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An Ethylene-Binding Protein, AzoETR1, in *Azospirillum brasilense* Sp7

Tiffany Nicole Thoms
University of Tennessee, Knoxville, tthoms@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Tiffany Nicole Thoms entitled "An Ethylene-Binding Protein, AzoETR1, in *Azospirillum brasilense* Sp7." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Gladys M. Alexandre, Major Professor

We have read this thesis and recommend its acceptance:

Brad Binder, Dan Roberts, Sarah L. Lebeis

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

An Ethylene-Binding Protein, AzoETR1, in *Azospirillum brasilense* Sp7

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DEDICATION

For my family, my friends, and especially my daughter.

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ABSTRACT

Ethylene is an important phytohormone produced by higher plants where it plays numerous roles in growth and development and in mediating responses to many environmental factors. The genome sequence of the soil alphaproteobacterium *A. brasilense* suggests that it encodes a homolog of an ethylene-binding protein (AzoETR1), similar to that found in plants. AzoETR1 also possesses a conserved histidine kinase domain with the *azoETR1* gene encoded upstream of a putative response regulator. Therefore, this ethylene binding protein may be part of a two-component signal transduction pathway. Recombinant expression of the ethylene-binding domain of AzoETR1 in *Pichia pastoris* followed by an *in vitro* ethylene binding experiment confirmed ethylene binding, which suggests that this domain in *A. brasilense* could bind gaseous ethylene. Our current goal is to identify the role of ethylene sensing in the life cycle of *A. brasilense.* Given that *A. brasilense* colonizes the rhizosphere, we are testing the hypothesis that binding of ethylene to AzoETR1 modulates plant-microbe interactions in the rhizosphere. We focused on motility and growth because ethylene perception is implicated in modulating motility and chemotaxis in another bacterial species. In the cyanobacteria, *Synechocystis,* ethylene is implicated in phototaxis. Our objective is to expand on the known functions for ethylene by uncovering new information on the effects ethylene has on soil-dwelling bacteria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AzoETRI	*Azospirillum* Ethylene-Binding Receptor 1
AzoETR1 RR	*Azospirillum* Ethylene-Binding Receptor 1-Response Regulator
PGPR	Plant-growth-promoting-rhizobacteria
ISR	Induced systemic resistance
TCS	Two-component system
HK	Histidine Kinase
RR	Response Regulator
REC	Receiver domain
CheA	Chemotaxis protein A
CheY	Chemotaxis protein Y
IAA	indole-acetic-acid (auxin)
ABA	Abscisic Acid
ACC	1-aminocyclopropane-1-Carboxylate
EBD	Ethylene-binding domain
ETR1	Ethylene Receptor I (*Arabidopsis thaliana)*
ERS1	Ethylene Receptor Sensor 1
ETR2	Ethylene Receptor 2
EIN4	Ethylene Insensitive 4
ERS2	Ethylene Receptor Sensor 2
AHL	*n*-Acyl Homoserine

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

One of the most profound revelations in recent biological research in the plant and microbiological sciences is the importance of plant-microbe associations that affect the overall health of higher plants. The presence of certain microbial communities in the rhizosphere directly affects host plants, and over time plants have evolved to cultivate beneficial rhizosphere microbiomes (Haney *et al.*, 2015 and Bakker, 2014). The rhizosphere is the interface between plant roots and the soil that directly surrounds roots; it is composed of three regions, the ectorhizosphere, defined as the region of soil that located directly adjacent to plant roots, the rhizoplane, the root surface, and the rhizodermal area, which is the inner area of the plant root (Benizri *et al.*, 2001). The rhizosphere is of profound interest because it is the place where symbiotic, commensal, and parasitic relationships are forged, ultimately influencing plant health (Berendsen *et al., 2012*).

Plant-growth-promoting rhizobacteria

Soil is abounding with various microscopic life forms including bacteria, fungi, protozoa, and algae (Glick, 2012). The rhizosphere is an area swarming with intense microbial activity driven by plant root exudation (Bowen and Rovira, 1999). Comparatively, the soil surrounding the rhizosphere has less microbial activity due to a general lack of nutrients in the bulk soil. Plant-growth-promoting-rhizobacteria (PGPR) are the soil-dwelling bacteria that pervade all regions of the rhizosphere, and share a common ability to provide beneficial effects in terms of growth and inhibition of the proliferation and deleterious effects of plant pathogens (Benizri *et al.*, 2001 and Ahemad,

2015). PGPR are a diverse group of rhizobacteria that include, but are not limited to, *Azospirillum, Azobacter, Bacillus, Burkholderia, Enterobacter, Herbaspirillum, Klebsiella, Pantoae Pseudomonas, Rhodococcus, Serratia, Streptomyces,* and *Rhizobia* species (Glick, 2012, and Babalola 2010).

PGPR as biofertilizers

PGPR are an effective tool used to increase plant growth and provide greater yields of many agriculturally and commercially important crops. PGPR, used as biofertilizers, have become a particularly attractive prospective alternative to traditional chemical fertilizers (Adesemoye *et al.,* 2009). Chemical fertilizers are hazardous to the environment and cause a host of negative side effects (Adesemoye *et al.,* 2009). Fertilizers bring essential nutrients, but typically in excess thereby causing an overall imbalance of nutrients in the soil. Nitrogen- rich fertilizers break down into nitrates that accumulate in the soil and travel deep causing groundwater contamination (Walsh, 2008). Water-soluble nitrates pollute our fresh water supply with devastating effects for many aquatic species including fish and crustaceans (Walsh, 2008). This not only affects ecosystems but also societies that depend on the water supply as a food source. Many smaller communities also suffer economically because they depend on a supply of fish as their primary source of income. An example is the "dead zone" in the Gulf of Mexico (Malakoff, 1998). In this area large amounts of excess nutrients from chemical fertilizers are washing into the Gulf of Mexico from farmlands across the Mississippi basin causing an influx in plant growth, but consequently massive oxygen starvation, which leads to essentially lifeless areas in the gulf (Malakoff, 1998 and Rabalais *et al.*, 2002).

Biofertilizers can remedy many of these undesirable effects. Biofertilizers do not cause an excessive release of specific nutrients. Additionally, biofertilizers often have beneficial effects on plant growth beyond the provision of essential nutrients. Due to the importance of maintaining the integrity of natural ecosystems and the high demand of producing sufficient crop yields to feed the world's growing population, commercially available PGPR biofertilizers have become an increasingly important in today's modern agriculture.

Mechanisms of growth promotion

PGPR can employ different physiological and molecular methods in order to facilitate plant growth (Lutenberg and Kamilova, 2009). For decades scientists have been able to conclusively prove that PGPR strains can promote overall plant health by activating a host plant's immune system (Pieterse *et al.*, 2014). One way that PGPR enhance plant growth is by mediating defenses against invading rhizosphere pathogens such as fungi. *Pseudomonas fluorescens* confers resistance to carnations against the fungal pathogen, *Fusariam oxysporum* when these PGPR colonize the root surfaces of the plant (Van Peer *et al*., 1991). In cucumber plants, induced systemic resistance (ISR) was demonstrated by root colonization by *Pseudomonas* and *Serratia.* These PGPR species cause a significant reduction in disease when the plants were exposed to *Colletotrichum orbiculare,* an anthracnose pathogen (Wei *et al.,* 1991).

Rhizospheric nitrogen is a limited nutrient and provision of this essential nutrient generally promotes plant growth. Aside from photosynthesis, nitrogen acquisition and utilization is the most important factor for plant development and growth (Pankievics, *et*

al., 2015), explaining why nitrogen is widely used in agricultural fertilizers. There is convincing evidence that the presence of nitrogen-fixing bacteria enhances plant growth under conditions where lack of organic nitrogen is a limiting factor to plant growth. Pankievicz *et al.* (2015) recently showed that it is not simply increased gene expression for the nitrogenase that accounts for growth enhancement in biological nitrogen fixation *in planta.* Using $^{13}N_2$ these authors measured the amount of nitrogen fixation and the incorporation of $^{13}N_2$ post-inoculation of *Setaria viridis,* a model C_4 grass, with *A. brasilense* and *Herbaspirillum seropedicae.* The uptake and incorporation of the $^{13}N_2$ in *S. viridis* were determined using radiometric analysis and decay on different plant tissues (Pankievics, *et al.*, 2015). Pankievicz *et al.* found an improvement in the height, seed number, shoot weight, root weight, root length and lateral root number in *S. viridis* that was inoculated with *Azospirillum* and *Herbaspirillum.*

PGPR directly promote plant growth by either synthesizing plant growth promoting substances or by facilitating nutrient uptake from the environment. Phosphate solubilization is a common property of many PGPR (Babalola, 2010 and Ahmad *et al.*, 2008). Additionally, PGPR aid in plant growth and development by producing siderophores, by producing plant phytohormones (Babalola 2010, and Ahmed *et al.*, 2008) such as auxins (including indole acetic acid (IAA)), gibberellins, and cytokinins, and by lowering the concentrations of ethylene in the rhizosphere (Ahmad *et al.*. 2008).

