

***Azospirillum brasilense* Signaling via PilZ-containing Chemotaxis Receptors
and
Chemotaxis Protein Paralogs**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**Lindsey Danielle O'Neal
May 2019**

Copyright © 2019 by Lindsey O’Neal.
All rights reserved.

DEDICATION

To all the people who have helped me along the way.
Especially those who provided support when they did not understand what I was doing or why I
was doing it.

ACKNOWLEDGEMENTS

This work would not have been possible without the unending support and patience of many, many people. If you are reading this page, I owe you a debt of gratitude I will never be able to repay.

First and foremost, I'd like to thank my husband and best friend, Sean Yoder. You have been with me since the beginning of this journey, and your support and love has never wavered. Thank you.

I also owe a massive thank you to Sadie. You always make sure I get daily walks, see the sunshine, and take breaks from science. So, thank you for your tail wags, sassy barks, and cuddles.

To Mike, Mom, Lacey, Austin, my grandparents, my numerous cousins and aunts and uncles: Thank you for your love and support. You always remind me to laugh, have fun, and where home is. You constantly remind me that I am kind, smart, and important even when science tells me otherwise. It means so much you support my passion, even when you don't understand it.

Jim, Denise, and Sarah: Thank you for always giving me a home away from home and helping me to achieve this dream. Thank you for your love and kindness. I couldn't ask for better in-laws.

Gladys, my fearless leader, you saw potential in me when I couldn't see it myself. Thank you for your patience and support. You have trained me to think critically and to love science. I could not have achieved this dream if you hadn't taken a chance on me 5 years ago.

To the rest of my committee, Brad Binder (my taco buddy), Liz Fozo, Liz Howell, and Jenny Morrell-Falvey: Thank you for giving me your time. I know that it is so precious, and all of you always made time for my questions, my grueling committee meetings, and my never-ending e-mails. You played an essential role in shaping me into the scientist I am today.

To my longest mentor, Angelia Gibson. You have known me for 10 years, first as a student and now as a friend. You helped get me through Intro Chemistry, Biochemistry, Molecular Biology, Senior Thesis, and Grad School. Thank you for the time, love, and encouragement you gave to me. When I grow up, I hope I have half the heart you do.

A lot of this work would not have been possible without some absolutely amazing undergrads. Shehroze and Zach, you always came into lab excited and ready to learn. You made science fun and tried to keep me up to date on all the latest trends. So thank you for helping me to achieve my dream and letting me be a small part of your journey. Part of this degree is because of you.

Finally, to my lab members, both past and present: Amber, Anastasia, Jessica, Kathleen, and Maddison. You made grad school fun (and bearable)! Each of you got me through the hard days and helped me to achieve this. We understand each other in a way that comes only from working together in science. Thank you for your help and being with me every step of this journey. I am so lucky to have been surrounded by such smart, strong, compassionate women. Go rock the STEM world!

ABSTRACT

Chemotaxis is the biased movement of bacteria in response to environmental stimuli. Chemotaxis is initiated with dedicated receptors that sense environmental signals, and these signals are transduced through a phosphorylation cascade that can influence flagellar rotation and swimming direction. Most knowledge of chemotaxis signal transduction comes from studies of *Escherichia coli*, which possesses a simple system consisting of five chemotaxis receptors and a single chemotactic signaling pathway. Unfortunately, *E. coli* is not representative of the diversity of chemotaxis signal transduction systems revealed by the analysis of completely sequenced bacterial genomes. Most sequenced bacterial genomes to date suggest most motile bacteria able of chemotaxis rely on at least two distinct chemotaxis signaling systems and a large number (>20) chemotaxis receptors. This dissertation focuses on the plant growth promoting alphaproteobacterium *Azospirillum brasilense* as a representative model for chemotaxis. The genome of *A. brasilense* encodes four chemotaxis operons and fifty-one chemotaxis receptors. Here we show that chemotaxis paralogs encoded in two of these chemotaxis systems, Che1 and Che4, interact to produce two functional chemotaxis signaling arrays segregated by chemotaxis receptor lengths. One of these arrays contains a subclass of receptors that possess an additional C-terminal domain and comprise the PilZ-containing chemotaxis receptors. In bacteria, the C-terminal PilZ domain was shown to bind the secondary messenger cyclic diguanylate monophosphate which affects enzyme activity, gene expression and protein-protein interactions. Here we develop a novel real time tool (slide-in-chamber) and assay (root-in-pool) for monitoring the role of *A. brasilense* chemotaxis and PilZ-containing chemotaxis receptors (Tlp1 and Aer) in bacterial behavior and colonization of wheat rhizospheres. We found that chemotaxis is essential

for *A. brasilense* to sense and subsequently colonize specific zones of wheat roots. *A. brasilense* exhibits an attractant response to the root hair and elongation zones of wheat and is repelled from the wheat root tip, and this response is dependent upon functional PilZ receptors Tlp1 and Aer. Furthermore, cyclic diguanylate monophosphate binding to Tlp1 and Aer is essential for bacterial response to roots. Taken together, these data reveal a new role for cyclic diguanylate monophosphate in modulating sensitivity to complex gradients.

TABLE OF CONTENTS

INTRODUCTION	1
<i>Escherichia coli</i> Chemotaxis signal transduction	1
Chemoreceptor structure and function.....	4
Receptor array structure and function.....	6
Chemoreceptor diversity	6
Chemotaxis Operon and Protein Diversity	11
Chemotaxis in the Rhizosphere	14
<i>Azospirillum brasilense</i> chemotaxis	15
<i>A. brasilense</i> chemotaxis receptors.....	15
C-di-GMP and Chemotaxis.....	17
References	19
CHAPTER I Distinct chemotaxis protein paralogs assemble into chemoreceptor arrays to coordinate signaling output.....	32
Abstract	33
Introduction.....	34
Materials and Methods.....	35
Strains, media, and chemicals.....	35
Electron Cryotomography.....	42
Fluorescence Microscopy	42
Bacterial two-hybrid assay.....	44
Protein pulldown.....	47
Sequence similarity and Conserved Residues.....	48
Measuring the height of chemoreceptor arrays.....	49
Bioinformatics datasets and analysis	50
Results.....	50
Cryo-EM reveals two distinct chemoreceptor arrays in <i>A. brasilense</i>	50
Assignment of chemoreceptors encoded in the <i>A. brasilense</i> genome to arrays of specific height.....	51
Localization of chemotaxis proteins depends on Che1 and Che4	53
Che1 and Che4 chemotaxis proteins interact with each other in the bacterial two-hybrid assay	57
Relative contribution of CheA4 and CheA1 to clustering of chemotaxis receptors into polar arrays.....	57
Contribution of Che1 and Che4 proteins, other than CheA paralogs, in clustering of chemotaxis receptors.....	62
Discussion	64
References.....	71
CHAPTER II Optogenetic Manipulation of Cyclic Di-GMP (c-di-GMP) Levels Reveals the Role of c-di-GMP in Regulating Aerotaxis Receptor Activity in <i>Azospirillum brasilense</i>	81
Abstract	82
Introduction.....	83
Materials and Methods.....	86

Bacterial Strains and Growth Conditions	86
Plasmid construction	89
Transfer of plasmid-borne light activated constructs into <i>A. brasilense</i>	89
Quantification of c-di-GMP by mass spectrometry	90
Free-swimming cells behavior and computerized assisted motion tracking	91
Spatial gradient assay for aerotaxis and density analysis	91
Temporal gradient for aerotaxis and swimming pattern analysis	92
Cell clumping and aggregation analysis	93
Statistical analysis of data	93
Results	93
The optogenetic system permits light dependent regulation of intracellular c-di-GMP levels	93
Optogenetic manipulation of c-di-GMP metabolism affects aerotaxis	97
C-di-GMP levels does not serve as an intracellular attractant or repellent	101
C-di-GMP levels modulate the ability of cells to detect the preferred oxygen concentration in a gradient	102
Altering c-di-GMP levels affects the time to respond and adapt to an oxygen gradient	106
Binding of c-di-GMP to a chemoreceptor, Tlp1, modulates its contribution to the aerotaxis response	108
c-di-GMP levels change with carbon metabolism.	111
Discussion	115
References	120
CHAPTER III A PilZ Containing Chemotaxis Receptor Mediates Oxygen and Wheat Root Sensing in <i>Azospirillum brasilense</i>	128
Abstract	129
Introduction	130
Materials and Methods	132
Bacterial strains growth and maintenance	132
Identification of genes encoding putative chemotaxis receptors with additional domains and PAS sequence alignment	132
Generation of markerless deletion of aer	134
Functional complementation of the Δ aer mutation	134
FAD quantification	136
Chemotaxis soft agar plate assays	136
Spatial gradient assay for aerotaxis	136
Root-in-pool assay	137
Wheat root colonization assay	138
Statistical analysis	138
Results	139
Identification of chemotaxis receptors containing PilZ domains	139
<i>A. brasilense</i> Aer PilZ domain is essential for aerotaxis	141
Aer contributes to competitive root surface colonization	143
Aer signaling is integrated with that of other chemotaxis receptors in a PilZ-dependent manner	145

Discussion	149
References	152
CHAPTER IV Monitoring Plant-Microbe Interactions in Real Time Reveals Role of <i>Azospirillum brasilense</i> Chemotaxis in the Rhizosphere.....	157
Abstract	158
Introduction.....	159
Materials and Methods.....	160
Bacterial Growth and Maintenance	160
Generation of GFP expressing <i>A. brasilense</i>	164
Germination of <i>T. aestivum</i> and <i>M. sativa</i>	164
Root-in-pool assay	164
Slide-in-chamber dimensions and assembly	165
Fluorescent Microscopy and image analysis	166
Long-term colonization.....	166
Root exudate collection and analysis	167
Chemical-in-plug assay.....	167
ROS exposure	168
Hydrogen peroxide zone of inhibition	168
Results.....	168
Slide-in chamber allows real time monitoring of plant-microbe interactions	168
Chemotaxis controls <i>A. brasilense</i> colonization of wheat	170
A chemoreceptor, Tlp1, drives wheat root sensing.....	174
C-di-GMP levels mediate response to hydrogen peroxide	183
Chemotaxis is beneficial in other plant-microbe interactions.....	185
Discussion	187
References	192
CONCLUSION.....	198
References	207
APPENDIX.....	211
Supplemental Dataset 1- 38H Receptors	212
Supplemental Dataset 2- 36H Receptors	253
VITA	255

LIST OF TABLES

Table 1: Strains and plasmids used in this study	38
Table 2: Primers used in this study	45
Table 3: Strains and plasmids used in this study	86
Table 4. Effects of temporal increases in c-di-GMP concentration on swimming behavior	102
Table 5: Effects of temporal decreases in c-di-GMP concentration on swimming behavior	102
Table 6. Effect of c-di-GMP on the adaptation time of <i>A. brasilense</i> to temporal changes in aeration.....	105
Table 7: List of strains used in this study	133
Table 8: Primers used in this study	135
Table 9: Strains and plasmids used in this study	161
Table 10: Chemicals identified in wheat exudates.	178

LIST OF FIGURES

Figure 1: <i>E. coli</i> chemotaxis signal transduction pathway.	3
Figure 2: <i>E. coli</i> chemotaxis receptors.....	5
Figure 3: Composition of chemoreceptor trimer of dimers and array	7
Figure 4: Multiple bacterial species form hexagonally packed receptor arrays with 12nm spacing	8
Figure 5: Diversity of chemotaxis receptors	10
Figure 6: <i>Azospirillum brasilense</i> encodes 4 chemosensory operons.....	16
Figure 7: <i>A. brasilense</i> PilZ containing receptors.....	18
Figure 8: <i>A. brasilense</i> encodes 2 chemotaxis operons and 2 membrane bound receptor arrays.	36
Figure 9: CheA2 is important for <i>A. brasilense</i> flagellation.....	37
Figure 10: Localization of YFP-tagged CheA proteins	55
Figure 11: Subcellular localization for YFP-tagged chemotaxis proteins encoded in Che1 and Che4	56
Figure 12: CheA2 and CheA3 involvement in chemotaxis array formation	58
Figure 13: Interaction of chemotaxis proteins from Che1 and Che4 in the bacterial two-hybrid assay	59
Figure 14: Role of chemotaxis proteins in localization of YFP-tagged chemoreceptors	61
Figure 15: Interaction of chemotaxis proteins from Che1 and Che4 with Tlp1 and Tlp4a	63
Figure 16: Interaction of chemotaxis proteins in pull down assays.....	65
Figure 17: Sequence alignments between <i>A. brasilense</i> chemotaxis paralogs and <i>E. coli</i> chemotaxis homologs.....	68
Figure 18: Domain architecture of Tlp1 and dichromatic optogenetic system for manipulating c-di-GMP levels in proteobacteria	85
Figure 19: Light-mediated changes in intracellular c-di-GMP concentrations in cells carrying pRed-DGC or pBlue-PDE.....	96
Figure 20: Light mediated real-time changes in c-di-GMP after aerotaxis band has formed.....	98
Figure 21: Light mediated reduction in c-di-GMP levels and cell-cell clumping and aggregation of the chsA mutant strain	100
Figure 22: Effects of light-mediated changes in c-di-GMP levels on aerotaxis in spatial oxygen gradients.....	104
Figure 23: Role of Tlp1 c-di-GMP binding in mediating changes in sensitivity to oxygen gradients upon optogenetic manipulation of c-di-GMP levels.....	110
Figure 24: Effect of carbon source-mediated changes in c-di-GMP levels on the formation of an aerotactic band.	114
Figure 25: <i>A. brasilense</i> encodes a chemotaxis receptor with a putative N-terminal FAD-binding PAS domain and a C-terminal PilZ domain	140
Figure 26: Effect of loss of Aer on oxygen sensing in a spatial assay for aerotaxis	142
Figure 27: Chemotaxis in a soft agar plate containing 10mM malate	144
Figure 28: Colonization efficiency of wheat seedling roots by <i>A. brasilense</i> wild type and mutant strains derivative lacking Aer and expressing an empty vector control, Aer or Aer Δ PilZ.	146
Figure 29: <i>A. brasilense</i> accumulation around wheat roots using a root in pool assay	147
Figure 30: The slide-in chamber for observing plant-microbe interactions in real time.	169

Figure 31: Accumulation and colonization of WT and nonchemotactic <i>A. brasilense</i> around wheat roots in the slide-in chamber.....	171
Figure 32: WT and chemotaxis null strains in the root-in-pool assay with intact roots of wheat root seedlings.....	173
Figure 33: $\Delta tlp1$ free swimming response and colonization of wheat roots.....	175
Figure 34: <i>A. brasilense</i> response to organic acids.....	177
Figure 35: Metabolomics profiles of wheat root exudates collected in the presence of absence of WT <i>A. brasilense</i>	180
Figure 36: The role of PilZ chemotaxis receptors in responding to reactive oxygen species and roots.....	182
Figure 37: The influence of intracellular c-di-GMP levels on the chemotaxis repellent response to hydrogen peroxide in a spatial gradient assay	184
Figure 38: Accumulation and colonization WT and chemotaxis null strains around alfafa roots	186
Figure 39: Free swimming and colonization behavior of motile environmental isolates with wheat roots.....	188
Figure 40: Model of <i>A. brasilense</i> chemotaxis signal transduction.....	199
Figure 41: C-di-GMP affects chemoreceptor signaling state	202

INTRODUCTION

Bacteria live in diverse, dynamic environments. To survive, bacteria must sense changes in the environment. When changes are sensed, bacteria may respond by adjusting gene expression, enzyme activities and/or protein-interactions (1). Motile flagellated bacteria adjust their swimming patterns using chemo- (chemical) taxis (motility) (2). Chemotactic bacteria sense environmental chemicals via dedicated chemotaxis receptors (or chemoreceptors) that directly bind to the cues, to cue-bound proteins, or indirectly, to cofactors that detect changes in the cell's metabolic state that the cue(s) causes. Motile bacteria are too small to detect chemical gradients through the cell, and instead detect temporal changes in the concentration of chemicals as they move through their environment (3). Beneficial chemicals (chemoattractants) cause the bacterium to bias movement up the chemical gradient, while detrimental cues (chemorepellants) cause the cell to change swimming direction more frequently and bias their motility away from the cue.

***Escherichia coli* Chemotaxis signal transduction**

The molecular mechanisms of chemotactic signaling is best-studied in the model *Escherichia coli*. In *E. coli*, when a chemoreceptor binds to a repellent, the signal is transmitted to a histidine kinase (CheA) that becomes phosphorylated using ATP (4). The signal transduction between the receptors and CheA is aided by CheW, a coupling protein that links CheA to the receptors (5). CheA-P can phosphorylate two distinct response regulators, CheY and CheB, with different outcomes. Phosphorylation transfer to CheY initiates signaling to the flagellar motor (4). CheY-P has an increased affinity (when compared with CheY) for the switch component, FliM, of the flagellar motor and upon interacting, causes a change in direction of rotation of the motor, which

changes the cell's swimming direction (known as a tumble) (6). If an attractant binds to a chemoreceptor, CheA phosphorylation is inhibited, the phosphorylation cascade is not initiated, and tumbles are suppressed (Figure 1).

Phosphorylation transfer from CheA-P to CheB increases the methylesterase activity of CheB (7). CheB-P deamidates/ demethylates chemotaxis receptors at specific glutamate residues countering the constitutive methyltransferase activity of CheR (8-10) and re-setting chemotaxis receptors (Figure 1). Increased methylation of chemoreceptors increases the rates of CheA phosphorylation, while lower levels of methylation inhibit CheA phosphorylation. Methylation and demethylation happen much more slowly than phosphorylation and thus confer the chemotaxis signaling system with a “memory” (11-13). If repellants bind to chemoreceptors, CheY-P and CheB-P increase, causing an increase in the cell tumbling frequency and an increase in receptor demethylation (which is slightly delayed). These events decrease the probability of CheA activation by dephosphorylation which causes cells to return to the pre-stimulus tumbling bias (14). If attractant levels are high, CheY-P and CheB-P levels decrease, receptor methylation increases, and pre-stimulus swimming behavior returns (11). If no chemical gradient is present, CheR and CheB activity generate an ambient level of CheY-P, which causes random runs and tumbles allowing the cell to explore the environment.

CheZ, a phosphatase, terminates the signal by dephosphorylating CheY (Figure 1) (15, 16). This phosphorylation cascade leads to a biased walk up attractant gradients or away from repellant gradients (5).

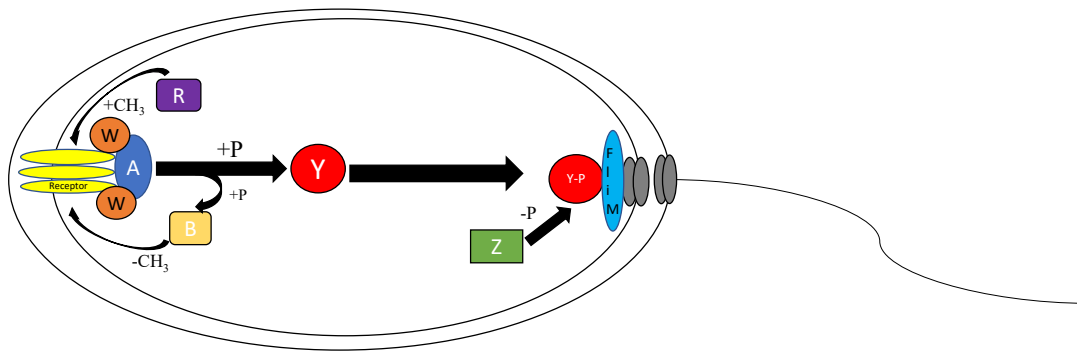


Figure 1: *E. coli* chemotaxis signal transduction pathway.

Membrane bound chemotaxis receptors are shown in yellow. The chemotaxis coupling protein CheW is shown in orange. The histidine kinase, CheA, is in blue. The receiver domain protein, CheY is red, and the flagellar motor switch protein, FliM, is shown in blue. CheR (purple) adds methyl groups to the chemoreceptors, while phosphorylated CheB (yellow) de-methylates receptors. CheZ (green) can reset the system by dephosphorylating CheY.

Chemoreceptor structure and function

Chemotaxis is initiated with chemotaxis receptors, also known as Methyl-accepting Chemotaxis Proteins (MCP), that sense attractants and repellants. MCPs have an N-terminal domain that varies greatly in sequence composition and typically contain a ligand bind region. Ligand binding domains are diverse, which reflects their ability to recognize a variety of cues. *E. coli* has 5 MCPs that are responsible for signaling: Tar, Tsr, Tap, Trg, and Aer (Figure 2). All of the MCPs in *E. coli* are membrane bound and each MCP has different sensory specificity. Tsr, Tar, Tap, and Trg contain periplasmic ligand binding domains that sense serine, aspartate, dipeptides, and ribose respectively (18-21). Aer is involved in oxygen sensing and is the only *E. coli* MCP with a cytoplasmic ligand binding domain (20, 22). MCPs also possess a highly conserved C-terminal signaling region that interacts with downstream chemotaxis proteins (23-29). This signaling region is an alpha helical coiled-coil that contains a methyl-accepting domain (MA), a flexible bundle region, and a conserved tip that binds the chemotaxis proteins CheA and CheW (26-29).

The propagation of signal from the ligand binding domain through the receptor has been studied extensively in the Tar and Tsr chemoreceptors of *E. coli*. Many membrane bound receptors have an additional domain called the HAMP domain that connects the input (LBD) domain to the output (signaling) domain (30). Ligand binding to Tar or Tsr causes a piston-like downward movement of one of the transmembrane domains, which changes the packing of the highly flexible HAMP domain (named for histidine kinases, adenyl cyclase, methyl-accepting proteins, and phosphatases) (31-33). The change in HAMP domain structure is propagated down the receptor to

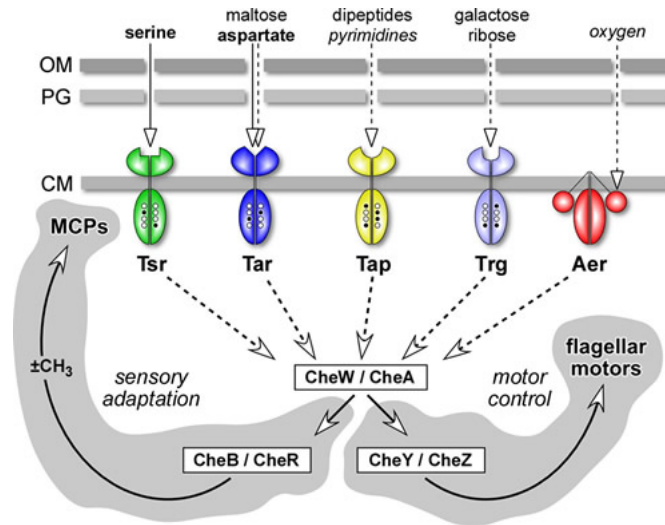


Figure 2: *E. coli* chemotaxis receptors

The five chemotaxis receptors (MCPs) Tar, Tsr, Tap, Trg, and Aer are able to sense specific cues and interact with chemotaxis machinery to signal to the flagella. Adapted from (17).

the conserved tip that interacts with CheA and CheW, and eventually causes a change in the phosphorylation state of CheA.

Receptor array structure and function

MCPs come together with CheA and CheW to form a trimer of dimers. CheA and CheW form rings that interact with the signaling region of the multiple trimers of dimers and form extensive receptor arrays that are commonly found at the cell poles (Figure 3) (34). These highly ordered arrays amplify the chemotaxis signal through receptor cooperativity (35, 36). All *E. coli* chemoreceptors are present in the array, but Tar and Tsr are the most abundant receptors (37). Array formation in *E. coli* is dependent upon the presence of CheA and CheW demonstrating that receptor-receptor interactions are not sufficient to form functional chemotaxis arrays (37). Cryo-EM has revealed a universal array architecture in chemotactic bacteria: the arrays pack in uniform honeycomb lattice structures with 12nm spacing (Figure 4) (39), which is unexpected given the great diversity in chemotaxis systems.

Chemoreceptor diversity

Receptor packing and array localization are conserved throughout multiple bacterial species (34, 39), but chemotaxis receptors and systems can vary dramatically. *E. coli* actually has a very small number of MCPs compared with other chemotactic bacteria. Bacteria with complex lifestyles or metabolisms have more MCPs on average, with some bacteria possessing over 60 chemotaxis receptors (40). Furthermore, *E. coli* MCPs are only representative of 2 topologies. MCPs can be soluble or membrane-anchored with a periplasmic or cytoplasmic ligand binding domain (41) and can be divided into 6 different classes based on the topology of the transmembrane

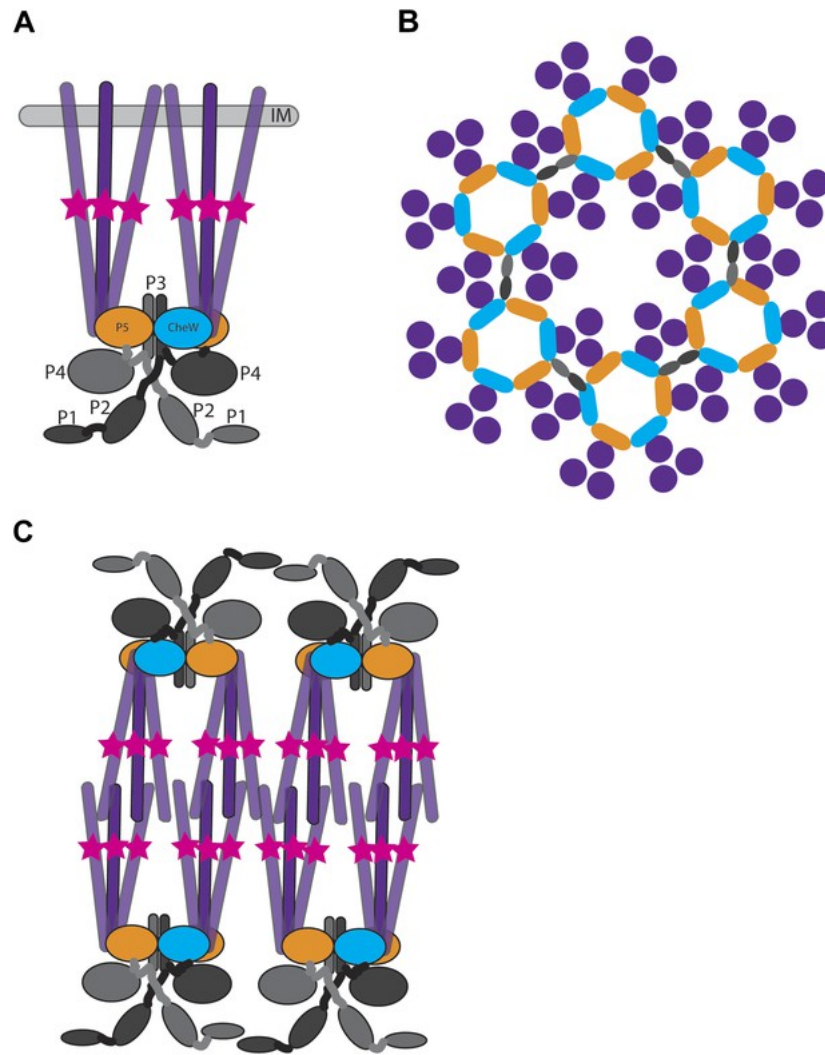


Figure 3: Composition of chemoreceptor trimer of dimers and array

MCPs (purple) dimerize and interact with the P5 domain of CheA (orange) and CheW (blue). The other domains of CheA (P1-P4) are shown in dark/ light grey. Adapted from (38).

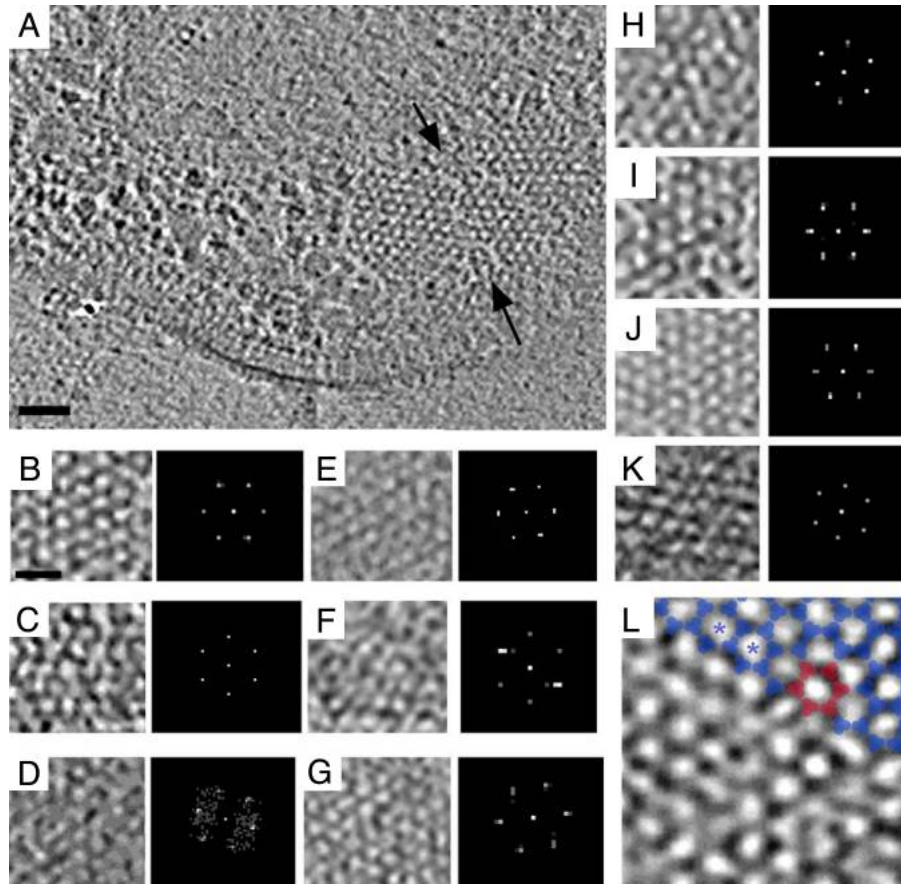


Figure 4: Multiple bacterial species form hexagonally packed receptor arrays with 12nm spacing

(A) Top view of receptor array for *Thermotoga maritima*. Top views (left) and power spectrums (right) of receptor arrays for (B) *T. maritima*, (C) *Acetonebma longum*, (D) *Campylobacter jejuni*, (E) *Helicobacter hepaticus*, (F) *Magnetospirillum magneticum*, (G) *Halothiobacillus neapolitanus*, (H) *R. sphaeroides*, (I) *E. coli*, (J) *V. cholerae*, (K) *Treponema primitia*. (L) Shows trimers of dimers (blue) outlined within the vertices of the hexagonal array. Adapted from (34).

and ligand binding domains (Figure 5A-B) (42). Seventy percent of chemotactic bacteria possess MCPs with a periplasmic ligand binding domain flanked by 2 transmembrane domains (Figure 5B), but these ligand binding domains vary greatly. Some MCPs possess known ligand binding domains: TarH, CHASE, CACHE, GAF or PAS domains, but despite having annotations, these domains can bind a range of ligands. Furthermore, the majority of ligand binding domains are unannotated (40), making predicting specificity difficult. Another level of chemotaxis complexity is added when MCPs have additional C-terminal domains. While the majority of ligand binding domains are at the N-terminus, 5% of chemotactic bacteria possess receptors with additional ligand binding domains at the C-terminus, such as zinc binding domains (CZB) and nucleotide binding domains (i.e. PilZ) (28). Little work has addressed how these C-terminal domains are involved in sensing and signal propagation. The variation in sequence composition and LBD topology has made predicting ligands difficult (41). MCPs also vary in the length due to 7 amino acid long insertions and deletions (heptads) in the conserved signaling region (Figure 5C) (43). These heptad repeats contain residues that facilitate interactions within the receptor and with other proteins (other MCPs, CheA, and CheW) (43). Receptors can be grouped into one of 7 major classes based on the amount of heptad repeats present in the signaling domain (24H, 38H, 34H, 36H, 38H, 40H, and 44H) (43), and only receptors of the same heptad-length can form a trimer of dimers and signal together (44, 45). All *E. coli* MCPs are of the same heptad class allowing them to interact and signal together with proteins encoded in the single chemotaxis operon (46), but many bacteria possess receptors of various heptad lengths (29, 43).

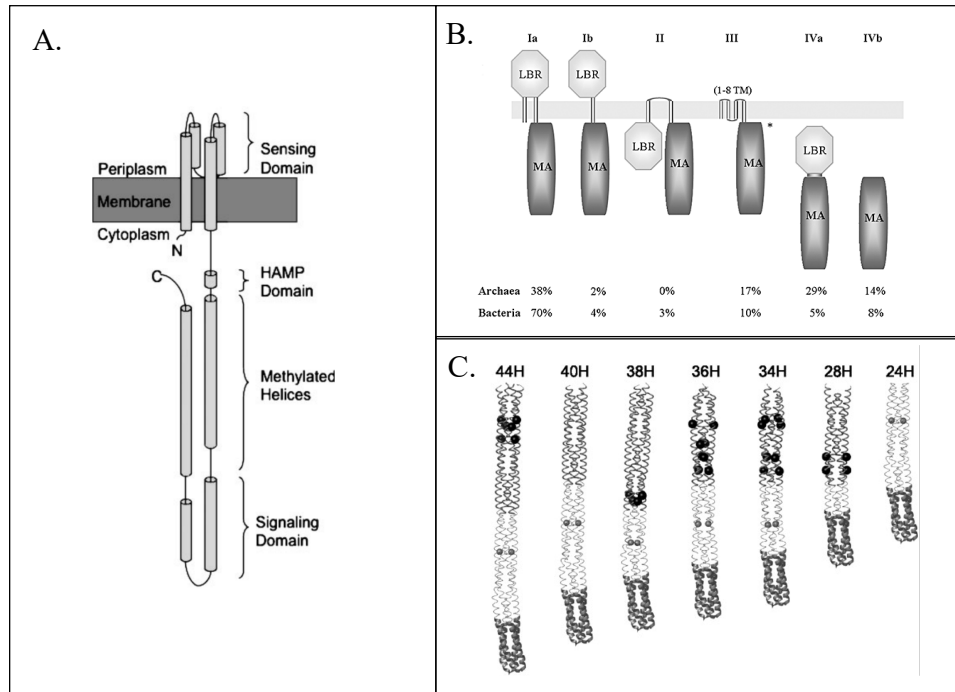


Figure 5: Diversity of chemotaxis receptors

Chemotaxis receptors, or MCPs, can come in a variety of topologies. The most common receptor topologies involve a periplasmic sensing/ ligand binding region (LBR) (A-B). B) Ligand binding regions (LBR) are indicated by octagons. Methyl accepting regions (MA) are shown as rectangles. Chemotaxis receptors also vary in length due to the insertion and deletion of heptad repeats in the C-terminus (C). Adapted from (14, 42, 43).

Chemotaxis Operon and Protein Diversity

Chemotactic bacteria can also possess multiple chemosensory operons (chemotaxis or chemotaxis-like operons that control flagellar mediated movement or alternative cellular functions) (47). Chemosensory systems are defined by possessing CheA, CheW, and an MCP (47). These systems vary in their composition and can be divided into 10 evolutionary classes based on gene order, presence of additional chemotaxis genes, conservation of protein sequences, and protein domain architecture (47). Seventeen of these classes control flagellar motility (F classes), one controls type 4 pili function (Tfp class), and one controls alternative cellular function (ACF class) (47). Only recently have we begun to understand how bacteria can coordinate receptors of different classes and specificities as well as multiple chemosensory pathways.

Receptors of the same heptad lengths signal through evolutionary classes with which they co-evolved (41), and this can be used to assign receptors to pathways. For example, *Pseudomonas aeruginosa* encodes 26 MCPs that belong to either the 40H (21 receptors), 24H (2 receptors), 36H (1 receptor) or are unclassified (2 receptors) (29). The majority of *P. aeruginosa* receptors are 40H, which are predicted to signal with F6, ACF, and Tfp evolutionary classes. This complicates pathway assignment since *P. aeruginosa* possesses F6, F7, ACF, and Tfp chemosensory operons (29, 47). Chemotaxis is controlled by the Che1 pathway, an F6 chemotaxis operon (48). The Che2 pathway (F7 class) also plays a role in optimizing chemotaxis (48). Che3 (ACF) and Che4 (Tfp) are responsible for the Wsp and PilChp systems which control biofilm formation and twitching motility, respectively (49, 50). Each of these systems has a distinct function and subcellular localization and dedicated MCPs. By looking at conserved chemoreceptor protein binding sites, methylation sites, and experimental evidence in addition to heptad class, each *P. aeruginosa* receptor was assigned to a specific pathway (29). The Che1 system uses 23 receptors: 19 from 40H

class, 2 from 24H class, and 2 unclassified receptors (29). The other sensory systems have one MCP each: Che2 utilizes the 36H receptor and both Wsp and Chp systems signal through a 40H receptor each (29). These results highlight a departure from the *E. coli* paradigm: receptors of the same length can signal in different pathways and control different cellular functions.

Chemotaxis can be further complicated by the fact that multiple chemotaxis paralogs can be essential for functional chemotaxis. For example, *Rhodobacter sphaeroides* is a photosynthetic heterotrophic bacterium that has 3 chemosensory operons, 2 of which are essential for chemotaxis. It encodes 4 CheA proteins, 6 CheY proteins, and 2 CheB proteins (51). Three of the CheA proteins (CheA₂, CheA₃, and CheA₄) are crucial for chemotaxis, and each of these CheAs has a different phosphotransfer specificity for downstream CheYs and CheBs (51, 52). CheA₃ and CheA₄ only phosphorylate CheY₆ and CheB₂ while CheA₂ can phosphorylate all CheYs and CheBs. CheA₂ cannot compensate for the loss of CheA₃ and CheA₄ due to spatial segregation in the cell (53). Bacteria possessing multiple chemotaxis systems are abundant, yet little is known about how other species avoid detrimental crosstalk between chemotaxis systems and how signals from multiple arrays are potentially integrated.

Finally, chemotactic bacteria may have additional chemotaxis proteins and adaptation systems. *Bacillus subtilis* is a gram-positive soil-dwelling bacterium that possesses additional proteins, adaptation systems, and an inverted phosphorylation cascade: binding of an attractant causes phosphorylation of CheA, initiating the phosphorylation of CheY, and binding of CheY-P to the flagellar motor decreases tumbling (54, 55). In *E. coli* receptors differential methylation levels act as a memory to compensate for changes in CheA phosphorylation, but in *B. subtilis* methylation of specific residues, not changes in methylation levels, result in adaptation. For

example, methylation of Glu637 of McpB causes adaptation to the attractants, while methylation of Glu630 causes adaptation to attractant removal (56). *B. subtilis* can also do methylation-independent adaptation using additional chemotaxis proteins, CheV, CheC, and CheD (57, 58). CheV can be phosphorylated when an attractant is sensed, and this phosphorylation is important in returning to pre-stimulus swimming and reversal levels (59). CheC is a phosphatase that can act on CheY-P, and CheD binding to CheC increases this activity. CheC and CheD are thought to be important for a feedback mechanism for CheY. CheV, CheC, and CheD are also present in the genomes of other bacteria, indicating a possible common methylation-independent adaptation mechanism (57, 58). Additionally, some receptors show no evidence of methylation-dependent adaptation, so other means of adaptation could exist (60, 61). Understanding receptor modification and adaptation in other systems would give a more complete understanding of chemotaxis.

Vibrio cholerae is a species that possesses additional chemotaxis proteins and can integrate them into the CheA-CheW baseplate of receptor arrays. *V. cholerae* has 3 chemotaxis clusters, each forming its own chemoreceptor array. Cluster II is not well understood, but it is known that ParP and ParC localize the chemotaxis proteins to the cell pole, and then ParP stimulates array formation and is integrated into the array (62, 63). Along with ParP, *V. cholerae* also encodes 4 copies of CheV, a CheW-CheY hybrid that can integrate into the baseplate like CheW (55). The baseplate in Cluster II arrays is highly variable, allowing *V. cholerae* to form 12nm arrays even in the absence of CheA. It can be composed of CheA-CheW rings, CheA-CheW-CheV-ParP rings, or CheW-CheV-ParP rings (64). The abundance of chemotactic systems that diverge from the *E. coli* model illustrate the need to study more bacteria to understand how multiple chemosensory systems and diverse receptors are used for environmental sensing.

Chemotaxis in the Rhizosphere

To understand complex chemotaxis systems, bacteria that live in diverse environments and have a wide metabolic versatility need to be studied. One such environment, the soil is enriched in chemotactic bacterial species, with the majority of soil bacteria encoding more than one cheA gene per genome (65, 66). The rhizosphere, or the zone of the soil directly surrounding the roots is a highly productive area where plant-microbe associations are abundant (67, 68). Chemotaxis is vital to many of these rhizosphere interactions (69). For example, the plant growth promoting bacteria *Rhizobium leguminosarum*, *Pseudomonas putida*, and *Sinorhizobium meliloti* are attracted to exudates of plants they are known to colonize (70-73). Furthermore, the colonization efficiency of *B. subtilis* to *Arabidopsis* and *P. putida* to maize depend upon functional chemotaxis systems and MCPs (74, 75). Chemotaxis and sensing are not only important for bacteria to colonize plants, but to initiate the plant growth promoting effects. In *R. leguminosarum* nodulation of pea roots is dependent upon functional chemotaxis machinery (76).

This dissertation will focus on another bacterium that is important in rhizosphere productivity: *Azospirillum brasilense*. *A. brasilense* is a microaerophilic alphaproteobacterium that promotes plant growth by colonizing the root surfaces of plants, producing auxin, and fixing nitrogen (77-79). *A. brasilense* is sold as a commercial biofertilizer and can increase the yield of cereals and grains up to 40 percent (80). Chemotaxis is essential for *A. brasilense*'s ability to find microaerophilic environments and colonize plant roots. *A. brasilense* is an excellent model for studying chemotaxis because it possesses many of the previously mentioned chemotaxis features that deviate from *E. coli*: multiple chemosensory operons and chemotaxis protein paralogs, a wide variety of chemoreceptors, additional chemotaxis proteins, and receptors that do not use methyl-dependent adaptation (61, 81-84).

***Azospirillum brasilense* chemotaxis**

A. brasilense swims through liquid using a single polar flagellum and can change swimming direction by switching the direction of flagellar rotation (85, 86). It encodes 4 chemosensory operons (Che1 through Che4), two of which are essential for chemotaxis (83) (Figure 6). In the presence of attractants, *A. brasilense* increases swimming speed and suppresses reversals (81). When a repellent is detected, *A. brasilense* does not increase speed, but changes swimming direction. Cells lacking the Che1 operon cannot modulate swimming speed in a gradient, indicating that Che1 is important in adjusting speed in response to environmental cues (81). On the other hand, loss of CheA4 or CheY4 impairs the cell's ability to reverse in a chemical gradient (83). Only when both Che1 and Che4 are knocked out is *A. brasilense* null for chemotaxis (Alexandre lab, unpublished). Changes in swimming speed and reversal frequency must be coordinated to generate responses to attractants and repellents. Additionally, loss of a functional Che1 has a slight defect on *A. brasilense*'s ability to reverse (81), and loss of Che4 affects swimming speed slightly (83). These results indicate that there may be cross talk between the signaling cascades, but it is unclear how *A. brasilense* integrates cues sensed by Che1 and Che4 proteins to coordinate one chemotaxis output.

***A. brasilense* chemotaxis receptors**

Along with the chemosensory operons, *A. brasilense* encodes 51 putative chemoreceptors that belong to the 36H and 38H classes (84). This large number of receptors likely reflects the diversity of the cues within the rhizosphere to which *A. brasilense* can respond. At the timing of this dissertation, only 2 of these chemoreceptors have been characterized: AerC and Tlp1(61, 94). AerC is a soluble chemoreceptor receptor that possesses an N-terminal PAS domain that binds flavin adenine dinucleotide (FAD). The ability of AerC to bind FAD is essential for *A. brasilense*

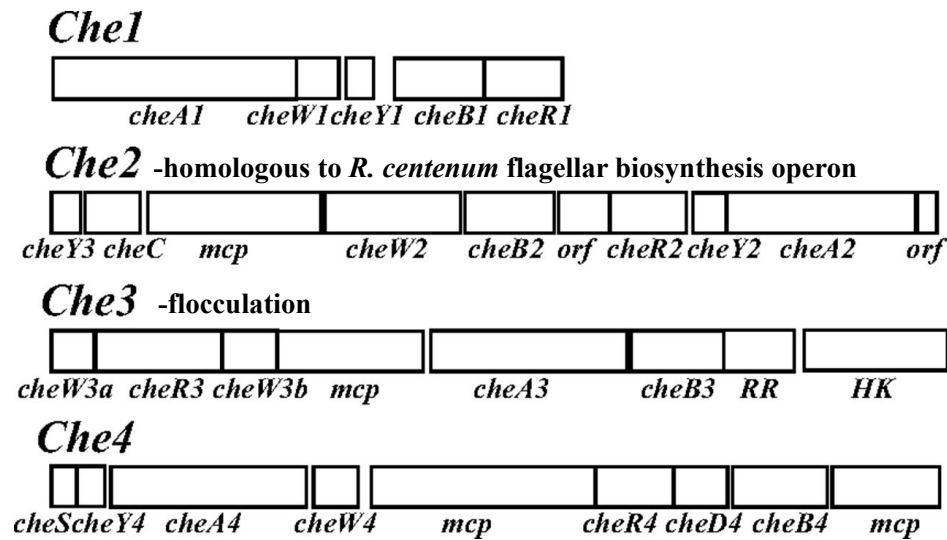


Figure 6: *Azospirillum brasilense* encodes 4 chemosensory operons

A. brasilense has 2 operons (Che1 and Che4) that control chemotaxis function and 2 chemotaxis-like operons that have alternative cellular functions. Adapted from (83).

to navigate oxygen gradients and respond to high oxygen levels by initiating cell-cell clumping (81, 94), demonstrating that AerC senses changes in metabolism via FAD binding. Despite AerC's role in sensing microaerophilic environments, work in the Alexandre laboratory has shown that AerC is dispensable in colonizing wheat (*Triticum aestivum*) roots.

Transducer like protein 1 (Tlp1) is a membrane bound energy taxis receptor that is essential for wheat root colonization (95). Tlp1 possesses a periplasmic ligand binding domain and an intracellular signaling domain with an additional C-terminal domain (Fig 7) (61, 95). The ligand binding domain is uncharacterized, but Tlp1 has been implicated in sensing organic acids and energy taxis, including aerotaxis (61, 95). The additional C-terminal domain is a PilZ domain that binds to cyclic diguanylate monophosphate (c-di-GMP). This domain is essential for aerotaxis, and binding of c-di-GMP to the PilZ domain of Tlp1 is critical for Tlp1's function as a chemoreceptor (61).

C-di-GMP and Chemotaxis

C-di-GMP is a bacterial secondary messenger that regulates the switch from a planktonic lifestyle to biofilm formation (96). Diguanylate cyclases and phosphodiesterases regulate intracellular levels by synthesizing or degrading c-di-GMP in response to various external cues. When c-di-GMP levels are elevated for long periods of time cells shut off motility, eject flagella, and will form a biofilm (96). In *A. brasilense*, c-di-GMP levels fluctuate rapidly with changes in aeration (61). When c-di-GMP is temporarily elevated, c-di-GMP binds to the PilZ domain of Tlp1 and regulates swimming speed and reversal frequency to changes in aeration (61). Cells expressing Tlp1 unable to bind to c-di-GMP do not adjust speed with the addition or removal of air and reverse much less than cells with the ability to bind to c-di-GMP (70). These results reveal that c-di-GMP

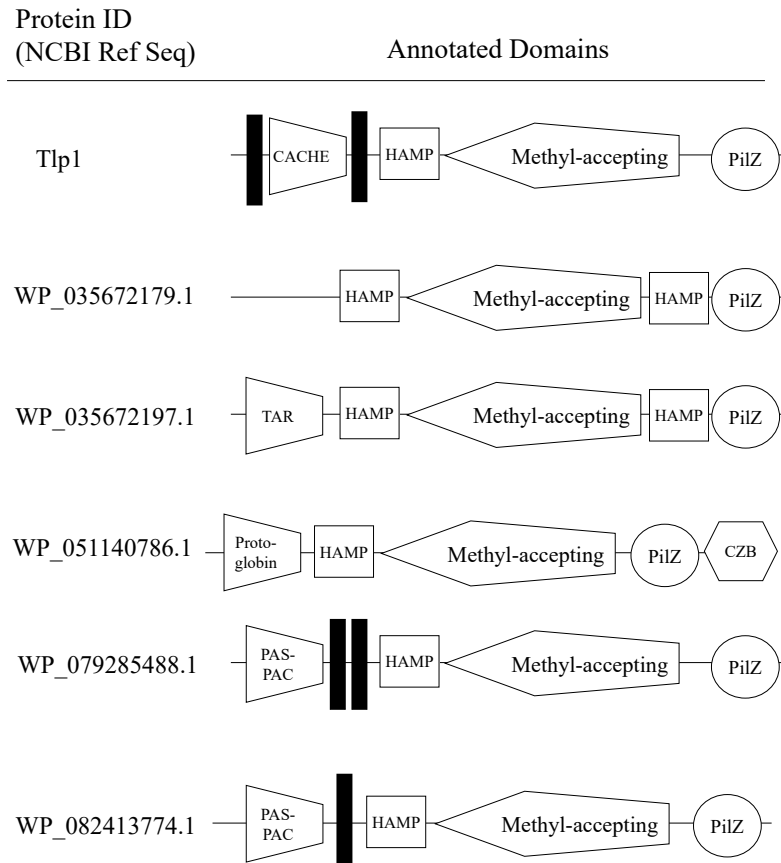


Figure 7: *A. brasilense* PilZ containing receptors

A. brasilense encodes 6 putative PilZ containing receptors, only Tlp1 has been characterized. The receptors containing multiple domains: CACHE, TAR, PAS-PAC, and Protoglobin domains are putative ligand binding domains. Transmembrane domains are represented by vertical black rectangles. HAMP domains are squares. Methyl-accepting domains are represented by oblong pentagons. Circles and hexagons signify C-terminal PilZ and CZB (chemoreceptor zinc binding domains) respectively.

binding to the PilZ domain of Tlp1 regulates the receptor's sensitivity (61). Currently, no evidence exists that Tlp1 is methylated, so it is hypothesized that c-di-GMP is acting as a methylation-independent adaptation mechanism (61). Tlp1 is potentially involved in sensing other cues besides oxygen, but it is unclear how c-di-GMP binding to the PilZ domain is involved in this or in cellular behavior in the rhizosphere (95). Besides Tlp1, *A. brasilense* encodes 5 additional PilZ containing chemoreceptors (84) (Figure 7), but their role in sensing is unexplored. However, understanding the role of c-di-GMP in chemotaxis and sensing is complicated by the fact that persistent elevated c-di-GMP levels shut off motility, and therefore chemotaxis. To understand the role of c-di-GMP in *A. brasilense*, more work needs to focus on characterizing PilZ containing chemoreceptors and developing tools to transiently manipulate c-di-GMP levels.

This dissertation aims to address several different aspects of *A. brasilense* chemotaxis. The first project will examine how *A. brasilense* integrates signals from the two chemotaxis operons by mixing chemotaxis paralogs encoded in the separate operons to form baseplates that interact with chemotaxis receptors (Chapter 1). The next project will address the problem of studying transient changes in c-di-GMP levels and their role in regulating chemotaxis receptor sensitivity (Chapter 2). Chapter 3 characterizes another PilZ containing receptor, Aer, in oxygen and root sensing. The final project examines the function of chemotaxis and 2 chemotaxis receptors (Tlp1 and Aer) in the rhizosphere and root sensing.

References

1. Guan N, Li J, Shin HD, Du G, Chen J, Liu L. 2017. Microbial response to environmental stresses: from fundamental mechanisms to practical applications. *Appl Microbiol Biotechnol* 101:3991-4008.
2. Adler J. 1975. Chemotaxis in bacteria. *Annu Rev Biochem* 44:341-56.
3. Macnab RM, Koshland DE, Jr. 1972. The gradient-sensing mechanism in bacterial chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America* 69:2509-2512.
4. Hess JF, Bourret RB, Simon MI. 1988. Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature* 336:139.
5. Wadhams GH, Armitage JP. 2004. Making sense of it all: bacterial chemotaxis. *Nature Reviews Molecular Cell Biology* 5:1024.
6. Barak R, Eisenbach M. 1992. Correlation between phosphorylation of the chemotaxis protein CheY and its activity at the flagellar motor. *Biochemistry* 31:1821-6.
7. Hess JF, Oosawa K, Kaplan N, Simon MI. 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. *Cell* 53:79-87.
8. Barnakov AN, Barnakova LA, Hazelbauer GL. 1999. Efficient adaptational demethylation of chemoreceptors requires the same enzyme-docking site as efficient methylation. *Proceedings of the National Academy of Sciences of the United States of America* 96:10667-10672.

9. Kehry MR, Bond MW, Hunkapiller MW, Dahlquist FW. 1983. Enzymatic deamidation of methyl-accepting chemotaxis proteins in *Escherichia coli* catalyzed by the cheB gene product. *Proc Natl Acad Sci U S A* 80:3599-603.
10. Sourjik V, Wingreen NS. 2012. Responding to chemical gradients: bacterial chemotaxis. *Curr Opin Cell Biol* 24:262-8.
11. Martin AC, Wadhams GH, Shah DSH, Porter SL, Mantotta JC, Craig TJ, Verdult PH, Jones H, Armitage JP. 2001. CheR- and CheB-Dependent Chemosensory Adaptation System of *Rhodobacter sphaeroides*. *Journal of Bacteriology* 183:7135-7144.
12. Ninfa EG, Stock A, Mowbray S, Stock J. 1991. Reconstitution of the bacterial chemotaxis signal transduction system from purified components. *J Biol Chem* 266:9764-70.
13. Parkinson JS. 1976. cheA, cheB, and cheC genes of *Escherichia coli* and their role in chemotaxis. *Journal of Bacteriology* 126:758-770.
14. Stock JB, Clarke S, Koshland DE, Jr. 1984. The protein carboxymethyltransferase involved in *Escherichia coli* and *Salmonella typhimurium* chemotaxis. *Methods Enzymol* 106:310-21.
15. Sanatinia H, Kofoed EC, Morrison TB, Parkinson JS. 1995. The smaller of two overlapping cheA gene products is not essential for chemotaxis in *Escherichia coli*. *Journal of Bacteriology* 177:2713-20.
16. Wang H, Matsumura P. 1996. Characterization of the CheAS/CheZ complex: a specific interaction resulting in enhanced dephosphorylating activity on CheY-phosphate. *Mol Microbiol* 19:695-703.

17. Parkinson S. An overview of *E. coli* chemotaxis, on Department of Biology- University of Utah. Accessed February 8.
18. Grebe TW, Stock J. 1998. Bacterial chemotaxis: The five sensors of a bacterium. *Current Biology* 8:R154-R157.
19. Parkinson JS, Hazelbauer GL, Falke JJ. 2015. Signaling and sensory adaptation in *Escherichia coli* chemoreceptors: 2015 update. *Trends in microbiology* 23:257-266.
20. Rebbapragada A, Johnson MS, Harding GP, Zuccarelli AJ, Fletcher HM, Zhulin IB, Taylor BL. 1997. The Aer protein and the serine chemoreceptor Tsr independently sense intracellular energy levels and transduce oxygen, redox, and energy signals for *Escherichia coli* behavior. *Proceedings of the National Academy of Sciences* 94:10541.
21. Watts KJ, Johnson MS, Taylor BL. 2005. Minimal requirements for oxygen sensing by the aerotaxis receptor Aer. *Molecular Microbiology* 59:1317-1326.
22. Bibikov SI, Biran R, Rudd KE, Parkinson JS. 1997. A signal transducer for aerotaxis in *Escherichia coli*. *Journal of Bacteriology* 179:4075.
23. Gegner JA, Graham DR, Roth AF, Dahlquist FW. 1992. Assembly of an MCP receptor, CheW, and kinase CheA complex in the bacterial chemotaxis signal transduction pathway. *Cell* 70:975-982.
24. Li X, Fleetwood AD, Bayas C, Bilwes AM, Ortega DR, Falke JJ, Zhulin IB, Crane BR. 2013. The 3.2 Å resolution structure of a receptor: CheA:CheW signaling complex defines overlapping binding sites and key residue interactions within bacterial chemosensory arrays. *Biochemistry* 52:3852-65.

25. Rowsell EH, Smith JM, Wolfe A, Taylor BL. 1995. CheA, CheW, and CheY are required for chemotaxis to oxygen and sugars of the phosphotransferase system in *Escherichia coli*. *Journal of Bacteriology* 177:6011-4.
26. Vu A, Wang X, Zhou H, Dahlquist FW. 2012. The receptor-CheW binding interface in bacterial chemotaxis. *Journal of molecular biology* 415:759-767.
27. Wang X, Vu A, Lee K, Dahlquist FW. 2012. CheA-receptor interaction sites in bacterial chemotaxis. *Journal of molecular biology* 422:282-290.
28. Ortega A, Zhulin IB, Krell T. 2017. Sensory Repertoire of Bacterial Chemoreceptors. *Microbiol Mol Biol Rev* 81.
29. Ortega D, Fleetwood A, Krell T, S. Harwood C, Jensen G, Zhulin I. 2017. Assigning chemoreceptors to chemosensory pathways in *Pseudomonas aeruginosa*, vol 114.
30. Parkinson JS. 2010. Signaling mechanisms of HAMP domains in chemoreceptors and sensor kinases. *Annu Rev Microbiol* 64:101-22.
31. Chervitz SA, Falke JJ. 1996. Molecular mechanism of transmembrane signaling by the aspartate receptor: a model. *Proceedings of the National Academy of Sciences* 93:2545.
32. Falke JJ. 2014. Piston versus scissors: chemotaxis receptors versus sensor His-kinase receptors in two-component signaling pathways. *Structure (London, England : 1993)* 22:1219-1220.
33. Yu D, Ma X, Tu Y, Lai L. 2015. Both piston-like and rotational motions are present in bacterial chemoreceptor signaling. *Scientific Reports* 5:8640.
34. Briegel A, Ortega DR, Tocheva EI, Wuichet K, Li Z, Chen S, Müller A, Iancu CV, Murphy GE, Dobro MJ, Zhulin IB, Jensen GJ. 2009. Universal architecture of bacterial

- chemoreceptor arrays. Proceedings of the National Academy of Sciences of the United States of America 106:17181-17186.
35. Lai RZ, Manson JM, Bormans AF, Draheim RR, Nguyen NT, Manson MD. 2005. Cooperative signaling among bacterial chemoreceptors. Biochemistry 44:14298-307.
 36. Liu J, Hu B, Morado DR, Jani S, Manson MD, Margolin W. 2012. Molecular architecture of chemoreceptor arrays revealed by cryoelectron tomography of *Escherichia coli* minicells. Proceedings of the National Academy of Sciences of the United States of America 109:E1481-E1488.
 37. Zhang P, Khursigara CM, Hartnell LM, Subramaniam S. 2007. Direct visualization of *Escherichia coli* chemotaxis receptor arrays using cryo-electron microscopy. Proceedings of the National Academy of Sciences of the United States of America 104:3777-3781.
 38. Briegel A, Ladinsky MS, Oikonomou C, Jones CW, Harris MJ, Fowler DJ, Chang Y-W, Thompson LK, Armitage JP, Jensen GJ. 2014. Structure of bacterial cytoplasmic chemoreceptor arrays and implications for chemotactic signaling. eLife 3:e02151-e02151.
 39. Briegel A, Ortega DR, Huang AN, Oikonomou CM, Gunsalus RP, Jensen GJ. 2015. Structural conservation of chemotaxis machinery across Archaea and Bacteria. Environmental microbiology reports 7:414-419.
 40. Lacal J, García-Fontana C, Muñoz-Martínez F, Ramos J-L, Krell T. 2010. Sensing of environmental signals: classification of chemoreceptors according to the size of their ligand binding regions. Environmental Microbiology 12:2873-2884.

41. Wuichet K, Alexander RP, Zhulin IB. 2007. Comparative genomic and protein sequence analyses of a complex system controlling bacterial chemotaxis. *Methods in Enzymology* 422:1-31.
42. Krell T, Lacal J, Muñoz-Martínez F, Reyes-Darias JA, Cadirci BH, García-Fontana C, Ramos JL. 2011. Diversity at its best: bacterial taxis. *Environmental Microbiology* 13:1115-1124.
43. Alexander RP, Zhulin IB. 2007. Evolutionary genomics reveals conserved structural determinants of signaling and adaptation in microbial chemoreceptors. *Proceedings of the National Academy of Sciences* 104:2885.
44. Herrera Seitz MK, Frank V, Massazza DA, Vaknin A, Studdert CA. 2014. Bacterial chemoreceptors of different length classes signal independently. *Mol Microbiol* 93:814-22.
45. Amin DN, Hazelbauer GL. 2010. The Chemoreceptor Dimer Is the Unit of Conformational Coupling and Transmembrane Signaling. *Journal of Bacteriology* 192:1193.
46. Gosink KK, Burón-Barral MdC, Parkinson JS. 2006. Signaling interactions between the aerotaxis transducer Aer and heterologous chemoreceptors in *Escherichia coli*. *Journal of bacteriology* 188:3487-3493.
47. Wuichet K, Zhulin IB. 2010. Origins and diversification of a complex signal transduction system in prokaryotes. *Science signaling* 3:ra50-ra50.
48. Güvener ZT, Tifrea DF, Harwood CS. 2006. Two different *Pseudomonas aeruginosa* chemosensory signal transduction complexes localize to cell poles and form and remould in stationary phase. *Molecular Microbiology* 61:106-118.

49. Cursino L, Galvani CD, Athinuwat D, Zaini PA, Li Y, De La Fuente L, Hoch HC, Burr TJ, Mowery P. 2011. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. *Molecular Plant Microbe Interactions* 24:1198-206.
50. Guvener ZT, Harwood CS. 2007. Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Molecular Microbiology* 66:1459-73.
51. Porter SL, Warren AV, Martin AC, Armitage JP. 2002. The third chemotaxis locus of *Rhodobacter sphaeroides* is essential for chemotaxis. *Mol Microbiol* 46:1081-94.
52. Wadhams GH, Warren AV, Martin AC, Armitage JP. 2003. Targeting of two signal transduction pathways to different regions of the bacterial cell. *Molecular Microbiology* 50:763-770.
53. Porter SL, Armitage JP. 2002. Phosphotransfer in *Rhodobacter sphaeroides* chemotaxis. *Journal Molecular Biology* 324:35-45.
54. Bischoff DS, Bourret RB, Kirsch ML, Ordal GW. 1993. Purification and characterization of *Bacillus subtilis* CheY. *Biochemistry* 32:9256-61.
55. Szurmant H, Ordal GW. 2004. Diversity in Chemotaxis Mechanisms among the Bacteria and Archaea. *Microbiology and Molecular Biology Reviews* 68:301.
56. Zimmer MA, Tiu J, Collins MA, Ordal GW. 2000. Selective methylation changes on the *Bacillus subtilis* chemotaxis receptor McpB promote adaptation. *J Biol Chem* 275:24264-72.
57. Rosario MM, Ordal GW. 1996. CheC and CheD interact to regulate methylation of *Bacillus subtilis* methyl-accepting chemotaxis proteins. *Molecular Microbiology* 21:511-8.

