class. Each square represents two heptads. Green indicates the methylation domain, pink indicates the flexible domain, and blue represents the signaling domain (Salah Ud-Din, A.I.M. and Roujeinikova, A. 2017).

CheA binding to the chemoreceptor and therefore is highly conserved (Li, X. et al 2013). MCPs can be classified into six different classes based on topology of their ligand binding domain, with Class I as the most abundant (Figure 4). Further classification of chemoreceptors is based on the signaling region sequence conservation and number of heptads (H) defined as symmetric seven amino acid-long insertions and deletions corresponding to two α -helical turns (Figure 5).

Organization of *E. coli* chemoreceptors

E. coli contains five chemoreceptors: Tsr (serine), Tar (maltose and aspartate), Tap (dipeptides and pyrimidines), Trg (galactose and ribose), and an MCP-like receptor Aer for redox sensing (Gosink, K.K., del Carmen Buron-Barral, M., and Parkinson, J.S. 2006, Lacal, J. et al 2010) (Figure 6). These five receptors condense into trimers of receptor dimers connected by CheA and CheW molecules in large patches largely localized to the cell pole (Draper, W. and Liphardt, J. 2017). Those patches not at the cell pole localize stably next to future division sites (Sourjik, V. and Armitage, J.P. 2010). The interactions between receptors in this ordered structure allows for signal amplification up to 36-fold (Sourjik, V. and Berg, H.C. 2002). Cryo-EM of *E. coli* shows that receptors form a hexagonal lattice with a regular 13.2 nm spacing (Li, X. et al 2013), each hexagon a trimer of receptor dimers (Briegel, A. et al. 2012), forming large patches called receptor arrays (Figure 7). The arrays are stabilized by interactions between CheW and the CheA P5 domain (Abedrabbo, S. et al 2017, Li, M. and Hazelbauer, G.L. 2014). The cytoplasmic tips of the MCPs interact with the P3 domain of CheA (Alvarado, A., et al 2017). In the absence of CheA and CheW, fluorescence microscopy has revealed that localization of *E. coli* chemoreceptors is diffuse or in multiple small clusters at the pole (Studdert, C.A. and Parkinson, J.S. 2004). This suggests that CheA and CheW are not only required for cluster formation but function to stabilize compact clusters. Organization in this way helps to amplify chemical signals (Duke, T.A.J. and Bray, D. 1999), broadening dynamic range (Sourjik, V. and Berg, H.C. 2002),

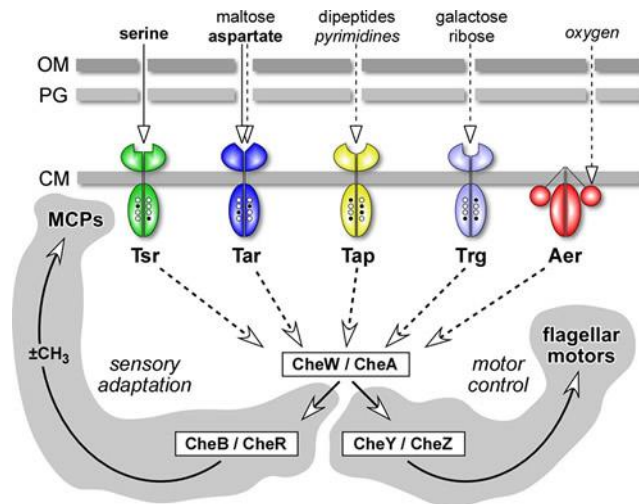


Figure 6: Chemoreceptors and downstream signaling components in *E. coli* (Parkinson lab website). *E. coli* contains five chemoreceptors, each sensing a subset of ligands. All five chemoreceptors signal to CheA and CheW which results in motor control and sensory adaptation.

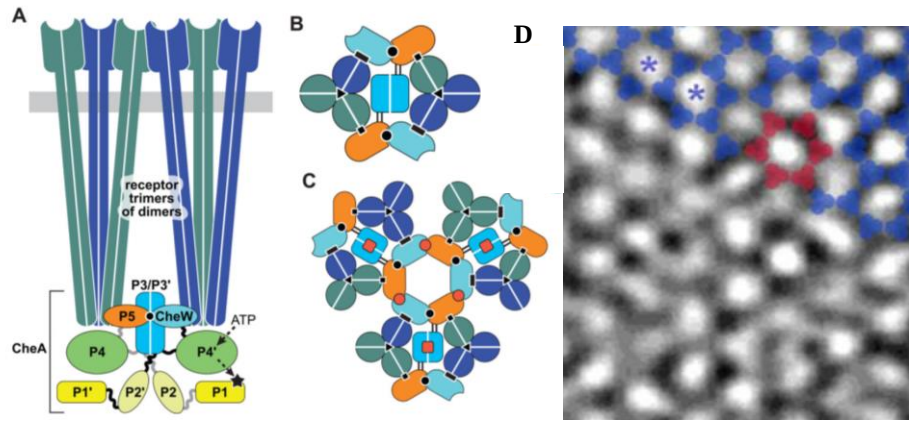


Figure 7: Spatial organization of chemoreceptors, CheA, and CheW in receptor lattice. A) Core complex of two receptor trimers of dimers, two CheW molecules, and a CheA dimer. B) Core complex viewed from the cytoplasmic membrane toward the cytoplasmic tips of receptors. C) Connections between core complexes in receptor array (Parkinson, J.S., Hazelbauer, G.L., and Falke, J.J. 2015). D) Electron tomography image of receptor clusters in *Vibrio cholerae*. Trimers of dimers fit onto TEM image (blue). Six trimers of dimers make up one hexagon (red). The asterisk indicates the consistent 12 nm spacing from the center of one hexagon to the center of the hexagon adjacent (Briegel, A. et al 2009).

increasing cooperativity between chemoreceptors (Li, G. and Weis, R.M. 2000), and timely adaptation (Endres, R.G. and Wingreen, N.S. 2006). Crosslinking experiments have shown that trimers of dimers can be made up of chemoreceptors of different types with their composition dependent of the relative abundance of the receptors (Studdert, C.A. and Parkinson, J.S. 2004). It has been previously shown that each chemoreceptor array visualized by cryo-EM consist of chemoreceptors belonging to only one major signaling class (34H, 36H, 38H, 40H, and 44H) (Briegel, A. et al 2009, Alexander, R.P. and Zhulin, I.B. 2007).

Chemotaxis in *Rhizobium leguminosarum* biovar *viciae*

Although all known chemotactic bacteria have similar chemotaxis proteins to *E. coli*, many have multiple chemotaxis operons as well as additional chemoreceptors and proteins involved in the signaling cascade (Lacal, J. et al 2010). One such species is *Rhizobium leguminosarum* biovar *viciae*, an α -proteobacteria found in the soil and a known symbiont of legumes. The genome of the wild type *R. leguminosarum* 3841 contains two chemotaxis operons (Che1 and Che2) and 27 chemoreceptors (Figure 8) (MiST database, Ulrich, L.E. and Zhulin, I.B. 2007). The Che1 pathway has been found to be the major chemotaxis cluster controlling motility pattern, with Che2 playing a minor role (Miller et al 2007). A previous study suggested that Che1 was the controller of swimming bias and Che2 functioned to decrease the probability of reorientation frequency via CheB_{II}, suggesting a possibility of “cross-signaling” between Che1 and Che2 that would depend on methylesterase, thereby implying a role of chemoreceptors. However, the exact signaling output of Che2 is not known. At the initiation of this study, nothing was known about the localization of chemotaxis proteins and the interactions between the chemotaxis proteins from each operon.

Che1



Che2



Figure 8: Organization of chemotaxis operons in *R. leguminosarum*. Modified from Miller, L. et al 2007. Each chemotaxis operon contains the core proteins necessary for the chemotaxis cascade as well as at least one chemoreceptor (mcp).

In addition to two Che operons, the genome of *R. leguminosarum* codes for 27 chemoreceptors, most of which are located outside of the two operons (Table 1). This is in stark contrast to the five chemoreceptors of *E. coli*. One of the cytosolic *R. leguminosarum* receptors is presumed to sense oxygen due to homology of its ligand binding domain to heme-binding chemoreceptors and was named HemAT (heme-containing aerotaxis transducer) (Miller, L.D. dissertation). None of the *R. leguminosarum* chemoreceptors have been fully characterized to determine ligand specificity. Analysis of chemoreceptor sequences from the *R. leguminosarum* biovar *viciae* 3841 genome also indicates that they belong to two primary heptad classes: 36H and 34H (Table 1). Previous phylogenetic and comparative analysis predicted that 36H chemoreceptors would interact with F7 chemotaxis classes and 34H chemoreceptors would interact with F8 chemotaxis classes (Scharf, B.E., Hynes, M.F., and Alexandre, G.M. 2016). Based on this, 36H chemoreceptors are predicted to signal through interaction with Che1 (F7) and 34H would signal through interaction with Che2 (F8) in *R. leguminosarum*.

Diversity of chemotaxis in Rhizobium species

Aspects of chemotaxis have been studied in other Rhizobium species in addition to *R. leguminosarum*. The two CheY proteins, one from each chemotaxis operon, in *Rhizobium meliloti* (also referred to as *Sinorhizobium meliloti*) play different roles: one mediates chemotaxis while the other serves as a phosphate sink (Sourjik, V. and Schmitt, R. 1996). *S. meliloti* utilizes eight of its nine chemoreceptors to chemotax toward amino acids found in alfalfa seed exudates (Webb, B.A., Helm, R.F., and Scharf, B.E. 2016). The ligand specificity of McpU (Webb et al 2014), McpV (Compton et al 2018), and McpX (Webb et al 2017) is known. Organic acids as chemoattractants are common for many soil bacteria; hydroxycinnamic acids are specifically strong chemoattractants for *Bradyrhizobium japonicum* (Kape, R., Parniske, M., and Werner, D. 1991). A *Cajanus cajan* isolate, Rhizobium sp. S2, may have two distinct

Table 1: Location of chemoreceptors in *R. leguminosarum*. Compiled from MiST database (Ulrich, L.E. and Zhulin, I.B. 2007; <https://mistdb.com/>).

MiST ID	Location	Protein Accession	Heptad Class
GCF_000009265.1-RL_RS25320	NC_008383.1 [31566..33389] plasmid pRL8	WP_011654661.1	36H
GCF_000009265.1-RL_RS36330	NC_008378.1 [734192..736336] Plasmid pRL12	WP_028742969.1	36H
GCF_000009265.1-RL_RS33280	NC_008378.1 [69635..71422] Plasmid pRL12	WP_028742558.1	36H
GCF_000009265.1-RL_RS33215	NC_008378.1 [complement(55461..57278)] Plasmid pRL12	WP_041936978.1	36H
GCF_000009265.1-RL_RS34485	NC_008378.1 [complement(330022..331899)] Plasmid pRL12	WP_011649086.1	36H
GCF_000009265.1-RL_RS32520	NC_008384.1 [complement(590672..591274)] Plasmid pRL11	WP_011655275.1	28H
GCF_000009265.1-RL_RS29420	NC_008381.1 [420034..421995] Plasmid pRL10	WP_011654428.1	36H
GCF_000009265.1-RL_RS22035	NC_008380.1 [complement(4537298..4539826)]	WP_011653672.1	36H
GCF_000009265.1-RL_RS20810 McpC	NC_008380.1 [complement(4262766..4264475)] Chromosome	WP_041936459.1	34H
GCF_000009265.1-RL_RS20805	NC_008380.1 [complement(4260574..4262496)] Chromosome	WP_011653454.1	34H
GCF_000009265.1-RL_RS20800 Mcp2A	NC_008380.1 [complement(4258583..4260400)] Chromosome	WP_011653453.1	34H

Table 1 cont.