PGPR can enhance plant growth by competing with pathogens for nutrients in the rhizosphere. *P. fluorescens* uses the siderophores it produces to capture iron from the rhizosphere, thereby making this essential element unavailable to phytopathogens such as

fungi. Iron sequestration by siderophores limits the available iron in the rhizosphere and serves to protect the plant from pathogen infection, which indirectly promotes plant health (Ahmad *et al*., 2008).

Azospirillum brasilense, a model PGPR for promoting growth of cereals

The alphaproteobacterium, *A. brasilense*, is a highly motile gram-negative bacterium capable of nitrogen fixation. It is closely associated with plant roots and classified as a PGPR (Steenhoudt and Vanderleyden, 2009). Several bacterial species in the genus *Azospirillum* are commercially available as biofertilizer inoculants to increase the yield of grasses and cereals used in food production for the world's ever-growing population (Okon and Itzigsohn, 1995). Within the rhizosphere, *A. brasilense* is thought to influence plant growth with reported increased crop yields up to 30% (Pedraza *et al.*, 2014)

Azospirillum species may affect plant growth in a number of ways. The additive hypothesis proposed by Bashan *et al.* (1991) underscores the multiple beneficial effects *Azospirillum* has on plant growth. This hypothesis proposes that there are multiple mechanisms that operate simultaneously to enhance plant growth. The most common explanation for the growth enhancing effects that *A, brasilense* has on plants is the production of phytohormones, such as auxins and gibberellins, which alter the plant metabolism and morphology, leading to increased mineral and water absorption. Many factors may influence how well *Azospirillum* is able to promote plant growth including: the condition of the soil, the composition of the microbiome within the rhizosphere, and the stage of growth of the plant and the *Azospirillum* inoculum. These factors influence the

ability of the *Azospirillum* inoculant to colonize the rhizosphere at sufficient levels, a step which is a prerequisite to the beneficial effects of the inoculated bacteria on plant growth.

Chemotaxis is Fundamental for Competitiveness in the Rhizosphere

The motility of *A. brasilense* is one of several traits crucial to its survival in the rhizosphere. *A. brasilense* are motile and capable of navigating through the rhizosphere using chemotaxis. Chemotaxis is characterized by movements or changes in orientation of motile cells along a chemical concentration gradient (Pedraza *et al.,* 2009). Chemotaxis is an important function of many PGPR, including *Azospirillum* sp., as it promotes rhizosphere competence and enhances plant root colonization (Babalola, 2010). The rhizosphere is a complex environment composed of many different microorganisms and chemoattractants that promote bacterial colonization of plant roots. The rhizosphere is a nutrient dense portion compared to the bulk soil, with several essential nutrients found in plant root exudates, which diffuse in the region immediately surrounding the roots (Faure *et al.*, 2009; Haicher *et al.,* 2014). Given the difference in nutrient availability between the rhizosphere and the bulk soil, it is not unexpected that bacteria able to quickly navigate in the complex root and soil environments, through motility and chemotaxis are typically more competitive than non-motile or non-chemotactic bacteria (Scharf *et al.,* 2016).

Bacterial two-component systems (TCS)

Archetypal bacterial TCSs are composed of a membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR) (Galperin *et al.*, 2001). The modular architecture of TCSs consists of diverse arrangements of conserved protein domains often found in combination with HK protein domains (Galperin *et al.,* 2001).

Sequence analysis of bacterial and archaeal genomes have identified many conserved protein domains such as PAS (heme- and flavin- binding), GAF (cGMP-binding), HAMP (linker), GGDEF (c-di-GMP synthesis), EAL (c-di-GMP degradation), and HD-GYP (c-di-GMP degradation) (Galperin, 2006).

TCSs mediate a wide array of functions in prokaryotes including cell division, antibiotic resistance, response to environmental stress, and taxis (Gao *et al.*, 2007). Pathways highly homologous to TCSs have also been discovered in eukaryotic species such as *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (Galperin *et al.*, 2001).

In the prototypical TCS, the HK becomes autophosphorylated in an ATP-dependent manner upon stimulation by a specific environmental cue that is detected by a receptor domain found associated with the HK. Once autophosphorylated, the HK transfers its phosphoryl group to a conserved aspartate residue on the N terminal of the REC domain of its cognate response regulator (West and Stock, 2000 and Robinson *et al.*, 2001). The response regulator, in turn, elicits some output response, which often includes modulating an enzyme activity or a pattern of gene expression (Gao *et al.*, 2007; Galperin, 2006; Galperin, 2010).

Phytohormones in the rhizosphere

Plant hormones have an integral role in regulating plant growth and development (Faure *et al.*, 2009). Phytohormones act as signaling molecules and can influence numerous growth parameters such as, plant cell elongation, cell differentiation and cell division (Merchante *et al.*, 2013). Phytohormones also influence how a plant responds to abiotic and biotic stresses. Several rhizosphere plant-associated bacteria have been shown to alter

plant hormone signaling pathways. Many plant-associated bacteria can also produce different phytohormones that affect plant growth and development, including IAA, absicisic acid (ABA), cytokinins, gibberelins etc (Ortiz-Castro *et al.*, 2009).

Other bacteria alter the plant hormone biosynthetic machinery. For example, pathogenic bacteria, such as *Pseudomonas syringae* stimulates plant hormone synthesis. *P. syringae* induces the biosynthesis of IAA and ABA in *Arabidopsis thaliana.* Another way rhizosphere bacteria interfere with plant hormone signaling pathways is by degrading plant hormones or their chemical precursors. For example, several rhizosphere bacteria possess an ACC (1-aminocyclopropane-carboxylase) deaminase (Glick, 2005). AAC deaminases degrade a precursor of ethylene thus lowering endogenous levels of ethylene, which is a phytohormone that functions to inhibit plant growth (Bowen and Rovira, 1999). ACC deaminases are beneficial to bacteria because they ultimately promote root growth by increasing the root volume available for colonization. These effects can also benefit plant nutrition.

Ethylene

Ethylene is an important phytohormone found in the rhizosphere that contributes to every part of a plant's life cycle. This simple hydrocarbon, C_2H_4, is essential for all major plant processes including: the regulation of seed germination; fruit ripening; petal and leaf abscission; organ senescence; stem elongation; and stress and pathogen responses (Schaller and Kieber, 2002). The role of ethylene is well established in plants, however, much less is known about how ethylene interacts with rhizosphere bacteria. Ethylene binding domains (EBDs) have been found in many organisms such as cyanobacteria,

proteobacteria, and fungi, however, EBDs are primarily found in plants (Wang *et al.*, 2006). SynETR1, an ethylene binding protein in the cyanobacteria *Synechocystis,* is involved in phototaxis in this species (Lacey and Binder, 2016).

Ethylene in the rhizosphere

Regulation of ethylene levels in the rhizosphere is at least partially regulated by rhizobacteria (Glick, 2014). Ethylene levels peak in response to a variety of stressors. Ethylene is involved in mediating many stress responses in plants, making ethylene particularly beneficial to most plants. However, too much ethylene leads to growth inhibition. Rhizobacteria promote plant growth by lowering ethylene levels thereby reversing growth inhibition (Glick, 2005 and 2014).

