Ethylene Perception and Response in *Azospirillum brasilense Sp7*

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Abstract

The plant hormone ethylene has been extensively studied for its role in many plant developmental processes. This has led to the discovery of five ethylene receptors that are all involved in a complex signaling pathway that overlaps with several other phytohormone pathways, as well as stress responses. The role that ethylene plays in stress responses makes it a central signaling pathway in plant-microbe interactions. This interface has been well studied from the plant perspective, but there is limited data on how bacteria respond directly to ethylene. This work shows that a plant growth-promoting bacteria, *Azospirillum brasilense,* has a functional ethylene receptor capable of binding ethylene and inducing a response, making it the first plant-associated bacteria to have a receptor identified. The application of ethylene to *A. brasilense* alters attachment behavior in both biofilm formation assays as well as root colonization of both *Arabidopsis thaliana* and tomato. These changes in attachment behavior are likely mediated by a large transcriptomic response to ethylene that leads to an upregulation of central carbon metabolism and a downregulation of genes involved in nitrogen fixation. In agreement with the changes in transcripts, ethylene treated cells also have a distinct metabolic profile when compared to untreated cells, and this change can be detected as early as 8 hours after exposure to ethylene. Together, this data show that ethylene perception by *A. brasilense* alters the nitrogen metabolism of the cell in a way that inhibits colonization of the root and upregulates carbon metabolism and storage.

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Chapter 1: Introduction

Ethylene Perception and Signaling in Plants

Ethylene responses were first identified in plants over 100 years ago (Neljubow 1901, Bakshi, Shemansky et al. 2015). It is now known that ethylene is a phytohormone that plays a role in several important processes in plant growth and development, as well as stress responses(Smith 1976, Mount and Chang 2002, Ju and Chang 2012, Muday, Rahman et al. 2012, Wilson, Kim et al. 2014, Bakshi, Shemansky et al. 2015, Bakshi and Binder 2017, Binder 2020, Van Der Straeten, Kanellis et al. 2020). Among these processes are seed germination, flowering, organ abscission, senescence, and fruit ripening. While fruit-ripening is one the most well-studied responses to ethylene due to its agricultural importance, ethylene is also involved in responses to stresses, both biotic and abiotic (Alonso, Hirayama et al. 1999, Wilson, Kim et al. 2014, Ibort, Molina et al. 2017, Nascimento, Rossi et al. 2018, Binder 2020). Ethylene's role in both growth and stress tolerance makes it of particular interest in understanding plant-microbe interactions where it plays a role in responses to both pathogenic and beneficial microbes. Ethylene's role in plant immune response to bacteria has been well studied (Felix, Duran et al. 1999, Kunze, Zipfel et al. 2004), (Zipfel, Robatzek et al. 2004), (Jones and Dangl 2006),(Boller and Felix 2009, Mur, Lloyd et al. 2009, Dodds and Rathjen 2010, Nascimento, Rossi et al. 2018, Li, Han et al. 2019, Yu, Pieterse et al. 2019, Kawa and Brady 2022). There is also a large body of work demonstrating how bacteria manipulate plant ethylene responses either to exploit the immune system by pathogenic bacteria or reduce stress and promote plant growth by beneficial bacteria (Glick 2005, Glick, Cheng et al. 2007, Contesto, Desbrosses et al. 2008, Ranf, Gisch et al. 2015, Nascimento, Rossi et al. 2018,

Li, Han et al. 2019, Scotti, D'Agostino et al. 2019). It is known that many bacteria harbor putative ethylene binding proteins, but few have been studied and the role that these proteins play in plant-microbe interactions remains unstudied (Lacey and Binder 2016, Lacey, Allen et al. 2018, Allen, Lacey et al. 2019, Carlew, Allen et al. 2019).

Ethylene receptors are defined by the presence of an ethylene binding domain (EBD). The EBD contains three transmembrane alpha helices with seven conserved amino acid residues that have been shown necessary for binding in AtEtr1 and are present in all confirmed ethylene receptors (Figure 1.1) (Schaller and Bleecker 1995, O'malley, Rodriguez et al. 2005, Wang, Esch et al. 2006, Lacey and Binder 2016). Most the research on ethylene signaling has taken place in the model organism *A. thaliana* which contains five ethylene receptor isoforms that contain an N-terminal transmembrane ethylene binding domain (EBD) comprised of three alpha helices, a GAF domain, and a kinase domain. Three of the five receptors also contain a receiver domain. The receptors form dimers which are stabilized at their N-terminus by two disulfide bonds (Schaller, Ladd et al. 1995).

The seven amino acid residues that are needed for binding create an ethylene binding pocket between the first and second helices of the monomers. Ethylene binds to a copper(I) ion which is coordinated by a conserved histidine and cysteine in the second transmembrane helix (Rodrıguez, Esch et al. 1999, Binder, Rodriguez et al. 2007). In *A. thaliana* the five receptors are divided into two subfamilies. Subfamily one consists of AtETR1 and AtERS1 which are predicted to contain functional histidine kinases, though AtERS1 is unlikely to function as a histidine kinase *in vivo* (Gamble, Coonfield et al. 1998,

Gamble, Qu et al. 2002, Wang, Hall et al. 2003, Qu and Schaller 2004, Voet-van-Vormizeele and Groth 2008). Subfamily two consists of AtETR2, AtERS2, and AtEIN4 appear to have diverged and display serine/threonine kinase activity (Zhang, Zhou et al. 2004, Binder 2020). In addition to the altered kinase activity, subfamily two receptors also have an additional N-terminal transmembrane domain. Three receptors, AtETR1, AtETR2, and AtEIN4 contain C-terminal receiver domains. Despite the general domain structure of these proteins being similar to bacterial two-component systems which function via His autophosphorylation and phosphotransfer, the role of phosphotransfer in ethylene signaling has not been well studied outside of AtETR1 (Chang, Kwok et al. 1993, Gamble, Qu et al. 2002, Wang, Hall et al. 2003, Binder, Mortimore et al. 2004, Kim, Helmbrecht et al. 2011, Bakshi, Wilson et al. 2015, Binder, Kim et al. 2018, Zdarska, Cuyacot et al. 2019).

These receptors are modeled to signal constitutively in air and ethylene functions as an inverse agonist, meaning that ethylene binding inhibits receptor activity (Hua and Meyerowitz 1998). In this model, in the absence of ethylene, the five receptors signal to a Ser/Thr kinase called CTR1 which phosphorylates and inhibits an N-RAMP-like (Natural Resistance-Associated Macrophage Protein), integral ER membrane protein EIN2 (Kieber, Rothenberg et al. 1993). EIN2 is required for responses to ethylene. When bound to ethylene, receptor activity is reduced leading to reduced CTR1 activity and increased EIN2 activity (Figure 1.2A). The mechanism of signal transduction from the five receptor isoforms to CTR1 is still unclear. Although CTR1 is known to associate with the five receptors, phosphorelay from the receptors to CTR1 does not seem to be essential to the signal transduction in the canonical ethylene response pathway (Gamble, Qu et al.

2002, Gao, Chen et al. 2003, Wang, Hall et al. 2003, Mason and Schaller 2005). It is suggested that when the receptors bind ethylene, there is a conformation shift that also alters CTR1 activity. In support of a mechanism that does not require phosphorelay, a kinase dead mutant of AtETR1 is still capable of responding to ethylene. Despite no known role in the canonical signaling pathway, ethylene does inhibit autophosphorylation of AtETR1. AtETR1 has been shown to interact with the phosphotransfer protein AHP1 which in turn phosphorylates ARR1 in the cytokinin signaling pathway. Mutants in AHP1, and ARR (Arabidopsis Response Regulator) proteins also have altered responses to ethylene, showing a functional consequence of this signaling pathway (Gamble, Coonfield et al. 1998, Mason and Schaller 2005, Voet-van-Vormizeele and Groth 2008). While the kinase activity of the other receptors is not well studied, it is thought that the kinase activity may be the basis for divergent functions between the isoforms (Shakeel, Wang et al. 2013, Wilson, Kim et al. 2014, Bakshi, Wilson et al. 2015).

Ethylene signaling and perception in Cyanobacteria:

Several cyanobacterial species have previously been shown to bind ethylene, but *Synechocystis PCC 6803* was the first prokaryote to have an ethylene receptor, SynEtr1, identified and characterized (Rodrıguez, Esch et al. 1999, Wang, Esch et al. 2006, Lacey and Binder 2016). Whereas the bacterium had been shown to bind ethylene, no functional analysis was done until Lacey et al 2016. Site directed mutagenesis of SynEtr1 amino acid residues shown to be important in *A. thaliana* binding resulted in loss of binding in SynEtr1. Although dimerization of the prokaryotic receptors has not been confirmed, loss

Figure 1.1 Sequence Alignment of Confirmed Ethylene Binding Domains

Sequence alignments of the Ethylene Binding Domains (EBDs) for the five *A. thaliana* receptors, the *Synechocystis* receptor, and two of the *Lycopersicon esculentum* receptors, all of which have been confirmed to bind ethylene. These binding domains are made up by three transmembrane alpha helices. In transmembrane domain one and two, there are 7 highlighted amino acids which have been shown to be necessary for ethylene binding in both *A. thaliana*, and *Synechocystis*. Alignments performed using Clustal Omega multiple sequence alignment tool.

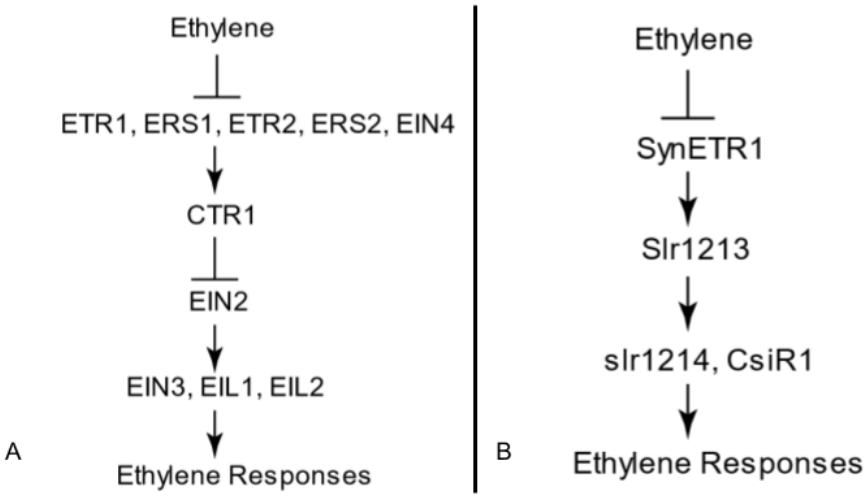


Figure 1.2. Ethylene Signaling in *A. thaliana* and *Synechocystis.*

A) In the absence of ethylene, the five receptors activate CTR1 which leads to the degradation of EIN2 and an inhibition of ethylene responses. In the presence of ethylene, the receptors are inactivated allowing EIN2 to localize to the nucleus and promote transcription factors leading to ethylene responses. B. SynEtr1 functions as a dual light and ethylene receptor. While light is an activating stimulus, ethylene functions as an inhibitor like the plant model. When light is absorbed by SynEtr1, the kinase domain undergoes phosphotransfer to slr1213 which functions as a transcription factor for slr1214 and the small RNA *CsiR1*. However, ethylene is thought to inhibit the kinase leading to alterations in phototaxis.

of binding after mutation of the histidine and cysteine residues in the predicted ethylene binding pocket support the idea that an ethylene binding pocket forms between dimers of the prokaryotic receptors to coordinate a copper(I) ion (Lacey and Binder 2016).

Interestingly, SynEtr1 has also been described as a photoreceptor that plays a role in phototaxis responses (Narikawa, Suzuki et al. 2011, Song, Cho et al. 2011). As a photoreceptor, studies show that both the phytochrome-like (cyanobacteriochrome) and histidine kinase domains are functional (Ulijasz, Cornilescu et al. 2009, Ramakrishnan and Tabor 2016). Exposure of SynEtr1 to UV-A light led to autophosphorylation of the receptor and subsequent phosphotransfer to the downstream response regulator slr1213. Slr1213 contains a DNA-binding domain which has been shown to bind to the promoter region of a small non-coding RNA, *csiR1* and *slr1214*. Phosphorelay from SynEtr1 to slr1213, and a functional slr1214 are required for wild type phototaxis responses (Hernández-Prieto, Schön et al. 2012, Klähn, Orf et al. 2015, Chandra, Joubert et al. 2017).

The effect of ethylene on SynEtr1 phosphorylation has not been directly tested but ethylene treatment results in a downregulation of the downstream genes, suggesting that ethylene inhibits kinase activity as seen in AtETR1 (Voet-van-Vormizeele and Groth 2008, Lacey and Binder 2016). The separate responses to light and ethylene are further strengthened by observations that ethylene responses do not require a functional cyanobacteriochrome domain. Application of ethylene was shown to increase biofilm formation and upregulate the production of type IV pili, leading to increased motility during phototaxis. Ethylene also caused wide-spread transcriptomic responses (Lacey, Allen et

al. 2018). These responses require a functional SynEtr1. Knocking out SynEtr1 phenocopied ethylene treatment, suggesting that this receptor also functions as a negative regulator of ethylene response (Figure 1.2B). This inverse agonist mechanism is consistent with the mechanism of the plant receptors (Hua and Meyerowitz 1998, Lacey and Binder 2016). The cyanobacteria *Geitlerinema sp. PCC 7105* has also been shown to contain two putative ethylene receptors, GeiEtr1 and GeiEtr2. These cells have also been shown to bind and respond to ethylene with an altered phototaxis response. The ability of the putative receptors to directly bind ethylene has not yet been tested. However, GeiEtr1 and GeiEtr2 are predicted to contain histidine kinase domains and GeiEtr2 is a hybrid histidine kinase encoding a C-terminal receiver domain, meaning that these receptors could represent two-component systems for ethylene response (Allen, Lacey et al. 2019).

Role of Plant Immune Responses in shaping Plant-Microbe Interactions

Plant-microbe interactions are an increasingly studied area of research, as microbial communities have been shown to play a role in resistance to both abiotic and biotic stresses, as well as leading to increased plant vigor, and yield (Van Wees, Van der Ent et al. 2008, Newman, Sundelin et al. 2013). Some microbes have been shown to promote plant health and vigor by providing nutrients such as fixed nitrogen, iron sequestration, and solubilizing phosphate as well as by producing and modulating phytohormones (Berendsen, Pieterse et al. 2012, Pieterse, Zamioudis et al. 2014, Pieterse, de Jonge et al. 2016, Bakker, Pieterse et al. 2018, Berendsen, Vismans et al. 2018, Yu, Pieterse et al. 2019). Another proposed mechanism of plant promotion is

biocontrol, the hypothesis that saturating the root surface with beneficial microbes will prevent pathogenic microbes from colonizing roots due to production of antibiotics, siderophores and other similar products as well as competing for nutrients in the root environment (Olanrewaju, Glick et al. 2017, El-Saadony, Saad et al. 2022). These characteristics make understanding the establishment of plant-microbe interactions very fruitful for agriculture. Within plant-microbe interactions, studies focused on beneficial interactions have focused on plant root associated bacteria in a region called the rhizosphere (Berendsen, Pieterse et al. 2012, Carvalhais, Dennis et al. 2015).

The rhizosphere is defined as the region of the soil around the root in which the plant is directly affecting the bacterial community. Rhizosphere soil has been shown to be significantly more densely populated by microbes than in regions without plants (Newman 1985). The formation of the rhizosphere is brought on by the release of root exudates into the soil. These root exudates can be composed of up to 70% of the carbon fixed via photosynthesis and so the investment of resources into recruiting microbes is substantial (Newman 1985, Vande Broek and Vanderleyden 1995, Van de Broek, Lambrecht et al. 1998, Fischer, Miguel et al. 2003, Bais, Weir et al. 2006, Carrillo, Dijkstra et al. 2014, Allard-Massicotte, Tessier et al. 2016, Banks 2016, Venturi and Keel 2016, Guyonnet, Cantarel et al. 2018, Canarini, Kaiser et al. 2019, Korenblum, Dong et al. 2020, Abedini, Jaupitre et al. 2021). The secreted carbon sources can be sensed by chemoreceptors in soil bacteria and lead them to a plant host (Rudrappa, Czymmek et al. 2008, Banks 2016, Scharf, Hynes et al. 2016).

The secretion of root exudates is a major portion of selection of a rhizosphere microbiome (Abedini, Jaupitre et al. 2021, Thoms, Liang et al. 2021). Recruitment to the rhizosphere seems to have some specificity. While bacteria have preference of different carbon sources, there are other compounds like volatile organic compounds, emitted into the rhizosphere to help select for a specific microbial community (Doornbos, Geraats et al. 2011, Berendsen, Pieterse et al. 2012, Zhang, Wang et al. 2014, Carvalhais, Dennis et al. 2015, van Dam, Weinhold et al. 2016, Venturi and Keel 2016, Korenblum, Dong et al. 2020, Korenblum, Massalha et al. 2022). Once recruited to the plant the microbes can remain in the rhizosphere, surviving in the soil around the root, colonize the root surface by attachment, or adapt an endophytic lifestyle by colonizing the interior of the root. If microbes colonize the plant host, then they must engage with the plant immune system (Yu, Pieterse et al. 2019).

Interestingly, both pathogenic and beneficial microbes upregulate plant defense pathways via pattern recognition-receptors that identify Microbial Associated Molecular Patterns (MAMPs) (Newman, Sundelin et al. 2013). MAMPs are typically highly conserved features such as flagellins, polysaccharides, and chitins. This leads to a relatively universal response to both pathogens and beneficial microbes. It has been shown that, just like pathogenic bacteria, beneficial microbes evade the plant immune system using effector proteins, divergent flagellin sequences, and altering the balance of hormone levels involved in stress, growth, and defense (Van Wees, Van der Ent et al. 2008, Yu, Pieterse et al. 2019, Nishad, Ahmed et al. 2020, Thoms, Liang et al. 2021, Kawa and Brady 2022). Outside of systems that are highly co-evolved and dependent on niche signaling, such as lipo-chitooligosaccharide Nod factors in the rhizobia-legume

symbiosis model systems, the plant is still able to determine which microbes are harmful and which are allowed to colonize(Haag, Arnold et al. 2013, Via, Zanetti et al. 2016, Buhian and Bensmihen 2018). The leading hypothesis for how the plant can defend against pathogens while welcoming other microbes which all elicit the same initial response is predicated on the synthesis of multiple signaling pathways, also referred to as dual receptor recognition (Thoms, Liang et al. 2021). While there are universal MAMP triggers, it is the recognition of a secondary factor, such as effector proteins for pathogens, symbiosis promoting signals, or the presence or absence of Damage Associated Molecular Patterns (DAMPs) for much of the microbiota, that dictates the level of immune response (Jones and Dangl 2006, Zipfel 2014, Zipfel and Oldroyd 2017, Monteiro and Nishimura 2018, Thoms, Liang et al. 2021, Yuan, Jiang et al. 2021).

MAMPs recognized by plant pattern recognition receptors (PRRs) lead to pattern triggered immunity (PTI) which is coordinated by several defense hormones, jasmonic acid (JA), salicylic acid (SA), and ethylene (Lu and Tsuda 2021, Ngou, Ahn et al. 2021, Yuan, Jiang et al. 2021). All MAMPs will lead to a low level of immune response, but it is the combination of MAMP, and either effector proteins, or DAMPs that engages the more robust responses necessary for the plant to protect itself (Kadota, Sklenar et al. 2014, Li, Li et al. 2014). While JA and SA play large roles in coordinating plant defense, pattern-triggered immunity and effector triggered immunity converge on ethylene dependent responses (Ngou, Ahn et al. 2021, Yuan, Jiang et al. 2021).

Not only does the detection of MAMPs lead to an increase in ethylene production, but exogenous application of ethylene leads to an increase in expression of PRRs,

suggesting that there is a feedback loop for upregulating defense pathways in response to MAMPs (Liu and Zhang 2004, Tintor, Ross et al. 2013, Augimeri and Strap 2015, Guan, Su et al. 2015, Hou, Liu et al. 2019, Li, Han et al. 2019, Nishad, Ahmed et al. 2020, Pattyn, Vaughan-Hirsch et al. 2021). Additionally, a functional ethylene signaling pathway is required in plants to fully induce several PRRs (Lu and Tsuda 2021). Ethylene insensitive *ein2* Arabidopsis mutants have reduced sensitivity to MAMPs (Boutrot, Segonzac et al. 2010, Mersmann, Bourdais et al. 2010). Interestingly, the reduced sensitivity to some MAMPs is not correlated with a reduction in PRR expression. This reduced sensitivity is instead due to an inability to transcriptionally respond to bound PAMPs without a functional downstream ethylene signaling pathway (Xu, Greene et al. 2017).

Ethylene in Plant Microbe Interactions

As previously stated, ethylene plays a role in regulating plant immune responses. As an integral part of these immune responses, inhibition of ethylene signaling is a common method utilized by bacteria in immune avoidance (Holguin and Glick 2003, Glick, Cheng et al. 2007, Contesto, Desbrosses et al. 2008, Nascimento, Rossi et al. 2018). The most direct action taken on ethylene signaling is the degradation of the ethylene precursor 1-aminocyclopropane-1-aminocarboxylic acid (ACC) by the enzyme ACC deaminase (ACCD) (Glick 2005, Glick, Cheng et al. 2007, Singh, Shelke et al. 2015). This allows for bacteria to reduce immune responses by lowering the biosynthesis of ethylene, and thus ethylene signaling. In addition to this, ACC breaks down into ammonium and alpha-ketobutyrate, yielding both carbon and nitrogen for bacteria, and chemotaxis towards ACC has been demonstrated in many bacteria (Glick, Cheng et al. 2007, Li, Zhang et al.

2019). ACC deaminase is also considered to be a plant growth-promoting mechanism, as the lowered ethylene levels allow for plant growth pathways to take priority over immune signaling. While there are other mechanisms used to inhibit ethylene biosynthesis and ethylene signaling cascades, most of these arise from bacteria avoiding the consequence of ethylene production (Nascimento, Rossi et al. 2018). Some of these mechanisms include effector proteins that target ethylene responsive transcription factors, production of polyamines, or, rarely, usage of ethylene as a carbon source via breakdown with a monooxygenase. Most of these mechanisms are in response to plant defense signaling, and not a direct response to microbial perception of ethylene (Nonaka, Yuhashi et al. 2008, Belimov, Dodd et al. 2009, Washington, Mukhtar et al. 2016, Blüher, Laha et al. 2017).

While some microbes have been shown to produce ethylene, the role of microbially produced ethylene in plant-microbe interactions remains unclear (North, Miller et al. 2017). In fact, ethylene has almost exclusively been studied from a plant perspective in plant-microbe interactions. The previously mentioned cyanobacterial responses to ethylene are the only cases where an ethylene receptor in a prokaryote has been specifically investigated (Lacey and Binder 2016, Lacey, Allen et al. 2018, Allen, Lacey et al. 2019). However, there are some responses in bacteria that have not been linked to an ethylene receptor, either because they are not predicted to encode an ethylene receptor or because receptors have not been examined for. For instance, the fruit colonizing bacteria *Komagataeibacter xylinus* ATCC 53582 was shown to have altered physiology and gene expression in response to the ethylene releasing compound ethephon (Augimeri and Strap 2015). Interestingly, the researchers showed that none of

the other by-products of ethephon degradation could cause the responses, suggesting a legitimate response to ethylene. In the case of *K. xylinus*, no ethylene binding protein has been put forward, and it is not predicted to contain a canonical ethylene binding protein, thus raising the question of a novel ethylene binding mechanism in other species (Carlew, Allen et al. 2019). Some interesting, but unsubstantiated work reported that several *Pseudomonas* strains exhibited altered chemotaxis in response to ethylene, although only one of these strains, *Pseudomonas fluorescens* is predicted to encode a putative ethylene receptor (Kim, Shitashiro et al. 2007). In this research, the authors identified the methyl-accepting chemotaxis protein TlpQ as a potential ethylene receptor and showed that in a *tlpQ* deletion line, the chemotactic response of *P. aeruginosa* in response to ethylene was abolished. They went on to show chemotactic responses of several other strains in response to ethylene. However, in this study the ethylene levels used were extraordinarily high, in excess of 10,000 parts per million (ppm). For context, most plant responses saturate between 1 and 10 ppm. Ethylene is a lipophilic molecule and non-specific responses at high concentrations are well documented (Luckhardt and Carter 1923, Fairlie 1929, Ujváry 2014). While the potential pleotropic membrane effects on bacterial chemotaxis have not been studied, another study was unable to detect any measurable ethylene binding from the TlpQ protein (Corral-Lugo, Matilla et al. 2018). In addition to these issues, the study also did not contain relevant controls, as no chemotaxis or motility deficient strain was used. A more recent study followed up on *P. putida*'s ability to respond to ethylene by knocking out a homolog of the TlpQ protein previously identified. In addition to the knockout of a *tlpQ* homolog, this study also used the high ethylene concentrations previously reported (Li, Zhang et al. 2019). In both cases, more work is needed to verify

an ethylene specific response and find the potential mechanism by which these bacteria are responding to ethylene.