MiST ID	Location	Protein Accession	Heptad Class
GCF_000009265.1-RL_RS20555	NC_008380.1 [complement(4211111..4213426)] Chromosome	WP_041936455.1	36H
GCF_000009265.1-RL_RS22630	NC_008380.1 [4664493..4666253] Chromosome	WP_011653768.1	36H
GCF_000009265.1-RL_RS13855 McpD	NC_008380.1 [2838031..2839905] Chromosome	WP_041936344.1	36H
GCF_000009265.1-RL_RS15160	NC_008380.1 [complement(3092943..3094760)] Chromosome	WP_011652452.1	36H
GCF_000009265.1-RL_RS07515	NC_008380.1 [complement(1509756..1510862)] Chromosome	WP_041936236.1	24H
GCF_000009265.1-RL_RS07220	NC_008380.1 [1448743..1450191] Chromosome	WP_011651091.1	40H
GCF_000009265.1-RL_RS05525	NC_008380.1 [complement(1145440..1147269)] Chromosome	WP_011650797.1	36H
GCF_000009265.1-RL_RS06875	NC_008380.1 [1382704..1385085] Chromosome	WP_011651033.1	36H
GCF_000009265.1-RL_RS03970	NC_008380.1 [812573..814420] Chromosome	WP_011650512.1	36H
GCF_000009265.1-RL_RS03965	NC_008380.1 [809810..812341] Chromosome	WP_011650511.1	36H
GCF_000009265.1-RL_RS02965	NC_008380.1 [complement(609477..611465)] Chromosome	WP_011650351.1	36H
GCF_000009265.1-RL_RS05060	NC_008380.1 [1049665..1051602] Chromosome	WP_041936183.1	36H

Table 1 cont.

MiST ID	Location	Protein Accession	Heptad Class
GCF_000009265.1-RL_RS04945	NC_008380.1 [1019449..1021272] Chromosome	WP_011650688.1	36H
GCF_000009265.1-RL_RS02375	NC_008380.1 [492347..494344] Chromosome	WP_011650243.1	36H
GCF_000009265.1-RL_RS02235	NC_008380.1 [468110..469606] Chromosome	WP_011650219.1	36H
GCF_000009265.1-RL_RS02220	NC_008380.1 [complement(462895..465234)] Chromosome	WP_028740524.1	36H
GCF_000009265.1-RL_RS03605 HemAT	NC_008380.1 [737051..738685] Chromosome	WP_041936171.1	36H

signaling pathways, one for sensing nutrients and the other for sensing of signaling compounds such as naringenin (Pandya, S. et al 1999). Many *Rhizobium* species contain at least two chemotaxis systems, each containing a complete set of chemotaxis proteins. However, this is not conserved across all members of the Rhizobiaceae family.

Chemotaxis in the rhizosphere

Chemotactic bacteria that live in the rhizosphere, the soil region surrounding plant root systems, rely on chemotaxis to navigate through a complex, heterogeneous environment to survive as free-living cells which is the dominant lifestyle as well as to localize and colonize plant roots (Bais, H.P. 2004). The chemical cues for colonization come from compounds exuded by the roots into the surrounding soil. Bacteria that live in complex environments such as the soil tend to have much more MCPs in their genome, particularly those that establish interactions with plant roots (Lacal, J. et al 2010; Alexandre, G., Greer-Phillips, S., and Zhulin, I.B. 2004). In the case of *R. leguminosarum*, colonization of non-legume hosts results in a biofilm on the root surface (Poole, P., Ramachandran, V., and Terpolilli, J. 2018) while colonization of legume hosts results in the formation of root nodules in the root hair region where nitrogen fixation by the bacteria will take place (Yost, C.K, Del Bel, K.L., Quandt, J., and Hynes, M.F. 2004). Flavonoids tend to be the cue for the nodulation process to begin (Oldroyd, G.E.D. and Downie, J.A. 2008). Nodulation factors appear to be highly specific to the plant and the bacteria, establishing a possible mechanism for host specificity in the rhizosphere (Hirsch, A.M., Lum, M.R., and Downie, J.A. 2001). The root exudate profile surrounding a plant may actively recruit specific microbes that have adapted to be able to colonize the root surface or form nodules; this may also be influenced by root architecture and redox conditions in the soil (Buchan, A., Crombie, B., and Alexandre, G.M. 2010). The root exudate profile of a plant likely varies both along the length of

the root as well as across various growth stages. Therefore, it is probable that distinct bacterial species preferentially colonize different areas of the root surface, with these patterns changing in time and space (O'Banion, B.S., O'Neal, L., Alexandre, G., and Lebeis, S.L. 2019).

Chemotaxis is highly important in the initial stages of colonization to optimize both the species and spatial preference of bacteria to root systems in the rhizosphere. While non-chemotactic *R. leguminosarum* can still colonize legumes, the process takes much longer and nodulation is less efficient (Gaworzewska, E.T. and Carlile, M.J. 1982), indicating that chemotaxis is necessary for competitive nodulation in the rhizosphere. Long term colonization by soil bacteria on the root requires the formation of a biofilm (Downie, J.A. 2010). Successful, long term interactions of *R. leguminosarum* with legumes, specifically pea plants, results in the establishment of root nodules containing dormant but nitrogen-fixing bacteria that receive nutrients from the plant (Long, S.R. 1989; Brenner, A. and Winans, S.C. 2005). Given the broad distribution of *R. leguminosarum* in soil and its large number of genome encoded chemoreceptors, many of the chemoreceptors in *R. leguminosarum* are likely tuned to recognize compounds found in root exudates from diverse plant rhizospheres, including its preferred symbiotic host.

CHAPTER II. MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 2. *Rhizobium leguminosarum* bv *viciae* strains were grown in either tryptone-yeast (TY) medium (Beringer 1974) supplemented with 1 mM CaCl₂ or Vincent's minimal medium (VMM) (Vincent 1970) at 28°C with shaking. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm. All *R. leguminosarum* stocks were routinely maintained on solid VMM (liquid VMM supplemented with 15 g/L agar) at room temperature. All *E. coli* stocks were routinely maintained on solid LB (liquid LB supplemented with 15g/L agar) at 4°C.

Plasmids/strain construction

Cyan fluorescence protein (CFP) fusions were constructed using Gateway cloning (Invitrogen) and the pRH004 vector (Hallez et al 2007). The pRH004 vector allows cloning of any gene to generate in-frame fusions of CFP at the C-terminus. Genes of interest (CheA1, CheA2, Mcp2A, McpC, McpD, and HemAT) were amplified with primers to add Gateway recombination sites to either side of the gene (Table 3) from *R. leguminosarum* bv *viciae* strain 3841 genomic DNA. PCR cleanup (Macherey-Nagel) was performed on the amplified DNA following manufacturer protocol. Recombination was performed using the Gateway BP reaction as described in the manufacturer's manual (Invitrogen) into entry vector pDONR221 and transformed into *E. coli* Top10 chemically competent cells. Colony PCR was utilized to screen transformed cells for the presence of the gene of interest using primers specific to the gene being inserted. Confirmed recombined vectors were extracted from colonies and sequenced to confirm the correct gene fusion using M13 primers provided by the University of Tennessee Knoxville

Genomics Core. Confirmed vectors were further recombined with the pRH004 destination vector using LR Clonase (Invitrogen) and transformed into *E. coli* Top10 competent cells. The resulting colonies were again screened by colony PCR for the gene of interest using primers specific for the gene inserted. The plasmids containing each gene of interest identified from colonies by PCR were isolated using Miniprep (Qiaprep) and transferred to *R. leguminosarum* strains 3841, $\Delta che1$, $\Delta che2$, $\Delta che1\Delta che2$ (Table 2) by triparental conjugation (Hauwaerts et al 2002).

A translational fusion between the genes encoding *CheA2* and *mCherry* was constructed as following. *mCherry* (Mignolet, J. et al 2010) was inserted into the vector pBBRMCS-2 by restriction digest using XbaI and SmaI. *CheA2* was amplified from the *R. leguminosarum* 3841 genome to add XmaI and XbaI (NEB) restriction sites at the 5' and 3' ends respectively (Table 3). Restriction digest of *mCherry-pBBR* and *cheA2* was performed with XmaI and XbaI followed by PCR cleanup to inactivate the enzymes and remove the buffer. Subsequently, the digested pieces were ligated using T4 DNA ligase (New England Biolabs) according to the manufacturer's recommendation and transformed into *E. coli* Top10 chemically competent cells.

Fluorescence microscopy

Strains of *R. leguminosarum* expressing fluorescent constructs were grown to stationary phase in 5 mL VMM with appropriate antibiotics and reinoculated three times to build up protein in the cells. 1% low melting point agarose pads were prepared and added on to cleaned glass microscope slides in order to immobilize cells. Ten μ L of fluorescent cells in stationary phase were placed on the agarose pads. Immobilized cells were covered with a cover slip and incubated on agarose pads at room temperature for two hours before microscopic observations. Images were taken on a DMI8 microscope equipped with Leica SP8 White Light Laser Confocal System using a 63X objective. The CFP filter (Excitation 420-450 nm, Emission 460-500 nm) was used to

observed fluorescence. At least 70 cells from four distinct fields of view were used for each sample. Images were analyzed to determine pattern of fluorescence: polar, nonpolar, or diffuse. The percentage of each fluorescent cell type was determined. Graphs were produced using GraphPad Prism software. All graphs display the ratio of polar: total cells, nonpolar: total cells, and diffuse: total cells. ImageJ (<https://imagej.nih.gov/ij/>) was used to create merged images of CFP and DIC channels. Statistical analysis between WT and mutants for each localization category was performed using two-tailed z-tests at significance level of 0.01.

Bacterial adenylate cyclase two hybrid assay

A bacterial adenylate cyclase two hybrid (BacTH) assay specific for membrane proteins was used to assess protein-protein interactions in vitro (Euromedex, Figure 9). Proteins of interest (CheA1, CheA2, Mcp2A, McpD, McpC, and HemAT) were fused on the C-terminus of the T18 and T25 catalytic domains of *Bordetella pertussis* adenylate cyclase found in Gateway compatible vectors pUT18C-DEST and pKT25-DEST respectively. The genes of interest were amplified with PCR from *R. leguminosarum* 3841 genomic DNA to add Gateway recombination sites to both ends of the gene. Recombination was performed using the Gateway BP reaction as described in the manufacturer's manual (Invitrogen) into entry vector pDONR221 and transformed into *E. coli* Top10 chemically competent cells. Colony PCR was utilized to screen transformed cells for the presence of the gene. Colonies containing the recombined vector were sequenced to confirm the correct gene fusion. Subsequently, recombination from the entry vector into the destination vectors pUT18C-DEST and pKT25-DEST (table 2) was performed using LR clonase (Invitrogen) and transformed into *E. coli* Top10 chemically competent cells. Colony PCR was used to screen for successful recombination. The vectors confirmed by colony PCR were isolated using Miniprep (Qiagen) plasmid isolation and sequenced.

Mcp2A fusion to the catalytic subunit T25 was made utilizing traditional restriction digestion cloning and ligation into pKT25 (Table 3). Mcp2A was amplified with PCR from *Rhizobium leguminosarum* 3841 genomic DNA with primers designed to add KpnI and SmaI (NEB) restriction sites to the 5' and 3' ends respectively. Restriction digestion of *Mcp2A* and pKT25 was performed sequentially with KpnI and SmaI enzymes then heat inactivated after one hour of digestion. Digested *mpc2A* and pKT25 fragments were ligated using T4 DNA ligase and transformed into *E. coli* Top10 chemically competent cells. Colony PCR was used to screen for ligated construct and candidate plasmids were extracted by Miniprep (Qiagen) and sequenced using M13 primers, as described above.

Two genes of interest, one tagged with T18 (cloned into the pUT18C vector) and one with T25 (cloned into the pST25 vector), were co-transformed into *E. coli* BTH101 chemically competent cells using the manufacturers protocol (Euromedex) with the addition of 1 μ L of β -mercaptoethanol to increase reaction efficiency (New England Biolabs). Three 5 mL LB cultures of each co-transformation with 0.5 mM IPTG and appropriate antibiotics were grown at 28°C overnight with shaking. The positive control was pUT18C zip and pKT25 zip. The negative control was empty pUT18C and pKT25. Square petri dishes of solid MacConkey media supplemented with lactose (10g/L) with appropriate antibiotics were prepared and dried 5-6 hours at room temperature on the bench. Ten μ L of the three biological replicates from co-transformed cells were spotted on MacConkey plates and dried in sterile conditions. Plates were then incubated one to four days at 28°C without shaking on a level surface until pink colonies appeared to indicate a positive interaction. Interactions were transferred by streaking onto fresh MacConkey plates with appropriate antibiotics that were dried 5-6 hours at room temperature and incubated overnight at 28°C. If protein-protein interactions took place, functional complementation occurs between the two catalytic domain halves and cyclic AMP (cAMP) is

produced. This activates the *lac* and *mal* operons in *E. coli*, allowing positive interactions plated on MacConkey to utilize the carbon source available in the agar and visually appear pink.