As discussed above, the enzyme AAC (1-aminocyclopropane-1-carboxylate) deaminase mediates rhizosphere concentrations of ethylene. ACC synthase catalyzes the formation of ACC, an ethylene precursor. Once levels of ACC peak, the transcription of *acds*, the ACC deaminase gene, is activated in many rhizobacteria. ACC deaminase cleaves ACC into ammonia and α-ketobutyrate (Penrose and Glick, 2003; Glick, 2014). The net effect is an overall lower concentration of endogenous ethylene, which relieves growth inhibition. Because ethylene affects plant growth and levels in the rhizosphere can fluctuate, adaptation to ethylene in this environment is necessary in order to regulate plant growth. The ACC deaminase/ACC synthase mechanism is one example that illustrates how rhizosphere bacteria may modify the composition of phytohormones in the rhizosphere to directly enhance overall plant health.

Ethylene receptors in *A. thaliana*

Ethylene receptors in *A. thaliana* are a family of five membrane-bound proteins located in the membrane of the endoplasmic reticulum (ER): ETR1, ERS1, ETR2, EIN4, and ERS2 (Merchante *et al.,* 2013). These receptors are further divided into two subfamilies based on structural features of the receptors and phylogenetic analyses of each protein (Shakeel *et al.,* 2013). Subfamily 1 includes ETR1 and ERS1, both of which have a conserved histidine kinase (HK) domain. Subfamily 2 includes ETR2, EIN4, and ERS2; these proteins have divergent histidine kinase domains, which lack the residues required for this kinase activity (Shakeel *et al.,* 2013). Structurally, these five receptors are functional as homodimers and possess a transmembrane domain at the N terminus where the ethylene-binding domain EBD is found. The cytosolic C terminus consists of the HK, cognate receiver domains, and GAF domains which link the EBD and His kinase domain (Schaller and Kieber, 2002). The EBD of the ethylene receptors in *A. thaliana* are located in the membrane of the endoplasmic reticulum (Merchante *et al.,* 2013). The HK and RR domains are involved in signal transduction and regulatory response, respectively, when the ethylene receptors in *A. thaliana* are stimulated.

Plant ethylene receptors have several conserved domains that are required for functional activity of the proteins. The conserved residues are mostly confined within the three transmembrane helices of the EBD (Wang *et al.,* 2006)). Conserved D, Y, I, and P residues from helix 1 and conserved I, C, and H residues from helix two of the EBD are crucial for binding ethylene (Wang *et al.,* 2006). These residues located in the hydrophobic transmembrane region form a binding pocket for ethylene and a copper cofactor

(Rodriguez *et al.*, 1999). The ethylene receptors in *A. thaliana* have both overlapping and non-overlapping functions in growth, development, and pathogen response. Ethylene signaling occurs in the transmembrane domain, which is believed to propagate an additional conformational change in the cytosolic domain of the receptor, which then elicits different downstream responses (Merchante *et al.*, 2013).

AzoETR1- a recently identified ethylene binding protein in the bacterium *A. brasilense*

Recently, a novel putative ethylene-binding protein, AzoETR1 (*Azospirillum* ethylene receptor 1) has been identified in the genome sequence of *A. brasilense.* This protein appears to be part of a two-component system similar to the ethylene receptors found in *A. thaliana*. The function of AzoETR1 in this bacterium is currently unknown. The AzoETR1 protein consists of a transmembrane EBD and a PAS domain. PAS domains are involved in protein signaling and act as sensor domains. A PAC domain is also present in AzoETR1. PAC domains are found at the C-terminus of PAS domains where they are believed to contribute to the PAS domain folding (Gu *et al*., 2000). AzoETR1 also has an HK domain with a HATPase_c domain, which is characteristic of ATP-binding proteins. Downstream of the gene coding for AzoETR1, is a gene predicted to code for a RR consisting of a single regulatory REC domain. There is no identified conserved output domain associated with the REC domain, making the prediction of the function that this putative RR controls challenging.

The research presented in this thesis aims to provide insight into the role of ethylene and the potential physiological effects it has on the behavior of the bacterium *A. brasilense.* The primary goals of this thesis were geared towards elucidating potential behavioral responses to ethylene under several growth conditions. In particular, changes in motility and growth patterns of *A. brasilense* were analyzed. Currently, there is little in the scientific literature that describes the role of ethylene in organisms besides plants.

CHAPTER II. MATERIALS AND METHODS

Strains and growth conditions

Bacterial cultures of wild-type *A. brasilense,* Sp7, and its mutant derivative strain *cheA1cheA4* were grown in 5ml either liquid MMAB (minimal media used for *A. brasilense*) or liquid tryptone yeast extract (TY, rich media used for *A. brasilense*) and incubated while shaking at 200rpm at 28°C until the cultures reached log phase (OD600 of ~0.4). Liquid MMAB media was prepared by adding 3g K_2HPO_4, 1g NaH_2PO_4, 0.15g KCl, trace amount of Na_2MoO_4, 5g of malate (as a source of carbon), and 1g of NH_4Cl (as a nitrogen source) per liter of deionized water and adjusted to a pH of 6.85-7.0 (physiologically relevant) prior to being autoclaved on for 30 min at 121°C. After autoclaving the following salts were added to the media: 250µl of $FeSO_4$ (0.631g $FeSO_4$□H_2O plus 0.592g of EDTA in 50 ml of H_2O), 500µl $CaCl_2$ (stock concentration 20g/l), and 5 ml of $MgSO_4$ (60g/l stock). Liquid TY cultures of *A. brasilense* were grown in media containing 10g/l tryptone and 5g/l yeast extract). Prior to autoclaving, the pH of the media was checked and brought to 6.85 to 7.0 for biological relevance. All stocks of bacterial cultures were maintained on solid TY plates (1.5% w/v agar).

Reverse-transcriptase Polymerase Chain Reaction

In order to verify *AzoETR1* and the *RR* genes were present we used PCR amplification (Table 1) with gene specific primers (Table 2) to verify the presence of each corresponding gene. A 1% agarose (w/v) DNA gel with ethidium bromide (stock concentration 10mg/ml, final concentration 36 mM) was used to visualize the DNA product of each reaction using an ultraviolet light after PCR.

Next, RT-PCR was used according to manufacturer protocol (Thermoscript Reverse Transcriptase, Thermo Fisher Scientific) to qualitatively test RNA expression. Cultures of *A. brasilense* Sp7 and SP245 were grown overnight in rich (TY) media and RNA was isolated from samples (Qiagen RNeasy). Once RNA was obtained, it was then used as a template for cDNA synthesis (Thermoscript Reverse Transcriptase, Thermo Fisher Scientific). Subsequently the cDNA was used as template for amplification of *azoETR1* and *azoETR1 RR*. The RT-PCR reactions were performed by an undergraduate student in the Alexandre lab, Quincy Banks.

Table 1. Steps in RT-PCR. Protocol adapted from the Thermoscript RT-PCR kit.

Step	Temperature	Time
Initial Denaturation	95ºC	5 min
35 Cycles	95º 62º 72º	1min 30 sec 2 min
Final Extension	72º	15 min
Hold	4ºC	∞

Table 2. Gene specific primers used for PCR amplification. Primers in this table were used for amplifying *azoETR1* and *azoETR1 RR*. Primer sequences and their genomic target sequences are provided.

	Forward Primers	**Reverse Primers**	**Genome Sequence Target**
azoETR1 (AZOBR_110076)	5'ATGTTCGGTGG C GTGGAAG 3'	5'TCAGGCATGAGCGCGCTC C 3'	Forward:5'ATGTTCGG TGGCGTGGAAG 3' Reverse:5'GGAGCG CGCGCTCATGCCTG A 3'
azoETR1 RR (AZOBR_110075)	5' TTGCATGTCCTG GTGGCC 3'	5'TCATGCGGGCCG CAGCTTCG 3'	Forward: 5'TTGCAT GTCCTG GTGGCC 3' Reverse:5'GAAGCT GCGGGCCCGCAT GATC 3'

Bioinformatic Analysis

In order to identify the domain architecture of the ethylene-sensing TCS, protein sequences for the HK (AzoETR1) and AzoETR1 RR were analyzed using BLAST (Altschul, *et al.*, 1990) and SMART (Schultz *et al.*, 1998) Previously, the Binder laboratory aligned the sequence of AzoETR1 with that of the five ethylene receptor isoforms from *A. thaliana* and Slr1212 (SynETR1) from *Synechocystis* to detect potential similarities (unpublished). This information was used as the premise of this study.