58. Szurmant H, Muff TJ, Ordal GW. 2004. *Bacillus subtilis* CheC and FliY are members of a novel class of CheY-P-hydrolyzing proteins in the chemotactic signal transduction cascade. *Journal Biological Chemistry* 279:21787-92.
59. Rosario MM, Fredrick KL, Ordal GW, Helmann JD. 1994. Chemotaxis in *Bacillus subtilis* requires either of two functionally redundant CheW homologs. *Journal of Bacteriology* 176:2736-9.
60. Stephens BB, Loar SN, Alexandre G. 2006. Role of CheB and CheR in the complex chemotactic and aerotactic pathway of *Azospirillum brasilense*. *Journal of Bacteriology* 188:4759-4768.
61. Russell MH, Bible AN, Fang X, Gooding JR, Campagna SR, Gomelsky M, Alexandre G. 2013. Integration of the Second Messenger c-di-GMP into the Chemotactic Signaling Pathway. *mBio* 4.
62. Ringgaard S, Yang W, Alvarado A, Schirner K, Briegel A. 2018. Chemotaxis Arrays in Species and Their Intracellular Positioning by the ParC/ParP System. *Journal of Bacteriology* 200:e00793-17.
63. Ringgaard S, Zepeda-Rivera M, Wu X, Schirner K, Davis BM, Waldor MK. 2014. ParP prevents dissociation of CheA from chemotactic signaling arrays and tethers them to a polar anchor. *Proc Natl Acad Sci U S A* 111:E255-64.
64. Yang W, Alvarado A, Glatter T, Ringgaard S, Briegel A. 2018. Baseplate variability of *Vibrio cholerae* chemoreceptor arrays. *Proceedings of the National Academy of Sciences* 115:13365.

65. Buchan A, Crombie B, Alexandre GM. 2010. Temporal dynamics and genetic diversity of chemotactic-competent microbial populations in the rhizosphere. *Environ Microbiol* 12:3171-84.
66. Scharf BE, Hynes MF, Alexandre GM. 2016. Chemotaxis signaling systems in model beneficial plant-bacteria associations. *Plant Mol Biol* 90:549-59.
67. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology* 57:233-266.
68. Walker TS, Bais HP, Grotewold E, Vivanco JM. 2003. Root Exudation and Rhizosphere Biology. *Plant Physiology* 132:44.
69. Levy A, Salas Gonzalez I, Mittelviefhaus M, Clingenpeel S, Herrera Paredes S, Miao J, Wang K, Devescovi G, Stillman K, Monteiro F, Rangel Alvarez B, Lundberg DS, Lu T-Y, Lebeis S, Jin Z, McDonald M, Klein AP, Feltcher ME, Rio TG, Grant SR, Doty SL, Ley RE, Zhao B, Venturi V, Pelletier DA, Vorholt JA, Tringe SG, Woyke T, Dangl JL. 2018. Genomic features of bacterial adaptation to plants. *Nature Genetics* 50:138-150.
70. Webb BA, Hildreth S, Helm RF, Scharf BE. 2014. *Sinorhizobium meliloti* chemoreceptor McpU mediates chemotaxis toward host plant exudates through direct proline sensing. *Applied and Environmental Microbiology* 80:3404-3415.
71. Gupta Sood S. 2003. Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. *FEMS Microbiol Ecol* 45:219-27.

72. Gaworzewska ET, Carlile MJ. 1981. Positive Chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *Journal of General Microbiology*:1179-1188.
73. Neal AL, Ahmad S, Gordon-Weeks R, Ton J. 2012. Benzoxazinoids in Root Exudates of Maize Attract *Pseudomonas putida* to the Rhizosphere. *PLOS ONE* 7:e35498.
74. Corral-Lugo A, De la Torre J, Matilla MA, Fernández M, Morel B, Espinosa-Urgel M, Krell T. 2016. Assessment of the contribution of chemoreceptor-based signalling to biofilm formation. *Environmental Microbiology* 18:3355-3372.
75. Allard-Massicotte R, Tessier L, Lécuyer F, Lakshmanan V, Lucier J-F, Garneau D, Caudwell L, Vlamakis H, Bais HP, Beaugard PB. 2016. Early Colonization of *Arabidopsis thaliana* Roots Involves Multiple Chemotaxis Receptors. *mBio* 7:e01664-16.
76. Miller LD, Yost CK, Hynes MF, Alexandre G. 2007. The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol Microbiol* 63:348-62.
77. Bashan Y. 1986. Enhancement of Wheat Root Colonization and Plant Development by *Azospirillum brasilense* Cd. Following Temporary Depression of Rhizosphere Microflora, vol 51.
78. Hauwaerts D, Alexandre G, Das SK, Vanderleyden J, Zhulin IB. 2002. A major chemotaxis gene cluster in *Azospirillum brasilense* and relationships between chemotaxis operons in alpha-proteobacteria. *FEMS Microbiol Lett* 208:61-7.
79. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology* 11:789.

80. Fibach-Paldi S, Burdman S, Okon Y. 2012. Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. FEMS Microbiol Lett 326:99-108.
81. Bible A, Russell MH, Alexandre G. 2012. The *Azospirillum brasilense* Che1 Chemotaxis Pathway Controls Swimming Velocity, Which Affects Transient Cell-to-Cell Clumping. Journal of Bacteriology 194:3343-3355.
82. Bible AN, Khalsa-Moyers GK, Mukherjee T, Green CS, Mishra P, Purcell A, Aksenova A, Hurst GB, Alexandre G. 2015. Metabolic adaptations of *Azospirillum brasilense* to oxygen stress by cell-to-cell clumping and flocculation. Applied and environmental microbiology 81:8346-8357.
83. Mukherjee T, Kumar D, Burriss N, Xie Z, Alexandre G. 2016. *Azospirillum brasilense* Chemotaxis Depends on Two Signaling Pathways Regulating Distinct Motility Parameters. Journal of Bacteriology 198:1764.
84. Ulrich LE, Zhulin IB. 2010. The MiST2 database: a comprehensive genomics resource on microbial signal transduction. Nucleic Acids Res 38:D401-7.
85. Hall PG, Krieg NR. 1983. Swarming of *Azospirillum brasilense* on solid media. Canadian Journal of Microbiology 29:1592-1594.
86. Zhulin IB, Armitage JP. 1993. Motility, chemokinesis, and methylation-independent chemotaxis in *Azospirillum brasilense*. Journal of Bacteriology 175:952.
87. Berleman JE, Bauer CE. 2005. A che-like signal transduction cascade involved in controlling flagella biosynthesis in *Rhodospirillum centenum*. Mol Microbiol 55:1390-402.

88. Qi X, Nellas RB, Byrn MW, Russell MH, Bible AN, Alexandre G, Shen T. 2013. Swimming motility plays a key role in the stochastic dynamics of cell clumping. *Phys Biol* 10:026005.
89. Sadasivan L, Neyra CA. 1985. Flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: exopolysaccharides and cyst formation. *Journal of bacteriology* 163:716-723.
90. Alexandre G, Greer SE, Zhulin IB. 2000. Energy Taxis Is the Dominant Behavior in *Azospirillum brasilense*. *Journal of Bacteriology* 182:6042-6048.
91. Taylor BL, Zhulin IB. 1998. In search of higher energy: metabolism-dependent behaviour in bacteria. *Mol Microbiol* 28:683-90.
92. Taylor BL, Zhulin IB, Johnson MS. 1999. Aerotaxis and Other Energy-Sensing Behavior in Bacteria. *Annual Review of Microbiology* 53:103-128.
93. Zhulin IB, Bespalov VA, Johnson MS, Taylor BL. 1996. Oxygen taxis and proton motive force in *Azospirillum brasilense*. *J Bacteriol* 178:5199-204.
94. Xie Z, Ulrich LE, Zhulin IB, Alexandre G. 2010. PAS domain containing chemoreceptor couples dynamic changes in metabolism with chemotaxis. *Proceedings of the National Academy of Sciences* 107:2235.
95. Greer-Phillips SE, Stephens BB, Alexandre G. 2004. An energy taxis transducer promotes root colonization by *Azospirillum brasilense*. *Journal of bacteriology* 186:6595-6604.
96. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology* 7:263.

CHAPTER I
DISTINCT CHEMOTAXIS PROTEIN PARALOGS ASSEMBLE INTO
CHEMORECEPTOR ARRAYS TO COORDINATE SIGNALING OUTPUT

Text and figures taken from the following:

O’Neal L, Gullett JM, Aksenova, A, Hubler A, Briegel A, Ortega D, Kjær, A, Jensen, G, and Alexandre G. 2017. Distinct chemotaxis protein paralogs assemble into chemoreceptor signaling arrays to coordinate signaling output. Manuscript submitted to mBio March 2019.

Author contributions to this work included: LO, JMG, GA designed research; LO, JMG, AH, AA, AB, AK performed research; LO, JMG, GA, AB analyzed data; LO, JMG, GA, AB wrote the paper.

Abstract

Most chemotactic motile bacteria possess multiple chemotaxis signaling systems, the function of which is typically uncharacterized. Chemotaxis signaling is initiated by chemoreceptors that assemble as large arrays, together with chemotaxis coupling proteins (CheW) and histidine kinase proteins (CheA) which form a baseplate with the cytoplasmic tips of receptors. These polar arrays mediate sensing, signaling, and signal amplification during chemotaxis responses. Membrane-bound chemoreceptors with different cytoplasmic domain lengths segregate into distinct arrays, as expected for ensuring functional assembly with baseplate proteins. Here we show that a bacterium, *Azospirillum brasilense*, possessing two chemotaxis signaling systems, each encoding a complete set of chemotaxis proteins with distinct effects on the motility pattern, coordinates its chemotactic responses through the production of two distinct membrane-bound chemoreceptor arrays by mixing paralogs within chemotaxis baseplates. The polar localization of chemoreceptors of different length classes was compromised unless baseplate signaling proteins from both chemotaxis systems were present. Furthermore, chemoreceptors and chemotaxis proteins from each of the chemotaxis signaling systems could physically interact with one another. The assembly of paralogs from distinct chemotaxis pathways into baseplates provides a straightforward mechanism for coordinating signaling from distinct pathways, which we predict is not unique to this system given the propensity of chemotaxis systems for horizontal gene transfer.

Introduction

In bacterial chemotaxis, chemoreceptors sense and propagate environmental signals via a conserved signal transduction cascade. Experimental evidence and mathematical modeling indicate that the stimuli detected by chemoreceptors are greatly amplified at the signaling complex, which indicates that one receptor can interact with multiple histidine kinases (1-3). The signal transduction system that controls bacterial chemotaxis has been best characterized in *Escherichia coli* (1). *E. coli* has five different transmembrane chemoreceptors (1, 4-8) that localize to the cell poles along with the kinase CheA and scaffolding CheW proteins to form large patches (9). Cryo-electron microscopy and tomography revealed that these polar patches are about 250 nm in diameter and remain mobile within the curvature of the cell pole while nonpolar patches form at the future division site (9-12). These patches correspond to hexagonally packed trimers of chemoreceptor dimers linked together by rings of interacting CheA and CheW proteins. These large assemblies of transmembrane chemoreceptors in polar arrays are universal features of chemotaxis found in Bacteria and Archaea. The high degree of cooperativity results from allosteric interactions between chemoreceptors, CheA and CheW, and this largely accounts for signal amplification (13).

Comparative analysis of the genome sequences of motile bacteria suggested that most species have more than one chemotaxis pathway encoded within their genome and a greater number of receptors than *E. coli*. (14). Experimental evidence from genetic approaches and cryo-EM analyses also demonstrated that bacteria may express more than one chemoreceptor array segregated into distinct assemblies (11, 15-17); only chemoreceptors of the same length class—those containing the same number of heptad (H) repeats in the signaling-terminal region—form

arrays (11, 18). The tip of chemoreceptors is conserved. This region mediates the stable assembly of chemoreceptors into trimers of dimers that interact with both CheA and CheW to form the structural unit of the chemotaxis array.

The genome of the nitrogen-fixing soil bacteria, *Azospirillum brasilense*, encodes four chemotaxis (Che) pathways (19), two of which (Che1 and Che4) are directly implicated in flagellar-driven motility (Figure 8) (20, 21), while the other two regulate functions unrelated to chemotaxis (Figure 9) (20). Signaling output from Che1 modulates transient changes in swimming speed (22), and the signaling output from Che4 controls transient changes in the probability of reversal in the swimming direction (21). The chemotaxis response of motile *A. brasilense* cells in a gradient depends on coordination of the signaling outputs from both Che1 and Che4 (21, 23, 24), with experimental evidence suggesting this is mediated at the level of chemoreceptor activity (24, 25). Here, we characterize the spatial organization of Che1 and Che4 chemotaxis proteins into two chemoreceptor arrays in *A. brasilense* and identify a mode of assembly that provides a mechanism for the integration of signaling from Che1 and Che4 into a coordinated output to control chemotaxis.

Materials and Methods

Strains, media, and chemicals

The bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth at 37°C supplemented with appropriate antibiotics (concentrations listed in footnotes of Table 1). *A. brasilense* strains were grown on plates at 28°C on minimal media for *A. brasilense* (MMAB) supplemented with 10 mM malate. Liquid cultures were grown by shaking (200 rpm)

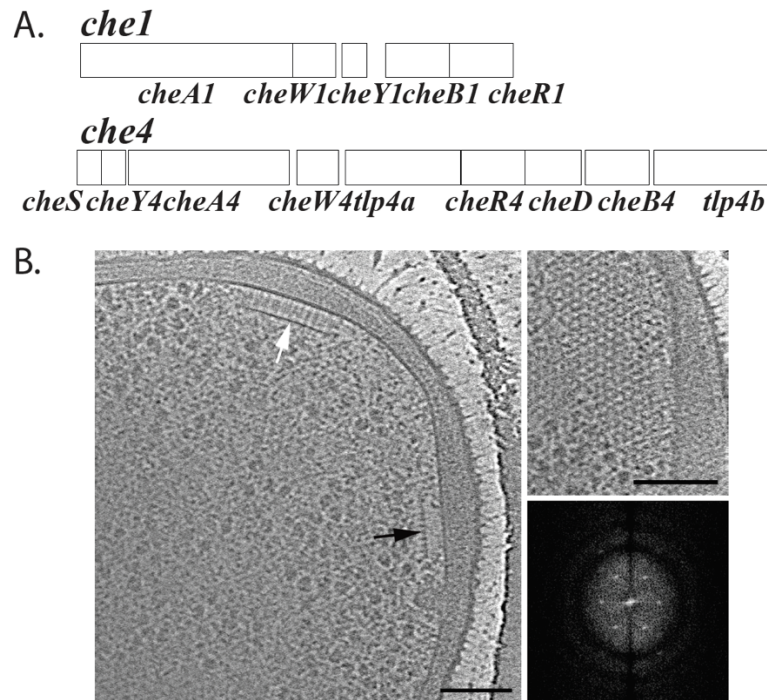


Figure 8: *A. brasilense* encodes 2 chemotaxis operons and 2 membrane bound receptor arrays

(A) The topology of Che1 and Che4, the 2 operons encoding the chemotaxis proteins that regulate swimming speed and reversal frequency, respectively. (B) Electron Cryotomography of wild type *A. brasilense*. Left: Slice through a cell showing side views of both tall (white arrow) and short array (black arrow). Top right: top view. Scale bars: 100 nm Bottom right: power spectrum from top view of the array (not to scale). This experiment was conducted by JMG and AB.

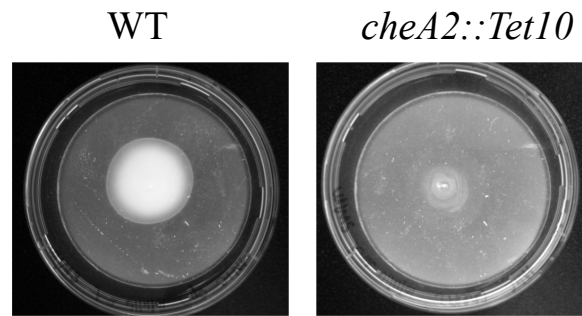


Figure 9: CheA2 is important for *A. brasilense* flagellation

Cells lacking CheA2 are impaired in swimming. This experiment was conducted by LO.

Table 1: Strains and plasmids used in this study

Strains or Plasmids	Genotype, relevant characteristics	Reference/source
<i>Strains</i>		
<i>E. coli</i> Top10	General cloning strain	Invitrogen
<i>E. coli</i> BTH101	F ⁻ <i>cya-99 araD139 galE15 galK16 rpsL1 hsdR2 mcrA1 mcrB1</i>	(58)
<i>E. coli</i> XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIq</i> ZAM15 Tn10 (Tet ^r)].	Agilent Technologies
<i>E. coli</i> S17.1	<i>thi endA recA hsdR</i> with RP4-2Tc::Mu-Km::Tn7 integrated in chromosome	(63)
<i>A. brasilense</i> Sp7	Wild type <i>A. brasilense</i>	ATCC29145
<i>A. brasilense</i> $\Delta che1$	$\Delta(cheA1-cheR1):: Cm (Cm^r)$	(23)
<i>A. brasilense</i> $\Delta che4$	$\Delta(cheA4-cheR4):: Gm (Gm^r)$	(21)
<i>A. brasilense</i> $\Delta che1 \Delta che4$	$\Delta(che1) \Delta(che4):: Cm (Cm^r), Gm (Gm^r)$	(21)
<i>A. brasilense</i> $\Delta cheA1 \Delta cheA4$	$\Delta(cheA1) \Delta(cheA4):: gusA-Km (Km^r), Gm (Gm^r)$	(21)
<i>A. brasilense</i> $\Delta cheA1$	$\Delta(cheA1):: gusA-Km (Km^r)$	(23)
<i>A. brasilense</i> $\Delta cheA4$	$\Delta(cheA4):: Gm (Gm^r)$	(21)
<i>A. brasilense</i> <i>cheA2::Tet</i> <i>cheA3::Tn5</i>	<i>cheA2::Tet (Tet^r)</i> <i>cheA3:: Tn5 (Km^R)</i>	This work
<i>A. brasilense</i> $\Delta che1$ (prhCheA1 Δ TMX)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>che1</i> with a plasmid containing CheA1 Δ TMX	This work
<i>A. brasilense</i> $\Delta che4$ (prhCheA1 Δ TMX)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>che4</i> with a plasmid containing CheA1 Δ TMX	This work
<i>A. brasilense</i> $\Delta che1 \Delta che4$ (prhCheA1 Δ TMX)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>che1</i> and <i>che4</i> with a plasmid containing CheA1 Δ TMX	This work

Table 1, Continued

Strains or plasmids	Genotype, relevant characteristics	Reference/ source
<i>A. brasilense</i> $\Delta che1$ (prhCheA4)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>che1</i> with a plasmid containing CheA4	This work
<i>A. brasilense</i> $\Delta che4$ (prhCheA4)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>che4</i> with a plasmid containing CheA4	This work
<i>A. brasilense</i> $\Delta che1\Delta che4$ (prhCheA4)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>che1</i> and <i>che4</i> with a plasmid containing CheA4	This work
<i>A. brasilense</i> $\Delta cheA1$ (prhTlp1)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA1</i> with a plasmid containing Tlp1	This work
<i>A. brasilense</i> $\Delta cheA4$ (prhTlp1)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA4</i> with a plasmid containing Tlp1	This work
<i>A. brasilense</i> $\Delta cheA1\Delta cheA4$ (prhTlp1)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA1</i> and <i>cheA4</i> with a plasmid containing Tlp1	This work
<i>A. brasilense</i> $\Delta cheA1$ (prhTlp4a)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA1</i> with a plasmid containing Tlp4a	This work
<i>A. brasilense</i> $\Delta cheA4$ (prhTlp4a)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA4</i> with a plasmid containing Tlp4a	This work
<i>A. brasilense</i> $\Delta cheA1\Delta cheA4$ (prhTlp4a)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA1</i> and <i>cheA4</i> with a plasmid containing Tlp4a	This work

Table 1, Continued

Strains or plasmids	Genotype, relevant characteristics	Reference/ source
<i>A. brasilense</i> <i>Sp7 (prhTlp1)</i>	Wild type <i>A. brasilense</i> with a plasmid containing Tlp1	This work
<i>A. brasilense</i> <i>Sp7 (prhTlp4a)</i>	Wild type <i>A. brasilense</i> with a plasmid containing Tlp1	This work
<i>A. brasilense</i> Δ <i>tlp1</i> (<i>prhTlp4a</i>)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>tlp1</i> and containing a plasmid with Tlp4a	This work
<i>A. brasilense</i> Δ <i>cheA1</i> (<i>prh005</i>)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>cheA1</i> and containing an empty pRH005 plasmid	This work
<i>A. brasilense</i> Δ <i>tlp1</i> (<i>prhTlp1</i>)	Sp7 mutant derivative of <i>A. brasilense</i> deleted for <i>tlp1</i> and expressing a plasmid containing Tlp1	(25)
<i>A. brasilense</i> <i>Sp7 (pKNOCKCheA2)</i>	Wild type <i>A. brasilense</i> with a plasmid containing CheA2 for insertional mutagenesis	This work
<i>A. brasilense</i> <i>cheA2::TetR</i> <i>cheA3::Tn5</i> (<i>prhCheA1TMX</i>)	Sp7 mutant derivative of <i>A. brasilense</i> with Tet insertion into <i>cheA2</i> gene and Tn5 insertion into <i>cheA3</i> gene and carrying a plasmid containing CheA1 Δ TMX	This work
<i>A. brasilense</i> <i>cheA2::TetR</i> <i>cheA3::Tn5</i> (<i>prhCheA4</i>)	Sp7 mutant derivative of <i>A. brasilense</i> with Tet insertion into <i>cheA2</i> gene and Tn5 insertion into <i>cheA3</i> gene and carrying a plasmid containing CheA4	This work
<i>A. brasilense</i> <i>cheA2::TetR</i> <i>cheA3::Tn5</i> (<i>prhTlp1</i>)	Sp7 mutant derivative of <i>A. brasilense</i> with Tet insertion into <i>cheA2</i> gene and Tn5 insertion into <i>cheA3</i> gene and carrying a plasmid containing Tlp1	This work

Table 1, Continued

Strains or plasmids	Genotype, relevant characteristics	Reference/ source
<i>A. brasilense</i> <i>cheA2::TetR</i> <i>cheA3::Tn5</i> (prhTlp4a)	Sp7 mutant derivative of <i>A. brasilense</i> with Tet insertion into <i>cheA2</i> gene and Tn5 insertion into <i>cheA3</i> gene and carrying a plasmid containing Tlp4a	This work
<i>Plasmids</i>		
TOPO 2.1	PCR cloning vector, Km	Invitrogen
pRH005	Gateway-based destination vector expressing proteins fused with YFP at their C-terminus; Km Cm	(57)
pRHTlp1	pRH005 containing a <i>tlp1</i> promoter region and ORF; Km Cm	(25)
pRHTlp4a	pRH005 containing a <i>tlp4a</i> promoter region and ORF; Km Cm	This work
pUT18	Derivative of pUC19 plasmid encoding T18 of CyA, Cb	(58)
pKNT25	Derivative of pSU40 plasmid encoding T25 of CyA, Km	(58)
pUT18C-zip	a derivative of pUT18C in which the leucine zipper of GCN4 is genetically fused in frame to the T18 fragment, Cb	(58)
pKT25-zip	a derivative of pKT25 in which the leucine zipper of GCN4 is genetically fused in frame to the T25 fragment, Km	(58)
pUT18cheA4	pUT18 containing <i>cheA4</i> , Cb	This work
pUT18cheW4	pUT18 containing <i>cheW4</i> , Cb	This work
pUT18tlp1	pUT18 containing <i>tlp1</i> , Cb	This work
pUT18tlp4a	pUT18 containing <i>tlp4a</i> , Cb	This work
pUT18tlp4S	pUT18 containing truncated <i>tlp4a</i> , Cb	This work
pUT18cheA1	pUT18 containing <i>cheA1</i> , Cb	This work
pUT18cheW1	pUT18 containing <i>cheW1</i> , Cb	This work

Km – kanamycin (50 µg/mL or 30 µg/mL), Cm- chloramphenicol (34 µg/mL), Gm – gentamycin (20 µg/mL), Cb – carbenicillin (50 µg/mL), Tet – tetracycline (10 µg/mL).

at 28 °C in MMAB supplemented with 10 mM malate and 18.7 mM ammonium chloride. To induce nitrogen fixation, cells were pelleted and washed three times with MMAB (supplemented with 10 mM malate, no nitrogen), and incubated in 5 mL of MMAB (supplemented with 10 mM malate, no nitrogen) at 28 °C without shaking to ensure low aeration for at least 6 h.

Electron Cryotomography

Azospirillum cultures were grown overnight in 5 mL MMAB + N with 200 µg/mL ampicillin at room temperature without shaking. The culture was spun down for 10 minutes at 3,500 x g, the pellet was resuspended in 5 mL MMAB - N, and spun down again for 10 min at 3,500 g. The pellet was then resuspended in 5 mL MMAB - N with 200 µg/mL ampicillin and left at room temperature (on bench) without shaking overnight. Cells were prepared on EM grids as described previously (1). Images were collected on either an G2 300 keV field emission gun microscope or TITAN Krios microscope with lens aberration corrector (both FEI company, now part of Thermo Fischer Scientific). Both microscopes were equipped with a GATAN K2 summit counting electron-detector cameras and GATAN imaging filters. Data were collected using the UCSFtomato software, using a cumulative dose of ~ 160 e/A². Tomograms were constructed using either automatically using RAPTOR (2), a program embedded in the Jensen lab pipeline and database (3) or imod software package (4).

Fluorescence Microscopy

Gateway cloning (Invitrogen) and the pRH005 vector were used to construct all yellow fluorescence protein (YFP) fusions (5). The pRH005 vector allows cloning of any gene to generate products fused in-frame with YFP at their C-terminus. Most YFP strains used in this study were

generated (Table 1) (6-8) using Gateway technology and adhering to manufacturer's protocol. Briefly, genes of interest were amplified using specific Gateway primers (Table 2) and *A. brasilense* strain Sp7 genomic DNA. Five microliters of PCR products were ran on a 0.8% gel for verification of insert and PCR clean-up (Macherey Nagel) was performed on the remainder of the PCR product. Resulting PCR products underwent a BP Clonase (Invitrogen) reaction with the pDONR2.1 vector (Invitrogen). Then, this reaction was transformed into Top10 chemically competent cells and plated on LB with kanamycin (50 µg/mL). Colonies from these plates were grown in 5 mL of LB with kanamycin (50 µg/mL), plasmid prepped (Qiagen) and resulting plasmids underwent an LR Reaction (Invitrogen) with the pRH005 plasmid. Resulting reactions were transformed into Top10 competent cells and plated on LB with kanamycin (50 µg/mL). All constructs were grown up in LB with kanamycin, plasmid prepped, and introduced into Sp7 and other strains (Table 1) by biparental mating as described in Hauwaerts et al., 2002 (9). One mL of cells grown as described above were pelleted at 5,000 rpm for 2 min. Twenty µL of the pelleted cells were resuspended in MMAB and mounted on a glass slide containing a 100 µL agarose pad (1% LMP agarose in 1X PBS buffer – NaCl 8g/L, KCl 0.2 g/L, KH₂PO₄ 0.24 g/L, Na₂HPO₄ 0.144 g/L, pH 7.0) and covered with a cover slip. Cells were left undisturbed for 2 h, or in some instances overnight, before being imaged. Images were captured using a Nikon ECLIPSE 80i fluorescence microscope equipped with a Nikon CoolSnap HQ2 cooled CCD camera. Measurements were taken from at least 80 cells from three independent cultures, and five fields of view were used for each sample. The results were graphed and analyzed statistically using GraphPad Prism software.

Bacterial two-hybrid assay

A bacterial two-hybrid assay specific for membrane proteins was used to investigate protein-protein interactions. In this assay, genes of interest were cloned into either pKNT25 (low copy) or pUT18 (high copy) vectors. These vectors each encode one half of a catalytic domain (T18 or T25) of the *Bordetella pertussis* adenylate cyclase. If protein-protein interaction takes place, functional complementation occurs between the two halves of the catalytic domain and cyclic AMP (cAMP) is produced. This activates the *lac* and *mal* operons in *E. coli*; positive interactions plated on MacConkey agarose utilize the carbon source in the agar and appear pink. From here, pink colonies can be grown in liquid media and subjected to a beta-galactosidase assay to quantify the strength of interactions. Proteins of interest (CheA1, CheA4, CheW1, CheW4, Tlp1, Tlp4a, and Tlp4S) were fused on the C-terminus of the T18 and T25 domains of *Bordetella pertussis* adenylate cyclase present in vectors pUT18 and pKNT25, respectively, as described by the manufacturer's protocol (Euromedex). The genes of interest were first PCR-amplified (Table 2) and cloned into a TOPO 2.1 vector (Invitrogen). The resulting vectors were digested with the following enzyme pairs (HindIII and EcoRI for *cheA4*, *cheW1*, *cheW4*, *tlp1*, and *tlp4a*; HindIII and KpnI for *cheA1*) and ligated into their destination vectors (high copy pUT18 and low copy pKNT25) that were previously digested with the same enzymes using T4 ligation (New England Bio Labs). Resulting plasmids were propagated in XL-1 Blue cells (Agilent Technologies), and the presence of an insert was confirmed by colony PCR. To test for protein-protein interactions, two plasmids expressing genes of interest were co-transformed into BTH101 competent cells and plated on Luria Broth (LB) plates with kanamycin (50 µg/mL) and carbenicillin (50 µg/mL). The plates were incubated for 48 h at 30 °C. Several colonies were picked from a plate, inoculated into

Table 2: Primers used in this study

Primer name	Sequence
CheA4 HindIII For BACTH	5' - AAG CTT ATG GAC GGG GTG CGC AAC AC - 3'
CheA4 EcoRI Rev BACTH	5' - GAA TTC GAC CGG TTC GAG TGC GGG GGC - 3'
Tlp4a HindIII For BACTH	5' - AAG CTT ATG GCG AAA GGG GTC GGT TCG - 3'
Tlp4a EcoRI Rev BACTH	5' - GAA TTC TGC CGC CCG TCC GCG GGC CAG - 3'
Tlp4S HindIII Fwd BACTH	5' -AAG CTT ATG CTC GCC CAC GAG GGC GC-3'
Tlp4S EcoR1 Rev BACTH	5' - GAA TTC TGC CGC CCG TCC GCG GCG G-3'
Tlp1 HindIII For BACTH	5' - AAG CTT ATG AAT CCC CTC CGC ACG TTC - 3'
Tlp1 EcoRI Rev BACTH	5' - GAA TTC GGC GAC CGC CGG AAG CGG GTG - 3'
CheA1 Δ TM HindIII For BACTH	5' - GC AAG CTTA GAC CGC CTG CCC TAC AAC - 3'
CheA1 Δ TM KpnI Rev BACTH	5' - GC GGT ACC TGC GGC ACC TTT CTG CTC - 3'
CheA2Tet insertion Fwd	5'- CCC CCC GGG TGC TGC GTG TCG AGG CCG-3'
CheA2Tet insertion Rev	5'-CCC CCC GGG ATG GCC GAG GTG CTG AC 3'

Table 2, Continued

Primer name	Sequence
CheA2 GW Fwd	5'- GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTG GCG CCT TTC ACC CGC A-3'
CheA2 GW Rev	5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA CAT CAG CTC CCG CAG ATT GAG
CheA3 GW Fwd	5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TAT GAA CGA CAT CCG CAC GCG CC-3'
CheA3 GW Rev	5'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA CAG CAG CCG CCG GAT GCC GGC C-3'

5 mL of liquid LB with kanamycin and carbenicillin (50 µg/ mL of each), and shaken (200 rpm) at 30 °C. Two microliters of the overnight cultures were spotted onto MacConkey plates with lactose as a carbon source and incubated at 30 °C for up to 96 h. Empty vectors (pUT18 and pKNT25) were used as negative controls while pUT18-zip and pKT25-zip (10) were used as positive controls. For quantification of interactions, cells were grown in 5 mL liquid LB with kanamycin and carbenicillin (50 µg/mL of each) at 30 °C while shaking at 200 rpm until they reached an OD₆₀₀ of 0.5- 0.6. A beta galactosidase assay was then performed as described in Ramsay, *et al* (11).

Protein pulldown

Protein pulldowns were used to confirm protein-protein interactions identified in the bacterial 2 hybrid assay. Gateway cloning and pDEST24 (Invitrogen) were used to generated CheA1ΔTMX-GST and CheA4-GST. Two liters of BL21 DE3 (pDEST CheA1ΔTMX-GST) and BL21 DE3 (pDEST CheA4-GST) cells were grown to OD₆₀₀=0.5 and induced with 1mM IPTG for 3 hours. Cells were collected and washed with 1x PBS (pH 8.0). Pellets were resuspending in RIPA buffer (50 mM Tris-HCl, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM PMSF, pH 8.0) and lysed using a French Press. Cell debris was removed by centrifuging at 17,000 rpm for 1 hour at 4°C. Total protein concentration was quantified using a Bradford. Lysate (2 mg total protein) was then applied to an equilibrated 2ml glutathione agarose resin and incubated together for 4 hours with rotation. Unbound proteins were washed off with 5 bed volumes of PBS. YFP tagged CheA1ΔTMX-YFP, CheA4-YFP, Tlp1-YFP, Tlp4a-YFP, CheW1-YFP, or CheW4-YFP were expressed in the corresponding mutant backgrounds and 500 ml cultures were grown to OD₆₀₀=0.8. Protein expression was confirmed using fluorescence microscopy. Cells were

collected via centrifugation (6000 rpm for 20 minutes at 4°C) and lysed in RIPA buffer using sonication with lysozyme (10 cycles of 15 second bursts followed by 10 second rest). Protein concentration was quantified using a Bradford assay (Biorad). Whole cell lysate (2mg total protein) was applied to the previously prepared CheA1 Δ TMX/ CheA4-GST resin and incubated overnight at 4°C with rotation. Unbound proteins were eluted using 10 bed volumes of 1xPBS. Column bound proteins were eluted with 1xPBS containing 10mM glutathione. Protein interactions were confirmed using western blotting: YFP- tagged proteins were detected using anti-GFP antibody (Abcam) at a 1:1000 dilution. CheA1 Δ TMX-GST and CheA4-GST column binding was confirmed using anti-GST polyclonal antibody (Invitrogen) (1:1000). The membranes were incubated with 1:5000 dilution of IRDye CW 800 donkey anti rabbit and developed using an Odyssey (Licor).

Sequence similarity and conserved residues

A. brasilense paralogs were aligned using BLAST Global align to identify sequence similarity. Residues 446-506 of CheA1 (Accession no.: AAL47021.1) and residues 334-397 of CheA4 (Accession no. WP_059399028.1) were used for aligning P3 domains. For P5 domains, residues 698-834 (P5A) and 860-984 (P5B) of CheA1 and residues 543-675 of CheA4 were aligned. The entire sequence of CheW1 (AAL47022.1) and CheW4 (WP_035675900.1) were aligned.

To identify conserved residues, the BLAST Multiple Alignment Tool was used to align CheA1 P5A and P5B domains with residues 519-659 of *E. coli* CheA (Accession no ANK01864.1). For CheW alignments, full length CheW1 and CheW4 of *A. brasilense* were

aligned with *E. coli* CheW (Accession no. ANK01864.1). Residues necessary for CheA-CheW interactions were identified based on (12, 13).

Measuring the height of chemoreceptor arrays

To measure the distance between the inner membrane and the CheA/CheW base plate, we used 3dmod v4.9.9 (4) to mark the inner membrane above the chemoreceptor array with model points. Next, we used a custom script written in Node.js that takes as input the tomogram and the model points to calculate the average pixel value in profiles running perpendicular to the model points but in the same plane as the model points to collect and generate a JSON formatted file with the average pixel intensity of each profile. Each file generated was named after the name of the tomogram, the name of the model and a unique number in case there were more than one profile per tomogram. The profiles were zipped and available in the Supplementary Dataset D2 (Appendix). The script is available in the GitLab repository at <https://gitlab.com/daviortega/sideview-profile-average>.

To visualize the profiles, we used the ObservableHQ notebook located at <https://beta.observablehq.com/@daviortega/generic-notebook-to-analyse-1d-averaged-electron-density-p>. For each profile, we measured the distance between the dips corresponding to the electron density of the inner membrane and the CheA/CheW baseplate in pixels. Uncertainty was estimated based on the precision to determine the center of each dip in pixels and propagated to the measure of distance between dips.

Bioinformatics datasets and analysis

The chemoreceptor sequences were collected from MiST (14). Multiple sequence alignments were conducted using L-INS-I algorithm from the MAFFT package version v7.305b (15) and manually edited using Jalview v2.10.1 (16). The alignments were only manually edited at the region between the transmembrane and the signaling domain. In the multiple sequence alignments, the sequences header contains two initial letters of the genus, the first three letters of the species, the NCBI accession number of the genome, the locus number and the heptad class of the receptor. Domain architecture of chemoreceptors were produced using CDVist (17). When missing, HAMP domains were identified manually using sequence signatures of HAMP domains in (18). Measurements performed in atomic models from the PDB (19) were obtained using VMD v1.9.1 (20).

Results

Cryo-EM reveals two distinct chemoreceptor arrays in *A. brasilense*

The *A. brasilense* genome encodes over 51 predicted chemoreceptors, from at least four different length classes (18) but the organization of the chemoreceptors contributing to flagella-mediated chemotaxis has not been characterized. We used electron tomography (cryo-EM) to characterize the chemoreceptor arrays formed by interaction with Che1 or Che4 signaling proteins. Because chemoreceptor arrays do not assemble as organized clusters in cells lacking CheA or CheW in *E. coli* (26, 27), we hypothesized that we could identify Che1- and Che4—associated arrays using a combination of wild type strain, $\Delta che1$, $\Delta che4$, and $\Delta che1\Delta che4$ mutant derivatives. In wild type cells, two structurally distinct types of chemoreceptor arrays were observed: out of 15 cell poles imaged, we found 6 short chemoreceptor clusters (27.5 ± 2.6 nm on average) and 2 tall

arrays (30.7 ± 1.8 and 31.3 ± 2.9 nm), as measured by the distance between the inner membrane and the CheA/CheW base plate (Figure 8B, Top left). Top views of the chemoreceptor arrays (Figure 8B, Top right, power spectrum (not to scale), Bottom right) revealed the typical, highly ordered hexagonal packing with a 12 nm spacing between the neighboring hexagons. In contrast, neither short nor tall arrays could be observed in the $\Delta che1\Delta che4$ mutant (20 cells imaged).

Assignment of chemoreceptors encoded in the *A. brasilense* genome to arrays of specific height

The height of chemoreceptor arrays is determined by the physical length of membrane bound chemoreceptor proteins present in the array, which in turn can be predicted from sequence based the heptad class of the signaling domain (49) and the arrangement of protein domains present between the transmembrane region and the signaling domain (50). To predict which set of chemoreceptors compose the two structurally distinct chemoreceptor arrays, we analyzed the sequences of the chemoreceptors from class 38H and 36H predicted to interact with Che1 (class F5) and Che4 (class F7) proteins respectively (51). The short arrays (28nm) visualized in the wild-type cells are likely formed by 38H chemoreceptors with a single HAMP and alpha helix linker, which comprised the majority of chemoreceptors encoded within the *A. brasilense* genome. Previous studies in *M. magneticum* have shown that 28 nm chemotaxis arrays have been reported to belong to 38H receptors with extra alpha helix linkers (50). The multiple sequence alignment of sequences of 38H chemoreceptors from both *A. brasiliense* and *M. magneticum* does not contain major gaps in the region between the transmembrane and the signaling domain, which indicates that they share the same domain architecture (Supplementary Dataset D1). As in a few chemoreceptors in *M. magneticum*, two 38H receptors in *A. brasiliense* have an extra HAMP instead of the alpha helix linker (AMK58_RS04445, AMK58_RS08090). Based on the alignment,

the alpha helix linker is approximately the same number of residues of one of the HAMP helices. According to the crystal structure of triple HAMP domains (PDB: 3LNR) (44) this difference increases 1.7 nm to the height of the chemoreceptor and thus, we expect that arrays formed by these chemoreceptors to be taller at 29.7 nm. These results suggest that the short array visualized in the wild-type cells are formed by 38H chemoreceptors with a single HAMP and alpha helix linker.

By exclusion, only 36H chemoreceptors can form the 31 nm tall array. The *A. brasilense* genome contains three 36H chemoreceptors and all of them have 3 HAMP domains. In addition, the chemoreceptor present in the *che4* operon, *tlp4a*, has also a PAS domain between the first and the second HAMP domain from the transmembrane. To predict the height of this receptor we measure the 11 nm length of the crystal structure of a triple HAMP. The signaling domain of 36H receptors alone in the HAMP + tsr fusion 3D crystal structure (PDB: 3ZX6) (45) is 18 nm. Finally, the PAS + HAMP structure available (PDB: 3VOL) (46) suggests that an additional PAS domain would add only 2 nm to the height of the receptor since the bulk of the PAS domain should be located aside of the long axis of the receptor.

Thus, we expect *tlp4a* receptor to have 31 nm which is consistent with the height of the array observed in wild type strains. Although the other two 36H chemoreceptors do not have PAS domains, they have a linker of 23 residues (Appendix- Supplementary Dataset 2). If these residues fold in a straight alpha helix, these 36H chemoreceptors are expected to form 32 nm arrays, however we expect that flexibility of the structure could accommodate this difference compared with *tlp4a*. Thus, we conclude that *tlp4a* is the receptor present in the observed 31 nm tall chemoreceptor array. Eleven cell poles of $\Delta che4$ cells still contained 14 short arrays averaging

27.7 ± 3.1 nm and 2 tall arrays with 30 ± 1.8 nm and 30.0 ± 3.6 nm. Interestingly, the tall arrays in $\Delta che4$ mutants are consistently shorter than in the wild type. This result is expected because the only chemoreceptor that make a 31 nm tall array, *tlp4a*, is encoded in the *che4* operon. We hypothesize that these 30 nm taller arrays are made by the 38H chemoreceptors with 2 HAMP domains. In $\Delta che1$ we only observed short arrays out of 7 cell poles averaging 28.1 ± 3.1 nm. Although our best estimates for the physical length of the chemoreceptor arrays matches the measurements obtained from the tomograms, these results are barely significant given the uncertainty inherent of the methods used. However, these results combined suggests that both operons *che1* and *che4* alone can form the most common, shorter array with 28 nm height. This unexpected result predicts that 38H chemoreceptors can form chemotaxis clusters with *che4* proteins. The *che1* operon appears to be needed to form taller arrays by either contributing to assemble the array containing *tlp4a* (31 nm) or in the absence of the operon *che4*, to build the taller arrays using 38H receptors with double HAMP domains (30 nm). At this point, we have not observed clusters that we can assign to the remaining Che operons, Che2 and Che3, suggesting they are either not expressed or simply not observed.

Localization of chemotaxis proteins depends on Che1 and Che4

The results of the cryo-EM analyses suggested that proteins from *che1* and *che4* could mix to form signaling arrays seen in wild type. To test this hypothesis, we analyzed the dependence of chemoreceptor clusters localization on Che1 and Che4 using fusions (CheA1 Δ TMX, CheW1, CheA4, CheW4, CheY4) to yellow fluorescent protein (YFP) tags and imaged each in wild type, $\Delta che1$, $\Delta che4$, and $\Delta che1\Delta che4$ mutant backgrounds. The functionality of all tagged proteins was verified through chemotaxis soft agar assays before imaging (29). *A. brasilense* Sp7 contains two

isoforms of CheA1: full length CheA1 (which is membrane anchored and localizes at the cell surface) and CheA1 Δ TMX (which lacks the transmembrane domains and is vital to chemotaxis) (29). In the wild type background, CheA1 Δ TMX-YFP localized as bright foci to the cell poles and was also found diffuse and throughout the cell surface (Figure 10A, C). This surface localization is probably due to CheA1 Δ TMX interacting with its full length CheA1 isoform, which localizes to the cell surface. In the Δ *che1* and Δ *che4* backgrounds, CheA1 Δ TMX-YFP localized to the cell poles (Figure 10A, C). In the Δ *che1* Δ *che4* background, cells were diffuse, and no distinct fluorescent foci were detected (Figure 10A, C). The ability of CheA1 Δ TMX-YFP to polarly localize in the absence of Che1 or Che4 proteins suggests that CheA1 Δ TMX-YFP is able to interact with proteins from both systems to form polar foci. Furthermore, the ability of CheA1 Δ TMX-YFP to mainly localize as polar foci in the Δ *che1* background indicates that the natively produced CheA1 Δ TMX may be part of the Che4 cluster.

CheA4-YFP localized as polar foci in the wild type, Δ *che1*, and Δ *che4* mutant strains and was diffuse in the Δ *che1* Δ *che4* strain background (Figure 10B, D). YFP fusions to CheY4 (CheY4-YFP), which functions downstream of CheA4 (21) and to the adaptor proteins, CheW1 or CheW4, which are encoded within the Che1 or Che4 pathways, respectively (CheW1-YFP and CheW4-YFP) also localized as polar foci in wild type, Δ *che1*, Δ *che4* backgrounds but were diffuse in the Δ *che1* Δ *che4* mutant background (Figure 11A-C). A CheY1-YFP fusion is diffuse since CheY1-YFP will interact with polarly-localized CheA1 Δ TMX and membrane-bound CheA1 (Figure 11D). We also looked at localization of CheA1 Δ TMX and CheA4 in cells lacking CheA2 and CheA3 (*cheA2::TetR cheA3::Tn5*) to determine if CheA2 and CheA3 are involved in polar foci formation. Both CheA1 Δ TMX-YFP and CheA4-YFP localized at cell poles similarly regardless

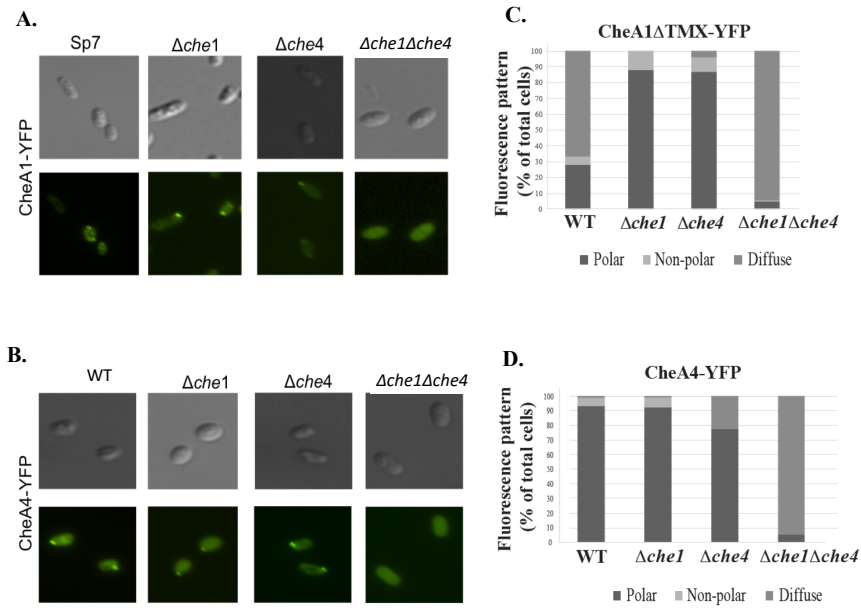


Figure 10: Localization of YFP-tagged CheA proteins

(A) CheA1ΔTMX-YFP and (B) CheA4-YFP cells in wild type (WT), $\Delta che1$, $\Delta che4$, and $\Delta che1\Delta che4$ mutant derivative backgrounds. (C) Quantification of the distribution of fluorescence in the population of CheA1ΔTMX-YFP cells analyzed in wild type and mutant derivative backgrounds. For each bar, $n \geq 100$ cells. (D) Quantitation of the distribution of fluorescence in the population of CheA4-YFP cells analyzed in wild type and mutant derivative backgrounds. For each bar, $n \geq 100$ cells. This experiment was conducted by JMG and AA.

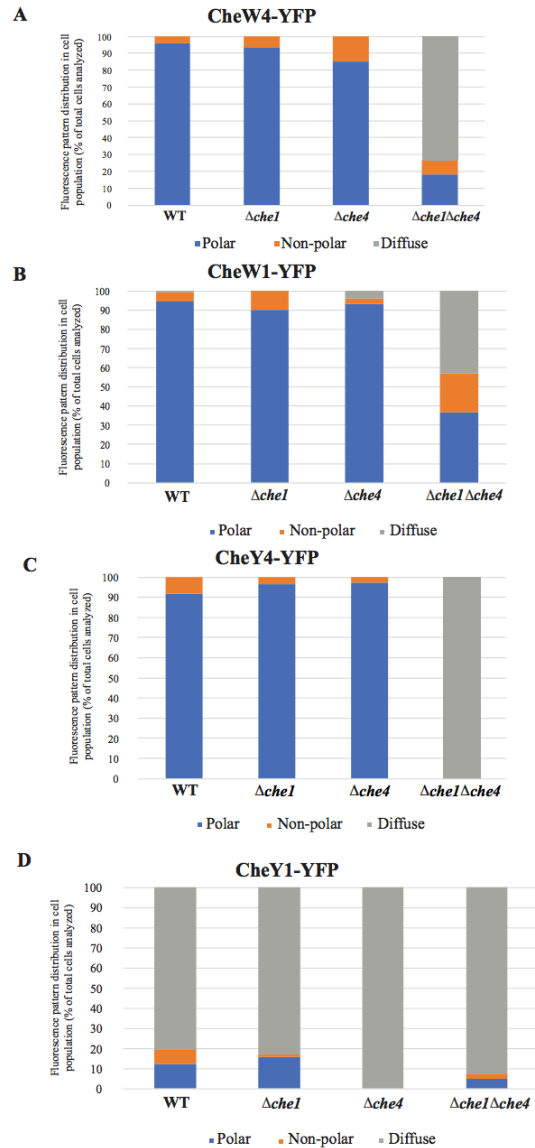


Figure 11: Subcellular localization for YFP-tagged chemotaxis proteins encoded in Che1 and Che4

(A) Quantification of the distribution of fluorescence in the population of CheW4-YFP cells analyzed in wild type (WT) and mutant derivative backgrounds. $N \geq 60$ cells for each bar. (B) Quantification of the distribution of fluorescence in the population of CheW1-YFP cells analyzed in wild type (WT) and mutant derivative backgrounds. $N \geq 73$ cells for each bar. (C) Quantification of the distribution of fluorescence in the population of CheY4-YFP cells analyzed in wild type (WT) and mutant derivative backgrounds. $N \geq 100$ cells for each bar. (D) Quantification of the distribution of fluorescence in the population of CheY1-YFP cells analyzed in wild type (WT) and mutant derivative backgrounds. $N \geq 60$ cells for each bar. This experiment was conducted by AA.

of the presence of CheA2 and CheA3—indicating they are not required for polar foci formation (Figure 12). Proper localization of chemotaxis proteins in the absence of either Che1 or Che4 proteins suggests Che1 and Che4 proteins interact and form polarly localized arrays.

Che1 and Che4 chemotaxis proteins interact with each other in the bacterial two-hybrid assay

To test whether Che1 and Che4 proteins physically interact, which would be required to form stable chemoreceptor arrays, we used a bacterial adenylate cyclase two-hybrid (BACTH) assay. Given that YFP fusions to the C-terminus of CheA1, CheA4, CheY1, CheY4, and Tlp1 are functional (25, 29), we tested all interactions with the catalytic domains fused at the C-terminus of the proteins expressed from both BACTH vectors.

We first determined that proteins encoded within the same operon are able to interact. CheA4 and CheW4 interacted with one another in this system, as expected since both proteins are encoded by genes found together within the *che4* operon, and previous work indicates they function in the same pathway (21) (Figure 13A). Furthermore, CheA4 and CheW4 were able to interact with themselves. We also detected positive interactions between proteins encoded for in Che1, CheA1 is able to interact with itself and CheW1. Proteins encoded in separate operons were able to interact with each other: CheA1 with CheA4, CheA1 with CheW4 and CheA4 with CheW1 (Figure 13A-B). These results concur with our microscopy observations and indicate that, in this assay, proteins from Che1 could physically interact with proteins from Che4. We did test these chemotaxis proteins against empty vectors and found no interaction, consistent with the absence of reports of auto-activation in this assay (30). False positives are rarely, if ever, reported with the BACTH (30), suggesting that positive interactions detected here are reliable.

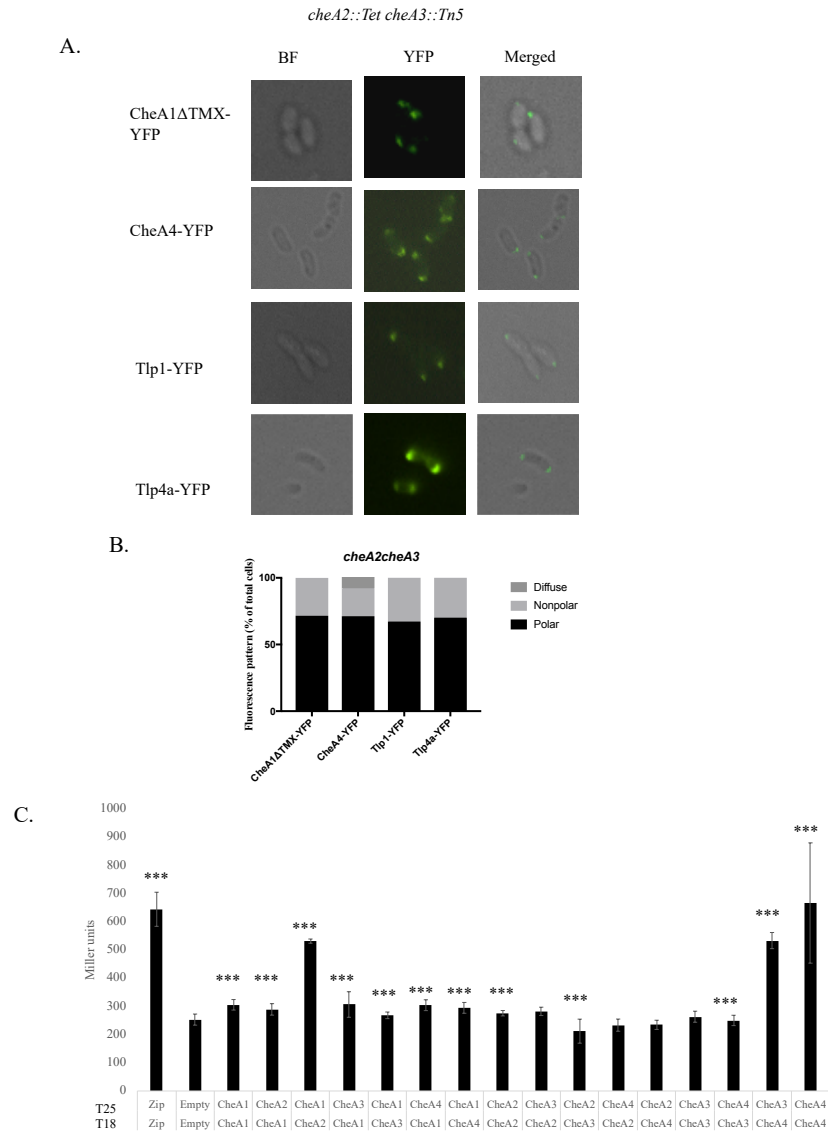


Figure 12: CheA2 and CheA3 involvement in chemotaxis array formation

YFP tagged CheA1ΔTMX, CheA4, Tlp1, and Tlp4a are all able to polarly localize in the absence of CheA2 and CheA3 (A-B). Interaction of *A. brasilense* CheA paralogs in the bacterial two-hybrid assay. All protein-protein interactions were tested bi-directionally since the vectors had different copy numbers (pKNT25, low copy; pUT18, high copy). Significance for each interaction is relative to the negative control ran alongside it on its individual 96-well plate. * denotes p value < 0.05; ** denotes p value < 0.005; *** denotes p value < 0.001. This experiment was conducted by LO.

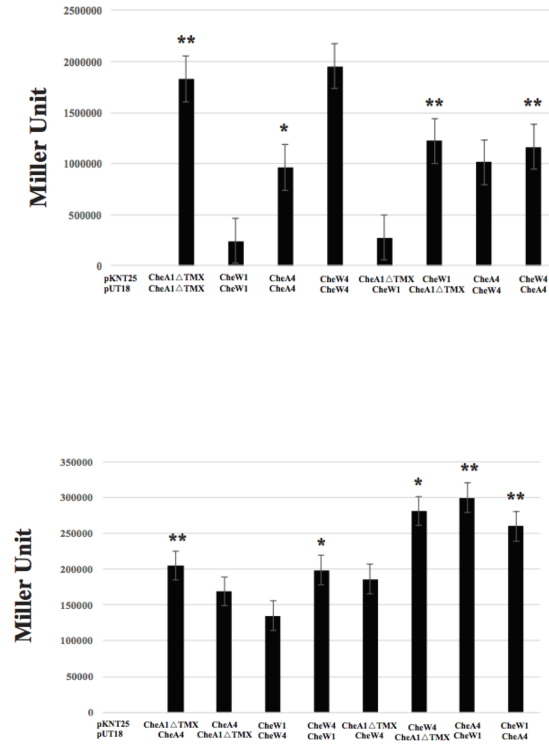


Figure 13: Interaction of chemotaxis proteins from Che1 and Che4 in the bacterial two-hybrid assay

(A) Interactions between chemotaxis proteins encoded within the same signal transduction pathway. (B) Interactions between chemotaxis proteins encoded in different signal transduction pathways. All protein-protein interactions were tested bi-directionally since the vectors had different copy numbers (pKNT25, low copy; pUT18, high copy). Significance for each interaction is relative to the negative control ran alongside it on its individual 96-well plate. * denotes p value < 0.05; ** denotes p value < 0.005. This experiment was conducted by AH.

Relative contribution of CheA4 and CheA1 to clustering of chemotaxis receptors into polar arrays

To further explore the possible interaction of Che1 and Che4 proteins within each of the two membrane-anchored arrays, we next characterized the interactions of these proteins with chemoreceptors using fluorescence microscopy and the BACTH assay. Che1 is the ancestral chemotaxis pathway controlling chemotaxis in *A. brasilense* (19) and most chemoreceptors predicted within the genome sequence, which belong to the 38H length class, are likely to interact with components of Che1. Furthermore, previous data indicated that Tlp1, a predicted 38H length class chemoreceptor, interacted with Che1 and perhaps another chemotaxis pathway, which we later identified as Che4 (25). On the other hand, *tlp4a* encodes for a predicted membrane-anchored chemoreceptor of the 36H class and is found within the *che4* operon and is thus likely to function with Che4 proteins (21). Therefore, we chose to test both Tlp1 and Tlp4a. Both Tlp4a-YFP and Tlp1-YFP, which are functional fusions, localized at the cell poles as punctate foci when expressed in the wild type strain (Figure 14). In the $\Delta cheA1$ strain, Tlp1-YFP was largely diffuse throughout the cell, with a small fraction of cells (29%) showing polar foci (Figure 14C, E). Tlp1-YFP localized to the cell poles in the $\Delta cheA4$ strain, in a pattern similar to that seen in the wild type strain background. Tlp1-YFP was diffuse in the $\Delta cheA1\Delta cheA4$ strain and fluorescent foci were seldom observed. These findings suggest that CheA1 plays a major role in polar foci localization of Tlp1-YFP while CheA4 plays a minor, if any, role. In strains lacking Che1 or Che4, Tlp1-YFP was mostly diffuse (Figure 14F), suggesting that other Che1 and Che4 chemotaxis proteins are required for Tlp1-YFP localization.

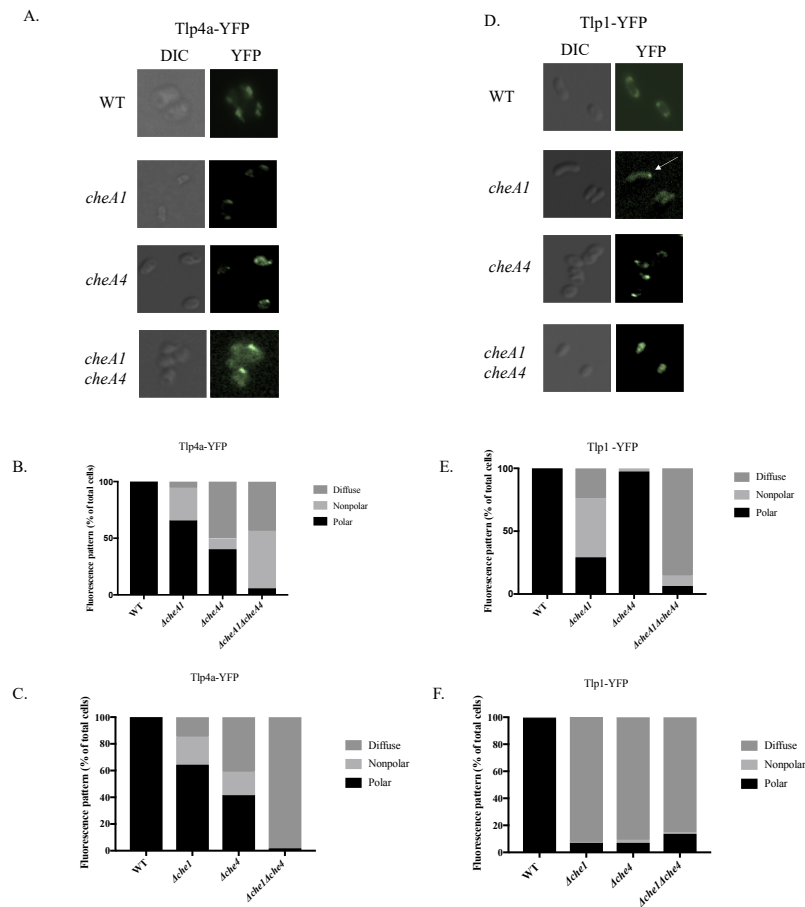


Figure 14: Role of chemotaxis proteins in localization of YFP-tagged chemoreceptors

(A,B) DIC and fluorescence microscopy images of Tlp1-YFP (A) and Tlp4a-YFP (B) in the wild type (WT) and $\Delta cheA1$, $\Delta cheA4$, and $\Delta cheA1\Delta cheA4$ mutant backgrounds. Relative polar foci fluorescence intensity with standard deviation is listed beneath the corresponding YFP image. Percentage of cells with Polar, nonpolar, and diffuse localization of Tlp1-YFP (C, E) and Tlp4a-YFP (D, F) clusters in cells lacking CheA1, CheA4, CheA1CheA4 (C, D) and cells lacking all Che1, Che4, Che1Che4 proteins (E,F). For each bar $n \geq 80$. This experiment was conducted by LO and AA.

In the $\Delta cheA1$ strain background, Tlp4a-YFP localizes as numerous foci, both lateral and polar, that also appeared smaller in size relative to the large polar foci of wild type. The average number of fluorescent foci per cell in the wild type background was 2.1 ± 0.7 while the number of fluorescent foci per cell in the $\Delta cheA1$ strain was 3.8 ± 1.3 (Student's *t* test, *p*-value < 0.0001, *N* = 90). In the $\Delta cheA4$, the Tlp4a-YFP fluorescent foci were mislocalized; foci were often slightly off-polar and detected at a greater distance from the cell poles (Figure 14D); while in $\Delta cheA1\Delta cheA4$, Tlp4a-YFP was mostly found as either non-polar foci or diffuse (Figure 14D). Together, these observations suggest that polar localization of Tlp4a-YFP in clusters seen as tight fluorescent foci require both CheA1 and CheA4. In strains lacking Che1 or Che4, Tlp4a-YFP localization produced patterns similar than the ones observed for strains lacking CheA1 or CheA4, including the loss of foci formation in the absence of both sets of chemotaxis proteins (Figure 14E).