Beta-galactosidase assay

For quantification of BacTH interactions, samples from overnight MacConkey plates were inoculated into 5 mL LB cultures with appropriate antibiotics in biological triplicates for each interaction, and shaken overnight at 28°C. 200 µL of overnight cultures was reinoculated into fresh 5 mL LB cultures with appropriate antibiotics and grown at 28°C with shaking to an OD₆₀₀=0.5-0.6. Samples were diluted to OD₆₀₀=0.2 in 200 µL in 96 well plates, three wells per biological sample. OD₆₀₀ reading was taken with a plate reader for analysis. Plates were either placed at -80°C for storage or continued to assay the same day (Euromedex). When ready for assay, the plate was removed from -80°C and placed at 37°C with the lid off for thirty minutes to lyse cells. 100 µL from the 96 well plate of each well was transferred to a new 96 well plate and freeze thawed in succession three times for thirty minutes each. The lid remained removed while in 37°C incubator every time. 4 mg/mL o-nitrophenyl-β-D-galactopyranoside (ONPG) was prepared in filter sterilized 0.1 M phosphate buffer at pH=6.85-7.0 and kept on ice until use. 20 µL of 4 mg/mL ONPG was added to each well and immediately placed into plate reader prewarmed to 37°C. The reaction was allowed to proceed for 30 minutes and the OD₄₂₀ was taken for analysis. Interactions were analyzed using the following equation:

$$\text{Miller Units} = 1000 * [(OD_{420}) / (T * V * OD_{600})]$$

T= time of reaction in minutes
V= volume of culture used in the assay in mL.

The positive control was pUT18C zip and pKT25 zip. The negative control was empty pUT18C and pKT25. A student's T-test was used to compare the negative control to each interaction. Significant differences ($p < 0.05$, $p < 0.0005$) indicates a positive interaction.

Root colonization and competition

Wheat, pea, and alfalfa seeds were sterilized with 70% ethanol and bleach sequentially for 30 seconds each. Seeds were then rinsed with double deionized H₂O until the smell of bleach was gone. Seeds were planted in semi-solid (0.4% agar) Fahraeus media and allowed to germinate in the dark for two days. After two days, germinated seeds were planted in fresh semi-solid Fahraeus media and incubated at room temperature under constant white light overnight. While seeds were germinating, three biological replicates of *R. leguminosarum* bv *viciae* samples were grown in TYC to stationary phase, approximately OD₆₀₀ of 1. One mL of each sample was washed and plated on TYC plates in serial dilutions, from 10⁻² to 10⁻¹⁰. Autoclaved growth chambers were filled with 50 mL of semi-solid Fahraeus and four germinated seeds were planted in each circular chamber in pairs on opposite sides, three chambers per bacterial strain. 20 µL of washed bacteria was inoculated into the center of each growth chamber and allowed to incubate five days. After five days, plants were removed from the growth chambers and the roots were weighed. Once weighed, each root was ground in 400 µL of sterile Che buffer (1.7 g L⁻¹ dipotassium phosphate, 1.36 g L⁻¹ monopotassium phosphate; 0.1 mM EDTA) in a sterile mortar and pestle and plated for colony forming unit (CFU) counts. Serial dilution plates were grown overnight at 28°C without shaking. CFU were counted and the log of CFU after colonization was divided by the log of CFU before inoculation to obtain a ratio of colonization.

For competition with root colonization, a distinguishing marker needed to be introduced to wild-type (WT) and mutant strains to select for each individually with antibiotics. Gentamycin resistance provided through pBBRMCS-5 (Kovach et al 1995) was introduced into *mcpC* and *mcpD* deletion strains (Yost et al 2004) and tetracyclin resistance provided through pBBRMCS-3 (Kovach et al 1995) was introduced into the WT strain by bacterial conjugation. *hemAT* was created using insertion of a gentamycin resistance cassette to disrupt the gene. Seeds were

germinated as described above and bacterial strains were grown individually in TYC to stationary phase, approximately $OD_{600}=1$. WT and receptor mutant strains were mixed in a 1:1 ratio and serial dilutions were plated on TYC plates with streptomycin only, streptomycin with gentamycin, and streptomycin with tetracyclin to be able to count the total number of colony forming units (CFU) in the mixture and differentiate between WT (streptomycin and tetracyclin) and mutant (streptomycin and gentamycin) cells. The mixture was washed and inoculated into the center of growth chambers with germinated seeds as described above. After five days, during which cells were allowed to navigate toward and colonize the roots of plants, roots were weighed and ground with mortar and pestle to perform serial dilutions on TYC plates with the same antibiotics as before colonization. Serial dilution plates were grown at 28°C overnight without shaking. Colony forming units (CFU) were counted and the log of CFU of WT cells was divided by the log of CFU of mutant cells to obtain a ratio of colonization.

Table 2: Plasmids and strains used in this study.

Strains or plasmids	Genotype, relevant characteristics	Reference or source
<i>R. leguminosarum</i>		
3841 (WT)	Biovar <i>viciae</i> , JB300 derivative, Sm ^R	Johnston and Beringer (1975)
$\Delta che1$	Deletion of <i>che1</i> operon, Km ^R , Sm ^R	Miller et al 2007
$\Delta che2$	Deletion of <i>che2</i> operon, Sm ^R	Miller et al 2007
$\Delta che1\Delta che2$	Deletion of <i>che1</i> and <i>che2</i> operons, Km ^R , Sm ^R	Miller et al 2007
3841 (pRH_CheA1)	CheA1 tagged with C-terminal CFP in WT background, Sm ^R , Kan ^R , Cm ^R	This study
3841 (pRH_CheA2)	CheA2 tagged with C-terminal CFP in WT background, Sm ^R , Kan ^R , Cm ^R	This study
3841 (pRH_HemAT)	HemAT tagged with C-terminal CFP in WT background, Sm ^R , Kan ^R , Cm ^R	This study
3841 (pRH_Mcp2A)	Mcp2A tagged with C-terminal CFP in WT background, Sm ^R , Kan ^R , Cm ^R	This study
3841 (pRH_McpD)	McpD tagged with C-terminal CFP in WT background, Sm ^R , Kan ^R , Cm ^R	This study
3841 (pRH_McpC)	McpC tagged with C-terminal CFP in WT background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1$ (pRH_CheA1)	CheA1 tagged with C-terminal CFP in $\Delta che1$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1$ (pRH_CheA2)	CheA2 tagged with C-terminal CFP in $\Delta che1$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1$ (pRH_HemAT)	HemAT tagged with C-terminal CFP in $\Delta che1$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1$ (pRH_Mcp2A)	Mcp2A tagged with C-terminal CFP in $\Delta che1$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1$ (pRH_McpD)	McpD tagged with C-terminal CFP in $\Delta che1$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1$ (pRH_McpC)	McpC tagged with C-terminal CFP in $\Delta che1$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che2$ (pRH_CheA1)	CheA1 tagged with C-terminal CFP in $\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che2$ (pRH_CheA2)	CheA2 tagged with C-terminal CFP in $\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che2$ (pRH_HemAT)	HemAT tagged with C-terminal CFP in $\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che2$ (pRH_Mcp2A)	Mcp2A tagged with C-terminal CFP in $\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che2$ (pRH_McpD)	McpD tagged with C-terminal CFP in $\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che2$ (pRH_McpC)	McpD tagged with C-terminal CFP in $\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1\Delta che2$ (pRH_CheA1)	CheA1 tagged with C-terminal CFP in $\Delta che1\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1\Delta che2$ (pRH_CheA2)	CheA1 tagged with C-terminal CFP in $\Delta che1\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1\Delta che2$ (pRH_HemAT)	HemAT tagged with C-terminal CFP in $\Delta che1\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study

Table 2 cont.

Strains or plasmids	Genotype, relevant characteristics	Reference or source
$\Delta che1\Delta che2$ (pRH_Mcp2A)	Mcp2A tagged with C-terminal CFP in $\Delta che1\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1\Delta che2$ (pRH_McpD)	McpD tagged with C-terminal CFP in $\Delta che1\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
$\Delta che1\Delta che2$ (pRH_McpC)	McpC tagged with C-terminal CFP in $\Delta che1\Delta che2$ background, Sm ^R , Kan ^R , Cm ^R	This study
3841 (pBBRMCS-3)	3841 with pBBR plasmid for root competition, Sm ^R , Tet ^R	This study
<i>mcpD</i>	Deletion of <i>McpD</i> from <i>R. leguminosarum</i> strain VF39, Sm ^R	Yost et al 2004
<i>mcpD</i> (pBBRMCS-5)	<i>mcpD</i> with pBBR plasmid for root competition, Sm ^R , Gm ^R	This study
<i>mcpC</i>	Deletion of <i>McpC</i> from <i>R. leguminosarum</i> strain VF39, Sm ^R	Yost et al 2004
<i>mcpC</i> (pBBRMCS-5)	<i>mcpC</i> with pBBR plasmid for root competition, Sm ^R , Gm ^R	This study
<i>hemAT::Gm</i>	Deletion of <i>hemAT</i> from <i>R. leguminosarum</i> strain 3841 by disruption with Gm resistance gene, Sm ^R , Gm ^R	Yost et al 2004
<i>E. coli</i> strains:		
Top10	General cloning strain	Invitrogen
BTH101	<i>F- cya-99 araD139 galE15 galK16 rpsL1 hsdR2 mcrA1 mcrB1</i>	Karimova et al 1998
XL1-Blue	<i>recA1 endA1 gyrA96 thi-I hsdR17 supE44 relA1 lac [F' proAB lacIq ZAM15 Tn10 (Tet^R)]</i>	Agilent Technologies
pUT18C_CheA1	CheA1 fused with T18C for BacTH, Cb ^R	This study
pUT18C_CheA2	CheA2 fused with T18C for BacTH, Cb ^R	This study
pUT18C_HemAT	HemAT fused with T18C for BacTH, Cb ^R	This study
pUT18C_Mcp2A	Mcp2A fused with T18C for BacTH, Cb ^R	This study
pUT18C_McpD	McpD fused with T18C for BacTH, Cb ^R	This study
pUT18C_McpC	McpC fused with T18C for BacTH, Cb ^R	This study
pST25_CheA1	CheA1 fused with T25 for BacTH, Sp ^R	This study
pST25_CheA2	CheA2 fused with T25 for BacTH, Sp ^R	This study
pST25_HemAT	HemAT fused with T25 for BacTH, Sp ^R	This study
pKT25_Mcp2A	Mcp2A fused with T25 for BacTH, Km ^R	This study
pST25_McpD	McpD fused with T25 for BacTH, Sp ^R	This study
pST25_McpC	McpC fused with T25 for BacTH, Sp ^R	This study
Plasmids:		
pRH004	Gateway-based destination vector expressing proteins fused with CFP at the C-terminus, Km ^R , Cm ^R	Hallez et al 2007
pBBRMCS-3	Expression vector, Tet ^R	Kovach, M.E. et al 1995
pBBRMCS-5	Expression vector, Gm ^R	Kovach, M.E. et al 1995
pBBRMCS-2	Expression vector, Kan ^R	Kovach, M.E. et al 1995

Table 2 cont.