Recombinant EBD from *Azospirillum brasilense* expressed in *Pichia pastoris*

To determine if the sequence of the predicted EBD of AzoETR1 binds ethylene, the sequence corresponding to the *azoETR1* EBD, identified through multiple sequence alignment and domain analysis as described above, was cloned and subsequently maintained in the high copy shuttle vector pGEM (Promega). The EBD from *azoETR1* was amplified using PCR and primers engineered with EcoRI and XhoI sites (Table 3) and the PCR fragment was then restriction enzyme-digested and cloned into the pGEM vector digested with the same enzymes. After verification by sequencing, the EBD from *azoETR1* was isolated from the pGEM vector by restriction digestion followed by ligation into the expression vector pPICZA (Invitrogen), digested with the same enzymes, resulting in a cloned recombinant EBD sequence in frame with the *AOX1* promoter present on the pPICZA and as a fusion to the C-terminal peptide containing the c-myc epitope and a 6xHis tag present on the same vector. The pPICZA containing the cloned sequence for the EBD of *azoETR1* was transformed into *P. pastoris*. Protein for recombinant protein expression.

Expression from AOX1 on the pPICZA vector is tightly regulated and methanol inducible (Invitrogen).

Table 3. Primers for cloning *azoETR1* EBD in pGEM. Using primers engineered with restriction enzymes for EcoRI and XhoI, the EBD of *azoETR1* was directionally cloned into pGEM. On the forward primer, an EcoRI site (underlined) was engineered to flank the 5' end of the sequence corresponding to the EBD. An XhoI site (underlined) was engineered on the reverse primer to flank the 3' end of the sequence corresponding to the EBD.

Forward Primer with EcoRI site	5'GTGAATTCATGTTCGGTGGCGTGGAAGCCTTC
Reverse Primer with XhoI site	5'GTCTCGAGGTCGGCGAGTTGCGTGGC

Induction of recombinant protein expression in *P. pastoris*

P. pastoris containing the putative EBD from *A. brasilense* AzoETR1 was grown in YPD media prepared by adding 20g/l bacto peptone, 10g/l yeast extract to 950 ml of H_2O and autoclaved at 121°C for 20 min. After autoclaving, 50 ml of sterile 40% (w/v) glucose was added to the medium. The culture was grown in 1 liter YPD to an OD_{600} of ~0.3-0.5 with shaking at 220 rpm at 30°C.

Cells were harvested by centrifugation and the pellet was resuspended in 250 ml BMMY. Liquid BMMY (buffered minimal media with methanol for yeast) was prepared by adding 5g yeast extract and 10g of peptone to 350ml H_2O and autoclaved at 121°C for 20 min and then cooled to RT. 50ml 1M potassium phosphate buffer (pH=6), 50ml 10X yeast nitrogen base stock, 1ml biotin (500X B: 0.02% biotin) and 50ml 10x M (0.5% methanol), yielding 500ml BMMY. Cells were grown for 48 hours and protein expression was induced by methanol. Following induction, (24 hours). Cells were harvested by centrifugation at 4000 rpms.

Ethylene-Binding Assay

Binding assays were performed with whole cells *A. brasilense* and with whole cells *P. pastoris* expressing the recombinant EBD from AzoETR1 from the pPICZA vector, as described above. Two samples of 3g of bacterial tissue cultures was spread on filter paper and placed in sealed glass containers. The samples were labeled hot, incubated with only ^{14}C ethylene or cold, incubated with ^{14}C ethylene with excess of ^{12}C ethylene. To each container 25 µl of 10X ^{14}C ethylene and 1.5 saturated LiCl was added and 250 µl of 12C ethylene was also added to cold jar. Both containers were then incubated for 4 hours. Samples were then removed from the containers and aired out for 10 min and then transferred to new sealed glass containers with 1ml mercuric perchlorate trap in a liquid scintillation vial. Both samples were incubated at 65°C for 90 min and then sealed containers were allowed to cool for 17 hours at RT. During this time any ethylene present in the jars is trapped in the mercuric perchlorate. After containers have cooled the mercuric perchlorate trap was removed from the jars and 10 ml of scintillation fluid was added to the scintillation vials previously removed from hot and cold jars. At this point radioactivity of each sample can be measured by liquid scintillation counts were quantify the amount of radiolabeled ethylene in each sample. (All work done to clone and express AzoETR1 in *P. pastoris*, as well as ethylene-binding assays where done in collaboration with Randy Lacey from the Binder lab)

Soft-agar assays

MMAB supplemented with NH_4Cl and malate as previously described with 0.3% (w/v) agar was used to test the motility of Sp7 wild-type strain and its derivative,

cheA1cheA4, a chemotaxis deficient mutant (Mukherjee *et al.,* 2016). Each strain was grown in 5ml TY overnight to an OD_{600} of ~0.4, then standardized to 0.4. Each sample was washed three times with sterile chemotaxis (che) buffer (0.85g $KHPO_4$ and 0.68g K_2HPO_4 per 500ml) to remove any residual media and antibiotics. Samples were resuspended after standardization and washing in 100µl che buffer. A volume of 5µl of these suspensions was used to inoculate the center of the soft-agar plates. Following inoculation, plates were incubated at ambient temperature in either air or 1ppm ethylene for 4 days. Two controls were used in every experiment: a non-inoculated plate, and a plate inoculated with only 5µl of che buffer to ensure there was no contamination. Three independent biological replicates were performed, each done in triplicate, for a total of 9 replicates in air and ethylene. Soft-agar plates were replicated in the dark to test for the possible abiotic production of ethylene (Buer, *et al.,* 2003) from light and water, by wrapping plates in aluminum foil prior to incubation.

Swarm plate assays

The Sp7 and *cheA1cheA4* strains were used to inoculate NB (nutrient broth) semi-solid 0.7% (w/v) agar plates. The NB medium was prepared by solubilizing 8g of nutrient broth (Difco) to one liter H_2O. Difco NB consists of 3g beef extract and 5 g peptone and is prepared at a pH of 6.85 to 7.0 prior to autoclaving at 121°C for 20 min. All cultures used in these experiments were prepared exactly as described for the soft-agar assays. 5µl of prepared suspensions were used to inoculate the surface of the center of each plate. Plates were incubated at ambient temperature in air or 1ppm ethylene for 2 days. Three biological replicates were performed in triplicate for each strain.

Growth assays under atmospheres of air or 1 ppm ethylene

To test the effect of ethylene on the growth of *A. brasilense,* two different assays were performed. In the first assay, 200µl of an overnight culture of Sp7 grown in TY was used to inoculate two flasks, each containing 20 ml of liquid TY media. One of these flasks was incubated in air and the other incubated under an atmosphere of ethylene created by flowing 1ppm ethylene in air/gas mixture in a tightly sealed container. Serial dilutions of these cultures were made from aliquoted samples taken at 0, 8, 16, 24, 32, and 40 hours post inoculation. Six 5µL drops of each dilution were plated on square plates with solid TY media 1.5% (w/v) agar. After spotting each sample, the plates were incubated in air and 1ppm ethylene for 2-3 days until individual colonies became visible and could be counted. Colony counts were documented and statistical analyses including, averages, standard deviations, and slopes were calculated. Colony forming units/ml (CFU/ml) were estimated and used to calculate doubling times. Some time points were omitted from final analysis because individual colonies could not be observed on the plates.

Statistical Analyses

Measurements acquired from the soft agar and swarm plate assays were used for statistical analyses. The diameter of the chemotaxis rings were recorded and means, standard deviations and P-values were recorded for soft-agar (Table 4) and swarm assays (Table 5). Under laboratory conditions, inoculating cells into solidified agar plates can characterize the swimming behavior of microorganisms. For *A. brasilense,* a low concentration of 0.3% (w/v) of agar is suitable for swimming (Moens and Vanderleyden, 1996). Swarming behavior, which occurs when the bacterium moves across the surface of

semi-solid substrate can be detected by inoculating cells onto agar plates solidified with 0.7% agar (w/v) (Moens and Vanderleyden, 1996). Chemotaxis and motility experiments included analysis of both swimming and swarming by using agar at different final concentration to mimic different viscosities.