Contribution of Che1 and Che4 proteins, other than CheA paralogs, in clustering of chemotaxis receptors

We next tested for possible physical interaction between CheA1, CheW1, CheA4, CheW4 with Tlp1 and Tlp4a in the BACTH assay. Tlp1 positively interacted with CheA1 Δ TMX, CheW1 and itself (Figure 15A). This finding was expected given that chemoreceptors are known to dimerize and that Tlp1 was shown to signal in a Che1-dependent manner (25). Tlp1 also positively interacted with CheA4 and CheW4 (Figure 15A). Tlp4a interacted with CheA4 and CheW4, as expected, but also interacted with CheA1 Δ TMX (Figure 15B). Full length Tlp4a was not found to interact with itself at detectable levels in this assay. However, given that Tlp4a had positive

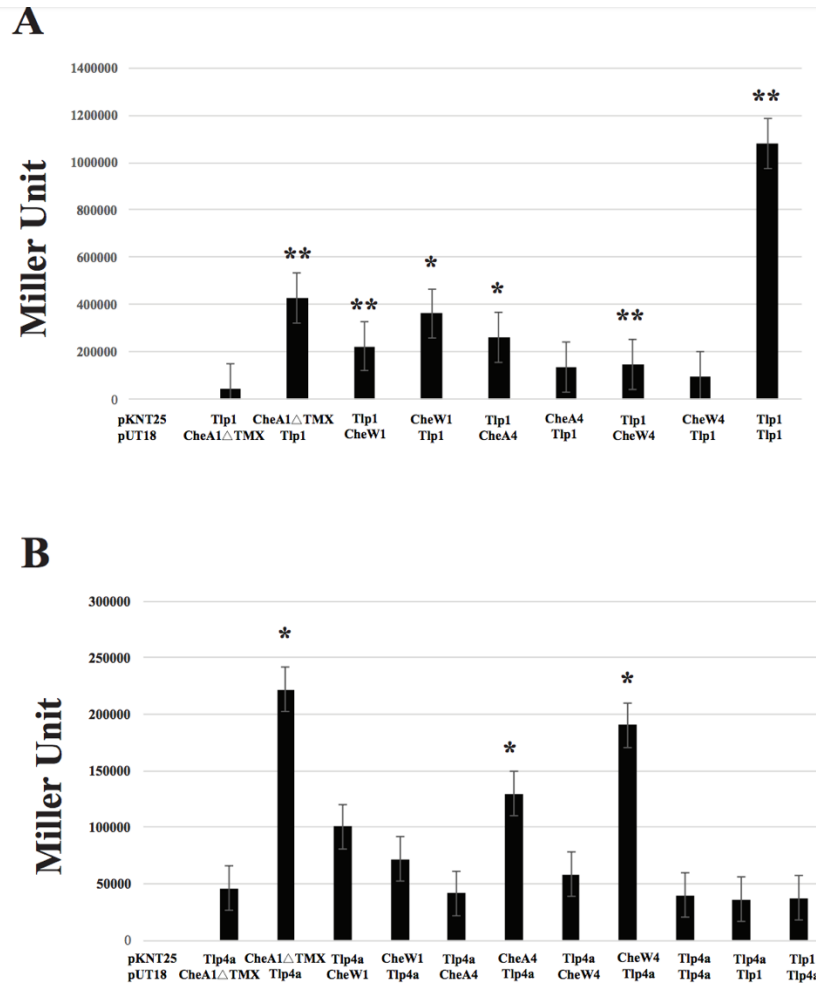


Figure 15: Interaction of chemotaxis proteins from Che1 and Che4 with Tlp1 and Tlp4a

Interaction of Tlp1 (A) and Tlp4a (B) with chemotaxis proteins from both operons in a bacterial two-hybrid assay. All protein-protein interactions were tested bi-directionally since the vectors had different copy numbers (pKNT25, low copy; pUT18, high copy). Significance for each interaction is relative to the negative control run alongside it on its individual 96-well plate. * denotes p value < 0.05; ** denotes p value < 0.005. This experiment was conducted by AH.

interactions with other proteins, it is unlikely that this is the result of a nonfunctional tagged protein, but the reason for this negative result is not known.

As expected from their different lengths, Tlp1 and Tlp4a did not interact. Together these data suggest that chemotaxis receptors from the 38H (Tlp1) and 36H (Tlp4a) classes can physically interact with Che1 and some of the Che4 proteins, in particular both CheA paralogs, in *A. brasilense*. While only predicted as possible physical interactions in the BACTH assay, these results are fully consistent with the fluorescence imaging data described above.

Next, we used pull-down assays to verify the physical interactions suggested by the BACTH assay (Figure 16). We found that CheA1 Δ TMX-GST could interact with itself as well as with CheA4-YFP, Tlp1-YFP and Tlp4a-YFP (Figure 16). CheA1 Δ TMX-GST intreracted with CheW1-YFP, albeit weakly, and CheW4-YFP. This finding is unexpected since CheA1 and CheW1 are encoded within the *che1* operon. CheW interacts with CheA through the P5 domain (31, 32) and the *A. brasilense* CheA1 possesses two P5 domains at its C-terminus, which could complicate the interactions tested here. In a similar assay, we found that CheA4-GST interacts with CheA1 Δ TMX-YFP, CheW1-YFP, CheW4-YFP, Tlp4a-YFP, and Tlp1-YFP (Figure 16). Together, these data validate the physical interactions between Che1 and Che4 chemotaxis proteins, including their interaction to form mixed chemotaxis signaling clusters.

Discussion

Our results indicate that in *A. brasilense*, each of the two membrane-bound chemoreceptor arrays detected by cryo-EM utilize paralogs from both Che1 and Che4 chemotaxis signaling pathways to build extended stable arrays. Our experimental evidence strongly suggests that CheA1 Δ TMX and CheA4, and likely, CheW1 and CheW4, are recruited to form the baseplates of

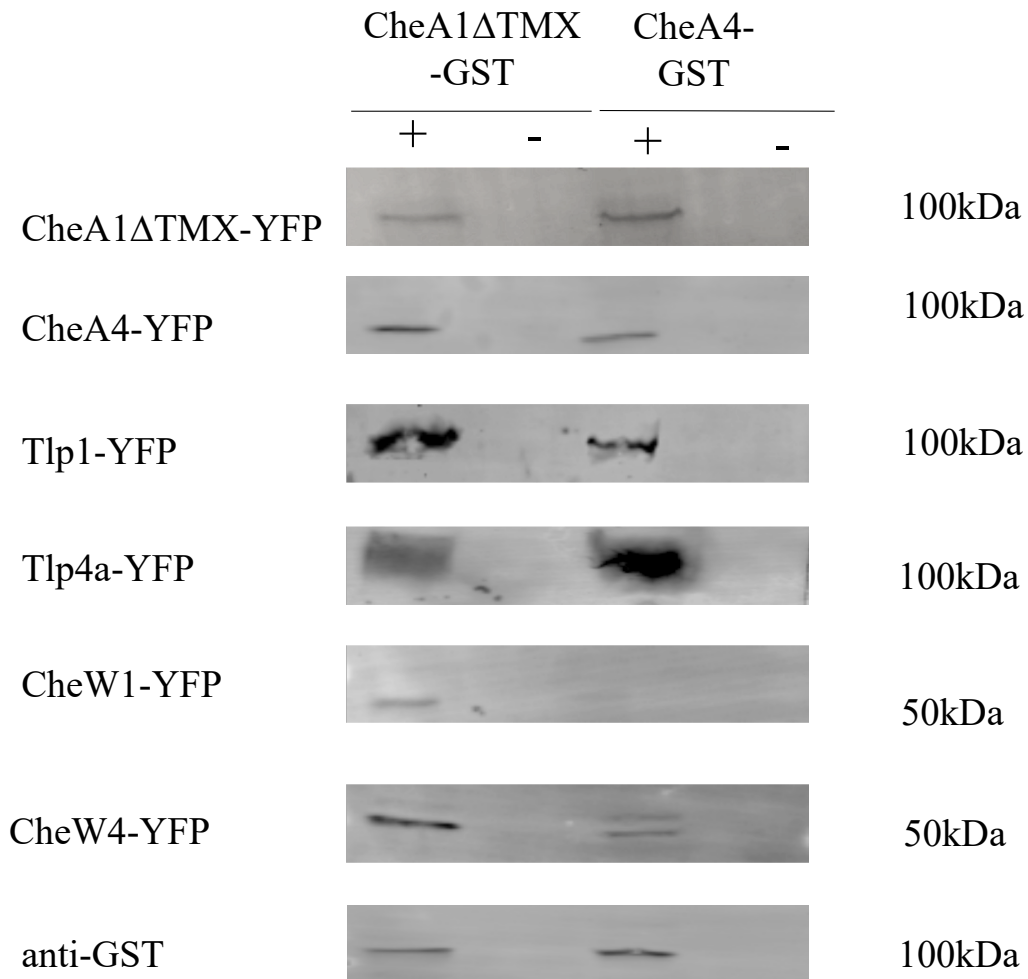


Figure 16: Interaction of chemotaxis proteins in pull down assays

Interaction of chemotaxis proteins in pull down assays. C-terminally GST-tagged CheA1ΔTMX and CheA4 were immobilized on an anti-GST column. Cell lysates containing YFP tagged CheAΔTMX, CheA4, Tlp1, Tlp4a, CheW1, CheW4 were flowed over columns containing GST-tagged CheA1ΔTMX and CheA4 (+) or GST (-). Unbound proteins were washed using 1XPBS and bound proteins were eluted with 10mM glutathione. Proteins were detected by western blots using Rabbit anti-GFP (Abcam) and Rabbit anti-GST (Invitrogen). This experiment was conducted by LO.

each of the two *A. brasilense* chemotaxis receptor arrays, each of which likely comprise Tlp1 and other 38H receptors or Tlp4a and other 36H receptors. First, cryo-EM data indicates that *A. brasilense* contains 2 chemotaxis arrays, even in the absence of Che1 or Che4 proteins. This suggests that Che1 and Che4 proteins can be used to build both arrays. Our fluorescence data also supports this: proteins encoded in Che1 and Che4 can properly localize as long as either the Che1 or Che4 system is present. We also show that proteins encoded in separate operons are capable of interacting with each in a bacterial two-hybrid and pull-down assay. Furthermore, our pull-down data verify that receptors from both arrays, Tlp1 and Tlp4a interact with both CheA1 Δ TMX and CheA4. These results provide a straightforward mechanism by which two chemotaxis signaling pathways, Che1 and Che4, could function in a coordinated manner to regulate chemotaxis responses in *A. brasilense*, despite each of Che1 and Che4 regulating different signaling outputs (21, 22). In support of this hypothesis, we have previously showed that mutations within Che1 affects reversal frequency which is the direct signaling output of Che4 (22) while mutations within Che4 also affect swimming speed, the direct output of Che1 (21). Additional evidence points to indirect interactions between chemotaxis signaling pathways at the level of chemoreceptors (24, 25). Given the propensity for chemotaxis signal pathways to be horizontally transferred between bacteria (14), a similar co-binding and mixing mechanism may be found in other bacteria.

Our data suggest that paralogs from Che1 and Che4 can physically interact, which would be necessary to form mixed baseplates. CheA dimerization is facilitated through the P3 domain. CheA1 P3 and CheA4 P3 have 59% sequence similarity, which could perhaps be sufficient to license dimerization by inter-subunit P3/P3 exchange. CheA paralogs possess P5 domains that interact with CheW, and the interaction interface is critical for stabilizing chemoreceptor arrays,

maintaining connectivity, and signal cooperativity (28, 32, 33). We detected positive interactions between CheA1 Δ TMX and CheA4 with both CheW1 and CheW4. CheA1 contains two P5 domains (P5A and P5B), while CheA4 has a single P5 domain. Previous work in *E. coli* identified conserved residues necessary for CheA P5-CheW interactions (32). CheA1 P5A contains 6 of the 16 conserved residues in *E. coli* CheA implicated in interaction with CheW, while CheA1 P5B only had 3 of these 16 residues. CheA4 P5 contains 5 of the conserved residues in *E. coli* CheA (Figure 17). Furthermore, while CheA1 P5A and P5B domains are most similar to one another (51%), CheA1 P5A, and not P5B, shares the most similarity with CheA4 P5 (47%). CheW1 and CheW4 share 50% sequence similarity with one another and both CheW1 and CheW4 possess all the conserved residues implicated in CheA-CheW binding in *E. coli* (Figure 17) (34). Given the role of CheA and CheW interactions with each other and receptors in cluster formation and the ability of P5-CheW to substitute with one another in chemoreceptor arrays (12, 35-37), it is likely that similar interactions between paralogous chemotaxis proteins are implicated in mixed clusters formation in *A. brasilense*.

The ability for different chemotaxis proteins to assemble in mixed baseplates within a single array is also suggested to occur via domains related to CheW in other model systems. CheV proteins, which structurally and functionally are CheW-REC hybrids (38), are widespread in bacterial genomes and recent evidence indicates that in *Helicobacter pylori*, CheV-comprising

chemoreceptor-kinase clusters can join a larger CheW-receptor-kinase membrane-anchored cluster. In *Salmonella enterica*, recent comparative genomics combined with analysis of experimental evidence also imply a role for CheV in bringing specific chemoreceptors to stable arrays (39). In *Vibrio cholera*, baseplate composition is dynamic, with CheW and CheV able to form baseplates in the absence of CheA (40). This variation is hypothesized to aid array function and receptor turnover. *A. brasilense* Che1 and Che4 proteins forming mixed baseplates is thus not incompatible with maintaining signal cooperativity within the chemoreceptor arrays. Physical separation of chemoreceptors into two distinct membrane-bound receptor arrays is dictated by segregation of chemoreceptors into length classes (15). This observation raises questions on the relative contributions of the different chemoreceptor arrays to sensing.

Signal processing and integration via multiple chemotaxis signaling pathways is unlikely to be unique to *A. brasilense* given the number of bacterial genomes with multiple chemotaxis pathways (14) and experimental evidence of signal integration between chemotaxis signaling pathways in other species (41). Evidence of signal integration implicating chemotaxis receptors and multiple signaling pathways exists in *Myxococcus xanthus* between the Che7, Dif, and Frz chemotaxis-like signaling pathways (42-44). In contrast, spatial segregation of chemotaxis signaling is required to prevent cross-talk in other species, including *R. sphaeroides*. In this species two functional chemotaxis clusters are found within the cells: a polar membrane-anchored cluster and a cytoplasmic one (45-48). These clusters are physically and spatially separated from each other and each of the chemotaxis proteins specifically assembles in a single chemotaxis signaling cluster, with experimental evidence suggesting this organization prevents detrimental signaling

cross-talk (44-47). The subcellular organization of chemotaxis signaling clusters in *R. sphaeroides* suggests stringent specificity in the interactions between chemoreceptors and baseplate proteins, CheA and CheW, to form signaling clusters which would be relaxed in the chemoreceptor-CheA-CheW clustering our results in *A. brasilense* suggest.

Despite recent examples of cross-talk at the level of phosphotransfer between histidine kinases and non-cognate response regulators (49), cross-talk is generally thought not to occur at the level of phosphotransfer between a histidine kinase and its non-cognate response regulator given the selectivity of the interaction between these signaling proteins (50-53) as well as the expected decrease in the specificity of response to a specific input (50, 54). Here, we present no evidence for cross-talk between paralogs but show evidence of co-binding of chemotaxis paralogs that form the signaling baseplate. This organization would allow for the integration and coordination of signaling from two otherwise independent chemotaxis signaling pathways without invoking the unlikely but potential disadvantages of cross-talk.

References

1. Hazelbauer GL, Falke JJ, Parkinson JS. 2008. Bacterial chemoreceptors: high-performance signaling in networked arrays. *Trends Biochem Sci* 33:9-19.
2. Sourjik V, Armitage JP. 2010. Spatial organization in bacterial chemotaxis. *Embo J* 29:2724-33.
3. Bray D, Levin MD, Morton-Firth CJ. 1998. Receptor clustering as a cellular mechanism to control sensitivity. *Nature* 393:85-8.
4. Springer MS, Goy MF, Adler J. 1977. Sensory transduction in *Escherichia coli*: two complementary pathways of information processing that involve methylated proteins. *Proc Natl Acad Sci U S A* 74:3312-6.
5. Manson MD, Blank V, Brade G, Higgins CF. 1986. Peptide chemotaxis in *E. coli* involves the Tap signal transducer and the dipeptide permease. *Nature* 321:253-6.
6. Liu X, Parales RE. 2008. Chemotaxis of *Escherichia coli* to pyrimidines: a new role for the signal transducer tap. *Journal of Bacteriology* 190:972-9.
7. Hazelbauer GL. 1975. Maltose chemoreceptor of *Escherichia coli*. *J Bacteriol* 122:206-14.
8. Hegde M, Englert DL, Schrock S, Cohn WB, Vogt C, Wood TK, Manson MD, Jayaraman A. 2011. Chemotaxis to the quorum-sensing signal AI-2 requires the Tsr chemoreceptor and the periplasmic LsrB AI-2-binding protein. *Journal of Bacteriology* 193:768-73.
9. Zhang P, Khursigara CM, Hartnell LM, Subramaniam S. 2007. Direct visualization of *Escherichia coli* chemotaxis receptor arrays using cryo-electron microscopy. *Proc Natl Acad Sci USA* 104:3777-81.

10. Liu J, Hu B, Morado DR, Jani S, Manson MD, Margolin W. 2012. Molecular architecture of chemoreceptor arrays revealed by cryoelectron tomography of *Escherichia coli* minicells. *Proceedings of the National Academy of Sciences* 109:E1481–E1488.
11. Briegel A, Ortega DR, Tocheva EI, Wuichet K, Li Z, Chen S, Muller A, Iancu CV, Murphy GE, Dobro MJ, Zhulin IB, Jensen GJ. 2009. Universal architecture of bacterial chemoreceptor arrays. *Proc Natl Acad Sci U S A* 106:17181-6.
12. Briegel A, Li X, Bilwes AM, Hughes KT, Jensen GJ, Crane BR. 2012. Bacterial chemoreceptor arrays are hexagonally packed trimers of receptor dimers networked by rings of kinase and coupling proteins. *Proceedings of the National Academy of Sciences* 109:3766-3771.
13. Pinas GE, Frank V, Vaknin A, Parkinson JS. 2016. The source of high signal cooperativity in bacterial chemosensory arrays. *Proc Natl Acad Sci U S A* 113:3335-40.
14. Wuichet K, Alexander RP, Zhulin IB. 2007. Comparative genomic and protein sequence analyses of a complex system controlling bacterial chemotaxis. *Methods Enzymol* 422:1-31.
15. Herrera Seitz MK, Frank V, Massazza DA, Vaknin A, Studdert CA. 2014. Bacterial chemoreceptors of different length classes signal independently. *Molecular Microbiology* 93:814-22.
16. Briegel A, Wong ML, Hodges HL, Oikonomou CM, Piasta KN, Harris MJ, Fowler DJ, Thompson LK, Falke JJ, Kiessling LL, Jensen GJ. 2014. New Insights into Bacterial Chemoreceptor Array Structure and Assembly from Electron Cryotomography. *Biochemistry* 53:1575-1585.

17. Briegel A, Ladinsky MS, Oikonomou C, Jones CW, Harris MJ, Fowler DJ, Chang YW, Thompson LK, Armitage JP, Jensen GJ. 2014. Structure of bacterial cytoplasmic chemoreceptor arrays and implications for chemotactic signaling. *Elife* 3:e02151.
18. Alexander RP, Zhulin IB. 2007. Evolutionary genomics reveals conserved structural determinants of signaling and adaptation in microbial chemoreceptors. *Proc Natl Acad Sci U S A* 104:2885-90.
19. Wisniewski-Dyé F, Borziak K, Khalsa-Moyers G, Alexandre G, Sukharnikov LO, Wuichet K, Hurst GB, McDonald WH, Robertson JS, Barbe V, Calteau A, Rouy Z, Mangenot S, Prigent-Combaret C, Normand P, Boyer M, Siguier P, Dessaux Y, Elmerich C, Condemine G, Krishnen G, Kennedy I, Paterson AH, González V, Mavingui P, Zhulin IB. 2011. *Azospirillum* Genomes Reveal Transition of Bacteria from Aquatic to Terrestrial Environments. *PLoS Genet* 7:e1002430.
20. Bible AN, Khalsa-Moyers GK, Mukherjee T, Green CS, Mishra P, Purcell A, Aksenova A, Hurst GB, Alexandre G. 2015. Metabolic adaptations of *Azospirillum brasilense* to oxygen stress by cell-to-cell clumping and flocculation. *Appl Environ Microbiol* 81:8346-57.
21. Mukherjee T, Kumar D, Burriss N, Xie Z, Alexandre G. 2016. *Azospirillum brasilense* chemotaxis depends on two signaling pathways regulating distinct motility parameters. *Journal of Bacteriology* doi:10.1128/jb.00020-16.
22. Bible A, Russell MH, Alexandre G. 2012. The *Azospirillum brasilense* Che1 chemotaxis pathway controls swimming velocity, which affects transient cell-to-cell clumping. *Journal of Bacteriology* 194:3343-55.

23. Bible AN, Stephens BB, Ortega DR, Xie Z, Alexandre G. 2008. Function of a chemotaxis-like signal transduction pathway in modulating motility, cell clumping, and cell length in the alphaproteobacterium *Azospirillum brasilense*. *Journal of Bacteriology* 190:6365-75.
24. Stephens BB, Loar SN, Alexandre G. 2006. Role of CheB and CheR in the complex chemotactic and aerotactic pathway of *Azospirillum brasilense*. *Journal of Bacteriology* 188:4759-68.
25. Russell MH, Bible AN, Fang X, Gooding JR, Campagna SR, Gomelsky M, Alexandre G. 2013. Integration of the second messenger c-di-GMP into the chemotactic signaling pathway. *MBio* 4:e00001-13.
26. Amat F, Moussavi F, Comolli LR, Elidan G, Downing KH, Horowitz M. 2008. Markov random field based automatic image alignment for electron tomography. *Journal of Structural Biology* 161:260-75.
27. Ding HJ, Oikonomou CM, Jensen GJ. 2015. The Caltech Tomography Database and Automatic Processing Pipeline. *Journal of Structural Biology* 192:279-86.
28. Kremer JR, Mastronarde DN, McIntosh JR. 1996. Computer visualization of three-dimensional image data using IMOD. *J Struct Biol* 116:71-6.
29. Hallez R, Letesson JJ, Vandenhoute J, De Bolle X. 2007. Gateway-based destination vectors for functional analyses of bacterial ORFeomes: application to the Min system in *Brucella abortus*. *Appl Environ Microbiol* 73:1375-9.
30. Xie Z, Ulrich LE, Zhulin IB, Alexandre G. 2010. PAS domain containing chemoreceptor couples dynamic changes in metabolism with chemotaxis. *Proc Natl Acad Sci U S A* 107:2235-40.

31. Hauwaerts D, Alexandre G, Das SK, Vanderleyden J, Zhulin IB. 2002. A major chemotaxis gene cluster in *Azospirillum brasilense* and relationships between chemotaxis operons in alpha-proteobacteria. FEMS Microbiol Lett 208:61-7.
32. Ouellette SP, Gaudiard E, Antosova Z, Ladant D. 2014. A Gateway((R)) -compatible bacterial adenylate cyclase-based two-hybrid system. Environ Microbiol Rep 6:259-67.
33. Karimova G, Pidoux J, Ullmann A, Ladant D. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc Natl Acad Sci U S A 95:5752-6.
34. Ramsay JP, Major AS, Komarovskiy VM, Sullivan JT, Dy RL, Hynes MF, Salmond GP, Ronson CW. 2013. A widely conserved molecular switch controls quorum sensing and symbiosis island transfer in *Mesorhizobium loti* through expression of a novel antiactivator. Mol Microbiol 87:1-13.
35. Zhao J, Parkinson JS. 2006. Mutational Analysis of the Chemoreceptor-Coupling Domain of the Escherichia coli Chemotaxis Signaling Kinase CheA. Journal of Bacteriology 188:3299-3307.
36. Boukhvalova M, VanBruggen R, Stewart RC. 2002. CheA kinase and chemoreceptor interaction surfaces on CheW. Journal of Biological Chemistry 277:23596-603.
37. Ulrich LE, Zhulin IB. 2007. MiST: a microbial signal transduction database. Nucleic Acids Res 35:D386-90.
38. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772-80.

39. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. 2009. Jalview Version 2-
-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189-
91.
40. Adebali O, Ortega DR, Zhulin IB. 2015. CDvist: a webserver for identification and
visualization of conserved domains in protein sequences. *Bioinformatics* 31:1475-7.
41. Dunin-Horkawicz S, Lupas AN. 2010. Comprehensive analysis of HAMP domains:
implications for transmembrane signal transduction. *J Mol Biol* 397:1156-74.
42. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN,
Bourne PE. 2000. The Protein Data Bank. *Nucleic Acids Res* 28:235-42.
43. Humphrey W, Dalke A, Schulten K. 1996. VMD: visual molecular dynamics. *J Mol Graph*
14:33-8, 27-8.
44. Airola MV, Watts KJ, Bilwes AM, Crane BR. 2010. Structure of concatenated HAMP
domains provides a mechanism for signal transduction. *Structure* 18:436-48.
45. Ferris HU, Zeth K, Hulko M, Dunin-Horkawicz S, Lupas AN. 2014. Axial helix rotation
as a mechanism for signal regulation inferred from the crystallographic analysis of the *E.*
coli serine chemoreceptor. *J Struct Biol* 186:349-56.
46. Sawai H, Sugimoto H, Shiro Y, Ishikawa H, Mizutani Y, Aono S. 2012. Structural basis
for oxygen sensing and signal transduction of the heme-based sensor protein Aer2 from
Pseudomonas aeruginosa. *Chem Commun (Camb)* 48:6523-5.
47. Maddock JR, Shapiro L. 1993. Polar location of the chemoreceptor complex in the
Escherichia coli cell. *Science* 259:1717-23.

48. Sourjik V, Berg HC. 2000. Localization of components of the chemotaxis machinery of *Escherichia coli* using fluorescent protein fusions. *Mol Microbiol* 37:740-51.
49. Alexander RP, Zhulin IB. 2007. Evolutionary genomics reveals conserved structural determinants of signaling and adaptation in microbial chemoreceptors. *Proceedings of the National Academy of Sciences* 104:2885.
50. Briegel A, Ortega DR, Tocheva EI, Wuichet K, Li Z, Chen S, Müller A, Iancu CV, Murphy GE, Dobro MJ, Zhulin IB, Jensen GJ. 2009. Universal architecture of bacterial chemoreceptor arrays. *Proceedings of the National Academy of Sciences of the United States of America* 106:17181-17186.
51. Natale AM, Duplantis JL, Piasta KN, Falke JJ. 2013. Structure, function, and on-off switching of a core unit contact between CheA kinase and CheW adaptor protein in the bacterial chemosensory array: A disulfide mapping and mutagenesis study. *Biochemistry* 52:7753-65.
52. Gullett JM, Bible A, Alexandre G. 2017. Distinct Domains of CheA Confer Unique Functions in Chemotaxis and Cell Length in *Azospirillum brasilense* Sp7. *J Bacteriol* 199.
53. Battesti A, Bouveret E. 2012. The bacterial two-hybrid system based on adenylate cyclase reconstitution in *Escherichia coli*. *Methods* 58:325-34.
54. Vu A, Wang X, Zhou H, Dahlquist FW. 2012. The receptor-CheW binding interface in bacterial chemotaxis. *Journal of Molecular Biology* 415:759-767.
55. Vu A, Wang X, Zhou H, Dahlquist FW. 2012. The receptor-CheW binding interface in bacterial chemotaxis. *J Mol Biol* 415:759-67.

56. Boukhvalova M, VanBruggen R, Stewart RC. 2002. CheA Kinase and Chemoreceptor Interaction Surfaces on CheW. *Journal of Biological Chemistry* 277:23596-23603.
57. Kentner D, Thiem S, Hildenbeutel M, Sourjik V. 2006. Determinants of chemoreceptor cluster formation in *Escherichia coli*. *Mol Microbiol* 61:407-17.
58. Levit MN, Grebe TW, Stock JB. 2002. Organization of the receptor-kinase signaling array that regulates *Escherichia coli* chemotaxis. *J Biol Chem* 277:36748-54.
59. Erbse AH, Falke JJ. 2009. The core signaling proteins of bacterial chemotaxis assemble to form an ultrastable complex. *Biochemistry* 48:6975-87.
60. Alexander RP, Lowenthal AC, Harshey RM, Ottemann KM. 2010. CheV: CheW-like coupling proteins at the core of the chemotaxis signaling network. *Trends Microbiol* 18:494-503.
61. Ortega DR, Zhulin IB. 2016. Evolutionary Genomics Suggests That CheV Is an Additional Adaptor for Accommodating Specific Chemoreceptors within the Chemotaxis Signaling Complex. *PLoS Comput Biol* 12:e1004723.
62. Yang W, Alvarado A, Glatter T, Ringgaard S, Briegel A. 2018. Baseplate variability of *Vibrio cholerae* chemoreceptor arrays. *Proc Natl Acad Sci U S A* 115:13365-13370.
63. Miller LD, Yost CK, Hynes MF, Alexandre G. 2007. The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol Microbiol* 63:348-62.
64. Xu Q, Black WP, Cadieux CL, Yang Z. 2008. Independence and interdependence of Dif and Frz chemosensory pathways in *Myxococcus xanthus* chemotaxis. *Mol Microbiol* 69:714-23.

65. Black WP, Schubot FD, Li Z, Yang Z. 2010. Phosphorylation and dephosphorylation among Dif chemosensory proteins essential for exopolysaccharide regulation in *Myxococcus xanthus*. *Journal of Bacteriology* 192:4267-74.
66. Campodonico EM, Zusman DR. 2010. Developments in Defining dif. *Journal of Bacteriology* 192:4264-6.
67. Wadhams GH, Martin AC, Warren AV, Armitage JP. 2005. Requirements for chemotaxis protein localization in *Rhodobacter sphaeroides*. *Mol Microbiol* 58:895-902.
68. Wadhams GH, Warren AV, Martin AC, Armitage JP. 2003. Targeting of two signal transduction pathways to different regions of the bacterial cell. *Mol Microbiol* 50:763-70.
69. Wadhams GH, Martin AC, Porter SL, Maddock JR, Mantotta JC, King HM, Armitage JP. 2002. TlpC, a novel chemotaxis protein in *Rhodobacter sphaeroides*, localizes to a discrete region in the cytoplasm. *Mol Microbiol* 46:1211-21.
70. Porter SL, Armitage JP. 2004. Chemotaxis in *Rhodobacter sphaeroides* requires an atypical histidine protein kinase. *J Biol Chem* 279:54573-80.
71. Howell A, Dubrac S, Andersen KK, Noone D, Fert J, Msadek T, Devine K. 2003. Genes controlled by the essential YycG/YycF two-component system of *Bacillus subtilis* revealed through a novel hybrid regulator approach. *Mol Microbiol* 49:1639-55.
72. Laub MT, Goulian M. 2007. Specificity in Two-Component Signal Transduction Pathways. *Annual Review of Genetics* 41:121-145.
73. Hoch JA, Varughese KI. 2001. Keeping signals straight in phosphorelay signal transduction. *Journal of Bacteriology* 183:4941-9.

74. Kojadinovic M, Armitage JP, Tindall MJ, Wadhams GH. 2013. Response kinetics in the complex chemotaxis signalling pathway of *Rhodobacter sphaeroides*. J R Soc Interface 10:20121001.
75. Mukhopadhyay D, Varughese KI. 2005. A computational analysis on the specificity of interactions between histidine kinases and response regulators. J Biomol Struct Dyn 22:555-62.
76. Rowland MA, Deeds EJ. 2014. Crosstalk and the evolution of specificity in two-component signaling. Proc Natl Acad Sci U S A 111:5550-5.

CHAPTER II
OPTOGENETIC MANIPULATION OF CYCLIC DI-GMP (C-DI-GMP)
LEVELS REVEALS THE ROLE OF C-DI-GMP IN REGULATING
AEROTAXIS RECEPTOR ACTIVITY IN AZOSPIRILLUM BRASILENSE

A version of this chapter was originally published by Lindsey O’Neal, Min-Hyung Ryu, Mark Gomelsky, and Gladys Alexandre:

O’Neal, L., Ryu, M.H., Gomelsky, M., & Alexandre, G. (2017). Optogenetic manipulation of c-di-GMP levels reveals the role of c-di-GMP in regulating aerotaxis receptor activity in *Azospirillum brasilense*. *Journal of Bacteriology*.

LO conducted the majority of the experiments and wrote the manuscript with the help of GA. MG and MR designed the optogenetic enzymes and the plasmids encoding them.

Abstract

Bacterial chemotaxis receptors provide the sensory inputs that inform the direction of navigation in changing environments. Recently, we described the bacterial second messenger cyclic di-GMP (c-di-GMP) as a novel regulator of a subclass of chemotaxis receptors. In *Azospirillum brasilense*, c-di-GMP binds to a chemotaxis receptor, Tlp1, and modulates its signaling function during aerotaxis. Here, we further characterize the role of c-di-GMP in aerotaxis using a novel dichromatic optogenetic system engineered for manipulating intracellular c-di-GMP levels in real time. This system comprises a red/near-infrared-light-regulated diguanylate cyclase and a blue-light-regulated c-di-GMP phosphodiesterase. It allows the generation of transient changes in intracellular c-di-GMP concentrations within seconds of irradiation with appropriate light, which is compatible with the time scale of chemotaxis signaling. We provide experimental evidence that binding of c-di-GMP to the Tlp1 receptor activates its signaling function during aerotaxis, which supports the role of transient changes in c-di-GMP levels as a means of adjusting the response of *A. brasilense* to oxygen gradients. We also show that intracellular c-di-GMP levels in *A. brasilense* change with carbon metabolism. Our data support a model whereby c-di-GMP functions to imprint chemotaxis receptors with a record of recent metabolic experience, to adjust their contribution to

the signaling output, thus allowing the cells to continually fine-tune chemotaxis sensory perception to their metabolic state.

Introduction

The bacterial second messenger cyclic di-GMP (c-di-GMP) has emerged as a key signaling molecule regulating the transition of bacteria from planktonic to sessile lifestyles. In addition to regulating this transition, c-di-GMP signaling pathways control biofilm formation, adhesion to surfaces, virulence, the cell cycle, and other processes (1). Intracellular c-di-GMP levels are set by diguanylate cyclases, which synthesize c-di-GMP, and c-di-GMP-dependent phosphodiesterases (PDEs), which degrade c-di-GMP. The diguanylate cyclase activity is encoded by a highly conserved GGDEF domain, while phosphodiesterase activity resides within conserved EAL or HD-GYP domains (1). c-di-GMP effectors that mediate changes in c-di-GMP concentrations come in many forms: transcriptional regulators, riboswitches, metabolic enzymes, and signal transduction proteins (1). The diversity of signal inputs and outputs associated with c-di-GMP signaling and effector proteins reflects the widespread role of this molecule in regulating bacterial metabolism and cellular behaviors.

The PilZ domain represents one of the best-characterized classes of c-di-GMP effectors (1). Binding of c-di-GMP to a PilZ domain changes its conformation and allosterically regulates activities of the output domains linked to it (2–4). A PilZ domain is present at the C terminus of a chemotaxis receptor, Tlp1, from the alphaproteobacterium *Azospirillum brasilense* (2, 5). Except for the presence of PilZ, Tlp1 possesses a prototypical structure of a chemotaxis receptor: an N-terminal membrane-anchored periplasmic domain that encodes the putative sensor region and a highly conserved C-terminal cytoplasmic kinase control module that interacts with the downstream

chemotaxis proteins to permit signal transduction (Figure 18A) (6). Tlp1 functions as an energy sensor. It mediates the ability of *A. brasilense* to navigate in gradients of compounds that affect intracellular energy levels, including oxygen and organic acids (7, 8). Recently, we showed that the PilZ domain of Tlp1 binds c-di-GMP with high affinity, which affects the ability of cells to navigate in oxygen gradients (5). *A. brasilense* grows best under microaerophilic conditions. Elevated oxygen concentrations cause stress to which motile cells transiently adapt by clumping, which is presumed to reduce their surface area and limit oxygen diffusion (9). Furthermore, the addition or removal of air in free-swimming cells triggers rapid (on the order of a few seconds) and transient changes in intracellular levels of c-di-GMP (5), indicating that oxygen is a major factor regulating c-di-GMP concentrations. Consistent with these observations, the binding of c-di-GMP to the PilZ domain of the Tlp1 chemoreceptor alters the aerotaxis (chemotaxis in gradients of oxygen) response (5). Aerotaxis is the strongest behavioral response in *A. brasilense*. Of 33 putative diguanylate cyclases and 25 phosphodiesterases encoded by the *A. brasilense* genome (10, 11), only 2, ChsA and CdgA, have thus far been functionally characterized (11, 12), and ChsA has been shown to contribute to oxygen-induced changes in intracellular c-di-GMP levels (5).

To date, most strategies for characterizing the role of c-di-GMP in cell physiology have relied on modulating the intracellular c-di-GMP concentrations via single and combinatorial mutations of diguanylate cyclases or phosphodiesterases (e.g., see references 13 and 14). Mutants lacking all enzymes involved in c-di-GMP metabolism have been described (e.g., see references 15–17). A complementary approach involving the use of the constitutive or inducible overexpression of diguanylate cyclases or phosphodiesterase has also been used (e.g., see reference 2). While these experimental approaches allow researchers to observe long-term

consequences of increased or decreased c-di-GMP levels, they preclude observations of short-term changes in c-di-GMP levels. However, transient changes in c-di-GMP concentrations, i.e., those occurring on a scale of seconds to minutes, can be profound in magnitude and significant (5, 18, 19).

Here, we use an optogenetic (synthetic photobiology) toolset for manipulating intracellular c-di-GMP levels within a seconds-to-minutes temporal resolution (20, 21), which is compatible with the time scale of chemotaxis signaling. This toolset comprises plasmids expressing a red/near-infrared-light-regulated diguanylate cyclase, BphS (21), and a blue-light-regulated c-di-GMP phosphodiesterase, EB1 (20) (Figure 18B and C). Using this toolset, we show that transient changes in c-di-GMP levels regulate the sensitivity of cells to oxygen gradients generated by the addition or removal of air. We also show that c-di-GMP binding functions as an activation mechanism for the Tlp1 receptor. Finally, we show that carbon metabolism modulates c-di-GMP levels and imprints chemotaxis receptors with a record of recent metabolic experience that allows them to ultimately adjust their preferred position for aerotactic band formation in oxygen gradients. Our data suggest that binding of c-di-GMP to a subset of chemotaxis receptors adjusts their signaling contribution, permitting cells to continually fine-tune chemotactic responses to their metabolic state.

Materials and Methods

Bacterial Strains and Growth Conditions

The wild type Sp7 strain of *A. brasilense* (herein, WT) and its mutant derivative *tlp1* (strain SG323; (22)), were used throughout this study (Table 3). Cells were grown in the dark at 28°C with shaking

Table 3: Strains and plasmids used in this study

Strain or Plasmid	Genotype or Relevant Characteristic	Reference or Source
Plasmids		
pIND4	inducible expression plasmid, Km ^r	(27)
pRK415	broad-host-range vector; Tc ^r	(9)
pRed-DGC	pIND4 containing red-light activated diguanylate cyclase; Km ^r	This work
pBlue-PDE	pIND4 containing blue-light activated diguanylate cyclase; Km ^r	This work
pRK-Tlp1	pRK415 containing a 2.7kb DNA fragment encompassing promoter and tlp1 ORF; Tc ^r	(5)
<i>A. brasilense</i>		
Sp7	Wild-type strain; ATCC 29145	(57)
SG323	<i>Δtlp1::Km</i> derivative of Sp7; <i>tlp1</i> , Km ^r	(22)
Sp74031	<i>chsA::Tn5</i> , <i>chsA</i> , Km ^r	(28)
Sp7 (empty vector)	Wild type strain carrying pIND4; Amp ^r Km ^r	This work
Sp7 (pRed-DGC)	Wild type strain carrying pRed-DGC; Amp ^r Km ^r	This work
Sp7 (pBlue-PDE)	Wild type strain carrying pBlue-PDE; Amp ^r Km ^r	This work
<i>tlp1</i> (pIND4)	SG323 carrying pIND4; Amp ^r Km ^r	This work

Table 3, Continued

Strain or Plasmid	Genotype or Relevant Characteristic	Reference or Source
<i>tlp1</i> (pRed-DGC)	SG323 carrying pRed-DGC; Amp ^r Km ^r	This work
<i>tlp1</i> (pBlue-PDE)	SG323 carrying pBlue-PDE; Amp ^r Km ^r	This work
<i>tlp1</i> (pRed-DGC)(pRK415)	SG323 carrying pRed-DGC and a control broad host range plasmid; Amp ^r Km ^r Tc ^r	This work
<i>tlp1</i> (pRed-DGC)(pRK-Tlp1)	SG323 carrying pIND4 and a broad host range plasmid expressing parental Tlp1; Amp ^r Km ^r Tc ^r	This work
<i>tlp1</i> (p-Red-DGC)(pRK-Tlp1 ^{R562AR563A})	SG323 carrying pRed-DGC and a broad host range plasmid expressing a variant Tlp1 with arginine 562 and 563 both replaced with alanine and unable to bind c-di-GMP; Amp ^r Km ^r Tc ^r	This work
<i>chsA</i> (empty vector)	Sp74031 Amp ^r Km ^r	This work
<i>chsA</i> (pRed-DGC)	Sp74031 Amp ^r Km ^r	This work
<i>chsA</i> (pBlue-PDE)	Sp74031 Amp ^r Km ^r	This work
<i>E. coli</i>		
S17.1 (pIND4)	Strain S17.1 carrying the empty vector, pIND4, Km ^r	This work
S17.1 (pRed-DGC)	Strain S17.1 carrying the empty vector, pRed-DGC, Km ^r	This work
S17.1 (pBlue-PDE)	Strain S17.1 carrying the empty vector, pBlue-PDE, Km ^r	This work

Amp^r: ampicillin resistance; Km^r: Kanamycin resistance; Tc^r: tetracycline resistance

in minimal media (MMAB) containing 10mM malate as a carbon source. In experiments with different carbon sources, cells were grown with shaking in minimal media with 10mM fructose or 10 mM succinate. All culture stocks were maintained on MMAB containing 1.5% agar with appropriate antibiotics: ampicillin (200 µg/mL), kanamycin (30 µg/mL), and/ or tetracycline (10 µg/mL). To determine doubling times, pre-cultures were from cells grown in MMAB containing 10mM of malate, succinate, or fructose as the sole carbon source, to stationary phase (OD₆₀₀ 1). A 96-well microplate was prepared with MMAB containing 10 mM malate, succinate, or fructose and wells were inoculated with a 1:10 dilution of the pre-cultures. Absorbance at 600 nm was recorded every 20 minutes for 20 hours using a BioTech Synergy 2 microplate reader with Gen5 2.09 Read Control and Data Analysis software. Doubling times were calculated as: Doubling time = $\frac{\text{Time}}{\text{generations}}$. The number of generations was calculated using: generations = $\frac{[\log(\frac{N_t}{N_0})]}{0.3}$, where N_t=OD₆₀₀ at the end of the log phase; N₀= OD₆₀₀ at the beginning of log phase; and time is the time elapsed between N_t and N₀.

Plasmid construction

The plasmids for optogenetic regulation of intracellular c-di-GMP levels were constructed using vector pIND4 that carries two replication origins, ColE1 for *Escherichia coli* and pMG160 for alphaproteobacteria (23). The *bphS-bphO* fragment from plasmid pMQbSH (21) that expresses the red/near-infrared light-dependent diguanylate cyclase BphS and the heme oxygenase BphO was ligated into pIND4 digested with XbaI and BamHI to yield pRed-DGC. In this plasmid, *bphS* and *bphO* are arranged in an operon that is expressed from the IPTG-inducible *Plac* promoter. The blue light-activated PDE gene, *eb1*, was amplified from plasmid pbSHE (20) and cloned into

pIND4 via digestion with XbaI and BamHI placed downstream of bphO as part of the three-gene *bphS-bphO-ebI* operon, to yield plasmid pBlue-PDE.

Transfer of plasmid-borne light activated constructs into A. brasilense

To transfer pIND4, pBlue-PDE or pRed-DGC, biparental conjugation was utilized as previously described (24) with *E. coli* strain S17.1 containing the plasmid of interest as a donor and *A. brasilense* strain Sp7 or its *tlpI* mutant derivative, as a recipient. The presence of the plasmid in transconjugants grown on selective medium was confirmed by colony-PCR where the *lacI* gene, which is specific to the plasmid, was amplified using primers pIND4-LacIq-Fwd (5'-GTGGTGAATGTGAAACCAGT-3') and pIND4-LacIq-Rev (5'-TCACTGCCCCGCTTTCCAGTC-3').

Quantification of c-di-GMP by mass spectrometry

For determination of whole cell intracellular c-di-GMP concentrations, *A. brasilense* was grown in tryptone-yeast media (TY) in the dark until OD₆₀₀ 1.0. Cultures were then re-inoculated into TY and grown to OD₆₀₀ 0.8. C-di-GMP was extracted and concentration was analyzed using LC-MS/MS at the Michigan State University Mass Spectrometry and Metabolomics Core, as previously described (24, 25). Intracellular c-di-GMP concentration was determined by dividing the total amount of c-di-GMP determined by LC-MS/MS by the number of cells, which was derived from plating assays and by the volume of an *A. brasilense* cell, which was determined as previously described (26).

Free-swimming cells behavior and computerized assisted motion tracking

Cells were grown to OD₆₀₀ 0.6-0.7 (exponential growth phase) in MMAB containing 10mM malate. Cultures were standardized to an equivalent number of cells, washed and concentrated 10 times in Che buffer (1.7 g L⁻¹ dipotassium phosphate, 1.36 g L⁻¹ monopotassium phosphate; 0.1 mM EDTA). All cells were motile. Cells were exposed to green light (505-575 nm) for 1 min, and then exposed to either red (610-730 nm) or blue light (450 nm) for 10 sec, then returned to green light. Digital recording of cells behavior continued through the experiment and lasted at least 5 min after the stimulus. Andover optical dichroic filters were used for exposure to green, red, and blue light. All swimming assays were filmed using a 40x objective of a phase contrast microscope fitted with a Leica MP120 HD. Videos were formatted using VLC media player software before using CellTrak version 1.5 (Motion Analysis Corp., Santa Rosa, CA) for swimming speed and reversal frequency analysis. One-second segments (30 frames) of video were analyzed before, during, and after stimulus pulse. At least 100 cells per time point were analyzed.

Spatial gradient assay for aerotaxis and density analysis

Cells were grown to an OD₆₀₀ 0.6-0.7 in the dark in minimal media containing malate. Cultures were standardized as described above under green light. The concentrated sample was then used to fill a 0.1 x 1 mm capillary tube (Vitro Dynamics, Inc., Rockaway, N.J.). Capillary tubes were placed in a gas equilibration chamber (23). Samples were allowed to equilibrate in humidified N₂ for 2 minutes inside the capillary tube under green light exposure. After equilibration, samples were exposed to either red or blue light for 10 sec and nitrogen gas was turned off and air was allowed to flow in the chamber. Aerotaxis was observed under green light for an additional 5 min. Images were captured using the 4x objective of a phase contrast Nikon

E200 microscope and C-mounted Nikon Coolpix digital camera. Aerotactic band distance from the meniscus and width were quantified using the measure tool in FIJI in ImageJ. The width of the tube was used as a reference for all measurements. Distance from the meniscus was measured from the center of meniscus to the center of the aerotactic band, and width of the aerotactic band was measured at the middle of the band upon formation of a stable band. The distribution of bacteria in capillary tubes was analyzed by intensity image analysis from digital pictures of aerotactic bands, using surface plot in ImageJ (imagej.nih.gov), after Gaussian adjustment.

Temporal gradient for aerotaxis and swimming pattern analysis

Cells were grown to OD₆₀₀ 0.6 and were washed with Che buffer before being concentrated 10 times in the same buffer. Ten μ L of the concentrated sample was placed on a slide inside of the gas equilibration chamber (23). All cells were motile and free swimming. Air or humidified N₂ was allowed to flow over the cells for 3 min. After equilibration, the atmosphere of the cells was switched to N₂ or air, respectively, thereby producing the stimulatory temporal gradient of oxygen. Under these conditions, free-swimming cells suppress reversals during the transition from N₂ to air or air to N₂, since the microaerobic oxygen conditions experienced by cells during these transitions are attractants for *A. brasilense* (23). When c-di-GMP metabolism was also manipulated in the temporal aerotaxis assay, wild-type cells carrying pRed-DGC, pBlue-DGC or pIND4 were first equilibrated in air or N₂ for 3 min, exposed to red or blue light for 10 sec, and then immediately exposed to N₂ or air, respectively. All assays were recorded using a 40x objective of a phase contrast microscope fitted with a Leica MP120 HD digital camera. Blind video analysis was used to determine the response time, i.e. the time required for the cell population to return to pre-stimulus swimming bias, following changes in aeration conditions. Short segments of

recordings were also analyzed by computerized motion analysis (CellTrak 2.0, Santa Rosa, CA) to verify the measurements. The response time was defined as the amount of time it took for 50 % of cells to return to a pre-stimulus reversal frequency.

Cell clumping and aggregation analysis

The *chsA* mutant carrying pIND4 or pBlue-PDE was grown in the dark to OD₆₀₀ 0.6, washed and concentrated as described above³. Ten µL of concentrated sample was placed on a slide and allowed to swim freely. Cells were exposed to green light for 3 min and then exposed to either red or blue light for 10 sec. After exposure, cells were placed back under green light. Images of cell suspension were taken before and after exposure to red or blue light. The area covered by the clumps relative to the total surface area imaged was calculated using the area measure tool in FIJI in ImageJ and used to determine the fold change in the surface areas covered by clumps.

Statistical analysis of data

To compare the effect of c-di-GMP increase or decrease using the optogenetic system and the different growth conditions on the intracellular c-di-GMP concentration or the swimming speed and reversal frequency, an ANOVA test with a post hoc (p value 0.05) (Excel Microsoft, Inc.) was used.

Results

The optogenetic system permits light dependent regulation of intracellular c-di-GMP levels

To manipulate c-di-GMP levels in *A. brasilense* with the temporal resolution of seconds to minutes, we adapted the dichromatic optogenetic module for c-di-GMP control recently

engineered for *E. coli* (20). This system consists of a chimeric red/near-infrared-light-regulated diguanylate cyclase, BphS (21), and a blue-light-activated c-di-GMP phosphodiesterase, EB1 (20) (Figure 18B). BphS senses light via the biliverdin IX α chromophore that is produced from heme by the heme oxygenase BphO. BphS is activated by red/far-red light (630 to 710 nm) and inactivated by near-infrared light (750 to 780 nm). It can also be activated by violet light of 400 to 420 nm (21). EB1 senses light via the flavin chromophore and is activated by violet-to-blue light (360 to 450 nm) (20). The engineered *bphS-bphO* operon expressing the diguanylate cyclase (plasmid pRed-DGC) or the *bphS-bphO-eb1* operon expressing both the cyclase and phosphodiesterase (plasmid pBlue-PDE) was cloned downstream of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible P_{lac} promoter into a broad-host-range vector, pIND4 (22). The plasmid containing the *bphS-bphO-eb1* operon was initially designed to provide the ability to control both c-di-GMP synthesis and degradation, with light, from a single plasmid (Figure 18B).

We transferred the corresponding plasmids into the *A. brasilense* wild-type (WT) strain and measured changes in intracellular c-di-GMP concentrations in cells exposed to green (505 to 575 nm), red (610 to 730 nm) or blue (450 nm) light (Figure 18C). Green light does not activate the diguanylate cyclase or the phosphodiesterase, based on the absorption spectra for these enzymes (20). As expected, we found that the intracellular c-di-GMP concentration of cells maintained in the dark did not significantly change when they were exposed to green light (Figure 19). Given that our experimental approach relies on the real-time observation of free-swimming cells and on the analysis of changes in motility patterns that occur within seconds, we initially tested the effect of pulses of red or blue light exposure ranging from 10 s to 1 min on c-di-GMP concentrations. This initial screening of optimum conditions showed that a 10-s exposure to red or

blue light was sufficient to induce statistically significant changes in intracellular c-di-GMP concentrations. Wild-type cells carrying pRed-DGC and maintained under green light had basal levels of c-di-GMP that were immediately increased almost 3-fold upon exposure to red light (Figure 19A). Under green light conditions, cells carrying the vector expressing both the red-light-activated diguanylate cyclase and the blue-light-activated phosphodiesterase (pBlue-PDE) had basal levels of c-di-GMP that were significantly decreased upon exposure to blue light (Figure 19B). No further increase of intracellular c-di-GMP concentrations beyond basal levels could be observed when these cells were exposed to red light (data not shown), possibly because of high *ebf* expression levels resulting in background phosphodiesterase activity and/or red light extending to near infrared (750 nm) that inactivates the diguanylate activity of BphS. We further confirmed the effect of blue light on intracellular c-di-GMP levels by using a *chsA* mutant derivative of *A. brasilense*. The *chsA* mutant possesses constitutively elevated c-di-GMP levels due to the absence of the ChsA phosphodiesterase (5, 11). The expression of the pBlue-PDE vector in this strain background also caused a significant decrease in the intracellular c-di-GMP levels (Figure 19C). In subsequent experiments, we used pRed-DGC and pBlue-PDE for elevating and lowering intracellular c-di-GMP levels, respectively. The changes in c-di-GMP concentrations induced by light were transient: 10 min after exposure to the inducing light, the intracellular c-di-GMP levels returned to prestimulus levels (Figure 19). Therefore, we established that the optogenetic system tested here allows the transient manipulation of intracellular c-di-GMP levels in *A. brasilense*.

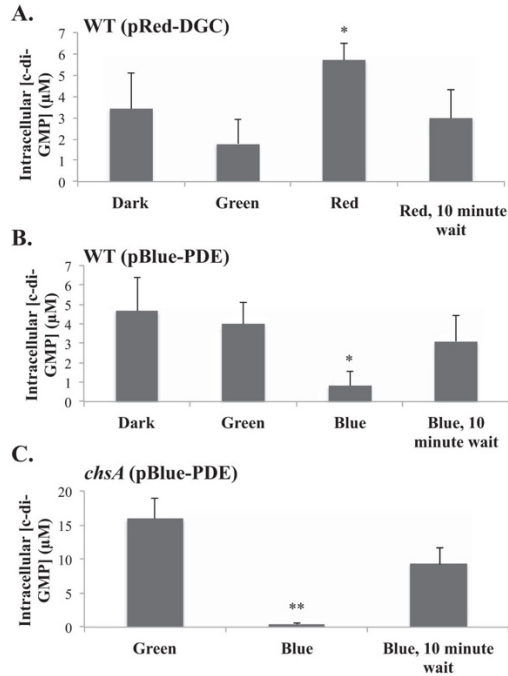


Figure 19: Light-mediated changes in intracellular c-di-GMP concentrations in cells carrying pRed-DGC or pBlue-PDE

(A and B) Changes in intracellular c-di-GMP concentrations in wild-type (WT) cells expressing a red-light-activated diguanylate cyclase [WT(pRed-DGC)] (A) or in wild-type cells containing a plasmid with both a red-light-activated diguanylate cyclase and a blue-light-activated phosphodiesterase [WT(pBlue-PDE)] (B). (C) Changes in intracellular c-di-GMP concentrations in a *chsA* mutant strain carrying the dually red-light-activated diguanylate cyclase and blue-light-activated phosphodiesterase [*chsA*(pBlue-PDE)]. Exposure to the light color indicated on the *x* axis was done for 10 s except for the controls, dark and green light, which correspond to conditions under which the samples were maintained. Quantifications are the averages of data from three biological replicates with standard errors of the means. *, *P* value of <0.05; **, *P* value of <0.01 (between treatment and the control [green light]). LO conducted this experiment.

Optogenetic manipulation of c-di-GMP metabolism affects aerotaxis

In oxygen gradients generated from the diffusion of air into a cell suspension of free-swimming cells, *A. brasilense* cells are attracted to specific low oxygen concentrations that support their microaerobic metabolism, and they form tight aerotactic bands at these locations (23). The spatial gradient assay for aerotaxis permits the real-time observation of the aerotactic response of motile cells to the gradient. The band pattern (position relative to the air-liquid meniscus and time to formation) is robust and highly reproducible for cells grown under the given conditions (7, 23, 24). An *A. brasilense tlp1* mutant expressing an empty control vector or a Tlp1 variant unable to bind c-di-GMP (Tlp1^{R562A R563A}) is also affected in its ability to locate the optimum position for metabolism in oxygen gradients (5), suggesting that c-di-GMP metabolism affects aerotaxis. To further test this hypothesis, we used the optogenetic system to modulate intracellular c-di-GMP levels in free-swimming cells exposed to a spatial gradient of air in the capillary assay for aerotaxis (Figure 20). Under these conditions, cells placed into the capillary tube formed a stable aerotactic band away from the air-liquid interface. Wild-type cells containing pRed-DGC formed a somewhat thicker aerotactic band than that formed by cells carrying pIND4, as a control. Under these conditions, exposure of cells to 10 s of red light did not visibly affect the position or shape of the aerotactic band (Figure 20A). To test the possible effect of elevated c-di-GMP concentrations on the thickness of the aerotactic band, we exposed these cells to red light repeatedly using two subsequent short pulses of red light to sustain an increase in c-di-GMP levels. In this case, the aerotactic band thickened, and an increase in the density of cell-cell clumps was also observed on the side of the aerotactic band closer to the air-liquid meniscus (Figure 20B).

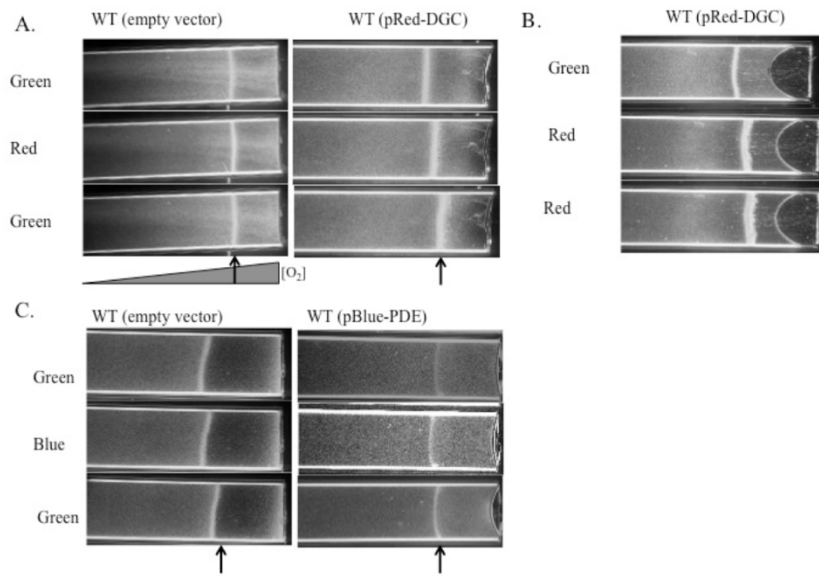


Figure 20: Light mediated real-time changes in c-di-GMP after aerotaxis band has formed

Cell suspensions standardized to equivalent numbers were placed in a capillary tube and allowed to equilibrate in green light. After 3 minutes, cells were exposed to 10 sec red (A) or blue (B) light and then placed back under green light or to three consecutive 10 sec red light exposure, prior to be placed back under green light (C). Representative images of 3 biological replicates are shown. LO conducted this experiment.

These observations suggest that as c-di-GMP levels increased, more cells navigated to the aerotactic band, also suggesting that more cells responded to the gradient. The increase in clumping on the side of the band exposed to elevated oxygen concentrations also suggested that the cells experienced an increased sensitivity to oxygen, since the clumping phenotype indicated that these cells were more susceptible to oxygen-induced stress under these conditions. Wild-type cells containing pBlue-PDE also formed an aerotactic band, but it was significantly thinner than that formed by cells carrying pIND4 (Figure 20C), suggesting that despite being motile, fewer cells responded to the gradient and joined the aerotactic band under these conditions. Decreasing the intracellular c-di-GMP concentration with blue light in cells containing pBlue-PDE did not cause any visible change in the position or shape of the aerotactic band, and no clumping was observed (Figure 20C). Decreasing the c-di-GMP concentration with blue light in a phosphodiesterase *chsA* mutant, which has constitutively high intracellular c-di-GMP levels (Figure 19C), completely dissipated the aerotactic band. Returning these cells to green light failed to restore the aerotactic band, although some accumulation of cells at the former position of the band could be seen (Figure 20D). Together, these data suggested that modulating intracellular c-di-GMP levels could alter transient responses in an oxygen gradient.

Next, we wanted to test if changes in c-di-GMP levels using the optogenetic system could modify stable adaptive responses. For this purpose, we used the *chsA* mutant. As we previously reported, free-swimming *chsA* cells swim more slowly than wild-type cells and form many stable aggregates (comprised mostly of nonmotile cells) in cultures (5) (Figure 21A). The *chsA* mutant carrying the pIND4 or pBlue-PDE vector exposed to noninducing green light formed aggregates in culture (Figure 21). When exposed to blue light, the density of aggregates within the suspension

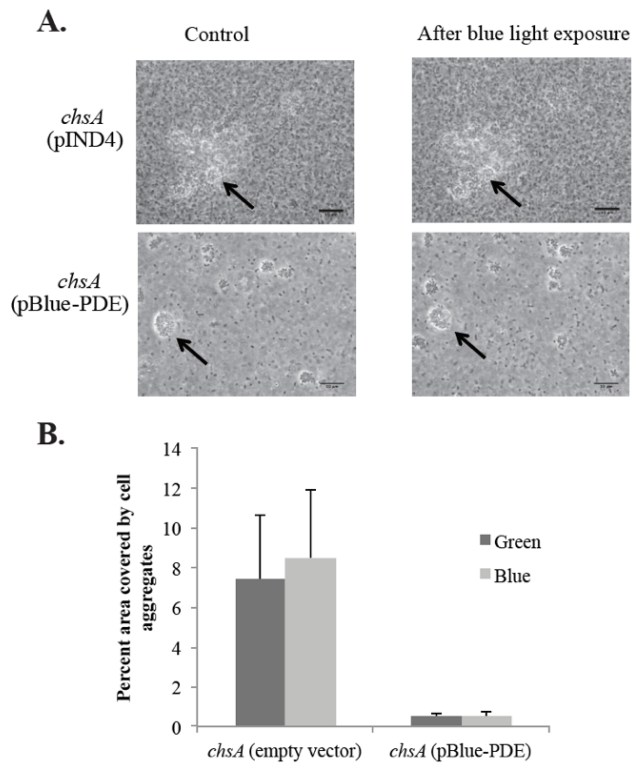


Figure 21: Light mediated reduction in c-di-GMP levels and cell-cell clumping and aggregation of the *chsA* mutant strain

Steady state cell clumping 30s before and 30s after blue light exposure for *chsA* mutant cells carrying an empty vector (*chsA*(pIND4)) (Top) and the pBlue-PDE vector (*chsA*(pBlue-PDE)) (Bottom). Percent area covered by cell aggregates (B). LO conducted this experiment.

of *chsA*(pBlue-PDE) cells did not change. Repeated short pulses (10 s each) of blue light did not change this outcome (data not shown). The presence of pBlue-PDE did not significantly decrease the amount of clumps formed in culture (*P* value of 0.1). These data indicate that the transient changes in c-di-GMP levels induced via the optogenetic system were insufficient to alter a more stable adaptive response such as the formation of aggregates.