Strains or plasmids	Genotype, relevant characteristics	Reference or source
pRK2013	Helper plasmid with RK2 tra region, Km ^R	Figurski, D.H. and Helinski, D.R. 1979
pUT18C	Derivative of pUC19 plasmid containing T18 of CyA, Cb ^R	Karimova, G. et al 1998
pKT25	Derivative of pSU40 plasmid containing T25 of CyA, Km ^R	Karimova, G. et al 1998
pUT18C-DEST	Gateway-based destination vector expressing proteins fused with T18 at the C-terminus, Cb ^R	Ouellette, S.P. et al 2013
pKT25-DEST	Gateway-based destination vector expressing proteins fused with T25 at the C-terminus, Sp ^R	Ouellette, S.P. et al 2013
pUT18C-zip	Derivative of pUT18C where the leucine zipper of GCN4 is genetically fused in frame to the T18 fragment, Cb ^R	Karimova, G. et al 1998
pKT25-zip	Derivative of pKT25 where the leucine zipper of GCN4 is genetically fused in frame to the T18 fragment, Km ^R	Karimova, G. et al 1998

Antibiotics used with working concentrations:

Sm – streptomycin (500 ug/mL), Km – kanamycin (50 ug/mL), Cm – chloramphenicol (34 ug/mL), Cb – carbenicillin (100 ug/mL), Sp – spectinomycin (100 ug/mL), Tet – tetracyclin (10 ug/mL), Gm – gentamycin (20 ug/mL)

Table 3: Primers used in this study

Primer name	Sequence (5' to 3')	Purpose
GW CheA1 Fwd	ggggacaagtttgtaaaaaagcaggctatggatatgaacgaaatc	Addition of Gateway sites to <i>CheA1</i>
GW CheA1 Rev	ggggaccactttgtacaagaaagctgggtctcccaccgctgctaacga	
GW CheA2 Fwd	ggggacaagtttgtaaaaaagcaggctatgagcacgctcgatccg	Addition of Gateway sites to <i>CheA2</i>
GW CheA2 Rev	ggggaccactttgtacaagaaagctgggtctcctgctacacgcaattg	
GW HemAT Fwd	ggggacaagtttgtaaaaaagcaggctatgccatcagatcaggct	Addition of Gateway sites to <i>HemAT</i>
GW HemAT Rev	ggggaccactttgtacaagaaagctgggtcgtaaacattccgccacc	
GW Mcp2A Fwd	ggggacaagtttgtaaaaaagcaggctatgcgcttcacaatcaaa	Addition of Gateway sites to <i>Mcp2A</i>
GW Mcp2A Rev	ggggaccactttgtacaagaaagctgggtctctgatggcggtcccga	
GW McpD Fwd	ggggacaagtttgtaaaaaagcaggctatgaaaaacgtaacaatc	Addition of Gateway sites to <i>McpD</i>
GW McpD Rev	ggggaccactttgtacaagaaagctgggtcagccgcccgcgaaaccgc	
GW McpC Fwd	ggggacaagtttgtaaaaaagcaggctgtgaccgcgttcaccttc	Addition of Gateway sites to <i>McpC</i>
GW McpC Rev	ggggaccactttgtacaagaaagctgggtctcgctctccctaaagtc	
BacTH Mcp2A Fwd	catcatggtaccatgcgcttcacaatcaaactt	Addition of restriction sites KpnI and SmaI to <i>Mcp2A</i>
BacTH Mcp2A Rev	attattgggcctcgggaccgcccacgcgag	
CheA2 XmaI Fwd	attgttccgggatgagcacgctcgatccg	Addition of restriction sites XbaI and XmaI to <i>CheA2</i>
CheA2 XbaI Rev	cgagcctctagatcctgctacacgcaattg	

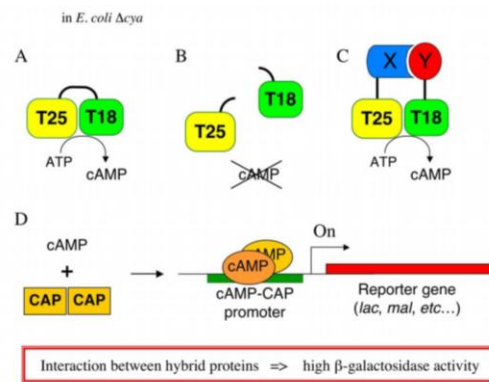


Figure 9: Schematic of BacTH assay (Euromedex). Each half of the catalytic domain of bacterial adenylate cyclase is fused to proteins of interest X and Y. If X and Y interact, the catalytic domain is brought together and catalyzes the formation of cAMP from ATP. This activates the reporter gene β -galactosidase and the activity of this enzyme can be quantitatively measured.

CHAPTER III. RESULTS AND DISCUSSION

In the *E. coli* model system, chemoreceptors form mixed arrays localized to the cell poles, held together by CheA histidine kinase and CheW coupling protein (Draper, W. and Liphardt, J. 2017). Cryo-ET studies of distinct bacterial species supports that receptor array architecture is universally conserved which leads to amplification and gain of signal (Sourjik, V. and Berg, H.C. 2002). To mediate chemotaxis, CheA must interact with chemoreceptors and CheW and tight chemoreceptor array formation is dependent on CheA and CheW binding, although loose receptor clusters can form in the absence of CheA or CheW (Studdert, C.A. and Parkinson, J.S. 2004). This clustering can be visualized by fluorescent microscopy as well as cryo-electron microscopy. Therefore, CheA localization is used as an indicator of the location of full receptor arrays within the cell. This pattern becomes more complicated when multiple CheA proteins are present within the same cell. Recently it was found that in *Azospirillum brasilense*, paralogs of chemotaxis proteins are coalesced into mixed baseplates, providing a mechanism for signal integration and coordinated response output from two distinct chemotaxis systems (O'Neal, L. and Gullett, J.M. et al 2019). It is unknown if this strategy is maintained in evolutionarily distinct bacteria living in a similarly complex environment.

The genome of *R. leguminosarum* contains two CheA paralogs (CheA1 and CheA2) as well as five CheW proteins and 27 chemoreceptors (MiST database, Ulrich, L.E. and Zhulin, I.B. 2007). It has been previously shown that there is a potential for signaling cross-talk between the two chemotaxis operons during chemotaxis at the level of receptor modification by CheB (Miller et al 2007). However, no additional work has been done to elucidate if other interactions between the two chemotaxis systems are occurring. It is known that chemoreceptors tend to cluster based on heptad length, which defines the distance between the periplasmic membrane and the signaling tips (Ortega, D.R. et al 2017). For proper baseplate signaling to occur, the baseplate must remain

level (Briegleb, A. et al 2014). Two distinct chemoreceptor arrays, composed of 36H and 34H receptors, respectively, are likely present within the *R. leguminosarum* cell. Given this organization, Che1 and Che2 could potentially combine in a variety of ways. The 36H receptors could interact with only Che1 proteins and the 34H receptors could contain only Che2 proteins, which has been seen in *Rhodobacter sphaeroides* (Wadhams, G.H., Warren, A.V., Martin, A.C., and Armitage, J.P. 2003). Another possibility is that chemoreceptors are organized into arrays with mixed baseplates of Che1 and Che2 proteins, similar to *A. brasilense* as described above. Che1 is an F7 system; therefore, it is predicted to interact with 36H receptors. Che2 is an F8 system predicted to interact with 34H receptors. If this is indeed the case, we expect to see Che1 and Che2 proteins segregating to distinct chemoreceptor arrays.

To understand chemoreceptor array organization in *R. leguminosarum*, we used fluorescently labeled proteins to determine the localization of chemoreceptors using live-cell fluorescence microscopy. We selected chemoreceptors encoded within each operon (36H HemAT from *Che1* and 34H Mcp2A from *Che2*) as well as two orphan chemoreceptors of two distinct heptad classes (34H McpC and 36H McpD). The localization of these proteins was compared in relation to each other as well as relative to fluorescently labeled CheA1 and CheA2. Fluorescence was classified as polar, membrane-associated, or diffuse. Polar localization in the scope of this study is defined as tight foci observed at one or both poles of the cell. Membrane-associated localization is defined as the absence of tight foci with fluorescence primarily on the membrane and not the cytosol. Diffuse fluorescence is the absence of localization, with fluorescent signal observed throughout the cell.

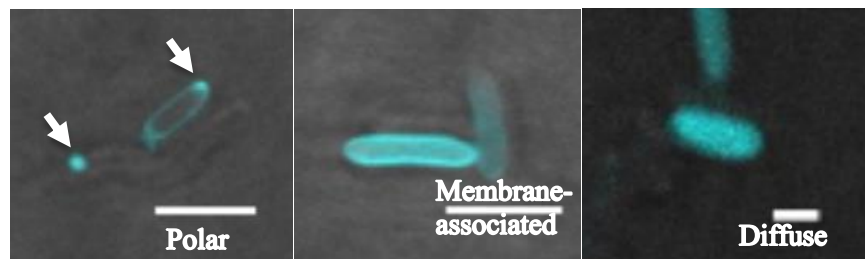


Figure 10: Representative images of polar, membrane-associated, and diffuse localization.

Che1 proteins localize to both poles while Che2 proteins localize to a single pole

When CheA1-CFP is expressed in the wild type background, localization is primarily to punctate foci at the cell poles (Figure 11, A). Interestingly, polar foci are found at both poles at almost twice the frequency as just one pole in this construct (Figure 11, B). This suggests that fluorescently labeled CheA1 localizes to large receptor arrays that are present and can be observed at both poles. However, upon deletion of either *che1* or *che2*, the only fluorescence observed was diffuse throughout the cell, suggesting that the CheA1-CFP construct no longer localizes in distinct foci. This observation implies that proper CheA1 localization to the cell poles is dependent on proteins from both operons being present in the cell.

In contrast, CheA2-CFP localization in the wild type background is detected at the cell pole in approximately 50% of cells (Figure 11, C). Of these cells, 20 times more cells have fluorescent foci at a single pole rather than at both poles (Figure 11, D). The other 50% of cells show primarily either membrane-associated or diffuse localization, suggesting that the polar array able to recruit CheA2-CFP may be comparatively smaller than the array that recruits CheA1-CFP. The number of cells with polar foci is greatly reduced when Che1, Che2, or both are deleted indicating that polar cluster formation depends on both Che1 and Che2 proteins. In the $\Delta che1$ background, localization of CheA2-CFP shifts heavily toward membrane-associated fluorescence with only 15% of cells showing polar localization, with a similar ratio of monopolar to dipolar localization as the wild-type background. Therefore, this polar localization represents the fraction of CheA2-CFP at the cell poles that is dependent on Che2 proteins.

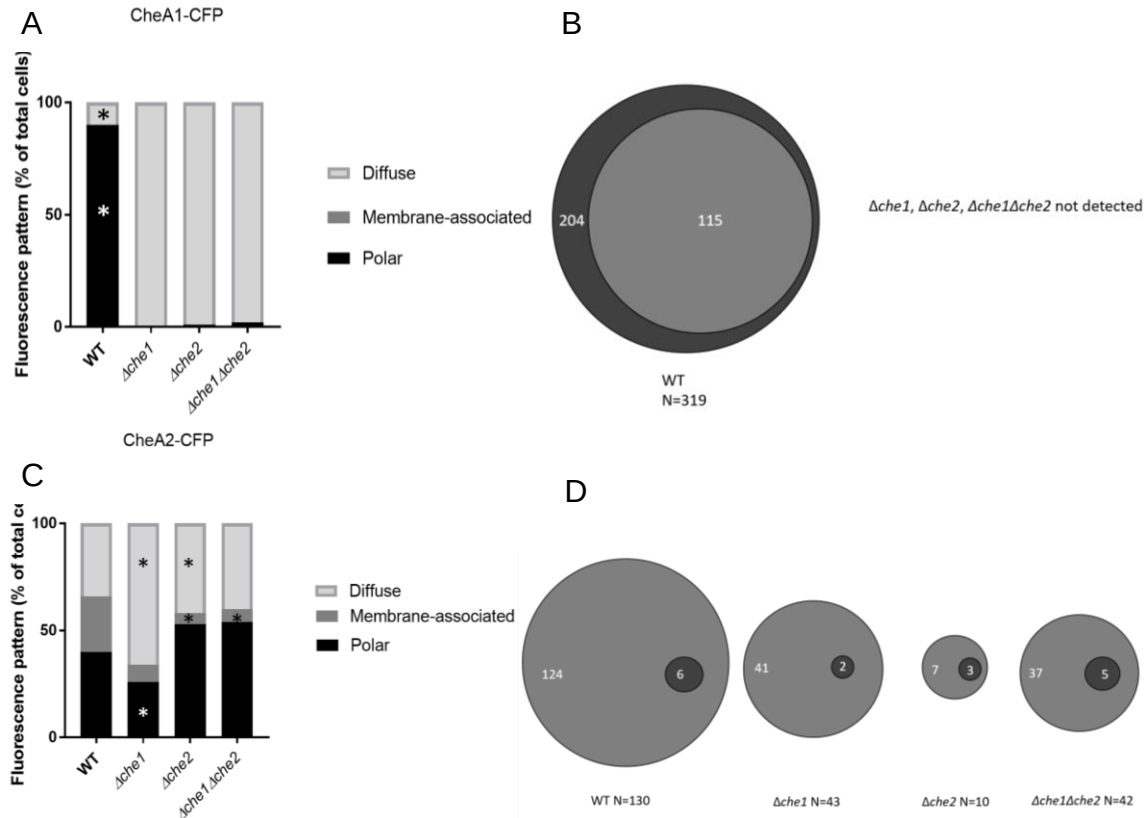


Figure 11: Fluorescent localization pattern of CheA histidine kinases in WT and chemotaxis mutant backgrounds. (A) Percentage of CheA1-CFP cells that localize into each fluorescence category. *: P-value<0.01 compared to WT. (B) Distribution of monopolar and dipolar foci for CheA1-CFP in each background where polar foci were detected. Light gray indicates monopolar, dark gray indicates dipolar. N is the total number of cells with polar foci. (C) Percentage of CheA2-CFP cells that localize into each fluorescence category. *: P-value<0.01 compared to WT. (D) Distribution of monopolar and dipolar foci for CheA2-CFP in each background where polar foci were detected. Light gray indicates monopolar, dark gray indicates dipolar. N is the total number of cells with polar foci.