Table 4. Statistical Analyses of Chemotaxis Rings on Soft-Agar Plates. Diameterof the chemotaxis rings were measured and recorded in mm. Measurements were acquired for plates grown in air and ethylene for strains *cheA1cheA4* (non-chemotactic) and Sp7.

	Light Avg (mm)	**Dark Avg (mm)**	**Light Standard Deviation**	**Dark standard deviation**	**Light standard error**	**Dark standard error**	**P-value: Light vs. Dark**
***cheA1cheA4* (air)**	0.00	0.00	0.00	0.00	0.00	0.00	n/a
***cheA1cheA4* (C_2H_4)**	0.00	0.00	0.00	0.00	0.00	0 .00	n/a
Sp7 (air)	31.80	29.11	6.70	2.72	1.93	0.91	0.20
Sp7 (C_2H_4)	30.00	30.33	6.13	2.84	1.77	0.95	0.99

Table 5. Statistical Analyses of Expansion Rings on Swarm Plates. Diameter of the expansion rings from the point of inoculation were measured and recorded in mm. Measurements were acquired for plates grown in air, ethylene, light and dark for wild-type Sp7 and *cheA1cheA4* strains.

	Average (mm)	**Standard Deviation**	**Standard Error**	**P-value: Air vs. C_2H_4**
***cheA1cheA4* (air)**	36.44	1.26	0.42	0.007
***cheA1cheA4* (C_2H_4)**	38.23	1.19	0.40	
Sp7 (air)	52.67	1.60	0.53	0.58
Sp7 (C_2H_4)	52.23	1.67	0.55	

CHAPTER III. RESULTS AND DISCUSSION

Ethylene perception is a phenomenon that is well-understood and reported in the literature as it relates to plants. Much less is known about how this gaseous hormone effects other organisms, such as bacteria. In plants, ethylene receptors are composed of structural elements characteristic of signaling proteins in bacteria (Bleeker, 1999). The arrangement of conserved domains and input and output components are similar to prokaryotic TCS (Figure 1) (Schaller and Kieber, 2002; McDaniel and Binder, 2012).

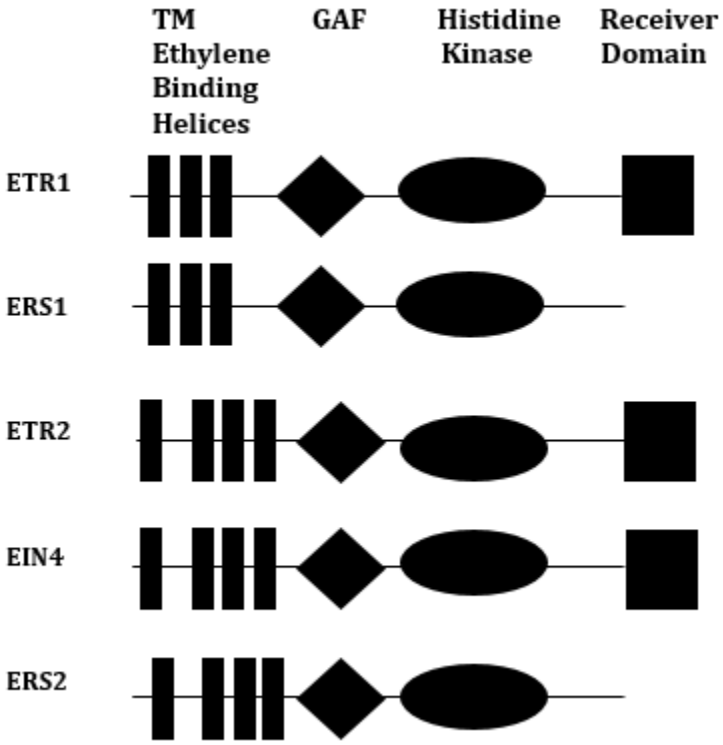

Figure 1. Ethylene Receptor Isoforms in *A. thaliana.* The ethylene binding domains are located in the transmembrane helices found at the N terminus of the proteins. GAF, histidine kinase and Receiver domains follow the EBDs. Figure adapted from McDaniel and Binder, 2012

Gene Expression and Bioinformatic analyses

PCR amplification (Figure 2) of *azoETR1* and its putative response regulator using primers specific for each gene verified that the genome of *A. brasilense* encodes for each protein in this ethylene-sensing TCS. The results from the RT-PCR indicate that each protein is transcribed into mRNA, and thus these genes are expressed under general laboratory conditions. This analysis also indicates that two related strains of *A. brasilense*, strain Sp7 and strain Sp245 both constitutively express *azoETR1* and *azoETR1 RR*, under the conditions tested (Figure 2). One limitation of RT-PCR is that it does not provide information on protein translation from mRNA transcripts. A logical next step to take would to assess translation of each protein and the function each protein has in *A. brasilense.*

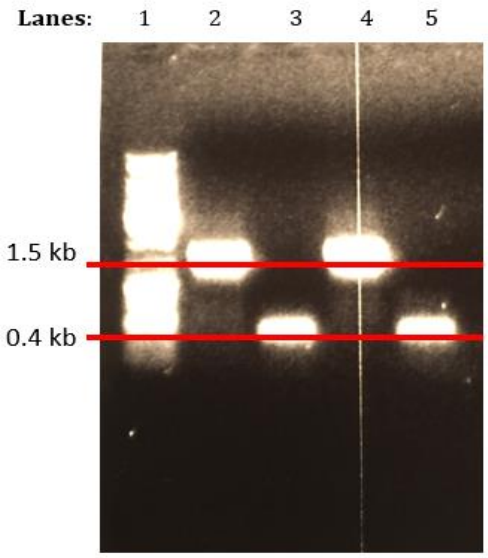

Figure 2. RT-PCR analysis of the expression of *azoETR1* and *azoETR1 RR*. Amplification of ***a****zoETR*1 and *azoETR1 RR* by PCR from genomic DNA of *A. brasilense* strains Sp7 and Sp245. Samples were run on 0.8% agarose ethidium bromide stained gel. The red lines highlight the expected molecular weight of the DNA sequences amplified. The *azoETR1* sequence is expected to be 1.5kb and the putative *azoETR1 RR* at 0.4kb. Lanes 2 and 3 are samples from strain Sp7 and lanes 4 and 5 are samples from strain Sp245.

The protein sequences of AzoETR1 and AzoETR1 RR were used as input sequences for a BLAST search to identify protein homologs. Each protein sequence was also used for a SMART (Schultz *et al*., 1998) database analysis in order to identify putative protein domains (Figure 3). The sequences used to probe BLAST for protein homologs originate from the translated genome of *A. brasilense* Sp245. The entire genome sequence for *A. brasilense* Sp7 was not available at the time of analysis. Protein sequences of AzoETR1, the 5 *A. thaliana* ethylene receptors, and slr1212 from *Synechocystis* were used for a multiple protein alignment in BLAST (Altshul *et al.*, 1990) (Figure 4). The conserved residues required for ethylene binding are highlighted in Figure 4.

SMART analysis of each protein sequence identified conserved protein domains in AzoETR1 and AzoETR1 RR (Figure 3). In AzoETR1, 3 transmembrane helices are present at the N-terminal domain of the protein. Following the transmembrane regions is a PAS domain. In other PAS domain containing proteins, these domains function as sensor domains involved in signaling (Taylor and Zhulin, 1999). The PAC motif located at the C-terminal end of the PAS domain contributes to PAS domain folding (Ponting and Aravind, 1995; Zhulin *et al.*, 1997). The histidine kinase domain (HATPase_c domain) is a hallmark element in TCS in bacteria (Perego and Hoch, 1996). The kinase domain includes a conserved histidine residue that becomes autophosphorylated from ATP upon stimulation. Phosphotransfer from the histidine kinase to a conserved aspartate residue of a response regulator elicits a cellular response (Davis and Vierstra, 1999). In AzoETR1, a prototypical HATPase_c domain is found at the C-terminal of the protein. The response regulator protein, AzoETR1 RR, analyzed by SMART is a single motif protein consisting of a REC

domain. The REC domain is a Che-Y-homologous receiver domain and it contains a conserved aspartate residue that is the phosphoacceptor site phosphorylated by a histidine kinase. REC domains are found in proteins that mediate cellular outputs from bacterial TCS.