Presence of Putative Ethylene Receptors in other Bacteria

In addition to the previously discussed cyanobacteria, recent searches of sequenced genomes show that putative receptors are widespread throughout bacterial genomes. Over 300 cyanobacteria species, almost 400 α-, β-, and γ-proteobacteria as well as representatives from several other phyla of bacteria such as Planctomycetes, Bacterioidetes, and Verrucomicrobia contain putative ethylene receptors (Lacey and Binder 2016, Carlew, Allen et al. 2019). The primary amino acid sequence of the ethylene binding domain is similar across plants, fungi, and prokaryotes. The predicted output domains vary widely with some similar to plants, but others quite different. Some of these microbes are predicted to have multiple ethylene receptor isoforms as found in plants. The diversity of species, as well as domain make-up of the putative receptors suggest diverse responses.

Since ethylene can be produced abiotically or by other organisms, some of these organisms may be using ethylene as an environmental cue, as is proposed in some cyanobacteria (Lacey and Binder 2016, Kuchmina, Klähn et al. 2017, Lacey, Allen et al. 2018, Allen 2019). Many of these microbes associate with plants, including several well-studied beneficial bacteria and symbionts, suggesting the intriguing possibility that ethylene is involved in cross-kingdom communication where the plant may be using ethylene to directly affect the microbes that associate with it (Figure 1.3). Interestingly, none of the identified microbes are pathogenic. This lack of pathogens could be indicative

of the role that microbial ethylene signaling is playing in plant-microbe interactions as a way for beneficial bacteria to monitor plant health. Support for this idea comes from a recent study showing that volatiles emitted by *Carex arenaria* roots attract certain bacteria such as *Burkholderia* sp. AD024, *Dyella* sp. AD056, *Paenibacillus* sp. AD087, and *Pseudomonas* sp. AD021 from a distance to the roots (van Dam, Weinhold et al. 2016, Schulz-Bohm, Gerards et al. 2018). The role of plant-emitted volatiles is increasingly being considered for a role in establishing the root microbiome, however the direct role of ethylene on bacteria has not been studied in this capacity (Ali, Alborn et al. 2010, Junker and Tholl 2013, Ahmad, Viljoen et al. 2015, Schmidt, Cordovez et al. 2015, van Dam, Weinhold et al. 2016, Gulati, Ballhausen et al. 2020).

Azosprillum brasilense Lifestyle and Behaviors

A notable member of the plant-associated bacteria harboring a putative ethylene receptor is the plant growth-promoting rhizobacteria *A. brasilense*. Bacteria of the genus *Azospirillum* are ubiquitous, motile, soil bacteria capable of colonizing the roots of a wide range of plants including legumes, cereals, grasses, and vegetables (Bashan and Levanony 1990, Croes, Van Bastelaere et al. 1991, Michiels, Croes et al. 1991, Bashan, Puente et al. 1995, Bashan and Holguin 1997, Okon and Vanderleyden 1997, Van de Broek, Lambrecht et al. 1998, Steenhoudt and Vanderleyden 2000, Bashan, Holguin et al. 2004, Pereg, de-Bashan et al. 2016). *Azospirillum* species are gram negative alpha proteobacteria that are known to establish associations with the roots of these diverse plants where they live as commensals (Pereg, de-Bashan et al. 2016). Preference for a

microaerophilic lifestyle, and ability to fix nitrogen make it well suited for life in the rhizosphere and the plant root environment where oxygen and nitrogen are both scarce.

A. brasilense are highly pleomorphic and are capable of differentiation in response to nutrient gradients and environmental stressors (Sadasivan and Neyra 1985, Sadasivan and Neyra 1987, Malinich and Bauer 2018). Amongst these differentiations is the process of flocculation which is induced under conditions of both low nitrogen and aerobic stress (Sadasivan and Neyra 1985). This process leads to cells swimming together and attaching to one another, losing their flagella, and forming polysaccharide heavy clumps which will protect the nitrogen fixing machinery (Aksenova 2014, Bible, Khalsa-Moyers et al. 2015). Other differentiation processes include biofilm formation which is induced under a high carbon to nitrogen ratio (Burdman, Okon et al. 2000, Siuti, Green et al. 2011).

Attachment to the surface of roots and biofilm formation are biphasic in *A. brasilense* (Michiels, Croes et al. 1991, Burdman, Okon et al. 2000, Bashan and De-Bashan 2005, Wheatley and Poole 2018*)*. This process first leads to cellular attachment to a surface via the flagellum. The flagellum of *A. brasilense* is glycosylated and this is believed to play a role in initial attachment to surfaces and plant roots (Moens, Michiels et al. 1995). After initial attachment via the flagellum, bacteria undergo a transition to an irreversible attachment called anchoring. Anchoring is defined by the production of extracellular polysaccharides (EPS) and EPS-mediated the anchoring of cells to the surface and to each other. The specific polysaccharides produced by *A. brasilense* during attachment behaviors is not known, but monosaccharide compositions are known to change in response to carbon to nitrogen ratios, root exudates and salt stress

(Burdman, Jurkevitch et al. 2000, Fischer, Miguel et al. 2003, Bahat-Samet, Castro-Sowinski et al. 2004, Sigida, Fedonenko et al. 2014, Rossi, Medeot et al. 2016).

A. brasilense Sp7 colonizes the surfaces of roots and is used agriculturally to boost crop production (Pereg, de-Bashan et al. 2016). While the plant growth promoting effects of *A. brasilense* have been described in most detail in cereals, the beneficial association of *A. brasilense* with plants is not restricted to cereals since they have been reported in many plant species across botanical families including *Solanum lycopersicum* (tomato) and *Arabidopsis thaliana* (Ribaudo, Krumpholz et al. 2006, Spaepen, Bossuyt et al. 2014, Pereg, de-Bashan et al. 2016).

A. brasilense has many proposed mechanisms of plant growth promotion, including nitrogen fixation and phytohormone production. *A. brasilense* produces auxin which is linked to its effect in remodeling roots (Reynders and Vlassak 1979, Barbieri and Galli 1993, Perrig, Boiero et al. 2007, Malhotra and Srivastava 2008). Like many beneficial bacteria, RNA-seq studies reveal that inoculation of plants with *A. brasilense* alters the expression of genes in the plant related to ethylene biosynthesis and signaling (Camilios-Neto, Bonato et al. 2014, Drogue, Sanguin et al. 2014, Spaepen, Bossuyt et al. 2014, Thomas, Kim et al. 2019). *A. brasilense* is known to produce auxin via the tryptophan biosynthesis pathway, and the indole pyruvate decarboxylase (*IpdC*) gene has been shown to be responsible for around 90% of auxin production (Malhotra and Srivastava 2008). This pathway has also been shown to be upregulated by the presence of auxin, creating a positive feedback loop to produce the phytohormone. In addition to the IpdC gene, treatment with auxin can produce broad transcriptomic changes by one hour of treatment with 100 micromolar IAA (Lambrecht, Vande Broek et al. 1999, Broek,

Gysegom et al. 2005, Van Puyvelde, Cloots et al. 2011). *A. brasilense* strains that are unable to produce auxin lose their ability to promote plant growth. The inability of *lpdC* mutants to promote plant growth is probably due to not only the reduced auxin production, but also to a change in the expression pattern of genes that respond to auxin.

The study of biological fixation of atmospheric nitrogen has been increasing in recent years as the ecological costs of chemical fertilizers become apparent. The fixation of nitrogen in *A. brasilense* is carried out by a dinitrogen reductase, NifH subunit (Liang, de Zamaroczy et al. 1992). As previously mentioned, nitrogen fixation in *A. brasilense* is linked to perception of the nutrient environment and the major regulators of nitrogen fixation monitor cellular carbon and nitrogen levels via the presence of 2-oxoglutarate and glutamine (Liang, de Zamaroczy et al. 1992, Arsène, Kaminski et al. 1996, de Zamaroczy, Paquelin et al. 1996, Huergo, Chubatsu et al. 2006, Gerhardt, Parize et al. 2020, Forchhammer, Selim et al. 2022). These metabolites allow for the cell to monitor flux through glycolysis and the TCA and nitrogen status of the cell with metabolites that are tightly linked. The GOGAT (Glutamine Oxoglutarate Aminotransferase) pathway makes glutamate from 2-oxoglutarate and glutamine from the glutamate that is produced (Temple, Vance et al. 1998, Huergo and Dixon 2015). These metabolites, glutamine and 2-oxoglutarate are detected by the PII nitrogen regulators GlnB and GlnZ (de Zamaroczy, Paquelin et al. 1996, De Zamaroczy 1998, Araújo, Monteiro et al. 2004, Araújo, Huergo et al. 2008, Kukolj, Pedrosa et al. 2019). Under nitrogen replete conditions, sensing of the carbon-nitrogen status of the cell will lead to modification of GlnB which promotes kinase activity of NtrB which is responsible for the phosphorylation of the translational regulator NtrC. NtrC is responsible for promoting the expression of not only *glnB* and the glutamine

synthetase *glnA*, but also for the *nif* operon, including *nifh*, the gene encoding for the dinitrogen reductase subunit (Broek, Michiels et al. 1992, Liang, de Zamaroczy et al. 1992, Huergo, Chubatsu et al. 2006, Huergo, Monteiro et al. 2008, Kukolj, Pedrosa et al. 2019, Forchhammer, Selim et al. 2022).

A. brasilense Putative Ethylene Receptor Binds to Ethylene

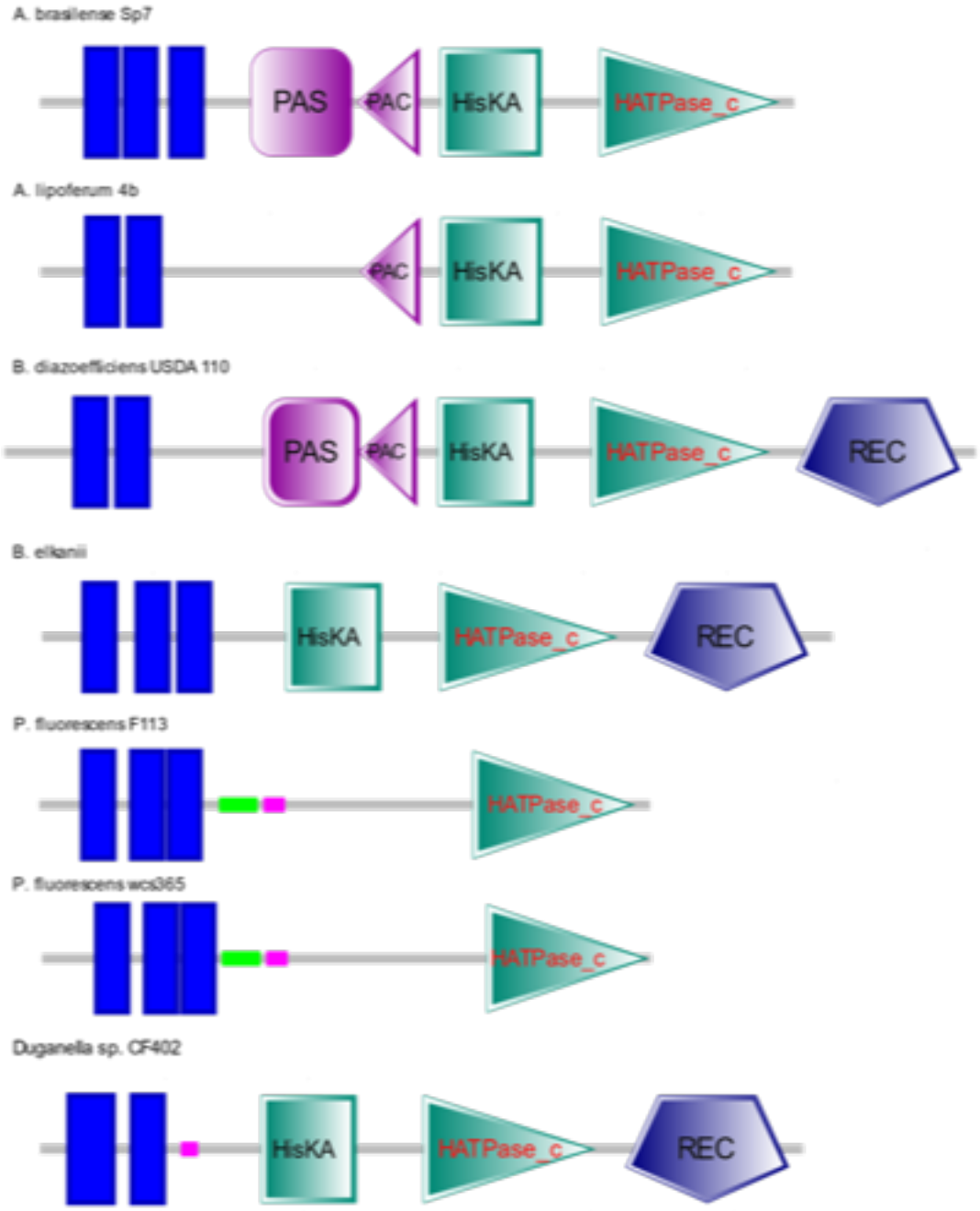
Sequence alignment of the AzoEtr1 predicted binding domain alongside the five *A. thaliana* receptors and SynEtr1 show that it has conserved the 7 amino acid residues shown to be needed for binding in *A. thaliana* (Figure 1.4A) (Wang, Esch et al. 2006). Previous experiments from the Binder and Alexandre lab, performed by Dr. Randy Lacey, shows that wild-type *A. brasilense* Sp7 binds ethylene above background levels, and that disruption of the putative receptor *AzoEtr1* results in a loss of ethylene binding (Figure 1.4B).

Expression of the EBD alone in *P. pastoris* cells also results in binding of ethylene above empty vector levels. Together this data shows that the EBD of AzoEtr1 is capable of binding ethylene, and that AzoEtr1 is responsible for ethylene binding observed by *A. brasilense* (Figure 1.4C). Two antibiotic resistance cassette insertional disruptions have been made in a*zoEtr1, azoEtr1::Tet*^{R} and *azoEtr1::Gm*^{R} (Figure 1.4D). *azoEtr1::Gm*^{R} was constructed due to some residual binding in *azoEtr1::Tet*^{R}*. azoEtr1::Gm*^{R} is disrupted directly between transmembranes 1 and 2 in the predicted EBD and thus should have no residual binding. Only a*zoEtr1::Tet*^{R} has been tested for ethylene binding, but phenotypically the two mutants are identical and will be referred to interchangeably as *AzoEtr1*$^-$ unless they are being directly compared. This thesis will investigate the role that

this receptor, and response to ethylene plays in the lifestyle of *A. brasilense* and the potential impacts on plant-microbe interactions.

Figure 1.3. Putative Ethylene Receptors in Plant Associated Bacteria.

Many plant-associated bacteria harbor putative receptors that contain the seven amino acids necessary for binding in a putative EBD. Among these bacteria the protein domains are less diverse, and are largely made up of histidine kinases, or hybrid histidine kinases. Additionally, there is some diversity in the EBD as some proteins have only two predicted transmembrane domains rather than the canonical three transmembrane domains. Domain predictions done using SMART.


A. brasilense Sp7
PAS
PAC
HisKA
HATPase_c
A. lipoferum 4b
PAC
HisKA
HATPase_c
B. diazoefficiens USDA 110
PAS
PAC
HisKA
HATPase_c
REC
B. elkanii
HisKA
HATPase_c
REC
P. fluorescens F113
HATPase_c
P. fluorescens wcs365
HATPase_c
Duganella sp. CF402
HisKA
HATPase_c
REC

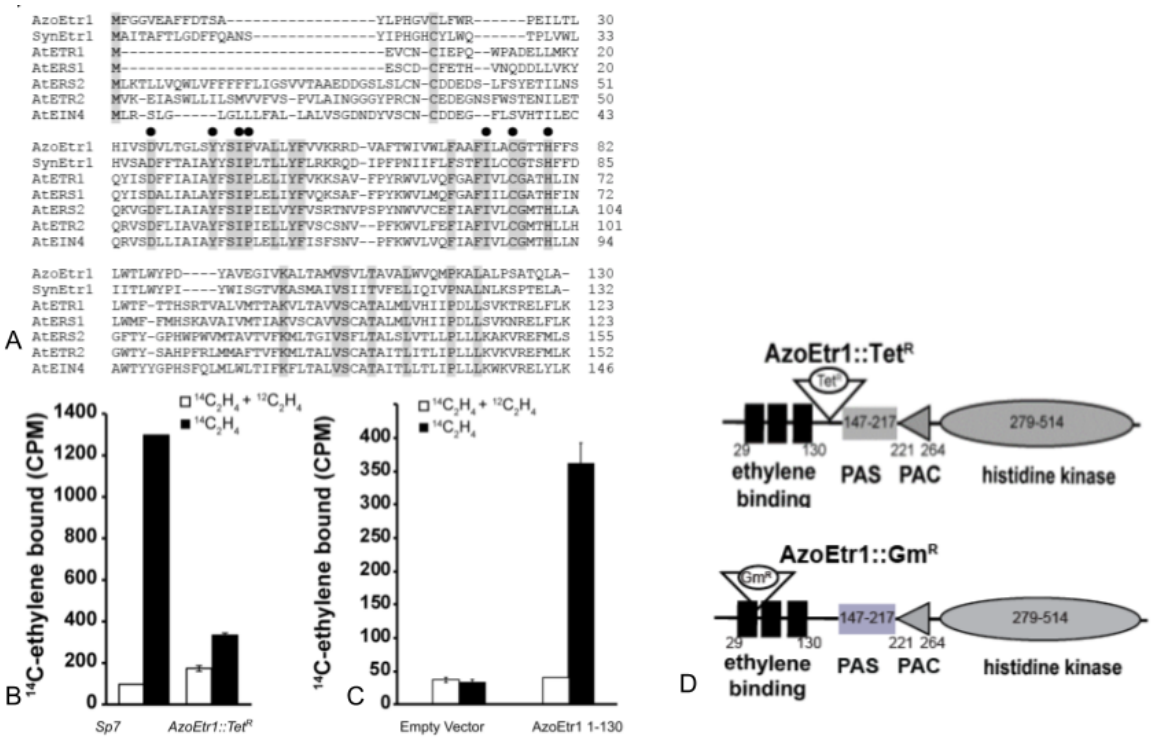


Figure 1.4 *A. brasilense* Ethylene Receptor, AzoEtr1, Binds to Ethylene

A) Sequence alignment of the EBD's from *A. brasilense*, *Synechocystis sp. 6803*, and the five *A. thaliana* ethylene receptors. Residues denoted with a black circle are necessary for binding of ethylene by AtEtr1. B) Radioactive ethylene binding assay showing results for whole cell *A. brasilense* Sp7, and the *azoEtr1::Tet*^{R} mutant. Black bars represent total binding, white bars represent non-specific binding. C) Radioactive ethylene binding assay for whole cell *P. pastoris* expressing either empty vector or the first 130 amino acids of AzoEtr1, representing the EBD alone. D) schematic showing the protein domains for AzoEtr1 and the location of antibiotic resistance cassette insertion for both mutant lines used (unpublished results). Sequence alignment's performed with Clustal Omega.

Chapter 2: Materials and Methods

Bacterial strains and culture conditions:

Azospirillum brasilense were grown in tryptone-yeast extract (TY) or minimal media for *A. brasilense* (MMAB) at 30°C overnight with shaking at 250 rpm except where noted otherwise (Grishanan 1991). MMAB was prepared as previously described with one exception, $NaNO_3$ was used as the available nitrogen source at 10mM rather than NH_4Cl. E. coli were grown in Luria Broth overnight at 37°C. For both *A. brasilense* *E. coli*, appropriate antibiotics were used as denoted in Table 1. For all assays, *A. brasilense* was grown overnight under these conditions, then washed three times in Che Buffer and resuspended to an OD^{600} 1.0 unless otherwise specified.

Cloning and Complementation:

DH5 alpha E. coli was used for subcloning. Gateway cloning was used to create the C-terminal YFP fusions of *AzoEtr1* using the pRH005 destination vector. Gateway cloning was done according to manufacturer recommendations. Gene specific primers were made and Sp7 genomic DNA was used as a template. PCR products then underwent BP and LR cloning steps to move the PCR product first into pDONR2.1, an expression vector before being moved into pRH005 the destination vector, yielding the YFP fusion constructs. To create site directed mutants *azoEtr1* C75A and D35A, Etr1 was ligated into the pUC19 plasmid and site directed mutagenesis was performed. After confirmation of mutagenesis the primers used to make the gateway primers used to make the original YFP fusion of *azoEtr1* were utilized. Primers were designed for

alanine mutations of residues shown to be important in ethylene binding. The primers used for this are listed in Table 2, lower case letters denote point mutations. Constructs confirmed by sequencing were transformed into *DH5 alpha E. coli* and then mated into *A. brasilense* strains using the triparental conjugation protocol for *A. brasilense* (Gullett, O'Neal et al. 2017).

Crystal Violet Biofilm Staining:

Performed as in (Suiti et al, 2011) with some modifications. Assays were performed in 12-well PVLC plates with 1mL of MMAB supplemented with 1mM $NaNO_3$ as the nitrogen source, inoculated with 30 µL of *A. brasilense* OD_{600} 1.0 for 3 days. Plates were placed in airtight chambers and treated with 0.1ppm ethylene or specified concentration. Lids of 12-well plates were pierced with a 27-gauge needle to ensure equal gas flow between wells. Holes made by needle were sealed with micropore tape that would allow gas exchange but not passage of potential contaminants. After 3-days, liquid was removed from wells and 500 µL 0.01% crystal violet is added and allowed to stain for 20 minutes. After 20 minutes, crystal violet is removed, and the plate undergoes 3 consecutive rough washes in DI water. After washes are complete 2 mL of 100% ethanol is added to each well and allowed to sit for 5 minutes. 1 mL of ethanol is removed and the OD_{600} is measured for each sample. Statistical analysis was done in Graphpad Prism.

Biofilm on Slide:

A. brasilense cultures were grown in TY media overnight with appropriate antibiotics and adjusted to OD_{600} 1.0 in the same low nitrogen MMAB used in the 12-

well biofilm assays but resuspended in biofilm media rather than Che buffer. 10 microliters were then plated onto Poly-L-Lysine coated slides without a coverslip and placed into 500 mL mason jars with injection ports for ethylene. Jars had Kimwipes dampened with DI water placed inside to prevent evaporation or drying of samples before being sealed with parafilm around the lids. Samples were then either injected with 0.5 mL 100 ppm ethylene to deliver an equilibrated concentration of 0.1 ppm or were injected with ethylene free air to maintain pressure equivalence between samples. Finally, samples were placed into a 28°C incubator overnight to allow for biofilm growth. After 24 hours the slides were removed, and a coverslip was placed on top of the spotted culture. This was then imaged on Zeiss Axio Observer Z1 at 100x.

Colonization Assays:

Performed as in (Mukherjee et al, 2016) with some modifications. Tomato seeds were surface sterilized by incubation in 50% bleach for 30 minutes, followed by 3 washes with dH_2O and plating on ¼ MS-NS agar for 3 days at 28°C for germination. 50mL chambers were filled with 25mL of Farhaeus media and two plants were placed in each chamber. Bacteria were grown to and OD_{600} 0.7 in MMAB with 10 mM $NaNO_3$ as the nitrogen source before being concentrated as previously reported. 20 µL of bacteria are inoculated into the center of each chamber. The inoculum culture is serially diluted to 10^-8 and plated for CFU counting to determine the number of cells input into the system. 4 chambers with two plants each were used for each condition per assay. Treatment conditions included treating the colonization chambers with 0.1 ppm ethylene for the duration of the assay, or growth in the presence of ethylene-free air. After 24

hours, the roots are removed and gently rinsed with DI water. After this root tissue is homogenized using a bead homogenizer. The resulting mixture is serially diluted to 10^-8 and plated to count CFUs to determine output of cells. Colonization index was calculated as previously, then normalized to either wild-type conditions or ethylene-free conditions.

For *A. thaliana* colonization, plants were sterilized under the same conditions as tomato, stratified for 3 days and then plated onto ¼ MS-NS and grown for 5 days at 22°C. After 5 days plants were dipped into bacteria concentrated as previously described. After dipping in bacteria, plants were patted dry with Kimwipes and placed onto a fresh ¼ MS-NS plate. Ethylene treatment occurred as previously described for tomato. Colonization assays were performed under the same temperature as plant growth. After the specified time point, whole plants were weighed and then homogenized before serial dilution and plating for CFU counting. Analysis was done by measuring CFUs per gram of fresh weight for whole plants.

Fluorescent Microscopy of Colonization:

YFP expressing *A. brasilense* were made by conjugation with the gateway destination vector pRH005 to express a Kan^R promoter-YFP fusion. This construct yielded high levels of fluorescence without antibiotic maintenance. For tomato cells were grown as previously described for colonization assays but were mixed with 0.6% agar Farhaeus media to yield 0.3% media mixed with concentrated bacteria. This was used to fill microscopy slide-in-chambers, previously described, containing one tomato

plant (Morrell-Falvey, Alexandre-Jouline et al. 2016). These chambers were kept at 28°C for 24 hours before imaging on Leica SP8 confocal microscope.