The reduced polar localization and membrane-associated localization of CheA2-CFP in the $\Delta che1$ background suggest that these CheA2-CFP foci may depend on the presence of Che1 proteins at the cell poles and elsewhere around the cell membrane. Membrane-associated localization of CheA2-CFP are also reduced in the $\Delta che2$ background relative to the wild-type, implying that localization also depends on some Che2 proteins. Polar localization of CheA2-CFP in the $\Delta che2$ and the double mutant backgrounds is very similar and increases relative to the localization observed in wild-type. This suggests that lack of Che2 proteins promotes polar localization of CheA2-CFP. It appears that eliminating Che2 removes a negative regulator of CheA2 localization. Interestingly, CheA2-CFP fluorescence, when not localized as polar foci or membrane-associated, is observed as large polar caps in all four backgrounds, but most strongly in the $\Delta che1$ and $\Delta che1\Delta che2$ backgrounds (Figure 14). This is distinct from the discrete polar foci and membrane-associated localization and could suggest that the polar cap fluorescence observed is due to CheA2-CFP localizing to inclusion bodies. This would need to be confirmed by analysis of protein stability through western blotting. These patterns of CheA1-CFP and CheA2-CFP suggest that polar clusters require proteins from both operons to form discrete foci. CheA2-CFP is prone to forming membrane-associated clusters when large polar clusters do not form.

Subcellular localization of chemoreceptors in wild-type and mutant backgrounds

When HemAT-CFP, 36H chemoreceptor encoded by the Che1 operon, is expressed in the wild-type background, almost 100% of cells contain polar foci (Figure 12, A), which is similar to the localization of CheA1-CFP, consistent with HemAT interacting with Che1 proteins. This is intriguing because HemAT is a soluble receptor; it does not necessarily need to be associated with the membrane. However, this has been observed in *Sinorhizobium meliloti* previously

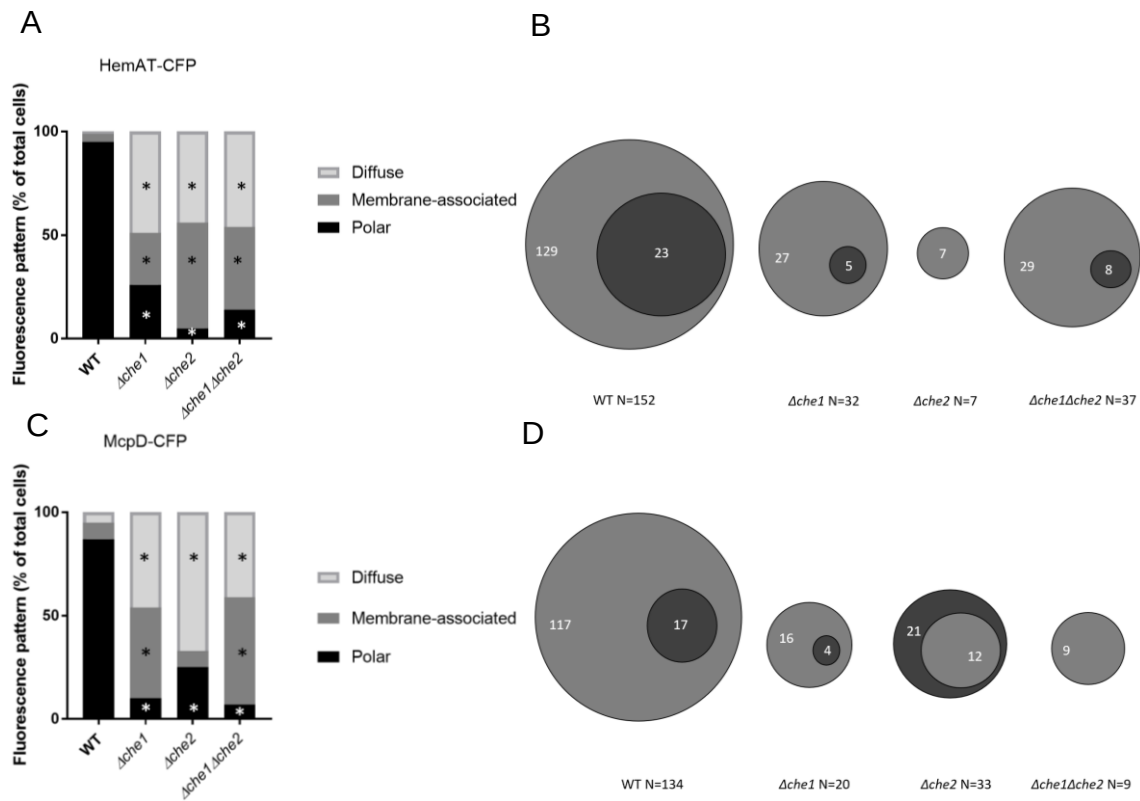


Figure 12: Fluorescent localization pattern of 36H chemoreceptors in WT and chemotaxis mutant backgrounds. (A) Percentage of HemAT-CFP cells that localize into each fluorescence category. *: P-value<0.01 compared to WT. (B) Distribution of monopolar and dipolar foci for HemAT-CFP in each background where polar foci were detected. Light gray indicates monopolar, dark gray indicates dipolar. N is the total number of cells with polar foci. (C) Percentage of McpD-CFP cells that localize into each fluorescence category. *: P-value<0.01 compared to WT. (D) Distribution of monopolar and dipolar foci for McpD-CFP in each background where polar foci were detected. Light gray indicates monopolar, dark gray indicates dipolar. N is the total number of cells with polar foci.

(Meier, V.M. and Scharf, B.E. 2009). Strong polar localization indicates that HemAT is brought to the membrane, probably through interactions with larger chemoreceptor arrays, presumably formed by other 36H chemoreceptors that are expected to rely on Che1 proteins for localization. In the $\Delta che1$ background, polar foci are found in approximately 25% of cells and the number of cells with membrane-associated fluorescence increased from 5% to 25%; HemAT-CFP thus becomes diffuse in or near the membrane when Che1 proteins are absent. Polar localization is almost abolished in the $\Delta che2$ deletion background, with 51% of cells observed to have membrane-associated fluorescence. These findings are consistent with results observed in CheA1-CFP and CheA2-CFP localization and further supports the hypothesis that polar and membrane-associated clusters are formed by both Che1 and Che2 proteins. In the $\Delta che1\Delta che2$ background, the localization of HemAT-CFP at the poles is similar to that observed in the $\Delta che1$ background while the membrane-associated localization is similar to that observed in the $\Delta che2$ background. This is consistent with both Che1 and Che2 proteins contributing to the formation of both polar and membrane-associated clusters. Polar and membrane-associated clustering remains rare in the $\Delta che1\Delta che2$ background confirming the role of both systems in the fluorescence observed.

When an orphan receptor in this study McpD-CFP (36H) is expressed in the wild-type background, 87% of cells express polar fluorescence (Figure 12, C). In the $\Delta che1$ background, fluorescence shifts to primarily membrane-associated fluorescence, similar to the localization of CheA2-CFP in wild-type cells. This pattern of localization suggests that McpD-CFP polar localization is dependent on Che1 proteins while membrane-associated fluorescent clustering depends on Che2 proteins. This is consistent with the prediction that Che1 interacts with 36H chemoreceptors. In the $\Delta che2$ background, 65% of cells have diffuse fluorescence, indicating that McpD-CFP fails to cluster into tight foci in the absence of Che2 proteins while polar and membrane-associated fluorescence is rarely observed. In the $\Delta che1\Delta che2$, most of the

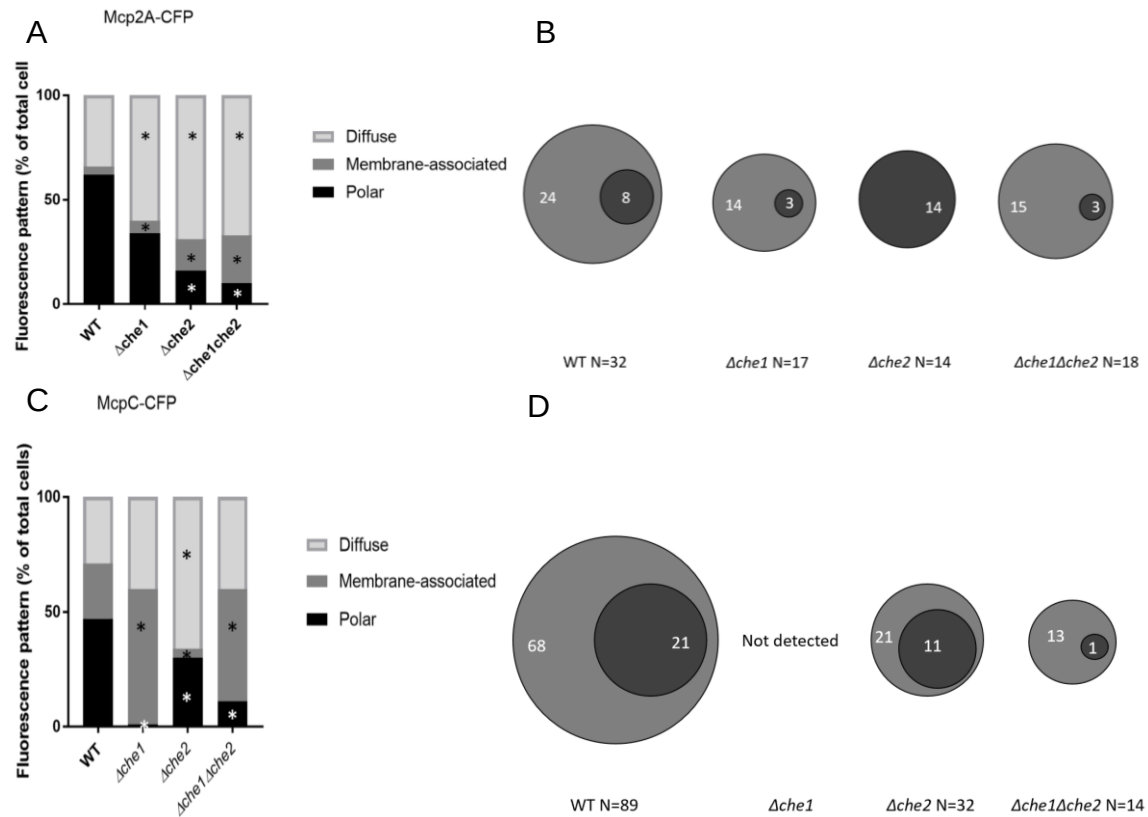


Figure 13: Fluorescent localization pattern of 34H chemoreceptors in WT and chemotaxis mutant backgrounds. (A) Percentage of Mcp2A-CFP cells that localize into each fluorescence category. *: P,0.01 compared to WT. (B) Distribution of monopolar and dipolar foci for Mcp2A-CFP in each background where polar foci were detected. Light gray indicates monopolar, dark gray indicates dipolar. N is the total number of cells with polar foci. (C) Percentage of McpC-CFP cells that localize into each fluorescence category. *: P,0.01 compared to WT. (D) Distribution of monopolar and dipolar foci for McpC-CFP in each background where polar foci were detected. Light gray indicates monopolar, dark gray indicates dipolar. N is the total number of cells with polar foci.

fluorescence observed is membrane-associated, suggesting that membrane-associated clusters assemble at random from membrane-associated chemotaxis chemoreceptors expressed in the cell. This is strikingly similar to the $\Delta che1$ background, suggesting that Che1 proteins control polar localization of McpD-CFP to the cell poles. It is important to note that fluorescence is found at both poles in most of the constructs observed in this study; therefore, change in foci localization may be more an indication of the size of the clusters changing rather than a distinct shift in localization pattern (Figure 12, D). Very small arrays are not likely to be observable with fluorescence microscopy.