C-di-GMP levels does not serve as an intracellular attractant or repellent

A. brasilense cells respond tactically to oxygen gradients via signaling by two Che systems that coordinately control transient changes in the swimming speed and in the probability of swimming reversals (25, 26). Therefore, we next examined the effect of transient changes in intracellular c-di-GMP levels on these motility parameters under steady-state conditions. We found that a temporal increase in intracellular c-di-GMP concentrations by a short 10-s pulse of red light did not change the swimming speed or the probability of reversals of wild-type cells carrying pRed-DGC, compared to the strain carrying an empty vector, pIND4 (Table 4). Therefore, triggering a temporal increase in c-di-GMP levels in cells carrying pRed-DGC with red light did not cause any change in the motility pattern; i.e., no chemotaxis signaling was induced. Similarly, decreasing the c-di-GMP level using blue light in cells carrying pBlue-PDE did not change these motility parameters. We observed that wild-type cells carrying pBlue-PDE exhibited a significantly lower swimming speed (p-value of 0.004), regardless of light exposure (Table 5). The reduced swimming speed is not due to a reduced growth rate since the wild-type strain from the growth rate of the wild-type strain carrying pIND4 (4.5 ± 1.0 h). Together, these carrying pBlue-PDE has a growth rate of 5.3 ± 1.3 h, which is not statistically different results demonstrate that c-di-GMP does not directly activate or inhibit chemotaxis signaling; i.e., c-di-GMP does not serve

Table 4. Effects of temporal increases in c-di-GMP concentration on swimming behavior

	WT (empty vector)		WT (pRed-DGC)	
	Swimming speed ^a	Reversal frequency ^b	Swimming speed ^a	Reversal frequency ^b
Green	27±1.5	0.8±0.1	22±1.1	0.9±0.1
Red	24±1.2	0.9±0.1	21±1.2	0.8±0.1
Green	26±1.4	1±0.1	24±1.3	0.8±0.1

^a Average swimming speed (µm/sec) ± standard error to the mean

^b Average reversal frequency (per second) ± standard error to the mean

Table 5: Effects of temporal decreases in c-di-GMP concentration on swimming behavior

	WT (empty vector)		WT (pBlue-PDE)	
	Swimming speed ^a	Reversal frequency ^b	Swimming speed ^a	Reversal frequency ^b
Green	27±1.8	0.8±0.1	16±1.5* ^c	0.6±0.1
Blue	31±4.4	0.6±0.1	16±0.8*	0.5±0.1
Green	25±1.9	0.8±0.1	16±1.2*	0.4±0.03

^a Average swimming speed (µm/sec) ± standard error to the mean

^b Average reversal frequency (per second) ± standard error to the mean

^c * indicates a significant difference compared to the uninduced cells under green light conditions (t-test; p<0.05)

as an intracellular attractant or repellent signal.

C-di-GMP levels modulate the ability of cells to detect the preferred oxygen concentration in a gradient

Although c-di-GMP does not directly activate changes in swimming motility patterns that would result from chemotaxis signaling, the data described above as well as from our previous work (5) suggest that changes in c-di-GMP levels modulated the ability of cells to sense and/or respond to oxygen gradients. To test this hypothesis further, we exposed cells containing pRed-DGC or pBlue-PDE to inducing light prior to exposing them to the oxygen gradient (see Materials and Methods). As a control, cells were stimulated with noninducible green light. When wild-type cells carrying pRed-DGC were maintained under green light, they started forming an aerotactic band at some distance from the meniscus within 30 s of exposure to the gradient, with a stable aerotactic band being established 40 s after exposure to the gradient. The formation of the band at this position and within this time frame is highly reproducible in cultures grown under similar conditions. Increasing c-di-GMP levels in these cells prior to exposing them to the oxygen gradient caused a reproducible and consistent lag of about 10 s, with a stable aerotactic band being formed by 60 s after exposure to the gradient (Figure 22A).

Wild-type cells carrying pBlue-PDE and exposed to noninducing green light prior to being exposed to the oxygen gradient formed a very faint and narrow aerotactic band 140 s after the introduction of the gradient, despite the cells being motile (Figure 22B). Incubation of these cells for longer times did not cause band widening (data not shown). Decreasing the c-di-GMP levels in these cells using blue light prior to exposure to the oxygen gradient licensed the cells to detect and respond to the gradient to form a stable aerotactic band at some distance from the meniscus at

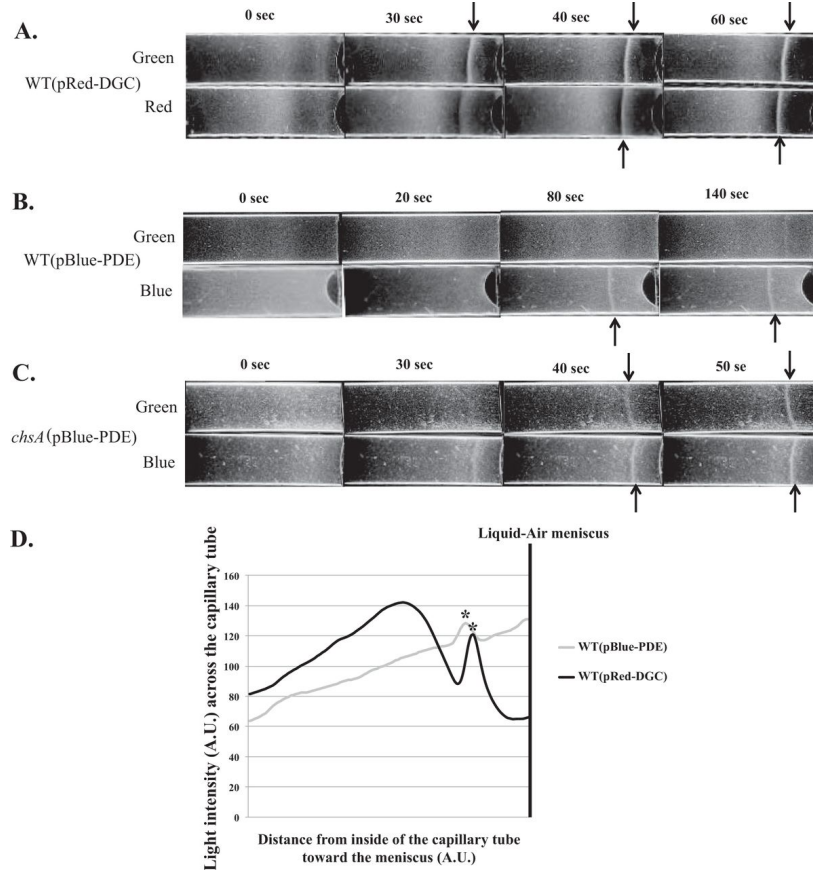


Figure 22: Effects of light-mediated changes in c-di-GMP levels on aerotaxis in spatial oxygen gradients.

(A to C) Behaviour of the WT strain carrying the optogenetic vectors pRed-DGC (A) and pBlue-PDE (B) in the gradient assay for aerotaxis, compared to that of the *ChsA* phosphodiesterase mutant (*chsA*) carrying pBlue-PDE (C). Cell suspensions standardized to equivalent numbers were placed into a capillary tube and exposed to N₂ for 3 min for equilibration under green light. Cells were next exposed to red or blue light for 10 s, and N₂ was immediately replaced with air that was allowed to flow into the chamber. Representative images of results from 3 biological replicates are shown. The time indicated at the top is in seconds after exposure to the air gradient. Arrows indicate the formation of a stable aerotactic band. (D) Distribution of cells throughout the capillary tubes after the formation of a stable aerotaxis band derived from image intensity analysis. The images of capillary tubes in which a stable aerotactic band formed under inducing conditions were analyzed by using ImageJ, as described in the text. Similar profiles were observed for biological replicates. A.U., arbitrary units. LO conducted this experiment.

80 s postexposure (Figure 22B). The aerotactic band formed by the cells under these conditions also formed farther away from the meniscus than the band formed by uninduced wild-type cells. Consistent with our above-described results, cells carrying pBlue-PDE formed an aerotactic band that was thinner than that formed by wild-type cells carrying pRed-DGC.

To confirm these observations, we performed a similar experiment using the *chsA* mutant carrying pBlue-PDE (Figure 22C). This strain, exposed to green light, formed a stable aerotactic band 50 s after the introduction of the gradient. When the c-di-GMP levels in the *chsA*(pBlue-PDE) strain were decreased with a short pulse of blue light prior to exposure to the gradient, a stable aerotactic band was formed after 40 s. These results are consistent with c-di-GMP modulating the ability of the cells to locate the preferred oxygen concentration in the gradient. Since the swimming speed or reversal frequency of wild-type cells carrying pRed-DGC or pBlue-PDE did not change upon exposure to red or blue light, respectively (Table 4 and Table 5), we interpret the changes in the time that it took cells to form a stable aerotactic band as reflecting differences in the abilities of the cells to respond to the gradient to which they were exposed.

We also analyzed the distribution of wild-type cells carrying pRed-DGC or pBlue-PDE, inside the capillary tubes, once a stable aerotactic band had formed (Figure 22D). This analysis confirmed that wild-type cells containing pRed-DGC and exposed to red light accumulated as a sharp aerotactic band. There was also a visible zone of clearing on both sides of the aerotactic band, indicating a depletion of cells. The clearing zone was also larger on the side of the band closest to the meniscus, suggesting that these cells responded strongly to the increased oxygen concentrations compared to cells located at lower oxygen concentrations relative to the aerotactic band. Beyond this region, cells accumulated in a broad zone, producing a visibly denser hump,

suggesting that cells failed to exhibit a strong and/or precise response to the gradient (Figure 22D). The distribution of wild-type cells carrying pBlue-PDE and exposed to blue light was in stark contrast: fewer cells accumulated at the preferred position in the gradient, yielding a thin aerotactic band, and there was no clearing zone in the vicinity of the band. These observations suggest that these cells had only a weak response to the oxygen gradient. These results thus support the hypothesis that changes in c-di-GMP levels affect the ability of cells to both detect and accumulate at the preferred oxygen concentration in a gradient.

Altering c-di-GMP levels affects the time to respond and adapt to an oxygen gradient

The results described above suggested that changes in c-di-GMP levels could affect the time that it took cells to form an aerotactic band and also excluded temporal changes in swimming speed in these behaviors. To further analyze this effect, we next determined the response time, which corresponds to the time that it takes motile cells stimulated with an attractant or repellent to return to a prestimulus swimming bias. The response time varies with the nature of the effector (a stronger attractant/repellent causes the cells to modify their swimming bias for longer times) and the adaptation rate, which depends on the level of signaling activity of chemotaxis receptors (27–29). To test the possibility that c-di-GMP modulates the signaling activity, and, thus, the contribution, of receptors to aerotaxis, we measured the time to response of wild-type cells carrying pRed-DGC or pBlue-PDE to a temporal decrease or increase in oxygen concentrations, under noninducing (green light) or inducing (red or blue light) conditions (Table 6). We found that changing c-di-GMP levels affected the response time of cells carrying pRed-DGC or pBlue-PDE (Table 6). For air-adapted cells experiencing a decrease in oxygen concentrations (air to N₂),

Table 6. Effect of c-di-GMP on the adaptation time of *A. brasilense* to temporal changes in aeration.

	Adaptation time following a change in aeration (sec)			
	WT(pRed-DGC)		WT(pBlue-PDE)	
	Green	Red	Green	Blue
Air→ N ₂ ^a	23 ± 3 ^b	40 ± 2* ^c	20 ± 1	29 ± 1*
N ₂ → Air	31 ± 2	19± 1*	21±1	17 ± 1*

^a Direction of the temporal gradient of air of free swimming cells observed

^b Average of three biological replicates with standard error.

^c * indicates a statistically significant difference from green light treatment (t-test; p<0.05).

increased (pRed-DGC) or decreased (pBlue-PDE) c-di-GMP levels significantly increased the response time compared to that of noninduced cells. In contrast, N₂-adapted cells experiencing an increase in oxygen concentrations (N₂ to air) responded to this temporal gradient by reducing the adaptation time compared to that of noninduced cells (Table 6). Consistent with the observed lag or lead time in aerotactic band formation seen for these cells (Figure 22), these results suggest that modulating intracellular c-di-GMP levels directly alters the response time of cells in response to oxygen gradients and support the notion that c-di-GMP alters the signaling activity of the chemotaxis receptors involved.

Binding of c-di-GMP to a chemoreceptor, Tlp1, modulates its contribution to the aerotaxis response

The effects of c-di-GMP on the aerotaxis response seen here are expected to depend on signaling by a chemotaxis receptor(s). Several chemotaxis receptors mediate aerotaxis in *A. brasilense*, and only a few of them have been characterized to date (5, 30). To test the role of chemotaxis receptors, we introduced pRed-DGC into the *tlp1* mutant. We also introduced an empty vector (pRK415), a vector expressing parental Tlp1 (pRK-Tlp1), or a vector expressing a Tlp1 variant that is unable to bind c-di-GMP (pRK-Tlp1R562A R563A) (5) into the *tlp1* mutant and analyzed band formation after the induction of c-di-GMP biosynthesis with red light (Figure 23). Under noninducing green light, the *tlp1*(pRK415; pRed-DGC) strain formed an aerotactic band at some distance from the meniscus ($654 \pm 86 \mu\text{m}$), 70 s after exposure to the gradient (Figure 23A). The time that it took the cells to respond to the gradient and to form an aerotactic band was significantly different from that of wild-type cells under similar conditions (Figure 22A). We previously showed that disrupting Tlp1 did not affect c-di-GMP metabolism (5), suggesting that

the lack of functional Tlp1 caused the slower response of *tlp1*(pRK415; pRed-DGC) to the gradient. Increasing c-di-GMP levels with red light in the *tlp1*(pRK415; pRed-DGC) strain caused the band to form earlier as well as closer to the meniscus ($405 \pm 84 \mu\text{m}$), with a stable band being formed 40 s after the introduction of the gradient (Figure 23A). These effects were not caused by the presence of the pRK415 vector alone, since cells lacking this vector behaved in a similar fashion (data not shown). The expression of parental Tlp1 in the *tlp1* mutant [*tlp1*(pRK-Tlp1; pRed-DGC)] restored the timing of aerotactic band formation seen for the wild-type strain containing pRed-DGC (Figure 23A and B). Furthermore, elevated c-di-GMP levels in the *tlp1*(pRK-Tlp1; pRed-DGC) strain with red light caused the aerotactic band to form later (>70 s post-gradient exposure) as well as further away from the meniscus ($521 \pm 57 \mu\text{m}$) than under green light control conditions ($339 \pm 30 \mu\text{m}$) (Figure 23B). Cells of the *tlp1* mutant expressing Tlp1R562A R563A possess a Tlp1 receptor that is unable to bind c-di-GMP and formed a stable aerotactic band by 30 s under noninducing green light (Figure 23C). Increasing c-di-GMP levels in this strain caused a small lag in the time that it took cells to form a stable aerotactic band (40 s), with the band also forming slightly farther away from the meniscus ($490 \pm 39 \mu\text{m}$ versus $341 \pm 35 \mu\text{m}$) (Figure 23C). The formation of the aerotactic band at a specific position occurs within a reproducible time frame in cultures grown under similar conditions (7, 8, 23, 24). Therefore, these results suggest that the lack of functional Tlp1 as well as modulations of c-di-GMP levels affected both the time to a response of the cells to the gradient as well as their ability to detect the preferred oxygen concentrations to accumulate as an aerotactic band.

We further compared the distributions of cells in the capillary tubes once the aerotactic band had formed (Figure 23D). Both the *tlp1*(pRK415; pRed-DGC) and *tlp1*(pRKTlp1^{R562A R563A};

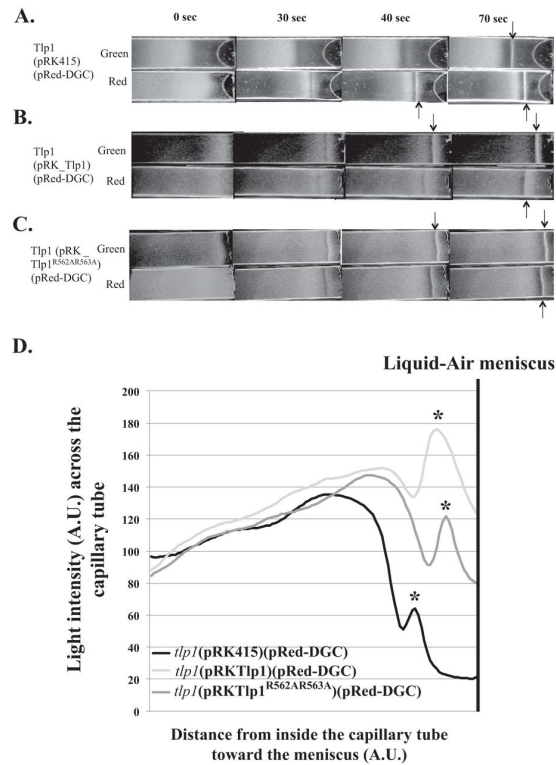


Figure 23: Role of Tlp1 c-di-GMP binding in mediating changes in sensitivity to oxygen gradients upon optogenetic manipulation of c-di-GMP levels.

(A to C) Spatial gradient assays for aerotaxis using an *A. brasilense* *tlp1* mutant carrying the optogenetic vectors (pRed-DGC or pBlue-PDE) as well as an empty vector (pRK415) (A), parental Tlp1 (B), or a Tlp1 variant lacking residues for c-di-GMP binding (Tlp1^{R562A R563A}) (C). (D) Image analysis of the distribution of cells throughout the capillary tube after the formation of a stable aerotactic band. Cell suspensions standardized to equivalent numbers were placed into a capillary tube and exposed to N₂ for 3 min for equilibration under green light. Cells were then exposed to red or blue light for 10 s, and N₂ was immediately replaced with air that was allowed to flow into the chamber. Representative images of results from 3 biological replicates are shown. The time indicated at the top is in seconds after exposure to the air gradient. Arrows indicate the formation of a stable aerotactic band. Cells that had formed a band at the latest time points were selected for image analysis. LO conducted this experiment.

pRed-DGC) strains formed similar aerotactic bands characterized by a dense accumulation of cells at the position of the aerotactic band that was followed by a broad accumulation of cells at lower concentrations of oxygen relative to the aerotactic band. These results suggested that the behavior of cells lacking Tlp1 was similar to that of cells carrying Tlp1 that were unable to bind c-di-GMP, implying that binding of c-di-GMP to Tlp1 promotes the contribution of this receptor to the aerotaxis response. The accumulation of cells that failed to locate the position of the aerotactic band further supports this notion. Consistent with the role of Tlp1 in supporting aerotaxis, the distribution of *tlp1*(pRKTlp1; pRed-DGC) cells was characterized by a larger aerotactic band that seemed to comprise more cells, while little to no accumulation of cells behind this preferred position in the gradient was observed. These data indicate that binding of c-di-GMP to Tlp1 is required for the ability of cells to respond to the oxygen gradient, including the ability to shift the preferred position for accumulation as a band in the oxygen gradient.

c-di-GMP levels change with carbon metabolism.

Aerotaxis is intimately linked to metabolism in *A. brasilense*, with the preferred position in an oxygen gradient corresponding to maximum intracellular energy production (8, 23, 31). We thus hypothesized that c-di-GMP could modulate the signaling contribution of some chemotaxis receptors as a function of metabolic status. This hypothesis implies that intracellular c-di-GMP concentrations vary with the carbon sources used for growth. Therefore, we first determined the c-di-GMP concentrations in wild-type cells grown in medium containing one of three preferred carbon sources for *A. brasilense* (8): malate, succinate, and fructose. Under conditions of growth in minimal medium, we found that intracellular c-di-GMP concentrations differed depending on the carbon source available for growth. Intracellular c-di-GMP levels were higher in cells grown

on malate ($2.7 \pm 0.5 \mu\text{M}$) and succinate ($2.1 \pm 0.7 \mu\text{M}$) than in cells grown on fructose ($1.3 \pm 0.2 \mu\text{M}$). Statistical analysis also indicated that c-di-GMP concentrations in fructose-grown cells were significantly different from the c-di-GMP levels detected in malate-grown cells (P value of >0.05). Doubling times were also lower on organic acids (4.3 ± 0.3 h for malate and 4.0 ± 0.4 h for succinate) than on fructose (6.2 ± 1.4 h), suggesting that different c-di-GMP levels could reflect different metabolic efficiencies. The average intracellular c-di-GMP concentrations that we obtained here were significantly higher under these conditions than those obtained with cells containing plasmids from the optogenetic system (Figure 19). We note that in addition to carrying plasmids, which affect the growth rate, as we describe above, the cells were grown under different conditions, specifically, a rich medium (Figure 19), compared to the minimal medium used here. We surmise that the distinct growth conditions and the presence of a plasmid together account for the discrepancy in intracellular c-di-GMP concentrations.

The dependence of *A. brasilense* aerotaxis on metabolism and the role of c-di-GMP in aerotaxis in this species, together with the results described above, suggested that changes in c-di-GMP levels could imprint the chemotaxis receptors of *A. brasilense* with a record of the cell's metabolic experience. We tested this hypothesis by comparing the preferred positions for aerotaxis band formation for the wild-type strain in the presence of various concentrations of malate or fructose. To evoke different metabolic experiences, we also used cells first grown on either malate or fructose (Figure 24). As expected, the position of the aerotactic band in the oxygen gradient was sensitive to the nature and the concentration of the carbon source available for aerotaxis (Figure 24A and B). There were striking differences between cells first grown on malate and cells first grown on fructose. First, the position of the aerotactic band formed by malate-grown cells changed

depending on the concentration of the carbon source available for aerotaxis. In contrast, cells first grown on fructose formed an aerotactic band at similar locations in the oxygen gradient, regardless of the nature or the concentration of the carbon source available for aerotaxis (Figure 24B). These observations suggest that cells that experienced recent conditions that caused elevated intracellular c-di-GMP levels (e.g., growth on malate) were able to adjust their aerotaxis response more precisely when they were subsequently exposed to an oxygen gradient. In contrast, cells that experienced conditions that decreased their c-di-GMP levels (e.g., growth on fructose) displayed an invariable subsequent aerotaxis response that was “blind” to changes in external conditions. Second, malate-grown cells failed to respond to the oxygen gradient in the presence of low concentrations of fructose, but they formed an aerotaxis band when higher concentrations of fructose were available for aerotaxis (Figure 24A). These results suggest that malate-grown cells rely on a more discriminative signaling input to select the preferred position for accumulation in a gradient by aerotaxis. This behavior would be expected if more receptors were contributing to the production of the aerotaxis response. In contrast, fructose-grown cells lacked the ability to fine-tune their preferred position for aerotaxis as a function of external conditions, as expected if fewer receptors were contributing to the signaling input (Figure 24A). To further validate this hypothesis, we analyzed the aerotaxis response of the *tlp1* mutant under similar conditions (Figure 24C). We found that the *tlp1* mutant first grown on malate was able to form an aerotactic band under all conditions, including low fructose concentrations. These data are consistent with Tlp1 contributing to the aerotaxis response of wild-type cells under these conditions and intracellular c-di-GMP levels altering the contribution of Tlp1 to the signaling output. Together, these findings suggest that c-di-GMP acts in a feedback loop that links recent metabolic experience to aerotaxis by

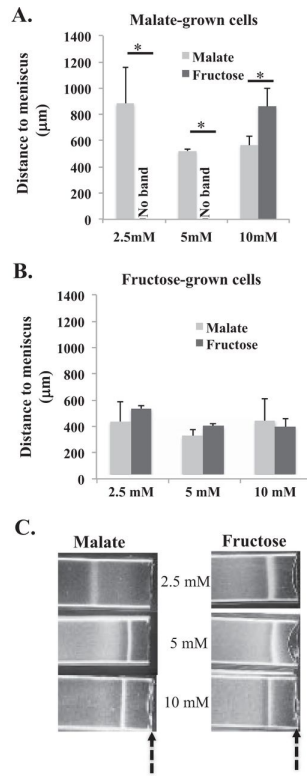


Figure 24: Effect of carbon source-mediated changes in c-di-GMP levels on the formation of an aerotactic band.

(A) Average position of the stable aerotactic band relative to the air-liquid meniscus for wild-type cells grown on malate (10 mM), washed, and incubated with malate or fructose at different concentrations for the spatial gradient assay for aerotaxis. (B) Average position of the stable aerotactic band relative to the air-liquid meniscus for wild-type cells grown on fructose (10 mM), washed, and incubated with malate or fructose at different concentrations for the spatial gradient assay for aerotaxis. (C) Representative images of results from at least 3 replicates showing the position of the stable aerotactic band formed by *tlp1* mutant cells grown on malate (10 mM), washed, and incubated with malate or fructose at different concentrations for the spatial gradient assay for aerotaxis. The arrows indicate the position of the air-liquid meniscus. An asterisk represents a significant difference between samples (P value of <0.05). LO conducted this experiment.

affecting chemotaxis receptors such as Tlp1.

Discussion

c-di-GMP is a second-messenger molecule in bacteria, and a range of assays and tools to characterize and manipulate c-di-GMP metabolism have been developed. Here, tools for manipulating c-di-GMP levels with high temporal resolution (seconds to minutes) have been tested. We adapted a recently engineered dichromatic optogenetic system for the real-time manipulation of c-di-GMP metabolism in bacteria (20) and used it to characterize the role of c-di-GMP signaling in the aerotaxis response of *A. brasilense*. We show that a brief induction of c-di-GMP biosynthesis, via a red-light-activated diguanylate cyclase, BphS, or the induction of c-di-GMP degradation via a blue-light-activated phosphodiesterase, EB1, leads to significant and transient changes in the c-di-GMP content of the cells within seconds. We also demonstrate that these transient changes are detected by the cells and affect the motility behavior in gradients of oxygen (aerotaxis). Specifically, we identify the binding of c-di-GMP to particular receptors as a mechanism to modulate the contribution of these receptors to the aerotaxis signaling output. Therefore, *A. brasilense* aerotaxis depends not only on signaling by dedicated chemotaxis receptors (5, 26, 30), as expected, but also on intracellular c-di-GMP levels.

We attribute the role of c-di-GMP in mediating changes in the aerotaxis response of *A. brasilense* to the ability of chemotaxis receptors, including Tlp1, to bind this molecule at its C-terminal PilZ domain. Mutants lacking Tlp1 or expressing a Tlp1 variant that is unable to bind c-di-GMP are not null for aerotaxis, which indicates that other receptors participate in this response and is consistent with previously reported findings (30, 32). The only other aerotaxis receptor for *A. brasilense* characterized to date is AerC, a soluble PAS domain-containing receptor that

lacks a PilZ domain (30). AerC has a significant role in modulating the aerotaxis of cells grown under nitrogen fixation conditions, but it does not contribute to the aerotaxis response, including aerotactic band formation, of cells grown under nitrogen-replete conditions (30), which we used here. A total of 48 putative chemotaxis receptors are annotated in the genome of *A. brasilense*. In addition to Tlp1, there are four additional genes for chemotaxis receptors containing PilZ domains. These additional genes also encode proteins with predicted PAS domains and a protoglobin domain. Given that PAS and protoglobin domains are often involved in oxygen sensing, including instances where they are present in chemotaxis receptors (33, 34), it is possible that some of these *A. brasilense* receptors participate in aerotaxis alongside Tlp1.

The results obtained here are consistent with the binding of c-di-GMP to receptors, such as Tlp1, affecting the ability of cells to accumulate at a preferred position within a spatial gradient of oxygen. We propose that intracellular c-di-GMP levels dictate the contribution of target chemotaxis receptors to the chemotaxis signaling output. In support of this notion, we showed that the binding of c-di-GMP to Tlp1 was required to support aerotaxis at the level of the wild-type strain: a mutant strain expressing a Tlp1 variant that is unable to bind c-di-GMP behaved like the *tlp1* mutant strain, which is consistent with previously reported findings (5). This further indicates that the binding of c-di-GMP to Tlp1 functions to increase the activity of this receptor. Our results show that changes in c-di-GMP levels occurring after an aerotactic band formed had little to no effect on the cell's behavior. This indicates that variation in the background levels of c-di-GMP does not affect the position of the aerotactic band once the cells have responded to the gradient. This is fully consistent with the lack of a role for c-di-GMP as an intracellular repellent

or attractant and with the robustness of the chemotaxis response that is a hallmark of the behavior of *E. coli* (35–37).

Within a cell, chemotaxis receptors are incorporated into large polar chemotaxis arrays with no turnover and, thus, little change in chemotaxis receptor composition between cell divisions (38). To modulate the activity of receptors and their contribution to the signaling output, most bacteria regulate the activity of chemotaxis receptors through differential methylation (39, 40). Our findings that c-di-GMP levels modulated the contribution of specific receptors (Tlp1) to the signaling output indicate that diverse small molecules can serve as intracellular cues to alter the activity of chemotaxis receptors. Small-molecule binding domains other than PilZ are found at the extreme C termini of diverse chemotaxis receptors (5, 41). A review of the predicted or demonstrated ligand binding specificities for these domains suggests that Zn^{2+} (41) and oxygen (5) might also directly modulate the signaling contribution of some chemotaxis receptors as a function of oxidative stress or ambient oxygen concentrations, respectively. For modifying the receptors by methylation, cells tap in to the intracellular *S*-adenosylmethionine (SAM) pool as a source of methyl groups, which also serves as a source of methyl groups for many other biological molecules, thereby linking general metabolism with chemotaxis signaling. By using c-di-GMP to modify the activity of the chemotaxis receptors, cells use a second-messenger molecule that signals the transition between planktonic and sessile lifestyles. Thus, the nature of the metabolic signal that is embedded in SAM and in c-di-GMP is distinct and thus likely expands the ways in which cells ensure that their chemotaxis signaling activity is fine-tuned with metabolism.

The formation of the tight aerotactic band at preferred, yet variable, positions in an oxygen gradient is reminiscent of the precision sensing behavior displayed during thermotaxis in *E.*

coli (42, 43). During thermotaxis, *E. coli* cells do not simply move up or down a temperature gradient but sense a particular temperature, and this behavior depends on chemotaxis receptor activity, set by differential methylation (44, 45). If aerotaxis is a form of precision sensing, then the accumulation of cells in the aerotactic band at a preferred position in the gradient is the result of the antagonistic signaling of receptors that sense oxygen (or a related cue such as redox) as an attractant versus those that sense oxygen as a repellent (or a related cue such as redox), with the summation of their signaling inputs ultimately determining the preferred position of the aerotaxis band in the gradient. This is consistent with data from previous experiments and mathematical modeling predictions (23, 24) that suggest that the formation of a stable aerotactic band in spatial gradients of oxygen by motile *A. brasilense* cells results from the ability of these cells to sense high and low oxygen concentrations as repellents and a precise, very low oxygen concentration as an attractant signal. The low oxygen concentration was estimated to be 0.4% dissolved oxygen when cells use malate as a carbon source (23). The effects of c-di-GMP on the position of the aerotactic band and on modulating the contribution of Tlp1 to aerotaxis together suggest that this molecule could tip the balance between the antagonistic activities of oxygen (or related cues)-sensing receptors implicated in determining the location of the preferred oxygen concentration in the gradient. Given that aerotaxis allows *A. brasilense* to locate positions in the gradients where energy generation via metabolism is maximal (23), this further suggests that c-di-GMP signals a particular metabolic status. In agreement with this hypothesis, the intracellular c-di-GMP levels changed with growth on different carbon sources and affected the ability of cells to locate the preferred oxygen concentration when cells were then exposed to an oxygen gradient. Our results also suggest that intracellular c-di-GMP levels may function in a feedback loop to imprint the

chemotaxis machinery with a “memory” of recent metabolic experience: cells that experienced growth on a readily metabolizable substrate, such as malate, adjusted their preferred position in the oxygen gradient depending on prevailing conditions, while cells that experienced lower growth rates prior to exposure to the gradient, such as growth on fructose, did not compute the prevailing conditions in determining their preferred position in the oxygen gradient. Given that this effect was linked to the signaling activity of Tlp1, the effect of c-di-GMP on increasing or decreasing the contribution of specific receptors to aerotaxis could thus provide a straightforward mechanism to tightly couple past metabolic experience with current metabolic status to ensure optimum energy generation by aerotaxis. Our observations that increased or decreased c-di-GMP levels affect the time that it took cells to respond to temporal changes in oxygen concentrations in the atmosphere directly support this notion and are consistent with aerotaxis being a metabolism-dependent behavior (5, 8, 33). In most bacteria studied to date, c-di-GMP is a major cue for signaling the transition from planktonic to sessile lifestyles, and this transition typically occurs as a result of metabolic challenges. It is thus not surprising to find a link between c-di-GMP levels and metabolism. The recent finding that a single-domain PilZ protein can modulate the activity of a chemotaxis-specific methyltransferase to control receptor signaling and chemotaxis in *Pseudomonas aeruginosa* (46) suggests that the mechanism that we suggest here is not unique to *A. brasilense*.

The dependence of c-di-GMP levels on carbon sources and, possibly, growth rates was also reported previously for *Vibrio cholerae* (47). Many other environmental cues, in addition to carbon sources, regulate c-di-GMP levels in bacteria, including oxygen, temperature, light, and amino acids (5, 48–50). It is thus possible that other small molecules also alter the contribution

of chemotaxis receptors to the signaling output.

References

1. Römling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial secondary messenger. *Microbiol Mol Biol Rev* 77:1-52.
2. Ryjenkov DA, Simm R, Romling U, Gomelsky M. 2006. The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. 30310-30314.
3. Amikam D, Galperin MY. 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* 22:3-6.
4. Benach J, Swaminathan SS, Tamayo R, Handelman SK, Folta-Stogniew E, Ramos JE, Forouhar F, Neely H, Seetharaman J, Camilli A, Hunt JF. 2007. The structural basis of cyclic diguanylate signal transduction by PilZ domains. *EMBO J* 26:5153-5166.
5. Russell MH, Bible AN, Fang X, Gooding JR, Campagna SR, Gomelsky M, Alexandre G. 2013. Integration of the second messenger c-di-GMP into the chemotactic signaling pathway. *MBio* 4:e00001-00013.
6. Hazelbauer GL, Lai WC. 2010. Bacterial chemoreceptors: providing enhanced features to two-component signaling. *Current Opinion in Microbiology* 13:124-132.
7. Alexandre G, Greer-Phillips S, Zhulin I. 2004. Ecological role of energy taxis in microorganisms. *FEMS Microbiology Reviews* 28:113-126.
8. Alexandre G, Greer SE, Zhulin IB. 2000. Energy taxis is the dominant behavior in *Azospirillum brasilense*. *J Bacteriol* 182:6042-6048.
9. Bible AN, Stephens BB, Ortega DR, Xie Z, Alexandre G. 2008. Function of a Chemotaxis-Like Signal Transduction Pathway in Modulating Motility, Cell Clumping, and Cell

- Length in the Alphaproteobacterium *Azospirillum brasilense*. Journal of Bacteriology 190:6365-6375.
10. Wisniewski-Dye F, Borziak K, Khalsa-Moyers G, Alexandre G, Sukharnikov LO, Wuichet K, Hurst GB, McDonald WH, Robertson JS, Barbe V, Calteau A, Rouy Z, Mangenot S, Prigent-Combaret C, Normand P, Boyer M, Siguier P, Dessaux Y, Elmerich C, Condemine G, Krishnen G, Kennedy I, Paterson AH, Gonzalez V, Mavingui P, Zhulin IB. 2011. *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. PLoS Genet 7:e1002430.
 11. Carreno-Lopez R, Sanchez A, Camargo N, Elmerich C, Baca BE. 2009. Characterization of chsA, a new gene controlling the chemotactic response in *Azospirillum brasilense* Sp7. Arch Microbiol 191:501-507.
 12. Ramirez-Mata A, Lopez-Lara-LI, ML X-V. 2016. The cyclic-di-GMP diguanylate cyclase CdgA has a role in biofilm formation and exopolysaccharide production in *Azospirillum brasilense*. Res Microbiol 167:190-201.
 13. Kulasakara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M, Hayakawa Y, Ausubel FM, Lory S. 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc Natl Acad Sci U S A 103:2839-2844.
 14. Le Guyon S, Simm R, Rehn M, Romling U. 2015. Dissecting the cyclic di-guanylate monophosphate signalling network regulating motility in *Salmonella enterica* serovar *Typhimurium*. Environ Microbiol 17:1310-1320.

15. Ha DG, Richman ME, O'Toole GA. 2014. Deletion mutant library for investigation of functional outputs of cyclic diguanylate metabolism in *Pseudomonas aeruginosa* PA14. *Appl Environ Microbiol* 80:3384-3393.
16. Gao X, Dong X, Subramanian S, Matthews PM, Cooper CA, Kearns DB, Dann CE, 3rd. 2014. Engineering of *Bacillus subtilis* strains to allow rapid characterization of heterologous diguanylate cyclases and phosphodiesterases. *Appl Environ Microbiol* 80:6167-6174.
17. Abel S, Fau BT, Nicollier M, Hug I, Kaeffer V, Abel Zur Wiesch P, Jenal U. 2013. Bimodal distribution of the second messenger c-di-GMP controls cell fate and asymmetry during the *Caulobacter* cell cycle. e1003744.
18. Christen M, Kulasekara Hd Fau - Christen B, Christen B Fau - Kulasekara BR, Kulasekara Br Fau - Hoffman LR, Hoffman Lr Fau - Miller SI, Miller SI. 2010. Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. *Science* 328:1295-1297.
19. Lori C, Ozaki S, Steiner S, Bohm R, Abel S, Dubey BN, Schirmer T, Hiller S, Jenal U. 2015. Cyclic di-GMP acts as a cell cycle oscillator to drive chromosome replication. 236-239.
20. Ryu M, Fomicheva A, Moskvina O, Gomelsky M. Optogenetic system for the dichromatic control of c-di-GMP signaling in bacteria (submitted).
21. Ryu M-H, Gomelsky M. 2014. Near-infrared light responsive synthetic c-di-GMP module for optogenetic applications. *ACS Synthetic Biology American Chemical Society*:802-810.

22. Ind AC PS, Brown MT, et al. 2009. Inducible-Expression Plasmid for *Rhodobacter sphaeroides* and *Paracoccus denitrificans*. *Appl Environ Microbiol* 75:6613-6615.
23. Zhulin IB, Bessalov VA, Johnson MS, Taylor BL. 1996. Oxygen taxis and proton motive force in *Azospirillum brasilense*. *Journal of Bacteriology* 178:5199-5204.
24. Mazzag BC, Zhulin IB, Mogilner A. 2003. Model of Bacterial Band Formation in Aerotaxis. *Biophysical Journal* 85:3558-3574.
25. Bible AN, Russell MH, Alexandre G. 2012. The *Azospirillum brasilense* Che1 Chemotaxis Pathway Controls Swimming Velocity, Which Affects Transient Cell-to-Cell Clumping. *Journal of Bacteriology* 194:3343-3355.
26. Mukherjee T, Kumar D, Burriss N, Xie Z, Alexandre G. 2016. *Azospirillum brasilense* chemotaxis depends on two signaling pathways regulating distinct motility parameters. *Journal of Bacteriology* 198.
27. Berg H, Brown D. 1972. Chemotaxis in *Escherichia coli* analysed by three dimensional tracking. *Nature* 239:500-504.
28. Carreno-Lopez R, Sanchez A, Camargo N, et al. 2009. Characterization of *chsA*, a new gene controlling the chemotactic response in *Azospirillum brasilense* Sp7. *Archives of Microbiology* 191:501-507.
29. Meir Y, Jakovljevic V, Oleksiuk O, Sourjik V, Wingreen N. 2010. Precision and kinetics of adaptation in bacterial chemotaxis. *Biophys J* 99:2766-2774.
30. Xie Z, Ulrich L, Zhulin I, Alexandre G. 2010. PAS domain containing chemoreceptor couples dynamic changes in metabolism with chemotaxis. *PNAS* 107:2235-2240.

31. Zhulin IB, Armitage JP. 1993. Motility, chemotaxis, and methylation-independent chemotaxis in *Azospirillum brasilense*. J Bacterio 125: 952-958. doi:10.1128/jb.175.4.952-958.1993.
32. Greer-Phillips S, Stephens B, Alexandre G. 2004. An energy taxis transducer promotes root colonization by *Azospirillum brasilense*. Journal of Bacteriology 186:6595–6604.doi:10.1128/JB.186.19.6595-6604.2004
33. Alexandre G. 2010. Coupling metabolism and chemotaxis-dependent behaviours by energy taxis receptors. Microbiology 156:2283-2293Alexandre G, Greer SE,
34. Henry JT, Crosson S. 2011. Ligand-Binding PAS domains in a genomic, cellular, and structural context. Annu Rev Microbiol 65:261-286.
35. Alon U, Surette MG, Barkai N, Leibler S. 1999. Robustness in bacterial chemotaxis. Nature 397:168-171.
36. Yi T-M, Huang Y, Simon MI, Doyle J. 2000. Robust perfect adaptation in bacterial chemotaxis through integral feedback control. Proceedings of the National Academy of Sciences 97:4649-4653.
37. Lazova MD, Ahmed T, Bellomo D, Stocker R, Shimizu TS. 2011. Response rescaling in bacterial chemotaxis. Proceedings of the National Academy of Sciences 108:13870-13875.
38. Schulmeister S, Ruttorf M, Thiem S, Thiem S, Kentner D, Lebiedz D, Sourjik V. 2008. Protein exchange dynamics at chemoreceptor clusters in *Escherichia coli*. PNAS 105:6403-6408.

39. Levit MN, Stock JB. 2002. Receptor methylation controls the magnitude of stimulus-response coupling in bacterial chemotaxis. *Journal of Biological Chemistry* 277:36760-36765.
40. Weis RM, Koshland DE. 1988. Reversible receptor methylation is essential for normal chemotaxis of *Escherichia coli* in gradients of aspartic acid. *Proceedings of the National Academy of Sciences* 85:83-87.
41. Draper J, Karplus K, Otterman KM. 2011. Identification of a chemoreceptor zinc-binding domain common to cytoplasmic bacterial chemoreceptors. *J Bacteriol* 193:4338-4345.
42. Oleksiuk O, Jakovljevic V, Vladimirov N, Carvalho R, Paster E, Ryu William S, Meir Y, Wingreen Ned S, Kollmann M, Sourjik V. 2011. Thermal Robustness of Signaling in Bacterial Chemotaxis. *Cell* 145:312-321.
43. Salman H, Libchaber A. 2007. A concentration-dependent switch in the bacterial response to temperature. *Nat Cell Biol* 9:1098-1100.
44. Jiang L, Ouyang Q, Tu Y. 2009. A Mechanism for Precision-Sensing via a Gradient-Sensing Pathway: A Model of *Escherichia coli* Thermotaxis. *Biophysical Journal* 97:74-82.
45. Micali G, Endres RG. 2016. Bacterial chemotaxis: information processing, thermodynamics, and behavior. *Current Opinion in Microbiology* 30:8-15.
46. Xu L, Xin L, Zeng Y, Yam JKH, Ding Y, Venkataramani P, Cheang QW, Yang X, Tang X, Zhang L-H, Chiam K-H, Yang L, Liang Z-X. 2016. A cyclic di-GMP-binding adaptor protein interacts with a chemotaxis methyltransferase to control flagellar motor switching. *Science Signaling* 9:ra102.

47. Koestler BJ, Waters CM. 2013. Exploring environmental control of cyclic di-GMP signaling in *Vibrio cholerae* by using the *ex vivo* cyclic di-GMP assay (TELCA). *Appl Environ Microbiol* 79:5233–5241. doi:10.1128/AEM.01596-13.
48. Bernier SP, Ha D-G, Khan W, Merritt JH, O'Toole GA. 2011. Modulation of *Pseudomonas aeruginosa* surface-associated group behaviors by individual amino acids through c-di-GMP signaling. *Research in Microbiology* 162:680-688.
49. Ryan RP, Fouhy Y, Lucey JF, Jiang B-L, He Y-Q, Feng J-X, Tang J-L, Dow JM. 2007. Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris*. *Molecular Microbiology* 63:429-442.
50. Townsley L, Yildiz FH. 2015. Temperature affects c-di-GMP signalling and biofilm formation in *Vibrio cholerae*. *Environmental Microbiology* 17:4290-4305.
51. Vanstockem MMK, Vanderleyden J, VanGool AP. 1987. Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn5 and Tn5-Mob insertion mutants. *Appl Environ Microbiol* 53:410–415.
52. Alexandre G. 2010. Coupling metabolism and chemotaxis-dependent behaviours by energy taxis receptors. *Microbiology* 156:2283-2293.
53. Bobrov AG, Fau KO, Ryjenkov DA, Waters CM, Price PA, Fetherston JD, Mack D, Goldman WE, Gomelsky M, Perry RD. 2011. Systematic analysis of cyclic di-GMP signaling enzymes and their role in biofilm formation and virulence in *Yersinia pestis*. *Mol Microbiol* 79:533-551.

CHAPTER III
A PILZ CONTAINING CHEMOTAXIS RECEPTOR MEDIATES OXYGEN
AND WHEAT ROOT SENSING IN *AZOSPIRILLUM BRASILENSE*

A version of this chapter has been published in *Frontiers in Microbiology* by Lindsey O’Neal, Shehroze Akhter, and Gladys Alexandre:

O’Neal, L., Akhter, S., & Alexandre, G. (2019). A PilZ containing chemotaxis receptor mediates oxygen and wheat root sensing in *Azospirillum brasilense*. *Frontiers in Microbiology*.

LO and SA conducted the experiments. LO and GA wrote the manuscript.

Abstract

Chemotactic bacteria sense environmental changes via dedicated receptors that bind to extra- or intracellular cues and relay this signal to ultimately alter direction of movement towards beneficial cues and away from harmful environments. In complex environments, such as the rhizosphere, bacteria must be able to sense and integrate diverse cues. *Azospirillum brasilense* is a microaerophilic motile bacterium that promotes growth of cereals and grains. Root surface colonization is a prerequisite for the beneficial effects on plant growth but how motile *A. brasilense* navigates the rhizosphere is poorly studied. Previously only 2 out of 51 *A. brasilense* chemotaxis receptors have been characterized, AerC and Tlp1, and only Tlp1 was found to be essential for wheat root colonization. Here we describe another chemotaxis receptor, named Aer, that is homologous to the *Escherichia coli* Aer receptor, likely possesses an FAD cofactor and is involved in aerotaxis (taxis in an air gradient). We also found that the *A. brasilense* Aer contributes to sensing chemical gradients originating from wheat roots. In addition to *A. brasilense* Aer having a putative N-terminal FAD-binding PAS domain, it possesses a C-terminal PilZ domain that contains all the conserved residues for binding c-di-GMP. Mutants lacking the PilZ domain of Aer are altered in aerotaxis and are completely null in wheat root colonization and they also fail to sense gradients originating from wheat roots. The PilZ domain of Aer is also vital in integrating

Aer signaling with signaling from other chemotaxis receptors to sense gradients from wheat root surfaces and colonizing wheat root surfaces.

Introduction

The rhizosphere is a chemical milieu, and rhizobacteria must navigate this environment to find habitable niches. Chemotactic bacteria adjust their motility in response to environmental chemicals, swimming toward favorable signals (attractants) and away from harmful chemicals. Chemotaxis receptors drive this recognition either directly, by binding to environmental cues or indirectly, by binding to proteins bound to these cues or by monitoring the effects that exposure to the cues has on metabolism (1, 2). The latter behavior is typically referred to as energy taxis (1). Upon recognition of chemoeffectors, chemotaxis receptors transduce this signal to downstream proteins (CheW, CheA, and CheY) that can relay this information to the flagellum machinery and alter the direction of rotation of the flagellum and hence, the swimming direction of the cell. Navigating an attractant gradient prevents changes in the swimming direction, causing the bacteria to keep swimming in the same direction (3). The molecular mechanism of chemotaxis is best known in *Escherichia coli*, and research in diverse Bacteria and Archaea has shown that molecular events during chemotaxis signal transduction are largely conserved across prokaryotes (4). The greatest diversity in chemotaxis signal transduction is seen in the chemotaxis repertoire with bacterial genomes encoding up to 80 chemotaxis receptors (5). Many chemotactic bacteria possess multiple chemosensory operons which can control chemotaxis or alternative cellular functions (5). Soil bacteria are characterized by both a large number of chemotaxis receptors and multiple chemotaxis signaling systems (6, 7), and plant-associated soil bacteria are more likely to encode

chemotaxis genes than non-plant associated bacteria (8). These features are thought to provide a competitive advantage within the chemically rich and complex environment of the rhizosphere (7).

Azospirillum brasilense is a motile, chemotactic plant growth promoting proteobacterium that inhabits the soil and colonizes the roots of diverse plants, including economically important cereals (9). The genome of *A. brasilense* encodes 4 chemosensory operons, named Che1 through Che4, and 51 chemotaxis receptors (10, 11). Only two of the chemotaxis systems, Che1 and Che4, are directly involved in chemotaxis, and the other two systems have functions outside of chemotaxis, including a likely role in flagellum biosynthesis (Che2 (unpublished data)) and cyst development (Che3 (12)). To date, the function of only 2 of the chemotaxis receptors have been characterized in this bacterial species: Tlp1 and AerC (13-16). Both are important in aerotaxis (taxis in an oxygen gradient), but only Tlp1 is necessary for plant root colonization (13, 16). Aerotaxis is a major behavioral response in *A. brasilense*, and the bacterium relies on sensing oxygen gradients to locate niches that are best suited for the microaerophilic lifestyle of the bacterium (17, 18). AerC is soluble chemotaxis receptor required for aerotaxis under conditions of nitrogen fixation, but not in presence of an organic source of nitrogen (16). Tlp1 is a prototypical chemotaxis receptor in that it is a membrane anchored protein with a periplasmic sensory domain at the N-terminus and a cytoplasmic signaling domain. The ligand sensed by Tlp1 is unknown, but the receptor also possesses an additional C-terminal PilZ domain (14, 15). PilZ domains are known to bind to the secondary messenger cyclic diguanylate monophosphate (c-di-GMP) (19-21). C-di-GMP metabolism is regulated by a variety of cues and has been implicated in modulating the lifestyle of bacteria inhabiting various environments, including the rhizosphere. In *A. brasilense*, c-di-GMP binding to the PilZ domain of Tlp1 regulates receptor sensitivity in an oxygen gradient,

and Tlp1 is essential for plant root colonization (14, 15). *A. brasilense* encodes several other PilZ containing chemotaxis receptors, each of different predicted topologies. Here, we characterize another PilZ-containing receptor, Aer, and its role in aerotaxis, sensing of root exudate gradients, and root surface colonization.

Materials and Methods

Bacterial strains growth and maintenance

Wild type *A. brasilense* (Sp7) and its derivative Δaer were maintained on Minimal Medium for *A. brasilense* (MMAB) supplemented with nitrogen (20mM) and malate (10mM) (16). All strains were maintained on 1.5% agar with appropriate antibiotics [ampicillin (200 μ g/ml), tetracycline (10 μ g/ ml), kanamycin (30 μ g/ml)] (Table 7).

Identification of genes encoding putative chemotaxis receptors with additional domains and PAS sequence alignment

The PilZ domain of Tlp1 (AAT76671.1) was used in a PSI-BLAST against all *A. brasilense* species to identify any receptors containing C-terminal PilZ domains. Each putative protein was verified using SMART domain search. Genes encoding each protein were identified in the Sp7 genome (GenBank project PRJNA265779). Residues 25-139 of the PAS domains of *K. pneumoniae* NifL (AAB26403.1), residues 8-129 of the PAS dsomain of the *E.coli* Aer (AYG17963.1), and residues 85-329 of the *M. capsulatus* MmoS (AAP80772.1) were aligned with residues 9-121 of the PAS domain of *A. brasilense* Aer using the COBALT multiple sequence alignment in NCBI (38).

Table 7: List of strains used in this study

Strain	Characteristics	Reference
<i>E. coli</i>		
TOP10	General cloning strain	
TOP10 (pk18mobsacB)	TOP10 cells carrying pk18mobsacB, KanR, SucS	
TOP10 (pk18mobsac_AerUpDown)	TOP10 cells carrying pk18mobsacB with Upstream and Downstream AZOBRv2_p28002 sequences, KanR, SucS	This Work
TOP10 (pRK415)	TOP10 cells carrying pRK415, TetR	This Work
TOP10 (pRKAer)	TOP10 cells carrying pRK415 encoding AZOBRv2_p280026 (<i>aer</i>), TetR	This Work
TOP10 (pRK AerΔPilZ)	TOP10 cells carrying pRK415 encoding truncated AZOBRv2_p280026 (<i>aer</i>), lacking the C-terminal PilZ domain, TetR	This Work
<i>A. brasilense</i>		
Sp7	Wild Type, AmpR	(41)
WT (empty vector)	Sp7 carrying the vector pRK415	(15)
Δ <i>aer</i>	Sp7 with AZOBRv2_p280026 (<i>aer</i>) deleted	This work
Δ <i>aer</i> (Aer)	Δ <i>aer</i> with Aer expressed from pRK415, AmpR TetR	This work
Δ <i>aer</i> (AerΔPilZ)	Δ <i>Aer</i> with truncated Aer expressed from pRK415, AmpR TetR	This work

Generation of markerless deletion of aer

The upstream and downstream sequences of AZOBR_p280026 were amplified using the primers listed in Table 8 and then cloned into the pk18mobsacB vector using restriction digestion. The plasmid containing the upstream and downstream sequences was confirmed via sequencing and transformed into *E. coli* TOP10. The plasmid was then transferred into WT *A. brasilense* via triparental conjugation, as previously described (39) and selected for on MMAB with 10 mM malate and kanamycin (30 µg/ml).

Single recombinants were screened on MMAB plates containing kanamycin (30 µg/ml). Once single recombinants were identified, they were passaged through TY without antibiotic selection 3-5 times. With every passage, serial dilutions were performed and plated on TY (Tryptone-Yeast) plates containing 10% sucrose (w/v). Colonies that appeared on TY containing 10% sucrose were rescreened on TY and TY with kanamycin (30 µg/ml) (39). Colonies with suspected gene deletions were PCR confirmed.

Functional complementation of the Δaer mutation

The gene encoding full length Aer or truncated Aer (Aer Δ PilZ) was amplified and cloned into pRK415 using restriction digestion and sequencing confirmed and transformed into *E. coli* TOP10 cells for maintenance. The plasmids were transferred into Δaer using triparental conjugation as described above.

Table 8: Primers used in this study

Primer name	Sequence
AZOBRv2_p280026-EcoR1-F	5'-CCCGAATTCACAATAGGGGCGAAGCGC-3'
AZOBRv2_p280026-ovl-R	5'- GCCGCCGTTGCGGATCTCGGCGTAGATGCGCTCG GC-3'
AZOBRv2_p280026-ovl-F	5'GAGATCCGCAACGGCGGCGCCATGCTGAGTGGG ATC-3'
AZOBRv2_p280026-Sal-R	5'-CCC GTCGAC CTTCGCCGCGACCGAGCC-3'
Aer complementation Fwd HindIII	5'-AAGCTTATGCGCGATAATGGACCGGT-3'
Aer complementation Rev BamHI	5'-GGATCCTCAGGCCGCCGCCGTCGCCA-3'
AerΔPilZ complementation Fwd HindIII	5'-AAGCTTATGCGCGATAATGGACCGGT-3'
AerΔPilZ complementation Rev BamHI	5'-GGATCCTCAGGCGTCGCTGGTGGAGGTGC-3'

FAD quantification

50 ml of wild type cells or Δ aer expressing the parental Aer were grown to OD₆₀₀=0.8 and pelleted. Intracellular FAD was extracted as previously described in (16, 32) using 0.6N perchloric D-alanine, 7.5 μ g horseradish peroxidase, and 25 μ M luminol and the reaction was initiated acid and sodium bicarbonate. The cell extract was combined with 23 mM with apo-D-amino acid oxidase 1 mg/ ml and luminescence was measured every second for 1 hour. Peak luminescence readings (18 minutes) were used for FAD quantification.

Chemotaxis soft agar plate assays

A. brasilense strains were grown in MMAB supplemented with nitrogen (20mM) and malate (10mM) to OD₆₀₀=0.8. One ml of the culture was centrifuged and washed with Che buffer (1.7 g L⁻¹ dipotassium phosphate, 1.36 g L⁻¹ monopotassium phosphate; 0.1 mM EDTA), and 10 microliters was inoculated into MMAB containing 0.3% agar. Chemotaxis rings were measured 72 hours post-inoculation.

Spatial gradient assay for aerotaxis

A. brasilense strains were grown and washed as previously described. Washed cells were used to fill an optically flat capillary tube. The capillary tube was equilibrated in N₂ for 3 minutes. N₂ was then shut off and air was introduced, and band formation was monitored using a 4X objective of a phase contrast Nikon E200 microscope (28). Images were acquired using a C-mounted Nikon Coolpix digital camera. Distance of band formation of the meniscus and band width was quantified using the measure tool in FIJI. Distance from the meniscus to the center of

the band at mid-height of the capillary tube. All measurements were performed at 180 seconds after air introduction.

Root-in-pool assay

Wheat seeds were sterilized with successive washes of bleach, 70% ethanol containing 1% Triton X-100, and sterile water. After sterilization, seeds were placed in a sterile petri dish containing a small volume of sterile water and placed in the dark. After 48 hours, plates were placed in the light and allowed to grow for another day. All assays were performed on 5-day old germinated seeds. *A. brasilense* strains were grown in MMAB with nitrogen (20mM) and malate (10mM). OD₆₀₀=0.4 and washed with chemotaxis buffer as previously described. Cells were starved in chemotaxis buffer (1.7 g L⁻¹ dipotassium phosphate, 1.36 g L⁻¹ monopotassium phosphate; 0.1 mM EDTA) for 30 minutes before exposure to the root. A black washer with a slit cut in it was secured to a depression slide using craft glue. The germinated root was placed in the slit and 400 µL of washed bacteria was used to fill the chamber created by the dip and washer, and then a cover slip was used to cover the pool chamber. All root-in-pool assays were recorded using the 4x objective of a Nikon E200 phase contrast microscope equipped with Nikon Coolpix digital camera. Assays were filmed for 3 minutes. FIJI was used for all image and video analysis. All root-in-pool assay videos were converted to 8-bit and background subtraction was performed. Intensity plots were generated out from the root to determine bacterial accumulation. Bacterial accumulation distance from the root surface was measured at the mid-point of each root zone. The average accumulation distance of 5 biological replicates is reported.

Wheat root colonization assay

Wheat seeds were germinated as previously described. *A. brasilense* strains were grown to OD600=0.4, washed and suspended in 400 µl of Che buffer (1.7 g L⁻¹ dipotassium phosphate, 1.36 g L⁻¹ monopotassium phosphate; 0.1 mM EDTA). Serial dilutions were performed to determine the number of bacteria inoculated. Plant growth chambers were filled with 50ml of semi-solid Fahraeus media(40) (0.9 mM CaCl₂·H₂O, 0.5 mM MgSO₄, 0.7 mM KH₂PO₄, 0.8 mM Na₂HPO₄·7H₂O, 20 µM Fe-citrate, 33 µg/l, a pinch of Na₂MoO₄ · H₂O, pH 7.0, 4g agar per liter), and four germinated seedlings were planted in the chamber. Twenty microliters of the bacterial suspension were inoculated into the center of the growth chamber, and after 5 days roots were harvested, homogenized, and serial dilutions were performed. Student's unpaired t-test was performed to determine if colonization levels were significantly different (p-value=0.05). Root colonization was calculated as a colonization index. The colonization index for an individual strain was the log₁₀(strain output/strain input), where the number of CFU extracted from roots after incubation was normalized to the number of CFU measured in the inoculated input. Competition assays were performed by inoculating equal amounts of WT and Δaer cells (confirmed by CFU and OD600 readings). After 5 days CFU counts were performed and 30 colonies from each replicate were screened for deletion of AZOBRv2_p280026 using primers in Table 2.

Statistical analysis

Average values from at least three independent experiments performed in duplicate and performed one-way analysis of variance (alpha level, 0.05), followed by pairwise two-sample *t*-tests assuming equal variances (alpha level, 0.05) using Prism (version 6) software (GraphPad Software Inc., San Diego, CA).

Results

Identification of chemotaxis receptors containing PilZ domains

To identify *A. brasilense* chemotaxis receptors with PilZ domains, we conducted a PSI-BLAST using the PilZ domain of Tlp1 (AAT76671.1) as a query. After 7 iterations, results converged to 7 unique PilZ-containing putative chemotaxis receptors. The most common PilZ containing chemotaxis receptor across all strains of *A. brasilense* identified by the PSI-BLAST is AZOBR_p280026. This chemotaxis receptor is predicted to be membrane anchored and to possess a cytoplasmic N-terminal sensory domain comprised of a PAS/PAC domain followed by, 2 transmembrane domains, a HAMP domain, a MA, and a C-terminal PilZ domain (Figure 25A). The PilZ domain contains the two strictly conserved arginine residues (R506, R507) required for binding c-di-GMP (Figure 25A) in homologs (21-23) indicating it is likely a functional c-di-GMP effector.

Except for the PilZ domain, the topology and domain organization of AZOBR_p280026 is similar to that of the *E. coli* Aer receptor, which functions in aerotaxis as an energy taxis receptor (24, 25). The *E. coli* Aer chemotaxis receptor binds FAD non-covalently within its N-terminal PAS domain and this cofactor binding is required for the receptor energy taxis function. Indeed, the FAD cofactor confers the *E. coli* Aer receptor with the ability to sense changes in redox that occur when cells navigate gradients of oxygen and other cues (26). A similar role for FAD non-covalent binding to the *A. brasilense* AerC chemotaxis receptor was previously found (16). In both the *E. coli* Aer and the *A. brasilense* AerC, FAD binding depends on a set of conserved residues (16, 24, 25, 27). Sequence analysis indicates that the protein encoded by AZOBR_p280026 contains all of the conserved residues necessary for FAD binding (Figure 25B). In *E. coli* and *A.*

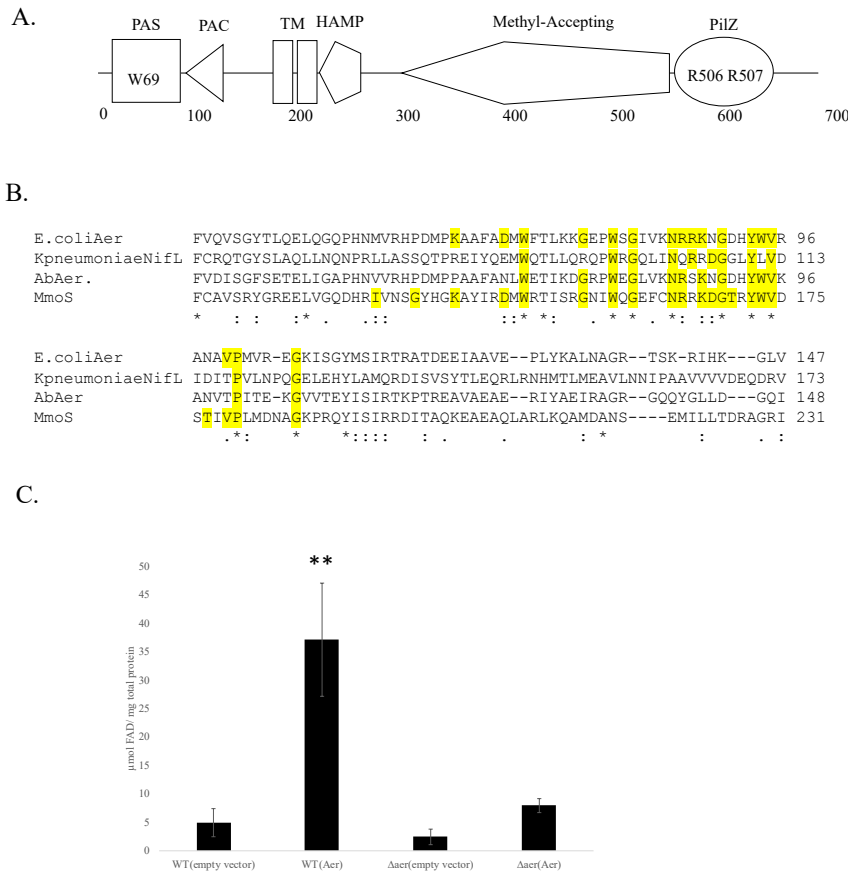


Figure 25: *A. brasiliense* encodes a chemotaxis receptor with a putative N-terminal FAD-binding PAS domain and a C-terminal PilZ domain

(A) Protein encoded by AZOBRv2_p280026 contains a PAS, PAC, Transmembrane domains, HAMP, MA, and PilZ domain. B) Conserved residues alignment between AZOBRv2_p280026 (Aer) PAS domains binding FAD in *E. coli* Aer, *Klebsiella pneumoniae* NifL and *Methylococcus capsulatus* Mmos. Below the sequence alignment, “*” indicates positions with strictly conserved residues, “:” indicates position with strongly similar residues and “.” indicates positions with weakly similar residues. Yellow highlighted residues are those implicated in FAD binding (24, 42, 43). (C) Total cytosolic FAD for strains of *A. brasiliense* overexpressing Aer. ** indicates statistically significant values (p-value < 0.005) relative to the wild type strain. WT (empty vector) corresponds to the wild type strain carrying pRK415, WT (Aer) is the wild type strain carrying pRK4Aer, Δaer(empty vector) is the Δaer mutant derivative carrying pRK415 and Δaer(Aer) is the Δaer mutant derivative carrying pRK4Aer. LO conducted this experiment.

brasilense, overexpression of Aer or AerC causes an increase in total intracellular FAD content (16, 24, 27). Similarly, when we overexpressed AZOBR_p280026 in *A. brasilense*, the total FAD content of cells increased, suggesting this chemotaxis receptor likely behaves like *E. coli* Aer and *A. brasilense* AerC (Figure 25C). Given these properties and the function of this receptor in *A. brasilense* aerotaxis (see below), we named it Aer.