Fluorescent Microscopy on *A. thaliana* roots was done by preparation of cells as previously described. Two 5-day old *A. thaliana* seedlings were placed into twelve well plates containing 5mL of Farhaeus media per well and inoculated with 20µL of bacteria in the center of each well. After 24 hours, plants were removed from the wells and placed onto slides with the addition of 5µL of water. Images were taken on a Zeiss Axio Observer Z1

Localization of AzoEtr1:

Using the AzoEtr1-YFP fusion construct conjugated into Sp7 cells, image stacks were taken on the Leica SP8 confocal microscope. Analysis of images was done by max intensity projection of 25 stacks. Processing of the image was done using Lightning deconvolution software.

Sequence alignment and domain prediction:

Sequence alignment was done using Clustal Omega alignment https://www.ebi.ac.uk/Tools/msa/clustalo/. Highlighted residues correspond to the 7 residues that were shown to be necessary for ethylene binding previously (Wang et al., 2006). Genomic predictions of Etr1 and RR^{Etr1} locations were made using BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi. Protein domain predictions were done using Simple Modular Architecture Research Tool (SMART) http://smart/embl-heidelberg.de/ (Schultz et al., 1998; Letunic et al., 2012).

RNA isolation, cDNA synthesis, qPCR:

For gene expression analysis, RNA was extracted from A. brasilense grown overnight in 10 mL of MMAB in the presence of air or designated ethylene concentrations (0.1ppm). RNA isolation was done by pelleting 2 mL of cells before resuspension in 550 µL of Trizol and dissolution of cell pellets. After cell pellets were dissolved, 250 µL of lysozyme was added, and samples were incubated for 20 minutes at 37°C. After incubation samples were placed on ice for 5 minutes before addition of 200 µL chloroform. Samples were inverted 5 times after chloroform addition and then centrifuged at 13,000 rpm for 15 minutes. After centrifugation the top layer of the biphasic sample was moved to a fresh tube, and an equal volume of isopropanol was added. Samples were incubated at -80°C for 15 minutes to precipitate nucleotides. After precipitation in isopropanol samples were centrifuged for 15 minutes at 13,000 rpm. Supernatant was carefully removed, and pellets were resuspended in 50 µL RNase free water. Samples were then treated with Turbo DNase according to manufacturer protocols. After DNase treatment, supernatant was moved to a fresh tube and an equal volume of isopropanol was added. Samples were then incubated at -80°C. After precipitation samples were centrifuged at 13,000 rpm for 10 minutes. Supernatant was carefully removed, and pellets were washed with ice cold 75% ethanol twice. After washing, ethanol was allowed to evaporate and finally pellets were resuspended in nuclease free water before measurement on a nanodrop.

cDNA synthesis:

cDNA synthesis was done with sensifast cDNA synthesis kits. RNA was normalized to 100ng/µL and 100ng/µL cDNA was made.

qPCR Experiments and Analysis:

qPCR was done using SYBR-NOROX kits. Primers for quantitative PCR were designed using IDTNA. Reference genes GlyA, and RecA were used as recommended previously (McMillan and Pereg et al., 2014). Primer sequences can be found in table 3. Analysis of qPCR data was done using the $-2^{\Delta\Delta Ct}$ method with samples normalized to expression in air.

Colony Morphology:

Cells were grown overnight in TY media and adjusted to OD_{600} 1.0. 10µL spots were made on TY plates and allowed to dry and grow for 3 days before imaging on a dissecting scope with a Nikon Camera.

RNAseq:

RNA was isolated as described after 4 hours of ethylene treatment, 16 hours of growth total and sent to GeneWiz for sequencing. RNAseq was performed as follows, protocol summarized from Genewiz. RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with 4200 TapeStation (Agilent Technologies, Palo Alto, CA, USA). Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate p-values and Log2 fold changes. Genes with adjusted p-values < 0.05 and absolute log2 fold changes > 1 were called as differentially

expressed genes for each comparison. A gene ontology analysis was performed on the statistically significant set of genes by implementing the software GeneSCF. The mgi GO list was used to cluster the set of genes based on their biological process and determine their statistical significance. A PCA analysis was performed using the Page 2 of 9 "plotPCA" function within the DESeq2 R package. The plot shows the samples in a 2D plane spanned by their first two principal components. The top 500 genes, selected by highest row variance, were used to generate the plot.

Metabolomics:

Samples were grown in TY overnight before being washed in Che Buffer and having OD_{600} normalized to 1.0. These samples were then used to inoculate 10mL of MMAB supplemented with $NaNO_3$. Samples grew for 24 hours in sealed tubes injected with 0.1ppm ethylene or equivalent volume of ethylene-free air. After 24 hours of growth, optical density was standardized, 10mL of each sample was moved to a 15mL falcon tube before centrifugation at 4,250rpm for 5 minutes. Supernatant was removed and samples were flash-frozen in liquid nitrogen before analysis by LC-MS/MS. A partial least squares differential analysis was performed to determine the separation of metabolic profiles.

Time course metabolomics were grown by inoculating 50 mL cultures from overnight controls grown in TY, washed, and adjusted to OD_{600} 1.0. 50 mL cultures were grown in flow through chambers with either 0.1 ppm ethylene or ethylene-free air. The major difference between conditions in the two metabolomic experiments is the

aerobic status. Flow through allows for constant refreshment of oxygen, as sealed tubes are likely anaerobic by 24 hours.

Congo Red:

Congo Red staining was done by adding Congo Red dye to the specified nutrient media at a concentration of 37.5 µg/mL. Colonies were grown overnight in TY media and had their OD_{600} adjusted to 1.0 after washing with Che Buffer.10µL spots were made on media and allowed to dry. For TY and MMAB plates growth was allowed to progress for 3 days before imaging. Biofilm plates were allowed to grow for 7 days.

PHB staining:

PHB granules were stained using 0.5% Nile Red. Cells were grown overnight in TY and washed in Che Buffer before adjusting their OD_{600} to 1.0. These samples were used to inoculate 50 mL cultures of MMAB supplemented with $NaNO_3$. The cultures were grown in flow-through chambers treated with either 0.1ppm ethylene or ethylene free air. Stirring was achieved using a stir bar set to 200rpm. Growth was allowed to occur for 24 hours. 2 mL of these cultures were taken and washed in Che Buffer before staining with Nile Red for 30 minutes. After staining, cells are again washed in Che Buffer and imaged on a Zeiss Axio Observer Z1. Images were analyzed in Fiji to determine the number of foci per cell. Statistical significance was determined using a Mann-Whitney Test.

Oxidative Stress:

Sp7 and *AzoEtr1*$^{-}$ cells were mixed 1:1 with solid TY agar. After 24 hours sterile filter paper disks were soaked in 0.3% hydrogen peroxide. After another 24 hours zone of inhibition diameters were measured. For ethylene treatment, plates were put in flow-through chambers and treated with 1ppm ethylene.

Carotenoid Extraction:

Cultures were grown for three days in TY at 28°C. After three days of growth cells were pelleted at 4,250 rpm and resuspended in an equal volume of 100% methanol. This was placed on a shaker overnight at room temperature at a speed of 180 rpm. After shaking overnight, 1mL was taken and measured at OD_{485}.

Auxin Production:

Auxin Production was measured by Salkowski reaction. Cultures were grown overnight in TY. They were washed, and OD_{600} adjusted to 1.0. Then 100µL were used to inoculate 10mL of MMAB supplemented with $NaNO_3$ and 5mM Tryptophan. Allow to grow overnight, Pellet cells and move supernatant to a fresh tube. To the supernatant ad 2mL 35% perchlorate and 10mM $FeCl_3$. Invert tubes and allow to incubate in darkness for 30 minutes.

Table 1: Strains/plasmids

Strain/Plasmid	**Relevant Characteristics**	**Source**
Azospirillum brasilense Sp7	Amp^R	Tarrand 1978
azoEtr1::Tet^R	Insertional disruption of *azoEtr1*, Amp^R, Tet^R	This work
azoEtr1::Gm^R	Insertional disruption of *azoEtr1*, Amp^R, Gm^R	This work
DH5 alpha	$RecA1^-$ $EndA1^-$ blue/white	Thermofisher
Azospirillum brasilense Sp7 (pRH005-Etr1)	Sp7 expressing Etr1-YFP fusion off plasmid, Amp^R, Kan^R	This work
azoEtr1::Gm^R (pRH005-Etr1)	Insertional disruption of AzoEtr1, Gentamicin Resistance expressing and Etr1-YFP fusion off plasmid, Gm^R, Kan^R, Amp^R	This work
azoEtr1::Gm^R (pRH005-D35A Etr1)	Insertional disruption of AzoEtr1, Gentamicin Resistance expressing a site directed mutation of D35A Etr1-YFP fusion off plasmid, Gm^R, Kan^R, Amp^R	This work
azoEtr1::Gm^R (pRH005- C35A Etr1)	Insertional disruption of *azoEtr1*, Gentamicin Resistance expressing a site directed mutation of C75A Etr1-YFP fusion off plasmid, Gm^R, Kan^R, Amp^R	This work
pRH005	Gateway-based destination vector expressing proteins fused with YFP at their C-terminus; Km^R, Cm^R	Kentner 2006

Table 2: cloning primers

Gene	**Primer Sequence**
Gateway Etr1 Native Promoter Region	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTCCGCTTCGAGGGAGCGGGTGTGGACG
Gateway Etr1 Reverse	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTGc GCT CAT GCC TGA CCC CGT Cac
C75A F	CATCCTGGCCgcCGGCACGACGC
C75A R	AAGGCGGCGAACAGCCAG
D35A F	CATCGTGTCCgctGTGTTAACCG
D35A R	TGCAGGGTCAGGATTTCC

Table 3: qPCR primers

Gene	Forward Primer	Reverse Primer	Source
RecA	GTCGAACTGCCTGGTGATCT	GACGGAGGCGTAGAACTTCA	Mcmillan 2016
GlyA	GGAGATCGCCAAGAAGATCA	GCTCTTGGCGTAGGTCTTGA	Mcmillan 2016
lpdC	AGTCGTCCAGGTCGTTGAAG	ACCCCATCGTGATCCTG	Palacios 2016
AzoEtr1	GCCGTGGSSGGCATCGTCAAG	GCGCTGAAGGGCTCCGTTGGC	This Work
RR^{Etr1}	TTCTTTCCCGCAAGGGATAC	GCAGATCGGTGATGACGAG	This Work
OH82_RS30850	TGGCCGTAGAGGATCTCGTT	AAGGTCGTCGCTCCCTACAG	This Work
OH82_RS14895	TCATGGGATGCCTGCTGTTC	TTTTCGCGAGATAGGGGACG	This Work
GlnB	TCACGAAGTCGGCATCAAGG	TTCACCTTCGGCAGGAAGTC	Moure 2019
GlnZ	GTCGAGGCGATCCAGAAGG	CAGAGAGCTTCGGTGTTGGT	Moure 2020
AmtB	TGGTCAGCATCCTGTGGTTC	TCAGGCTGTCCTTGGTGATG	Moure 2021

Table 3: qPCR primers continued

Gene	Forward Primer	Reverse Primer	Source
NifH	AACAAGGCGCAGGAAAATC TACA	ATGCCCCTTGGAGATGTT GTTG	Camilios-Neto 2015

Chapter 3: *A. brasilense* Physiological Responses to Ethylene

Additional authors that contributed research in this work: Eric Brenya, Brianna Sexton, Lauren Donnelly, Eric Heinze, Josie King.

Abstract

Ethylene perception and disruption of the receptor alter the extracellular polysaccharides present and inhibit biofilm formation, and root colonization. Both ethylene-treated cells, and *azoEtr1* have a distinct lack of cell aggregation under biofilm conditions. Light microscopy reveals that these conditions lead to single cell attachments to surfaces unlike the robust aggregation under control conditions. *A. brasilense* biofilm formation is robustly inhibited at ethylene concentrations as low as 0.008 ppm ethylene which is below the threshold for most plant responses. Root colonization of both tomato and *A. thaliana* is reduced by *azoEtr1* disruption and ethylene treatment. Inhibition of colonization by ethylene treatment seems to be temporal as colonization is recovered on *A. thaliana* roots after 48 hours. Colonization inhibition of *azoEtr1* is not recovered by 48 hours. In addition to inhibition of colonization, treatment with ethylene reduces auxin production by *A. brasilense*. This change in auxin production is not related to transcript levels of the *IpdC* gene which is responsible for about 90% of auxin production in *A. brasilense*. Together, the data here show that ethylene inhibits attachment of *A. brasilense* to plant roots, at least in early stages of colonization, and alters other plant associated traits.

Introduction

It has been previously documented that many non-plant species contain putative ethylene receptors (Rodrıguez, Esch et al. 1999, Wang, Esch et al. 2006, Lacey and Binder 2016, Allen, Lacey et al. 2019, Carlew, Allen et al. 2019). Here we show evidence that the putative receptor in *Azospirillum brasilense Sp7*, AzoEtr1, is a functional ethylene receptor. This protein is predicted to encode three N-terminal transmembrane domains that contain the residues required for ethylene binding, making up a canonical ethylene binding domain (EBD). This protein is also predicted to encode a PAS/PAC domain and a c-terminal histidine kinase. Downstream from the encoded *AzoEtr1* there is a predicted response *AzoRR*^{Etr1} regulator with an overlapping start codon, forming a proposed operon. Together, these likely represent a two-component system that responds to ethylene (Figure 3.1)(Stock, Robinson et al. 2000). Response to ethylene via a two-component system is consistent with the existing receptors that have been studied (Chang, Kwok et al. 1993, Narikawa, Suzuki et al. 2011, Binder, Kim et al. 2018)

Amongst the many bacteria with putative receptors, *A. brasilense* was of particular interest due to its plant interactions. Ethylene has a known role in plant-microbe interactions that has been covered earlier in this work, and so we analyzed the physiological response of *A. brasilense* to ethylene in the context of plant-microbe interactions and survival in the soil.

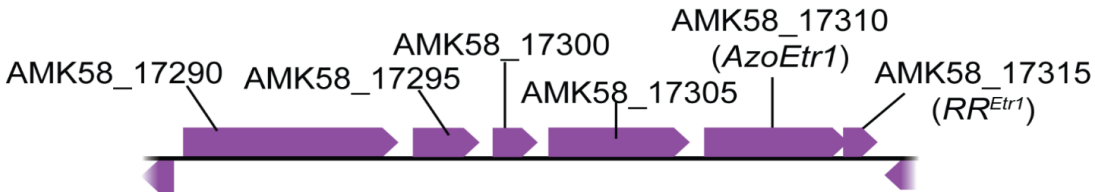


Figure 3.1 Genomic Region around *AzoEtr1*

The genomic region near *azoEtr1* (AMK58_17310) reveals a downstream gene encoded to predict a single-domain response regulator (AMK58_17315) denoted as RR^{Etr1}.

Results

Ethylene perception alters surface characteristics and biofilm formation of *A. brasilense*.

The ability of AzoEtr1 to bind to ethylene was demonstrated by previous unpublished work in the lab, but the response to the signal had not been investigated. Using the *azoEtr1* mutant line we began investigating potential defects that were related to plant attachment. Previous work has shown the ability of *A. brasilense* mutants to bind to Congo Red is correlated with their ability to form aggregates both in flocculation and biofilm(Del Gallo, Negi et al. 1989, Michiels, Verreth et al. 1990, Burdman, Jurkevitch et al. 1998, Burdman, Jurkevitch et al. 2000, Burdman, Okon et al. 2000, Sheludko, Kulibyakina et al. 2008). To determine if ethylene treatment or *azoEtr1* mutants caused changes in the surface characteristics of *A. brasilense*, we used Congo Red dye under different nutrient media conditions. Congo Red is a dye that binds to polysaccharides and has been used as an indicator for the abundance of ß1-3, and 1-4 glucans polysaccharides(Teather and Wood 1982). When grown on both rich and minimal media there was no observable difference in the amount of staining in the *azoEtr1* and *Sp7* colonies (Figure 3.2 A, B, D, E). However, when grown in low nitrogen minimal media that has been shown to be optimal for biofilm formation, *azoEtr1* colonies uptake less dye than *Sp7* suggesting that there is a difference in polysaccharides between the two under these conditions (Figure 3.2C, F) (Siuti, Green et al. 2011). Addition of ethylene to *Sp7* did reduce the amount of dye uptake but this phenotype was less severe than in *azoEtr1* (Figure 3.3 A, B).

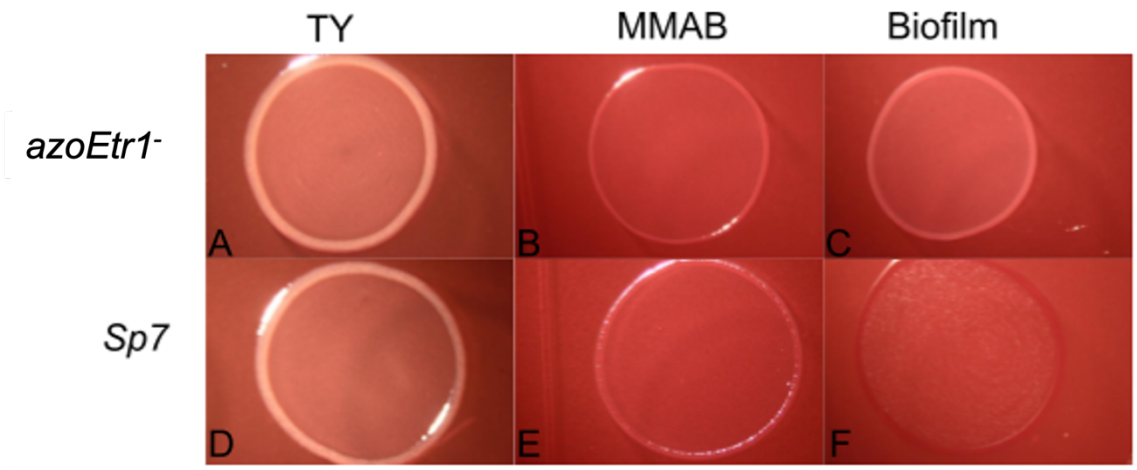


Figure 3.2 Disruption of azoEtr1 reduces Congo Red binding under biofilm conditions.

Congo Red dye binding of colonies on TY (A, D), MMAB (B, E) and biofilm media (C, F) for *Sp7* (bottom) and *azoEtr1* (top). Congo Red binding is signified by an increase in red coloration of the colony. Paler colonies have lower Congo Red binding.

With changes in Congo Red binding occurring under conditions of biofilm formation we tested the ability of *A. brasilense* to form biofilm in the presence of ethylene. The ability of *A. brasilense Sp7* to attach to hydrophobic surfaces and build biofilm was tested over the course of three days in under the same nutrient conditions used in Congo Red binding assays. Samples were either treated with 0.1ppm ethylene or ethylene-free air in a flow through system that allowed for constant concentration of ethylene to be delivered. It was observed that in the presence of ethylene, *Sp7* had significantly reduced biofilm formation. Neither *azoEtr1::Tet*^{R} nor *azoEtr1::Gm*^{R} formed biofilm in either air or ethylene conditions. Both treatment with ethylene and the disruption of the receptor resulted in decreased biofilm formation (Figure 3.4). To examine the loss of biofilm formation more closely, a biofilm on slide assay was developed and *Sp7* and *azoEtr1* were imaged after 24 hours of biofilm formation under either air or ethylene treated conditions. Wild-type cells in the absence of ethylene were shown to form robust biofilms, making identification of single cells impossible (Figure 3.4B). However, in both the ethylene treated conditions and the mutant lines no aggregation or biofilm formation was visible (Figure 3.4B, C respectively). Instead, single cells were easily distinguishable from one another, and no aggregation was easily discernible. We examined biofilm formation at different levels of ethylene (0.08 ppm, 0.07 ppm, .29 ppm and 10 ppm) and saw biofilm significantly reduced at concentrations as low as 0.008 ppm (Figure 3.5). The lowest concentration was not significantly different from any other dosage of ethylene, but all dosages were significantly lower than air. This range of ethylene concentrations was chosen due to

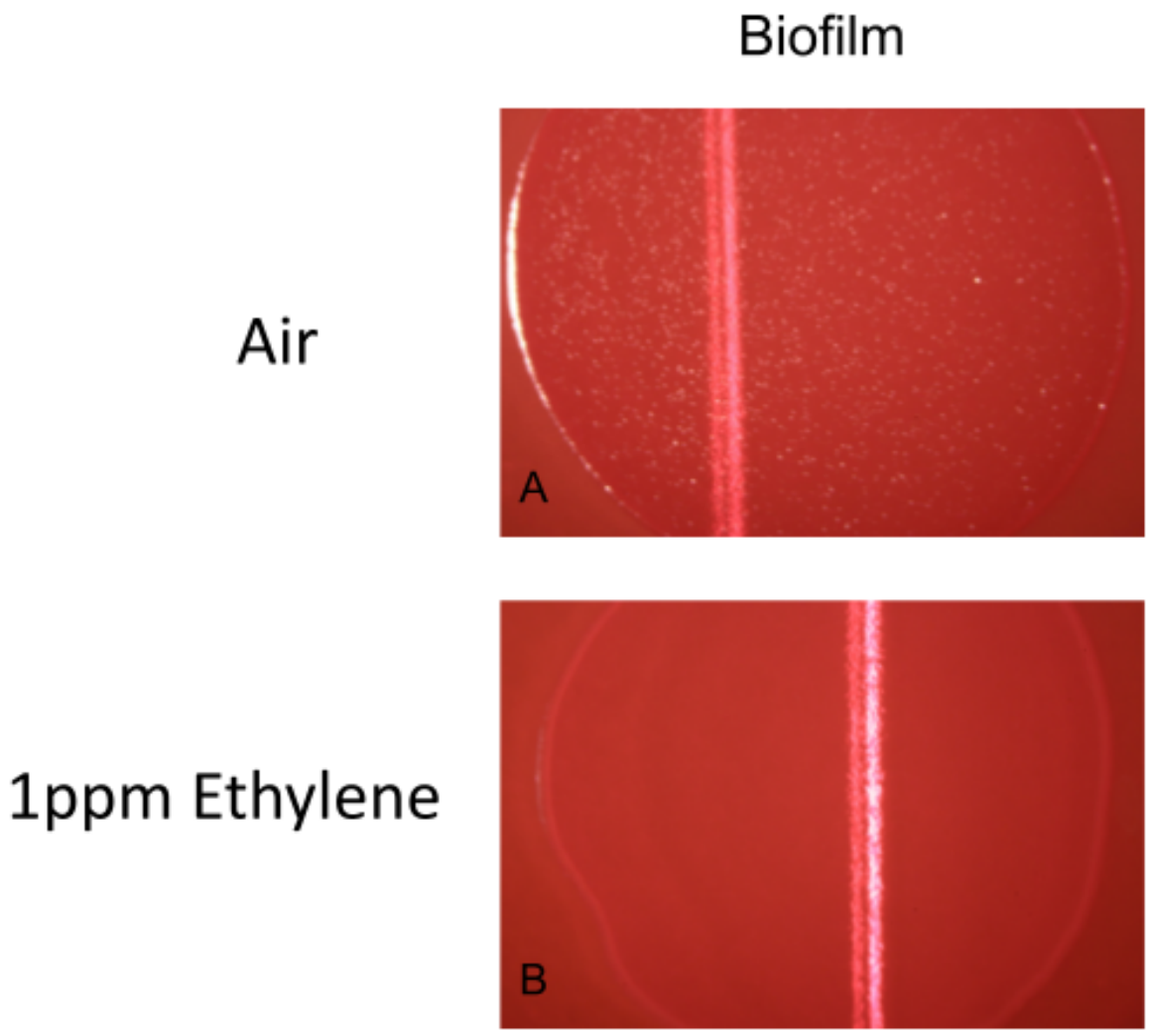
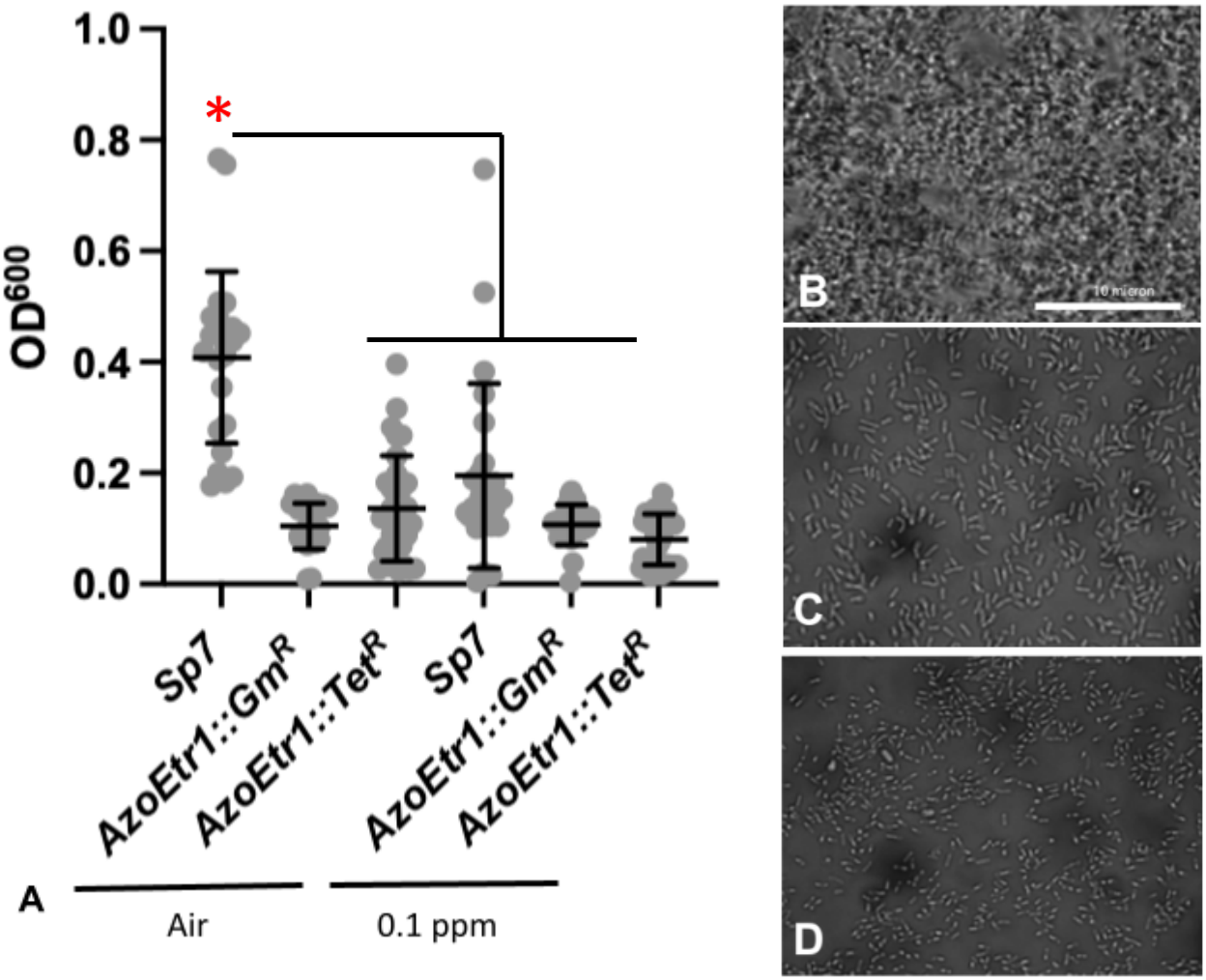


Figure 3.3 Ethylene reduces Congo Red dye binding of Sp7 colonies.