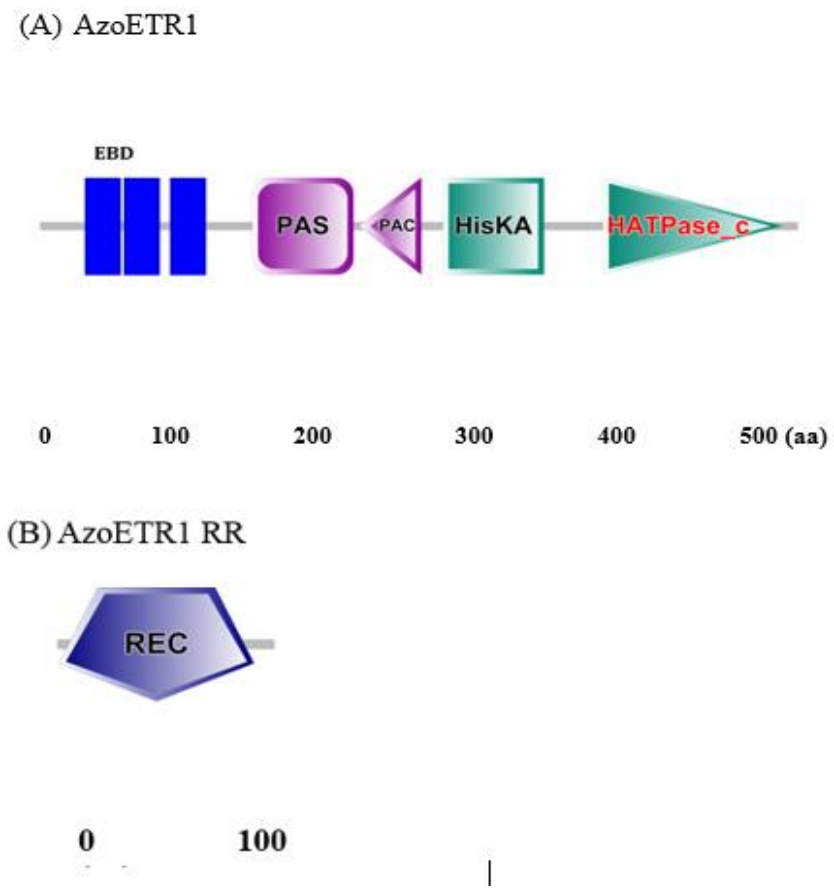

Figure 3. SMART Database predictions of protein domains found in AzoETR1 (A) and its putative RR, AzoETR1 RR. Amino acid number is shown below each protein domain(s).

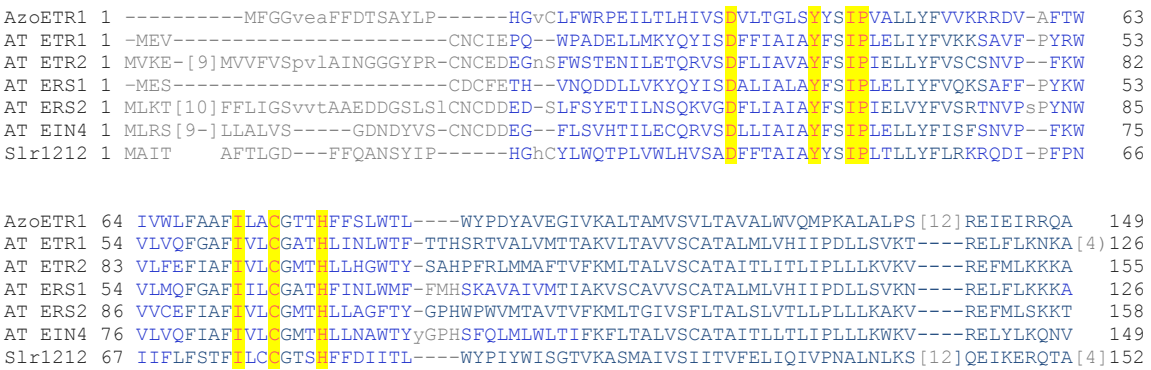

Figure 4. Multiple Protein Alignment of EBDs. The EBDs of AzoETR1, AT ETR1, AT ETR2, AT ERS1, AT ERS2, AT EIN4, and slr1212 from *Synechocystis* were aligned using COBALT from BLAST (Altschul, *et al.*, 1990). The highlighted sequences indicate conserved residues in the first and second transmembrane helices of the EBDs that are required for binding ethylene. The numbers in brackets represent non-conserved residues omitted in the alignment for better visualization.

Ethylene-Binding of AzoETR1

In vivo measurements of ethylene binding to ethylene binding sites can be obtained using an isotope displacement assay developed by Edward Sisler. In this assay, binding of ethylene to biological material is measured by determining the displacement of ^{14}C ethylene from labeled biological sample with non-radiolabeled ^{12}C ethylene (Sisler, 1979).

Following sequence analysis of the TCS proteins, we sought to experimentally verify ethylene binding. The data in this section can be considered preliminary because further replications of the ethylene binding assay must be performed to verify ethylene binding. Using the isotope displacement assay, we verified that ethylene bound the predicted EBD domain from AzoETR1 in both whole cells of *A. brasilense* and in whole cells of *P. pastoris* expressing the recombinant EBD of the AzoETR1 protein by measuring radioactivity of samples using liquid scintillation counts. (Figure 5). Control experiments used *P. Pastoris* containing an empty pPICZA vector because *P. pastoris* cells does not

bind ethylene (Binder and Lacey 2016). Binding of gaseous ethylene appears to be greater in *P. pastoris* compared to *A. brasilense.* This may be due to the level of recombinant protein production being higher in *P. pastoris* compared to the endogenous levels in *A. brasilense*. This discrepancy could also result from the excessive production of EPS in *A. brasilense* compared to *P. pastoris* under the conditions of the experiments. We observed that the *A. brasilense* samples used in the binding assays were much more wet and sticky than those from *P. pastoris*, which were very dry when harvested. It is possible that ethylene, a very hydrophobic molecule, could not diffuse through the hydrophilic EPS and thus was not as effective at saturating putative ethylene receptors in *A. brasilense.* The ethylene binding assays were performed with Randy Lacey in the Binder lab.

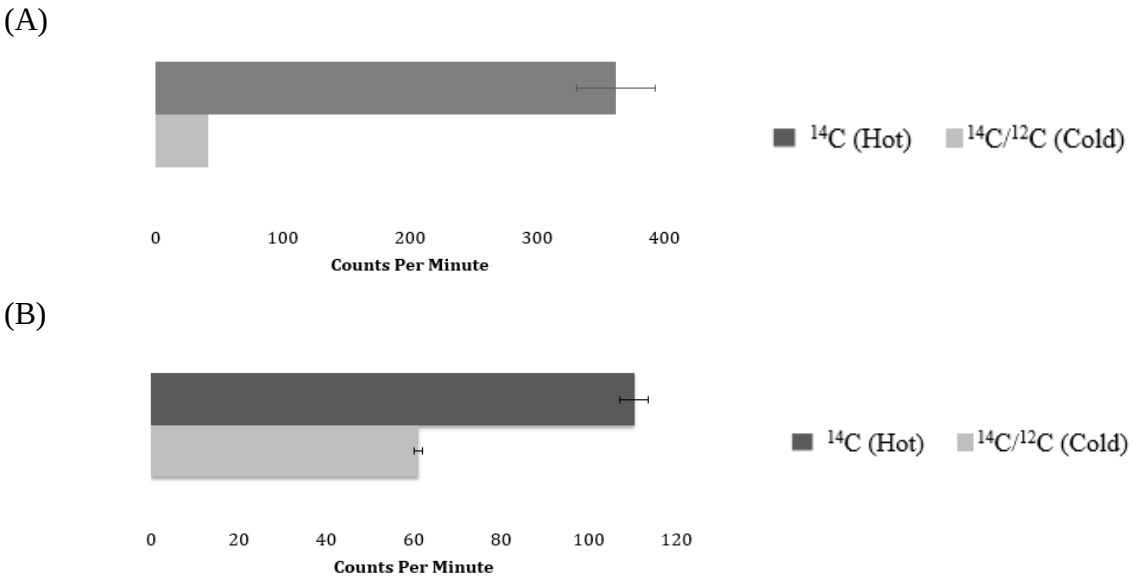

Figure 5: Ethylene-binding assay in whole cells *A. brasilense* (A) and in whole cells *P. pastoris* expressing the EBD from AzoETR1 (B). Counts per minute measure radioactive activity of each sample. The dark grey bars represent the measure radioactivity in hot samples and the light grey bars represent the amount of radioactivity in the cold samples.