***A. brasilense* Aer PilZ domain is essential for aerotaxis**

To probe the function of Aer in *A. brasilense* sensing, we created a markerless deletion mutant, named Δaer (Table 7). Given the predicted FAD binding by the *A. brasilense* Aer receptor and the role of aerotaxis as the strongest behavioral response in this species, we predicted Aer would function as an aerotaxis receptor. To characterize the role of Aer in aerotaxis, we used the spatial gradient assay for aerotaxis and compared the behavior of the wild type strain, the Δaer mutant derivative and its complemented strains expressing either full length Aer or Aer Δ PilZ that were either grown with ammonium chloride as a nitrogen source or grown under conditions of nitrogen fixation (Figure 26). In the spatial aerotaxis assay, an open-ended capillary tube is filled with motile cells, and an oxygen gradient is established through diffusion of air from the atmosphere into the cell suspension (28, 29). Under these conditions, motile *A. brasilense* cells accumulate in a tight band at a location that corresponds to an oxygen concentration optimum for metabolism (18, 28). When cells were grown with ammonium prior to being exposed to the gradient, the Δaer mutant cells formed an aerotactic band further away from the air-liquid interface compared to the WT cells (Figure 26, left panel). The aerotaxis defect could be restored by expressing the parental Aer *in trans* from a low copy vector; however, expressing a truncated form of Aer that lacked the C-terminal PilZ domain (Aer Δ PilZ) failed to restore aerotaxis band

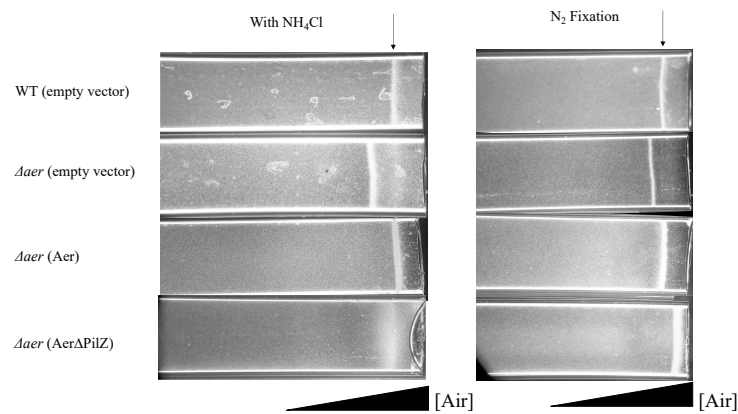


Figure 26: Effect of loss of Aer on oxygen sensing in a spatial assay for aerotaxis

Cells grown in presence of ammonium chloride or grown in the absence of combined nitrogen and under condition of nitrogen (N_2) fixation before being placed in a capillary tube. Over 95% of cells are motile under these conditions. An oxygen gradient is generated by diffusion of air into the suspension of motile cells. WT cells accumulate in a tight band at an oxygen concentration optimum for metabolism (arrow). Representative images are the result of 3 biological replicates. LO conducted this experiment.

formation, though there was cell accumulation at the position of the aerotaxis band should have formed. A similar behavior was observed for cells grown under conditions of nitrogen fixation, with the notable exception that Δaer mutant cells expressing Aer Δ PilZ accumulated as a tight band closer to the air-liquid meniscus (Figure 26, right panel). We also observed that Δaer cells expressing Aer Δ PilZ began to clump together and lose motility after band establishment when they were close to the air-liquid interface (at about 3 minutes), suggesting that cells accumulate at oxygen concentrations that are too high and trigger stress responses (12). Together these data show that Aer is required for aerotaxis regardless of nitrogen availability. Our data indicate that cells can form an aerotactic band in the absence of Aer, suggesting other receptors like Tlp1 and AerC can mediate the behavior. In the absence of Aer, the aerotactic band forms further away from the meniscus, suggesting that Aer's signaling is required to guide cells into regions of higher oxygen concentrations. Strains expressing Aer Δ PilZ form the aerotactic band at high oxygen concentrations under nitrogen fixation conditions, but the band is abolished when organic nitrogen is available. Together these findings suggest that the contribution of the PilZ domain to Aer signaling during aerotaxis depends on growth (organic nitrogen availability) conditions.

As expected for its role in aerotaxis, cells lacking Aer or expressing the variant lacking PilZ did not have any defect in chemotaxis toward organic acids (Figure 27), indicating that Aer is dispensable in chemotaxis.

Aer contributes to competitive root surface colonization

Given the role of Aer in aerotaxis, we next tested how this response contributed to colonization of wheat (*Triticum aestivum*) root seedlings. The Δaer strain colonized the roots at

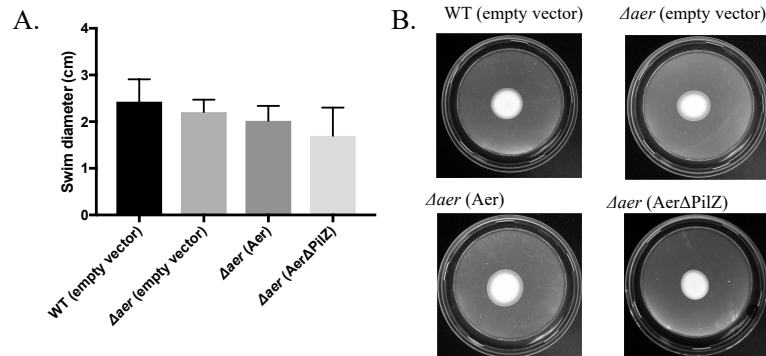


Figure 27: Chemotaxis in a soft agar plate containing 10mM malate

A) Average chemotaxis ring diameter of cells in a soft agar plate containing malate with standard deviation. Given in the average of 3 biological replicates. B) Representative images of WT (empty vector), Δaer (empty vector), and Δaer (pRK Aer), and Δaer (pRK Aer Δ PilZ). LO conducted this experiment.

levels similar to wild type, when inoculated alone while the Δaer strain expressing the parental Aer colonized at higher levels than the wild type strain (Figure 28). These differences in the colonization ability of the strains inoculated alone were unexpected and could not be attributed to changes in the level of motility between the strains. We hypothesized that functional Aer would provide a competitive advantage in colonization of the root surface and tested this idea by inoculating the wild type and the Δaer strains in competition at a 1:1 ratio (See Methods). After 5 days, the strains were recovered from homogenized wheat roots at a ratio of wild type to Δaer mutant of 92:8. These findings indicate that the Δaer mutant is severely impaired in its ability to compete with the wild type strain for wheat root surface colonization despite being able to colonize the root surfaces when inoculated alone. Surprisingly, the Δaer strain expressing Aer Δ PilZ was severely impaired in colonization of the root surfaces when inoculated alone (Figure 28). These observations hint at a complex contribution of Aer and PilZ-mediated signaling in the vicinity of the roots.

Aer signaling is integrated with that of other chemotaxis receptors in a PilZ-dependent manner

To gain further insight into the apparent complex contribution of *A. brasilense* Aer and Aer PilZ in wheat root surface colonization, we sought to develop an assay permitting real time observation of chemotaxis responses, which we call the root-in-pool assay. In this assay, a 5-day old germinated wheat seedling's root is placed into a pool of concentrated motile cells (Figure 29A). The immediate response of motile bacteria to the presence of the roots is recorded for up to 5 minutes and the preferential accumulation of bacteria in different regions around the roots can be inferred from image intensity analysis (see Methods). When exposed to wheat roots, the wild type *A. brasilense* cells accumulated in a tight band away from the root hair ($129 \pm 18\mu\text{m}$) and

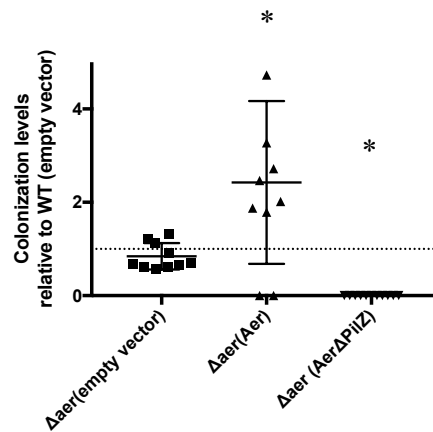


Figure 28: Colonization efficiency of wheat seedling roots by *A. brasilense* wild type and mutant strains derivative lacking Aer and expressing an empty vector control, Aer or Aer Δ PilZ

The colonization index was the $\log_{10}(\text{strain output}/\text{strain input})$, where the number of CFU extracted from roots after incubation was normalized to the number of CFU measured in the inoculated input. The dotted line value at 1 indicates the colonization index of the WT (empty vector). Points above the line are representative of a higher colonization efficiency, while points below the line are indicative of a lower colonization efficiency. * indicates significant difference from WT (empty vector) (p-value < 0.05). SA conducted this experiment.

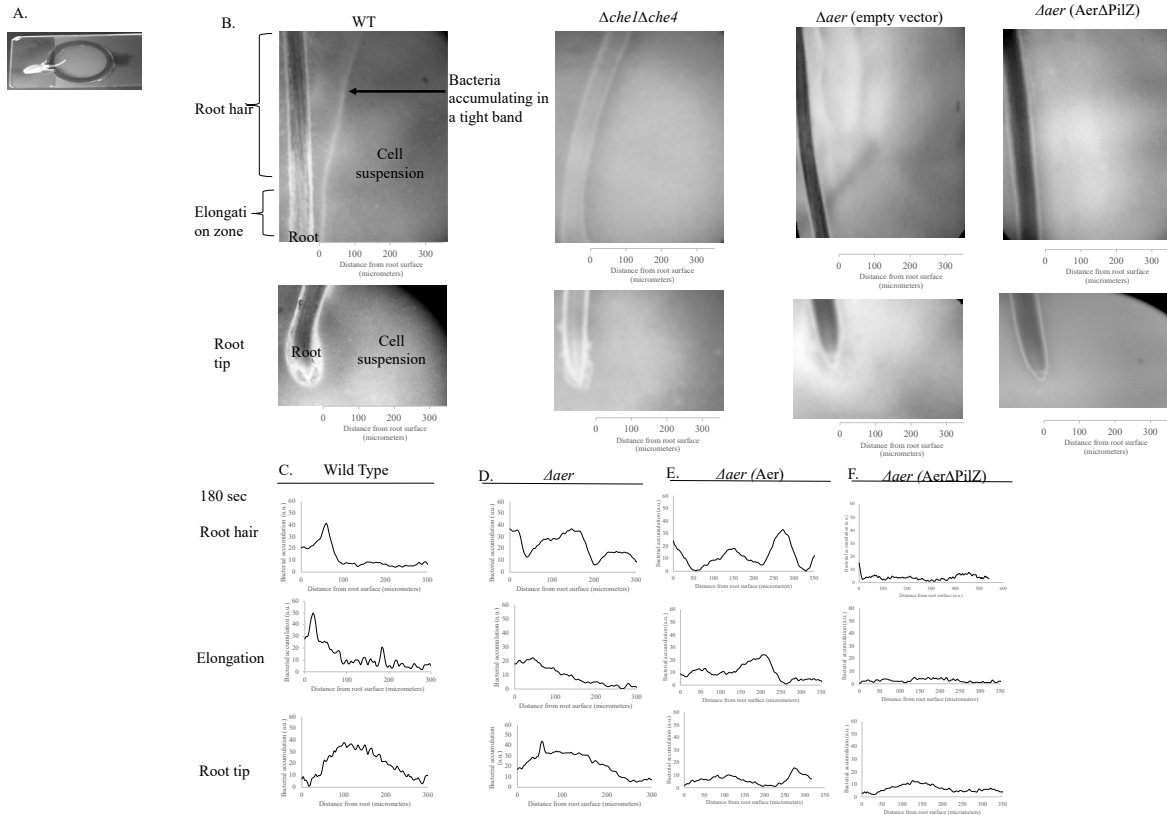


Figure 29: *A. brasilense* accumulation around wheat roots using a root in pool assay

The root in pool consists of a divet slide with an attached O-ring. The pool of bacteria is placed in the O-ring and the root is dropped in. The cell-root suspension is covered with a coverslip and 4x magnification on a phase contrast scope is used to film (A). This set up allows for monitoring of bacteria-root interactions at the root hair, elongation and tip zones (B). Bacterial cell accumulation (as image intensity in arbitrary units) away from root zones indicated in the different strains of *A. brasilense* (C-F). Shown are representative image intensity plots (bacterial accumulation) of 5 biological replicates of the strains. LO conducted this experiment.

elongation ($80 \pm 7 \mu\text{m}$) zones (Figure 29B), but they depleted a region of about $100 \mu\text{m}$ out from the root tip (Figure 29C). This response was stable for at least 10 minutes and was strikingly similar to aerotaxis band formation. To further establish that the response seen was a chemotaxis-dependent response, we performed the same assay with a motile, but non-chemotactic strain of *A. brasilense* (10). This strain was motile but failed to accumulate in any region around the roots indicating that the accumulation of motile cells as a band is a chemotactic response (Figure 29B). Cells lacking Aer did not accumulate in a tight band in either the root hair or elongation zones. Instead, local depletions of cells away from the root hair zone were followed by a broad accumulation at some distance from the root hairs, in a pattern that was distinct from that of the wild type cells (Figure 29C). In the elongation zone, a broad but weak accumulation of cells close to the root surface could be detected. These results suggest that Aer is required for the discrete accumulation of cells in bands in the vicinity of the root hair and root elongation zones. The Δaer strain was not repelled from the root tip and accumulated closer to the root tip (Figure 29C). The nonchemotactic strain showed no response in this region. Expressing full length Aer from a plasmid did not restore the accumulation and banding pattern seen for wild type cells in the root hair and elongation zones, suggesting that the chemotaxis response observed in the wild type integrates signaling from other chemotaxis receptors in addition to Aer. While this is not unexpected given the chemical complexity of root exudates (30), the behavioral differences likely account for the surprising difference in the ability of the strains to colonize the root surface when inoculated alone as seen above. Complementing

with full length Aer abolished the small accumulation of the cells at the root tips and thus partially rescued the phenotype, suggesting a greater contribution of Aer to this particular response. The $\Delta aer(Aer\Delta PilZ)$ strain was not able to accumulate as tight bands of motile cells at any location and showed no response in the vicinity of the root tips, despite being fully motile. Cells from this strain remained distributed around the roots without any specific accumulation pattern which was similar to that of a non-chemotactic mutant (Figure 29B). Lack of PilZ in Aer thus abolishes the ability of cells to sense chemical gradients originating from the roots, suggesting a critical role of PilZ from Aer in ensuring chemotaxis signaling function.

Discussion

The plant rhizosphere is a nutrient rich environment compared to the surrounding soil and it is characterized by a plethora of chemical gradients (31). Bacterial chemotaxis is functionally enriched in the genomes of soil and rhizosphere bacteria suggesting it provides a critical competitive advantage (6). With its 51 chemotaxis receptors and 4 chemotaxis signaling systems, *A. brasilense* is a model representative of the complex signaling diversity found in bacteria that colonize the rhizosphere of various plants. Of the 51 chemotaxis receptors encoded within the *A. brasilense* genome, Tlp1 and AerC have been previously characterized (13, 16). Interestingly, both modulate aerotaxis responses but only Tlp1, and not AerC, is critical for wheat root surface colonization. Here we show that *A. brasilense* Aer, a membrane anchored chemotaxis receptor that is likely to bind FAD, modulates aerotaxis, as expected from its similarity with *E. coli* Aer (24, 25). Evidence obtained here also suggest that *A. brasilense* Aer is critical to adjust the position of the cells in a gradient in response to elevated oxygen concentrations.

Given that *A. brasilense* Aer likely binds FAD as a cofactor, it likely functions as a redox sensor rather than an exclusive aerotaxis receptor, i.e. it monitors changes in redox that alter the flux of FAD/FADH₂ in the cell (26, 32). Further evidence for this broader role includes the complex, albeit unclear, role of Aer in sensing chemical gradients emanating from wheat roots in the root-in-pool assay. Chemicals exuded by roots are likely to affect metabolism and thus the intracellular redox status of the cells, which would be sensed by Aer and other energy taxis receptors.

Here we also show that PilZ domain contributes to Aer function and likely, to the integration of Aer signaling with other chemotaxis receptor signaling input. Chemotaxis signaling receptors are organized as large arrays of interacting trimers of chemotaxis receptor dimers (33, 34). This organization is required for signal integration, amplification and for chemotaxis signal sensitivity. This array organization is universally conserved in bacteria (6) and accounts for the summative operation of chemotaxis receptor information to produce an integrated response (35, 36). We hypothesize that it is this computation of signals from diverse receptors that explain some of the puzzling effects we have observed when deleting PilZ from Aer. We show that lack of Aer yields aerotaxis defects that are fully complemented by expression of the parental Aer *in trans*, suggesting the defect is due solely to lack of signaling by Aer. In the absence of Aer, other chemotaxis receptors, for example Tlp1 and AerC, integrate signaling information together with that of other chemotaxis receptors to produce a response that ultimately guide the cells in the oxygen gradient to form a band at a specific position. These aerotaxis defects are not rescued by expressing an Aer variant lacking the PilZ domain and in fact produce additional defects that depend on the environmental conditions: in presence of ammonium, but not under conditions of

nitrogen fixation, PilZ-mediated Aer function was critical since no band formed. These results suggest that (i) a different set of chemotaxis receptors contribute to aerotaxis signaling and response depending on the nitrogen nutrition status and, (ii) that PilZ from Aer is required for the ability of this receptor to integrate signaling with other chemotaxis receptors. *A. brasilense* AerC is a major aerotaxis receptor under conditions of nitrogen fixation but not under conditions when ammonium chloride as nitrogen source (16), an observation fully consistent with the defects seen here. The effect of Aer PilZ on integration of Aer signaling with other, unknown receptors, is most strikingly observed in response to the complex gradients exuded by wheat roots. Under these conditions, functional PilZ was essential for the ability of cells to detect chemical gradients and to display any chemotaxis response. This, in turn suggests that c-di-GMP metabolism and its effect on receptors such as Aer is likely a critical factor for chemotaxis and competitiveness in the rhizosphere in this bacterial species. *A. brasilense* encodes 34 diguanylate cyclases and phosphodiesterases that are predicted to sense a variety of cues to modulate c-di-GMP metabolism (37). Oxygen is a major regulator of c-di-GMP metabolism in *A. brasilense* (15), and findings here indicate that c-di-GMP signaling and integration with chemotaxis is likely critical for the ability of *A. brasilense* to detect chemical gradients in the rhizosphere. This hypothesis is further supported by the observation that Tlp1, which is also a PilZ-domain containing chemotaxis receptor plays a key role in root surface colonization (13).

References

1. Schweinitzer T, Josenhans C. 2010. Bacterial energy taxis: a global strategy? *Archives of microbiology* 192:507-520.
2. Wadhams GH, Armitage JP. 2004. Making sense of it all: bacterial chemotaxis. *Nature Reviews Molecular Cell Biology* 5:1024.
3. Adler J. 1975. Chemotaxis in bacteria. *Annu Rev Biochem* 44:341-56.
4. Briegel A, Ortega DR, Huang AN, Oikonomou CM, Gunsalus RP, Jensen GJ. 2015. Structural conservation of chemotaxis machinery across Archaea and Bacteria. *Environmental microbiology reports* 7:414-419.
5. Wuichet K, Zhulin IB. 2010. Origins and diversification of a complex signal transduction system in prokaryotes. *Science signaling* 3:ra50-ra50.
6. Buchan A, Crombie B, Alexandre GM. 2010. Temporal dynamics and genetic diversity of chemotactic-competent microbial populations in the rhizosphere. *Environ Microbiol* 12:3171-84.
7. Scharf BE, Hynes MF, Alexandre GM. 2016. Chemotaxis signaling systems in model beneficial plant-bacteria associations. *Plant Mol Biol* 90:549-59.
8. Levy A, Salas Gonzalez I, Mittelviefhaus M, Clingenpeel S, Herrera Paredes S, Miao J, Wang K, Devescovi G, Stillman K, Monteiro F, Rangel Alvarez B, Lundberg DS, Lu T-Y, Lebeis S, Jin Z, McDonald M, Klein AP, Feltcher ME, Rio TG, Grant SR, Doty SL, Ley RE, Zhao B, Venturi V, Pelletier DA, Vorholt JA, Tringe SG, Woyke T, Dangl JL. 2018. Genomic features of bacterial adaptation to plants. *Nature Genetics* 50:138-150.
9. Pereg L, de-Bashan LE, Bashan Y. 2016. Assessment of affinity and specificity of *Azospirillum* for plants. *Plant and Soil* 399:389-414.

10. Mukherjee T, Kumar D, Burriss N, Xie Z, Alexandre G. 2016. *Azospirillum brasilense* Chemotaxis Depends on Two Signaling Pathways Regulating Distinct Motility Parameters. *Journal of Bacteriology* 198:1764.
11. Ulrich LE, Zhulin IB. 2010. The MiST2 database: a comprehensive genomics resource on microbial signal transduction. *Nucleic Acids Res* 38:D401-7.
12. Bible AN, Khalsa-Moyers GK, Mukherjee T, Green CS, Mishra P, Purcell A, Aksenova A, Hurst GB, Alexandre G. 2015. Metabolic adaptations of *Azospirillum brasilense* to oxygen stress by cell-to-cell clumping and flocculation. *Applied and environmental Microbiology* 81:8346-8357.
13. Greer-Phillips SE, Stephens BB, Alexandre G. 2004. An energy taxis transducer promotes root colonization by *Azospirillum brasilense*. *Journal of bacteriology* 186:6595-6604.
14. O'Neal L, Ryu M-H, Gomelsky M, Alexandre G. 2017. Optogenetic manipulation of c-di-GMP levels reveals the role of c-di-GMP in regulating aerotaxis receptor activity in *Azospirillum brasilense*. *Journal of Bacteriology*.
15. Russell MH, Bible AN, Fang X, Gooding JR, Campagna SR, Gomelsky M, Alexandre G. 2013. Integration of the Second Messenger c-di-GMP into the Chemotactic Signaling Pathway. *mBio* 4.
16. Xie Z, Ulrich LE, Zhulin IB, Alexandre G. 2010. PAS domain containing chemoreceptor couples dynamic changes in metabolism with chemotaxis. *Proceedings of the National Academy of Sciences* 107:2235.
17. Barak R, Nur I, Okon Y, Henis Y. 1982. Aerotactic response of *Azospirillum brasilense*. *Journal of Bacteriology* 152:643.

18. Zhulin IB, Beshpalov VA, Johnson MS, Taylor BL. 1996. Oxygen taxis and proton motive force in *Azospirillum brasilense*. *Journal of Bacteriology* 178:5199-204.
19. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology* 7:263.
20. Römling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiology and molecular biology reviews* : MMBR 77:1-52.
21. Ryjenkov DA, Simm R, Romling U, Gomelsky M. 2006. The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* 281:30310-4.
22. Amikam D, Galperin MY. 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* 22:3-6.
23. Schirmer T, Jenal U. 2009. Structural and mechanistic determinants of c-di-GMP signalling. *Nat Rev Microbiol* 7:724-35.
24. Bibikov SI, Barnes LA, Gitin Y, Parkinson JS. 2000. Domain organization and flavin adenine dinucleotide-binding determinants in the aerotaxis signal transducer Aer of *Escherichia coli*. *Proc Natl Acad Sci U S A* 97:5830-5.
25. Watts KJ, Johnson MS, Taylor BL. 2005. Minimal requirements for oxygen sensing by the aerotaxis receptor Aer. *Molecular Microbiology* 59:1317-1326.
26. Repik A, Rebbapragada A, Johnson MS, Haznedar JO, Zhulin IB, Taylor BL. 2000. PAS domain residues involved in signal transduction by the Aer redox sensor of *Escherichia coli*. *Mol Microbiol* 36:806-16.

27. Bibikov SI, Biran R, Rudd KE, Parkinson JS. 1997. A signal transducer for aerotaxis in *Escherichia coli*. *J Bacteriol* 179:4075-9.
28. O'Neal L, Mukherjee T, Alexandre G. 2018. Analyzing Chemotaxis and Related Behaviors of *Azospirillum brasilense*. *Current Protocols in Microbiology* 48:3E.3.1-3E.3.11.
29. Wong LS, Johnson MS, Zhulin IB, Taylor BL. 1995. Role of methylation in aerotaxis in *Bacillus subtilis*. *Journal of Bacteriology* 177:3985.
30. Walker TS, Bais HP, Grotewold E, Vivanco JM. 2003. Root Exudation and Rhizosphere Biology. *Plant Physiology* 132:44.
31. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* 57:233-66.
32. Watts KJ, Ma Q, Johnson MS, Taylor BL. 2004. Interactions between the PAS and HAMP domains of the *Escherichia coli* aerotaxis receptor Aer. *J Bacteriol* 186:7440-9.
33. Briegel A, Ladinsky MS, Oikonomou C, Jones CW, Harris MJ, Fowler DJ, Chang Y-W, Thompson LK, Armitage JP, Jensen GJ. 2014. Structure of bacterial cytoplasmic chemoreceptor arrays and implications for chemotactic signaling. *eLife* 3:e02151-e02151.
34. Frank V, Piñas GE, Cohen H, Parkinson JS, Vaknin A. 2016. Networked Chemoreceptors Benefit Bacterial Chemotaxis Performance. *mBio* 7.
35. Sourjik V, Berg HC. 2002. Receptor sensitivity in bacterial chemotaxis. *Proc Natl Acad Sci U S A* 99:123-7.
36. Sourjik V, Berg HC. 2004. Functional interactions between receptors in bacterial chemotaxis. *Nature* 428:437-41.

37. Ramírez A, Millán Pacheco C, F. Cruz Pérez J, Minjárez Sáenz M, Baca B. 2018. In silico comparative analysis of GGDEF and EAL domain signaling proteins from the *Azospirillum* genomes, vol 18.
38. Papadopoulos JS, Agarwala R. 2007. COBALT: constraint-based alignment tool for multiple protein sequences. *Bioinformatics* 23:1073-1079.
39. Gullett J, O'Neal L, Mukherjee T, Alexandre G. 2017. *Azospirillum brasilense*: Laboratory Maintenance and Genetic Manipulation. *Curr Protoc Microbiol* 47:3e.2.1-3e.2.17.
40. Gosta F. 1957. The Infection of Clover Root Hairs by Nodule Bacteria Studied by a Simple Glass Slide Technique. *Microbiology* 16.
41. Tarrand JJ, Krieg NR, Dobereiner J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol* 24:967-80.
42. Schmitz RA. 1997. NifL of *Klebsiella pneumoniae* carries an N-terminally bound FAD cofactor, which is not directly required for the inhibitory function of NifL. *FEMS Microbiol Lett* 157:313-8.
43. Ukaegbu UE, Rosenzweig AC. 2009. Structure of the redox sensor domain of *Methylococcus capsulatus* (Bath) MmoS. *Biochemistry* 48:2207-2215.

CHAPTER IV
MONITORING PLANT-MICROBE INTERACTIONS IN REAL TIME
REVEALS ROLE OF *AZOSPIRILLUM BRASILENSE* CHEMOTAXIS IN
THE RHIZOSPHERE

Text and figures taken from the following:

O'Neal, L., Akhter S., and Alexandre, G. Monitoring plant-microbe interactions in real time reveals role of *Azospirillum brasilense* chemotaxis in the rhizosphere. Manuscript in preparation.

Author contributions to this work included: LO and GA designed research; LO and SA performed the research and analyzed the data; LO and GA wrote the paper.

Abstract

Plant roots shape the rhizosphere community by secreting compounds that recruit diverse bacteria. Colonization of various plant roots by the motile alphaproteobacterium *Azospirillum brasilense* causes increased plant growth, increased root volume, and increased crop yield. Bacterial chemotaxis in this and other motile soil bacteria is critical for competitive colonization of the root surfaces. The role of chemotaxis in root surface colonization has previously been established by end-point analyses of bacterial colonization levels detected a few hours to days after inoculation. More recently, microfluidic devices have been used to study plant-microbe interactions, but these devices are size-limited. Here we present a slide-in chamber that allows real-time monitoring of plant-microbe interactions that are not limited by seedling size. We use *A. brasilense* and *Triticum aestivum* (wheat) to investigate plant-microbe interactions. We show that chemotaxis is essential for *A. brasilense* colonizing wheat roots. Furthermore, we use this chamber to illustrate the role of Tlp1, a previously characterized energy taxis receptor, in responding to attractants and repellants exuded by wheat roots. Tlp1 is essential in sensing both malate and hydrogen peroxide, which are produced by wheat roots. Together these results suggest that *A. brasilense* chemotaxis dictates initial root surface colonization patterns. We also demonstrate that chemotaxis provides an advantage in other plant-microbe associations.

Introduction

Crop production is dependent upon plant-microbe interactions in the rhizosphere. Beneficial microbes increase plant yield by producing phytohormones and fixing nitrogen (1, 2). Motility and chemotaxis, the directed swimming in chemical gradients, are vital for beneficial bacteria to find desirable niches, especially in the soil where bacterial chemotaxis function is enriched (3, 4). Chemotaxis is initiated when chemicals in the environment bind to chemotaxis receptors. A repellent-bound chemotaxis receptor changes the phosphorylation state of the histidine kinase, CheA. CheA-P interacts with and phosphorylates CheY. CheY-P diffuses through the cytoplasm, interacts with the flagellar motor, and causes a change in the rotation of the flagellar motor, which reorients the cell in a new swimming direction through a tumble or a reversal (5, 6).

The soil is a complex environment and dynamic chemical milieu (3, 4). Soil bacterial species, such as *Bacillus amyloliquefaciens* and *Sinorhizobium meliloti*, exhibit chemotactic responses to plant root exudates (7, 8), and previous work in *Bacillus subtilis* has shown that chemoreceptors are necessary for initial colonization of *Arabidopsis* roots (9). Another soil-dwelling plant growth promoting bacterium, *Azospirillum brasilense* must colonize the roots of plants to exhibit its plant growth promoting effects (10). *A. brasilense* can colonize a variety of plants and is sold as a commercial inoculant (11). The genome of *A. brasilense* encodes 4 chemotaxis signal transduction systems, with two of these modulating flagellar motility and controlling chemotaxis and 51 chemotaxis receptors (12, 13). Chemotaxis plays a vital role in sensing chemical gradients in the soil, but swimming and chemotaxis in the rhizosphere is largely unstudied.

The role of motility and chemotaxis as well as other cellular functions in root surface colonization by *A. brasilense* and related species have been well-studied using mutant strains, end point assays and microscopy observations (11, 14, 15). With respect to motility, these experiments have established a role for the polar flagellum and flagellar motility (16, 17), some chemotaxis receptors (18, 19), and chemotaxis signaling (12) in root surface colonization. Similar to what is known in other motile plant-associated bacteria, the emerging picture of root surface colonization events by *A. brasilense* is relatively static due to the techniques used. To address this shortcoming, we developed a novel slide-in chamber that allows the plant and bacteria to be monitored in real time for up to 1 week, without disrupting the interaction. This chamber allows monitoring of free-swimming behavior, colonization along the roots, and temporal changes in plant-microbe associations. Using this tool here, we track *A. brasilense* initial wheat root sensing (< 3 minutes) and accumulation in root zones that depend on chemotaxis signaling. We found chemotaxis attractant and repellent responses in discrete regions of the roots that underlie long term colonization patterns and further identify a subclass of chemotaxis receptors that mediate these responses.

Materials and Methods

Bacterial Growth and Maintenance

All strains used throughout this study are detailed in Table 9. All *A. brasilense* strains were maintained on 1.5% agar minimal media for *A. brasilense* (MMAB) containing 10mM malate as a carbon source with appropriate antibiotics: ampicillin (200 µg/mL), kanamycin (30 µg/mL), and/or tetracycline (10 µg/mL). Environmental isolates were maintained on 1.5% agar LB.

Table 9: Strains and plasmids used in this study

Strains/ Plasmids	Relevant genotype/ phenotype	Citation
Plasmids		
pHR GFP	Tc ^R , constitutive GFP expression	(21)
pRK415	broad-host-range vector; Tc ^r	(22)
pRKTlp1	pRK415 containing a 2.7kb DNA fragment encompassing promoter and tlp1 ORF; Tc ^R	(23)
pRKTlp1 ^{R562A R563A}	pRK415 containing a 2.7kb DNA fragment encompassing promoter and tlp1 ORF with a site directed mutations at residues 562 and 563; Tc ^R	(23)
pIND4	Inducible expression plasmid, Km ^R	(24)
pRED-DGC	pIND4 expressing a red-light activated diguanylate cyclase (bphS-bphO); Km ^R	(25)
pBLUE-PDE	pIND4 expressing a red-light activated diguanylate cyclase and a blue-light activated phosphodiesterase (bphS-bphO-eb1); Km ^R	(25)
Bacterial Strains		
<i>E. coli</i>		
S17.1	General cloning strain	Invitrogen
S17.1 (pHRGFP)	General cloning strain carrying pHRGFP	This study
<i>A. brasilense</i>		
WT	Wild type strain (Sp7); Amp ^R	ATCC29145

Table 9, continued

Strains/ Plasmids	Relevant genotype/ phenotype	Citation
<i>Δtlp1</i>	<i>Δtlp1::Km</i> derivative of Sp7; Amp ^R Km ^R	(18)
<i>Δaer</i>	WT with Aer receptor deleted using markerless deletion; Amp ^R	(26)
<i>Δche1Δche4</i>	<i>Δ(cheA1-cheR1)Δ(cheA4-cheR4)::Cm</i> (Cm ^R), Gm (Gm ^R)	(27)
WT (pHR GFP)	Wild type strain (Sp7) carrying pHR GFP; Amp ^R Tc ^R	This work
WT (pIND4)	Wild type strain carrying pIND4; Amp ^R Km ^R	(25)
WT (pRED-DGC)	Wild type strain carrying pRed-DGC; Amp ^R Km ^R	(25)
WT (pBLUE-PDE)	Wild type strain carrying pBLUE-PDE; Amp ^R Km ^R	(25)
<i>Δtlp1</i> (pHR GFP)	<i>Δtlp1</i> carrying pHR GFP; Amp ^R Km ^R Tc ^R	This work
<i>Δtlp1</i> (pRK415)	<i>Δtlp1</i> carrying the broad host range vector pRK415; Amp ^R Km ^R Tc ^R	(23)
<i>Δtlp1</i> (pRK Tlp1)	<i>Δtlp1</i> carrying pRKTlp1; Amp ^R Km ^R Tc ^R	(23)
<i>Δtlp1</i> (pRK Tlp1 ^{R562A R563A})	<i>Δtlp1</i> carrying pRKTlp1 ^{R562A R563A} ; Amp ^R Km ^R Tc ^R	(23)
<i>Δaer</i> (pRKAer)	<i>Δaer</i> with Aer expressed from pRK415, Amp ^R Tet ^R	(26)

Table 9, continued

Strains/ Plasmids	Relevant genotype/ phenotype	Citation
Δaer (pRKAer Δ PilZ)	Δaer with truncated Aer expressed from pRK415, Amp ^R Tet ^R	(26)
$\Delta che1 \Delta che4$ (pHR GFP)	carrying pHR GFP; Amp ^R Tc ^R	This work
<i>Environmental Isolates</i>		
106-RC-6W	<i>Ochrobactrum</i> sp.	This work
104-OG-2	<i>Stenotrophomonas</i> sp.	This work
106-RC-2	<i>Stenotrophomonas</i> sp.	This work

Generation of GFP expressing A. brasilense

pHR-GFP was transferred into Sp7 (WT), $\Delta tlp1$, and $\Delta che1\Delta che4$ using biparental conjugation as previously described in (20). Transconjugants were selected for on MMAB containing tetracycline and confirmed using fluorescent microscopy.

Germination of T. aestivum and M. sativa

T. aestivum (wheat) and *M. sativa* (alfalfa) were utilized throughout this study. Wheat and alfalfa were sterilized with successive washes of bleach, 70% ethanol containing 1% Triton X-100, and sterile water. After sterilization, seeds were planted into 0.3% agar and placed in the dark for 48 hours to germinate. Then, plates were placed in the light and allowed to grow for 24 hours. All assays were performed on germinated seedlings 3-5 days old.

Root-in-pool assay

Cells were grown to O.D.600 0.4 in MMAB containing malate as a carbon source (*A. brasilense*) or LB liquid (environmental isolates). Five ml of cells were washed 3 times with Che buffer (1.7 g L⁻¹ dipotassium phosphate, 1.36 g L⁻¹ monopotassium phosphate; 0.1 mM EDTA) and re-suspended in Che buffer. A black washer with a slit cut in it was secured to a depression slide using vacuum grease. The germinated root was placed in the slit and 400 μ L of washed bacteria was used to fill the chamber created by the dip and washer, and then a cover slip was used to cover the pool chamber (previously described in (26)).

All root-in-pool assays were recorded using the 4x objective of a Nikon E200 phase contrast microscope equipped with Nikon Coolpix digital camera. Assays were filmed for 3 minutes.

FIJI was used for all image and video analysis. All root-in-pool assay videos were converted to 8-bit and background subtraction was performed. Bacterial accumulation was measured by measuring pixel intensity out from the root over the course of the video. All intensity measurements were standardized by dividing by the intensity of non-responding cells (cells at least 200 μm away from the root at 0 s).

Slide-in-chamber dimensions and assembly

The microscope slide-in chamber (85 mm x 35 mm x 8 mm) was designed in Autodesk Inventor and printed from ABS thermoplastic filament with a Fortus 250MC 3D printer (<https://www.kerafast.com/product/2029/slide-in-plant-chamber>). The print layer height was set to 0.007 inches (28). Microscope slides and coverslips were attached to the slide-in chamber using E600 industrial strength adhesive. A string wick was threaded through the 0.4 cm hole in the bottom of the chamber to hydrate the chamber over the course of a week.

Cells were grown to late log phase in MMAB containing malate, washed with Che buffer, and suspended in Che buffer and mixed in a 1:1 ratio (v/v) with molten Fahraeus semi-solid media (29)(CaCl_2 , 100 mg l^{-1} , $\text{MgSO}_4 \bullet 7\text{H}_2\text{O}$, 120 mg l^{-1} , KH_2PO_4 , 100 mg l^{-1} , Na_2HPO_4 , 150 mg l^{-1} and ferric citrate 5 mg l^{-1} 0.3% agar). Molten Fahraeus was used to plug the bottom of the chamber and then the bacteria-Fahraeus mixture was used to fill the interior of the chamber. Germinated seedlings were planted at the top opening with roots directed downward. Chambers were stored in humidified vessels and imaged for up to 1-week post inoculation.

Fluorescent Microscopy and image analysis

All chambers were imaged using confocal microscopy. Images were analyzed using FIJI. All distance measurements were obtained using the measure tool. Short-term colonization (0 hpi to 72 hpi) measurements were obtained by measuring fluorescence of root-attached cells and normalizing to root area. Calculated total fluorescence was measured by splitting the channels and using the Time Series analyzer plugin to measure intensity on each channel. The root surface was identified through the z-plane series, and an area of interest was selected. The area, mean gray value, and integrated density were measured on both the red and green channels in the area of interest. Calculated Total Corrected Fluorescence (CTCF) for each channel was calculated using: Integrated Density—(Area of selection x Mean Fluorescence of Background).

Long-term colonization

Long term colonization (5-day) was determined using a previously described assay (12). Cells were grown to OD₆₀₀= 0.5, and 2mL of the culture was washed with Che buffer and resuspended in 400 µL of Che buffer. A 6.5 cm diameter growth container was filled with semi-solid Fahraeus and four plants (germinated, as previously described) were placed in the media. Twenty microliters of the washed and concentrated bacteria were inoculated into the middle of the chamber. Growth chambers were incubated at 25°C for 5 days. After 5 days, roots from all 4 plants were combined, massed, homogenized in 400 µL Che buffer and CFU counts were performed.

For competition assays, WT and $\Delta che1\Delta che4$ were grown to O.D.₆₀₀=0.4-0.6 and serial dilutions were performed. WT and $\Delta che1\Delta che4$ were normalized to O.D.₆₀₀=0.5 and mixed in a 1:1 (O.D.₆₀₀:O.D.₆₀₀) ratio and inoculated into the center of the growth chamber as previously described. After 5 days, roots were homogenized and CFU counts were performed in parallel on

TY medium ampicillin (all *A. brasilense*) or ampicillin and kanamycin ($\Delta che1\Delta che4$). The amount of WT was determined by $CFU_{total} - CFU_{\Delta che1\Delta che4}$.

Root exudate collection and analysis

Seeds were sterilized and germinated as previously described. Plantlets were separated into 12-well plates with 2ml sterile water per well (2 plantlets/ well). Exudates were collected for 24 hours. After collection, exudates were filter-sterilized and lyophilized and the plant roots were weighed. For consumption data, after 24 hours of exudate collection, 500 μ l of WT was placed in the wells with plantlets and water and allowed to incubate for 30 minutes. After 30 minutes, exudates were filter-sterilized and lyophilized. The lyophilized exudates were resuspended in 1 ml of water and separated by Hydro RP HPLC. Samples were ionized by electrospray in negative mode on an Orbitrap Mass spectrometer. Data was processed and peaks picked via Maven software. Metabolite area counts were normalized to dry sample weight.

Chemical-in-plug assay

To generate chemical-in-plugs, malate (10mM), pyruvate (10mM), succinate (10mM), or root exudates were mixed with molten 1% low melting point agarose. Ten microliter plugs were allowed to solidify on a divet slide, and 100 μ l of bacteria was placed on the divet slide and covered with a coverslip, once the plug had solidified. Bacterial behavior was monitored using the 4x objective of a Nikon E200 phase-contrast microscope for 10 minutes. Behavior was videoed using a C-mounted Nikon Coolpix digital camera. Frames from each video were obtained using FIJI.

ROS exposure

Twenty-five ml of cells were grown to O.D.₆₀₀=0.5 in TY liquid with appropriate antibiotics. Cells were mixed with 25 ml of TY semi-solid (0.3% agar). Plates were allowed to solidify overnight, and cells were exposed to filter paper soaked in 20 µl hydrogen peroxide, cumene peroxide, or Che buffer. Cells were also exposed to various concentrations of hydrogen peroxide (3%, 0.3% or 0.03%) by mixing hydrogen peroxide with Che buffer.

For cells carrying plasmids encoding light activated enzymes, cultures were grown and maintained in the dark, and plates were exposed to red or blue light immediately before exposure filter paper. Response was observed every 5 minutes for 2 hours.

Hydrogen peroxide zone of inhibition

WT and *Δtlp1* were grown in liquid culture until O.D.₆₀₀ 0.8. Five hundred microliters of cells were spread on TY solid with ampicillin. Once the plates dried, sterile filter paper discs were soaked in buffer or hydrogen peroxide (3%, 0.3%, or 0.03%) and placed on the plates. After 48 hours of growth, zones of growth inhibition were measured.

Results

Slide-in chamber allows real time monitoring of plant-microbe interactions

To observe real-time plant-microbe interactions we developed a novel slide-in chamber which allows for plants and bacteria to be monitored in real time, undisturbed, for up to 1 week using confocal microscopy (Figure 30) (28). The chamber consists of 85 mm x 35 mm x 8 mm rectangle with a 20 mm x 62 mm rectangular opening. Sterile seedlings can be planted in the 21 mm x 5 mm opening at the top of the chamber. A slide is secured on the back of the device

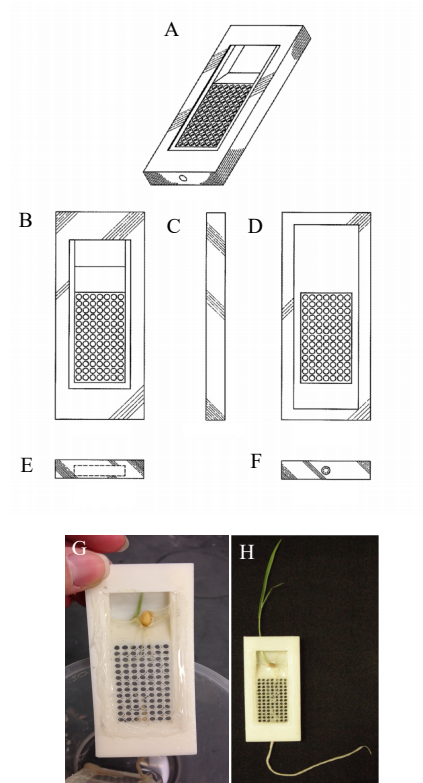


Figure 30: The slide-in chamber for observing plant-microbe interactions in real time.

The chamber consists of a grid, wick, coverslip, and slide. A-F depict the front (A-B), side (C), back (D), top (E), and bottom (F) of the chamber. The inside is filled with a semi-solid medium and inoculated with microbes and germinated seedlings are planted at the top. Here we show a germinated wheat seedling (G) planted in the semi-solid medium. The chamber allows for the seedling to grow and be monitored for up to a week (H).

(Figure 30D) and a coverslip on the front (Figure 30B) to create an internal cavity with a volume of ~3 ml. The internal grid allows for monitoring of specific areas over time. We filled the chamber with a semi-solid (0.4% Gelzan) Fahraeus medium that supports both plant growth and bacterial motility while remaining transparent for microscope observations. The microscope slide in-chamber can be stored in a humidified vessel and plant-microbe interactions can be observed repeatedly over several days. Here, we utilized confocal microscopy to monitor GFP-expressing *A. brasilense* behavior in real time in the vicinity of the roots over several days.

Chemotaxis controls A. brasilense colonization of wheat

Previous work has shown the *A. brasilense* can colonize wheat (18), so we sought to monitor free swimming behavior in physiologically distinct root zones of wheat: the maturation (root hair), elongation, and the root tip zones. We observed that wild type *A. brasilense* collects around wheat roots within 5 hours post inoculation (hpi) with cells numbers increasing over time (Figure 31A). At 24hpi, WT accumulates in tight bands within 150 μm of the root surface in the root hair and elongation zones (Figure 31A, brackets). Within these bands, cells swim in long runs, with less frequent changes in direction, but on the edges or outside of the bands, cells frequently reverse and swim in shorter runs. WT accumulation at the root tip zone is significantly less than other areas of the root at all observed time points. The accumulation of motile cells in specific zones suggested a role for chemotaxis. We tested this hypothesis using a chemotaxis null strain ($\Delta che1\Delta che4$). Cells of the $\Delta che1\Delta che4$ strain are still motile but are unable to sense changes in the environment and cannot respond to those changes by modulating swimming speed and reversal frequency (12, 13). We found this strain did not display any accumulation in any of the three root zones and there was no band of highly motile cells and does not actively avoid the root tip

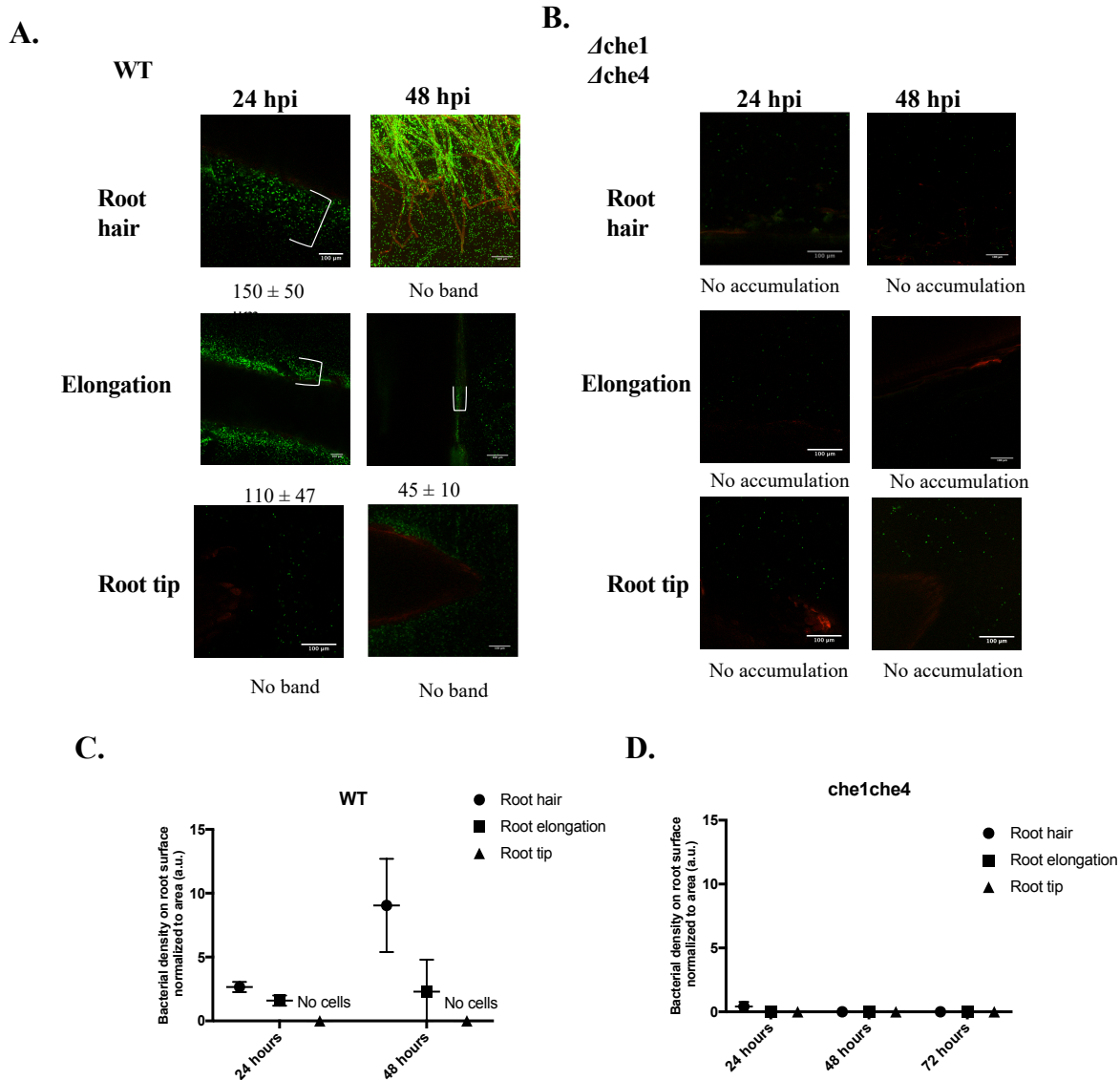


Figure 31: Accumulation and colonization of WT and nonchemotactic *A. brasilense* around wheat roots in the slide-in chamber.

WT and chemotaxis null ($\Delta che1 \Delta che4$) bacteria expressing a constitutively active GFP were inoculated into the slide-in chamber and free swimming behavior was observed over time. Bacteria are visible in green, while the root is red (autofluorescence). (A,B) WT and $\Delta che1 \Delta che4$ accumulation close to the wheat root in the root hair and elongation zones. Bands of motile fluorescent bacteria are denoted by brackets and the average bandwidth in micrometers is noted below the picture. (C, D) Colonization levels on each root zone were determined by measuring fluorescence of surface attached cells normalized to the area of the root observed. LO conducted this experiment.

(Figure 31B). In fact cell distribution was random regardless of the areas of the roots considered. This finding indicate that a non-chemotactic mutant cannot preferentially accumulate in any region around the wheat roots, suggesting chemotaxis mediates accumulation of bacteria in the vicinity of wheat roots.

At 48 hpi, the majority of WT cells are attached to the root surface in the root hair and elongation zones, and fewer free-swimming cells are observed, suggesting accumulated cells transitioned to root surface attachment. In contrast, there was no measurable colonization at the root tip (Figure 31C). This indicates that the accumulation of the WT in certain regions of the roots depends on chemotaxis toward these regions and it precedes colonization of the root hair and elongation zones. Non-chemotactic cells do not strongly colonize any zone, suggesting that *A. brasilense* uses chemotaxis to identify root regions suitable for subsequent colonization.

Next we used a modified spatial assay which we called, the root-in-pool assay to monitor bacterial chemotaxis responses upon initial root sensing that occur within 15 minutes of exposure to roots, as a complementary approach to the slide-in-chamber, allowing us to bridge initial root sensing and spatial accumulation and colonization captured by the slide-in-chamber assay. Motile wild type bacteria accumulate in reproducible patterns within seconds of exposing them to wheat roots in this assay. In the root hair zone, WT cells gather in a tight band at some distance from the root hairs, and within a short time (~90 seconds) this band of motile cells moves closer to the root surface (Figure 32A). Conversely at the root tip, the WT bacteria form a band at about $100 \pm 50\mu\text{m}$ away from the tip, and it remains static over at least 15 minutes observation (i.e. it does not move closer to the root surfaces). This band at the root tip is not only stable over time but also attracts an increasing number of bacteria (Figure 32B). The $\Delta che1 \Delta che4$ strain does not exhibit

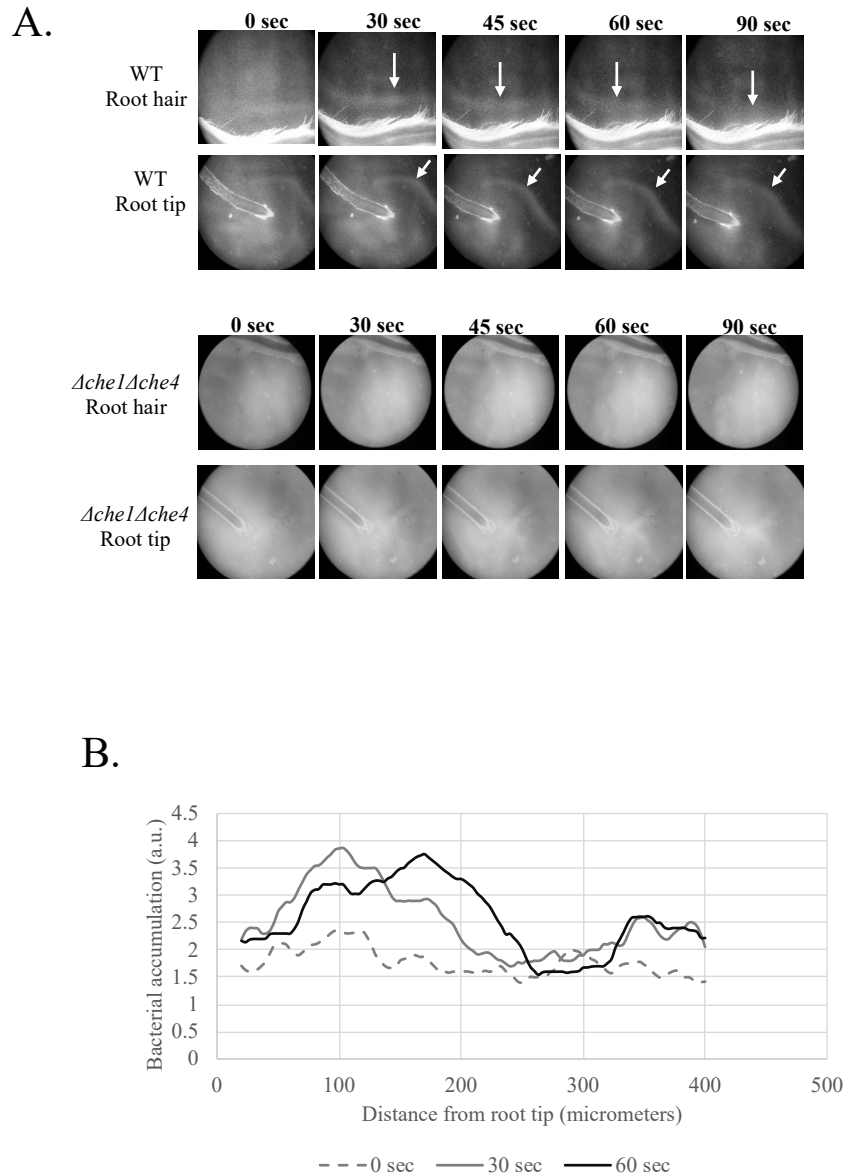


Figure 32: WT and chemotaxis null strains in the root-in-pool assay with intact roots of wheat root seedlings.

Wheat roots from intact seedlings were placed in a suspension of motile WT or chemotaxis null bacteria ($\Delta che1\Delta che4$) and free-swimming behavior in the vicinity of the roots was observed and recorded over time. A) Intensity profile analysis of images shown for WT top panel. WT *A. brasilense* accumulates in a band in the root hair and tip zones (indicated by the white arrow), while the chemotaxis null strain forms a homogenous pool that does not change within 10 minutes of observation. B) WT accumulation over time at the root tip. LO conducted this experiment.

this banding pattern and no specific accumulation is seen over time. The chemotaxis-dependent accumulation in all 3 zones hints at the presence of attractants and repellants. We hypothesized that the root hair and elongation zones exude attractants, which is why *A. brasilense* forms bands that move closer to the root over time and that the root tip exudes repellants which would be responsible for the bacteria accumulating away from the root.

A chemoreceptor, Tlp1, drives wheat root sensing

Since WT cells exhibited an attractant response in the root hair and elongation zones and a repellant response at the tip, we sought to understand which receptors could be driving this behavior. Previous work in this lab has shown that a chemoreceptor, Tlp1, is essential for wheat root colonization and plays a role in organic acid sensing (18). To further characterize the role of Tlp1 in wheat root surface sensing and colonization, we used $\Delta tlp1$ mutants constitutively expressing GFP in our slide-in chamber. In the slide-in chamber, $\Delta tlp1$ was absent in the wheat root hair and elongation zone, but significantly accumulated at the root tip (compare Figure 33A). Eventually $\Delta tlp1$ colonizes the wheat root hair, elongation, and unexpectedly, the root tip (Figure 33C). Within 48hpi, $\Delta tlp1$ exhibits the highest colonization levels at the root tip. In the root-in-pool $\Delta tlp1$ mimics its slide-in chamber behavior, cells form a band close to the root tip, and do not accumulate in the root hair zone (Figure 33B). Thus, the behavior of the *A. brasilense* in all wheat roots regions is dependent on chemotaxis and Tlp1.

$\Delta tlp1$ does not preferentially accumulate or colonize in the root hair and elongation zones in the root in pool and slide-in chamber assays, hinting that Tlp1 senses attractants exuded from wheat. Previous work has shown that Tlp1 is important in responding to organic acids in a soft agar assay (18). We tested the role of Tlp1 in sensing organic acids using a chemical-in-plug assay

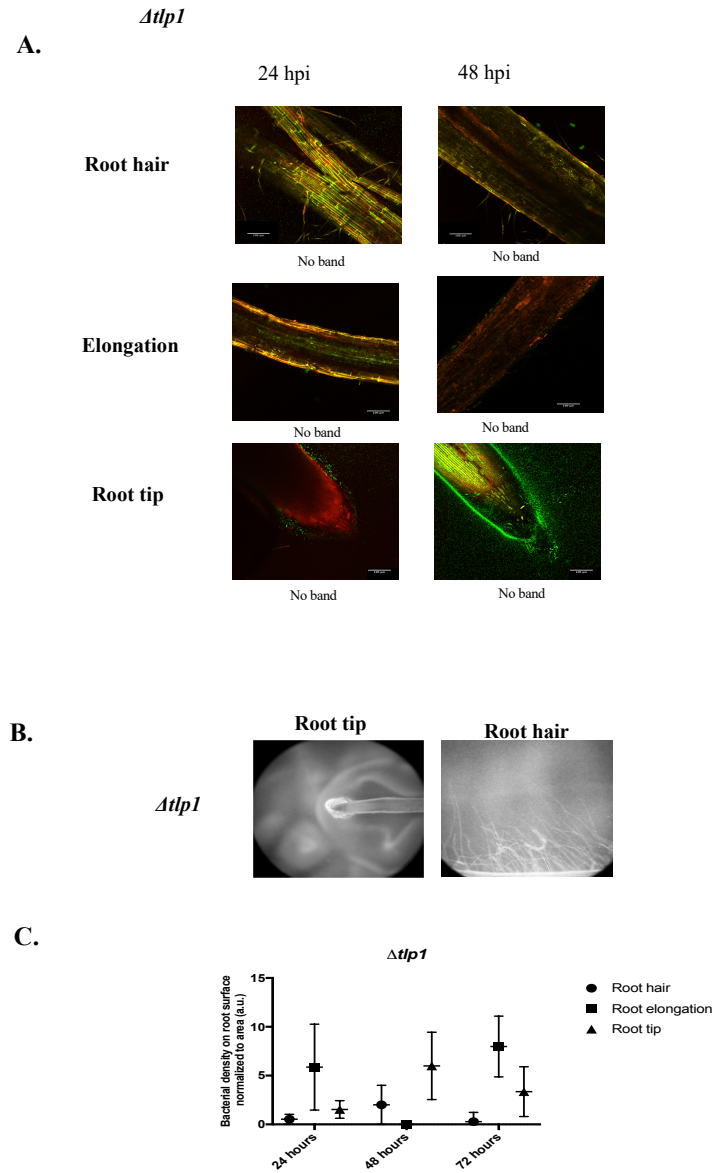


Figure 33: *Atlp1* free swimming response and colonization of wheat roots.

Atlp1 in the slide in chamber with wheat seedlings (A). A root-in-pool was used to observed behavior of cells lacking Tlp1 or expressing Tlp1 impaired in binding to c-di-GMP in the presence of wheat seedlings (B). Time course colonization of wheat roots by *Atlp1* (C). *A. brasilense* constitutively expressing GFP colonization was inoculated into a slide-in-chamber containing 3-day old wheat and imaged over the course of 72 hours post inoculation (hpi). *A. brasilense* is visible in green and the wheat root is in red (autofluorescence). Images are representative of 3 biological replicates in different chambers. Colonization levels were quantified by calculating CTCF (Calculated Total Corrected fluorescence) of the bacteria and normalizing to root area. LO conducted this experiment.