When Mcp2A-CFP, a 34H chemoreceptor encoded in the Che2 operon, is expressed in the wild-type background, 60% of cells show polar fluorescence (Figure 13, A). This percentage is reduced in the $\Delta che1$ background to approximately 20% of cells containing foci at the cell pole. The percentage of cells with membrane-associated fluorescence remain about the same as is seen in the wild-type background. This suggests that the localization of Mcp2A-CFP to the cell pole depends mostly on Che1 proteins while membrane-associated fluorescent localization does not depend on these proteins. This is not consistent with the prediction that 34H chemoreceptors should interact with the Che2 system. In both the $\Delta che2$ and $\Delta che1\Delta che2$ backgrounds, polar localization is reduced even further (15% and 10% of cells respectively) and the amount of membrane-associated localization is increased (15% and 23% respectively) compared to WT. This suggests that the default localization for chemoreceptors is membrane-associated independently from Che1 or Che2 proteins. Consistent with this idea, in the wild-type, $\Delta che2$, and $\Delta che1\Delta che2$ backgrounds there is a similar ratio of monopolar to dipolar with the majority being found only at one pole, although the fraction of cells with polar localization remains low.

When McpC-CFP, a 34H orphan receptor, is expressed in the wild-type background, roughly 50% of cells show polar localization (Figure 13, C). However, in the $\Delta che1$ background, all polar localization is abolished, and the majority of cells have membrane-associated

fluorescence. This suggests that proper McpC-CFP localization to the cell poles is dependent on Che1 proteins and that the membrane-associated fluorescence observed in the WT background is due to Che2 proteins. In the $\Delta che2$ background, polar localization of McpC-CFP is significantly decreased as compared to WT. This indicates that McpC-CFP localization is somewhat dependent on Che2 proteins as well as Che1 proteins. In the $\Delta che1 \Delta che2$ background, fluorescence is primarily membrane-associated (approximately 50% of cells) with only 10% of cells containing polar localization. This suggests that the default localization pattern of McpC-CFP is membrane-associated and the presence of Che1 proteins is required to shift this localization to the cell poles. This is again not consistent with the prediction that Che2 would interact with 34H chemoreceptors. However, the localization pattern of McpC-CFP in the wild-type background is very similar to that observed in CheA2-CFP, suggesting that both Che1 and Che2 proteins are involved in localization.

Che2-associated proteins show non-canonical subcellular localization

An interesting phenomenon is observed in the localization of CheA2-CFP and Mcp2A-CFP, the two chemotaxis proteins from the Che2 operon. In the wild-type and all three deletion backgrounds, CheA2-CFP polar localization occurs as canonical foci as well as large caps of fluorescence (Figure 14). All fluorescence data for CheA2-CFP and Mcp2A-CFP is reported without the inclusion of the cap phenotype to minimize the inclusion of potentially false localization. In the wild-type background, cells with the cap phenotype make up 24% of the polar localization observed. This jumps to 43% in the $\Delta che1$ background and 69% in the $\Delta che2$ background. In the $\Delta che1 \Delta che2$ background, the percentage of capped cells is reduced to 57% which is an intermediate phenotype between the two single deletions. This phenomenon is also

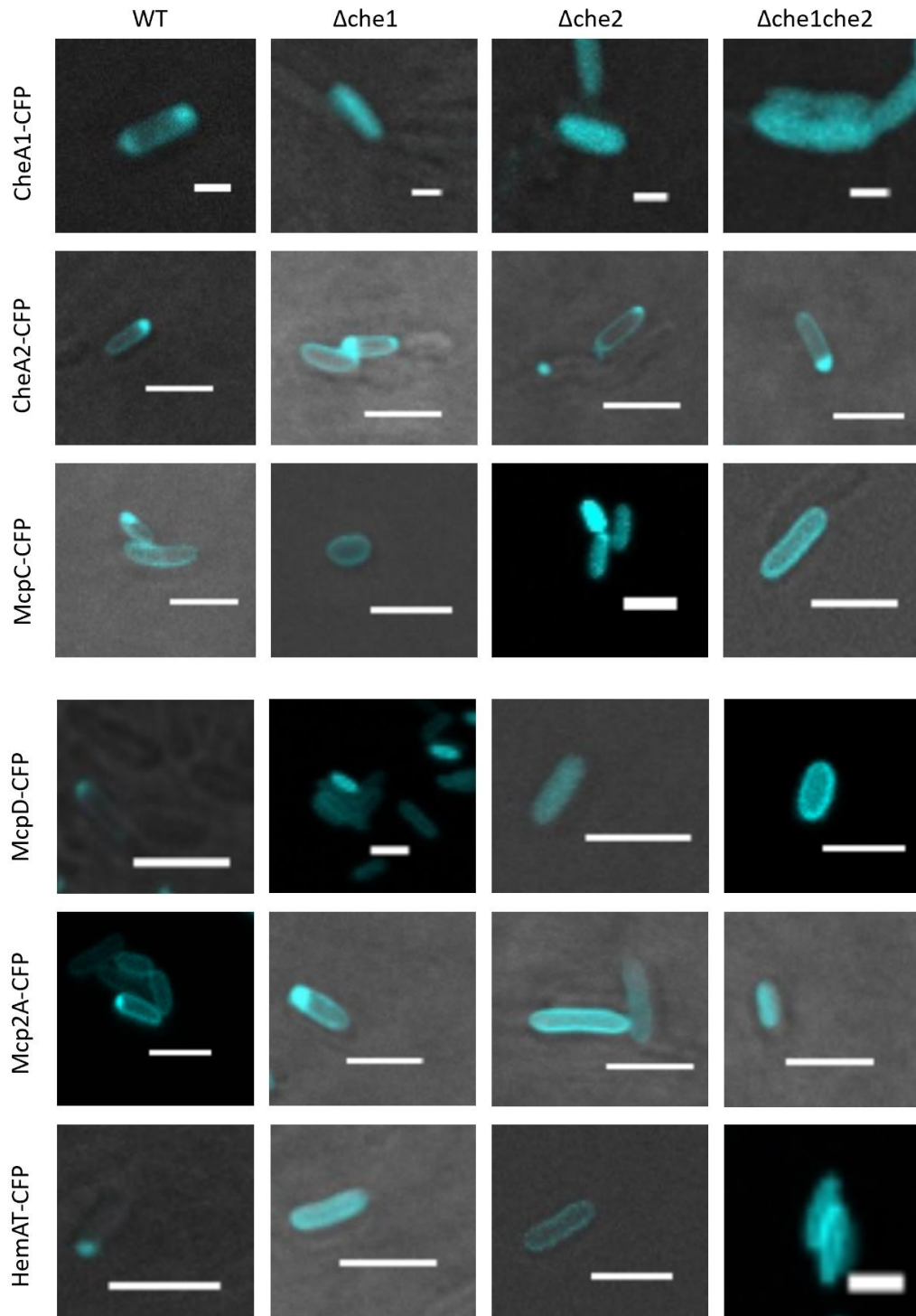


Figure 14: Representative images of each CFP fluorescent construct in WT, $\Delta che1$, $\Delta che2$, and $\Delta che1\Delta che2$ backgrounds. Scale bar represents 3 micrometers in each image.

Table 4: Statistical analysis of mutant strains from microscopy data using two-tailed Z-test. *:

P<0.01.

Name	Polar			Membrane-associated			Diffuse		
	$\Delta che1$ vs. $\Delta che2$	$\Delta che1$ vs. $\Delta che1 \Delta che2$	$\Delta che2$ vs. $\Delta che1 \Delta che2$	$\Delta che1$ vs. $\Delta che2$	$\Delta che1$ vs. $\Delta che1 \Delta che2$	$\Delta che2$ vs. $\Delta che1 \Delta che2$	$\Delta che1$ vs. $\Delta che2$	$\Delta che1$ vs. $\Delta che1 \Delta che2$	$\Delta che2$ vs. $\Delta che1 \Delta che2$
CheA1-CFP	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	*	N.S.
CheA2-CFP	N.S.	*	*	*	*	N.S.	N.S.	N.S.	N.S.
HemA-T-CFP	*	*	*	*	*	N.S.	N.S.	N.S.	N.S.
McpD-CFP	*	N.S.	*	*	N.S.	*	*	*	*
Mcp2A-CFP	N.S.	*	N.S.	N.S.	*	N.S.	N.S.	N.S.	N.S.
McpC-CFP	*	N.S.	*	*	N.S.	*	N.S.	N.S.	N.S.

observed for Mcp2A-CFP localization in the $\Delta che1$ background, with 60% of cells having large caps. However, this is the only background in which Mcp2A-CFP has this phenotype.

The capping fluorescence is not observed in the proteins from the Che1 operon or the orphan receptors; therefore, it appears to be unique to proteins encoded by the Che2 operon. This could be due to large amounts of protein located at the pole, making polar foci appear much larger. However, it is perhaps more likely that this fluorescence is representative of inclusion bodies being pushed toward the poles by the bulky chromosome that takes up most of the intracellular space in the middle of the cell (Badrinarayanan, A., Le, T.B., and Laub, M.T. 2015). This should be investigated by western blotting of membrane and cytosolic fractions for the presence of fluorescently labeled protein. If confirmed, this would suggest that Che2 proteins do not form stable foci at any location within the cell.

In addition to the capping phenotype, CheA2-CFP is often found to be localized as membrane-associated fluorescence. To observe fluorescence signal, CheA2-CFP must be clustered at the cell membrane, most likely clustering with membrane-bound chemoreceptors that are not localized to the cell pole. This is not expected for CheA proteins; not being integrated into the clusters at the cell pole would be expected to result in diffuse localization of these proteins. This is not observed in Mcp2A-CFP suggesting that this is not a phenotype consistent across the Che2 operon. However, McpC-CFP in the wild-type does show a large fraction of cells with membrane-associated localization. Because McpC-CFP is completely localized in a membrane-associated manner in the absence of Che1 proteins, it is possible that CheA2-CFP is able to sequester some 34H chemoreceptors out of the arrays at the cell pole.

***In vitro* interactions of chemoreceptors with CheA1 and CheA2 proteins**

Based on the fluorescent microscopy data described above, it appears that chemoreceptor clusters are present at both cell poles, with clusters associating with CheA1 more abundant or

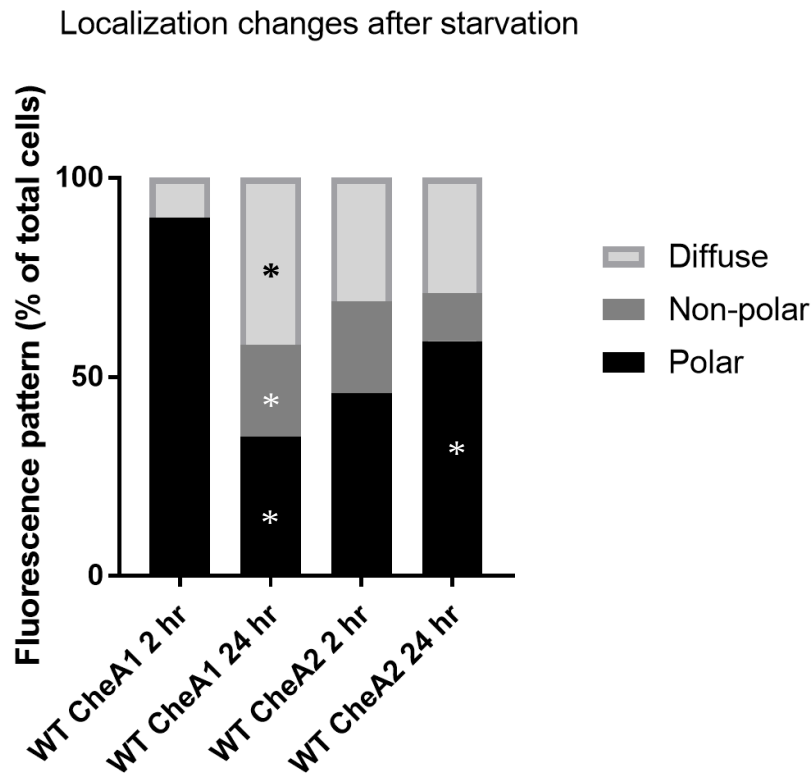


Figure 15: Changes in fluorescence localization of WT(CheA1-CFP) and WT(CheA2-CFP) after starvation on low melting point agarose. Fluorescent constructs were starved on nutrient limiting low melting point agarose pads for two or twenty-four hours. *: $P < 0.01$.

Table 5: Results of B-galactosidase assays. **: P< 0.0005 (strong interaction), *: P<0.05 (weak interaction), NI: no interaction.