Further experiments using the ethylene binding assays should include replicates of the assays already performed to confirm the findings. Additionally, it would be ideal to create an *azoETR1* mutant lacking the sequence corresponding to the EBD to verify that the ethylene binding observed in whole cells *A. brasilense* was due to the presence of AzoETR1 and to confirm its role as an ethylene binding protein.

Motility of *A. brasilense* in the presence of air and ethylene

Chemotaxis and motility are important characteristics used by soil bacteria in colonizing plant roots (Scharf *et al.*, 2016). *A. brasilense* has two different types of flagella that it uses to move throughout environments of different viscosities. In a liquid medium, *A. brasilense* uses a single polar flagellum to propel through the solution (detected in soft 0.3% agar plates), while on more viscous media, additional lateral flagella are produced to move across these surfaces (detected in swarm 0.7% agar plates) (Khammas *et al.*, 1989; Tarrand *et al.*, 1978; Moens and Vanderleyden, 1996).

After statistical analysis, there is no discernable difference among the conditions tested (Figure 6): motility by swimming or swarming is not affected by exposure to 1 ppm of ethylene. Preliminary results of the soft-agar assays performed in light initially suggested that ethylene may have a small inhibitory effect on motility, but, after all collected data were combined no a statistically significant difference among samples was detected.

We note that in the soft agar plate assay, there was no ring formed for swimming cells of the *cheA1cheA4* strain, although there was some growth (not shown), as expected since this strain is non-chemotactic (Figure 6). In the swarm assay, the non-chemotactic

cheA1cheA4 strain showed some swarming behavior (Figure 7), suggesting that translocation across the surface under the conditions of the experiments does not depends solely on chemotaxis. However, chemotaxis likely contributes to wild-type level swarming since our data showed a significant difference in swarming between the wild type strain and the *cheA1cheA4* strain. Similar to swimming, however, we did not detect any effect of ethylene on this behavior (Figure 7).

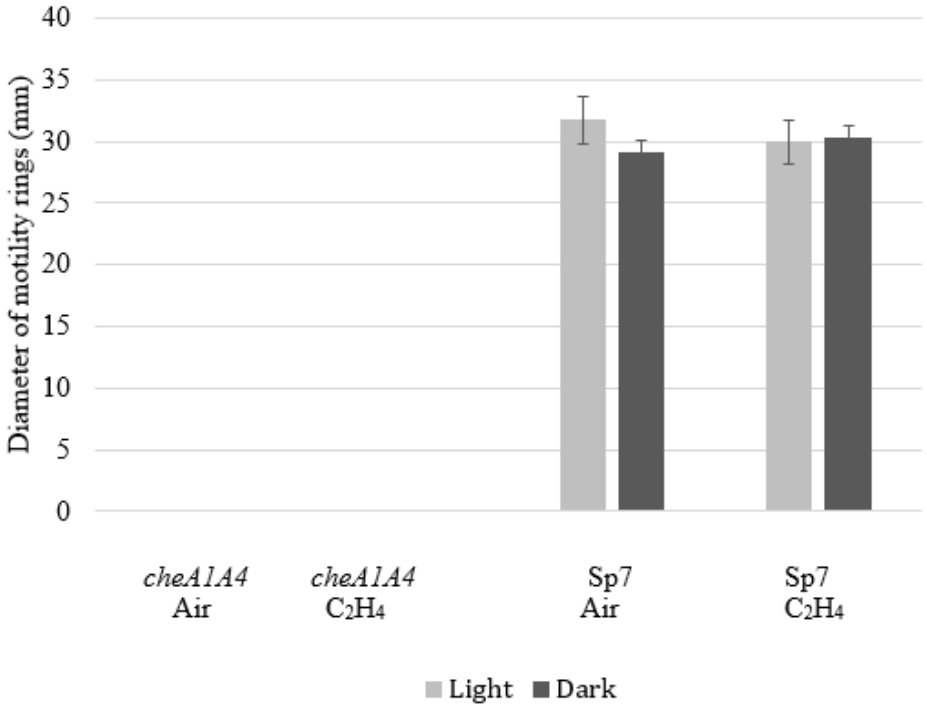

Figure 6: Soft-agar motility assays in light and dark condition in air and 1ppm ethylene. Motility assays were performed on 0.3% agar (w/v) and the diameter of the rings were measured. Statistical analysis revealed there was not a significant difference in light and dark conditions in either air or ethylene.

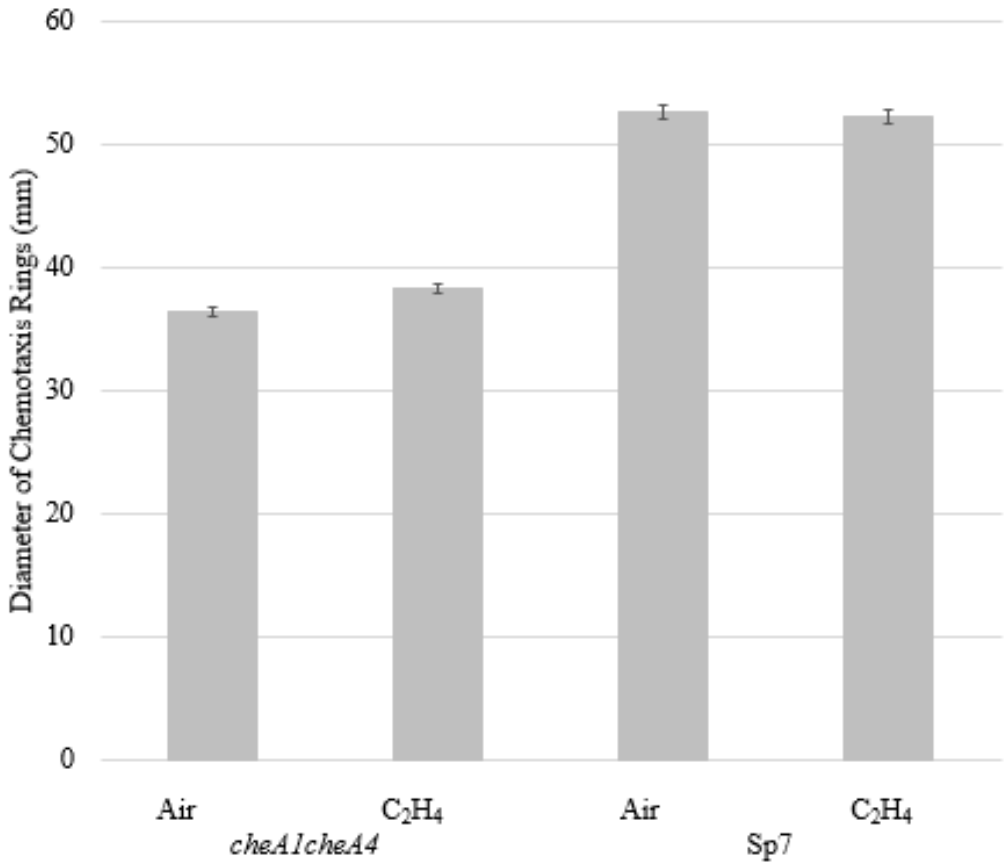

Figure 7. Swarm plates of Sp7 and *cheA1cheA4* in air and ethylene. Plates with 0.7% agar (w/v) were inoculated on the surface of the media. Measurements were recorded and statistical analyses showed there is no difference in swarming in air and ethylene.

Growth Assays

Growth assays were performed to assess whether or not ethylene had an effect on the growth of *A. brasilense* Sp7. Growth of strain Sp7 in air and ethylene followed the same trend, indicating a small inhibitory effect on growth in presence of ethylene. Linear regression analysis was used to predict the estimated number of colony forming units per ml. This assay revealed that there was increased number of colony forming units when Sp7 was grown in air. There was a slight inhibition of growth when Sp7 was grown in the presence of 1ppm ethylene (Figure 8).