Congo Red dye binding of Sp7 colonies under biofilm conditions treated with either ethylene free air (A) or 1ppm ethylene (B). Reduced Congo Red binding can be seen on the leading edge of colonies under ethylene treatment. Bright lines in the middle of colonies are a reflection of grids in the plate, not a feature of the colony.

Figure 3.4 Ethylene Inhibits Biofilm Formation

A) Crystal violet staining of *A. brasilense Sp7* and *azoEtr1* biofilms after three days of growth under treatment of either ethylene-free air or 0.1 ppm ethylene. DIC imaging of *A. brasilense Sp7* and *azoEtr1* cells grown under biofilm conditions on poly-L-lysine slides for 24 hours. Sp7 cells were treated with either ethylene-free air (B) or 0.1ppm ethylene (C). The *azoEtr1* cells were grown in ethylene-free air (D). *= p-value <0.05 determined by two-way ANOVA. Data in panel A is representative of 3 biological replicates with 12 technical replicates per biological.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*
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AzoEtr1::TetR
Sp7
AzoEtr1::GmR
AzoEtr1::TetR
A
Air
0.1 ppm
B
10 micron
C
D

data on plant physiology where most plant responses have a threshold around 0.01 ppm saturate at 1 ppm (Binder, Mortimore et al. 2004). Because *A. brasilense* responds robustly to varying concentrations of ethylene without obvious physiological differences, 0.1 ppm ethylene was chosen as a standardized concentration for further experiments to remove any concern about chaotropic effects that ethylene may have on membrane fluidity.

Since ethylene is a stress signal from plants, and was capable of inhibiting biofilm formation, we wanted to know if it also had the ability to disperse *A. brasilense* biofilms. To do this we allowed for biofilm to form in the absence of ethylene for three days, and then moved the plates to a chamber where they would be exposed to 0.1 ppm ethylene for two days. In these experiments, plates that were transferred from air to ethylene showed significantly less biofilm than plates that were allowed to grow in the absence of ethylene for five days (Figure 3.6). To determine if biofilm formation had been reversed, we also compared the air to ethylene transition to biofilm plates grown for three days in air or ethylene. Air to ethylene transition plates displayed significantly more biofilm than plates grown in ethylene for three days, and there was no difference when compared to plates that grew biofilm for three days in the absence of ethylene. This suggests that ethylene is not capable of dispersing biofilms but does prevent growth of a biofilm.

To explore the idea of ethylene as a priming signal for *A. brasilense*, we grew cells in TY with and without ethylene treatment before inoculating into biofilm formation

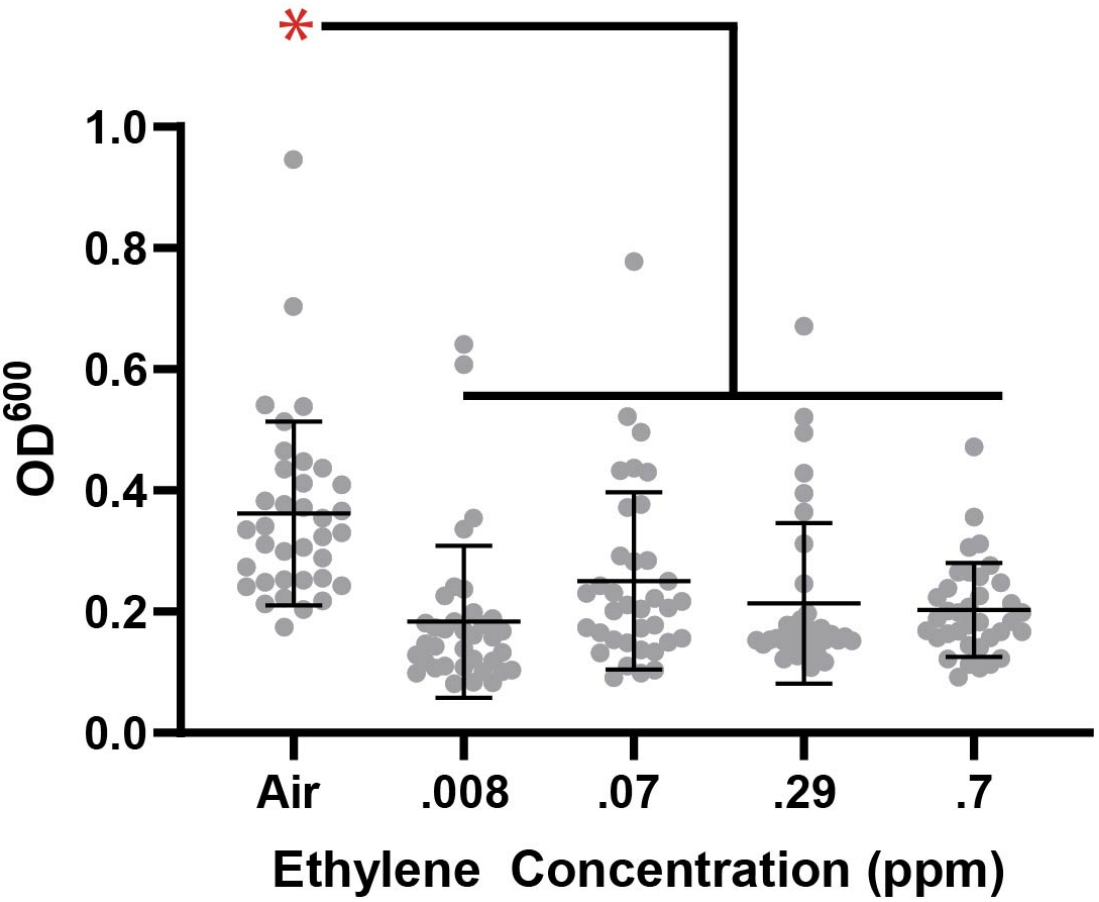


Figure 3.5. *A. brasilense* Cells Respond to Low Levels of Ethylene

Crystal violet staining of *A. brasilense* biofilms grown under treatment of varying ethylene concentrations from 0.7 ppm to 0.008ppm. *= p-value <0.05 as determined by two-way ANOVA. Data is representative of 3 biological replicates with 12 technical replicates per biological replicates.

assays. Pretreatment with ethylene during growth did not cause any effect on biofilm formation (Figure 3.7).

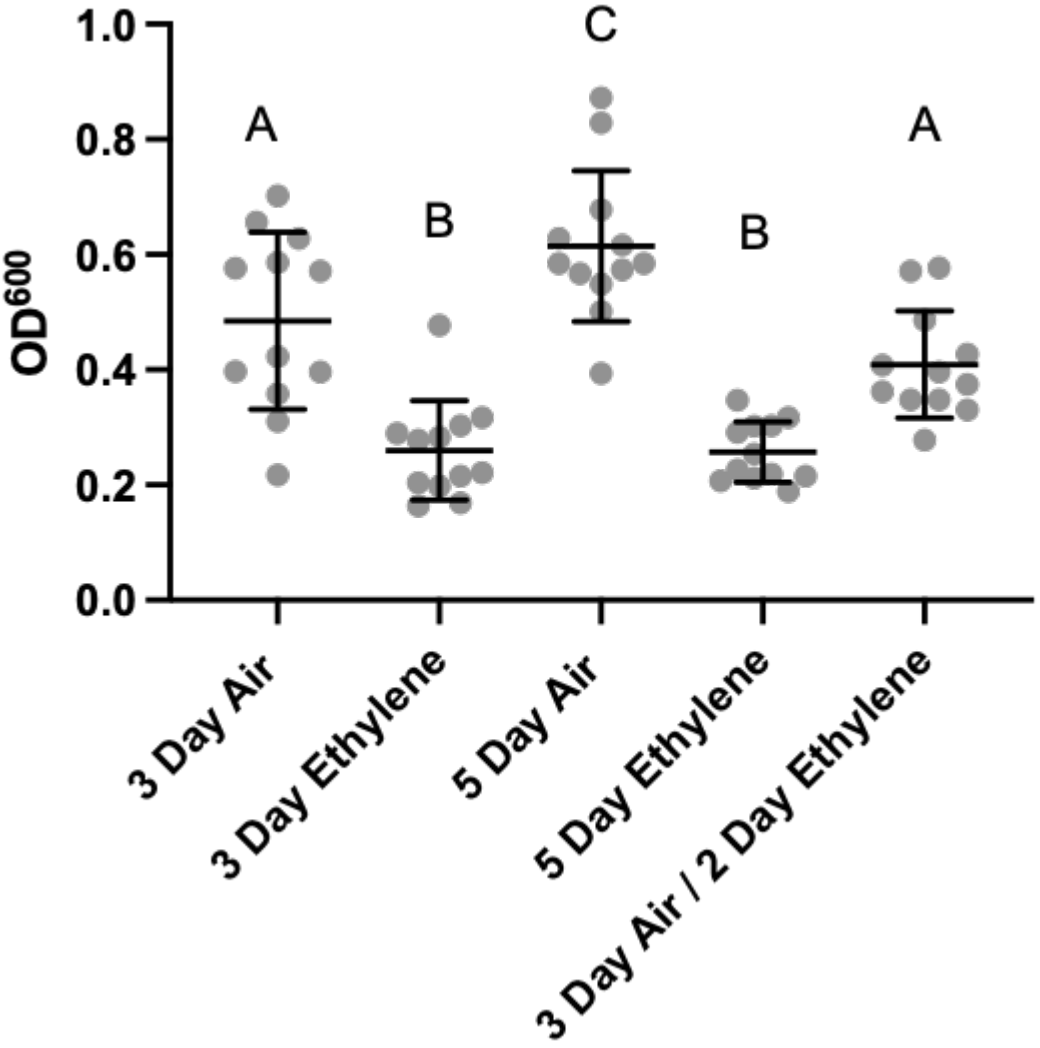
Ethylene Alters Plant-Associated Behaviors

After ethylene was shown to inhibit the formation of biofilm formation, we decided to test its effect on root colonization, due to analogous mechanisms mediating both responses (Michiels, Croes et al. 1991, Rodríguez-Navarro, Dardanelli et al. 2007, Bianciotto, Andreotti et al. 2009, Wheatley and Poole 2018). To determine a potential role in plant-microbe interactions, and to determine if root attachment behavior was affected by ethylene, we conducted colonization assays as previously described with some modification (Mukherjee, Kumar et al. 2016). Using a Microtom and Floradade varieties we saw that the *azoEtr1* had reduced colonization of roots after 24 hours as compared to the wild-type (Figures 3.8, 3.9). Imaging of fluorescently labeled *Sp7* and *azoEtr1::Tet*^{R} on tomato roots showed that the mutant strains did not form aggregates on the root surface as can be seen in the wild type (Figure 3.8 B,C).

To investigate the effect of ethylene on colonization Alisa Craig *NeverRipe (Nr)* and *ein2-5 A. thaliana* were used. Colonization of *Sp7* on *Nr* ethylene insensitive tomato is inhibited by application of 0.1ppm ethylene after 24 hours (Figure 3.10). Colonization of *ein2-5* inoculated with wild-type *A. brasilense* was treated with air or 0.1ppm ethylene. At 24 hours ethylene treated samples had significantly reduced colonization (Figure 3.11 A). When fluorescently labeled *Sp7* was imaged on roots of ethylene insensitive *ein2-5 A. thaliana* roots there was a dramatic reduction in root coverage, but

Figure 3.6 Biofilm Dispersal by Ethylene

Crystal violet staining of *A. brasilense* biofilms grown under treatment with ethylene-free air, 0.1 ppm ethylene, or a transition from air to ethylene after 3 days. Biofilm formation was allowed to occur in the absence of ethylene for 3 days before transition to an ethylene chamber where biofilm formation is inhibited. 3- and 5-day air and ethylene samples were used as controls to monitor biofilm formation over time in both air and ethylene conditions as compared to the transition samples. Letters represent statistical significance between groups with p-value <0.05 as determined by two-way ANOVA. Data is representative of 3 biological replicates with 4 technical replicates per biological.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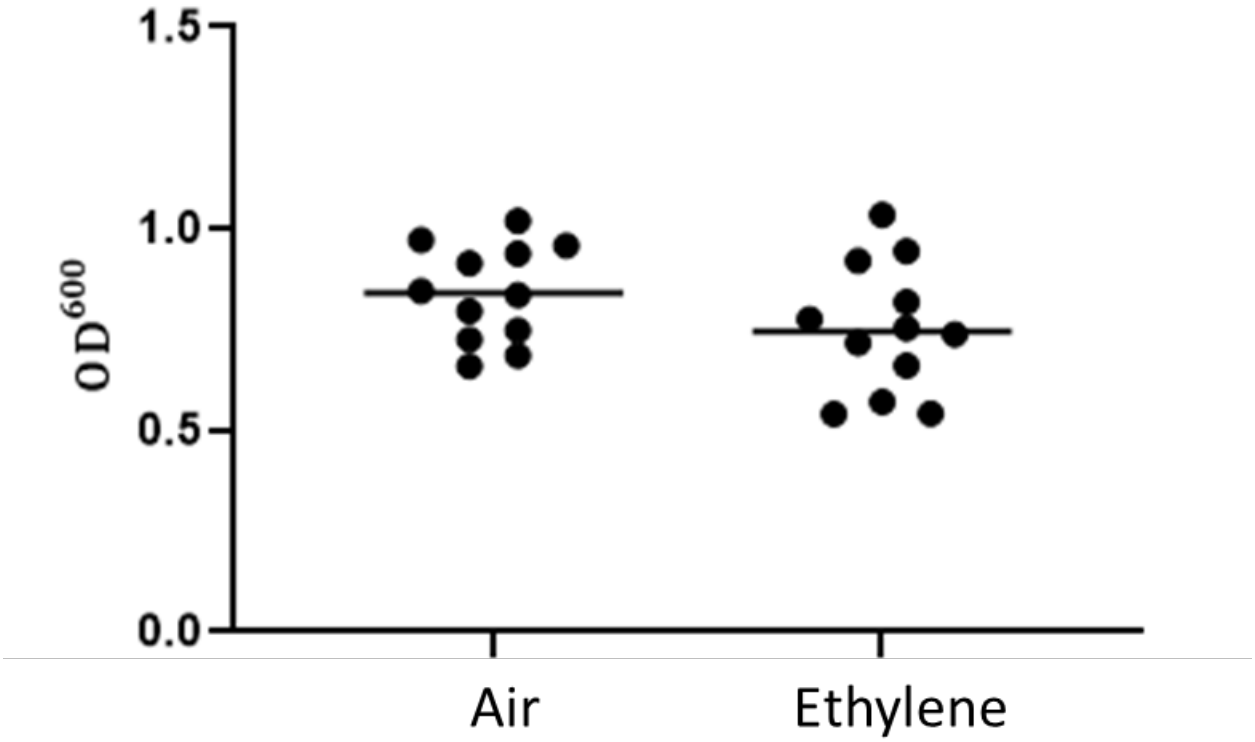


Figure 3.7 Biofilm Formation after Ethylene Pretreatment.

A. brasilense cells were grown overnight in rich media and were either untreated or injected with 0.1 ppm ethylene during overnight growth. Cells were then inoculated into biofilm media as done previously. Data represents 3 biological replicates with 4 technical replicates per biological replicate.

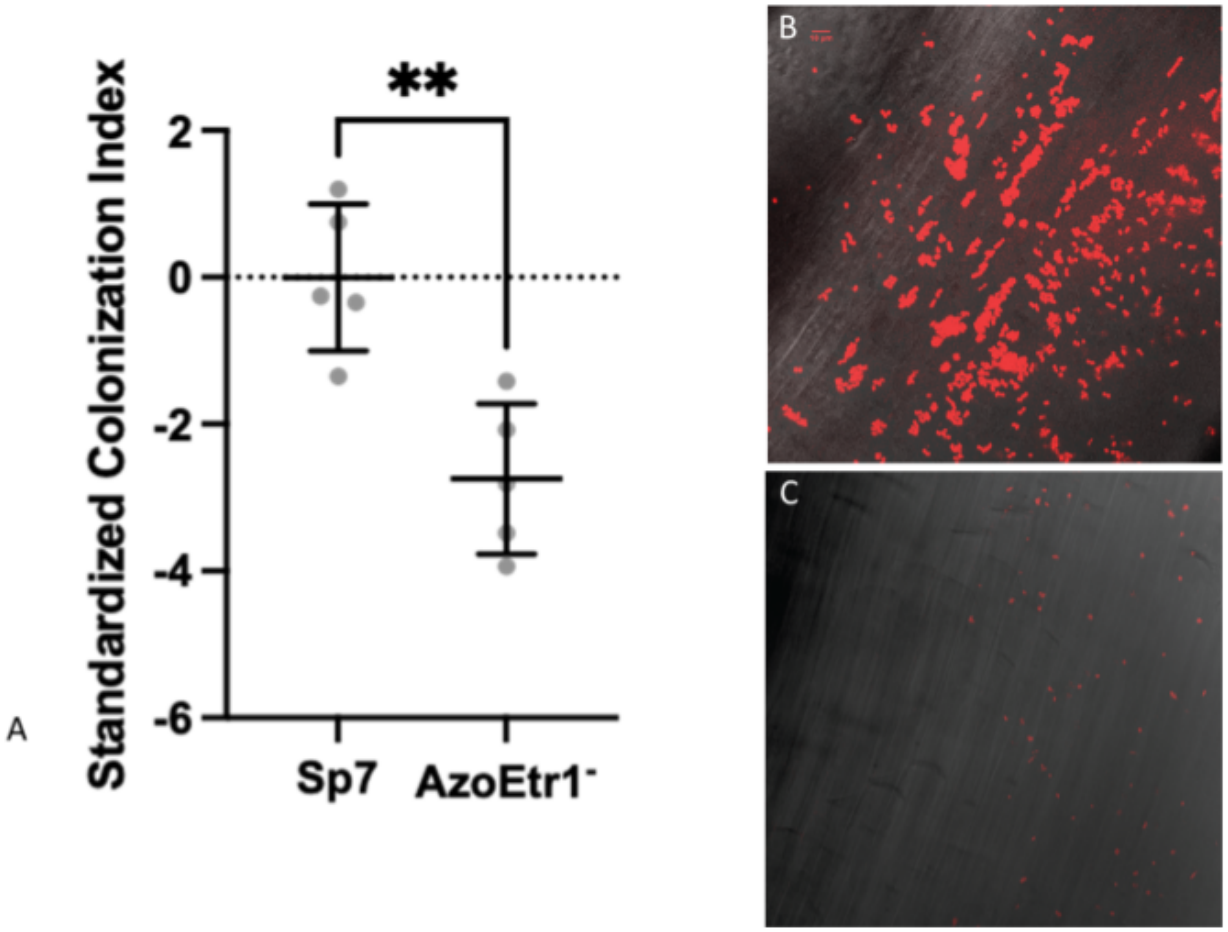


Figure 3.8 *azoEtr1*⁻ has Impaired Root Colonization and Does Not Aggregate on Tomato Roots

A) Root colonization of Sp7 and *azoEtr1* on Floradade tomato roots after 24 hours, normalized to wild-type colonization. (B,C) Fluorescent microscopy of YFP expressing strains of Sp7 (B) and *azoEtr1* (C) on Floradade roots overlaid with DIC imaging of the roots. Colonization index is the result of calculating the number of cells input into a system against the number of cells extracted using the formula log10 (strain input/strain output). ** = p-value < 0.01 as determined by a t-test.

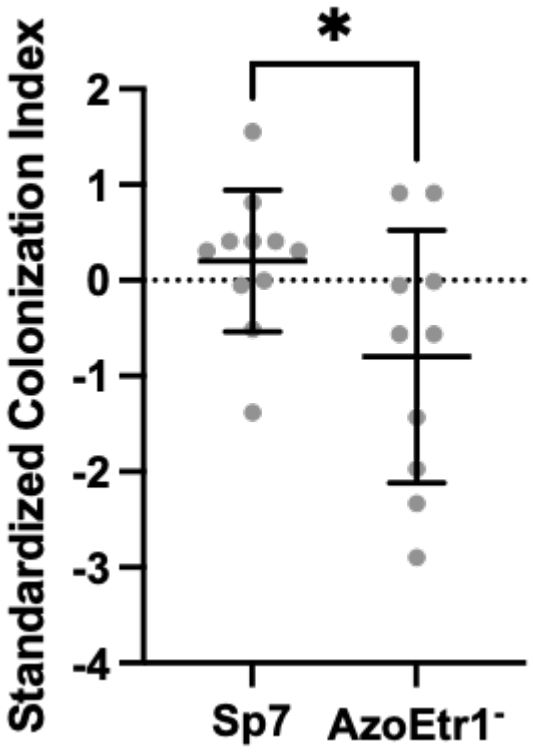


Figure 3.9 *azoEtr1* has Impaired Colonization of *Microtome* Roots.

Colonization of microtome tomato roots by Sp7 and *azoEtr1* after 24 hours, normalized to wild type colonization levels. *= p-value <0.05 as determined by a t-test. Data represents 3 biological replicates with 3 or 4 technical replicates per biological. Total N=10 per condition.

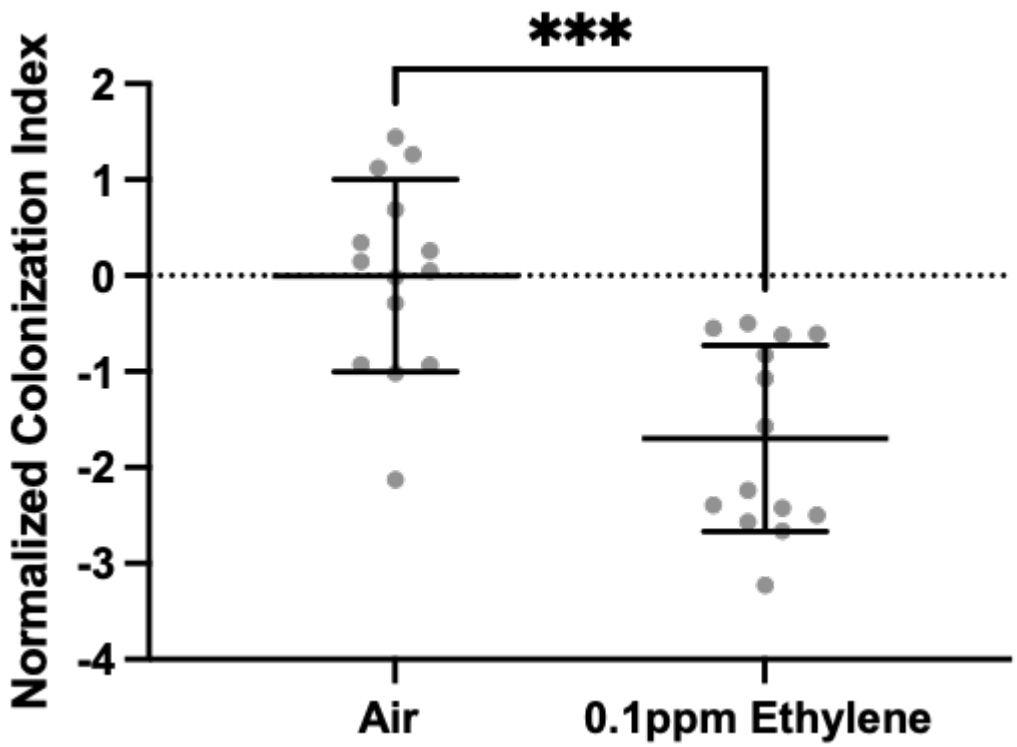


Figure 3.10 Ethylene Inhibits Colonization of Sp7 on *Neverripe* Tomato Roots

Sp7 cells were inoculated onto ethylene insensitive *neverripe* tomato (Alisa Craig) roots and either treated with ethylene-free air or 0.1ppm ethylene. ***=p-value <0.001 as determined by a t-test. Data represents 4 biological replicates with 3 or 4 technical replicates per biological. Total N= 13 per condition.