(Figure 34). WT cells exhibited attractant responses to malate, pyruvate, and succinate when placed in a plug, and this response was dependent upon chemotaxis (Figure 34). Cells lacking Tlp1 displayed altered responses to pyruvate and succinate but were completely null in sensing malate. To determine if organic acids, and specifically malate, are present in wheat exudates, we performed metabolomics on exudates collected from wheat roots in the presence or absence of *A. brasilense*. We were able to identify 121 metabolites (Table 10), and the majority of these were organic acids (Figure 35). Malate is abundant in collected wheat exudates (Figure 35) and is rapidly depleted when *A. brasilense* is incubated with wheat roots. Thus, Tlp1 plays a role in responding to metabolizable attractants exuded by wheat roots. Cells lacking Tlp1 are no longer repelled from the wheat root tip, but accumulate closer and colonize this zone preferentially, indicating that loss of Tlp1 results in a loss of repellent sensing at the root tip. No obvious repellents were identified in our metabolomics (Table 10), but previous work has shown that wheat roots exude reactive oxygen species (ROS), presumably hydrogen peroxide, and this is concentrated at the root tip (30). Since Tlp1 is vital in responding to wheat root tips and it has been implicated in responding to redox (18), we hypothesized that Tlp1 is essential in guiding *A. brasilense* away from the ROS exuded from the tip. We exposed motile cells to sterilized discs soaked in hydrogen peroxide, cumene peroxide, or buffer. WT cells move away from hydrogen peroxide discs within 20 minutes post exposure, and this response depends on functional chemotaxis (Figure 36A). On the other hand, $\Delta tlp1$ cells are blind to hydrogen peroxide and do not respond. This response can be complemented by expressing Tlp1 *in trans*. Migration away from ROS is specific to hydrogen peroxide, as all strains tested are null for response to cumene peroxide. Furthermore, cells lacking

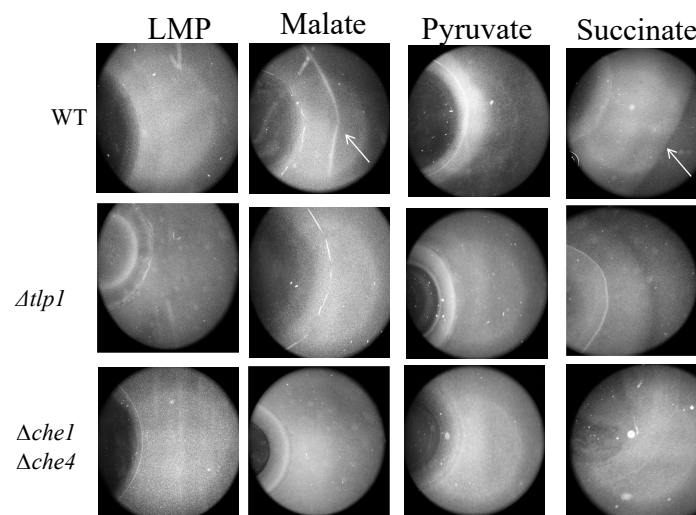


Figure 34: *A. brasilense* response to organic acids.

Motile *A. brasilense* cells were exposed to low melting point agarose plugs (LMP) containing 10mM malate, pyruvate, or succinate. LO conducted this experiment.

Table 10: Chemicals identified in wheat exudates.

Metabolites		
Dimethylglycine	Methionine	Indoleacrylate
hydroxybutyrate	xylose	N-Acetylglutamine
Serine	3-Methylphenylacetic acid	Acetyllysine
Glycerate	Guanine	N-Acetylglutamate
Cytosine	Xanthine	homocitrulline
Uracil	Hydroxyphenylacetate	Citrate/isocitrate
Creatinine	Xylitol	2-Dehydro-D-gluconate
Proline	Dopamine	D-Gluconate
Fumarate	Histidine	Tryptophan
2-Oxoisovalerate	Orotate	Pikatriptan
Valine/betaine	Allantoin	D-Glucuronate
Succinate/Methylmalonate	pimelic acid	Pantothenate
3-Hydroxyisovalerate	Indole-3-carboxylate	N-Acetylglucosamine
Homoserine/Threonine	2-Aminoadipate	deoxycytidine
3-Methylthiopropionate	Phenylpyruvate	Deoxyuridine
Nicotinate	Phenylalanine	Ribose phosphate
Taurine	Phenyllactic acid	homocarnosine
Thymine	Phosphoenolpyruvate	Thymidine
Pyroglutamic acid	Uric acid	Cytidine
Citraconate	3,4-Dihydroxyphenylacetate (DOPAC)	Uridine
N-Acetyl-beta-alanine	Pyridoxamine	6-Phospho-D-gluconolactone
Hydroxyproline	Pyridoxine	Glucosamine phosphate
Leucine/Isoleucine	Sulfolactate	Glucose phosphate
aminocaproic acid	sn-Glycerol 3-phosphate	S-Ribosyl-L-homocysteine
methyl succinic acid	Aconitate	Adenosine
Asparagine	N-Acetylmethionine	6-Phospho-D-gluconate
Hydroxyisocaproic acid	Arginine	1-Methyladenosine
Ornithine	tricarballic acid	Guanosine
Aspartate	Ascorbate	Xanthosine
Malate	N-Carbamoyl-L-aspartate	N-Acetylglucosamine
Adenine	Allantoate	1/6-phosphate
Hypoxanthine	2-Isopropylmalate	Octulose 8/1P
4-Aminobenzoate	Gluconolactone	dTMP
Salicylate	Glucosamine	CMP
		cAMP

Table 10, continued

Metabolites		
Hydroxybenzoate	myo-Inositol	Fructose-1,6-bisphosphate
Phosphorylethanolamine	Tyrosine	Trehalose/Sucrose
alpha-Ketoglutarate	Homovanillic acid (HVA)	Riboflavin
Glutamine	4-Pyridoxate	UDP-glucose
3-Phosphoglycerate	UDP-N-acetylglucosamine	N-Acetylglutamine
Indoleacrylate	Glutathione disulfide	Acetyllysine

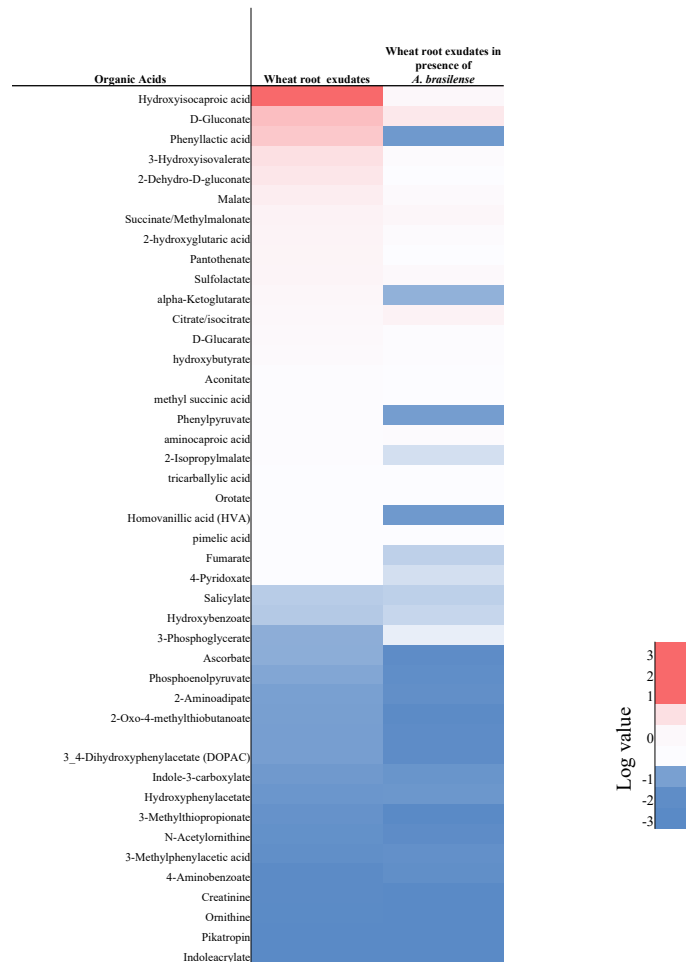


Figure 35: Metabolomics profiles of wheat root exudates collected in the presence of absence of WT *A. brasilense*.

LO conducted this experiment.

Tlp1 are not more resistant to hydrogen peroxide stress because WT and $\Delta tlp1$ have the same susceptibility to 3% , 0.3%, and 0.03% hydrogen peroxide. Only 3% hydrogen peroxide kills both WT (9.9 ± 1.4 mm zone of inhibition) and $\Delta tlp1$ (9.9 ± 2.2 mm zone of inhibition). These data indicate that Tlp1 is essential in responding to attractants and repellants in the wheat rhizosphere.

PilZ domains are necessary for responding to wheat roots

Tlp1 is important in sensing both attractants and repellants exuded by wheat roots, but it is unclear how one receptor mediates sensing to both attractant and repellant cues. Tlp1 is a prototypical chemotaxis receptor with an uncharacterized ligand binding domain. It also possesses an additional C-terminal domain, PilZ domain that binds to cyclic diguanylate monophosphate (c-di-GMP) (23, 25). C-di-GMP binding to Tlp1 adjusts its sensitivity (23, 25), so we examined the role of the PilZ domain and c-di-GMP in responding to gradients originating from roots. *A. brasilense* cells expressing a form of Tlp1 unable to bind to c-di-GMP (Tlp1^{R562A R563A}) is blind to all zones of the root in a root-in-pool assay. On the other hand, cells expressing a functional Tlp1 respond to all zones like WT, accumulating close to the root surface in the root hair and elongation zones and exhibiting a repellant response at the tip (Figure 36). Thus, the c-di-GMP binding to the PilZ domain of Tlp1 facilitates wheat root sensing.

Tlp1 is not the only PilZ receptor that is important in responding to roots. We have previously shown that another PilZ receptor, Aer, is important in responding to wheat roots (26). Aer is an energy taxis receptor that possesses a putative N-terminal FAD binding domain and a C-terminal PilZ domain (26). Cells lacking Aer can partially respond to and colonize wheat roots (26). In a root-in-pool assay, cells expressing a version of Aer unable to bind c-di-GMP (Aer Δ PilZ) are completely null in responding to wheat roots (Figure 36) and are unable to colonize wheat roots

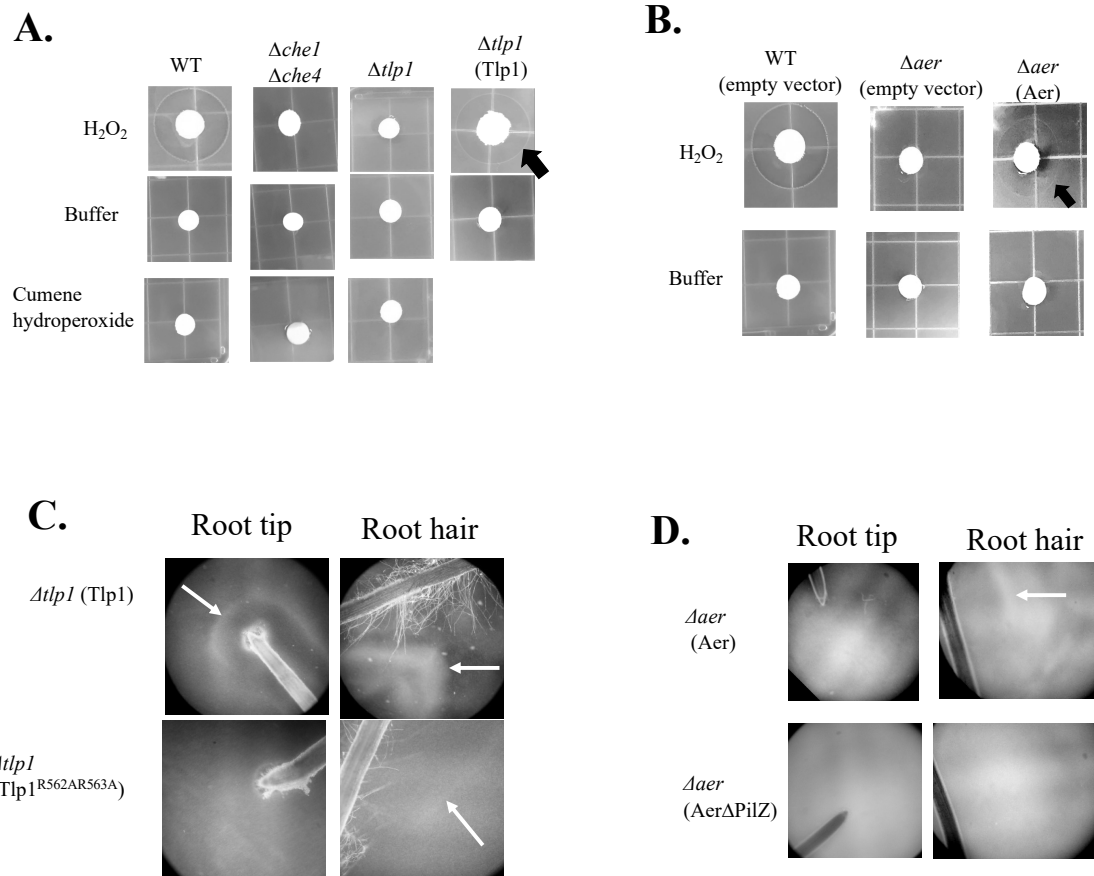


Figure 36: The role of PilZ chemotaxis receptors in responding to reactive oxygen species and roots

Motile *A. brasilense* cells lacking Tlp1 (A) or Aer (B) were exposed to filter paper soaked in buffer, hydrogen peroxide, or cumene peroxide. A response is indicated by a ring forming away from the filter paper. A root-in-pool assay was used to observe the motility behavior of cells expressing Tlp1 (C) or Aer (D) impaired in binding to c-di-GMP in the vicinity of the roots of wheat seedlings. Arrows indicate an accumulation of motile cells seen as a band. LO conducted this experiment.

(26). Despite having different ligands, both Tlp1 and Aer are essential in responding to wheat roots, suggesting that their PilZ domains facilitate this response.

C-di-GMP levels mediate response to hydrogen peroxide

Previous work has shown that Aer is dispensable for malate sensing (26), so we hypothesized Aer would have a role in repellent and ROS sensing. In an ROS disk assay, Δaer is blind to hydrogen peroxide (Fig 8). Both Tlp1 and Aer modulate the response to ROS, so we hypothesized that the c-di-GMP binding to the PilZ domains and thus, intracellular availability of c-di-GMP is crucial to this response. c-di-GMP is a secondary messenger that is made by diguanylate cyclases (DGCs) and broken down by phosphodiesterases (PDEs) in response to environmental cues (31). *A. brasilense* encodes over 30 DGCs and PDEs, only 2 of which are characterized (32). Here, we used an optogenetic system that transiently changes c-di-GMP levels on a timescale consistent with chemotaxis (25). This system uses a red-light activated diguanylate cyclase and a blue-light activated phosphodiesterase. The enzymes are encoded on separate plasmids, and by illuminating cells carrying these plasmids with red or blue light for 10 sec intracellular c-di-GMP levels can be changed significantly (25, 33). These changes in intracellular c-di-GMP level are rapid and temporary, which is advantageous when studying behavior dependent on motility (25, 33). Temporarily (10 minutes; (25)) lowering c-di-GMP levels in WT cells renders them unable to respond to hydrogen peroxide stresses (Figure 37). On the other hand, cells with increased c-di-GMP levels respond 10 minutes sooner to all concentrations of hydrogen peroxide (Figure 37C). Thus, intracellular c-di-GMP availability is essential in setting the sensitivity of cells to respond to hydrogen peroxide in a spatial gradient assay.

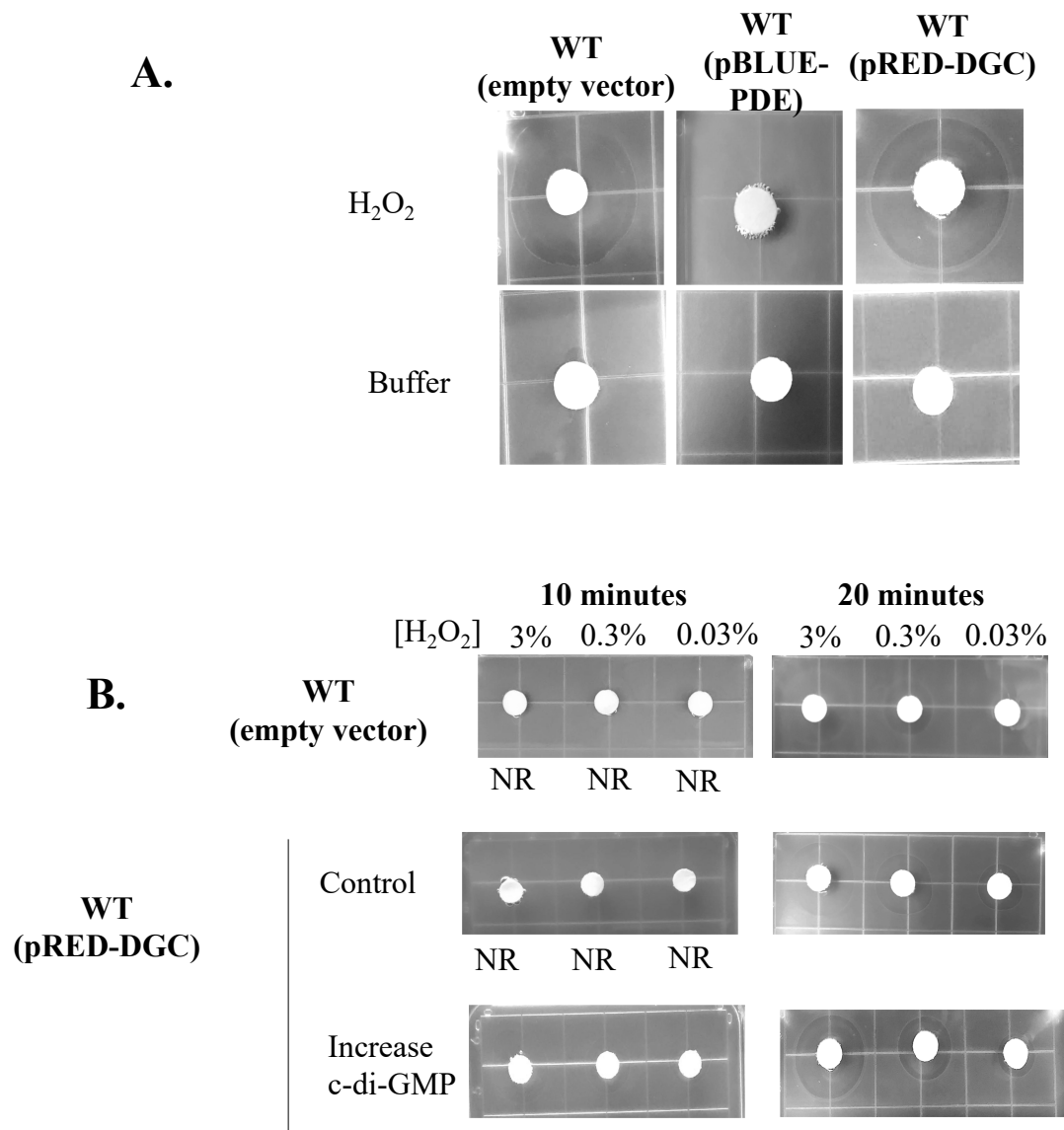


Figure 37: The influence of intracellular c-di-GMP levels on the chemotaxis repellent response to hydrogen peroxide in a spatial gradient assay

Intracellular c-di-GMP levels were manipulated using an optogenetic system, as described in Materials and Methods (A). Motile cells are exposed to filter paper soaked in buffer or in hydrogen peroxide solution. A repellent response is indicated by a ring forming a certain distance away from the filter paper within the time indicated. Modulating the intracellular c-di-GMP concentrations altered the response time and the sensitivity of the cells to the concentration of hydrogen peroxide as can be seen in (B). NR indicates no response. All assays were observed every 5 minutes for 2 hours. Shown is the timing of initial response (10-20 minutes). LO conducted this experiment.

Chemotaxis is beneficial in other plant-microbe interactions

Here we have detailed a mechanism in which *A. brasilense* uses chemotaxis and specific chemotaxis receptors to respond to wheat roots. We then examined the role of chemotaxis in other plant-microbe interactions. We monitored *A. brasilense* accumulation and colonization of *Medicago sativa* (alfalfa) roots, another plant that *A. brasilense* is known to colonize (11). WT accumulates in the root hair and elongation zones of alfalfa and does not accumulate at the root tip (Figure 38A). Chemotaxis-null cells ($\Delta che1\Delta che4$) do not exhibit preferential accumulation in any zone of alfalfa (Figure 38B). Furthermore, colonization of alfalfa roots is not observed until 1-week post inoculation, a delay compared with *A. brasilense* colonization of wheat, which is observed at 48hpi (Figure 38C-E). When placed in competition, WT was able to colonize alfalfa roots, whereas $\Delta che1\Delta che4$ was not recovered from alfalfa roots (Figure 38C-E). So chemotaxis provides *A. brasilense* with an advantage when colonizing roots of various plant species. We hypothesized that other bacterial species would also use chemotaxis to find desirable root zones and colonize.

To test this, we used environmental isolates (general lab stock) in a root-in-pool assay with wheat. These isolates were obtained from soil of various cereals and grains (red clover and orchard grass) at the Agricultural Station at the University of Tennessee. Isolates were selected blindly and their response to wheat roots were tested. Root-in-pool and 1-week colonization assays were used to determine if short term accumulation mimicked long term colonization patterns. After determining colonization levels, strains were identified using 16S sequencing. We selected 3 isolates and found that only 1 isolate (Genus *Ochrobactrum*) preferentially accumulated in any wheat root zone in the root-in-pool assay. *Ochrobactrum* had high levels of accumulation at the

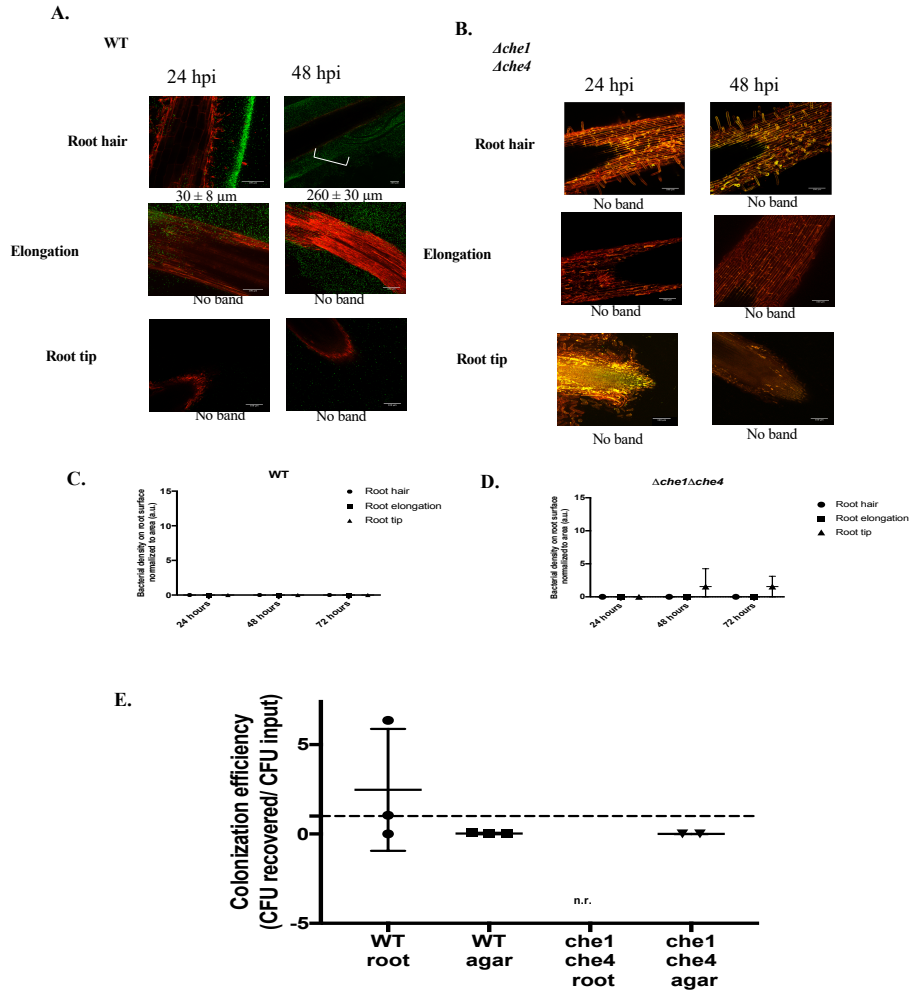


Figure 38: Accumulation and colonization WT and chemotaxis null strains around alfalfa roots

Free swimming cells of the WT (A) and the $\Delta che1 \Delta che4$ mutant derivative (B) accumulation around alfalfa roots. Time course of the colonization of the surface of alfalfa roots by WT (C) and $\Delta che1 \Delta che4$ (D). 5-day colonization levels of WT and chemotaxis null when inoculated at a ratio of 1:1 for competition (E). *A. brasilense* constitutively expressing GFP colonization was inoculated into a slide in-chamber containing 3-day old wheat seedlings and imaged over the course of 72 hours post inoculation (hpi). *A. brasilense* is visible in green and the wheat roots are in red (autofluorescence). Images are representative of 3 chamber replicates. In chamber (C, D) colonization levels were quantified by calculating CTCF (Calculated Total Corrected fluorescence) of the attached bacteria and normalizing to root area. Five-day colonization was measured by CFU counts from homogenized roots. NR indicates no response because no bacteria were recovered under these conditions. LO conducted this experiment.

elongation zone. The other 2 isolates (both Genus *Stenotrophomonas*) both formed homogenous pools with no strong accumulation in any zone. We also noticed that *Ochrobactrum* had higher levels of colonization on wheat, while *Stenotrophomonas* could colonize, but colonized at much lower levels (Figure 39). Both *Ochrobactrum* and *Stenotrophomonas* genuses encode chemotaxis gene, but only *Ochrobactrum* exhibits an attractant response to wheat. Thus, chemotaxis provides multiple species with the ability to preferentially recognize and colonize root zones and species.

Discussion

Here we use a novel chamber to analyze the role of bacterial chemotaxis in plant-microbe interactions in real time. Using this chamber, we show that bacterial chemotaxis modulates the root colonization pattern of *A. brasilense*. Here we show that chemotaxis and individual chemotaxis receptors mediate both attractant and repellant responses to distinct regions of roots. The different patterns exhibited by bacteria in the root hair/elongation versus the root tip zones suggest these are chemically distinct environments.

Using end-point analyses and microscopy, bacterial chemoreceptors were found to facilitate plant-microbe interactions in the association of *B. subtilis* with roots of *A. thaliana*: mutant derivatives lacking individual chemoreceptors were impaired in their ability to colonize the root surfaces (34). Other studies have used metabolomics and binding assays to show the importance of receptors in plant-microbe interactions. In *Sinorhizobium meliloti*, McpU senses proline which is abundant in alfalfa exudates (8). A chemoreceptor in *Pseudomonas putida*, McpG, binds to GABA which is exuded from plant roots, such as tomatoes (35, 36). These studies establish the role of chemotaxis receptors by linking behaviors of mutants to specific compounds found in the root exudates that are also ligands for these chemotaxis receptor (4). Here, we

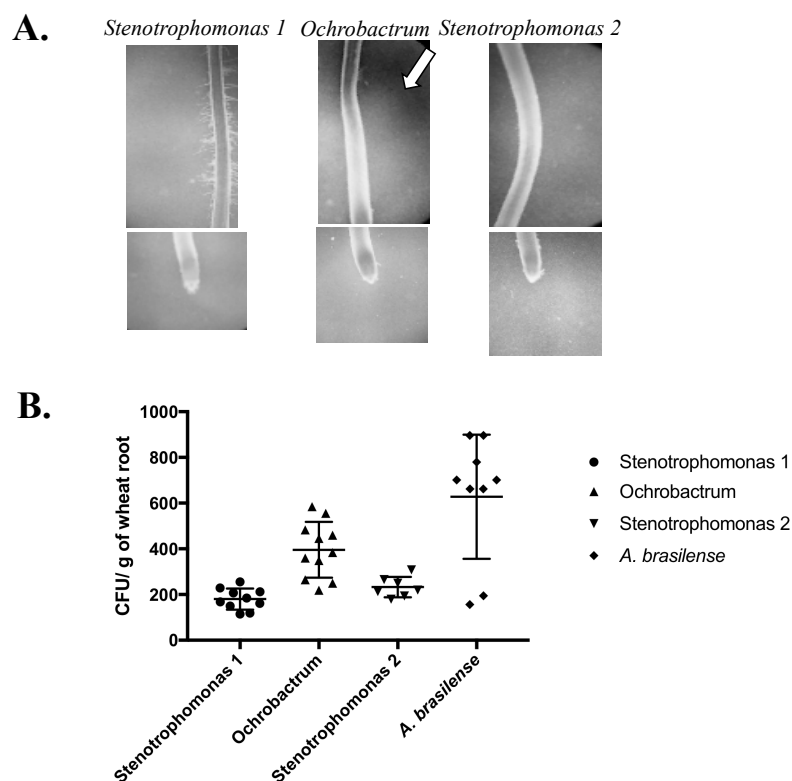


Figure 39: Free swimming and colonization behavior of motile environmental isolates with wheat roots

Accumulation of free-swimming cells of each isolate around wheat roots in the root-in-pool assay was observed and quantified as described in Fig 3 (A). Suspension of the isolates were also inoculated to sterile wheat seedlings and colonization of wheat root surfaces 4 days post inoculation were determined after CFU counts of homogenized roots to determine colonization level (B). SA conducted this experiment.

characterize how real time motility patterns that are regulated by chemotaxis signaling alter the interaction with and colonization of plant roots over time. In addition to the role of *A. brasilense* chemotaxis in association with wheat roots, we provide evidence that these approaches can be employed to characterize bacterial chemotaxis in other plant-microbe associations. The tools and results presented here thus supplement these studies by expanding the temporal resolution for the analyses of plant-microbe associations.

Here we identify two chemoreceptors, Tlp1 and Aer, that when deleted, render *A. brasilense* mutants blind to the repellent signal(s) originating from the root tips. Root tips are actively growing regions that are known for producing ROS, and ROS are critical to plant growth, including at the tip (37, 38). We show that *A. brasilense* chemotactically responds to spatial gradients of hydrogen peroxide as a repellent but does not respond to ROS generated via organic peroxide, suggesting that this response is specific to hydrogen peroxide. These types of ROS are abundant in plant roots (37, 38). Tlp1 and Aer have distinct sensory specificity, but both possess a C-terminal PilZ domain (23, 25, 26) predicted (Aer) or shown (Tlp1) to bind c-di-GMP. Previous work has shown that c-di-GMP binding affects the sensitivity of *A. brasilense* to oxygen gradients (25) and that c-di-GMP binding to Tlp1 (23) and to Aer (26) impairs chemotaxis receptor activity. Therefore, we hypothesized that the ROS repellent response is mediated by binding of c-di-GMP. It follows that the repellent response should change with intracellular c-di-GMP levels. Consistent with this hypothesis, we showed that lowering c-di-GMP levels using optogenetics abolished the repellent response to hydrogen peroxide while increasing c-di-GMP levels caused a precocious response to a hydrogen peroxide gradient, suggesting an increased sensitivity. Together this evidence supports a role for c-di-GMP in modulating chemotaxis receptor sensitivity to ROS. The

only previously reported repellants, changes in oxygenation and redox changes have been linked to c-di-GMP metabolism in *A. brasilense* (23, 25, 26, 39). The results obtained suggest that these repellent responses are mediated via c-di-GMP binding to the PilZ domain of chemotaxis receptors such as Tlp1 and Aer. The genome of *A. brasilense* encodes a total of 6 PilZ-containing chemotaxis receptors (26), suggesting c-di-GMP levels are major cues to integrate metabolism with chemotaxis in this species.

In addition to repellant response, we also observed that a strain expressing a Tlp1 variant unable to bind c-di-GMP was blind to attractant gradients originating from the root elongation and hair zones. This diverges from strains that just lack Tlp1, which can still partially respond to and will colonize these zones. We previously observed a similar effect on wheat root colonization and sensing of gradients in the roots with Aer: a strain lacking Aer could respond to and colonize wheat, while one expressing an Aer variant lacking the PilZ domain was severely impaired in colonization and root sensing (26). On the other hand, a strain lacking Aer or Tlp1 or expressing a variant unable to bind c-di-GMP can respond to gradients of single chemicals such as oxygen or single organic acids (23, 25, 26, 40). These observations suggest that functional PilZ domains and c-di-GMP binding to these domains are necessary for recognizing and integrating cues from complex gradients found in the vicinity of roots.

How could c-di-GMP binding to one receptor influence the integration of multiple cues? One possibility may lie in the organization of chemotaxis receptors in large clusters at the cell poles. *A. brasilense* cells express two chemotaxis arrays that are spatially distinct at the cell pole (27). Chemotaxis receptors segregate based on length into distinct arrays (41), and *A. brasilense* encodes 2 receptor lengths, 38H and 36H which separate into distinct operons (27). All PilZ

containing receptors in *A. brasilense* belong to the same class, 38H, indicating that these receptors are likely signaling together and may control sensitivity of the 38H cluster (27). If Tlp1 or Aer are missing from the cluster, the cluster can signal. On the other hand, if Tlp1 or Aer are present, but unable to bind c-di-GMP, the cluster appears “off”. So, c-di-GMP binding to PilZ receptors primes the array to an “on” state to allow integration of all sensed cues. Whether this is true for all PilZ receptors is unclear. It could be that Tlp1 and Aer are highly abundant and control signaling of the array through interactions with other receptors, but the fact that all PilZ receptors are in the same array hints at a universal function for c-di-GMP controlling sensitivity of the 38H array.

References

1. Souza Rd, Ambrosini A, Passaglia LMP. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and molecular biology* 38:401-419.
2. Glick BR. 2012. *Plant Growth-Promoting Bacteria: Mechanisms and Applications*. Scientifica 2012.
3. Buchan A, Crombie B, Alexandre GM. 2010. Temporal dynamics and genetic diversity of chemotactic-competent microbial populations in the rhizosphere. *Environ Microbiol* 12:3171-84.
4. Scharf BE, Hynes MF, Alexandre GM. 2016. Chemotaxis signaling systems in model beneficial plant-bacteria associations. *Plant Mol Biol* 90:549-59.
5. Bren A, Eisenbach M. 2000. How Signals Are Heard during Bacterial Chemotaxis: Protein-Protein Interactions in Sensory Signal Propagation. *Journal of Bacteriology* 182:6865.
6. Wadhams GH, Armitage JP. 2004. Making sense of it all: bacterial chemotaxis. *Nature Reviews Molecular Cell Biology* 5:1024.
7. Feng H, Zhang N, Du W, Zhang H, Liu Y, Fu R, Shao J, Zhang G, Shen Q, Zhang R. 2018. Identification of Chemotaxis Compounds in Root Exudates and Their Sensing Chemoreceptors in Plant-Growth-Promoting Rhizobacteria *Bacillus amyloliquefaciens* SQR9. *Mol Plant Microbe Interact* 31:995-1005.
8. Webb BA, Hildreth S, Helm RF, Scharf BE. 2014. *Sinorhizobium meliloti* chemoreceptor McpU mediates chemotaxis toward host plant exudates through direct proline sensing. *Applied and environmental microbiology* 80:3404-3415.

9. Allard-Massicotte R, Tessier L, Lécuyer F, Lakshmanan V, Lucier J-F, Garneau D, Caudwell L, Vlamakis H, Bais HP, Beaugerard PB. 2016. Early Colonization of *Arabidopsis thaliana* Roots Involves Multiple Chemotaxis Receptors. *mBio* 7:e01664-16.
10. Bashan Y. 1986. Enhancement of Wheat Root Colonization and Plant Development by *Azospirillum brasilense* Cd. Following Temporary Depression of Rhizosphere Microflora, vol 51.
11. Pereg L, de-Bashan LE, Bashan Y. 2016. Assessment of affinity and specificity of *Azospirillum* for plants. *Plant and Soil* 399:389-414.
12. Mukherjee T, Kumar D, Burriss N, Xie Z, Alexandre G. 2016. *Azospirillum brasilense* Chemotaxis Depends on Two Signaling Pathways Regulating Distinct Motility Parameters. *Journal of Bacteriology* 198:1764.
13. Bible A, Russell MH, Alexandre G. 2012. The *Azospirillum brasilense* Che1 Chemotaxis Pathway Controls Swimming Velocity, Which Affects Transient Cell-to-Cell Clumping. *Journal of Bacteriology* 194:3343-3355.
14. Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P. 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil* 378:1-33.
15. Calvo P, Nelson L, Kloepper JW. 2014. Agricultural uses of plant biostimulants. *Plant and Soil* 383:3-41.
16. Rossi FA, Medeot DB, Liaudat JP, Pistorio M, Jofré E. 2016. In *Azospirillum brasilense*, mutations in *flmA* or *flmB* genes affect polar flagellum assembly, surface polysaccharides, and attachment to maize roots. *Microbiological Research* 190:55-62.

17. Croes CL, Moens S, Bastelaere Ev, Vanderleyden J, Michiels KW. 1993. The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. *Journal of General Microbiology* 139.
18. Greer-Phillips SE, Stephens BB, Alexandre G. 2004. An energy taxis transducer promotes root colonization by *Azospirillum brasilense*. *Journal of bacteriology* 186:6595-6604.
19. Xie Z, Ulrich LE, Zhulin IB, Alexandre G. 2010. PAS domain containing chemoreceptor couples dynamic changes in metabolism with chemotaxis. *Proceedings of the National Academy of Sciences* 107:2235.
20. Gullett J, O'Neal L, Mukherjee T, Alexandre G. 2017. *Azospirillum brasilense*: Laboratory Maintenance and Genetic Manipulation. *Curr Protoc Microbiol* 47:3e.2.1-3e.2.17.
21. Ramos HJ, Roncato-Maccari LD, Souza EM, Soares-Ramos JR, Hungria M, Pedrosa FO. 2002. Monitoring *Azospirillum*-wheat interactions using the *gfp* and *gusA* genes constitutively expressed from a new broad-host range vector. *J Biotechnol* 97:243-52.
22. Keen NT, Tamaki S, Kobayashi D, Trollinger D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 70:191-7.
23. Russell MH, Bible AN, Fang X, Gooding JR, Campagna SR, Gomelsky M, Alexandre G. 2013. Integration of the Second Messenger c-di-GMP into the Chemotactic Signaling Pathway. *mBio* 4.
24. Ind AC, Porter SL, Brown MT, Byles ED, de Beyer JA, Godfrey SA, Armitage JP. 2009. Inducible-expression plasmid for *Rhodobacter sphaeroides* and *Paracoccus denitrificans*. *Applied and environmental microbiology* 75:6613-6615.

25. O'Neal L, Ryu M-H, Gomelsky M, Alexandre G. 2017. Optogenetic manipulation of c-di-GMP levels reveals the role of c-di-GMP in regulating aerotaxis receptor activity in *Azospirillum brasilense*. *Journal of Bacteriology*.
26. O'Neal L, Ahkter S, Alexandre G. 2019. A PilZ-Containing Chemotaxis Receptor Mediates Oxygen and Wheat Root Sensing in *Azospirillum brasilense*. *Front Microbiol* doi:10.3389/fmicb.2019.00312.
27. O'Neal L, Gullett J, Aksenova A, Hubler A, Briegel A, Kjeaar A, Jensen GJ, Alexandre G. 2018. Distinct chemotaxis protein paralogs assemble into chemoreceptor signaling arrays to coordinate signaling output. Submitted.
28. Morell-Falvey JL, Alexandre GM, Sarles SA. April 26, 2016 2016. Microscope Slide-In Chamber. United States patent US D754,871S.
29. Gosta F. 1957. The Infection of Clover Root Hairs by Nodule Bacteria Studied by a Simple Glass Slide Technique. *Microbiology* 16.
30. Mendez-Gomez M, Castro-Mercado E, Alexandre G, Garcia-Pineda E. 2016. Oxidative and antioxidative responses in the wheat-*Azospirillum brasilense* interaction. *Protoplasma* 253:477-86.
31. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology* 7:263.
32. Ramírez A, Millán Pacheco C, F. Cruz Pérez J, Minjárez Sáenz M, Baca B. 2018. In silico comparative analysis of GGDEF and EAL domain signaling proteins from the *Azospirillum* genomes, vol 18.

33. Ryu M-H, Fomicheva A, Moskvina OV, Gomelsky M. 2017. Optogenetic Module for Dichromatic Control of c-di-GMP Signaling. *Journal of Bacteriology*.
34. Allard-Massicotte R, Tessier L, Lécuyer F, Lakshmanan V, Lucier J-F, Garneau D, Caudwell L, Vlamakis H, Bais HP, Beauregard PB. 2016. *Bacillus subtilis* Early Colonization of *Arabidopsis thaliana* Roots Involves Multiple Chemotaxis Receptors. *mBio* 7:e01664-16.
35. Ortega A, Zhulin IB, Krell T. 2017. Sensory Repertoire of Bacterial Chemoreceptors. *Microbiol Mol Biol Rev* 81.
36. Reyes-Darias JA, García V, Rico-Jiménez M, Corral-Lugo A, Lesouhaitier O, Juárez-Hernández D, Yang Y, Bi S, Feuilloley M, Muñoz-Rojas J, Sourjik V, Krell T. 2015. Specific gamma-aminobutyrate chemotaxis in pseudomonads with different lifestyle. *Molecular Microbiology* 97:488-501.
37. Considine MJ, Foyer CH. 2014. Redox regulation of plant development. *Antioxidants & redox signaling* 21:1305-1326.
38. Gapper C, Dolan L. 2006. Control of plant development by reactive oxygen species. *Plant Physiol* 141:341-5.
39. Carreno-Lopez R, Sanchez A, Camargo N, Elmerich C, Baca BE. 2009. Characterization of *chsA*, a new gene controlling the chemotactic response in *Azospirillum brasilense* Sp7. *Arch Microbiol* 191:501-7.
40. O'Neal L, Mukherjee T, Alexandre G. 2018. Analyzing Chemotaxis and Related Behaviors of *Azospirillum Brasilense*. *Current Protocols in Microbiology* 48:3E.3.1-3E.3.11.

41. Herrera Seitz MK, Frank V, Massazza DA, Vaknin A, Studdert CA. 2014. Bacterial chemoreceptors of different length classes signal independently. *Mol Microbiol* 93:814-22.

CONCLUSIONS

The goal of this dissertation was to further characterize complex signaling amongst two chemotaxis systems in *Azospirillum brasilense*. This dissertation first examined the organization of proteins within two chemotaxis arrays found at the cell pole. We have found that chemotaxis paralogs encoded in separate operons are able to interact and form mixed baseplates (Chapter 1). This finding diverges from previously observed systems that spatially segregate chemotaxis paralogs into separate arrays (1). The mixed baseplate is advantageous for *A. brasilense* because the Che1 and Che4 operons regulate two independent parameters of chemotaxis. Che1 controls swimming speed (2), while Che4 is responsible for modulating reversal frequency (3), so integrating these proteins via mixing into signaling arrays, as opposed to spatial segregation, ensures that the cell integrates signals both of these pathways to produce a coordinated response to attractants or repellants without detrimental cross-talk.

We also showed that *A. brasilense* possesses two separate chemotaxis receptor arrays that are segregated by receptor class length (38H and 36H) (Figure 40). Work in this dissertation has focused on the function of a subclass of 38H receptors: PilZ containing receptors that can bind to the secondary messenger cyclic diguanylate monophosphate (c-di-GMP). Cells expressing receptors impaired in c-di-GMP binding can still partially respond to oxygen, suggesting there are other c-di-GMP independent oxygen sensors, like AerC (6). These *A. brasilense* PilZ-receptor mutants are null for sensing complex gradients such as root exudates in the rhizosphere. Loss of c-di-GMP binding to either Tlp1 or Aer renders cells completely blind to wheat roots, a phenotype more profound than loss of the receptor. (Chapter 3-Chapter 4). This “blindness” hints at an important role for PilZ containing receptors: integrating information in complex gradients. It is

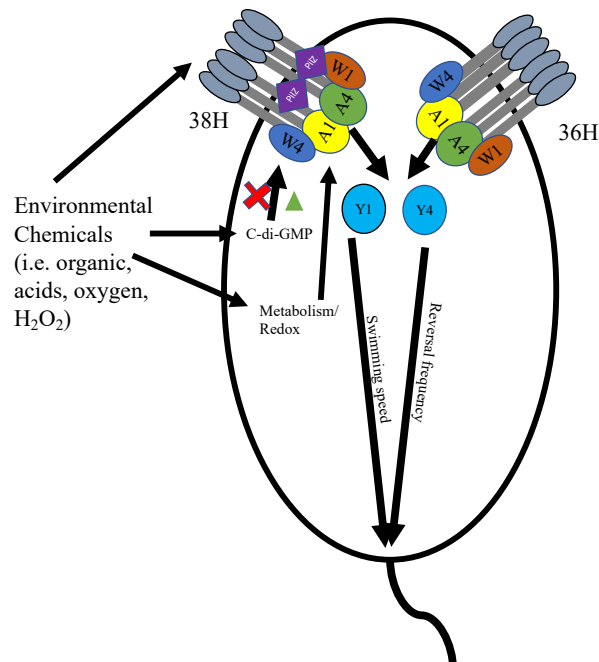


Figure 40: Model of *A. brasilense* chemotaxis signal transduction

A. brasilense possesses two chemotaxis receptor arrays, 38H and 36H. The arrays receive signals via extra- or intracellular cues. Upon binding to a ligand, the signal is transduced down the receptor to a baseplate composed of protein encoded in both the Che1 and Che4 operons. CheY proteins then transduce the signal to the flagellar motor and modulate swimming speed and reversal frequency. The 38H receptor contains a subclass of receptors, PilZ containing receptors (purple triangles) that bind the secondary messenger cyclic diguanylate monophosphate (c-di-GMP) and play a role in signaling integration of multiple receptors.

currently unclear how loss of c-di-GMP binding to a single receptor would effectively blind cells to the multitude of chemicals present in the rhizosphere, but one possibility could be chemoreceptor organization. Chemotaxis receptors associate and signal with receptors that are the same heptad length (7), and these associations form large signaling arrays (8-10). Tlp1 and Aer appear to be interacting and signaling with other chemotaxis receptors responsible for sensing rhizosphere cues and loss of c-di-GMP binding to either Tlp1 or Aer produces the same phenotype: the cells are unable to sense any gradient and behave as a non-chemotaxis mutants would. This suggests that loss of c-di-GMP binding by Tlp1 and Aer not only turns off these PilZ-containing receptors but also the other receptors with which they interact in signaling arrays. *A. brasilense*'s dependence on PilZ domains differs from how other organisms use receptor interactions to modulate output. In *E. coli*, two minor receptors, Tap and Trg, do not signal when both major receptors, Tar and Tsr are missing (11). This is due to Tar and Tsr's role in binding to CheR, the methyltransferase. When Tar or Tsr is present, it can recruit CheR where CheR can then methylate neighboring receptors and modulate their sensitivity. Thus Tar or Tsr is necessary for Trg or Tap to be methylated and to be able to sense and respond to gradients (12, 13). This loss of function can be rectified by creating chimera receptors: Tap/Trg with the CheR binding domain from either Tar or Tsr. While *E. coli* minor receptors depend on the presence of at least one major receptors, *A. brasilense* differs from this model by needing a small secondary messenger to bind to receptors to coordinate signaling.

But how could c-di-GMP binding to a receptor be causing a change in signaling state? PilZ domains undergo extreme conformation changes when binding c-di-GMP in other organisms. *Vibrio cholerae* and *Pseudomonas putida* express VCA0042 and PP4397, respectively, which is a

two-domain protein that contains a C-terminal PilZ domain. When both VCA0042 and PP4397 bind to c-di-GMP, the protein undergoes a conformation change which tightens the packing and brings the C-terminal PilZ domain closer to the N-terminus (24, 25). Though unstudied in *A. brasilense*, c-di-GMP binding to the C-terminal PilZ domains of chemoreceptors is likely inducing extreme conformation changes that could change the receptor conformation and signaling state and this conformation change could be transduced to neighboring receptors. PilZ-containing receptors could influence the signaling states of other receptors in two possible ways: either by influencing the signaling state locally (affecting signaling of a small receptor neighborhoods) or universally (by changing the signaling state of the entire array) (Figure 41). Since all 6 PilZ receptors are 38H and loss of Aer and Tlp1 c-di-GMP binding result in the same phenotype (Chapter 3-4), PilZ receptors are likely associating and signaling together in a 38H chemotaxis signaling array. Each PilZ receptor has a different ligand binding domain and thus, a likely different specificity. It could be that the cell needs the information from all PilZ receptors to compute an appropriate response and turning one of these receptors “off” affects the cell’s signal (Figure 41). Each PilZ receptor has a different ligand binding domain, and likely a different specificity (See Introduction, Figure 7). In this model, PilZ receptors form a subcluster within the signaling array and signaling together in an all-or-none response. Receptors binding to c-di-GMP could affect the signaling state of its neighboring receptors via a large conformation change that influences the CheA conformation and phosphorylation state. Receptors locked in the “off” state (unable to bind c-di-GMP) would lock this subcluster in a non-signaling state. This all-or-none response has been previously modeled with *E. coli* chemoreceptors (14-16). If this model is correct, loss of c-di-GMP binding to any of the other 4 PilZ receptors should yield similar results

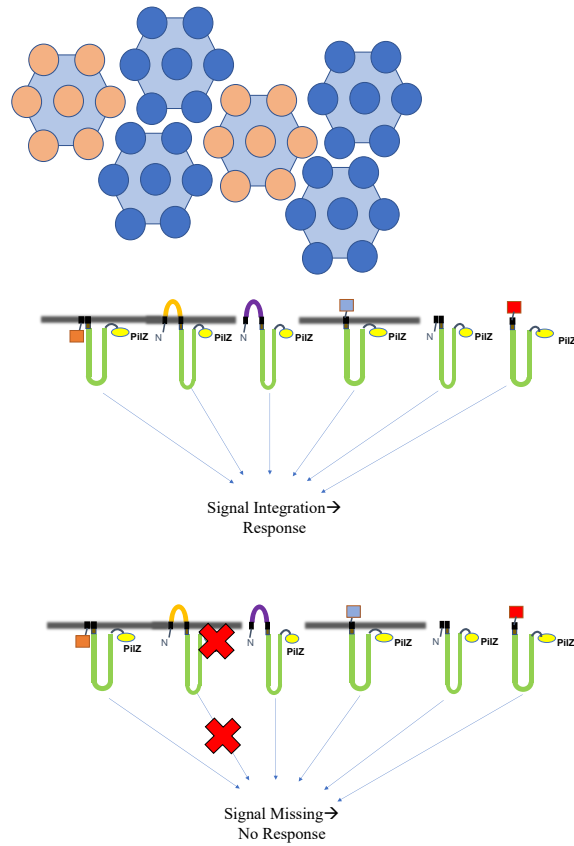
Figure 41: C-di-GMP affects chemoreceptor signaling state

PilZ containing receptors can either exhibit effects locally as a signaling team (A) or throughout the array (B). In A all PilZ receptors form a subcluster within the array and signal as a team. When all receptors are on (c-di-GMP bound), the signal is amplified, and the cell responds. If one PilZ receptor is off though it changes the ability of the signal to be amplified and the cell is impaired in response.

In B, PilZ receptors are distributed throughout the array, and c-di-GMP binding to these receptors causes a conformation change in the receptor which primes the entire array for signaling. Depicted are the ligand binding domains (top down view) of the array. Receptors in the array can change from a tensed (T) state to a relaxed (R) state. T-state receptors cause CheA to be kinase on, while R state receptors lead to kinase-off. PilZ receptors could mediate this transition by undergoing an conformation change that changes the T/R state of neighboring receptors.

Circles represent dimers of receptors. Orange are PilZ-containing receptors and blue are non PilZ receptors.

A. Response dependent upon PilZ receptors being “on”



B. PilZ domains influence Receptor Packing and Signaling State

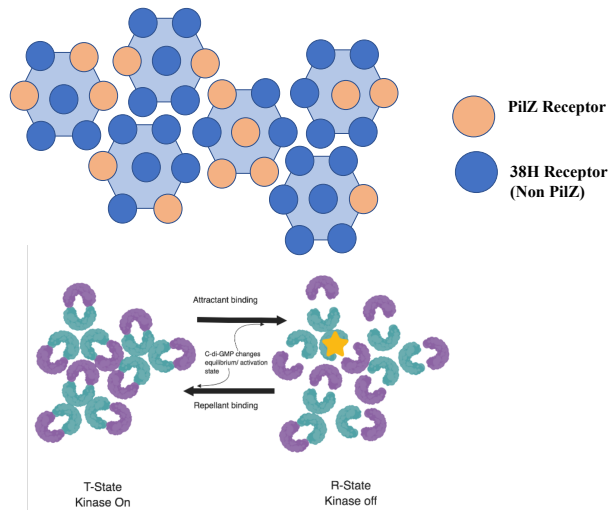


Figure 41

to loss of binding to Tlp1 or Aer: cells blind to complex, rhizosphere gradients. To understand if this is even possible, future studies should look at the potential for interaction between PilZ receptors using bacterial two-hybrid assays and/ or protein pulldowns. We should also investigate the ligand specificity of each PilZ receptor and determine if they are involved in sensing rhizosphere cues and/ or energy taxis.

Another possibility is that c-di-GMP binding to chemoreceptors could prime the entire array for signaling. Previous studies have hypothesized that signal amplification is achieved by changes in array packing state. For instance, in *E. coli*, chemoreceptors undergo top-down transitions during ligand binding that ultimately change the array conformation. These transitions are initiated with the sensing domain going from Tensed (T) state to Relaxed (R) state upon attractant ligand binding (17, 18), which spreads to the rest of the receptor (17, 19-21) and alters the signaling state of CheA by influencing the receptor-kinase interaction face (21). These transitions are proposed to affect large areas of the chemoreceptor array, priming the array for cooperative signaling (17, 19). Receptors in the tense/ compact state are in a kinase-on state. Binding of an attractant causes the sensing domain to change conformation and thus the rest of the receptor changes state and influences the CheA-receptor interface and forces CheA into a kinase-off state (19). Methylation of receptors reverses the kinase state by changing receptor conformation in *E. coli* (19). To date, we have only observed methylation-independent taxis in *A. brasilense* (22, 23), but this does not disqualify the ability to change receptor conformation and thus the T/R state of the array. If PilZ receptors are distributed throughout the entire array, changes in c-di-GMP binding state could have far reaching effects (Figure 41). Results presented in this dissertation show that c-di-GMP binding to Tlp1 and Aer play a major role in the bacterial response to complex

gradients, but for this model to be true PilZ receptors would need to be spread throughout the array and undergo significant conformation changes that can influence neighboring receptors. Here we would expect PilZ receptors to be promiscuous: able to interact with any other 38H receptor, not just other PilZ receptors. We would also expect all PilZ receptors to be highly expressed as they would constitute the majority of receptors within the 38H array. Finally, we would expect loss of c-di-GMP binding to affect sensitivity to many more cues than previously observed. For instance, the majority of *A. brasilense* receptors are predicted to be within the 38H array, with only 3 receptors predicted to be within the 36H array, none of which are PilZ containing receptors. This model implies that the 36H and 38H signaling arrays are functionally different. So we should be able to identify 38H and 36H specific cues, and thus c-di-GMP dependent and independent cues. So even if the 38H receptor is off due to low c-di-GMP levels, the 36H array is still functional and able to signal. We currently have a clue as to one of these cues: oxygen. Results in this dissertation show that altering c-di-GMP levels or expressing receptors impaired in binding c-di-GMP impairs *A. brasilense*'s ability to sense oxygen, but cells are not null. This suggests that WT cells integrate cues from both the 38H and 36H signaling arrays when sensing oxygen, but when the 38H array is off, then the 36H array can partially compensate.

When looking at this dissertation as a whole, we are able to generate a model of chemotaxis in *A. brasilense* (Figure 40). *A. brasilense* possesses two chemotaxis receptor arrays, each with different length class of receptors. These receptors can receive information from extracellular cues (i.e. carbon or nitrogen sources) or intracellular cues (i.e. FAD). The mixed baseplate integrates cues from multiple receptors to modulate both swimming speed and reversal frequency. One of these arrays (38H) contains all of the PilZ-containing chemotaxis receptors (including Tlp1 and

Aer), and c-di-GMP binding to the PilZ receptors in this array control signal integration and receptor contribution. The 38H receptor array signaling is c-di-GMP dependent, while we would expect c-di-GMP to have no role in 36H array signaling, due to the lack of PilZ-containing receptors. This model implies that the 38H and 36H signaling arrays are functionally different and modulate while the role of c-di-GMP, if any, in affecting the 36H receptor array remains unclear.

References

1. Ringgaard S, Zepeda-Rivera M, Wu X, Schirner K, Davis BM, Waldor MK. 2014. ParP prevents dissociation of CheA from chemotactic signaling arrays and tethers them to a polar anchor. *Proc Natl Acad Sci U S A* 111:E255-64.
2. Bible A, Russell MH, Alexandre G. 2012. The *Azospirillum brasilense* Che1 Chemotaxis Pathway Controls Swimming Velocity, Which Affects Transient Cell-to-Cell Clumping. *Journal of Bacteriology* 194:3343-3355.
3. Mukherjee T, Kumar D, Burriss N, Xie Z, Alexandre G. 2016. *Azospirillum brasilense* chemotaxis depends on two signaling pathways regulating distinct motility parameters. *Journal of Bacteriology*.
4. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology* 7:263.
5. Jenal U, Reinders A, Lori C. 2017. Cyclic di-GMP: second messenger extraordinaire. *Nat Rev Microbiol* 15:271-284.
6. Xie Z, Ulrich LE, Zhulin IB, Alexandre G. 2010. PAS domain containing chemoreceptor couples dynamic changes in metabolism with chemotaxis. *Proceedings of the National Academy of Sciences* 107:2235.
7. Herrera Seitz MK, Frank V, Massazza DA, Vaknin A, Studdert CA. 2014. Bacterial chemoreceptors of different length classes signal independently. *Mol Microbiol* 93:814-22.
8. Briegel A, Li X, Bilwes AM, Hughes KT, Jensen GJ, Crane BR. 2012. Bacterial chemoreceptor arrays are hexagonally packed trimers of receptor dimers networked by rings of kinase and coupling proteins. *Proc Natl Acad Sci U S A* 109:3766-71.

9. Briegel A, Ortega DR, Huang AN, Oikonomou CM, Gunsalus RP, Jensen GJ. 2015. Structural conservation of chemotaxis machinery across Archaea and Bacteria. *Environmental microbiology reports* 7:414-419.
10. Briegel A, Ortega DR, Tocheva EI, Wuichet K, Li Z, Chen S, Müller A, Iancu CV, Murphy GE, Dobro MJ, Zhulin IB, Jensen GJ. 2009. Universal architecture of bacterial chemoreceptor arrays. *Proceedings of the National Academy of Sciences of the United States of America* 106:17181-17186.
11. Feng X, Baumgartner JW, Hazelbauer GL. 1997. High- and low-abundance chemoreceptors in *Escherichia coli*: differential activities associated with closely related cytoplasmic domains. *J Bacteriol* 179:6714-20.
12. Le Moual H, Quang T, Koshland DE. 1997. Methylation of the *Escherichia coli* Chemotaxis Receptors: Intra- and Interdimer Mechanisms. *Biochemistry* 36:13441-13448.
13. Li J, Li G, Weis RM. 1997. The Serine Chemoreceptor from *Escherichia coli* Is Methylated through an Inter-Dimer Process. *Biochemistry* 36:11851-11857.
14. Keymer JE, Endres RG, Skoge M, Meir Y, Wingreen NS. 2006. Chemosensing in *Escherichia coli*: Two regimes of two-state receptors. *Proceedings of the National Academy of Sciences of the United States of America* 103:1786.
15. Sourjik V, Berg HC. 2004. Functional interactions between receptors in bacterial chemotaxis. *Nature* 428:437-41.
16. Tu Y. 2013. Quantitative Modeling of Bacterial Chemotaxis: Signal Amplification and Accurate Adaptation. *Annual Review of Biophysics* 42:337-359.

17. Baker MD, Wolanin PM, Stock JB. 2006. Signal transduction in bacterial chemotaxis. *Bioessays* 28:9-22.
18. Ottemann KM, Thorgeirsson TE, Kolodziej AF, Shin Y-K, Koshland DE. 1998. Direct Measurement of Small Ligand-Induced Conformational Changes in the Aspartate Chemoreceptor Using EPR. *Biochemistry* 37:7062-7069.
19. Khursigara CM, Wu X, Zhang P, Lefman J, Subramaniam S. 2008. Role of HAMP domains in chemotaxis signaling by bacterial chemoreceptors. *Proceedings of the National Academy of Sciences* 105:16555.
20. Mowbray SL, Sandgren MOJ. 1998. Chemotaxis Receptors: A Progress Report on Structure and Function. *Journal of Structural Biology* 124:257-275.
21. Ottemann KM, Xiao W, Shin Y-K, Koshland DE. 1999. A Piston Model for Transmembrane Signaling of the Aspartate Receptor. *Science* 285:1751.
22. Hauwaerts D, Alexandre G, Das SK, Vanderleyden J, Zhulin IB. 2002. A major chemotaxis gene cluster in *Azospirillum brasilense* and relationships between chemotaxis operons in alpha-proteobacteria. *FEMS Microbiol Lett* 208:61-7.
23. Zhulin IB, Armitage JP. 1993. Motility, chemokinesis, and methylation-independent chemotaxis in *Azospirillum brasilense*. *Journal of Bacteriology* 175:952.
24. Benach J, Swaminathan SS, Tamayo R, Handelman SK, Folta-Stogniew E, Ramos JE, Forouhar F, Neely H, Seetharaman J, Camilli A, Hunt JF. 2007. The structural basis of cyclic diguanylate signal transduction by PilZ domains. *The EMBO Journal* 26:5153.

25. Ko J, Ryu KS, Kim H, Shin JS, Lee JO, Cheong C, Choi BS. 2010. Structure of PP4397 reveals the molecular basis for different c-di-GMP binding modes by Pilz domain proteins. *J Mol Biol* 398:97-110.