T25 18C	CheA1	McpD (36H)	HemAT (36H, soluble)	CheA2	Mcp2A (34H)	McpC (34H)
CheA1	**	**	**	**	**	**
McpD (36H)	*	NI	*	NI	NI	NI
HemAT (36H, soluble)	**	*	*	NI	NI	NI
CheA2	**	NI	NI	*	**	*
Mcp2A (34H)	**	NI	NI	*	*	NI
McpC (34H)	**	*	NI	NI	**	*

larger than clusters associating with CheA2. Both Che1 and Che2 proteins seem to be essential for observing polar foci. It also appears that CheA2 localizes equally to the cell pole and elsewhere on the membrane. Furthermore, reduced polar localization of some proteins in the mutant backgrounds correlate with increased membrane-associated localization, suggesting these subcellular localizations are functionally related. One potential explanation competition for integration into clusters located at the cell pole between CheA1 and CheA2. Interestingly, when CheA1-CFP and CheA2-CFP are starved for 24 hours on agarose pads rather than 2 hours, CheA1-CFP is observed to localize to the cell pole in only 35% of cells while CheA2-CFP polar localization is observed in 60% of cells (Figure 15), suggesting these conditions stabilize CheA2-dependent polar clusters. This localization pattern could suggest that CheA1-CFP begins to degrade when cells are starved. Consistent with this hypothesis, the expression of Che1 proteins decreases in root nodules when cells are not growing (Yost, C.K., del Bel, K.L., Quandt, J., and Hynes, M.F. 2004). Interestingly, $\Delta che2$ mutations do not have chemotaxis behavior under conditions of sustained growth (Miller et al 2007). Further work should examine if mutations in Che2 affect chemotaxis under conditions where cells are starved.

Our data thus far indicate that both Che1 and Che2 protein localization to the cell poles may depend on proteins from both clusters. To further evaluate the interactions of CheA1, CheA2, and the selected subset of chemoreceptors, we utilized the Bacterial Two-Hybrid assay, which is optimized for analyzing membrane protein interactions. In this assay, protein-protein interactions lead to the reconstitution of the catalytic domain of adenylate cyclase (*cya*) from *Bordetella pertussis* which then produces cAMP and thus leads to the activation of expression of the *lac* operon. *E. coli* strains in which the adenylate cyclase is functional as a result of positive interactions of proteins of interest will be able to utilize lactose as a carbon source. This can be quantified by measuring the activity of the β -galactosidase enzyme, which breaks down lactose into monosaccharides.

Interactions between CheA and chemoreceptors are required for stable chemoreceptor array formation in *E. coli* (Alvarado, A., et al 2017). We first determined that proteins encoded by the same operon are able to interact, as we would expect. CheA1 and HemAT interact strongly, as do CheA2 and Mcp2A (Table 5). In addition, CheA1 and CheA2 strongly interact, supporting the subcellular localization data in which we determined that localization of CheA1-CFP and CheA2-CFP depend on both Che1 and Che2 proteins. In contrast, the chemoreceptors tested interacted in a distinctive pattern. The 36H receptors HemAT and McpD strongly interact with CheA1 but not with CheA2 or the 34H receptors. However, the 34H receptors Mcp2A and McpC were able to interact with both CheA1 and CheA2 but not with the 36H receptors. Because 36H and 34H receptors do not interact within this assay, there appears to be two spatially distinct chemoreceptor arrays within the cell: one assembled through 34H chemoreceptors and one from the 36H chemoreceptors. Furthermore, CheA1 is likely found in both arrays as it can interact with all proteins tested while CheA2 is likely to be found only in the array containing 34H receptors.

Model developed from fluorescence and interaction data

Based on subcellular fluorescent localization patterns and *in vitro* protein-protein interactions, the following preliminary model has been developed (Figure 16 and 17). Based on the fluorescence data discussed previously, chemoreceptor arrays are found at both cell poles. Protein-protein interactions suggest that one array contains a mixed baseplate of CheA1 and CheA2 proteins and the other array seems to exclude CheA2 in favor of CheA1. This is consistent with the fluorescence pattern observed. CheA1-CFP is observed exclusively at the cell poles in the wild-type background while CheA2-CFP is found equally at the cell poles and on the lateral cell membrane as membrane-associated localization. The CheA2-CFP that are localized in a membrane-associated manner are likely clustering with 34H chemoreceptors embedded in the cell

membrane. While the BacTH data suggests a sharp division between 34H and 36H chemoreceptors in regard to their interactions with the CheA proteins, the fluorescence localization suggests that all four chemoreceptors tested are dependent on both chemotaxis operons for localization to the cell pole. 36H chemoreceptors HemAT-CFP and McpD-CFP appear to be heavily dependent on both chemotaxis operons for polar localization, which is consistent with the *in vitro* interactions of 36H receptors with both CheA proteins. Under the conditions of these experiments, it appears that interaction with CheA1 is favored over interaction with CheA2. Mcp2A-CFP (34H) does appear to be more dependent on Che2 proteins than Che1 proteins which is in line with the interaction data, while McpC-CFP (also 34H) appears to be much more strongly influenced by the presence of Che1 proteins for polar localization than Che2. When the selected chemoreceptors in this study are not found at the cell pole, localization is often shifted to membrane-associated fluorescence rather than diffuse. Therefore, 34H chemoreceptors that are not localized to the cell pole are likely clustering with CheA2 in the cell membrane. However, CheA1 is not seen localized anywhere in the cell other than the cell pole in the wild-type. Therefore, any 36H chemoreceptors present in the membrane outside of the cell pole are likely loosely clustered with each other but it is unlikely that CheA1 or CheA2 are involved in this interaction.

It is likely that other proteins from each operon could be influencing the fluorescent localization pattern in wild-type *R. leguminosarum* cells which is not observed when testing protein-protein interactions *in vitro*. The fluorescent localization observed could also be influenced by timing or nutrient availability, with a different pattern emerging over time or in stress conditions. Localization and *in vitro* protein-protein interactions of the five CheW scaffolding proteins with the two CheAs and the chemoreceptors could help to solidify this model by giving additional resolution to how proteins behave within the baseplate. Pull-downs of interacting proteins *in vivo* would also be largely beneficial in the refining of the current model.

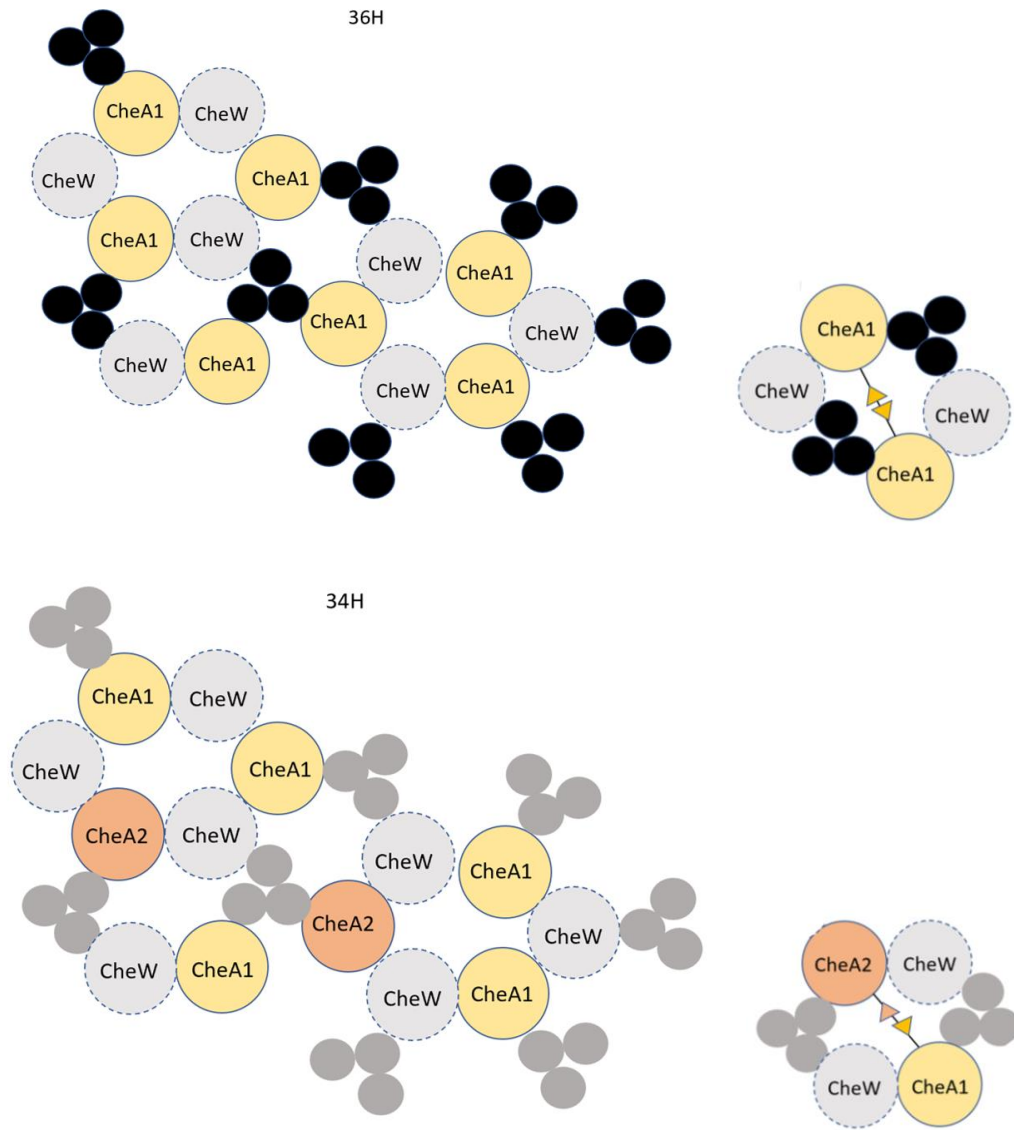


Figure 16: Proposed baseplate model for the 36H and 34H receptor clusters. The 36H cluster contains only CheA1 (yellow). The 34H cluster contains both CheA1 (yellow) and CheA2 (orange). CheW shown in gray to fill out baseplate but currently the five CheW proteins in *R. leguminosarum* are uncharacterized. Sets of three small circles represent trimers of dimers color coded based on heptad class, 34H (gray) and 36H (black). Dimerization of CheAs depicted on right.

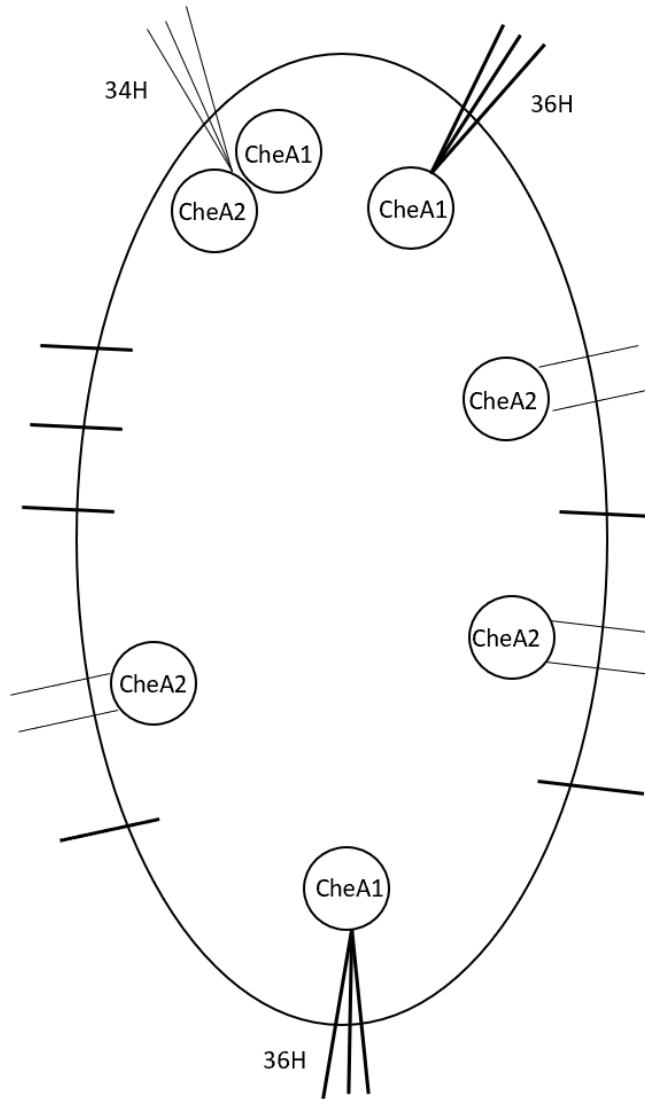


Figure 17: Model of *R. leguminosarum* localization of chemoreceptors and CheAs based on this study. 36H chemoreceptors are indicated by thick black lines. 34H chemoreceptors are indicated by thin black lines. CheA2 and receptors represented on the lateral membranes are found around the full cell. 36H chemoreceptors interact with only CheA1. 34H chemoreceptors interact with both CheA1 and CheA2.