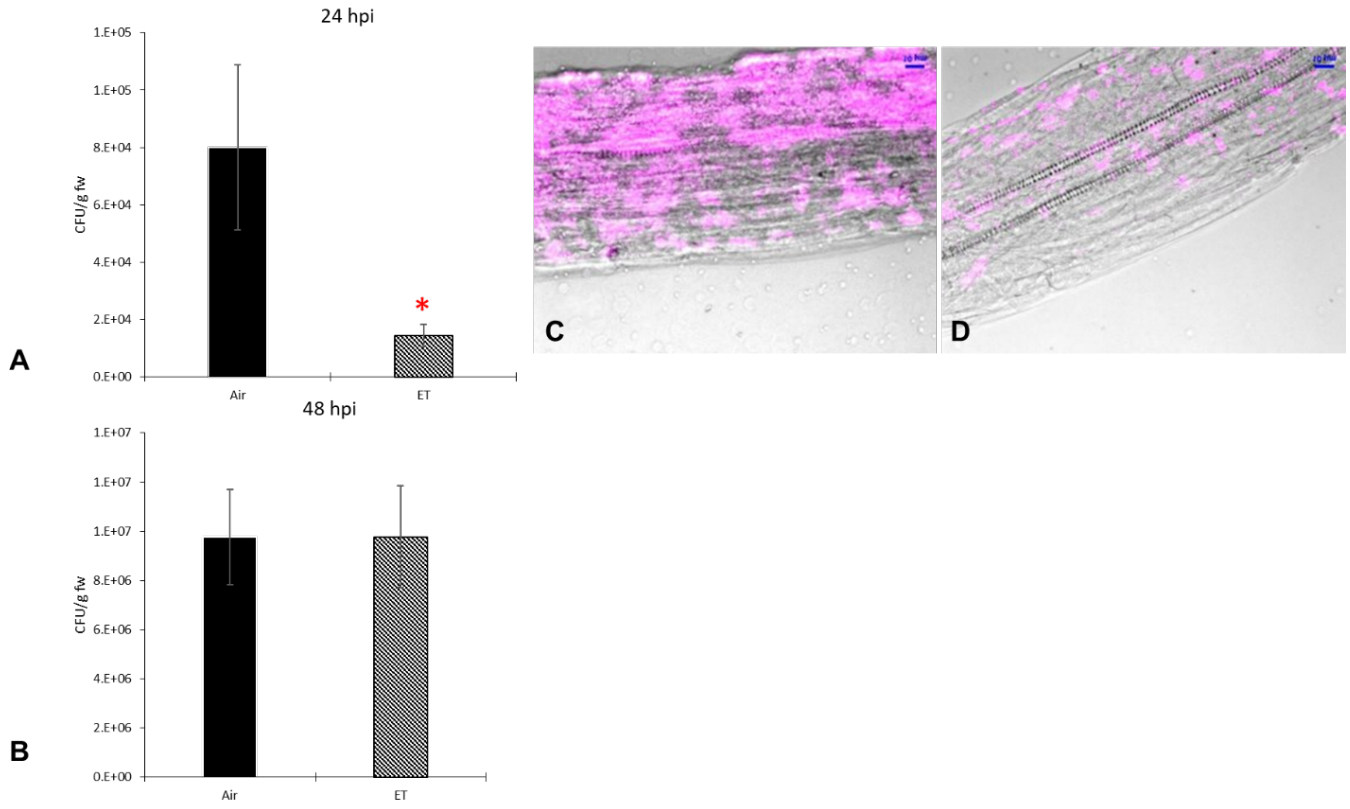


Figure 3.11 Ethylene Inhibits Sp7 Colonization of *ein2-5*.

CFUs per gram of fresh weight from *ein2-5* plants inoculated with *Sp7* after 24 hours(A) and 48 hours(B) of colonization in air or 0.1ppm ethylene. Fluorescent microscopy of YFP expressing Sp7 cells after 24 hours of colonization on *ein2-5* roots in air (C) and 0.1ppm ethylene (D). Scale bar in top right corner of images represents 10mm. *= p-value < 0.05 as determined by a t-test. N=11 for each condition.

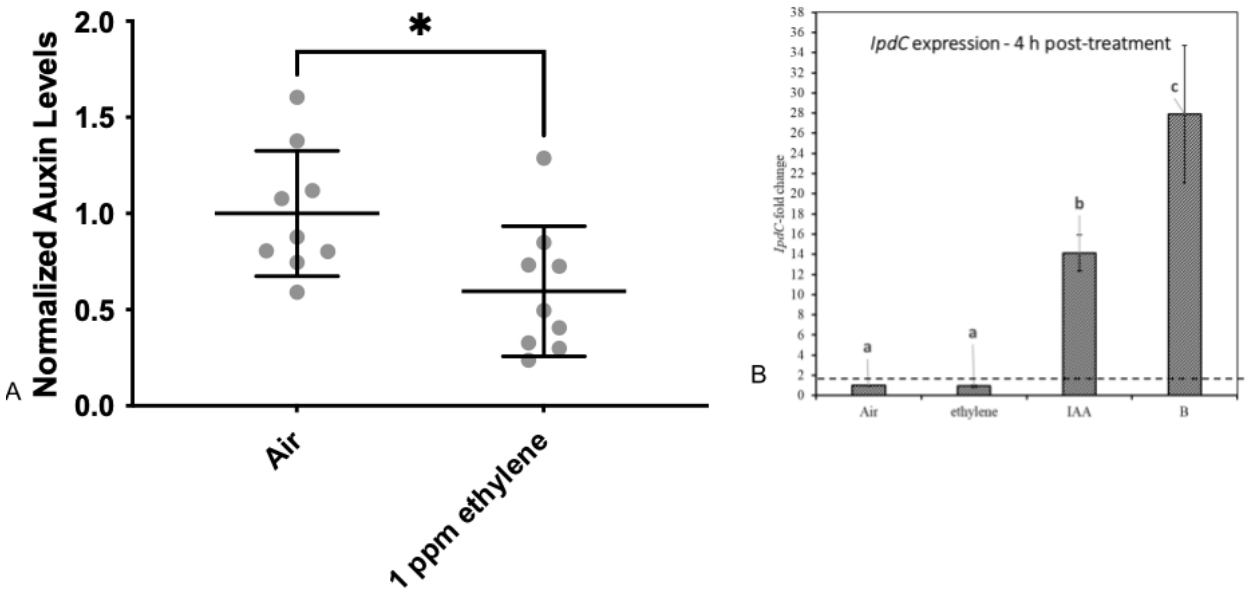


Figure 3.12 Ethylene Inhibits auxin synthesis, but does not alter *lpdC* transcripts

A) Salkowski reaction colorimetric results normalized to air sample OD^{530} nm measurements. B) qPCR data showing the expression of *lpdC* when cells are grown under control conditions, in the presence of 0.1ppm ethylene, in the presence of 100μM IAA, or both ethylene and auxin (B). *= p-value <0.05 as determined by a t-test, letters indicate statistical significance between groups, as determined by a two-way ANOVA.

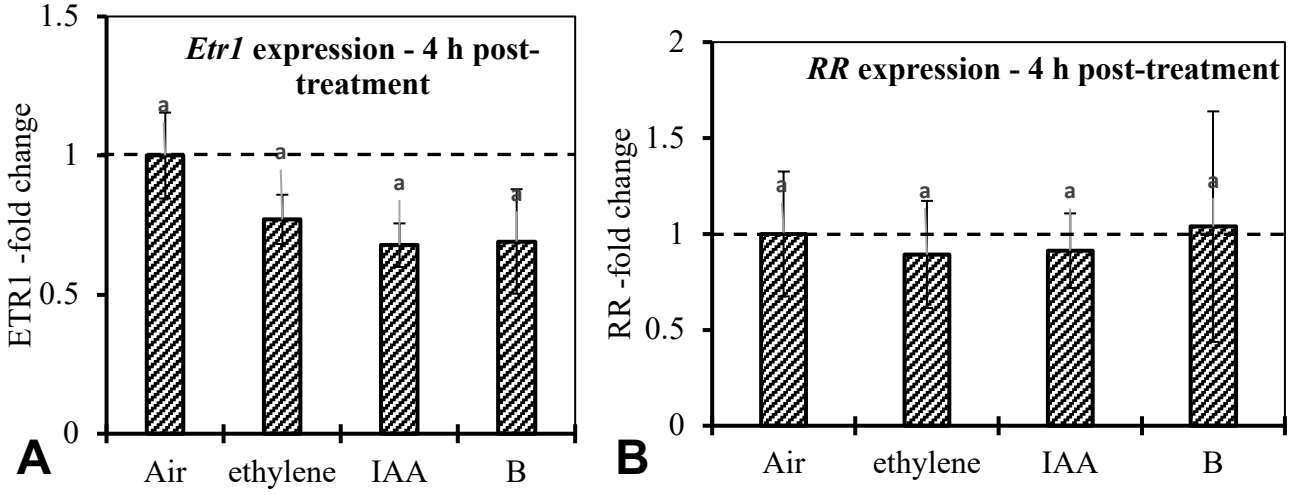


Figure 3.13 Auxin Does Not Alter *azoEtr1* or *RR*^{Etr1} Transcripts

Transcript levels of *azoEtr1* (A) and *RR*^{Etr1} (B) after 4-hour treatments in the presence of 0.1ppm ethylene 100 micromolar IAA or both. Letters represent statistical groups as determined by a two-way ANOVA. Data represents 3 biological replicates with 3 technical replicates per biological replicate.

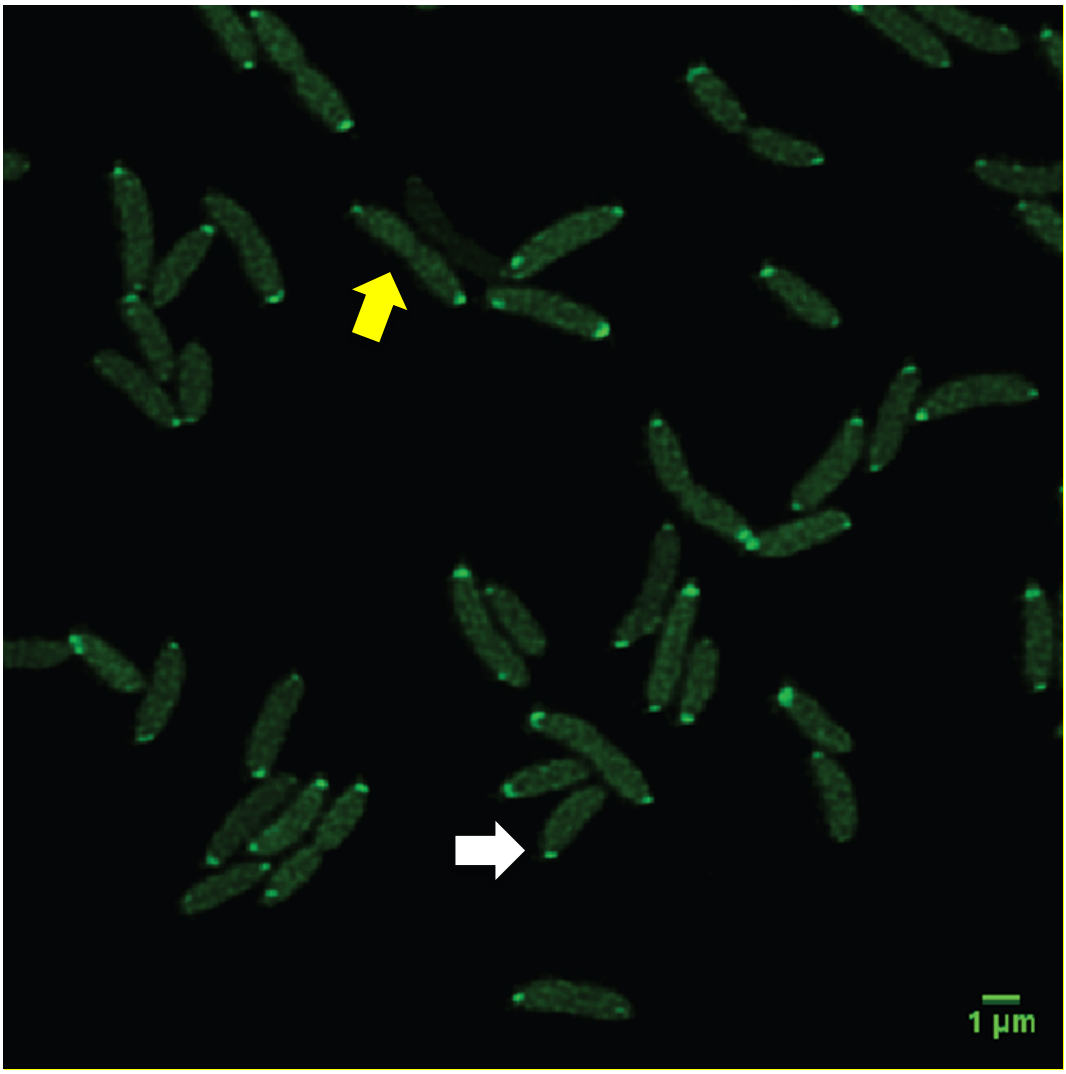


Figure 3.14 AzoEtr1 Localizes to the Flagellar Pole

AzoEtr1 with a C-terminal YFP fusion expressed in the *Sp7* background. Fluorescent microscopy shows foci at a single pole of a non-dividing cell, and in the pole away from the division plane in dividing cells. White arrow indicates single cell with foci at one pole. The yellow arrow indicates dividing cell with foci away from division plane.

aggregates did still form on the root as compared to the mutant lines (Figure 3.11 C, D). Interestingly in the *ein2-5* colonization experiments, at 48 hours there was no longer a difference in colonization between ethylene treated and untreated samples (Figure 3.11 B).

Further examining A. brasilense plant-associated behaviors, we decided to investigate auxin production. Auxin and ethylene interact in complex signaling pathways in root growth and development and auxin is used by many soil bacteria to suppress plant immune signaling (Jones and Dangl 2006, Stepanova, Yun et al. 2007, Bielach, Duclercq et al. 2012, Muday, Rahman et al. 2012, Spaepen, Bossuyt et al. 2014, Leyser 2018, Hildreth, Foley et al. 2020, Nakano, Omae et al. 2022, Shekhawat, Fröhlich et al. 2023). *A. brasilense* is known to produce auxin and mutants incapable of producing auxin lose much of their ability to promote plant growth and remodel roots (Malhotra and Srivastava 2008). Ethylene treatment reduced auxin production by *A. brasilense* in MMAB supplemented with tryptophan (Figure 3.11A). To further investigate this phenotype, I used qPCR to evaluate transcript levels of the *indole pyruvate decarboxylase (ipdC*) enzyme which is the rate limiting step in auxin production by *A. brasilense*. Interestingly, the addition of ethylene did not alter *ipdC* expression (Figure 3.11B). However, addition of both IAA and ethylene together, showed a slight but significant increase in *ipdC* expression over the IAA treated positive control.

To begin investigating potential mechanisms for the attachment phenotypes exhibited by *azoEtr1* mutants and *Sp7* cells in the presence of ethylene we tagged AzoEtr1 with a C-terminal YFP to localize the protein. Fluorescent microscopy showed

foci at a single pole in *A. brasilense* cells (Figure 3.12). Imaging of dividing cells showed that these foci localized to the pole away from the division plane, which is known to be the flagellar pole in *A. brasilense*. The flagella, in addition to its role in motility and chemotaxis in the soil, is also a glycoprotein that is important in the early stages of colonization (Croes, Moens et al. 1993, Moens, Michiels et al. 1995, Belyakov, Burygin et al. 2012, Meshcheryakov, Barker et al. 2013, Merino and Tomás 2014, Shelud'ko, Filip'echeva et al. 2015, Rossi, Medeot et al. 2016). It is possible that AzoEtr1 interacts either directly with flagellar components or with other regulatory proteins that dictate the response to flagellar attachment.

Pleiotropic phenotypes in a*zoEtr1* mutants

Disruption of *azoEtr1* induces multiple stress phenotypes that are not phenocopied by application of ethylene. The first phenotype that was observed in the *azoEtr1* lines was a corrugated colony morphology (Figure 3.15). Application of ethylene up to 10ppm did not induce this response even after 5 days of incubation in Sp7 colonies. *azoEtr1* lines were also observed to be redder both on plates and in cell pellets and so carotenoid content was measured. The a*zoEtr1* mutants accumulate more carotenoids after two days than wild-type (Figure 3.16). The wild type did not show any signs of carotenoid accumulation after ethylene treatment. Because both carotenoid accumulation and corrugated colony morphology are associated with oxidative stress, we examined sensitivity to the reactive oxygen species hydrogen peroxide (Gupta, Kumar et al. 2013). *azoEtr1* showed increased sensitivity to hydrogen peroxide but treatment with ethylene did not induce any increased sensitivity in the wild-type (Figure 3.17A, B). The colony morphology phenotype, while not induced by

ethylene, was used to screen rescue when the Etr1-YFP construct used to localize AzoEtr1 was conjugated into *azoEtr1* backgrounds. Complementation with *azoEtr1* alone was not enough to rescue this phenotype. This suggests that RR^{Etr1} may also be knocked out in the insertional disruption mutants. Because colony morphology was not rescued, these lines were not characterized.

Discussion

AzoEtr1 is a functional ethylene receptor and both disruption of this receptor, and application of ethylene induce extensive physiological responses in *A. brasilense.* Because both treatment with ethylene and disruption of *azoEtr1* resulted in the same phenotype for biofilm formation and root colonization, we hypothesized that ethylene acts as an inhibitor to the ethylene receptor in *A. brasilense* as is seen in both *A. thaliana* and *Synechocystis sp.* PCC 6803. This is consistent with the inverse agonist model that has been used to describe the mechanism of signal transduction in all studied ethylene receptors (Hua and Meyerowitz 1998).

Congo Red dye binding, and biofilm formation assays show that ethylene is playing a role under biofilm forming conditions where carbon is available, but nitrogen is limiting. The rhizosphere environment is nutritionally similar to this, and the root colonization and biofilm phenotypes are likely tied (Fischer, Miguel et al. 2003, Wheatley and Poole 2018, Korenblum, Dong et al. 2020, Korenblum, Massalha et al. 2022, Liu, He et al. 2022). As adhesion to both abiotic and biotic surfaces follow similar mechanisms, the nutrient signals to induce both are also similar. This suggests a change in the way that *A. brasilense* responds in a low nitrogen environment. Though

root colonization of *A. thaliana* under ethylene treatment is rescued by 48 hours suggesting that the root environment may include additional signals that further encourage attachment that are not present in abiotic environments. Interestingly, despite the ability to inhibit biofilm formation even at low concentrations, application of ethylene to existing biofilms does not cause dispersal but does inhibit further growth. *A. brasilense* is known to travel from root to root and ethylene production from the plant could serve as a potential signal for *A. brasilens*e to limit colonization of one host plant and allow newly divided cells to move back into the soil and look for other hosts (Bashan and Holguin 1994). In contrast to this idea, ethylene signaling may only be critical at the initiation of attachment and once transition from transient initial attachment has occurred, the significance of ethylene perception changes.

The attachment phenotypes shown in the light microscopy for both ethylene treated and mutant cells suggest that *A. brasilense* is never transitioning past initial attachment as single cells (Michiels, Croes et al. 1991, Hinsa, Espinosa-Urgel et al. 2003, Bashan and De-Bashan 2005). On tomato roots, azoEtr1^{-} exhibits the same phenotype, however, ethylene treated cells on *A. thaliana* roots display small levels of aggregation. This again suggests that root colonization is a more complex environment, but the core phenotype of reduced attachment and reduced aggregation is consistent across conditions.

Decreased levels of auxin secretion by *A. brasilense* in response to ethylene is somewhat counterintuitive. Many soil bacteria produce auxin in response to plant immune signals due to its ability to inhibit ethylene biosynthesis and downregulate

plant immune signaling. A decrease in auxin production suggests that *A. brasilense*'s response to ethylene is not to manipulate the plant immune system. The mechanism by which auxin production is decreased is not clear. This phenotype is difficult to interpret, and the complexity of the interaction is highlighted by inability of ethylene to alter *ipdC* expression alone, but large increase in expression induced by the simultaneous presence of both. Taken together, ethylene perception by *A. brasilense* leads to inhibition of colonization, at least at early time points, and a reduction in plant-growth promoting behaviors.

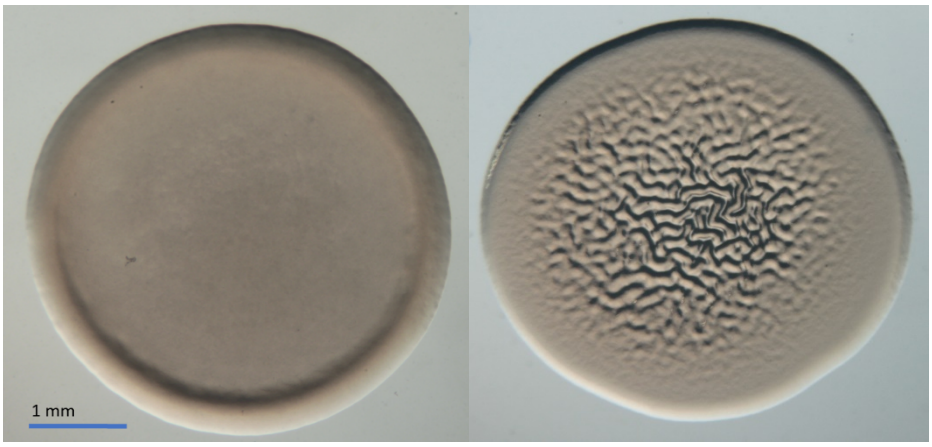


Figure 3. 15 *azoEtr1* Displays Corrugated Colony Morphology

Colony morphology for Sp7 (Left) and *azoEtr1* (Right) grown for 72 hours at 28ºC on solid TY medium.

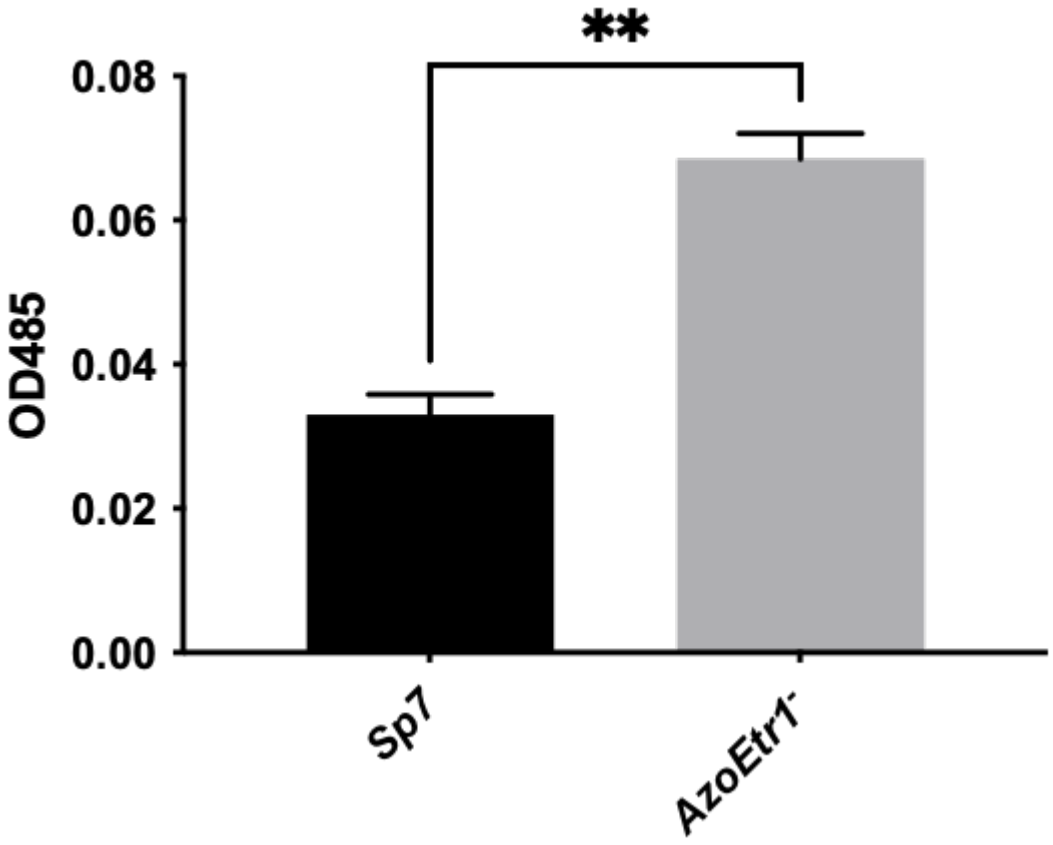


Figure 3.16 *azoEtr1* Accumulates Carotenoids

Optical density (485 nm) from methanol extractions of carotenoids from *A. brasilense* cultures. * =p-value <0.01 as determined by a t-test.