APPENDIX

Supplemental Dataset 1- 38H Receptors

>Az_bra|GCF_001315015.1-AMK58_RS04445--38H

```
-----
-----
-----
-----MTN-----
-----SSSL-----SKAFALGA-----
-----VAALLV-----
-----AAL-----
-----
-----
-----L-----VTDALDVG
-----G-----APL-----
-----RI-----GIGAAALAA-LVGV-M-LCMVR--AR
A---AVGQAVAVCQAVSRGDFEARVVNVREKGD LGELMHALNDSIDRTDAFIREATASMSYVSANKYFRRI
VLKGMQGNFLHASRTINTATD-AIAVKVKGFAGVTDRFETQIRAVCEQVSGTAHQLEMSARTMESDAQTATG
KASSVAAAASQTGSSVGSVAVATEELSASIREIARQVEEVAKVAANAAGEAERSNALIAELSGAAKTVGEVV
TLINDIASQTNLLALNATIEAARAGEAGKGFVVAQEVKRLADQTAKATGDIAGQIAGIQSSTDVAVASIVG
IGHTIQDINQIAGGIAAGIGQQGSAIRSIVSNMDQAAAGTRAVTVDIQDVTTAAERTSGTAHEVFAAASERMT
VQADRLTREVQLFL-DEMHKV--A-----
-----
-----
```

>Az_bra|GCF_001315015.1-AMK58_RS08090--38H

```
-----
-----
-----
-----MTS-----
-----LSSV-----SKSLAAAA-----
-----SAALLS-----
-----
-----
-----
-----G-----
-----
-----AQAVYGFMTGDAV-----AAALGLLAI-LPCL--AAIRW--LA
L-TNRTIHKAIAVIAAAEKGDLQPRILNIQGASPIADMLRTLNRLLDRVESFGKEANAAMQHAAEGQYYRRI
VMTGMVGEFGAYAHQINDGLAAM-DGKSREFVESATRIGANIKEVAQSLASAAQLEASSTAMTATAATASE
QSFSASAAAEQVSSNVDGVAAATGEVSGAIGEVAQGVSRADLARSSVEKVRADATIRSLAASDQIGAVV
QLINDIASQTNLLALNATIEAARAGEAGKGFVVAATEVKNLANQTAKATEDISAQISQVQSVTRDTAAVIQH
VGGMIHDIDQIAVGIAGAAEQQSAAIDEISRSIREASAGVRTVADAVTSVSSGAQDASAAASQVLSSAGELA
RRAETLNGDIDKFV-ARVCGGQR-----
-----
-----
```

>Ma.mag.767-amb0586-YP_419949.1--38H

```

-----
-----
-----
-----MNSS-----
-----SL-----SRALAATA-----
-----LAALLC-----
-----
-----
-----
-----
-----G-----
-----
-----VGAVIALSR--GA-----LLDAGAMGLAALGA-L-GALLF--IN
R-ARRSVHRACTILAEAGAGRLDVRVVGITESGVLGQLDKAINRLLDLTEAFTKEADAAMALTSEGRYFRHI
LTDGLVGEFADHARLINQALSQM-EKRALAFSTEATGVGNTIKHVTQVVASTATELEATAQQMSDIASQTSE
QSTRVAGAAEDAYSHVESVAAAAEQVSSGIRDVAARIQDSARMAQETVRVATETDDAINGLNTAAQKIGEVV
SLITEIAAQTNLLALNATIEAARAGEAGKGFAVVANEVKHLANQTARATDEISSQIDGMRHATGQAVEAVRN
IAGKIREINDNATGIAATTEQQSAAVAEMSRSIRTVAADVQTVADTIGEVASTAGTATDAAGQVLIAGDLA
SRTVSMNDDIDAFI-SRVCSGIKG-----
-----
-----G-----
-----
-----
>Ma.mag.767-amb2196-YP_421559.1--38H
-----
-----
-----
-----MES-----
-----TLGSY-----ARTLSLGMLVP-----
-----SAICLLA-----
-----
-----
-----
-----G-----
-----
-----TFGLLGSS-IAL-----WVVITVSLIGV-VGGV--KIGGS--AR
R-MAGDLSTAIHVLRSASGDLNARILDVRGSDGIGALQRSINRLLDLAEAFGKEAFAAVESANHGRYYRRI
ITTGLRGDFVLYAKTINHALKRM-EARDAEFIAFA--NNQVKPVVNAVAAAATELEASSGAMSAQSTDTS
QAMTVAAAAEQASVNVQAVASAVEEFSASIKEISAQVHRAAAVASEAAGVASRTDTTVHGLNEAAERIGAIV
SLINDIAAQTNLLALNATIEAARAGDAGKGFAVVANEVKNLNQTARATEDITSQVAHIQQVAAEAIQAIDE
ITRTVSQIEETSSAVAGAVEEQNAVTVETIARNVAEAATGTSSVSSAIITVQATAAEATESAGQVADAASELS
RQSENLSREVDGFI-ARIGGR-----
-----
-----
-----
>Ma.mag.767-amb3701-YP_423064.1--38H
-----
-----
-----
-----M-----LPN-----

```



```

-----IDSR-----Q-----ASSLLIVI-----
-----VIALTV-----
-----
-----
-----
-----LAIISL-----
-----
-----TGGPLGGLQ-----WMELILCLVLG-GAGLLIGASLRVR-VG
K-TAEFVDRATRVVAAAAQGDLNARITNIDRRHPIASLLSGINRVLDLSEAFKEANTAMQYANQRKYFRTI
LTKGLRGDFVTFATTINRSLAMM-ETNEKAFVAF-----EEKVRPVARAVGGASSALADNASNLSAQASDTSQ
QAMTVAAAAEQASVNVQAVASAVEEFSASIREITQQVNRAASTAQEASGKVEQTNSVVQTLGNAAQRIGDVV
SLIQDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKNLANTARATEDITQQVSQIQDATKEAVQAMSE
IGVTVAQIEETSAAVAGAVEEQNAVTQEIARNVAEAATGTQSVSEAI SLVQAAAKNALDGAAEVSHSSQSMS
RDSSNLIENIDSFL-DRLRA-----
-----
-----
-----
-----

```

>Ma.mag.767-amb4008-YP_423371.1--38H

```

-----MQN-----
-----LSSV-----SKARR-----ACQITFGL-----
-----ALALVL-----V-----AL-----
-----
-----
-----LLQN-----WVAAGFAVVLA-IPAW-----IPVGL-----LN
R-SNASIEKAAQVCAAAAQGNLGVRIMDIKGQGNIGQMLRSINRLLDLTEAYCRESQAAMEHANQRQYFRKI
IPTGLRGDFARYAGIINTSLDLM-KGRDADALNFA-----EQNVRVLVQEVSSASTQLRQSSDRLMNNATQTVE
QAMTSAAAAEEASVNVQSVAGAAEELAASFGEINMQTTRATAISSEAMVTAQRTDQTVQDLGHAAAQIGSVL
SLIQDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKTLANTARATEEISAHVSQIQLASEGAAAAIRE
IAQIVGTIQETSTAVAGAVEEQNAVTMEISRNVQEAATGTASVSEAVAYVKDTAETTDQEAREISAAAASLA
ARADDLEAQIGGFI-AKIKQAA-----
-----
-----
-----
-----

```

>Ma.mag.767-amb0994-YP_420357.1--38H

```

-----MET-----
-----TLGSY-----ARTLSLGMLVP-----
-----SAICLLA-----
-----
-----

```


R-----NMQGIAQANDE----LSAVNSLMT--TL-----MMVVGIGGG-LGAL-A-LSVLV--AR
IGISRPIAGITDVMARLATGDTSVTPGLGRGDEIGGMAKAV-----AVFKDNAVDREMRVAEEAERR
-----AKERRTHAVEELVQSFDRNVSGILRTVASAATELEATAQSMLTTADQTKG
QAASTAAAAEQASVNVQTVAAATNELSASINEISGQVTRSTNIASHAVGEAQQTNERVQGLVAQAQRIGDVV
KLITDIASQTNLLALNATIEAARAGEAGKGFVAVASEVKNLANQTAKATEEIASQIASMQEATGGAAAAING
IGDTIGTISEIATIIASAVEEQGSATGEIARNVQQAQGTHLVTTNITEVSDGATQTGSAATQVLGAAGELS
RQSEVLRTEVERFL-AGIRAA-----

>Az_bra|GCF_001315015.1-AMK58_RS05235--38H

MT-----
-----IGTRIA-----LGFAAVL-----LMTVA-----VAFVGWNSL-----RTYAGRVDLAA
-----HTAELDARLKTURI-----EEAR-FVTERDSK-----
-AADNVPGMLDRLRDEAQGTRSALEDAGSIRLVDEI-----LSGIAGYRSAFAN-----FVAQ-D--
KEARARTQSMETRAQALREIAEKIGNQQSD---RYDQNMVSLKDAEHAVRQSRDTSRADRLIEMVLEARRL
QSDFAHTRNPATMAAAGEAIAALLANAE-----
-----TIEKDLVGTNDEEL-AQRIVTIAGAYRMV
F-----EQAGAE---GVGDSASGRIQALDRHAHEIQN-----
-----LAHEMQENQAMVSSALQEAA-----N-----FAQSEVNEA-
-----V-----QLRGIAMRL-----IQGAQAA-----MLGQRDFILMSGEDARGRV
HGAVKETLALAT---QAGAVLVDSE-----GRALIAAITEAAQAFDGE-----FAALVTAS
-----ENQ-----RTASRTM-AKAAGDVSE
Q-----VARLVSIQRDD---RENGRSGAE--LI-----IIIGAVAL-ALGM-V-MAWII--DR
A-ITHPMHAMTKAMGRLEAGDLTVDIPGGDRRDELAMADAL-----SVFKENALEMQRMEGEREEMRR
Q-----IDADRRRTMNEFANGFEQAVSGVVLSTESAGNLGRDAQEMSSDAALTTA
KSSAVAAAASEQASSNVQTVAAAAEELSSSIAEISRQLNSSSQVAVGAAAKATETNGIVEGLAEAAQRIGQVV
DLIGEIAEQTNLLALNATIEAARAGEAGKGFVAVATEVKNLAGQTAKATEEISSQVQMQAATGGAVGAIRT
ISDAVGTISSTVSEIARAMEQQGLATREIAQN VNQAAEGTQEVMMHIAEVTNAATKTGGAADAVLSASRTL
AQAHLRSEVQGFL-NKVRTA-----

>Az_bra|GCF_001315015.1-AMK58_RS07770--38H

-----M-----LSN-----
-----I-SI-----GTRIALLV-----
-----AASVVTLGL--LGGTVTVGAQRMFEATAELDQFRDAYEHTAAIERRASRLRFQ
ALRFVADRDETAEEAFKNAEEASRLDLLDLKERGVERLPQE-----ELDHLSQG-----IGTLKERFSSV
V-----A---RAKELGLSDDSGLRGVLRASARAIEDELKQW-----
-----PNADKLTGRMESMRKMEK-----DFIIYSDES-
-----LLSGHRKA-----FNEFTF-----
-----FLADS-----GL-DTATQTKLEKQARTYRTD-----LGRFVDAT
KA---FRAE-----VQTF-----NDAFQAM-VPRFEALLD
S-----ANAGMAGAVET---QNRVRGEVI--AN-----VLQVGSVLL-VGFA-I-ISLLV--AR
S-ITRPLRLIEGVMERLAGGDRTATVPETGRRDEIGAMARAV-----SVFKENLQRTHELEQESRRAER
R-----AEEERESALRAIAHDFDSAFGRVLHTVSHAADQIRNGAHILRDTAEKMEK
QALDTSEKAEQTSEVVGIVNNVSRTLSASIGEIGGRVTTSSSTAIRRAVDHARQSDTAVAALADSSQRIGEIV

RLINDIAGQTNLLALNATIEAARAGEAGKGFVAVASEVKVLANQTAKATEDIAGQVGAIQEATGSVVEAIRA
IRTTVEEVVAHLSEDVSAAVRDQLEQTEEIVGAVGRANDNTHEVTESVSTMAITAAETGKSAIEMIYSAAQLA
DELRLQLEADADRFV-SSIKI-----

>Az_bra|GCF_001315015.1-AMK58_RS05280--38H

-----M-----RFQD-----
-----F-RI-----VNKVLLVI-----
-----GLMA-----
-----VL-----AVALST-----
-----NGLVRMGSLDQSYTNLINGEAKAS-----
-----LWLARGTSTLNETGRLLY-----TMISEADPA-----
-----RMRSLAEE-----IVRQEK-----LL
DQRLDMAGK-----TL-----PS-----LAADIAAAHQQTGVMSKI-----AEEVRSLT
-----LGG-----KDQEAMA-----VMGKRY-----EPAFLDL-LSRMRALLE
R-----ADKDLDSAADM-----ATAGYQDSR--LW-----TMIIGAVGI-VVCLGL-AVLLT--RR
T-IAGPIERLTGAMRALADGNLDTIVEGQGRGDEIGGMSTAL-----QVFKDSAIEKRRLEAAEKEESA
-----RRGERQRRIEEFTRRFDGIVTGLLDRVTASVRTLHTSSDSLSTAEETQR
QSATVSSAAEQATANVETVAAAGNELTASIEHIAQVSDGSAIATTADRDAANHKIGSLADAVARIGEVT
SLIQNIASQTNLLALNATIEAARAGEAGKGFVAVASEVKSLANQTAKATEEIAAQITAIQTETAVAVAAIRG
ISATTVRMRELTTSIAGAVEQQGAATTEIVRNVDAAQGTRVVADNIIIDVARAASDTGRMAGDVFKAASEVQ
EESETLRVEIERFL-QQMHA-----

>Az_bra|GCF_001315015.1-AMK58_RS24680--38H

MTTSL-----RAFAN-----
---LRTATKVY-----TGFGVTL----ALLVT-----LGAVSWSGLRNSNDTALRSYAAQSDVT-
-----VTLGEADTALS-----DALGAA-----AE-FLVSGTDG-----
-AANRFRATTGDFRRHLDKAAPGIGDSADRQA-----VDE-----
-----IRGL-----ERDFLTGF-----
--D-----RLVA-----
-----LRTERDG-----IV-----AEIVNK-----
-----LGADIRRTLSDLVTAERQTGNLDR-----
-----TVQAAGVSEQYLLVRLVA-----RFVTETKPE-----
-----DLVRIRQD-----LASVAA-----DV
EGEAKGWAD-----SP-----GAAKRTEVLAKLPRYTAG-----IERIAAIA
---E-----EMARVNS-----DTL-----TRAGAQI-NGRIGSIRQ
H-----STEVLKGLERS----AATAVTAAE--RQ-----GTAVTAAAV-ALGL-L-LAWMI--SR
A-ITRPLGAI TGAMGR LAEGDRTVQVDEGWRKDEIGALARAL-----QVFKSNAEEMERMKAQEEAER
Q-----AAEQRRATMMRMADTFETTQVGIVEAVASAAGGMHNAASTLSGTAADASE
RSLLVASASEEASANVQTVASATEELSASIAEIGQVEISTRIAGQAVADAEGADRTMRALVTAAEQIGQVV
EIIISGIAAQTNLLALNATIEAARAGEAGKGFVAVAGEVKALANQTAKATDEIQSKVQEIQTGTGAQKAIGS
IGQITGRMSEIATTIAAAIEEQAAATREIASSVSQAQGTVEVSRNIAGVSTAVSETGEAASNVTHTSEELA
VEAERLRSEVRSFI-TTVRA-----


```

-----
-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS17420--38H
MV-----
-----AALAVFGGMT-----AMEAT-----
-----RIADGNAELAQRVA-----
-----ENQRIAL-----EAERLAKLGE-----AVIVSG-----
-----DEATRGDALTSL-----DAH--AARMSANA-----
-----APEIAQTVVK-----
-----AV-----
-----AASREAAAIGAKVEALDKK-----
-----FGFNISRAESLSGDIARGLS-----A-----AMRATTQPA-
---DEQ-----ALATLFST-----IRDAKFL-----LTRLPSQLYP-----PAV
GNDLRQFRE-----HM--AKAMEL-----RRHLPSADEFESVADDIASFAALDEAF--TQRTETLRLT
-----TDA-----HAHNQEV-RTLVDGLVQ
G-----MNAASDAVTAR----SQEGAAALT--AVLDR----LTLLIALGAFVLIGLIGGLNMLLG-YR
F-IMQPVRDASRALQALTRGEGAVPLRPSPLR-EIADIHGAV-----EAFREALDARQRAEETRRDQER
R-----AEEERRALMAKLADGLERTVQAVAGSLSVAAEEVTGSARAVAGMASDTAQ
RTRAAADAAGTATSNVGAVASASEQLSASIDGIGRQIAQSRVAEDAVAESRRADGLTKGLSDSAQKIGEVV
AIITAIAQQTNLLALNASIEAARAGDAGKGFVAVATEVKALASQTASSTEEIATLVHGIQQSTMGVVDIAIR
IGSTIGVIGESVSGIAAAVEQQRQATSEITHNVRIVECMNTDVSSNIGDVSRAVDVTDGRSADAMMGAADRLS
ELAGTLNGAVDDFL-RQVRA-----
-----
-----
-----

```

```

-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS26410--38H
MLRSTWSGGARTRFWPTRPGSADGKWGQGPRTACGGANEKQDRRWGRDDM--SKPLGIAQDKVVRPS----
-RLGGVQGRFLF-----TGVGAVMC--CTLVAIG-----VALTGYAGFGRTLQVITAEAVPSALGA
-----LRAAQHAGRID--ALAPAFRSVTSK--EEKAD-----
-----LAARIAM-----ERHDFERELRD-----
-----LAELEQNSANAASHAGG-----QSETAAAS-----
-----SAAQALLANVT-----
-----LLDQAA-----DKRIGL-----
---SDRRAELL---EQVVIATRRLSQLVAPWKSIHSSGVDMQRAAFR-----
-----DDTRPAEERLQAGEEYLQTEEQ-----LTLVRSIDDGA
TTVRG-----LLTEAAAS-----FDESRL-----SI
IEAQTRILV-----TT-----MTASSSQLPEASRQAMAEP-----LKAJETLS
-----IEPD--GLIPTRLAELEILKA-----QRDLLTNN-----LSLSETL-TMAVVNLVH
T-----QEGAITDAAEE---SEKALSNGT--IL-----QIGVGVVSLLSAL-V-ILVFV--RR
M-VVRRLTLQGGMEKIAAGDLDTVPSPGGR-DEISAMARTV-----EVFRENALAKRRLEAEQIESAR
R-----AEEDRKRSLEEIASSFEGAVGSVVSFRFVAESTEMEESARSMSATAEETNR
LATSVAAAATEQTSANVETVAAASEQLTSSIEEITRQITESTNIVREAMTLAERANGQIGGLADSVQSIGRVV
DLINDIASQTNLLALNATIEAARAGEAGKGFVAVANEVKALAAQTARATGEIAAQAAGIQSATGEAVQEIQA
VVQVVTRVGTIGATVAAAVEQQSAATKEIARNVHQAALGTTEVTQTIAGVNAAADRSGTAARTLLDKAHDLA
DGANALNRELSGFL-QRVRAS-----
-----
-----
-----

```

```

-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS01380--38H

```

```

-----
-----
-----
-----M-----TCNVNASWNE-----P-
-----MSTV-----KARLLVAL-----
-----SVLC-----
-----FF-----LFAIAA-----
-----TGWLALGQAHRGMETVFKDRVVPLRDL-----
-----KVVSDMFAVNIVDT-----THKVRGGAE-
-----TWEGGLKS-----IDAALG-----EI
ATRWSAFTA-----T-----SMNAR-----EKDLANEAEERHMGKARAA-----ATTARALM
-----QAK-----DQAALDA-----FAVSTL-----YPSIDPV-AAAVGKLV
L-----QLKEAEAEYGR-----TNAAYDTSR--WI-----LGIAVLLGV-LAAA-F-AVTTT--LF
R-VVGPLRAMAELMQRLAKGDLGITVNGTDRKDEVTGLARSL-----AVFKDSAVANRRMEEEEQRQEQA
-----RKEARQALVENYIVGFDSTVHATLKMLASAATEMRATAKSMSSSTAEETSR
QASTVAAASEQATTNVQAVAGATEELSASITEIGRHVATSTTIAGNAVDEAARTDRTVQGLAEAGQKIGEVV
GLIKAIADQTNLLALNATIEAARAGEAGKGFAVVASEVKSLANQTARATEEIATQIQTIQGVTTETVRAIKT
IGGTIGEINGIATTIAAAVEEQGAATGEIARNVQQAAGTTEVSGSIVQVHQAAVETGSAASQVLSSAEELS
RQAETLRLEVERFL-VNIRAA-----
-----
-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS05565--38H
MA-----
-----
-----HPIRVH-----
-----KKERTTM-----LLSN-----
-----L-KI-----STKILSII-----
-----ILLG-----
-----AI-----CAGITA-----
-----TSVSGLDALSRATDEIKASGDDMR-----
-----VGARLNRLVAELLRD-----EYRLAANPA-
-----DLPEVLPS-----ITTGRE-----TL
STNLTRVKE-----TADPE-----QKRLADFETALSLYKTQ-----LEQTLAVA
-----RQINAKGAVGF--HQDILSS-----VENS-----AVAARKL-QDLSVKYTA
Y-----TDDKAARLSDD--ASQLAERRA--VQ-----LIAAAVGI-LVGL-L-LGWLMS-QF
G-IVRPVTAIVACLRGLADGKLDTEVYGTDRKDEVGAIAATA-----DVFKRNLLHSRAMEREAKEAEA
R-----SQAEKRASMLALAGRFESQVGVIVNDVGGATELQATAAQLASAVEEVGA
QCNVAGASEEASANVQTVASASEEMSASIHelaERVTRAAARSKAAADGANLAQAQLDSLAAIEQVDQIV
ASINAVAAQTNLLALNATIEAARAGEAGKGFAVVASEVKSLANQTHAMTEQIGNQIAAVKSASGRTVDAMRA
IITQVEDIDRSTAEMAASVEQQSAATGEISRNAQQAASGTAEVSSNVAGIQQAESSTATHSVKEAADQLA
ERASSLKREVDQFL-SEVRAA-----
-----
-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS10585--38H
MS-----
-----
-----
-----V-----LSN-----

```

```

-----I-TI-----KAKIVSSF-----
-----SILV-----
-----MF-----I IALGL-----
-----FSIDRISTVNDAGKDIRDNWLPSV-----
-----GEIARMTDYFEFYRILES-----AHILSTEP-----
-----QQAEEEKT-----MAATLE-----NF
QDAVRKYES-----M-----LTPGY-----ETETFQKLMGHWERYQELS-----HRKLLPLS
-----RDD-----RNNEATA-----LYKGES-----RVEFRKA-QGLMRDLIQ
F-----NIRNGRIAADQ-----GEATYGSSK--TL-----LIVAISVVT-LLGA-M-MGLAI--IA
G-VSKPIQQLTGVMNRLAGRELTVTVDGAQRKDELGAMARAV-----QVFKDGLIEADRLAAVQAEESA
-----TKQRRSEAVERLVNRFQDSSAASLRTVAAAASELDATEHSMTSMAERTNI
QATTVQAAAEQTSANVQTVASATEEMASSIREIATQVSRSSQIAGQAVEQATRTNETVRGLVEAAQKIGEVV
SLITNIASQTNLLALNATIEAARAGEAGKGFVAVAGEVKNLASQTAKATDEIAAQIAAIQGATDGAVRDISA
IGGIIGQINDISTAIAAAIEEQGAATNEISRNQVEAATGTHQVSGSITEVTLAAGETGAAAGQVMSASGELA
RQSETLRKEIESFL-AEIKAA-----

```

>Az_bra|GCF_001315015.1-AMK58_RS08765--38H

```

-----M-----TVSGAADGMQD-----
-----SGNQGGGL-GL-----RLRALLLI-----
-----G-----GVLL-----VYQ-----VLAIW-----
-----GVHSSANQAKETLAARADMVA-----
-----SLQARAAAI-PLF-----
-----DFDTE-----QVREVAKAPSSDPDFLGAQVRD--DKAKVVAEVG
-----DTKAAKG-----FIEVVKPI-----VGGQAGQ-RKTIGEFVL
RLR-----T-----DRVEARVTADA--VT-----QIAGVAVAF-LAIM-A-ALYLV--VS
A-ITRPLMQITAMVGRLAQGDYAVAVPALDRRDEMAMARTI-----DMLRQNAQHRQLEAEKLARQA
-----EEAQRGERLTRLAAAFDRSVQATVGEASTAASQMKGSAGSMLDSALRADQ
CNASVAGAADETSRTVQTASAAAEQLTQSIRSIAESVKQSVMSAQAIADRADASRKTVEALAAGAAKIGEIT
SLITSIAGQTNLLALNATIEAARAGEAGKGFVAVASEVKNLASQTAKATEEIIATQIGAIQSVTQETVSAIGA
ITETIGQLSQRSIAEIAASVQQQLAATGEIADKVRTVAGEADTVTRSIASVSGASSEVATAARESVEVAQTLQ
ARFVDLRDEVQRFL-ASIKAA-----

```

>Az_bra|GCF_001315015.1-AMK58_RS11605--38H

```

MS-----LFNH-----
---LRVGTKIT-----AANAGVL-----LFLVA-----IGGIGVYALTDKDGAFATYQTLARQT-
-----TEVGRIQATML-----EVQLQA-----KT-FLLTGSEE-----
-AAHTVDSLAAADLDGRIDEAKTLFTEPTLNRT-----VTQMDN-----
-----ELGQYREAFARI--M-----EFQ-----ATRNEASA-----
-----ELLD-----
-----LGVKMEKALSGVMSAYTDGDTEA-----

```

```

-----AYQAGMASRHFLAARNTAN-----RFLTDGSAE-
-----SADIFRKE-----LQSACE-----RG
NAMLAKLTE-----LERRRGASSFLAYQRFATQ-----FEKVVVAT
---T-----KRDELVA-----QVLETL-GPTVNARVE
QL-----RDASARQQAALGTA----ATANIDRAR--TT-----TTAAALVAV-LLGL-I-SALLI--GR
G-LSRPIVSMTDTMTRLAGGDRRVDVPGLGRGDEIGGMKAV-----EVFKHSLIEADRMAAEQAVEHE
-----TRRERSERIDSLTREFDRMVMEVLSRVSSAATQLNSTAQGMSATAEQTR
QASAVAAASTQATANVETVAAAAEELTSSIQEISRQVAQSNSIAGQAVSTAERTDATVRGLVDAAQRIGEVV
QLINDIASQTNLLALNATIEAARAGEAGKGFVAVASEVKSLANQTAKATEDISGQIAGIQQVSREAAGAI AE
IGRVIGEISHISTTIAAAVEEQGAATREISRNQQAARGTQEVSGNIAGVTQAAEATGDASRQVLDAAADGLT
GEAEGLRGFVSRFL-TDMRAA-----
-----
-----
-----

```

>Az_bra|GCF_001315015.1-AMK58_RS13805--38H

```

-----M-----TLTK-----
-----L-SI-----RAKLWALV-----
-----VAAS-----VA-----
-----SFAIAGAGLYLNYTRMHADRLDTL-----
-----YSIVETGVTLATALEAEAA-----AGKITREEA-
-----QARFKA AV-----AGIRYA-----GGKEYL-----FA
HTMDGYGFA-----HVNAK-----L-IGADVKPLKSANGVHM-----IVEFIRMV
-----RATG-DGLLEYDWPRTVGGSD-LAVKLG-----Y-----VRGFEPW-NIFIGTGIF
I-----DDIRAAFLDQL--RT-----LAFVILALA-VP AV-G-LIAWV--GT
D-ISRVLRGLGAKMRSLADGDLSTRFPEAERGDEIGAMAKAV-----LVFQTNARDKQRLEAEQAASAQ
R-----AGEEKRRAMLALAASFEQSIGAVVRTVSDAAATMEERAAEMSRAAAQTDE
LATSVAAAATEQTSANVQTVAAASEELAGSIQEISRQVAESSRIAGEAVTLSGRANGKVGGLAEAVQKIGAVV
QLINDIASQTNLLALNATIEAARAGEAGKGFVAVASEVKALANQTAKATEEIAAQVANIQTVTGDAVGEIQG
VTQVILRVNEIATSIASAVEEQGAATREISRNQQAAGGTQEVSANISGVTSAAATRSATARDVLEMSRQLG
HQLDTLHREVGGFL-QQLRAA-----
-----
-----
-----

```

>Az_bra|GCF_001315015.1-AMK58_RS16940--38H

```

MI-----
-----VIVTVT-----
-----I-----FSLAVGIAALTT-----
-----KSGSDIETLAFQSGEQLGH--RYGE-MV-----HARLGTAM-----
-----EAGRFIATSLVG-----
-----LKAAGRADREQLSTWLKSI AEANPDFLG VVW-GMEPNALDGRDAE----FANKLGSDASGR
FLPYWNRG-----SGTVALES LVGYDDPGSDGAYYQIPKRTGHAM-----
-----VVEPYNYTVAGRKVLMSVMSVPIVENG-----RVIGVAGIDLST
DGIW-----SMLKTVKP-----FDSGSI-----HL
VSNDGVWAG-----HPDAE-----R---MGQSIGASDPALDAAKPAIRA----GRSFEQMA
VA-----DGQPVKQ-----LFLPVKV-----AGTDTPW-SLLVNLPLD

```


K-----INAPVRELR-----NATIIGGLVL-LAAL-VSALLFA--SR
SIVGLPLRRTIATLDAVVAGERGVVIADTGRADEIGAINKAL-----KLFQDNNTRMAELEEEERHREEQ
R-----AAERRKRDLDVASRFEASVGGVVRNVSEQAGEMRSTAQSLSAIAAQTDR
QAAAVSTAAEEASQNVQTVAAAAEELSCSIREINERIGHSSQMAAEVAEVERTNTTLEGLSAAAQKIGDVV
NLIQGIAGQTNLLALNATIEAARAGEAGKGFVAVASEVKNLANQTAKATEDISRQIADMQTVSGTVVSVIGT
VGRSIIAINETITAIAAAAEQQGAATQEISRVVQASMGTAASVNIIGGVTQAAGSTGSMADQALNAAGDLS
READQLRQEVERFV-AMIRAA-----

>Az_bra|GCF_001315015.1-AMK58_RS19995--38H

-----M-----NWFRN-----A-----
-----KV-----GLRLMLSF-----
-----SSIL-----
-----VL-----MVALAA-----
-----IAIYKVNSINDSLSTINDVNNVKQ-----
-----RYAINFRGSVHDRAISLR-----DVTLLSDAA-
-----ALKAEELAT-----IDRLAA-----DYA-----RS
AEQLDRMFAVG-----T-----HITAK-----ERDILASIKETEAKTLPL-----ARGVIEAR
-----QAG-----DAARAVK-----ILVEQA-----RPGFTEW-LARINAFID
L-----QEAESQAVAKH-----ARTVSESFQ--VL-----MISLCAVTV-LLGA-G-LAWW---SI
L-TVRPLRRLTDTTLKLAEGDLSIEVPQATSREVEGEIIRAV-----QVFKDNMVRARRMESEKEETER
R-----AEAEREAMNGLADQFERSVGSIVELVSHAAAELEGAAQTLNATMERAND
QAGTVAAAATQATANVESVATACGELAGSVSSIGQQVRQSADIANRAVRNAEDTQATAEGLVSTSQKIGEVV
QLINSIAQQTNLLALNATIEAARAGEAGKGFVAVASEVKNLANQTAKATEDITAQIAGVQDVTLRVQSIRE
IAQVIGESSQIADDIAQAVEQQGIATQEIARNVQASAGTAEVSGAIVQVSGAASQGGTAAGRVLGSQAQELS
RSAARLRAEVSGFL-AKVRAA-----

>Az_bra|GCF_001315015.1-AMK58_RS02635--38H

MS-----

-----M-----LSS-----
-----M-TI-----RTKVVAAT-----
-----VCVL-----
-----LM-----SVALGL-----
-----FAVDRLSTVNHAAEEIRSNWLPSV-----
-----TAIGEIDGAANDYRILEA-----SHILALYPA-
-----EMAEVEKE-----MTTVLN-----RL
AEARRKYEP-----L-----LAPGW-----ETQTFRRWEAAWDGYLKIS-----QGKLLPPS
-----RAN-----ENEKATG-----IYRDES-----RKAFDEC-SALVNDLTT
S-----NVQNATKAADS-----GRATYVGAR--NW-----ILGAIAFVA-VLCL-F-AGIAM--TK
S-VARPIVALTSIMDRLARRDLGVAVEGGGRQDEIGAMARAV-----QVFKDGLIEAERLTAAQAAEEE
-----NKRRRTERIDSLISNFDGAVHSLVLTQTVGAAASQLDTTARSMTDIAEHTRA
EAGSAAAAAELTSANVQTVAAASEEMAASITEISQQVSQSANIAGKAVDEADRTNATVQGLSEAAQRIGDVV

DLIQNIAAQTNLLALNATIEAARAGEAGKGFVAVASEVKSANQTSKATEEIAQQIGAIQAATGGAVGAIRG
IGATISSINDISSIAAAIEEQGAATQEISRNQQAAQSTQEVTSNIGRVSQAATETGSAATQVMSASGDLN
QGAMRLKSEVENFL-VAIKAA-----

>Az_bra|GCF_001315015.1-AMK58_RS19255--38H

-----M-----LKALRN-----
-----L-KI-----GPKVYLLV-----
-----GLQA-----
-----VV-----AAVTGL-----
-----MGLAAMQDLAGKSEAIERASDRQV-----
-----LGEQLNGLVLAVVMDSR-----GVYMARDRA-----
-----EVDKFGKP-----LLDNLN-----TL
GERLGT LKR-----LTPAE-----GQAALAPVATKTEDFIRF-----RTETVRLA
-----REK-----SPAERT-----YGDNDAN-----RSNRQAL-NAAIQTLSA
D-----QDGMIDRLHDE-----LVSARNHWT--TL-----MLAIGVGGV-LFGI-L-LAVLV--AR
DMIARPIARMTAAMKTIAAGNTGAEVPGTGQGDEVDMAEAV-----LVFRDGLRRATDLAEQERREID
-----VRRQRQERLEALTHGFTQKIEGLCRTLNGEADSIRNSAESLTHSAADASR
RSSTVAAAAEQATGNVQTVAAATEELSTSIGSISQRLGEAARIASEAEREQRTNSTIQGLAQAAEKIGAVV
NLITEIASQTNLLALNATIEAARAGEAGKGFVAVAGEVKSANQTARATEEISAQIVAIQNETQQAVGAISG
IVGTIERISDISTNVAAVEQQGSATQEIARNVQEAAGTQEVSTISGVSQSADHTGEAAGGLLSAAKGLS
VHARTLQSDVDDFT-RQMKAV-----

>Az_bra|GCF_001315015.1-AMK58_RS26645--38H

MT-----
---GRFRTKII-----IGMTAVL-----AVGAA-----AVTSVS-----
-----LLLSRDAA-----
-----ERAVTTLVAETADHHGARVAADISAALAD-----
-----ARSAAALVEVERSTGAPRRET V NRY-----LNRLMAVG-----
-----PLYAGVWV-----
-----DMADDGFDGRDRDVAGLDRA-----GEILGLPG-----TGRMSLLWL--
-----PGPKADDSEGYPFSDVEEKEYYRAAAAAGKPVL-----
-----TEPYLDDLT KALMTSAAAPVKDGD-----RVIGVAGVDLTL
SGLT-----DLVRRVKP-----YGDGHA-----AV
LTGAGVYVA-----HPDAG-----R-----LSQPADDLPEDARRAIAEGRA-----YDGVVTLG
-----GAPHYLRLSPVRFTGADG
A-----WSFLVAAPQAS-----VMADANRLT--LL-----TVLVGVGCI-LLGG-L-VAWRV--GD
G-IARPIAGLTVAMDRLAAGDLDAAI PGADRKDEAGAMARAV-----GVFKQGLVQARDLGRQQEA EWR
-----AREARTATLLDLQRGFEEERAGGLTGALASAAQQLSTARALTGIAEQTHG
RSEMTLSAHSAADNVQTAAAATEELSASVEEIGQQVGESARIAEAAVADVKRADS AVVVLADSAERIGAVV
GLINDIAGQTNLLALNATIEAARAGEAGKGFVAVAGEVKSANQTARATEDIVGQIKGIRDATGEAVEAVQS
ISQTIAQVSRIASGIAAAVDQQSAATRD IARSVLQAADGAREVSGGMGEIRAGAGDTGTAADQLLAAAAGVA
GQSKELAREIDRFI-AGVHKA-----

>Az_bra|GCF_001315015.1-AMK58_RS14830--38H

-----M-----KALGN-----
-----L-RI-----GQRLSIGF-----
-----AAVI-----AV-----LVVTVG-----
-----TGLLLTDNGKTLNRLVADV RMPTA-----
-----LAGQGIVAAVTATNAAMQ-----GYLLTNRE--
-----DLKQERNV-----AWDRID-----AL
RKELDGLSG-----R---WSDPQ-----VQATWDAVKPLLDHLRRDQDM-----AESLGTIG
-----DQLGALN-SLNDDA-----I-----PRANQVM-SLLVGDARS
NDSSG-LIPRQRARLQADTEE----MIQGADRLV--MV-----QAVLLTLAL-LIAA-A-AVLLT--SR
S-IVRPLGAMTEAMRRLADGDSATPVPAAGRGDEIGQMAKAV-----LVFKDNMIAARQAAEREHAEQD
-----VRARRARAIEELTGRFDREAAAVLGSVSAAAVQMKATASQLAATAEQTTR
QSVTVAGASEQASANVQTVATATEELAASVQEIGRQVASSTAIADAEVHKAERADQAMRSLATASAEIVAVI
DLITQVASQTRLLALNATIEAARAGEMGKGFVVAEVEVKALADQTTTRATDEIAAKIVSVRSETEGAVASIGD
VMGTIGRISEVASTIAAAVEQQQAATREIARNIQQAAGTQDVTSNIAVDVNRAASETGSAARNVLEAAGSL
DQADGLRSKVEVFL-SAVRTA-----

>Az_bra|GCF_001315015.1-AMK58_RS02615--38H

-----MND-----
-----I-SV-----RTKTFAVF-----
-----AFLS-----LL-----LAGIGA-----
-----LGINRLSVVNDSSSTEIATFWMPRV-----
-----VQVNTVNDVSNFRILQS-----AHILSTSEP-
-----EMAAAEKR-----MDEMEA-----NI
ANARRTYEA-----T---LRTAE-----GRALFTQFEQQWAQYLTL-----QRQITALS
-----RRN-----ENAAANA-----LFRDAA-----DKGFQEV-GRTLDAMID
H-----NNRGAAKASDD---ADAVYAQSS--TM-----LIGALLIGV-AVCL-G-GALMM--IR
G-VSSPIGAMTEAMRRLAGGDKTTAIPFASRGDEIGAMATAV-----QVFKDGLIEADRLAAEQAAEQ
-----AKLRRTEAVERLIASFEEQVADALRNVAAAATELDTTAQSMAVTARQTND
QAANAAAAAEQTSANVQTVASAAEEMSSSIGEIGSQVTRSTGIAGQAVQEAGRTTDSVRGLADAAHRIGAVV
QLITNIAGQTNLLALNATIEAARAGEAGKGFVAVASEVKQLANQTARATDEIASQIQAIQEATAGSVGAIEG
IGRTIAAINEISTSIAAAIEEQSAATNEISRNVQQAAGTREVSSNIVQVTEAAGTTGAAAHQVLGAAGGLA
SQAENLRRDVESFL-AAIRAA-----

>Az_bra|GCF_001315015.1-AMK58_RS14235--38H

```

-----
-----
-----
-----M-----QLKN-----
-----V-RI-----AGKILGIV-----
-----VVLG-----
-----TV-----SVSTAL-----
-----VSGYGLATLGGELNRVEEAGREAT-----
-----QGARLNRYLVELSRA-----EYQMGANPA-
-----SVATIEAV-----VADRKA-----EV
RAHLEHARA-----GADAQ-----QRPLLDGIESKYAAYVSS-----LDDTVAAA
-----KKHADMGITYA---RREVLNN-----VSGS-----QELVETL-IQAVQDYN
L-----TVAKTARISEE---AEALAGRIT--WL-----IAAVALLGILASGL-I--GRWLS--AR
G-IVGPIVEAVSCLRRLAGGDLTVVITGADRRDEVGDIARAL-----AVFKDNALDRQRIQAEQAEAR
-----HKLDRAEAVGATILRFDREVGEALHVMKDAAAALATANSLSAGAEQASE
RMTVVGAAAEQTAANVQTVAAATEEMTSTVQEVSRQMNEARGFADEATRQAQQAQQRVGALTESGQRINEVI
DLIQGIASQTNLLALNATIEAARAGEAGKGFVAVSEVKQLANQTAQATDEIRGQVGAMQETIGGAVSAIQT
IATVIFRLNEMATAVAAAVEQQGAATAEIGRNAVQAAQGTAEVTGTIGTLRVASESTAAGSAQVLQSAREVA
GRAVALKGSVDAFI-AEVKTA-----
-----
-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS04410--38H
-----
-----
-----M-----FER-----
-----I-SI-----KGKISVIL-----
-----GVAA-----
-----VG-----
-----LALIAAVSLFGLRAEMMKDRQDKT-----
-----RSVVEVAHSLVAHYEEMER-----KGALTRDAA-
-----QTAAKDAL-----RRLRYA-----GSEYF-----FV
IDRAQRMIM-----H-----PIRPE-----M-DGQDQSRTADPNGKLL-----FVEMTRVG
-----VAAE-GGFVDYLW-PKPGQAE-PVPKIS-----Y-----VRGFEPW-GWLIGSGIY
V-----DDVAAAFREAA--LF-----LGGLGLLIA-LVAT-G-IALLV--SR
A-IVSPLHAMTGVMQTLAGGDTAVTVSGTRRSDEIGVMARTL-----EIFRKNQIDLQRHWERQQVEHR
-----VTGQRARALERLTERFDATVSAMVGTVGQAVQALENTARSLSDTADRNM
EAASVAGASQQASVNVQTVAAAAEELSSAIAEIGTQVATSSHISLEAVTASRQASERIDGLGHAAQKINEVA
DLITSIASQTNLLALNATIEAARAGEMGKGFVAVAGEVKNLQAGTAKATEEIAAQISEVQHASNGAVTAIRE
IAGIIARSDEIGTSIAAAVQQGAATGEIARSAGEAAQGTRQVSTSIDEVSASALETEKAANSLLDAAESLA
QQAGALRALVDAFL-VNVEAINSAELATL--FAPA-----GEEVFMPWNDTLAVGQEDIDN---
---DHMILVALINRAARR-IRG-----GEVPPQAIGSAIDQ-----
-LVAYTILHFEEEEERVMQRS-----GYPDFVQH-----KAQHDALRKRVAELKQRFEGEARVGDLLA-
-----LFRDWLTGH-----IQR
FDKRIGH---HLTRPVE-----PTSKAA
>Az_bra|GCF_001315015.1-AMK58_RS22205--38H
-----
-----
-----M-----PLHRWFSN-----R-----

```

```

-----KL-----LHKILVPV-----
-----LVLL-----
-----IV-----IASLVW-----
-----TARSAISDLTSSTARITELVADRL-----
-----GATIAVQSALGDAMDTEA-----NAIVASSRE-
-----AIDAANTQ-----LTANIA-----KA
LSAIDTLAA-----A-----YDDPA-----ERESIQAIKSIVGEYERV-----AQTTVQLA
-----RGF-----DTDGAIR-----VSIDVG-----RPVRQKL-TAALGERVE
Q-----GMAETREAER-----ATALSQQVM--TR-----LYTVAGAGL-LFAFGL-LGSIL--LF
F-VVRPLHRLTSEMTSIAAGDLTVSIQGTERTDEVGLLARAL-----QVFKSNGLDMRRLQEESDAQKR
Q-----MEADRRSAMLALADQFDANVQGVVQAVSQAARQLQSNAQTMGTGAETTTE
QASSVAAATEQASANVATVAAASEQLGSSIGEIGRQVNGAATVARDAVAEGERTNALVERLASAAQKIGDVV
NLIQNIASQTNLLALNATIEAARAGEAGKGFTVVAHEVKALATQTSQATEEIASQITEIQAITEGTVTAIRN
IARTIADVDAIAGAIAAAVEEQTAATHEIGRNIQQAQGTRLVSGNIGGVSQSATDVREAAAEVLDAANSLS
RDSDLLRDEIHGFI-ARVRAG-----
-----
-----
-----

```

>Az_bra|GCF_001315015.1-AMK58_RS06645--38H

```

-----M-----FDN-----
-----L-SI-----RSKLWGLV-----
-----ALAS-----
-----IA-----
-----SAAIVGAGLWLNQYQRMHADRVSLS-----
-----RFMVEAAHSMMAAYEAEAT-----AGQITREEA-
-----QARFKRSL-----LGMRYG-----GQEYL-----FA
ITLDGFGFA-----HPSPK-----L-MGQDVSGVKDTKGAPI-----LMDMARIA
-----KTTG-EGTYGYFWNVAPNSNE-TAAKLS-----Y-----IKLFQPW-GIYIGTGVEF
I-----DDIRGAFLNTL--WT-----VLAVAGLLA-VPVAV-C-LIALV--GV
R-LSATIRSLSTRMHALAEGDLAVAFPEASRRDEIGAMASAA-----RVFLGNAEEKRLEADQAAALR
R-----SEDDKRRTLGLAERFERSVGEVMKSVSLETEEMEREAQEMTRAAGQTD
LAGAVTAATEQTSVNVQTVAVATEQLSGSVGEISRRVADASRIAREAATASERATGKVTGLAEAVGRIGTVV
SLINDIAAQTNLLALNATIEAARAGEAGKGFAVVAGEVKSLSQATARATEEIAAQVSGVQSATGEAVAEIGG
VARVIEQVNGIATIIASAVEQQGAATQAISRNVQQAQAEVTREVARDISGVTAAAGQCGKMAYAVLEASRQLA
QQSETLNGEIGGFL-RTLRTQ-----
-----
-----
-----

```

>Az_bra|GCF_001315015.1-AMK58_RS22815--38H

```

-----M-----LSVSEGRIVRLFS-----
---IKVILPVI-----V--CGLA-----LLGIF-----MAGDAAFTALRGVEAASAYRNVNQVA-
-----GGLVAAAGEFA-----LERGA-----TN-GALNGSTP-----IP-
-----SERRARLQQRDAADAA-----LRTALDA-----LPT--IPE-----
-----MVPH-----GARLNEVE-----
-----ASLA-----
-----SFRNYRRGVDEALARDAGERTPDVVSFGGTI-----TAHIER-----
-----VGALRL-----LLEAVAPPPKPKETLQLV-----

```

```

-----QLRGYAADMAEHAGRERALSALVAGSR-----PVAPDQAEA-
-----LAKSRG-----RL
EQAWDGIRA-----L-----RTRPDIT-----PDL-ARAI DGVEVAYFQAF AET-----RQEV LKGG
-----ADG-----RYPLTSD-EWMGRA-----TVG-----IDSILT L-AGTVGA AID
A-----AVAGTAEKARS-----DL--LV-----SLAVFTLSL-LLGV-G-GSLLV--IR
R-VVRPLTAMTGAMRRLADGDLAVTIPGTGRRDEIGAMAQAV-----EIFQEHAVARERLEAEQRRELE
-----ARERRAVVLDRLTRGF EAKVSGLVGTLSSAATEMEATAGSMSTAAQQTNQ
QSFAVAAASEQASTNVQTVAAATEQLTASIREIGNRAAQSQAITTRAVADARRTDETVRLMAGCAQRIGEVV
GLIQAISSQTNLLALNATIEAARAGDAGKGF AVVAHEVKT LASQTAQATDDIAAQIVQVQTVTRDAVA AIQD
IAGVIGEVNEIATAIAAAVEEQGAATQEITRNVQQA AVGTQEVASNIAGVQQAANDTGTAASHVLSAAQELS
QQAERLTGEVGEFL-TGVRSA-----
-----
-----
-----

```

>Az_bra|GCF_001315015.1-AMK58_RS23860--38H

```

-----M-SL-----SGKTVSLCVG-----
-----GLVV-----
-----LT-----
-----SATFAVNETLLSRYAERMAVERQETNMRVA-----
-----WDVLNQYG-----RDISVR-----DGALYVGDR-
-----ALNGFFEP-----VDRV KAL-----VGGTA-----TL
FMGDTRVTT-----N-----VQKPD-----GGRAVGTKLAKGPVYDAVL-----
-----VRGEPYRG-----QADILGV-----PF-----YTAYDPI-KDRSGKVVG
VL-----YGVPRAEF-----FAPIRDTQ--TM-----IGGLGAVVT-LLVA-G-GSLLL--SR
R-IFRPLSAMRGAMERLATGTL SVEVPALGRKDEIGGMARAL-----SVFKENAQRVARLDAAQAEERA
R-----AERDRRDALEAVARNFEGTMLS VVETLSATTAQLESNAQRLHGIAENSSG
QAAAVSGAAGEASDDVRTVAGATEELTASIVEVSRQIDESSRMVRNAVEEVERTNHTVEGLAAAAGKIGEVV
TLIQSIAAQTNLLALNATIEAARAGEAGKGF AVVANEVKGLANQTARATDEIAQQVAEIQAVTGS AVTAIGG
IGRTVVAVNDLVGRIAGAADRQNATTGEIARSVQRAADRTSEVTYSVRDVSASAHETGTMASEVRSAAADLG
RQALALREQAGAF L-RQIRSA-----
-----
-----
-----

```

>Az_bra|GCF_001315015.1-AMK58_RS02155--38H

```

-----M-----TFLNH-----
-----L-HI-----PTKMGI PA-----
-----ATAILG-----
-----MLAIAL-----
-----LGGS AITEQSRLLDTLFNRSFTREADV-----
-----QALTDTLTVAHAGLYRTVI-----LSTANASPK-
-----AVEDESKA-----LTEQIT-----KL
KTQADKMKG-----QS--AATEE-----EGQILQRFGSDTAAYQAK-----VTSFLDLL
-----KMG-----VDPLDF-----LQEV-----QAAYGRL-NGTARDYLT

```

Y-----QRQQSADAYAN---VNASVDATT--QA-----FIASAIVAL-LITV-G-VALFI--GF
N-IARPVVRLTTVMERLAQGRLEDEVPAAERGDEIGQMARTV-----RVFKENALRVQEMAREQEAMRA
R-----ATEEQRRAMNSLAADLEASVKAMMGEVVRSATSMRGEANVMLENARQTS
HSDSVAHAVQEATSEVESVAAGAEQLRASIDEITRSITQSTQLARGAVDEAGRTDSIVQGLSEASRKIEEVV
GLINNIAGQTNLLALNATIEAARAGEAGKGFVAVQEVKSLANQTAKATDEIGAEIAAVQAATTAAVNAIRA
IVNTIRQVDESLSVAAAVEEQDAATRDISERSQRAATDTVAVLQEMRLVQQAETTGHSAAGAVQTTTEELS
RSFNRLDNEIEAFI-TRITAA-----

>Az_bra|GCF_001315015.1-AMK58_RS04505--38H

MK-----
--IRTIQQKI-----AGLAGLC-----LIGTV-----GVLVGFVLSANTT-----
-----NTYVNTQVADILERKTKESSL-N-L-----
-----ASSQAGL-----IRAEFDTALNA-----
-----ARTMAHSFASI-----VDPGN-----TGSAPVATRDAIN-----
-----GILL-----
-----NVLQNNELF-----NGTYTA-----
-----WEPNALDGDDAAFNGHRESGNATGRFIPYWNDRNGRIAMQPLVEYDSRDLH
PNGVMKGGWYIGPKETGKESVLDPLPYVVQKQVFLATLSVPVVVGG-----KFQGVAGADFNL
DFVQ-----QLTAKVSK-----AVFDGRSE-----VVI
ISNMGLIVA-----H-----SGKPN-----L-IGQSISSFDAWQDDLAYVRG-----GTAHVEVQ
-----AESGLLRT-----FAPIPM-----GRTGKPW-SVLIQVPKD
I-----VLADANALGGA-----LNDRADSGM--LW-----QVGVGLLVI-VAAV-G-LMWM--AG
R-IARPVRACVRFAEGIDGDFNQTLEV-RQEDEIGTLADAL-----RKMLTDLQRMIAQRAADQR
Q-----AEAERTAMLTTLADDLESNVMSVVEGVDTAAKAMGVTAQAMTSTATQTSQ
QAAVVASAAADDANVNVQTVAAATEELSGSIREIGERVNRSAEIARDAVTAAQRANDQVLGLTEAAGKIGMVV
QLIQDIAAQTNLLALNATIEAARAGEAGKGFVAVAGEVKHLANQTAKATEDIALQVNEMQVRVTGDTATVIK
VGTIIAQIDGIATNIAAAVEEQSAATLEIARNVQQAATGTQDVSANITGVRGAATEAGHSAQEVLSVSGQLS
VESERLRGVVHGF-LGKIRAA-----

>Az_bra|GCF_001315015.1-AMK58_RS23955--38H

ML-----

-----L-----
-----VSRT-----DT---RGKITFVN-----
-----K-----AFVD-----
-----ISGFSETELIGAPHNVVRHPDMPAAFA-----

-----NLWETIKDGRPWEGLVKNRSK-----NGDHY-----WV
KANVTPITEKGVV---TEYI-SIRSK---P-----SRDAVAEAERIYAEIRAG-----
-----RGQQ-YGL-----LDGQIVRR-----GFGVWL-----GDLASSI-AGRIVATSG
LLL-----LTMVLGWLSTGSDSVEA-----FAGLMLFGL-LVAG-V-GTSLV--LG
T-VRRPLGLVETHLDAIARGDLMTNPSSG---VAEFARIR-----SQIRAVKAKLNYSTQERAERQR
Q-----AEEERITALQSMATVEREASHAVEQVALRTGGMAQDADAMATSAERVSI
NAQNVAIAAEEALANAQTVAATEELAASIREISAQIAHSSAVTRRAVENGQRTQGTIQSLSEAVGRIDEVV

KLITDIASQTNLLALNATIEAARAGEAGKGFVVAQEVKNLANQTARSTEEITRLIAEIQGVSTSTAVGAVAE
IGDTIGEIDQISSAIAAAMEEQAAATQEISRNVVETSNAAQEVSVRIAASVSSDADQTGSQASGVRAGSDEVA
TSIDELRRVLVRVV---RTSTSDADRR---RSPRYRVNEACGVVVH--GRDQSGTLTDLNNGGAMLSGIAG
LGV-GDRGTLR-----LDRFG-----LTVAFEVRAIDK-----
---TAIHVRFAESDVALPR-----FRDAFAQLTRGLQPVATAAA-----

>Az_bra|GCF_001315015.1-AMK58_RS04540--38H

-----M-----
-----RF-----TIKFILLS-----
-----VLSLLG-----AL-----LALSDDL-----
-----AGLRALNVANGNLRTVHDERVLPLRDL-----
-----KIISDAYAVFIVDASHKARNGNF-----
-----TWNESLSS-----IRKAKD-----DI
RERWNAYLG-----T---NLDQE-----ETALVNGIKPEMAKADAA-----VERLIGVL
-----SGR-----DAAALDA-----YVKNEL-----YQTIDPV-TDGISQLID
L-----QVRVAGERYAE---AQATHATAN--VT-----SLVLLGIGAV-MVIL---GMAVV--VL
R-VVRPVARLTETMRRIVGGDYDTMVPATDRRDEIGAMAQAV-----EVFKANGLENQRMREQERQRE
Q-----AEIIKRDALENMAQTVERETRNAVVDQVSERTGQMERNAAAMAQSASAVGA
NSQSVATAAEQALRNAQTVAAASEELTASIREIGAQVAQASSITRIAVEKGDLAGTIQSLSDAVQIRIGDVA
QLIQNIASQTNLLALNATIEAARAGEAGKGFVVAQEVKNLASQTAQATEDIAQQIGEIQAAATSSAVAVVST
ITGSITEVDQVAGAI AAMEEQAAATQEISRSVSQTADAAREVSRRIA EVSSEAKATGDHAADV RVIADDVS
GSIDELRGILVRVV---RTSMSEVD RR---REARYAVELPARADTS--AGNRICRVQNLSSSGAALQVWND
ATN-GSRGTLH-----IDGFG-----MPLPFTVVTAGA-----
---QSCHVRFEVPDEAKAG-----FEQRFNALVRDRALRPMMA-----

>Az_bra|GCF_001315015.1-AMK58_RS04510--38H

-----M-----
-----IANI-----

-----RYDVAQVQQWLT DV SATRG-----LDGLDDGPA-----
-----KADEFKA-----FAQHAE-----NA
HAVARELGL-----SE-----MVR SIAATQAAFP PFYEL-----GQRMSRAY
VDGGPVTGNAMMG-----QFDVAE-----SM-----DKEMERL-LSTMEEVRG
E-----RLNSLDAAVTD---IEASAATLK--TL-----LVTTATVTL-LLIA-A-SIVFL--RL
A-VVGPI DRMRLTMTRMATGDLRAEVGFERRGDEIGQMADAV-----RVFREAGLENERLRAEQERARV
A-----GEEERRQSLAAMADTVERETR TAVEQVARHAGQMASNAEAMAASAGMVSD
NSQTVA A AANQALVNAQTVAAANELSASIREIGRQVTASVSVTGQAVEKAGSASAIIGHLSEAVERIGAVA
RLIGDIASQTNLLALNATIEAARAGEAGKGFVVAQEVKNLANQTARSTEEIATLITDVQSVTRQAVDRVGE
VAATIDQVSDISSIAAAVEEQDAATREIARTVNETSHAAQEVSSRIALVSDEANQTGERADGMRAAAGDVA
QSIDDLQSVLVRVV---RTATSDVDRR---RRARFQIDL PCTIDGAVAGTDCKARIANLSSGGAMIVDAPM
IAA-GTPGT LR-----APGLS-----RPVPFTVKSCPE-----


```

---GRLHVKFHVPEADQPA-----LDHDLETLAGMAPNRSAA-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS23785--38H
-----
-----M-----NPLRT-----
-----F-SL-----ATKLTILC-----
-----TLGLIA-----LSG-----
-----AL-----
-----TYATIVKVTASISKDAAERREQAITNF-----
-----RMLLDQKGS-----EYRLK-----DGALYIDDF-
-----KLDAANDI-----VDTVRSV-----TGGVA-----TI
FRGDTRVAT-----N-----IHNP-----GSRAVGTKLARGPVYSAVI-----
-----DGKEPFRG-----EADILGE-----AY-----FTAYDPI-IDRNGEVIG
VLFVGLKKSVLRVLDEVVFN-----IIQVAAVIT-VGMA-L-VMLLV--VR
R-QFRTLDQIRGTMFQLADERYEVTVPGLDRQDEIGAMAKTV-----EVFRLKGIDNQRLREAQERERR
E-----AEAIAAIALESMARTEQETRTAVDRVAERSVSMDSNAQAMASSAETVSA
NSQSVAAAAEQALGNAQAVAAATDQLTASIAEITGQVTFASQISRRAVESGRRTENVVLSLSEAIGHIGEVA
TFIQDIASQTNLLALNATIEAARAGEAGKGFVAVAEVKNLATQTSKSAEEISRQITDLQSMTAGAVDAVQD
IGRTITEIDGVASSIASAMDEQGAATSEISRNVGQTAAAAQEVSSRIAHVSEEAAVTGNRADEVRRVASDVA
GSIDHLREMLVRVAV----RTATPEVDNR----RAPRFEVNLPTISGA--VGSLSGHLTNLSEGGATISGLDR
SQV-GNRGVL-----IPGCT-----VSIPFAVLGGKE-----
---QDLHVRFELTPDVADR-----FATDVQRLTAGLHPLPAVA-----
-----
>Az_bra|GCF_001315015.1-AMK58_RS23780--38H
-----
-----MPD-----
-----SL-----R-----
-----ALD-----VTKTAY-----
-----FDLGPESVGSRLRQALPIISR-----
-----AL
PGILDRFYA-----RT--MAQPE-----LAALFAN-----PAQVEGAK
-----RAQIAHWTR
L-----FEGRFDDGYKES-----VRAVGIAHH-RIGL-S-PSWYVG-GY
AFI-----LGEIMAALAQ-SMGGTLLTGSRIGSIVTMQRAVVS-----AVLMD-----LDAALSVYWD
R-----LIDERLQAVDAMVDSIDHEANDVVDSVASYTADLRRTVGDLDSVCASVKD
SITGTSADTAEALDAAQTVAAGAEQLQAAVGEISVQVSGASRIAGEAVEKSGEARQIMAQLSTAAQEIGGIL
NLIRAIAGQTNLLALNATIEAARAGEAGKGFVAVATEVKNLAGQSAKADEIAAKVGAIQAVAEQAITGMDG
VATTITTMQEINSSIAAAVEEQSSASREIARNIGEIVAGIFRGIASRMTEVGGETERATTVAITVGESANRMQ
EALDTLPSLLARAI----RTSSDLANRR--HTRRRPVLLDGSVDQT-----PALVRDISECGCYAETKGA
APT-GGQVTVS-----LPGYK-----LSLRGSVVARHD-----
---NRLHIRFDERKIGREEVDNRSDRGVGLVRLA-----KQDHLAFVDKIMEAVGGQRTLLPAD-----
-----LSTHHSCLGRWYDSVNDPRTMRLPTYKGMATPHRTVHESGRETLALQAGNRMEAER
HAAEMKRASAEVVRLLE---MERDFARTLAEATE
>Az_bra|GCF_001315015.1-AMK58_RS28610--38H

```

MCE-----D-----
---RRIRHEVSD-----VQVHHVLA-----
-----LDLAPMRSAYR---KRAEARSSGKLP-----SAGSFALDGDQGCLRPFQGGHI IPL
VFCDKIRREACRV-----PIRSTAMSFSLPD---SPRNVQEAERLTEVKVERPVCSQLVSETVPC
RNGERTPIGSSAGRRELM SI-----KLKILLPV-----
-----GLLGALLIIASAYRAMVSWEALTVARHAASVNV TADRLYIAAAAWAVERGS
TAVALGDPQR-----PNP--VIRAKRLEGNQAFEEAIGA-----
----IDRLPR-T---PRIDVAVKQLARTGRLADEARTEVDAALRNLASEGLRQ TWIPTVTALIM-----
-----AG-----QDLRAALEVTVSTGVVAPVVQAFELKAA-----L-----WRAAEFAGRERA-
-----TLAAVISG-----GKPL-----SL
RELEVVLTA-----RGEVEGS-----LARVAEAKDAFGPAFADA-----YGAIMRVY
LD----KIDPLRMAV----FQSSGTAQ-----PYTVTG-----DDWFAAA-THGIDTIFA
A-----QRAASAAAKVVL DGTVETAFNSVL-----LSIATLAAV-LLATTM-AGWTA--AA
G-VARPLRAMTESMRALADGR LDTEIVGGDRHDEVGDMAAAM-----DVFKAQAIENAH LRAEQERERA
A-----SEAAKIAALHRMAETVERETRVAVDSVAERTHRMDANAQAMADSAGR MSS
NSQAVATAAAQALTNAQTVA AATEELTGAI REIGAQVSHATEVTKRAVEKGRSTQNTISSLSEAVAKIGDVS
KLISDIASQTNLLALNATIEAARAGEAGKGF AVVANEVKHLANQTAKATEDIAAQIAAVQSVTQDAVHAVQD
IGGTVLEIEAVSGAVAAAMEEQSAATSEISRNVVETTVA AQDVSCKIAEVSDEVRATGSRAGDV RNVASDVA
LSIEELRNVLVRVV---RTSMSEVDRR---HKPRHLVHAPCRVEIE--GHKSVALLGDI SEGGA MLKGIAA
AGA-GSIGILR-----IDGIP-----MALKFVVR SVGKD-----
---DQAHLAFDLEPEQRDR-----LSQAIRDLLRSKGA-----ED-----

>Az_bra|GCF_001315015.1-AMK58_RS07920--38H

-----MS-----H-----
-----SDQTQSI-----LDR-----
-----IS-----
-----FL-----
-----RIDEPSKAILREF-----

-----QPHLAQRIDQVLEDFYGY-----LRSVPQIA
-----TLL-----ANPTVVS-RAREMQ-----KQHWLK-NVFTGTFFDD
A-----YMAQVLKIGQA-----HQ RIGLEPRWYT GAYCF-TLNK-L-IDLAYQVYR
K-KPEKLAQLMQAINKAVFLDM-----DLATSVYVDLN
-----TAAIISREL GTTADSFEREVKSVVQAVASAAQQLQGT AQNMSRTAEETSA
QSTQVAAAAEEASVNIQTVA AAEELTASIGEISHQVTQSA AIAGEAMSQAERTNTTVQGLADAASRIGQVV
KLIHDIASQTNLLALNATIEAARAGEAGKGF AVVASEVKSLANQTAKATEEINSQIGAVQGATQDAVLAI RS
ISETIARINDISTSIASAMEEQGAATTEIARNVQQASIGTREVSETIVRVNSSASDTGAEARSVLSATNELS
VQSNNLHAEVD RFL-ARVRAG-----

>Ma.mag.767-amb2795-YP_422158.1--38H

-----ME-----
-----LDR-----


```

-----DILPPPEYIIIESYLVASQ-----ALAAEPS-
-----KLAPLKEA-----MIRLRR-----DY
DDRHOYWQG-----Q-----DLEAS-----VRDGLLVESYKPAVQFFDHA-----FSTYFPAL
-----EKG-----DREAARA-----SFAVL-----GGLYATH-RAAIDTLVT
R-----SDALSKAVEAQ-----AAASESSYK--LL-----VIVLTLASA-LAAL-A-LVIGM--SR
A-IVAPMARLGEAVGRLGGGALDAGVPETERVDEIGPLARAL-----DGWRQGLIAAEARRRDEAEEQS
-----RLQARQRRVEAATRFRDEAIITMLARIRDSAENLHAASNSLSANAEQTHA
QGAAVSAATEQSAASVESVASAGNQLSSSIEEISSQVRQSSAIVAAASHEAEAANGRIAGLTEAVERIGQVL
SLINDIASQTNLLALNATIESARAGEAGKGFVAVVAHEVKNLAGQTSRATEDIARQIAAVRDEAGTAVAALAG
IAQTIARINEMSASIAGAVEQQGAAASEISRNVQAAAGSREVVHNITGVVGAAGETGAMARNVFAAANQLL
QSTELEREVA AFL-REMAA-----
-----
-----
-----