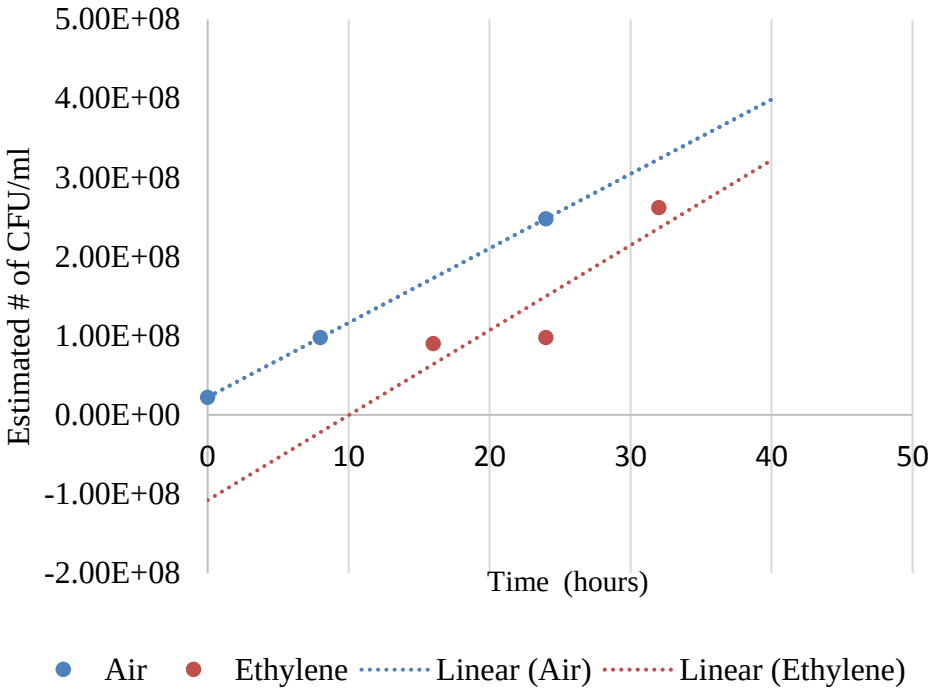

Figure 8: Growth of *A. brasilense* Sp7 in air and ethylene. Growth assays were performed by inoculated solid rich media plates with *A. brasilense* at different time points during growth. Plates were incubated until visible colonies could be counted. Colony counts were used to determine CFU/ml. CFU/ml was used to calculate the number of viable cells per ml bacterial culture.

CHAPTER IV. FUTURE DIRECTIONS

The scope of this thesis was to determine whether the AzoETR1 protein from the soil bacterium *A. brasilense* could bind ethylene and to characterize a putative function for ethylene in the life cycle of *A. brasilense*. We were able to predict and verify that AzoEtr1 possesses a functional EBD domain able to bind ethylene, similar to EBD of ethylene plant receptors. However, much remains to be discovered regarding the function this gaseous plant hormone elicits in the bacterium.

The REC domain of the AzoETR1 RR indicates that this protein likely functions in a phosphorylation cascade. Given that this protein is a single REC domain protein without a DNA binding domain it is unlikely that this response regulator acts as a transcription factor in regulating gene expression. We assume, given the genome organization, that AzoETR1 RR is phosphorylated by the HK found on AzoETR1. However, this assumption should be experimentally tested in the future. Typical TCS response regulator proteins activate cellular responses to environmental cues. The presence of a sole REC domain in AzoETR1 RR, with no detectable additional output domain, raises further questions regarding whether AzoETR1 RR regulates a cellular response directly, or perhaps, through phosphorylation of additional proteins that would be encoded from genes found elsewhere in the genome.

Initially, our hypothesis was that ethylene perception by AzoETR1 could modulate the motility of *A. brasilense,* since SynETR1, which is homolog to AzoETR1, modulates phototaxis in *Synechocystis* (Lacey and Binder, 2016). However, the effect ethylene has on motility in *A. brasilense*, if any, is too subtle to be observed, with the techniques we

have used. We have observed the motility of free-swimming cells of *A. brasilense* in air and in ethylene but did not detect any difference (not shown). Therefore, it is likely that AzoETR1, upon ethylene binding, regulates different functions. In this respect, characterization and analysis of a mutant strain lacking functional AzoETR1 and AzoETR1 RR should be productive in providing insight into the biological functions ethylene mediates in this bacterium.

Recent studies are providing emerging evidence of interkingdom crosstalk between plants and bacteria. Plant-associated bacteria can interact with their host plants through chemical interactions with compounds produced by plants (Brencic and Winans, 2005). Many of these compounds specifically interact with regulatory proteins to influence gene expression in bacteria. Recently, a class of *N*-acyl homoserine lactones (AHLs) have been discovered that require plant interference (Scaehfer *et al.*, 2008). *P*-coumaroyl-AHLs signals produced by the rhizobacteria *Rhodopseudomonas palustris* require *p*-coumarate. However, *p*-coumarate is not synthesized by the bacterium, but by the plant as it is the precursor for lignin, and it is present in the rhizosphere. To produce the AHLs, *R. palustris* relies on plant-derived p-coumarate as a precursor, illustrating a plant-microbe crosstalk that ultimately regulates gene expression in *R. palustris* (Scaefer *et al.*, 2008). The AzoETR1 system in *A. brasilense* could be functioning similarly to the *p*-coumaryl-AHL system in that a bacterial ethylene receptor in *A. brasilense* could specifically interact with exogenously, plant-derived ethylene, in the rhizosphere with some yet unknown effect(s) on bacterial behavior. Alternatively, it is plausible that ethylene is produced in the rhizosphere by other bacteria or from abiotic sources, although this should be

demonstrated. We note, however, that given the hydrophobicity of ethylene, we do not expect it to affect rhizosphere bacteria located at some distance from the root surfaces. Thus, it is likely that if plant-produced ethylene serves as a signal for *A. brasilense*, then we would expect it to act once the bacteria are in contact or close proximity to the root surfaces. Considering the role of *A. brasilense* in the rhizosphere as a plant growth enhancer and the role that ethylene plays in the growth and development of plants, it is possible that ethylene is involved in regulating PGPR-dependent plant growth promotion by influencing the colonization pattern of *A. brasilense* on the root surfaces.

In conclusion, our study reveals an ethylene binding protein in *A. brasilense* that appears to be part of an ethylene sensing TCS encoded within the genome of this bacterium. We were unsuccessful in linking ethylene to motility in *A. brasilense*, however, there are numerous other functions in which this phytohormone could affect the life cycle of the rhizospheric bacterium that remain to be tested. The ethylene sensing system in *A. brasilense* can potentially provide a model for probing crosstalk between rhizosphere bacteria and their plant partners.

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VITA

Tiffany Nicole Thoms was born in New Orleans, Louisiana. She completed elementary and middle school, and had to leave high school three months before graduation due to unpredictable circumstances. She went on to earn her G.E.D. in 2002 and completed vocational school to earn her Certification and Licensure as an Emergency Medical Technician (EMT) (Level Basic and Intermediate) with the National Registry of Emergency Medical Technicians in Louisiana in 2004. She also earned her reciprocity licensure to work as an EMT in the state of Tennessee when she moved to Tennessee in 2009. In 2009, she decided to switch her focus to science instead of medical care. Tiffany enrolled at Lincoln Memorial University in Harrogate, Tennessee in 2009 and majored in Biology and minored in Chemistry. She discovered her love of basic science research while completing an undergraduate research project under the guidance of Dr. Ashleigh Prince and Dr. Cynthia Ryder at LMU. Additionally, she participated in a Research Experience of Undergraduate program at the University of Tennessee in Knoxville during the Summer of 2012. At UTK she worked under the guidance of Dr. Engin Serpersu. Tiffany then graduated from LMU cum laude with a Bachelor of Science degree. After graduation in 2013 she completed an internship through Oak Ridge Associated Universities at the National Center for Toxicological Research in Little Rock, Arkansas with Dr. John Sutherland. In August of 2013 Tiffany accepted a graduate teaching position in Department of Biochemistry, Cellular, and Molecular Biology. Tiffany worked in the lab of Dr. Gladys Alexandre where she earned her Master of Science degree in 2017.