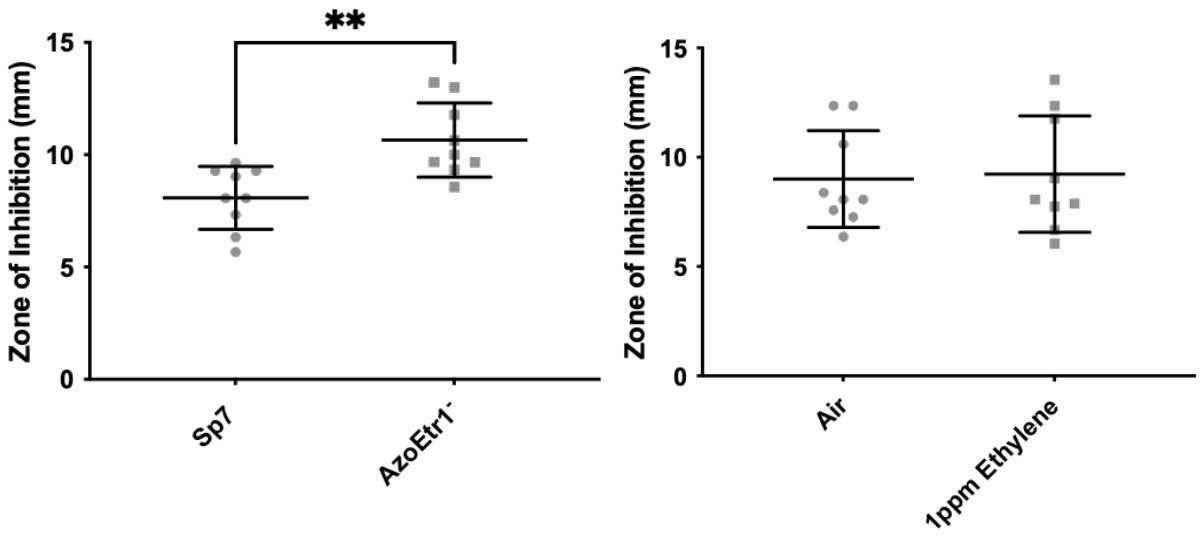


Figure 3.17 Ethylene Does Not Phenocopy *azoEtr1*$^{-}$ Hydrogen Peroxide Sensitivity.

Measurement of zone of inhibition caused by filter paper discs soaked in 0.3% (v/v) hydrogen peroxide after 24 hours of growth on 2% solid TY media. **=p-value < 0.01 as determined by a t-test. Data is representative of 3 biological replicates with 3 technical replicates per biological.

Chapter 4: *A. brasilense* Metabolism Changes in Response to Ethylene

Additional authors that contributed research to this work: Lauren Donnelly, Josie King.

Abstract

A. brasilense was shown to undergo extensive transcriptomic changes in response to ethylene treatment. Pathway analysis of differentially expressed genes (DEGs) revealed that carbohydrate metabolism, amino acid metabolism, and energy production and conversion were the most highly affected pathways. This suggested a change in metabolism in response to ethylene. Metabolomic analysis revealed a distinct metabolism from air samples with accumulation of metabolites involved in nucleotide metabolism as well as amino acid metabolism. This shift in metabolism was shown to occur within 8 hours of treatment with ethylene. Among the altered metabolites were 2-oxoglutarate and glutamate which are important signals in carbon to nitrogen ratio signaling. This, along with decreased expression of nitrogen regulator GlnB and the glutamate to glutamine synthetase GlnA suggested a role in nitrogen metabolism. Expression of transcripts under the control of nitrogen transcriptional regulator NtrC were shown to be downregulated under nitrogen fixing conditions. Additionally, accumulation of Poly-hydroxybutyrate granules (PHB), which is also controlled by NtrC, was observed. *azoEtr1* was shown to respond transcriptionally to nitrogen source, strengthening the relationship between ethylene and nitrogen signaling in *A. brasilense*.

Introduction

Azospirillum brasilense has been shown to undergo rapid and extensive transcriptomic and proteomic changes in response to the phytohormone auxin, and nitrogen starvation (Broek, Gysegom et al. 2005, Kukolj, Pedrosa et al. 2019). Proteomic responses to nitrogen starvation were investigated in *A. brasilense* using changes in nitrogen status and mutants in the nitrogen regulator NtrC. The nitrogen regulatory cascade in *A. brasilense* is dependent on two PII proteins GlnB, and GlnZ (Figure 4.1) (Liang, de Zamaroczy et al. 1992, de Zamaroczy, Paquelin et al. 1993, de Zamaroczy, Paquelin et al. 1996, De Zamaroczy 1998, Huergo, Souza et al. 2003, Araújo, Monteiro et al. 2004, Huergo, Chubatsu et al. 2006, Araújo, Huergo et al. 2008, Forchhammer, Selim et al. 2022). These proteins monitor 2-oxoglutarate levels, ATP availability, and glutamine status in the cell. The measurements of these resources allow PII proteins to function at the center of regulation of metabolism in the cell Forchhammer and Lüddecke (2016). Under nitrogen starvation 2-oxoglutarate levels are high, while glutamine levels are low (Radchenko, Thornton et al. 2010, Huergo and Dixon 2015). This causes uridylation of PII proteins which leads to a trimeric form and prevents inhibition of NtrB kinase activity in *A. brasilense (Huergo, Souza et al. 2003, Araújo, Huergo et al. 2008)*. NtrB phosphorylates NtrC and this allows NtrC to form a holoenzyme with the RNA polymerase RpoN (Liang, de Zamaroczy et al. 1992, Zhang, Burris et al. 1997, Steenhoudt and Vanderleyden 2000). This allows for the expression of the ammonium transporter *amtB*, *glnB,* glutamine synthetase *glnA*, and the nitrogen

fixation operon including *nifH* (Broek, Michiels et al. 1992, Arsène, Kaminski et al. 1996, Huergo, Souza et al. 2003).

Analyses of proteomic changes in wild-type and *ntrC* mutants of *A. brasilense* led to the identification of proteins involved in amino acid metabolism as the most differentially regulated under low nitrogen conditions, as well as the implication of *ntrC* in the regulation of ABC transporters and many nitrogen transporters. Changes in nitrogen status also led to large changes in the metabolic profiles of *A. brasilense* with amino acid metabolism and carbon metabolism being the most affected pathways (Kukolj, Pedrosa et al. 2019). GlnB, and NtrC have also been implicated in the regulation of fatty acid biosynthesis and PHB accumulation in other bacterium and closely related species (Sun, Peng et al. 2000, Sun, Van Dommelen et al. 2002, Sacomboio, Kim et al. 2017, Gerhardt, Parize et al. 2020, Ganusova, Vo et al. 2021). Together, this analysis links the regulation of nitrogen and carbon metabolism of *A. brasilense.*

In response to auxin treatment a rapid change in ribosomal RNAs suggest the response to auxin is to slow translation in what appears to be a stress response. While changes in ribosomal structures were the largest change in response to auxin, there were also changes in amino acid metabolism, as well as energy production and conversion (Broek, Gysegom et al. 2005). Of particular interest were changes in the expression of ABC transporters as well as secretion systems. These changes are indicative of alterations of the cell surface and suggest a potential role in mediating plant interactions, or adhesive

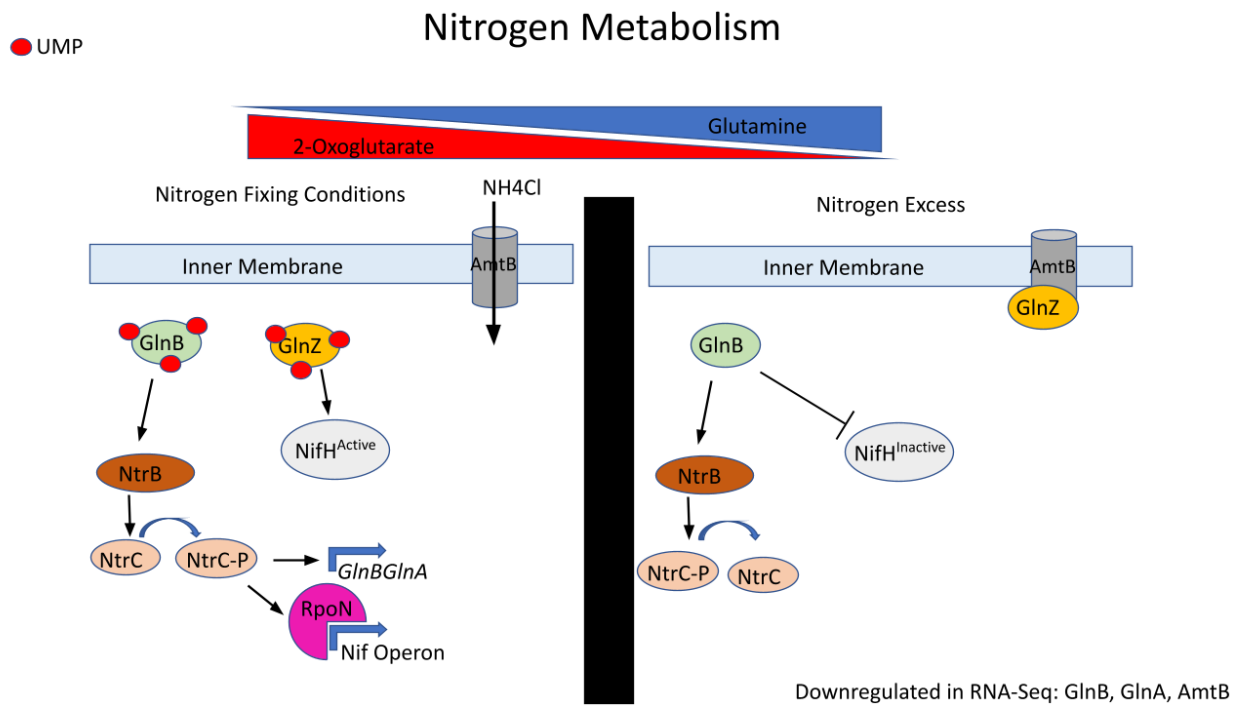


Figure 4.1 Simplified Model for *A. brasilense* Nitrogen Metabolism

Represented are relevant biochemical pathways for *A. brasilense* sensing of carbon to nitrogen ratios in the cell, and the resulting signaling cascade under nitrogen fixing conditions and conditions of excess nitrogen. Under Nitrogen fixing conditions, 2-oxoglutarate is abundant and the PII proteins GlnB and Glnz are uridylated. GlnZ then interacts with the dinitrogenase NifH to promote nitrogen fixation, while GlnB allows for NtrB to function as a kinase and phosphorylate NtrC which then promotes expression of glutamine synthetase, GlnA, the PII protein GlnB, the nitrogen fixation operon, and more. Under nitrogen replete conditions, GlnB and GlnZ are not uridylated, leading GlnZ to bind and inhibit AmtB, while GlnB promotes phosphatase activity in NtrB, and inhibits NifH activity.

interactions with other cells consistent with the stress response indicated by the downregulation of rRNA (Burdman, Jurkevitch et al. 1998, Burdman, Jurkevitch et al. 2000, Burdman, Okon et al. 2000, Bahat-Samet, Castro-Sowinski et al. 2004, Camilios-Neto, Bonato et al. 2014, Levy, Salas Gonzalez et al. 2018, Malinich and Bauer 2018, Vargas-Blanco and Shell 2020).

Together these studies illustrate *A. brasilense* ability to robustly respond to environmental cues from both nutritional status and phytohormones. Ethylene is a central cue for plants in plant-microbe interactions and immune responses, and thus would be advantageous for bacteria to perceive (Elías, Guerrero-Molina et al. 2018, Nascimento, Rossi et al. 2018, Ravanbakhsh, Sasidharan et al. 2018, Li, Han et al. 2019, Shekhawat, Fröhlich et al. 2023). Additionally, ethylene levels in soil can be indicative of plant stresses and soil conditions. Ethylene levels in soil can persist among a wide range from sub part per billion (ppb) to over 16 (ppm) (Abeles and Heggestad 1973). The retention of ethylene in soil is also dependent on moisture level, and other soil characteristics. High ethylene concentrations in soil also tend to coincide with low oxygen levels, making it increasingly relevant for microaerophilic nitrogen fixing bacteria like *A. brasilense* (Abeles and Heggestad 1973). Given the physiological responses that ethylene treatment causes in *A. brasilense* and the lack of easily identifiable interacting partners for AzoRR^{Etr1} transcriptional analysis of ethylene responses were performed to determine the pathways that are affected.

Results

Ethylene Induces Transcriptomic and Metabolic Changes in *A. brasilense*

The putative ethylene response pathway of *azoEtr1* and *azoRR*^{Etr1} is proposed to function by phosphorylation of the response regulator via the histidine kinase. AzoRR^{Etr1} is a single domain response regulator, meaning that there is no effector domain that can be used for further analysis and thus it likely functions either via protein binding or phosphotransfer to another protein (Galperin 2006, Gao, Mack et al. 2007, Jenal and Galperin 2009). This limits our ability to investigate the mechanisms by which the ethylene response pathway is altering physiology. In lieu of protein pulldowns and searches for interacting partners with AzoRR^{Etr1} we opted for RNA sequencing to determine what pathways are being affected by ethylene (Figure 4.2). Samples were grown in MMAB with $NaNO_3$ as the nitrogen source and grown in sealed tubes injected with either 0.1 ppm ethylene or 0.03 mL ethylene free air to mimic any changes in pressure caused by injection with gas. Samples were grown for 12 hours prior to ethylene treatment, and an additional 4 hours during treatment. Treatment with ethylene under these conditions led to a significant change in expression in 395 genes. 344 of these genes were upregulated, and 51 downregulated. Analysis of 222 of these genes that could be sorted into pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed that amino acid transport and metabolism with 45 significantly altered genes and energy production and conversion were the most highly affected pathways. The next category was carbohydrate metabolism, containing genes in the glycolysis and gluconeogenesis pathway, the pentose phosphate pathway, and

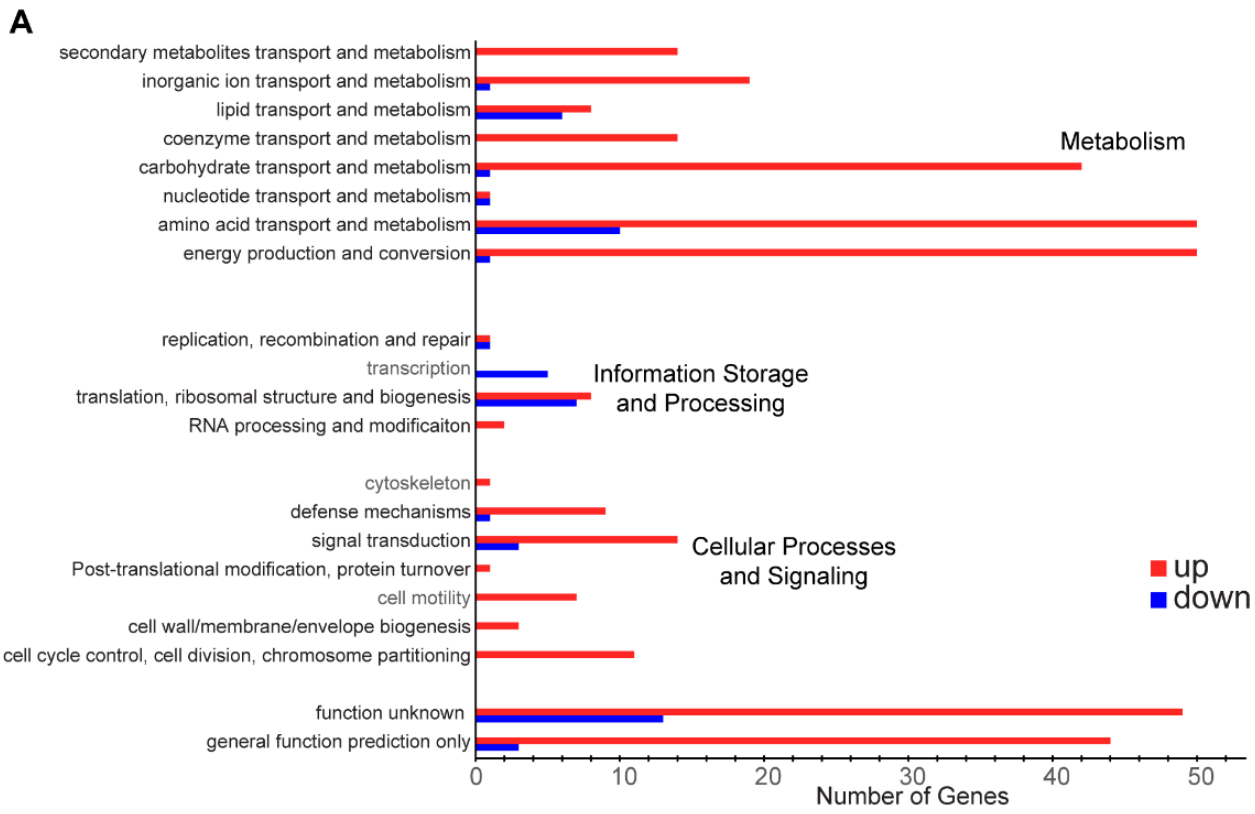


Figure 4.2 Ethylene Induces Transcriptomic Changes in *A. brasilense*

Pathway analysis for differentially expressed genes after 4 hours of ethylene treatment. This pathway analysis represents the 222 genes of the 395 total that could be mapped to a pathway by the Kyoto Encyclopedia of Genes and Genomes. Red Bars indicate upregulated genes, blue bars indicate down-regulated genes.

the TCA cycle. In addition to large sets of genes involved in carbon metabolism, there was also a downregulation of several important genes in regulation of nitrogen metabolism including the PII protein GlnB, Glutamine Synthase GlnA*,* and the ammonium transporter AmtB. These genes were of interest not only due to their known roles in nitrogen metabolism, but also because the pathways regulating the expression of these genes are well studied (Liang, de Zamaroczy et al. 1992, de Zamaroczy, Paquelin et al. 1993, De Zamaroczy 1998, de Zamaroczy and Elmerich 1998, Araújo, Monteiro et al. 2004, Moure, Siöberg et al. 2019). Together this information suggested a large change in the metabolism of the cell, and potential alteration of how *A. brasilense* balances carbon and nitrogen levels.

In response to the results of the RNA sequencing we performed untargeted metabolomics under the same conditions that the RNA-seq was performed (Figure 4.3). This analysis showed that ethylene treated cells have a distinct metabolism as compared to cells grown in the absence of ethylene. The analyses showed 70 metabolites with a significantly altered abundance when compared to the air controls. Of the 70 metabolites, 63 were significantly upregulated, and 7 were downregulated. Interestingly, the pathways most altered in the metabolomic analyses are nucleotides and analogues. Amino acid metabolism was the second most altered pathway.

Within the TCA cycle, 2-oxoglutarate is significantly enriched in ethylene samples. This is of particular interest as 2-oxoglutarate is the carbon molecule that PII proteins like GlnB and GlnZ use to determine the carbon-nitrogen balance in the cell (Radchenko, Thornton et al. 2010, Huergo and Dixon 2015). Additionally, glutamate

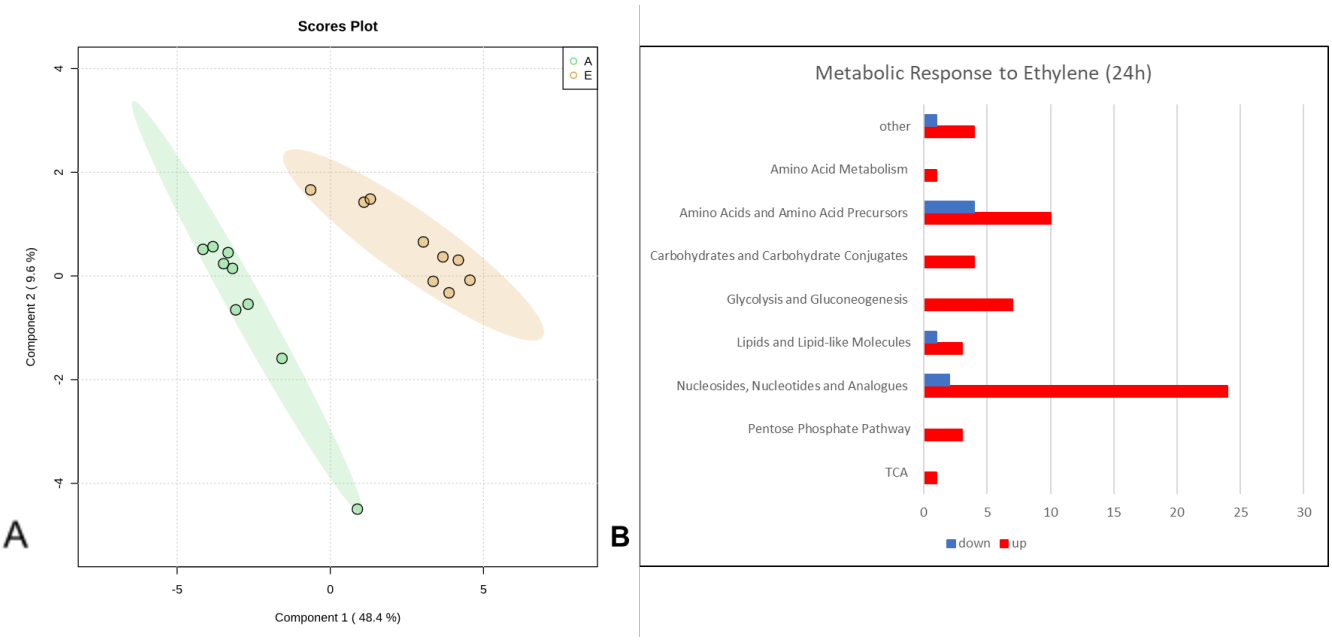


Figure 4.3 Ethylene Induces a Distinct Metabolic Profile in *A. brasilense*

A) Principal Component Analysis of ethylene-free air- and 0.1 ppm ethylene- treated metabolomic samples showing clear separation. B) Pathway analysis of the 70 differentially accumulated metabolites. Red indicates a higher abundance of a metabolite, blue indicates a lower abundance of a metabolite. Samples were grown in sealed tubes and treated with ethylene by injection, as done in the RNAseq experiments. However treated samples were grown in the presence of ethylene for 24 hours, rather than the 4 hours of exposure used in the RNAseq experiments.

is significantly depleted in ethylene samples, although glutamine, the metabolite used as an indicator for nitrogen status, is unchanged.

The RNAseq samples were collected after 4 hours of exposure to ethylene, and the metabolomic samples were collected after 24 hours of exposure. To determine if the changes in the metabolomics represented the initial response to ethylene or an adaptation to the stimuli, we sampled at both 8 and 24 hours of exposure to ethylene (figure 4.4). After 8 hours of exposure, ethylene treated samples showed a distinct metabolism from control samples with 36 metabolites significantly altered. 35 of the 36 metabolites were enriched with nucleotides, and amino acid metabolism showing the most changes. This is consistent with the original metabolomics sample and the concurrent 24-hour samples which showed 24 metabolites significantly altered with 23 enriched with nucleotide and amino acid metabolism the most affected pathways. The difference in the number of metabolites altered between the 24-hour samples is likely due to changes in growth conditions. To allow for time course samples to be taken from the same biological replicates, samples were grown in 50 mL cultures with flow-through treatment of ethylene rather than injection into sealed tubes. This likely changed the aerobic status of samples and could account for the change in altered metabolites, though the relevant pathways are consistent.