```

>Ma.mag.767-amb0263-YP_419626.1--38H

```

MLRR-----
-----
-----KIRRSSLQG--VSWHGR-----
----MRAPSTGTRCRRSGVRAGICRARDRLM-----LGN-----
-----L-RI-----AARLMVGF-----
-----GVLV-----LL-----IAGLSG-----
-----YGVYSGSNTGKAFARAVRLVSNQA-----
-----MVEKLEKEIFQGRMLVW-----VALATGEDA-
-----KMGAARAS-----FDSARG-----
--NLDRLRA-----A-----TVTPE-----RLARIDELSRLLGDYDRE-----IAKIKVSS
----GRAA--SL-----DNAETAA-----AAPAA-----AAIATKL-DLVGEDLAA
N-----FHQVALEVQAG----AERQIDEAI--DA-----SLLVGSLSV-ILGL-V-LSFFI--GR
S-ISAPVIAMTNAMEALAAGDTTIHVPATGNRDEIGDMAKAV-----QVFKDNAIRVAALQTEQAEAKA
R-----AEADRRRAMLGMA DSFEAQVMGLVKGVSVQATQMEAVSQGVSSAAHQQA
QAGTIAAAAGQATGNVQAVAAAAEELSSSISEIGRQVADAASISRQAAEETERTNDMVRALAEAGRIDQVV
SLITDIASQTNLLALNATIEAARAGEAGKGFVAVVAGEVKNLANQTARATEEIGTQIGAVQEETRRAVEAIRN
IGTVIGQVREISGGIADAVEEQSAATAEIARNVQQAVQGTQDVS LNIAGVSEAA SSTGAASRQMLAGAGELA
GNSARLRDEVVRFL-DRVRAG-----
-----
-----
-----

```

>Ma.mag.767-amb0552-YP_419915.1--38H

```

MER-----
-----
-----M-----GVSVSHAWQN-----
-----L-RL-----SSRFMLIV-----
-----GAGVII-----LI-----AGVVLT-----
-----IARFERTEMERQLRQISANEMTSLHALI-----
-----LNVMKRPE-----
----DSD-----NI-----GITVFNNW-----FDSRNI-----HYPGKVWSAWG-----PKV
AAHMKEVEP-----ERAPKLPKDDIDREAFETKVAVGRFDGGFYRYS LPI-----VLGVTDGA
-----KEEV-----C-----HACHGGM-GMVDGDVIA

```

V-----LSSTLSTAE-----SEARLNQVL-TWL-----VVGVIATI-LAVF-G-VRTIL--KR
I-IADPIEDMTERMEQLARGDTSIGVPALDRKDEVGDIARAV-----QVFKDNTIAKQQMEVEAQAAAA
-----ARDRRMHHLEDLIAAFDKAVTAVLDSVSASAAQMIQTARSMVDSADQALE
KANSAARQASDANQNVSTVASAAEELSSSISEIAQQVGMSNQVAVSATKEADSVNTRVQGLAGAADKIGAI
V
EMITGIAEQTNLLALNATVEAARAGEAGKGFNVVASEVKNLARQTVGATEDIASQVNTIQQETDKTVRAIRG
INTTISMDGITTATAIASAIEEQGAATQEIARNIDHAASGTFVYENINAVTQTIHETDQSAKDVLGAVETLQ
RQAQTLRAEVDRFL-QGIREA-----

>Ma.mag.767-amb0554-YP_419917.1--38H

-----MAKWPLPG-----GC
RPSRNRKTEVAMGGLKL-GI-----GGKIWALV-----
-----ALLV-----
-----AG-----
-----LGLLAVQSLSSTHETLIEDRRQAL-----
-----KQVLDSSASVVESEFRARAA-----KGEFSEEQG-
-----KRLAMETL-----RAWRYG-----NNDYV-----FI
NSYDMRPLM-----H-----PLRPE-----M-EGQDQSEMKDPNGVYV-----TRAMIEVA
-----RSPAGAGFTSYHW-ARVKDKP-PVPKLV-----Y-----VINYPKW-NWVLGTGVY
I-----DDIDEAYAACA--LD-----LGTKAALLG-VLAA-V-IAVLI--AR
S-ITGPLGQLVSRMRALAEGDIERQVEGTGRKDEIGALARAM-----EVFRGNARENRRLLDEQEQLKL
A-----AAQERNRTLRLDMAEGLERRVKSASVDAINNVGQRLNGASSAMTQTADQTS
QTGAVVAATQQTSTNVQTVASAAEELSASGNEISRQVSLTADIARAAAAEASQTNGMVAALAAAAGRIGEVV
QLIDAIAGQTNLLALNATIEAARAGEAGKGFVAVAGEVKTLANQTAKATQEIITTQIAAVQTETTRAVAAIRH
IGETITRVDEATSSIASAVEEQNAAIHEITRSVQEAARGTREVTEHISRVSDGARTSKSAEEVAGSAREMV
TQNGTLTREIDGFL-REIRTQAS-----

>Ma.mag.767-amb0854-YP_420217.1--38H

-----M-----LKN-----
-----L-RI-----AVRLALALIIP---
-----LVAVIV-----YSGLALSEKSRQANEMEQUEELARLAPAVSALVHELQKERGT
SAGFVGSKEKEF-----KDRLEPQRKATDGQL-----SGLRTALSHFP-----AADYGTSEFK--
-----AKLDAAEIALKGLDDKRMATLSLPLADTTGYYTG-----TIARLLA-----
-----VIEEMAVVSRDTRVTKAIIAYVQLLQGER-----AGQERATAS-
-----GGFAAKAFAPA---LHRSFTQLIA-----RQ
EVFFDTFAK-----NAEPE-----LRQAFEKIQTDPAVKE-----VDRMRAVA
L-----DAPFTND-----LGGIEA-----PVWFDTI-TRKIDLLKQ
V-----EDMASAALVDMADK---GHDATLSAL--YA-----SLGITLAVL-TLGI-I-LISVI--AR
G-ITRPLAAMTFDMTRLAEGDKTVLIEGLERRDEIGAMARAV-----EIFKDGLIRADRLEQERHQTEV
-----AKDRRARVIDNLLREFNDEVGDALSGMASTATELEATSRSLSSTAEGASA
QASAVAAGIEETAVNMRTAAGSAEQLAHTGKDITRKVQDSVRISEEASAEARRTTQLIDGLASAVDKIGAVV

ALINDIAAQTNLLALNATIEAARAGDAGKGFVAVNEVKHLANQTAKATDEIAGQISTVQKVTGEAVSAITA
ITSVIEQVHDVATGIAQAVQGQDSATGEIAANVTQVATATGEISANVAGVNHAAEETRQASAEVLQTAQDVS
ERANRLRDRVDSFL-TSIRTS-----

>Ma.mag.767-amb1217-YP_420580.1--38H

-----M-----LGK-----
-----S-GI-----LLKLMTTA-----
-----VLAL-----VG-----
-----FAILSAVALTQLRQSMNDRIEKV-----
-----AALAEVARGTLKSFHDRAK-----AGEFDEATA-----
-----KAQAIETL-----RPLRYQ-----GDEYF-----FI
YDFDGVNVL-----H-----PVRPE-----R-EGKNFIDSKDADGKAY-----IRGMIEGS
-----KTGR-A-VEFYKF-PRPGSDA-PVPKVS-----V-----ALSHAPW-KLMVGTGIY
I-----DDIDTAFNAAA--LR-----FAGIALVVC-LVAL-G-LVTVL--SR
D-IAAPLRRRLAEVAQRLARQDYSAQVEKTERKDEIGLLIAAV-----AVLRDEAASAAEARAQQESYR
T-----ASIQRKQARLQLADDIESSIKRVTDVVVQAVGEMESAAAIVSGAVGEAGA
KAGAAASSAEQASGNVATVAAAAEQLSASISEISRQVHESDTSGRAVAEAERTNALVQGLAEAAAGHIGEVV
TLINDIAGQTNLLALNATIEAARAGEAGKGFVAVAGEVKHLATQTAKATDEIYNQVNTVQARTREAVDAIAS
IAATIGRINEIAGAIAAVEEQGAATAEIARNVQQASAGTQEVSSAMGSLAAATASAGSSADSVQGIAKRLT
SEAQALDREVHQFM-VSMRSDQGAES-----

>Ma.mag.767-amb1538-YP_420901.1--38H

-----M-----
-----SL-----RSLILFGF-----
-----AVCL-----
-----AI-----TAALAG-----
-----TAIVTIGNQAAPLETIETKADGTANLGI-----
-----PLLLALKDLRYDVVQVQQFLT-----DVSATGNRE-----
-----GFGEAE EYAQR-----FASDLA-----QA
RDLSGRLGL-----AE-----LSATLGQLPGLFDAYYAS-----GRQMAEAY
VSG--GQEAG-----NVKMEAFDA-----TAKAL-----AERMEEA-GTKVNDYAW
A-----GMGELLEQTHS----VRAANDSLK--ST-----LMITCALGV-LVNL-L-VALAI--GA
Y-ATGLFRRLGTDIAVVMEEEREAAPHLSDRHDEFGPIARAL-----ALFRERSAEVKALQARRQSAEA
A-----AEAERRAALLSMADTVEIETASAVENVASEGDRVSHSASAMAQSAQSVGE
LSQAVAAAAEQALSNAQTVAGASEELSASIQEIARQVAQSNAVERVVSQANETSSIVDSLGTGAMDKIGDVA
SMIAEVANHTNLLALNATIEAARAGDAGKGFVAVNEVKHLANQTSKSTEEISRQVGTQGVGREVAASISQ
MIDTVHEVEGMASSIAAAVRQQDCATREIARNVVETSAAAKEVSEHIATVAAEASSTGQRASEVQDLLHHMA
GRIRELRSVNAAV----RSATPEVNR--RNPRVEIGQIATVELG--DHNIEAELVDLSLCGGRLAAVPE
DIK-GTGGSLR-----VQGLG-----AKVPFHVVEMGD-----

```

---GSARLRFAEAGAPSGEL-----S-----RFIAERARQVRGAA-----
-----
-----
>Ma.mag.767-amb1578-YP_420941.1--38H
MA-----LDQTLR-----
---FTVRARIF-----LGFGLVL-----LLLAL-----IAIIGANGLSSATDQVEEFSRVSDNT-
-----VRVAVLVGDFT-----EARRNV-----RN-YANEGDAA-----
-SAKAAREQLAKVRKDIAEITGKTVNPARREA-----LTKM-----SG-----
-----EI-----DGY--AANFEKMA-----
--E-----ARAA-----KEK--
-----LI-----NEQLSV-----
-----LGQQAREAMASIVSGAMASGDFFA-----
-----AAQSSQTQEALMQARLLAS-----RFTSTPTPK-
-----LADASRQQ-----MTAFLG-----LV
HSLEERLRD-----PA-----RKRLAKDAAAQAESYAAA-----FGQMVDRT
M-----QVEQLAN-----TVM-----RNEAMEI-AKLANQVRT
A-----QVERLDTLKGQ-----TISGMGRAT--VS-----GLVVSGAAL-AVGL-L-LALLI--AG
S-IVTPVQAMTDTMTKLASGDKTVAIPATENKDEIGEMARAV-----QVFKDGLIRAEQLEAEARADQE
-----REVARGRKRELLTADFDMIRRVIAKVDNTVQNVHSTSTSLHAAAEQTSR
QSAAVAAAAEQATANIQTVASAAEELGASTHEISRRVQDTRITQEAVDGVQTADATVEGLSTAAQKIGEIV
SLINDIAAQTNLLALNATIEAARAGEAGKGFVAVNEVKHLATQTAKATSEIAEQIGGIQNSTQSAVNAIKT
VGAAIGRVDEVVSSIAAAVEEQNAATQEIVRNVQEAANGNEHVTSTNISEVSSAAHMTGEMASNMFKVAEVL
ESGTSGLGKHVETFL-ASVQAV-----
-----
-----
-----
>Ma.mag.767-amb1806-YP_421169.1--38H
-----MGQSGPN-----
-----FTYLR-----
-----RTNSM-----LDR-----
-----L-SV-----AKKLILSF-----
-----GAIL-----
-----SV-----VIGLSV-----
-----FAVNRLAAVNQLSTDLDNLWLPV-----
-----DVSRELDGLLAKQRATVF-----RHILTTDEA-
-----QMAEVEKV-----LNDQHS-----AF
VAALERYGK-----L-----LSSPE-----EKANFDDWAKVAVEYDAM-----TARALDFS
-----RKN-----ENDKAKE-----VILSNL-----PLYANA-QKHAAKAVE
I-----NVRGGKDASLA-----GDALYHLSR--MI-----LLIAVAVVI-VMVV-I-FGLLL--RK
S-IAEPIIAMTAAMNRLADKDFAVAIPAVGRVDEVGRMASAM-----QTFKDNMLRSEQMESEQRAAQE
-----RNVARGKTIESLTRNFDEAVSSMLNAVSRASDMEHAAQGMSTAEQTSR
QAVMVAATEEASSSVQTVASAAEELAASIHEIGRQVTQSSTVARMAAEEASKTNQTVNGLAESSARIGDVV
SLINDIASQTNLLALNATIEAARAGDAGKGFVAVAGEVKNLANTGRATEEISQQIAAVQEATRQAVDAIGG
IVKRIEENQISAAIASAVEEQSAATAEIARNVQQAASGTQEVSATIGGVTKAAEETGTAAGVVLGSIKSVN
QQADALKGVVANFL-TGVKSA-----
-----
-----
-----
>Ma.mag.767-amb1868-YP_421231.1--38H

```



```

-----SL-----AARITVAP-----
-----FLVL-----
-----AL-----TGILLV-----
-----MADRQAATALSIDSIHQAERRNQV-----
-----DDLIIATIIYQTHSDVSRHLA-----LVDSGTSEA-
-----KLEAMRKA-----IGANLA-----KA
GGLIDKLKA-----DPA-----AAEAMADIQTRLATYAKA-----VAQMNDMA
-----QSD-----RLIAIPL-----MTHV-----DKQFAEM-AAKIEAAQG
L-----ITAAAGNAAQA-----TRDTAEAAG--QR-----FWMVTAALM-LVFFV-G-ISLLV--VK
S-ITGPLMHQVQAMRSISKHLEVPVDGTEAGDEVGSMARAL-----KVFKDNALETERLRAEQAGMAA
T-----AERAKRQALDTMAATVEREAAAAGRVGEHTGAVSDRADEMQVSASRVDA
KAQSVAAAAEQSLANAQTVAAAAEELTASIGQISSQIGHSAQVTRQAVSTAGRAQDTIGELTRAVARIGDVA
ALIADIASQTNLLALNATIEAARAGDAGKGFVAVNEVKLANQTSKSTEEINRQVAEIRSVTDNTVVAVQD
VIGAITEIEHIGETIAQAVGQQGEATQEIARNIVQTTAAAEVSSNIGEVSQEAANTGEHAARVREMAREVS
SSIADLQQVLVQVVHDSVRKA-----

```

```

-----G-----
-----
>Ma.mag.767-amb2104-YP_421467.1--38H
-----

```

```

-----M-----MIAALTS-----
-----NL-KI-----GRRVWLAILLPAM-
-----AAVVSATMID-----LDKFREMDKAERTGRLAGLTIEIGATVHELQKERGL
SSAFLA--SK--GARM-AGERGKQMETTNPRLA-----TL-----SGAMGG----L
GDGIGEEELAW-----SIDKANRDLGGLQALRGSVDRQDLTAPQAVGRYSV-----LIESLLG-----
-----VAEKLTTATETGQETGNLIAAYLELMRAKEN-----AGIERATGA-
-----A-----ALTAGLDEG--FRQRIALAA-----VQ
DTHIRAYAV-----R--ALPAQ-----RRALQTVLESSAATEVAGY-----RKLILDGA
-----PGDLTPE-----RWFTAA-TRRIDLMKG
V-----EDALAADLSAGI-AAIRAEAAARAA--AA-----ITAALVLAL-AAGA-A-VVIVL--TR
S-ITRPVGDLTTAMLRLAERDLVQVTGGDRGDEIGDMARAV-----AVFKENAAAVARLEEEQERARA
L-----AEAERRAALQAMASTIETETGQVVAKEGGSRQAVSAAESMAASAIRVED
NSQRVAAAAEQSLANAQTVAGAAEELTASIREIASQVASSSSSLVAEAVVAADDANTTVATLLDAVSRIDTVV
AMIAAIIAGQTRLLALNATIESARAGEAGKGFVAVASEVKRLADQTTGGQTGEIAGRIEELKMAAAHVGEAISA
TVGVIRKVTETIASSVAAAVEEQDAATGEIARNVHQSAEAAEVTERIIVVADEARRSGAEATSVTALLGEMA
AQVGELGLVLNRVV---RTAAPEVDRR---QVPRPEPQPRSTASVV--QPRPAAESLDMA-----

```

```

-----LTPEMQPA-----

```

```

>Ma.mag.767-amb2134-YP_421497.1--38H
MN-----K-----
--VSINARVITMA-----ATGISLIL-----GLAA-----LIFWSLGTLDKMDARRKAFADIAAGSK
ALQ-----IKSLEARRE-----KD-LLIRHEIK-----YAA
MAAKAGEETI-----AMATAM-----GRLPEL-----
-----APAAAHIRSV-----ETGMATYV-----
-----RNLN-----
-----AV-----AAAMTT-----
-----AGLDEESGLQGELRTAVHKVEKAVA-----

```

```

-----GQGRQDLVNHMLMLRRHEK-----DYLRLGNLE-
-----YRAKIEAEAAAF--LD-----
-----N---LQRSD-----L-PSSQKSELRLIEAYALA-----IGKMIDAD
-----QSK-----RKAVAE-----M-----SSAYASF-APAFDKIAD
F-----AIAMGNQVDAE----DDAIHNLV--GL-----STFIAIGAT-VAFL-I-LSWVI--SR
S-IVLPVRGITGVM TALAGGDRAIMVPYADGHDEIAEMARSV-----QIFKDGLIRSEKLEDEARAARE
-----EQLARSHKRELLTADFDVMIRRVIGKVEGAVESVHTTSTNLHAAAEQTSR
QSSAVAAAAEEASSNIQTVASAAEELGASTQEISRRVQDTTRITQDAVDGVHTADATVEGLSTAAQKIGEIV
GLITDIAAQTNLLALNATIEAARAGEAGKGFVAVAGEVKHLATQTAKATSEIAEQIGGIQSSTQNAVAAIKA
VGVAIGRVDEVVSSIAAAVEEQNAATQEI VRNVQEAANGNQEVT SNISEVSSAAHMTGEMASTMFKVADELK
VAGTSLGRHVETFL-GSVKTV-----
-----
-----
-----

```

>Ma.mag.767-amb2333-YP_421696.1--38H

```

-----MEGP-----YQRLPW-G-----IW
RMSIS-----NW-RF-----RAKIFLIV-----
-----VLSL-----LG-----
-----MGAIVAVNLANLHNELMAARKIKT-----
-----QHVVETAHSLIGHYVKLSQ-----SGQMSTDAA-
-----QAAAIEAI-----KTMRYA-----GTEYF-----WI
NSLAGKMVV-----H---PIRPD-----M-LGKDLMLGLKDPAGKLF-----FEAMIDVV
-----KKDK-AGFVDYLW-PKPGLDQ-PVPKVS-----Y-----VKGIEEW-GWLVGSGIY
V-----DDVDSAFRAEV--MN-----LGGIVTGVV-LLVL-L-VSWWI--GK
N-VVDGMRNATGGIRKLAEGDTSVEIKGHERGDEIGELVQAA-----EIFREHSLTMKRMSEERAEQRR
Q-----AEAERRSTLAGLATELERGVKSTVTVSESAGMRSTATGMAGAI DNASQ
ESQAVAAAAQQTSSNVEAVAAAAEELSSSIRGIGSQVAESTQIAKEAVDAANRTDGVVRGLSEAADRIGEVV
RLINDIAGQTNLLALNATIEAARAGEAGKGFVAVANEVKHLASQTAKATEEIGQQIASIQSTTADAVGAIES
IGKTIGRMDEIANAIAEAVEQQGAATQEIARNVHEAADGAQEVSHHISISRTASEAGVAARELLGAAAELA
GESETLRNGVDRFL-GEVRAM-----
-----
-----
-----

```

>Ma.mag.767-amb2652-YP_422015.1--38H

```

-----MSG-----
-----RAI-SL-----RFRLLAIP-----
-----ALAA-----LG-----
-----LFAFAVVTVLTVRNGQLETRRVQI-----
-----HSVVESAMKVAEAYHGKAR-----KGEMSEDAA-
-----KAAALEAI-----GMIRFD-----GDNYL-----WV
NDLEGKLLM-----H---PFRPK--E-----VGKSMLEVKDSAGKFI-----YRAFVDAG
-----RSG--GGLVDYVG-RRPGADSYDSPKLA-----Q-----IIPYAPW-GWGIGTGVY

```

I-----DDVEAVTRAAV--VK-----VGLIALAIL-ALVT-G-VSAWI--GM
R-IGSRVHRQAETMLRLAEGDLEAEVELDGARDEIGEMAEAV-----AVFKRNAIEKRQLEAESLADQE
-----ARNRRQEAIERLTADFNQSV EGV LHTVSTSTS QLR LAAESLTHVADGT SR
NASSVAAAAEQASVNVETVAAAAEEMSASQGEIAAQVSRSTQIAERAANDVHHIDEIVQGLSVATGRIGAIA
GIINDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKNLANQTAKATEEIGVHIQAVQDVSRETAEAVRA
IGETIATVHETASAIATAVEEQSAATHEIARNVHEASIGTRSVTETITEVSAGAGTTGDSAQQVFATANDLN
TRTAELASDIKDFL-QALRQT---GNRR---QFERFPVSI PVVIDGR-----PYRTQDLSAGGACLDVDLG
LQS-GVAVKVS-----LDG-G-----AAIRARVVAVEG-----
---GHTHLQFALDDTTQAG-----LESRLGWRRQAA-----

>Ma.mag.767-amb2660-YP_422023.1--38H

-----M-----LARVADA-----
-----RI-----TVKFFIAP-----
-----LLLV-----
-----AC-----IMVLGA-----
-----VFHVAMERQEQSM EGMVTVSFANS-----
-----RTAAELDGMAATIESNIY-----RLLGWQAAKEEK-
-----DRIRALDTQ-----VRADLK-----TL
GEKSKALLD-----SLKAE-----PATSKQIRDYILA-----AGDVLDMY
-----QSD-----HVTALAM-----MGAT-----ELEYDGI-RAKLREMTT
K-----AAASAAEDYHE-----AKASADSTR--TQ-----YFLVLAIFL-ALGV-V-VTLGM--AR
L-TARPVTQLTGIMGR LAEGSTDVEIPSQDGRDEVGEMARAV-----AVFRDGMKRAADLESQQRQRQRE
-----QQT ELLARRDRMIAEFNGAMEKIMATVSSS IERVHTLSSSLQSTAEQTS A
QGS AVAGAAEHSAANVATVASAAEQLGSSVQEISRRVTETATITSEAVSGIHTANTTMDGLAEAAARRIGEVV
QLINDIAGQTNLLALNATIEAARAGEAGKGFVAVASEVKTLANQTARATDEIAQQIAGIQSISQEAVQ TIRN
VGTTIDRVNEVVSSIAAAVEEQSAATDEIVRSVQEASSGNAEITRNIADVSKAATATGEMASGMFKAADLV
EEAGHLRSEVSGFL-GNMRQIRIRHEKSRTRHHRPRSGSHHADLIPSC--GDRNRSPQRPHAAGRRAPLPPA
RKARGPLGLTN-----

-----PR-----

>Ma.mag.767-amb2770-YP_422133.1--38H

MT-----VKQDAV-----
-----TADFLGFRPIC-----
-----WTWAREAF-----
W-----AGELPF-----LAD-----
-----L-PI-----RARFVLLV-----
-----VLALVASVV--FGSVYLAAGQRIEAMLQAQDSYRRLNDLAGDVRGLSAAAQNL
MEIFARERDPAVAEALKRDLGQIRERLDRVAELSAGGPMAQ-----AVGEAQVG-----VEAIAQSFQGL
E-----S---H MVRLGLTPQSGLRARLNESSRAVERELAMW-----
-----PASAPLSLAMARMRLAER-----DFMLDGLED-
-----ALGRHRAA-----TGQLDI-----
-----ALDSS-----AL-PNSTRDDLRLLSAAYGEE-----LQAF AAGS
LA--VKAE-----LAEV-----RSGFLAL-QPVMQRVLA
Y-----AREGMAEAI AA---QEAERRATG--RQ-----VALVGLLAG-LSFL-L-ACLVI--AH
S-ITRPVRLIQDAMERLSHGDHGI AVPGSARRNEIGDMARAV-----GVFKENAIAMVRLQEEQRSIRS
Q-----ADAANRAHLLALAEGFEQAVKSTADLVASNAVGI RHTAERMTSGTDQGKS
SAMAVAEAAARQCRTTVQAVAEATGVLGDSVREISAGTEDAAA IARDAVGRLASTTERIQSLSLVAGRIGRVV

TMIEEIAQRTNMLALNATIEAQ RAGEAGKGF VVAGEVKHLASQTARSTREIAAQVAEIQGATADTEVAIEG
IGDTILRMDRIAGEVADAVSRQ RDVTARIQRCVGEMDRDAAVVGDGVASVTQAAVRYCAAAIRVIWAAKDLA
RPATALNHEVDSFL-TTVRR-----

>Ma.mag.767-amb2788-YP_422151.1--38H

-----MKVR-----SLFVLFVALVAAV-----FL-----
-----VASI-----TMV-----MSAYQE-----RAQVAAAY-----
-----RG-----VG--LQGALLR-----
-----LAERINFERGLYNVSLIN-----KQTLSP-----ELTARLKG-----
-----LADDS---DTSFAAAQAAAKGGA AVNDLLRAQQDRIKASREMVVRQ-----
-----LG-----SGAPEGHA EVAKAYSALNISIGDAIRHEAQGLIPELNKQSGPLSDYVVLAHAAS
RMR-----NLAGQRNT--PLLPVIQAGVQMPDHLE RHYRLNG-----RL
AEAWEDVQAK-----SKTLALSSPE-----IDQGLKNVETAFFGAVESM-----VRELEKAH
-----WQKQ-----PYPF DVN-----DYRTRT-----VEKFATF-SQLGDAYIA
T-----ALRLGEQ----RLAGLSVSL--AL-----LSVGI VVV-ALFC-V-ATIVF--NR
R-VVSP LIGTAGTITHLANDQLDIDIPWRDRGDEIGEIGRAL-----ETLRGNAKAARSVAEDRTRERL
-----ASDEV RHGVDVAVRTFADRIAGLMKSSDDSMGSLRQYARDLSRLAGDASG
GSGEVARAADGAATNVQGIASATSELLSSIGEISQVVADMAGISRQAVDEARASRQAVEELTQSAGRIGEIV
ALINDIAAQTNLLALNATIEAARAGEAGKGF VVANEVKVLANQTGKATEQIQGQVGAIQRG TATASHS IAG
IDSTVGRISEMATS IASAVEEQNAATSEIASSIQQASDGVGAVAGTITHVRENAASTDRAAESVQAAAERLA
ANTAALHQEFDQMV-ELIRRSA-----

>Ma.mag.767-amb2821-YP_422184.1--38H

ME-----

-----L-----LRN-----
-----L-RI-----GRRILLAFLLP-----
-----VFGLVA-----FSGYVIVLRWL VVDDTSGLIRMAATANSV SAVVHELQKERGA
SSLYVASGRKQF-----GDRVEAQ RKLSDE-----AARRFEEQT-SDPVATLGP DFAAS
QAKARDALAGRAKMRADIDSGAMDRNALFQAYTALIKVQLDMVGQMAR-----
-----LTPDKKAAEAVGAYLSFMEAKER-----AGQERATGA-
-----A---GFAGTFDPV-----IYRRLV-----SL
IADQEMLFA-----HFVR-TASPE-----LVAFFREKMGDP IVAE-----VNTLREAA
-----HAKALSG-----GEGVPA-----PKWFEAT-TKRIDL MKV
V-----EDRIATDLVAS----ATKTTS DAR-LFL-----WGQIIAVIGGL-GITF-A-AAGIL--AK
G-ITGPIAHITNAMSRLATGDTAISVEGVDLPTEE GEMARAV-----QVFRNNHLAAEK LAAEQKAEQE
-----AKERRRQAIERLTAAFRQEVSGALAAVG DATRLLNNSANSLGSSAQSMEG
HAAAVAAAAEEASINVETVAGAAEELSASINEISRQVATS AQVSKQAVAEAE RTNALVHGLADAARNIGE VV
TMIGDIAGQTNLLALNATIEAARAGEAGKGF VVANEVKHLATQTARATSQITEQVGAVQAATDQAVTAIQG
IGAIIERINEVSSAIAAAVEEQDATTRDIARNVAEAAAGGTREVS RHVSDVTSEAGETGKTATDVLGAVTALG
SQSDALNTSVQRFL-AGVETA-----

>Ma.mag.767-amb3054-YP_422417.1--38H

MQT

-----TKRGIAM-----LGHRIAT-----
-----M-GI-----VPKIMALA-----
-----VSGVLL-----
-----L-----GL-----SVTLLS-----
-----RSLHSAADAARERVETNMKVAVDVLKS-----
-----KG-----TAFSVA-----DGKILAGDH-----
-----VLNGNFDV-----VDKIKAL-----VGGSA-----TV
FMGDTRVTT-----N-----VIKPD-----GSRAVGTQLAKTAAEAVF-----
-----GRKTSFRG-----EVEILGE-----PY-----MTAYDPI-LDGAGNVIG
VLYV-----GIKKAD-----FLVAAERTL--QL-----MVGVTVLVG-ALSI-L-VSWTIA-KR
N-LATPLKRSILAMRDLADGNLDVAIRHTERRDEIGEMIRAL-----EIFKENGQARHRLEAAQAAEQA
-----ARNRRQEAIERLTTFNSSFVAVLNGVTESAHHLRDSAQSMTSVAEDTSR
QSTVVAAAAEQASVNVETVAAAAEQLAASEHEIARQVANSSEISHRASEEAERVNSIVLSLSEATNRIGQVV
TLINDIAAQTNLLALNATIEAARAGDAGKGFVAVNEVKHLATQTARATEDIVVQINSVQAVTRDAVSAIGG
IGHTISSISESASAIASAVEEQTAATAEIARNVQEASSGTREVTSSITLVNQGAATTGSAAHQLLGTADEL
SQQSEQLATEVSEFL-AAIKAA-----

>Ma.mag.767-amb3067-YP_422430.1--38H

-----M-----LAK-----
-----L-GI-----ANRLLVGF-----
-----VVLI-----
-----AL-----IAGLAT-----
-----FSRISAGDIAAAVAASRRGSDNAL-----
-----ALESIERHIYEARFNIW-----VYFGTDDTS-----
-----RLQKGISL-----TNDLIK-----
-EVEALHAA-----T-----LFAPE-----RQRQVREMAELIAAYKVR-----VEKLSELR
-----ARK-AAF-----DDPELAA-SRSDAG-----KISAAI-DKIGASLSE
G-----YRATSDAEAAQ-----VESLIALVS--RL-----SVIVGLLAT-VFGL-V-LALAI--GR
S-ITNPLGDLVGAVRRLAGGDTGLAVPGVERRDEFGLAQAAL-----NGWRSSIMEAAARAEEERLART
-----QREARQQVIEVATQRFDDQSIETTSLRVKAAVQALHGSADTLSANAEQTQR
QSSAVSTATDEATANVGTVASASTELASSIHEISRQVRQSSDIAQAAETEVMETRKVTGMAEAAQRIGNVV
KLISDIASQTNLLALNATIESARAGDAGKGFVAVVAHEVKNLAQGTAKATEEITLQISAVQAETQAAVEAISG
ISHTISRINELATAIAGAVEEQGAATAEIARNVELANEGTSEVARNIAGVAQAASETGGMAQAVFTSANGLL
DESARLESEVREFL-TSVRTA-----

>Ma.mag.767-amb3102-YP_422465.1--38H

MPNGVI-----LSDRPAGWSKQNRECP-----MRDNGAITNRE-----VELKDDDM
 VSRTDAGGRITFVN---KAFIDISGFTE--QELLGSPHNLVRHPMPKEAFADLWASIKAGRPWEGYVKNRT
 KTGDHYWVRANVTPFMEKGQIAGFISIRSKPTRAQINA-----
 -----AEQAYALFREGKARGLAIRDGR-VVSTGLGGRVSALKN-----
 -----SI-----AGRLSGVL-----
 -----AVLG-----
 -----VL-----MLVVGW-----
 -----LGISGMRGIGEDMREYVLENTMPAIDL-----
 -----AEVADLSQENYRQIASIAV-----QMLANQHWV-
 -----PVSERLAA-----IESNSQ-----II
 NRKWEHYAQG-----SHPAE-----ELALIEKFAKARTAFRDQG-----LIPAVDLV
 -----RKN-----DAAALSK-----HFGEAM-----RPLYNAN-HVALKGLID
 Y-----QSKDAGNTYAR----AEQHLSSRT--FL-----VIGALVVAV-VLGF-I-LAVWI--LA
 G-IRNPLKAMERHFDIAIASGNQAHEIPVADM-QEFGQLHSL------RALKAKL---GYSALEKAELD
 R-----AEENRRAELNRVAKSLEDVRVGVVDLIDVSSGSLLGNAQTLSSANAQQTMV
 QAGNVTAMTGQVTANVQSVSAATQELSSSVTEISRQVAHAATISGDAVRQAGETDRMVRSLAEAAQKIGEVV
 NLITDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKHLANQTAKATEEIGQQVTAIQGETRSAVEAIRS
 ISGTIENISELSSAIAAAVEEQGAATAEAIARSVEQAAHGTASAAENVEVVSAGAAEETKLMSDQVYEAAANGLK
 GASNQLAQEVAGFI-REIRSA-----

>Ma.mag.767-amb3267-YP_422630.1--38H

-----M-----
 -----L-SI-----L-----

 -----NSLHTVYEDRTVCL-----
 -----VQLGTMQRNLFRIVRLL-----K-----MLDLNAYDA-
 -----TMVAQ-----IADAE-----II
 NKEWKAYLT-----T---ELAAE-----EKVIADKIKTALPDYLSE-----KNKVIELL
 -----KAN-----DPDKATS-----LANGPA-----LQKFMHL-DEQIVANIE
 L-----QERIAKEEYEE---GKASASQKI--NF-----SLTLGGLAL-ALGA-A-IAFVI--AR
 S-ITGPVNGIKGCMEQLTAGNLSAEVPGVDRGDELGQMAKAV-----GVFKEGLLRVKEMEAEQEAQKA
 R-----AEAERKAAMRQLANTFEGSVGKVIQTVTSAATQLQVSSTQMASTAAETSA
 QATTVASASQQASANVETVAAATEELSSSITEIAKQVERSQSVASRAQDEAANTNVQIRALSENANKIGEIV
 NLINDIASQTNLLALNATIEAARAGDAGKGFVAVNEVKHLATQTGKATEEIASQIRAVQDGTNNAVHAIDG
 ITKVISEMGEISASVASAVEEQAAATQEIARNVEQAAAGTAEVSSSVTVTEQAARDTGAAAEQIHSAAATDLS
 QQSEFLRAEVGRFL-DQVRSDQSDVKLLN-----WDNALNMGIGNIDR---
 ---HHREMFDEVNRLYAKMSQGNG-----AEAGQGMITLID-----
 ---TVMGSHFREEEVEMSRN-----SYPHLDEH-----RRHRDFLDRAKSHKGAVQSGDPTAAAKMFE-
 -----YVATWLTNH-----IQM
 EDGKLAN---YLKA-----KKVA-

>Ma.mag.767-amb3314-YP_422677.1--38H


```

-----LSSDARVGRMIGAYLKLME-----G-----KEKAGQERA-
-----TAAGAFSSG-KFEPEVYRRFV-----AI
VAEQQLFLG-----E-----FQESA-----APAQVDFFRITLDDEASRT-----VERMRKVG
L-----DSPATGT-----VGDVTG-----PAWFAAA-TSRINLLKK
V-----EDHMAADVQALAAQ-----VNGDARGVL--LT-----TVLGVVAAL-VIAL-A-VAWTV--VR
Q-LTSAIGSLVGTMERLAADDFSVSVGGTERADEMGAMARSV-----QIFKDAMMRGHD LARKQLAEAH
-----NGELRGRAIEALVNRFGSAERMVA AVSSAAQRLQGTAAAMSAAAASDTND
RATVVAGATELASANVQTVAAAAEELTSSIQEIGRQVHRS AEISGNAVAEAGDAQNDVTGLATMVGKIGEVV
TLINDIAAQTNLLALNATIEAARAGEAGKGF VAVAGEVKNLANQTARATGEIGQQISSVQQQTEKVVGAISD
IVTIIREVGEITASIASAVEEQSAATQEIARSVEQAAAGTAEVSANVGGVQDAAGRTGGA AVEVLDASRAMG
EEAVNLKTTIDGFL-GEIRAV-----
-----
-----
-----

```

>Ma.mag.767-amb3467-YP_422830.1--38H

```

-----M-----AGR-----
-----L-RI-----GVKIQLLV-----
-----GVMA-----VV-----AVVIAA-----
-----LGQWAISSYDSRTDAILDASRRAA-----
-----FGQKAGSAIYAIVMSR-----GVYMARNAD-
-----EVRKFAEP-----MAKSLD-----AF
EKMAAEWRG-----VVP AE-----QTAEFAKL N KELVDFIRF-----RRELIRLG
-----LEQ-----GAPAA RV-FGDNDA-----N-----RSNRQKL-GKLVDEYSA
M-----ADREVDENHLA-----MDAFAGQLK-IGL-----LLGALTALL-GGGI-V-TLIMV--RR
S-VSGPLGRVAAATHRMAAGYLDEPVADAERGDEAGEIARAL-----QVFRDTMRRTRELEAAALAEQR
-----RRAERA EYLERRTAEFDRTVQSL LGDVGEVVRTVSTTADNLHRNVDDAGH
ESASVAVAAAQSCANVQEVASATGQLRSSSMGIAERVRETTAIVRETVDVMEGAGATMASLDDGAKRIGEVV
SLINDIAAQTNLLALNATIEAARAGEAGKGF VAVAGEVKNLANQTARATDEIGRQVAAMQDTVAQVVATNHR
VAESIGRIESVAGAVASAAVEQGHVTD AIVQNVQEA AQGGRSVTDSISKVSRQVEAGGAMAGDLAHMAHSLA
DRAQSLRAAVTGFL-VDAKTV-----
-----
-----
-----

```

>Ma.mag.767-amb3474-YP_422837.1--38H

```

-----MDLEF-----LTG-----
-----ETEM-RI-----GKRLWLGF-----
-----GILC-----TL-----IAVVTG-----
-----TII FEALGVNKAADRMIGLRMPVA-----
-----QTSAGIEGHMYASLAALR-----GYLLTGKDA-
-----FKAERAEA-----WGD-----L--D-VL
IADMDKLAL-----R-----FTNSA-----NQQAWKDARTL LLELKAA-----QAKAEGLG
-----QGEEAIK-----VLVTEAVPRVTRLVT-VFSGEKAADGKRSGGMID

```


N-----QKRMLETDATE---VEGLVNTMI--TV-----AVGALIAGL-LIAL-V-TALRT--AA
S-IVPPLVAMTGAMDSLKGDTSVAIPAASTADEVGEMAAAM-----EVFRANLIHQRELEEKQKQADE
-----AARQALRIASLTTFDNAASAMVQQVAAAAGQLQATAGAMSATAQQTSH
QATSVAAAASEEASVNVQTVAAAAEELSGSINEIGRQVAHSSAISQDAVGEATKAADVNNELADTAQKIGEVV
SLITDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKNLANQTARATDDIGRQIAAIQDQTQRVVDTIGA
IVKVIEEIGHISGDVAASVEEQSAATQEIARNVEQAAAGTAEVSGNVVQVQDAADQTGTSSREVLEASRTLA
EQSGNLRSTIEAFL-KDVRAA-----

>Ma.mag.767-amb3539-YP_422902.1--38H

-----M-----SDGVWQN-----
-----L-SL-----RRRFMIVV-----
-----GLGVIV-----
-----MA-----TVIVVF-----
-----IARFEERALERKLHEL SVNEMTSLHALI-----
-----VNVMATRPE-----
---DGD---NI---GIKVFENKW---FDSRNV---HYAGKVWSVWG---PKV
TAHMKDVEP-----DRAPKAAQDDIDREALATGKPVGRLVGDSYRYAMPI-----VLGVTDGA
-----RDEV-----C-----HSCHGGM-GMTDGEVIA
V-----LSSSLSTTA-----EKAELNNVL-MGL-----FAFGVLATL-ISVL-G-VKSIL--TR
V-ITRPIGRITSLMGRADGDTSVIEAQERRDEVGDMARTV-----QVFKEHMLEAEHLRAAQEQERI
R-----AAEERARTMRDMADHFEATVKAKVAEVEESTTGIHKTAMNMATRSGQSGS
RSLDVSEAAANITTERAGTVSDATRQLALSUNEIAQQVGESSRIAQQAVANVNNTASQMSGLSDSVQAIGNVV
QLINDIAAQTNLLALNATIEAARAGEAGKGFVAVNEVKNLANQTAKATEEISNQVSAVQTSTREMTASIEG
VVETIRSIDNISTAIAEAVQHQETTTTRHIASNIDEVAAQAREVSSSVSTLSRSSARSACAGSVRVIWSAKTLA
KAVSDLKTEAEDFL-RSVRG-----

>Ma.mag.767-amb3554-YP_422917.1--38H

MR-----VNEPITNHE-----IELPEGTIL
VSKTDLGGRITFVN---QAFIEISGYTE--EELIGAPHNLVRHPMPVAFADLWNTIKAGKPWEGIVKNRA
KNGDHYWVRANATPEMEGGQITGYISIRTAPTRAQVEA-----
-----AEHLYEQVRSGKARNIKVEEGR---VLSTTAAARLGR-----
-----ALNSI-----SGRLALII-----
-----AIMV-----
-----MV-----MGTVAW-----
-----FTLDGMADSNEALKRVYQQRTVPAGQF-----
-----VEIIDRMRENLLL VHQM-----QIDLNNGQA-----
-----DAIPRRTL R-----VRANAE-----HI
SAVWSAYRA-----N---PLPAE-----EAEAGDFETRKRKDFVEQG-----LLVALDLA
-----EKG-----DAATLGR-----HIATGV-----QPQFEQV-HEVLKDLS
L-----QLRQASELYEG---ARTDYGHM--RL-----GIGTLVGGT-LLAT-L-MALVL--IR
Y-LRRPIATLEYHFDIAIARGDFTHEIQA---EEVREFQRSS-----ALLRAMEAKLAYAVQERAENAR
K-----AQEKL RSEMLNLTELLEGEVDNTVAEISTQAERLKEGAHLLATAEELRD
KSETVARAIEITSGNVQTVAGATEELEASSREITARIQSSSEHSEAARHRVDEASSSVGTLTEATARIGDVV

SLIQAIAGQTRMLALNATIEAARAGDAGKGFVAVASEVKGGLAEQTEDAIGRVNAQARDIESTTQKAVATVEA
VADTIRDMEQIAGQIAASADEQRAATGEIMSSAVQAADHTRDVADAAADV LKASEQTGVTARKVSELSMLVN
HDIGALQRRNLNVIL----RTSYGG-NRR--AVERIPASLEFTATFA--GRTFSGHTGDISLMGALLVVGDA
PKLEGEIGSIT-----FPGVGEIPTKAILGSRLGIQCQYLKVGQDIKQRLSAAIQAAQANDAPFIATA
QKAAADVVARAFEQAVNAGRI-----SLAELFECEYTPVLDTDPQOMLAHHTQVADQVIPAIIEPALASDSR
AVFCCVADR--NGYIATHNK-----IYSQPQRPNDPVWNTANCRNRRMFDDRTGILARNT-KEYLAQT
YARDMGGGNFVVLKEIDCPINVGNRHWGSVRYGIKL

>Ma.mag.767-amb3690-YP_423053.1--38H

-----M-----SLGN-----
-----L-KI-----AARLMVGF-----
-----GLLV-----
-----VL-----IASLSG-----
-----YAVHSGRANLESVGNVFSFLDNGA-----
-----LMERIDKRMFSGRMQLW-----IALATGEPT-
-----YWEKSDAD-----FARSRE-----
--NLKKLIE-----N----TSHPE-----RRAKAEAVSRDFEYLVK-----VAKLRTIG
-----GKN--PAL-----ETPEAKA-----LAADV-----MSARVKL-DEKAESLTT
E-----YRQAGTEQGEV----AVKSINRGI--DL-----SILVGAI SV-VIGM-V-LAFFI--SR
S-ITNPVNAMTRAMGVLAGGDT SVEIPATGQKDEIGEMAHAV-----QVFKDNAIRVKAMQKEQEDAKA
R-----AEAERKQAMLMADSFESAVMGLVKGVSAQASEMQATSQSMSAGAQQTSA
QAATVAAAAQEATANVQTVAAAAEELSSSISEISRQVAEAAQVSATASEETARTNAMVQGLAHAADKIGEVV
SLINDIAAQTNLLALNATIEAARAGDAGKGFVAVANEVKNLANQTARATEEISSQVGSVQEETRRRAVD A I R N
IATVIEQVQQISSG IASAVEEQGAATAEIARNVQEAAKGTQDVSANVQGITQTASETGAASQQVLAASGELA
RNSETLRAEVDKFL-DGVRAG-----

>Ma.mag.767-amb3793-YP_423156.1--38H

MAGR-----
-----AGPAGASGKGSRPRQGESKKGKVDIQP
DVE-----IPIRFLS-GGDFAFSTDKWNTNRNHIGHWYVSWSAN-FRETPTLPSHK-----IIMT
FALELTNNNRPVILPRIGDASP--GPRRAAM-----FAN-----
-----L-KI-----GTRLFAGF-----
-----ALVL-----
-----SL-----TAFLGV-----
-----NADIAGDALSEQTNKLYRHPFTVT-----
-----NALQAANTHIVAMHRSMK-----DVALAKTPE-
-----ELDRAVAD-----VDAREK-----KVYE-----KF
ALARERFLG-----DKADMEAAAKAFADWKPI-----RDEVITAF
-----REG-----RRDDAAT-----ITKTKG-----AAQVAKI-SETLEKVIA
W-----AMDKAEAFMAN----AEQVNDRAD--LI-----SRSGLAAAL-ILGA-L-VAWLI--TR
S-ISRPINAMTGVMNELSGNNLSVDVPYADRGDEIGAMAKSV-----GHFKNQLMRVHQLAEAEQEEQKK
R-----AEADRMTAMRKMADTFEESVGKVIETVTSATELQAASSQMSGTATETSA
QATTVATSAHQASANVQTVASATEELAASIREIAHQVERSQA VSAHAGQEVNTTTSQVRALSES VSKIGEIV
NLINDIAAQTNLLALNATIEAARAGDAGKGFVAVANEVKNLANQTARATSEIAGQIQAVQDGTNAAVHAIDA
ISSVIGEMGEISSAVAAAVEQQSGATTEIARNVEQAATGTEEVSSNITSVEQAARETGAAAEQIKDSSADLS
RQAEFLRHEVGQFL-AQVRADKKDKMLLV-----WDGALNTGVASVDR---
----HHQAMYDQVNEFYRQMMSGDG-----SRAAAAMLAE LN-----


```

-----
-----
-----
-----MSFW-----
-----TI-----TRKLQAMT-----
-----VLML-----
-----LL-----LTATSG-----
-----FGILGMSRMHDGFRAVTEDTTPAL-----
-----IHLSGTVDALHRIRVRVI-----GAAMETDSA-
-----RIAALRDE-----YVKQLA-----DL
NKTWNAYAA-----S-----RLSPE-----EAALAREAEETGIKSYQGFI-----QESWARIA
-----AGE-----ANGVLAD-----LVGGKG-----VDKFREA-ATPLRKLLD
F-----QSREAATMFAE-----GEQNYDRDR--SA-----SLGLVGLGL-VLGF-A-LSALI--GR
S-VARPIHRIIAVMGRLAANDTQVEVTGQDRGDEVGEIARAV-----QVFKLNALEKGRLEAEAEQENKR
L-----AESRHHQEMKSLAADFESGVSVVVDKVAGASAEMEHTAQALSTLSEQVAV
RAEEVAGASEHAAANVETVAAATEELAASVAEIGRQVSESARVARDAVDEAAQANTIVHGLSEATKRIGEVV
TMITDIAAQTNLLALNATIEAARAGEAGKGFAVVAGEVKNLANQTARATDEISSQIASVQAETGRAVDAIRG
VGTTIGRIDEISAAIASAVEEQGAATREIARNVEEAAQGTQSVSGNIAGVTSAAARDTGQASATVLASATELA
AEAHTLRRIVQDFV-AKVRAS-----
-----
-----
-----

```

>Ma.mag.767-amb3970-YP_423333.1--38H

```

-----
-----
-----
-----M-KI-----AIRLWLIV-----
-----ATAL-----
-----CG-----
-----IAAVVAASLAQMNHDLMDREVKT-----
-----RHIVEVGQSLLSHYEEMER-----AGRLSRDEA-
-----QAAAIAAV-----SKLRYE-----GTEYL-----WI
HRLDESVML-----S-----HPNAK-----L-IGQPILDMKDPNGLFL-----FREMNRMV
-----KEKS-VGFVYYMW-PKPGATE-PVAKLS-----Y-----VQGFAPW-GWVIGSGIY
I-----DDVSAAFRTRA--VE-----FGAAALGIL-ALVW-L-IAAWV--GR
G-ITRPLGDVTGALDRLTSDDHSVEIRHTGRTEIGALARGL-----KVFQTHIEAAARAAEEKLRQQE
-----ADLARQSRIAALTSEFDAKVAGVIKSVGAAAAQMQSTSQSMSAIADQTAR
QSTAVAAAAANHAAMSVQTVAAATEELHASEAEIARQVEVSTSRSTAVQEAERSSEIVSGLNSAAGRIGEVV
ALINDIASQTNLLALNATIEAARAGEAGKGFAVVANEVKNLANQTARATDEISSQIGEVQSAATLAAEIAA
IVNTIAEIDETSSVVAIAVDQQAATTEIARSIEQAATGTAEVSNNIGEVSDAARHAGSTASEVLAAAAELT
GQADALRTEVEQFL-NAIHQQRPTQAP-----
-----
-----HLHLVAAE-----
-----
-----

```

>Ma.mag.767-amb3991-YP_423354.1--38H

```

-----
-----
-----
-----M-----HSGLTiarLTN-----
-----

```

```

-----LFAV-----
-----SLVA-----
-----GFLAIMAAGY-----
-----LAIGELKVGGPVYQRIVQGKDLVA-----
-----DILPPPEYVIEAYLE-----TNLALANPD-
-----SLPERKAA-----LAKLRK-----EY
DERHAYWLA-----Q-----DMEAG-----LRDRLVVGHAHAPAMRLWAVI-----DQGFLPAL
-----ERG-----EMDAARA-----AFAEI-----SAAYAABH-RAEIDEVVK
G-----ADKLTDTTEAY-----AAQREQTFM--LI-----LWSVSGVVL-AVTL-A-GVAGV--IF
G-MVAPVKAMTGAMTALADGELSTRIPSVRRGDEIGAMARAV-----EVFKENALKILQLRQDQAEAEA
R-----AKVEQRQSLHRMADDLENKVGEVIGVVASAATQLQASAQGMSSVSEQTVR
QSSAVAAASEQAAANVSTVAAASEELSASSREIGSQVSLASAIQDAASEAATTTDLVRGLAEAGRIGDVV
SLINDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKTLANQTARATEEITAQINQVQDRDQAVEAIA
IARTIERMNEISGAIAVAVEEQGAATQEIARNIQQAHVGTSEVAGNIAGVSDGAQESNAAALHVLDAAGALH
RQTDTLRGALGEFL-SGVRAASASS-----

```

-----C-----

>Ma.mag.767-amb4076-YP_423439.1--38H

```

MS-----RVLSR-----
---FSLKVQIGSL-----VALAGLVL--GICMAVL-----WTARGATEEVNHHNLNRETAIA-
-----QRAASLDSALL-----NARRNE-----KD-FLLRRSDK-----
YAAEHAKTIA-----AALAAL-----AGIAEALPE-----
-----GDGRRLQLEAV-----RKG---AADYTQAF-----
-----RTVT-----
-----ENEIR-----
-----VGLSEKDGLLGLALRTSVHEVETALK-----
-----AHDEPRLQILMLMMRRHEK-----DFLARLDSK-
-----YMDDLAKRGTE-----FDKALA-----
-----A-----SA---LSSAD-----RAPIAERMAAYQRDFRLA-----AQAMLDLR
-----DSAKVM-----SETYAAM-APAVQALGD
A-----ARQGMEEARDE---ADRVNLSAG--RA-----MTWVMLAGF-AAML-V-IGTAI--AR
S-IYRPLTAMTGVMGRLAKGDMTVEIPDQDRGDEVGAMARSV-----QRFKEDLTEVERLRAAQEEERR
K-----AERDKIAALQGMAETVERETRAAVDRIAEMTRMADNAGGMALSASAVGD
NSQSVAAAATQALANAQTVAAAEEELSASIEIANQVGTASTVTSTAVEASNQASRTISQLSETVNRIGEVA
NLINDIASQTNLLALNATIEAARAGEAGKGFVAVNEVKLANQTAKATEEITAQIVAIQTTTQDAVRSVGE
ITQAIGDVQGVSAAVATAIEEQGAATQEIARNVAQTSDAANEVADRIAKVSTEAHTGERAAQVGSVSSEVA
GGIDHLREILIRVV---RTATKEVNRR---HKPRYRLDADGTITAG--GSVMAARMVNCSEGGFTAAGDFP
GLRASSRVELT-----LPGIP-----FPLAAVIKDFEH-----
---GRLHGKFEFPEAQMGN-----WTEAFARLVAGLSPLEDAA-----

```

>Ma.mag.767-amb4156-YP_423519.1--38H

```

-----MRNPLSRFS-----
---LKIQIGSL-----VALAGLVL--STLLAVD-----LMEQSIADRANAIAARETIIG-----
-----DQAGAVDRALL-----NARRHE-----KD-FLLRGEAK-----YIA
QHAESIRAAARAALDG---MTSAMVTDDVRHPQ-----IDA-----
-----VRKG-----LTTYVDFAF-----
-----RV-----LTERHILV-----
-----GL-----TVNDGL-----
-----TGALR-KSVHEIESALKSHDDPPL-----

```


K-----PAAVVEIVMDS--RDYAAEYVEAR--TN-----AIIGAVIIL-ALGL-L-AVWLL--AR
G-ISLPLQGITRVMHTLAAGDLSVEAPFTHRGDEVGEMARAV-----EVFKRNALDKRRMEDEAQELRR
-----QEDLARAARDEAAAAEHALGVQAKVASVDKATDAIRSTAKAMSQRSERSGS
LSLDMGDAAQITSERATVVSEATRQLALSVD EIAHQVSCANDVTQKTVASVEETASQMEGLSTAVRAIGDIV
HLISDIASQTNLLALNATIEAARAGEAGKGFVAVAGEVKHLANQTARATEDISRQVEGIQDSTQGMAERIGE
VVGLIRTLDGVS SAIAGAIQQQDAATREIASNVEQVARQADKVSGAVS QLAQASAKTCAGTIRVMWSAKGLA
QTV DGLTSETESFL-GRLHGQ-----

>Ma.mag.767-amb0728-YP_420091.1--38H

-----MNRIG EVA
NLIQDIASQTNLLALNATIEAARAGEAGKGFVAVANEVKNLANQTAKATEEISAQIGEVQASTVDAVASVAE
IAAAIKEVEGVSVSVAGAIRQQDAATQDIARNIAQTTSAREVAERIAEVSEEAGITGERAARVNHISVDVA
RSIESLKSIVRVV---RTATKEVDRR---NAPRYALGVKGLVDMG--GAEVPVVFDEVSEGLTIQGDVC
PVAKGMRVKVR-----IEGCS-----VALSMVGRECEN-----
---GRLHGNLELTPDVETR-----WQEEFQRLILGRHPIRDAA-----

>Ma.mag.767-amb2994-YP_422357.1--38H

MP-----PP-----RPP-----

-----TTSI-----

-----PVAAGAEELSASIKEIGRRVSHSSSMARNAARESEEATEIVRGLAEAAQRIGEVV

DLINSIAAQTNLLALNATIEAARAGEAGKGFVAVAGEVKTTLATQTAKATEEIASQIGVVQSRTSAAVGAIAH
 ISGTISEIDAISSAEITQAVDQQDAATQEIARNVQQAAGVGTVDTSIQSVSAATSSSGQIATDVLDSARQLV
 AKADSLHAGVDSFL-AAIKNEEQITHSDD-----LAFADYVRA-----
 --GAADLARRLEGAVERGEI-----GLDDLFDDSYQPIPGTSPQQVTTRFTELADRLFPEIQEKALVQFPK
 AVFCVGVDR--NGYLPHTNA-----KFSQPQ-GNDPVWNDANCRNRRMFTDPTGLAAARNAAKPSLVQS
 YRRQMGDKS-VLMIDVSSPIMIRGRHWGALRLGYAV

Supplemental Dataset 2- 36H Receptors

>Az_bra|GCF_001315015.1-AMK58_RS02395--36H
 MAKGVGSFVGSMSGISKRLWLAFALLLALLAAQVAASLLSIRSLNGSVDAVLTGGELATFAKDL SARLANQRI
 HGRDYLVS GDPAALNRQRA-----LRTEFDQALAAHEGAIQRSSQAAFAELARLHSEYHTQFETIRVV
 RDRYDAAIRQTMDPLGTQITETLERVVEGAQATGDKDAALVGEEMEKHWVLSRLFANRALGMRDAAAAAPME
 KNLDLEMLEHVREAQALLTEPRVAALLREVAERAPGYRAAFRDAVAADKEIDRLRAEIVAKIVTALTATIDSI
 AIAIQAEQK-GIETRAEAEVENS VLLALLLGGLSLLLGIGAAQVIARSITGPVQGIRKVM TDLSDGHL SVTV
 PHTDGRDEL GAMARAVAAFKEDAVTAVRAGIALDRVSSC IMTVGEDGTVTYCNAAALAMFKA AESDLRQALP
 AFDAAGLIGQTL DALDTEAPRLRSSLSGLASPYKGMVKTGRRSFEITASPVAGR HGEKLG LVVEWRDLTAE L
 SIEAEIAGMVDSAVRGDFSQRIALDGKSGFFRLVSEGMNRLAGNVSGVTEDLAAMLESLSQGDLSRRIDKPY
 EGVFLRLKDDFNGTAEKLSEIVRRINGAAESIAAASREIADGSLDL SERTEQQASSLEETAASMEELAATVR
 SNADNAQQVNAFANDARRAAERGGQVAASAVEAMRGIERSSQKISDIIGVIDEIAFQTNLLALNAAVEAARA
 GDAGRGFAVVAQEVNLAQRSAQASKEIKTLILD SGAQVKDGV ELVRSAGSSLT DIVAGIARVADLVSEIAR
 ATAEQASGLDEVNTAVAQMDEMTQKNAALVEESTAAARSLEE QAGQLRQQMAFFSLD-----AGHAHG
 GSELSRGRAA-----

>Az_bra|GCF_001315015.1-AMK58_RS20975--36H
 MLK-NLSLTAKLSLLNVLGLFILAIVLT LASSQVLSDRSAASAREQRERSMALAHMLLKQNGADYSLRDGAL
 YVGSVRIDGDTRMVDQIRELTGGAATVFR-----GDTRVATTVQN-----
 -----PDGSRANGT-----KLGAGPVF----DSVITQ--
 -----RKP YRGEADILGERYFTSYDPLLDASG SVV-----
 ----GVL FVGLKRADVLRVVD EVERTILVVAPLVTLAFGLVSFFLVRRQMGAVRAMSATMHDLAADKLDVAV
 PGLDRGDEIGQMAQAVQVFKD NAIQK-----KAMDEAERERLE
 AERRA-----DEAQRAR-----ET
 AIGEEIAALIDGVSKGDL SRRLDLTGKDG FYKTMSAGINRLTDTVEAVIADLGAVLSALA QGDLNKRVERDY
 EGAFQTLKTDVNATS AKLSEIVGQITQSADTIASAAGEVSIGSSDLAERTEQQASSLEETAASMEELGATVR
 SNADNAQRANGMAADARTAAESGGTVADSAIDAMKRIEASSRKITDIIGVIDEIAFQTNLLALNAAVEAARA
 GDAGRGFAVVAQEVNLAQRSAQASKEIKGLI LD SNAQVKDGV ELVKKAGDALEGIVSGVQQVAGLIAEMAS
 ASSEQAAALDEINATVSQMDEMTQKNAALVEET TAAQAMAGQANDLKGLIGFFKLDPRAMPVAVPV-----
 -----AVATTAAAPRHVAPVVRP TRAAAKPSARAVSGAALRRNVSEDDDWKEF-----

>Az_bra|GCF_001315015.1-AMK58_RS04195--36H
 M-Q-TSSLAGRFKVGTRIYFGFLVVL LLLAVVVAIGVRGLGVARDGFESYAAVSNHSIQVSSIDAQVAQMRR
 LALTFTNTFGDPKVADQVRVQATLVKDLRSALDGT TGERRALLERMNQVFA-----SYVTDFTNLAE L
 RLRRDRLYAEQLVPAGEKARETVS QLVSTLIADGEHEAANAALAEQQLMARLAASRFFTS PNESIITEVS
 ARDDAFGEAIRRATERLNNPARRAAALADQLSDRYVSLFRETA AVMLENTRLVDVMGARGVE--FADLSDR
 TVAIEGKDRDGVLAETTGSM DRTL SANVTIAAGAF LFGLLAWAVARSIVKPVVSM TETMTNLAGGDLTVTI
 PALANRDEIGRMAQAVQVFKD NAIQK-----KAMDEAERERLE
 AERRA-----DEAQRAR-----ET
 AIGEEIAALIDGVSKGDL SRRLDLSGKDG FYRTMSEGINRLTDTVEAVIADLGAVLSALA QGDLNKRVERDY

QGAFQTLKTDVNTTS AKLSEIVGQILRATDAISGAASEVSLGSSDLAERTEQQASSLEETAASMEELGATVR
SNADNAQRANGMAADARTAAESGGTVADSAIDAMKRIEASSRKITDIIGVIDEIAFQTNLLALNAAVEAARA
GDAGRGFVAVVAQEVRNLAQRSAQASKEIKGLILDSQVKDGVVELVKKAGDALEGIVSGVQQVAGLIAEMAS
ASSEQAAAALDEINATVSQMDEMTQKNAALVEETTAAAQSLANQATDLRSLSVFFKLDAAAF-MAAPAGHQGG
GAPALRRSIAPTKPAP--AKPAARKAAPPARPAAGKAASATLQRASADEDDWKEF-----

VITA

Lindsey O’Neal was born in Sparta, TN. She attended White County High School and graduated with honors. She then continued her education at Maryville College in Maryville, TN where she received her Bachelor of Arts in Biochemistry. At Maryville, she earned the honor of Exemplary Senior Thesis for her work with Dr. Angelia Gibson on the thermostability of luciferase, the enzyme responsible for bioluminescence in several bacterial species. Lindsey then chose to attend the University of Tennessee at Knoxville for her doctoral degree. There, she joined the lab of Dr. Gladys Alexandre and worked to understand chemotaxis of soil bacteria. She has presented her work at several conferences including Bacterial Locomotion and Signal Transduction, Gordon Research Conference for Sensory Transduction in Microorganisms, and the annual Women in STEM Day at the University of Tennessee. While at the University of Tennessee, she received several honors including the Robert and Nell Keenan Fellowship and a UTK Summer Research Assistantship. Along with her scientific publications, she has also published articles for SisterSTEM.