Contribution of chemoreceptors to initial root colonization

Chemotaxis is required for competitive root colonization of *R. leguminosarum* in the rhizosphere (Miller et al 2007). This is hypothesized dependent on sensing by specific chemoreceptors of signals being exuded from plant roots (Scharf, B.E., Hynes, M.F., and Alexandre, G.M. 2016). We tested the ability of three chemoreceptor deletion mutants of *R. leguminosarum* to compete for colonization of plants with different root architecture and, presumably, root exudate patterns: pea (symbiotic host), wheat, and alfalfa. The chemoreceptor mutants available were *hemAT*, *mcpC*, and *mcpD*. *R. leguminosarum* strains were grown in rich media and allowed to colonize for five days. This is a long enough time frame that attachments to the roots can form and colonization of the root surface may take place.

After five days of colonization, *hemAT* colonized at approximately the same level as the wild-type on wheat roots (Figure 17). On alfalfa roots, *hemAT* generally colonized at the same level as the wild-type. Only on pea roots was the colonization ratio significantly different between the wild-type and *hemAT* strains, with wild-type colonizing better than *hemAT* consistently. This indicates that HemAT is important for competitive colonization on pea roots but not for alfalfa or wheat roots. HemAT is known to bind oxygen (Miller et al 2007), implicating oxygen sensing in the pre-nodulation colonization of pea roots by *R. leguminosarum*. This is interesting because low oxygen availability is a hallmark of root nodule conditions. These data also hint at the different role of chemoreceptors in various rhizospheres.

When *mcpC* was allowed to compete with wild-type for colonization of pea and alfalfa roots, both strains have an equal presence on the roots after five days (Figure 18). There is a larger level of variation in colonization of wheat roots, with several roots showing much higher amounts of *mcpC* as compared to wild-type. However, this difference was not statistically

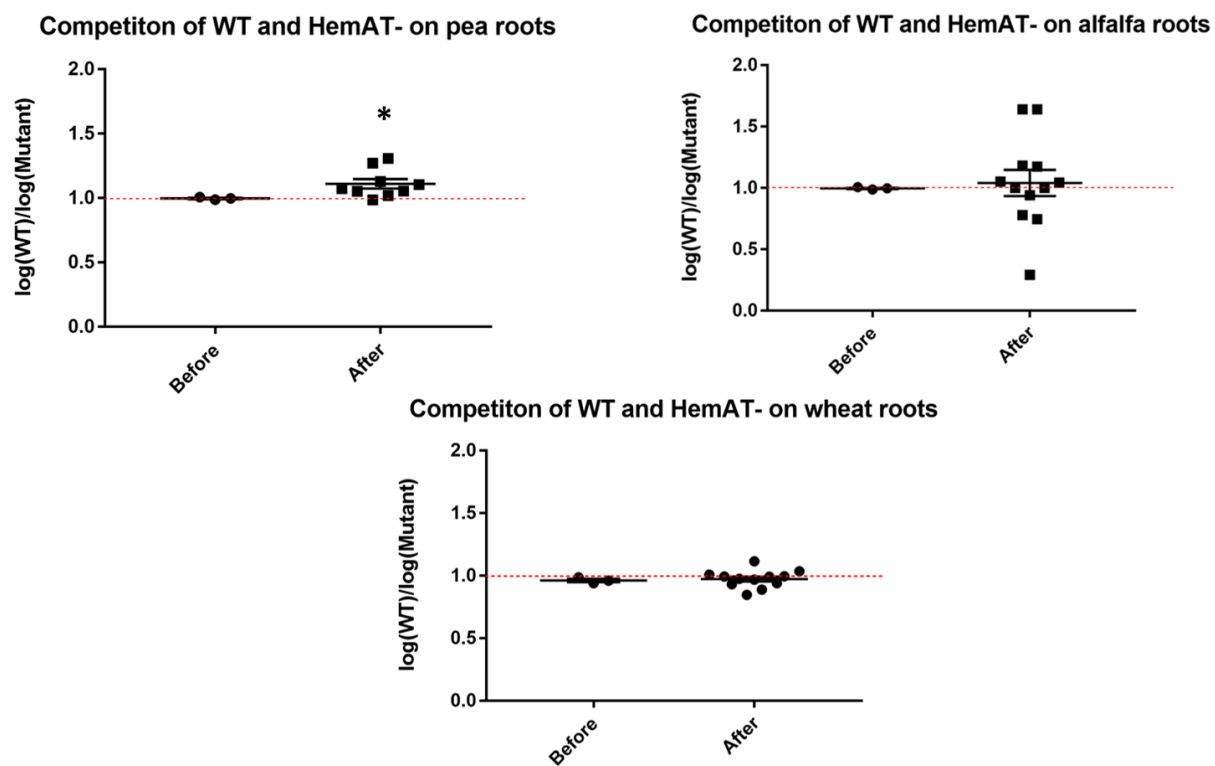


Figure 18: Competition of WT and *hemAT* on pea, alfalfa, and wheat roots. The red dotted line indicates equal ratio of WT and *hemAT*. *: $P < 0.05$ in T-test between WT and mutant colonization.

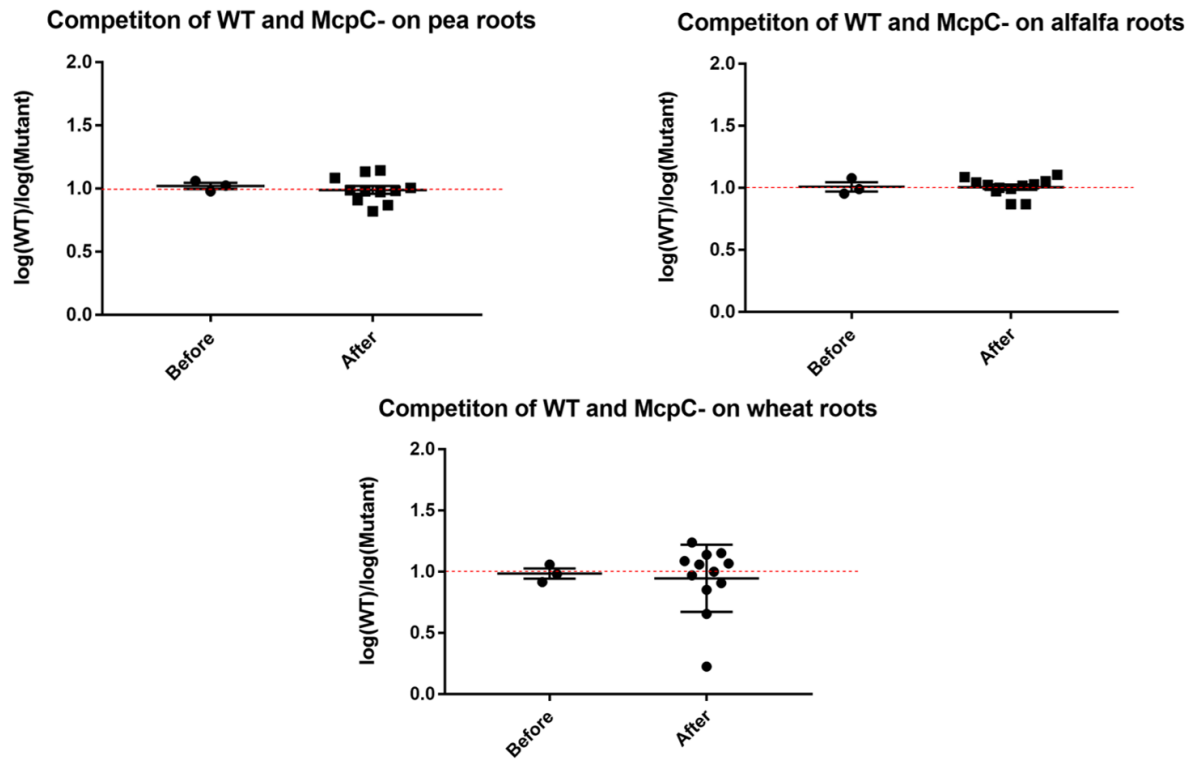


Figure 19: Competition of WT and *mcpC* on pea, alfalfa, and wheat roots. The red dotted line indicates equal ratio of WT and *mcpC*.

significant. Without any information regarding the sensing specificity of the McpC chemoreceptor, conclusions are difficult. However, these preliminary data also support the hypothesis of different roles for chemoreceptors contributing to competitive root surface colonization of different plant hosts.

After five days of colonization, *mcpD* is present in equal amounts to wild-type on pea and wheat roots (Figure 19). However, wild-type colonizes significantly better in competition with *mcpD* on alfalfa roots. This suggests that McpD is responsible for sensing a ligand that is either present in the root exudate profile of alfalfa only or in higher amounts from alfalfa.

This experimental setup does not give information of the spatial distribution of the wild-type and chemoreceptor mutant strains on different root systems. It provides a proof-of-principle that chemoreceptors may have different contribution for the root surface colonization of different plants and that differences can be detected in competitions between wild type and mutant.

Linking these patterns of rhizosphere colonization with specific ligand(s) sensed by the chemoreceptors could provide insights into the specific chemical gradients that bacteria respond to in different rhizospheres. This approach could provide a first step toward identifying effectors that modulate the colonization of *R. leguminosarum* in different rhizosphere. Fluorescent microscopy of wild-type and chemoreceptor mutants during the colonization of different plants would give additional information of the chemical effector sensed by specific chemoreceptors.

CHAPTER IV. CONCLUSIONS

In this study, we have established that *R. leguminosarum* contains two chemotaxis clusters, one of which contains a mixed baseplate of CheA1 and CheA2 proteins. These clusters are separated in the membrane based on the heptad class of the chemoreceptors involved. This is a similar organization to that found in *A. brasilense*, although *A. brasilense* contains a mixed baseplate in all chemotaxis clusters studied. *R. leguminosarum* and *A. brasilense* are genetically distant species within the α -proteobacteria family; therefore, this shared organization could point to a conserved mechanism for integrating multiple chemotaxis systems within the same living environment, the rhizosphere. However, it is unusual that not all chemotaxis clusters in *R. leguminosarum* contain a mixed baseplate. This could be a limitation of the assays utilized in this study and will need to be confirmed *in vivo* through protein pulldowns using CheA2 as bait. We have also provided preliminary evidence of potential competition between CheA1 and CheA2 for integration into the baseplate. This has not been seen previously in chemotactic species studied to date and it is unclear why this phenomenon is occurring. Another interesting aspect of chemotaxis in *R. leguminosarum* that has not been seen in other species is the tendency for fluorescent localization to be observed across the cell membrane rather than just at the cell poles or diffuse throughout the cell in the absence of chemotaxis proteins. This could point to an additional protein other than CheA and CheW that is involved in holding chemoreceptors in clusters. However, the protein ParP that has been established to serve this role in other species is distinctly absent in *R. leguminosarum*. A putative protein domain to fill this function in *R. leguminosarum* is the Sulfate Transporter and Anti-Sigma factor antagonist (STAS), which is a domain found in a family of scaffolding proteins. There is a STAS domain containing protein present in both chemotaxis operons of *R. leguminosarum* that is not deleted in any of the deletion mutants utilized in this study and can be investigated in this capacity in the future.

Organization of chemoreceptors and integration within chemotaxis clusters impacts the ability of chemoreceptors to pass on information pertaining to the resources available in the environment. We have established that removing a single chemoreceptor from the cell alters the behavior of *R. leguminosarum* within the rhizosphere. This study particularly looked at root colonization behavior, but it is likely that many of the chemoreceptors present within the cell influence free-living navigation through the soil. Therefore, understanding both the organization and ligand specificity of chemoreceptors will give clues as to how bacteria navigate their environment. Several chemotactic bacteria are being developed as commercial fertilizers. Therefore, understanding of how the chemotaxis signaling cascade functions allows for the possibility of manipulation of the system to recruit bacteria for beneficial purposes.

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VITA

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