Crosstalk Between Ethylene and Nitrogen Signaling

As previously mentioned, the regulation of GlnA, GlnB, and AmtB are well studied (de Zamaroczy, Paquelin et al. 1993). The expression of these transcripts is driven by the activity of the nitrogen regulator NtrC. NtrC is regulated by the kinase NtrB

which also acts as the phosphatase for NtrC. NtrB activity is regulated by GlnB, and thus the activity of this pathway is modified by the nitrogen status of the cell and is activated under nitrogen starvation. Interestingly, both the RNAseq and metabolomics data were recorded under conditions of nitrogen excess when NtrC should not be activated, although the 2-oxoglutarate levels in the cell are elevated which may mimic signals in the cell during nitrogen starvation. The repression of NtrC targets prompts further investigation into pathways it regulates. Polyhydroxybutyrate (PHB) is a carbon storage molecule that is associated with stress tolerance and persistence in the rhizosphere for *A. brasilense* (Kadouri, Jurkevitch et al. 2003). PII protein GlnB has been shown to regulate fatty acid synthesis by monitoring 2-oxoglutarate levels in the cell, where increasing 2-oxoglutarate levels relieve inhibition of fatty acid synthesis by GlnB (Sun, Van Dommelen et al. 2002, Gerhardt, Rodrigues et al. 2015). Accordingly, accumulation of PHB is higher under conditions of nitrogen fixation and, in a closely related species *Herbaspirillum*, is regulated by NtrC (Sacomboio, Kim et al. 2017). Poly-hydroxybutyrate depolymerase which is involved in the breakdown and utilization of PHB is also downregulated in the RNAseq data set (Kadouri, Jurkevitch et al. 2003). Together, PHB accumulation is an interesting target due to its role as a carbon storage polymer that is regulated by nitrogen metabolism. Staining of PHB granules in the presence of ethylene showed that cells treated with ethylene accumulated more PHB granules per cell, and that more cells contained PHB granules than under control conditions (Figure 4.5). In *ntrC* knockouts, accumulation of PHB is observed under conditions of nitrogen availability similar to the ethylene treated samples (Sun, Peng et al. 2000). This further supports the hypothesis that ethylene is altering the regulation of

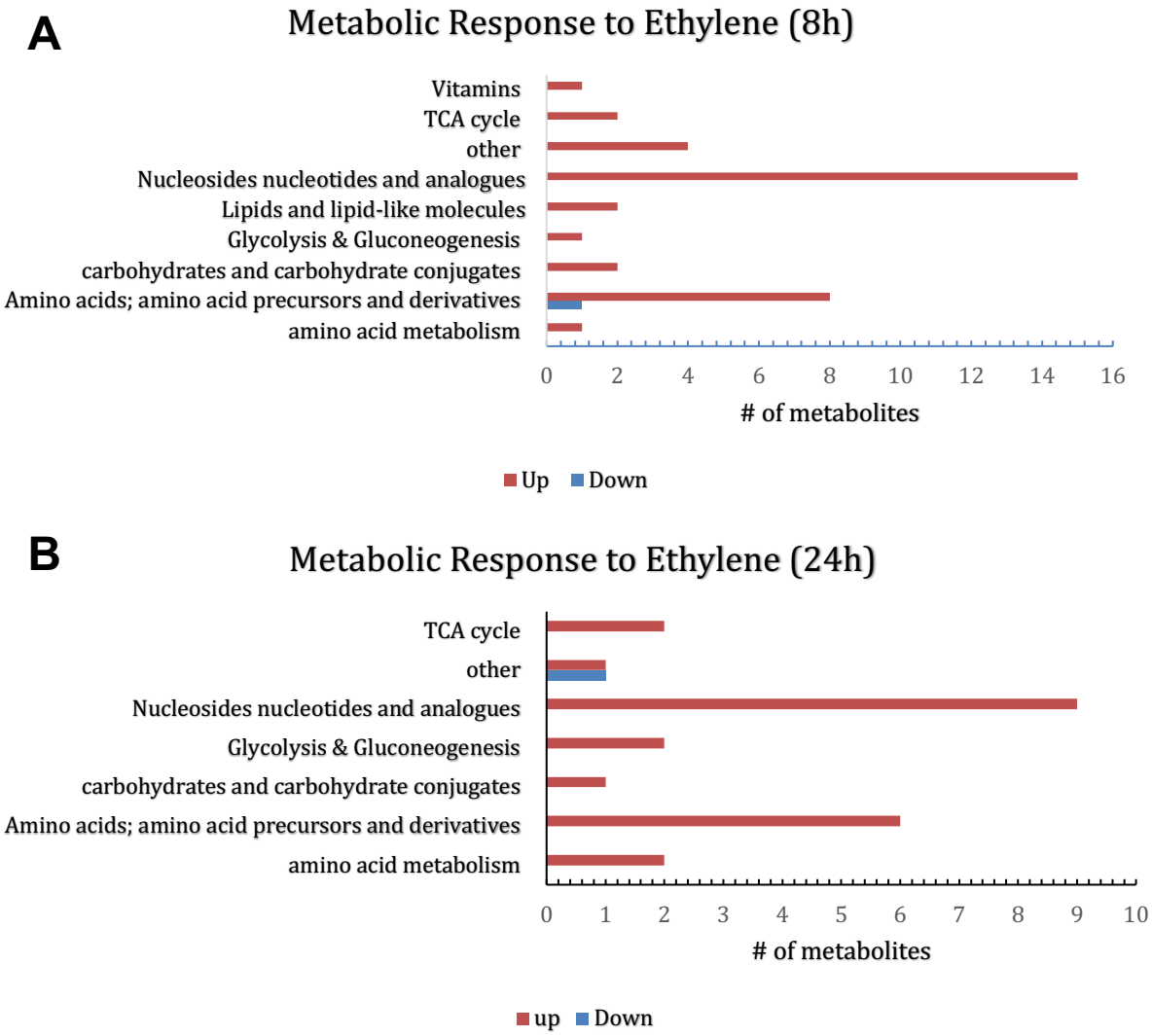


Figure 4.4 Ethylene Induces a Metabolic Shift as Early as 8 Hours.

Pathway analysis of metabolites differentially accumulated at 8 (A) and 24 (B) hours. Samples were grown in flow through chambers rather than sealed tubes with injection as in RNAseq and initial metabolomics experiments.

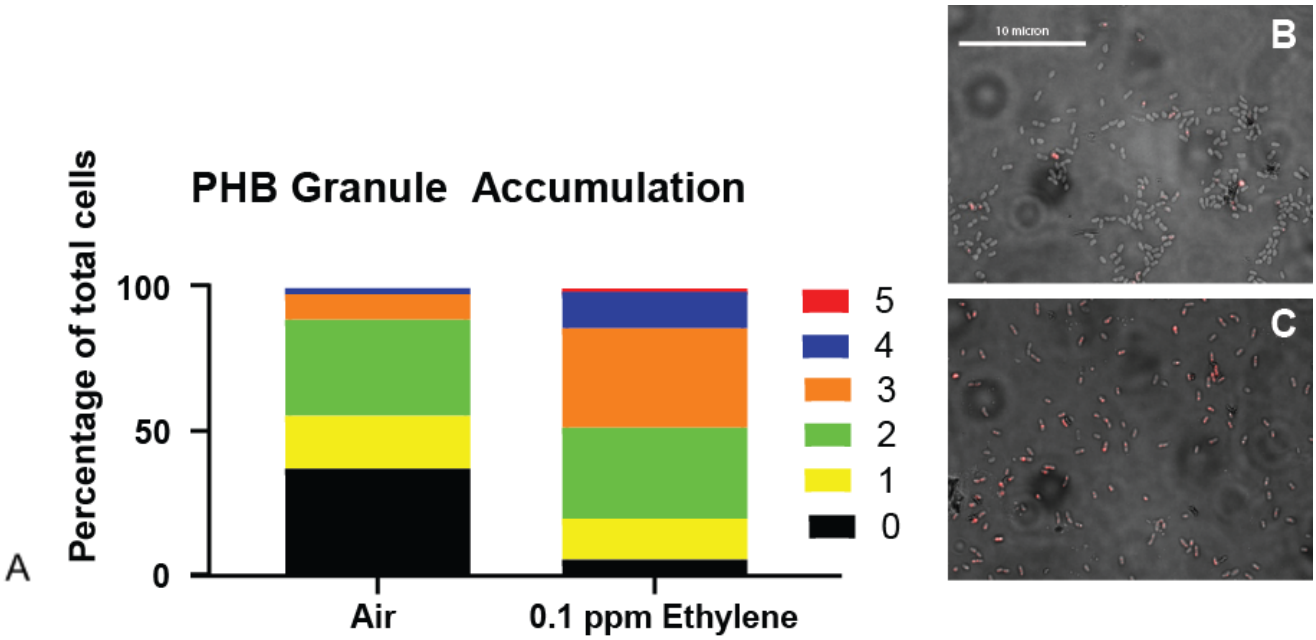


Figure 4.5 Ethylene Causes Accumulation of Poly-Hydroxybutyrate Granules

A) Stack graph showing the percentage of cells with the specified number of granules under either air, or 0.1ppm ethylene treatment. B, C Nile Red fluorescent microscopy overlaid with DIC imaging of *A. brasilense* cells grown in either air (B) or 0.1ppm ethylene (C). N=386 cells for ethylene-free air samples, and N=351 cells for ethylene treated samples. Mann-Whitney U-test determined a p-value <0.05 between groups.

many genes and behaviors that are controlled by NtrC. Analyses of the RNAseq, metabolomics, and physiological data all suggest a change in nitrogen metabolism and genes under the control of NtrC. In addition to the traits investigated above, the nitrogen fixation operon and nitrogenase *nifH* are regulated by NtrC.

To determine if ethylene can also repress the expression of *nifH*, we conducted qPCR analysis of these nitrogen regulatory genes under nitrogen fixing conditions where expression should be driven by nitrogen repletion. First, expression of both PII genes *glnB* and *glnZ* were measured, and only *glnB* expression is reduced by ethylene (Chen, Guan et al. 2001). This is consistent with the hypothesis that this regulation is occurring via changes in NtrC activity. Next, the ammonium transporter *amtB* whose expression should be driven by NtrC activity under conditions of nitrogen starvation is also repressed by ethylene treatment (Dommelen, Keijers et al. 1998). Finally, *nifH* expression is also reduced by ethylene. This suggests that ethylene is also capable of reducing nitrogen fixation by *A. brasilense*. Direct evaluation of nitrogen fixation under ethylene treatment is difficult as this is most commonly assayed by acetylene reduction which yields ethylene (Soper, Simon et al. 2021).

Investigation of the timing of ethylene responses led to examining if the duration of ethylene exposure led to changes in expression patterns. Two genes *OH82_RS14895*, encoding an amino acid permease, and *OH82_RS30850*, encoding pyruvate dehydrogenase subunit B from the RNAseq were analyzed by qPCR in a time course with samples taken at 2, 4, 6, and 8 hours of ethylene treatment (Figure 4.7). These genes were chosen due to a log2 fold change > |1.0} in the RNAseq and high

expression levels under control conditions similar to the reference genes used. At 2 hours, no change was seen in transcript levels for either of these genes but at 4-, 6-, and 8-hours transcripts were significantly higher than at air samples. Additionally, there was no significant difference in transcript abundance between different time points, excluding the 2-hour samples.

In the RNAseq data *azoEtr1* did not see its expression change. This is consistent with a time course for *azoEtr1* and RR^{Etr1} performed in different concentrations of ethylene (Figure 4.8 A, B). In the promoter region of *azoEtr1*, a binding site for the sigma-54 factor RpoN was identified. RpoN controls many nitrogen related behaviors, along with flagellar assembly in *A. brasilense* and is part of the holoenzyme with NtrC that promotes *Nif* operon expression (Milcamps, Van Dommelen et al. 1996). Given the nitrogen behaviors associated with ethylene, we wanted to know if nitrogen sources altered *azoEtr1* expression. (Figure 4.9). Using MMAB with NH_4Cl, $NaNO_3$, KNO_3, glutamate, alpha-ketoglutarate (AKG), and no nitrogen, we measured *azoEtr1* expression using qPCR. These nitrogen sources were chosen either due to their common use in studying *A. brasilense* nitrogen behaviors, or a general role in carbon: nitrogen metabolism like alpha-ketoglutarate. *azoEtr1* expression did not change between the $NaNO_3$, glutamate, or NH_4Cl conditions and all three of these are known to be suitable nitrogen sources for *A. brasilense* that do not change growth patterns (Sadasivan and Neyra 1985). Growth in KNO_3 did not cause a statistically significant change (p-value = 0.06) but a strong negative trend was seen with this nitrogen source. Interestingly, no nitrogen and supplementation with a poor nitrogen source, AKG, resulted in an increase in *azoEtr1* expression.

Discussion

The transcriptomic response to ethylene highlights changes in carbon and nitrogen metabolism in *A. brasilense*. This suggests that *A. brasilense* is functioning as an environmental cue informing the cell about the nutrient status of the surrounding, and potentially of plant immune signaling and competition for nutrients. The upregulation of central carbon metabolism in the RNAseq data may be attributed to the influence of the nitrogen regulatory pathways (Sun, Peng et al. 2000, Sacomboio, Kim et al. 2017, Kukolj, Pedrosa et al. 2019). While few metabolites central to carbon metabolism were enriched in the metabolomics data the abundance of nucleotide intermediates suggest that the pentose phosphate pathway may be the output of the upregulated carbon metabolism induced by ethylene leading to the accumulation of nucleotides. The accumulation of nucleotides should also be viewed in the context of translation. The RNAseq data also shows a downregulation of several ribosomal RNAs, as well as an increase in several ribosomal modifying genes. This could suggest that a decrease in translation is also contributing to the abundance of nucleotides and their analogues. The accumulation of nucleotides could be indicative of a general stress response in response to ethylene or the induction of a stringent response leading to a decrease in translation (Traxler, Summers et al. 2008, Battesti, Majdalani et al. 2011, Vargas-Blanco and Shell 2020).

A link between nitrogen metabolism and carbon metabolism is well established in many rhizobacteria and NtrC specifically has been implicated in regulating expression of transporters, and enzymes involved in glycolysis and gluconeogenesis. The

transcriptomic and metabolomic data here, along with physiological analysis represent a new avenue by which nitrogen metabolism is regulated in *A. brasilense*. Under conditions of nitrogen availability, 2-oxoglutarate is elevated, and genes regulated by NtrC are repressed (Liang, de Zamaroczy et al. 1992, De Zamaroczy 1998, de Zamaroczy and Elmerich 1998). This suggests that either the ethylene signaling pathway is differentially regulating different aspects of the regulatory pathway, i.e., GlnB is bound by 2-oxoglutarate and unable to inhibit NtrB, but NtrC is not allowed to be activated, or that there are additional signaling elements that have not been investigated. The accumulation of PHB granules in conjunction with repression of other NtrC transcriptional targets is consistent with increased PHB accumulation in NtrC mutants (Sun, Peng et al. 2000). The link between nitrogen metabolism and ethylene signaling is reinforced with the discovery of a RpoN binding site in the promoter region of *azoEtr1*. Interestingly, growth in no nitrogen media or poor nitrogen conditions increased expression of *azoEtr1*. Given the proposed mechanism of ethylene functioning as an inhibitor to the signaling pathway, it is hard to determine if increased expression of the receptor would increase or decrease sensitivity to ethylene under these conditions. It is possible that under nitrogen fixing conditions, the cell decreases sensitivity, responding only to robust or prolonged presence of ethylene to allow for nitrogen fixation in the presence of transient signals in the rhizosphere or on the root. Alternatively, the increased expression of the receptor could amplify the signal and make the cells more sensitive to low dosages to stop the cell from devoting energy to nitrogen fixation, and to instead create carbon stores in PHB granules. In either case, the role of ethylene in nitrogen fixation requires more study to determine the mechanism

of interaction from both ends of this signaling pathway, ethylene to nitrogen and nitrogen to ethylene.

The timing of responses is noteworthy in that the original RNAseq analysis contained two time points, 1 hour and 4 hours, were chosen. After 1 hour, no transcripts were significantly altered. In contrast with auxin, ethylene takes a comparatively much longer time to induce changes. This may be due to the diffuse nature of ethylene, and the likelihood of transient perception in the rhizosphere. In the time course experiments, this relatively slow response was confirmed in that no expression changes were seen until 4-hours. Interestingly, expression seems to plateau at this point and there is no further increase by 8-hours.

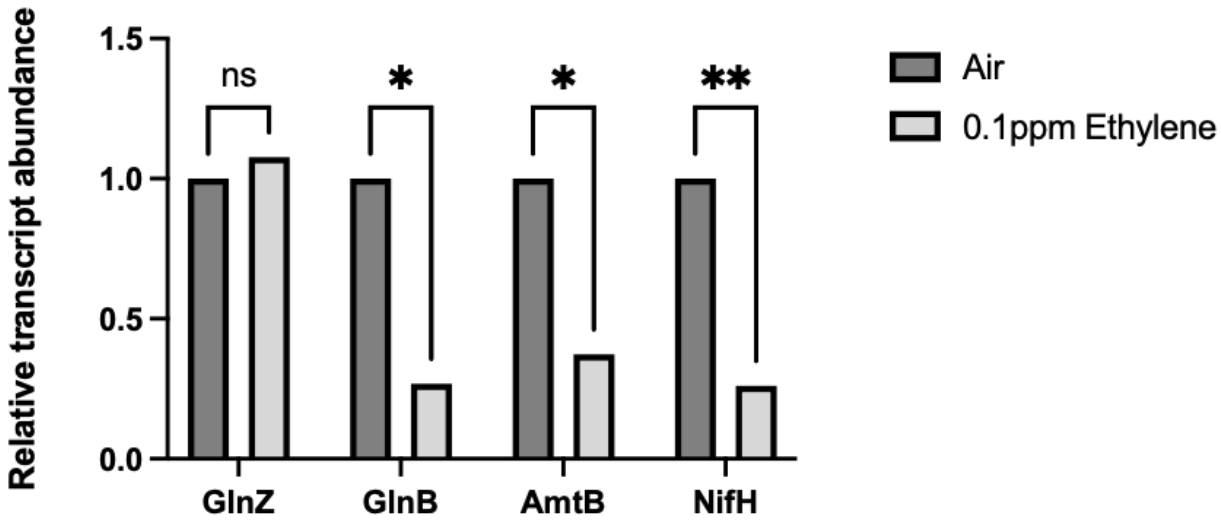


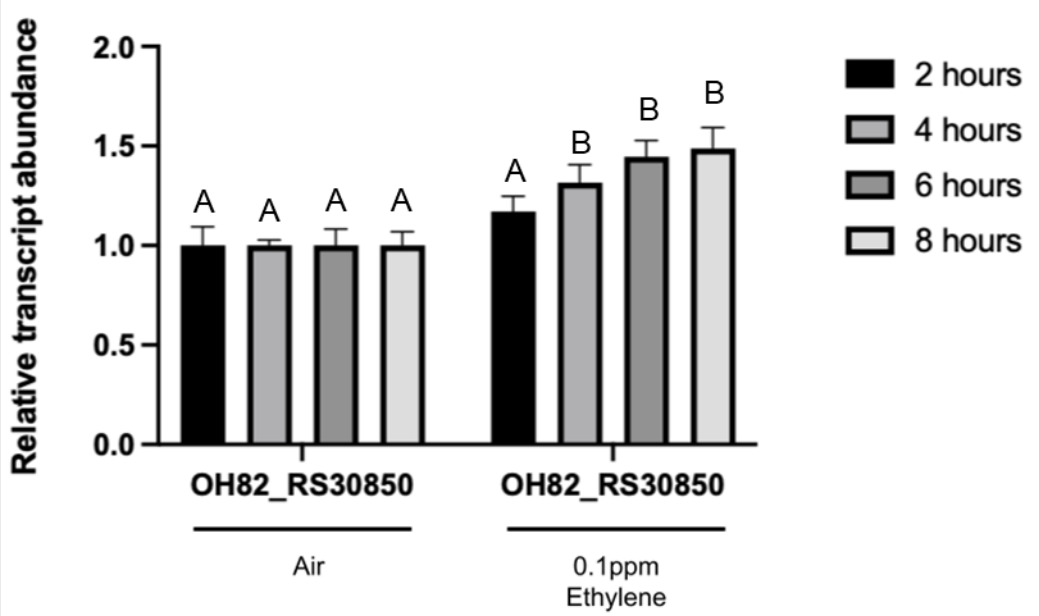
Figure 4.6 Ethylene Inhibits Expression of NtrC-Regulated Transcripts under Nitrogen Fixing Conditions.

qPCr Results showing the expression of *glnZ*, *glnB*, *amtB*, and *nifH* after 24 hours of growth under nitrogen fixing conditions in the presence of ethylene-free air or 0.1 ppm ethylene. *glnz* is not under the control of NtrC and serves as a control. * =p-value <0.05, **=p-value <0.01 as determined by t-test. Data represents 3 or 4 biological replicates with 3 technical replicates per biological replicate.

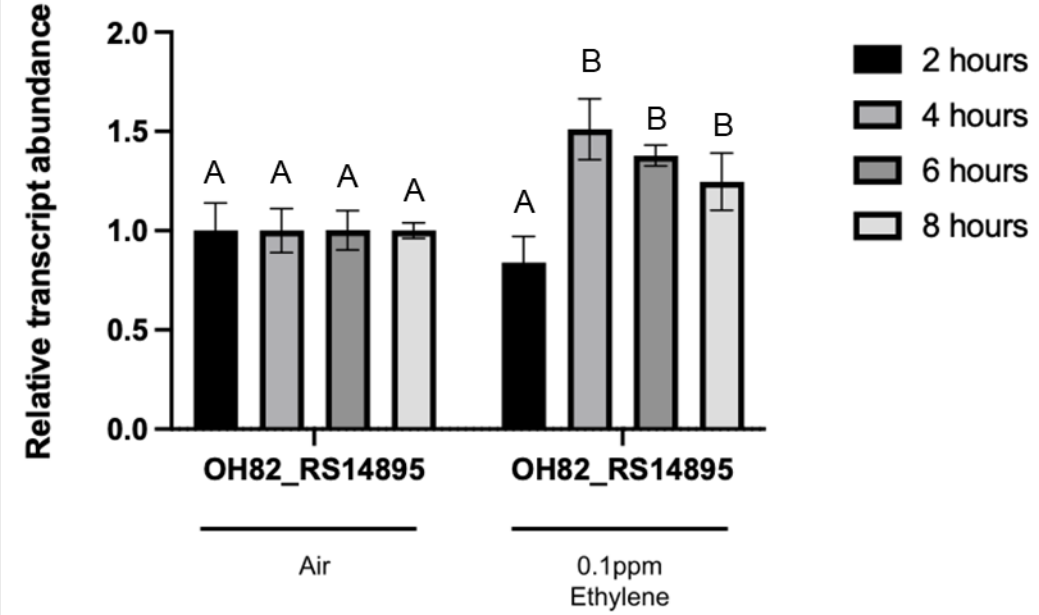
Figure 4.7 Time Course Expression of Ethylene-Induced Transcripts.

Time course expression data for OH82_RS30850 (A) and OH82_RS14895 (B) at 2, 4, 6, and 8 hours. Ethylene-free air treated samples on the left, and ethylene treated samples on the right. Letters denote grouping of statistical significance as determined by two-way ANOVA. Data represents four biological replicates with 3 technical replicates per biological.

A


Relative transcript abundance
2.0
1.5
1.0
0.5
0.0
A
A
A
A
A
B
B
B
OH82_RS30850
OH82_RS30850
Air
0.1ppm
Ethylene
2 hours
4 hours
6 hours
8 hours

B


Relative transcript abundance
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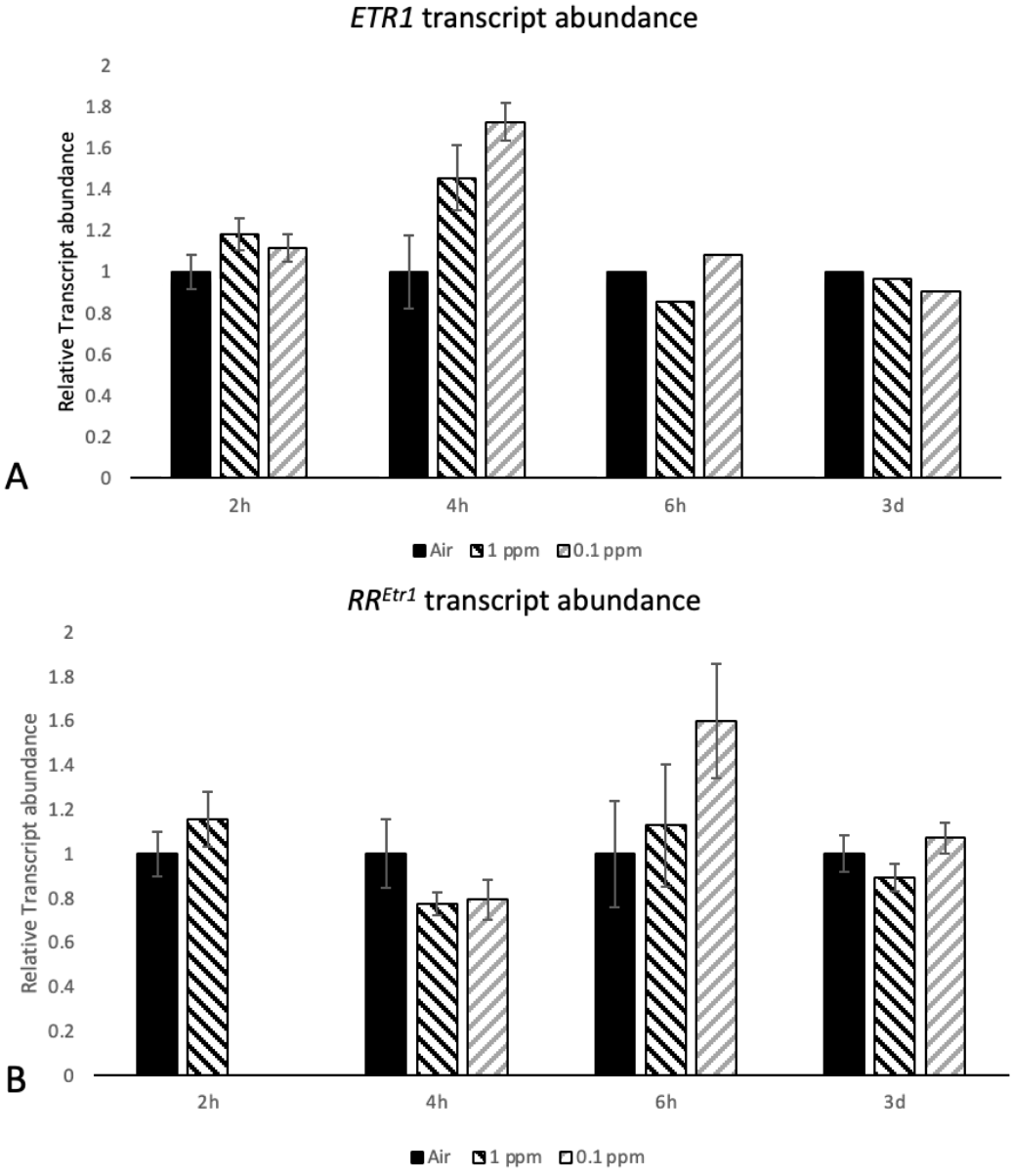

Figure 4.8 Neither *azoEtr1* nor *RR^{Etr1}* Transcript Levels Respond to Ethylene

azoEtr1 (A) and *RR^{Etr1}* transcript levels at 2-, 4-, 6- and 72-hours under treatment with ethylene at either 1ppm or 0.1ppm. RR^{Etr1} 2-hour sample failed for 0.1ppm. No significant difference was seen at any time point. Data represents 4 biological replicates with three technical replicates.

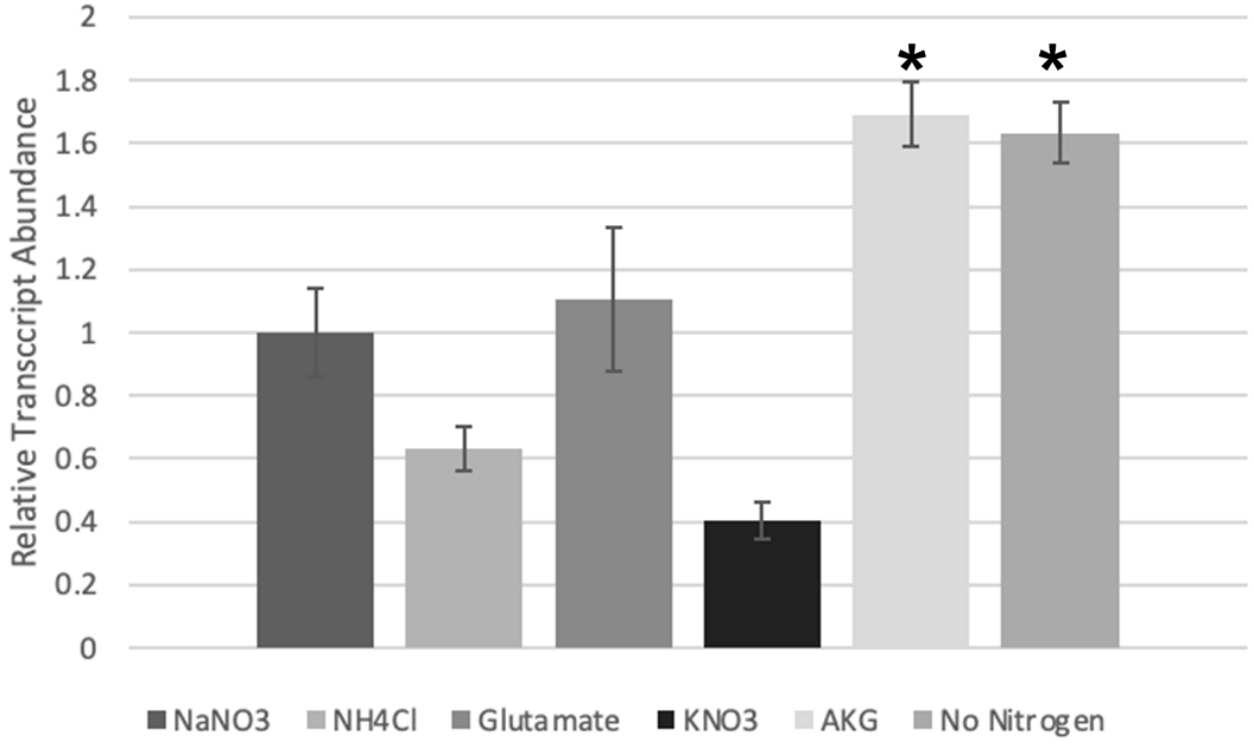

Figure 4.9 Nitrogen Source Influences *azoEtr1* Expression

qPCR analysis of *azoEtr1* expression after 24 hours of growth under different nitrogen sources. All samples were grown in MMAB supplemented with the indicated nitrogen source. All values are normalized to $NaNO_3$ as this was the media used in the RNAseq experiments. Nitrogen concentrations are standardized to 10mM. No nitrogen samples were grown without shaking. AKG (alpha-ketoglutarate) samples flocculated during growth. * = p-value < 0.05 as determined by a two-way ANOVA. Data represents 4 biological replicates with 3 or 4 technical replicates per biological.

Chapter 5: Conclusions and Future Directions

AzoEtr1's ability to bind ethylene had previously been confirmed by the Binder lab and so the primary goal of this thesis was to determine if the ethylene binding protein, AzoEtr1, is a functional ethylene receptor. Here, it was demonstrated that the application of ethylene, and the disruption of *azoEtr1* induces many responses in *A. brasilense*, indicating that AzoEtr1 is a functional ethylene receptor. Biofilm formation and root colonization are inhibited in either the presence of ethylene or the absence of *azoEtr1*. As ethylene and receptor disruption lead to the same phenotype, it is posited that ethylene negatively regulates AzoEtr1. This is in agreement with the proposed mechanisms in both plant and cyanobacteria ethylene signaling pathways (Hua and Meyerowitz 1998, Lacey and Binder 2016).

A. brasilense's plant association was the main consideration for its selection as an interesting organism to study bacterial ethylene responses and this research shows that ethylene alters plant associated behavior of the bacteria. This opens the interesting possibility that many bacteria which harbor putative ethylene receptors can respond directly to plant-produced ethylene to alter colonization patterns and behavior in the rhizosphere. In response to ethylene *A. brasilense* colonization is inhibited, at least temporally. Initial attachment to the root seems to occur as the mutant lines still show single cell attachment to roots. In ethylene treated wild-type cells, low levels of aggregation can be seen on the roots at 24-hours suggesting some equilibration in response to prolonged ethylene exposure in the presence of a plant, that does not occur during abiotic attachment. In lieu of equilibration to the response, the root may produce

additional signals which promote attachment despite the presence of ethylene. Both hypotheses could explain the recovery of colonization in the presence of ethylene by 48 hours. In favor of the latter hypothesis, a MerR transcriptional regulator is highly downregulated in response to ethylene. MerR proteins are a highly diverse family of mostly transcriptional repressors. One such MerR family protein is NolA (Nodulation protein A) which is involved in the regulation of Nod factors in *Bradyrhizobium* (Loh, Stacey et al. 1999, Loh and Stacey 2003, Fang and Zhang 2022). *nolA* is a part of the regulon for Nod factor production in *B. diazoefficiens* that is regulated by population density as well as plant signals. This MerR in *A. brasilense* could be responsible for regulating the production and secretion of molecules involved in dampening plant-immune responses and eliciting a further signaling exchange encouraging colonization.

Production of auxin by *A. brasilense* is also inhibited in the presence of ethylene via an unknown mechanism. While there is no change in *ipdC* transcript levels in response to ethylene, the auxin precursor molecules anthranilate and tryptophan are both depleted in ethylene treated samples (Reynders and Vlassak 1979). This could provide a mechanism by which ethylene indirectly inhibits auxin biosynthesis. The mechanism by which auxin production is inhibited may be relatively straight forward as the influence of ethylene on nitrogen fixation is altering amino acid metabolism and this could explain the reduction of the precursor molecules. Additionally, the precursor molecules may be shunted toward purine and pyrimidine biosynthesis via PRPP production. Interestingly a combination of auxin and ethylene greatly increases the transcript level of *ipdC*. *A. brasilense* undergoes large transcriptional changes in response to auxin and thus it is possible that one of the pathways in auxin response is

to ensure the promotion of auxin biosynthesis in an environment where both auxin and ethylene are present (Malhotra and Srivastava 2008, Van Puyvelde, Cloots et al. 2011). This also highlights the potential risk of considering transcriptomic data without also measuring metabolites, or protein expression as analysis of a single factor can lead to improper assumptions.

In addition to auxin biosynthesis, nitrogen fixing behavior is also influenced by ethylene. *A. brasilense*'s ability to fix nitrogen is well-studied and in conjunction with auxin production, is among the primary reasons why it is broadly used as a biofertilizer (Reynders and Vlassak 1982). The major transcriptional pathways affected by application of ethylene are amino acid metabolism and energy production and conversion. These pathways are highly influenced by nitrogen availability and NtrC signaling (Kukolj, Pedrosa et al. 2019). Metabolic analysis revealed that in addition to amino acid metabolism, nucleotide metabolism was heavily altered. Interestingly, this category was not heavily regulated by nitrogen availability and may indicate that the mechanisms by which ethylene is regulating cellular metabolism and behavior is more complicated than regulation of NtrC activity alone. However, the accumulation of nucleotides and their analogues may also indicate either a shunting of carbon towards the pentose phosphate pathway and accumulation of nucleotides (Stincone, Prigione et al. 2015). It is also possible that this accumulation is a result of reduced translation, as several rRNAs are downregulated by ethylene treatment, and a breakdown of purines as a nitrogen source (Schultz, Nygaard et al. 2001, Izaguirre-Mayoral, Lazarovits et al. 2018). This hypothesis is potentially supported by downregulation of nitrogen fixing genes in the presence of ethylene.

The downregulation of nitrogen-fixing genes and nitrogen metabolism may also play a role in the physiology of biofilm formation and root colonization. *A. brasilense* attaches to surfaces in response to the nutrient environment, in that carbon should be readily available and nitrogen scarce (Sadasivan and Neyra 1985, Burdman, Okon et al. 2000, Siuti, Green et al. 2011). This allows for the cell to dedicate carbon to building biomass to protect the nitrogen fixation machinery and to power the dinitrogen reductase. In the presence of ethylene, cells are neither attaching nor upregulating nitrogen fixing pathways under no nitrogen conditions. It seems likely that the presence of ethylene alters either the perception of carbon:nitrogen status in the cell, or at least the response to low nitrogen (Figure 5.1). *A. brasilense* has been shown to express *nifH* when attached to a plant root, and for ethylene to inhibit both nitrogen fixation and root attachment agrees with the noted behavior (Broek, Michiels et al. 1993). The upregulation of *azoEtr1* transcripts under nitrogen poor conditions suggests that ethylene may be a signal that a surface is not suitable for colonization, and thus not suitable for the establishment of a colony and sinking carbon into nitrogen fixation. This hypothesis is supported by the accumulation of the carbon reserve material PHB. PHB accumulation is associated with stress endurance and soil survival in *A. brasilense* (Kadouri, Jurkevitch et al. 2003). In some legume-rhizobia symbiosis, accumulation of PHB during symbiosis is also downregulated by plant-hosts as it takes available energy away from nitrogen fixing machinery (Trainer and Charles 2006). Together, treatment with ethylene appears to downregulate events in *A. brasilense* when associated with plants including inhibiting attachment, phytohormone production, and nitrogen fixation.

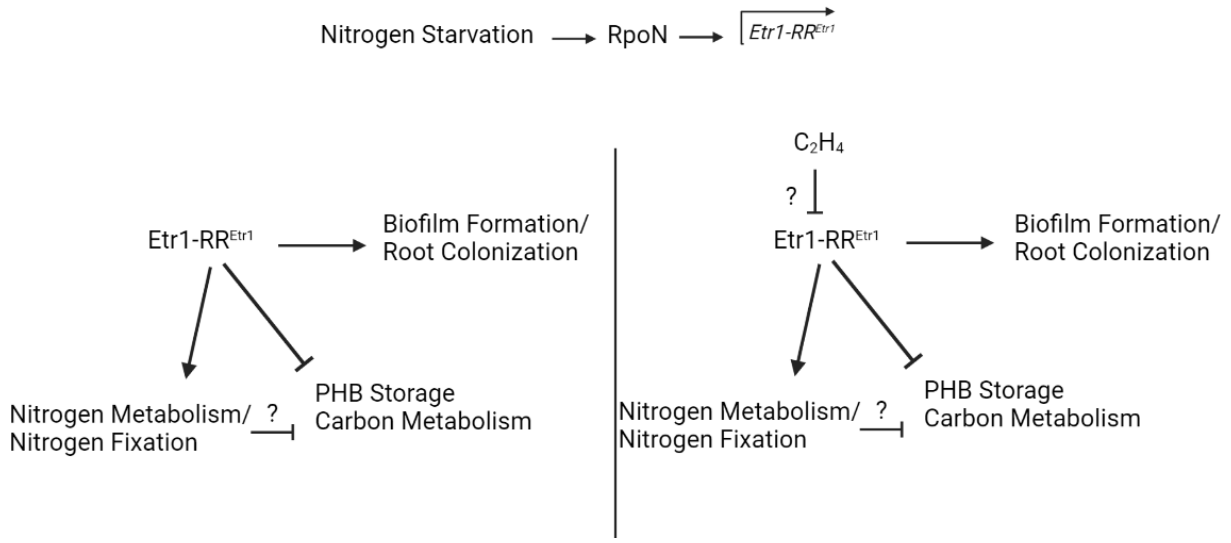

Figure 5.1 Model for Ethylene Signaling in *A. brasilense*

A summary of the experimental results obtained by this work. Transcription of *azoEtr1* is induced by nitrogen starvation, likely regulated by the RpoN binding site in the promoter region. In the presence of ethylene, biofilm formation and root colonization are inhibited, along with transcripts associated with nitrogen metabolism and nitrogen fixation. In addition, PHB storage, along with transcripts associated with central carbon metabolism are upregulated. If AzoEtr1 is inhibited by ethylene binding, then this model suggests that AzoEtr1 signaling is a required component for attachment and nitrogen behaviors.

The phenotypes in response to ethylene span across several time points and the timing of these phenotypes may give some information about the function of this signaling pathway in the soil and in plant interactions. Changes in transcript levels are not seen prior to 4 hours of treatment with ethylene. It is possible that this timing is artificial as all RNA in this study was extracted from liquid grown cultures and as ethylene is hydrophobic, this timing may be more indicative of the timing of ethylene reliably diffusing across an aqueous barrier before reaching cells. Given sufficient aeration and previous experiments performed similarly in *Synechocystis* this is unlikely (Lacey, Allen et al. 2018). More likely, *A. brasilense* encounter fluctuating and brief levels of ethylene while navigating the soil and the root and undergoing a robust response to a very short-lived stimulus would not be advantageous. Requiring a sustained perception of the stimulus before altering central metabolic responses increases the likelihood that the ethylene production is local and not a product of typical ethylene production during growth and development of the host or diffusion of ethylene through the soil. Though the requirement for a sustained exposure has not been tested, a temporal exposure to ethylene may still be capable of inducing a response, but that response is not visible by two hours, the shortest duration tested here. The timing of metabolic shifts is also relevant to the transition to a plant-attached lifestyle. As outlined previously in this work, reversible attachment to roots can happen quickly, between 15 minutes and 2 hours, but irreversible anchoring to the root does not occur before 8 hours (Michiels, Croes et al. 1991). Here we see that ethylene has already shifted the metabolism of *A. brasilense* cells by 8 hours, and that this shift likely interferes with the perception or response of the carbon to nitrogen ratio that encourages attachment

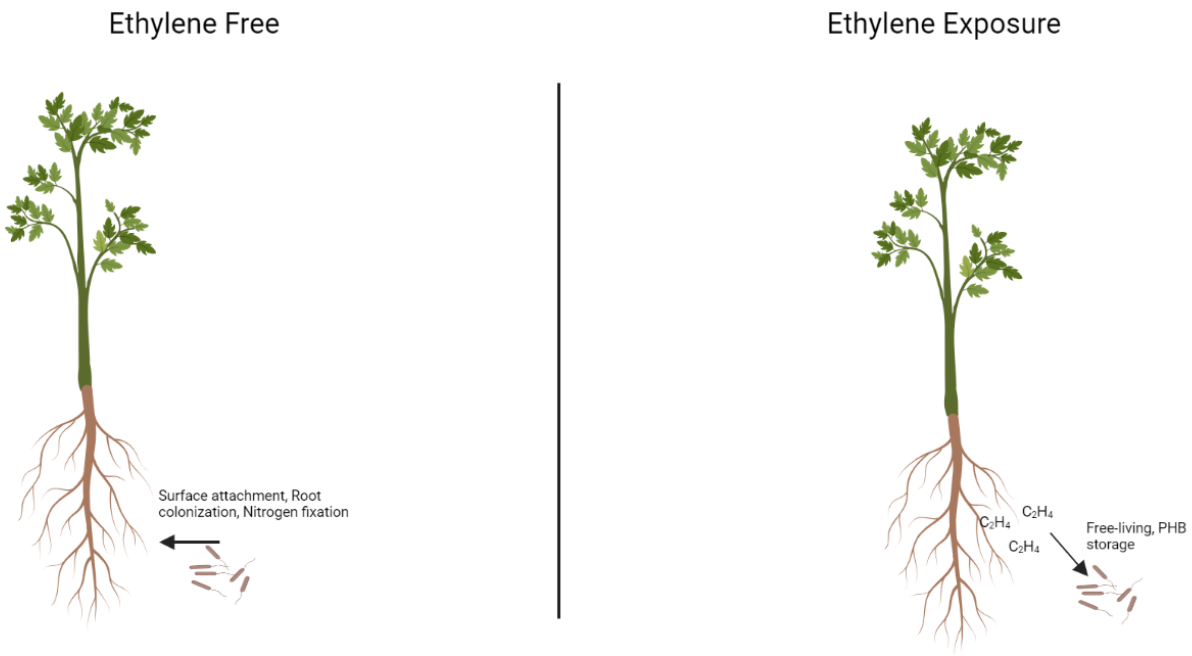

Figure 5.2 *A. brasilense* Response to Ethylene in Soil

In the absence of perceived ethylene, *A. brasilense* responds to plant roots by attaching to the surface, forming dense aggregates along the root and fixing nitrogen. When Ethylene is perceived, *A. brasilense* do not colonize and form aggregates along the roots, but instead remain free-living, does not associate with plant roots and store carbon as PHB polymers, down-regulating nitrogen fixation as a response to nutrient scarcity.

(Burdman, Okon et al. 2000). The advantage of perceiving ethylene in the rhizosphere is at this point unclear, however, the data does give us some suggestions. Perceiving ethylene inhibits colonization and nitrogen fixation in favor of storage of carbon in PHB and a free-living lifestyle (Figure 5.2).

One major assumption this work makes is that *A. brasilense* is responding specifically to plant produced ethylene, despite many potential sources in the rhizosphere (Smith 1976, North, Miller et al. 2017). The assumption made in designing experiments is that the ethylene perceived by *A. brasilense* is due to production by a plant that is either under experiencing abiotic or pathogen stress and is thus reacting to an immune response. It is also possible that ethylene is a signal of a high density of microorganisms in the rhizosphere as it is known to accumulate in anaerobic soils. In either the context of unfavorable plant hosts or a highly competitive root environment, a free-living cell that is storing carbon is likely to be more viable than a sessile nitrogen fixer as *A. brasilense* do not seem to be strong competitors on the root, as they are typically found in low abundance (Nievas, Coniglio et al. 2023). It is also possible that the transcriptional and metabolic changes that ethylene induces in *A. brasilense* promote a distinct role in a rhizosphere microbiome and fill a role in the production and breakdown of different carbon and nitrogen products (Flamholz and Newman 2022).

This work takes a large step forward in identifying the responses of *A. brasilense* to ethylene and attempting to place them in context of plant association and survival in the rhizosphere. These responses will require more work to understand the context in which they are relevant in the soil, but these characterizations outline some potential

mechanisms to probe. As previously stated, much of the physiological, transcriptomic, and metabolic responses to ethylene tie into pathways known to be regulated by NtrC. As RR^{Etr1} is a single-domain response regulator, it likely transduces signal either via phosphorelay or protein-protein binding (Galperin 2006, Jenal and Galperin 2009). Direct biochemical approaches will need to be performed to determine interacting partners but, proteins in the signaling cascade from GlnB, and it's modification, to NtrC provide interesting candidates to screen for protein-protein interactions with RR^{Etr1}. As previously outlined, low nitrogen conditions lead to a reduction in intracellular glutamine and an accumulation of 2-oxoglutarate (Forchhammer, Selim et al. 2022). The PII protein GlnB is uridylated in response to increasing 2-oxoglutarate levels and the uridylated state promotes NtrB kinase activity, this leads to the phosphorylation and multimerization of an NtrC hexamer (De Zamaroczy 1998, de Zamaroczy and Elmerich 1998, Araújo, Huergo et al. 2008, Bonatto, Souza et al. 2012, Wojnowska, Yan et al. 2013). Under ethylene treatment an accumulation of 2-oxoglutarate is seen under nitrogen replete conditions, however there is not a resultant increase in expression of NtrC regulated transcripts, and instead a repression. This suggests that RR^{Etr1} may be a required signaling component for this phosphorelay if ethylene is regulating kinase activity of AzoEtr1, as it has been shown to do in A. thaliana Etr1, then an altered phosphorylation state of RR^{Etr1} could inhibit phosphorylation of NtrC (Gamble, Coonfield et al. 1998, Wang, Hall et al. 2003, Voet-van-Vormizeele and Groth 2008). This model is consistent with the loss of AzoEtr1, and presumably RR^{Etr1} in a polar knockout, phenocopying ethylene treatment.

Alternatively, the ethylene signaling pathway may not interact directly with the nitrogen signaling pathway at all. 2-oxoglutarate levels are elevated under ethylene treatment, but glutamine levels are not altered. Ethylene signaling may exert an influence on the nitrogen signaling pathway by elevating the carbon status of the cell and differentially regulating the PII proteins GlnB, and GlnZ. GlnB is known to be more sensitive to modification in response to 2-oxoglutarate levels, whereas GlnZ is more sensitive to glutamine levels (De Zamaroczy 1998, de Zamaroczy and Elmerich 1998, Araújo, Huergo et al. 2008, Bonatto, Souza et al. 2012). Additionally, GlnB mediated inhibition of fatty acid biosynthesis is repressed by accumulation of 2-oxoglutarate(Gerhardt, Rodrigues et al. 2015). This may suggest that the accumulation of fatty acids and carbon stores such as PHB is the major function of ethylene signaling and this is done by altering levels of 2-oxoglutarate and secondary pathways influence nitrogen fixation and metabolism. In either case, ethylene has a significant impact on nitrogen signaling in *A. brasilense*.

The convergence of ethylene and nitrogen signaling in *A. brasilense* is extremely interesting and the role that it plays in the soil will likely provide insight into the fundamentals of *A. brasilense* -plant attachment. One area that will likely be fruitful is the examination of ethylene response under nitrogen-fixing conditions. In this study, all qPCR done under nitrogen fixing conditions allowed cells to acclimate to both signals over 24 hours. It would be interesting to determine if the cells response more quickly, or more robustly to ethylene under nitrogen starvation.

Investigation into the interplay between *A. brasilense* ethylene and nitrogen signaling will very likely yield novel signaling pathways that influence the association of a common biofertilizer with plants and open the field of exploration into direct response to ethylene by other plant-growth promoting bacteria.

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Vita

Scott Carlew was born in Memphis, Tennessee to Tim and Dawn Carlew. He is the oldest of two children, with a younger sister, Haylee. After graduating from Brighton High School in 2012 he went on to pursue a Bachelor of Science at the University of Tennessee-Knoxville where he worked as an undergraduate researcher in the lab of Dr. Tessa Burch-Smith. After graduation in 2016, he was admitted to the Doctoral Program at the University of Tennessee-Knoxville in Biochemistry and Cellular and Molecular Biology. There he joined the lab of Dr. Brad Binder where he worked on ethylene signaling in plant-growth promoting rhizobacteria.