

Effect of Ethylene on *Azospirillum brasilense*
Lauren Donnelly, Scatt Carlew, Gladys Alexandre, Brad Binder

INTRODUCTION

Ethylene is a gaseous, symmetrical two-carbon phytohormone (Arshad & Frankenberger, 2002; Binder, 2020). Ethylene acts as a stress hormone, and it is responsible for several plant processes including fruit and flower ripening, flower and leaf senescence, the release of dormancy, leaf and fruit abscission, and many more functions (Arshad & Frankenberger, 2002). The rhizosphere is a rich environment featuring many microorganisms, and some of the microorganisms create beneficial symbiotic relationships with the plant, such as *Azospirillum brasilense*. *A. brasilense* is a nitrogen-fixing plant growth-promoting rhizobacterium that belongs to the alpha subclass of proteobacteria (Arruebarrena Di Palma et al., 2013). The production of indole-3-acetic acid (IAA) by *A. brasilense* promotes plant growth and leads to improved plant health (Arruebarrena Di Palma et al., 2013; Zimmer et al., 1988). Additionally, *A. brasilense* improves plant growth through nitrogen fixation and secretion of phytohormones, such as gibberellin, auxin, and cytokinin-like substances (Zimmer et al., 1988). Secretion of phytohormones has been demonstrated to increase the number of lateral roots and the development of root hairs (Zimmer et al., 1988). To promote plant growth, *A. brasilense* must attach to the roots. There are two phases of *A. brasilense* attachment to the plant root (Michiels et al., 1991). The biphasic attachment process consists of an absorption phase followed by an anchoring phase (Michiels et al., 1991). The first attachment phase, absorption, is a weak and reversible attachment style mediated by the polar flagellum (Croes et al., 1993). The second phase, anchoring, is an irreversible attachment mediated through fibrillar material and polysaccharides (Bashan & Holguin, 1993).

Recently the Binder lab discovered that *A. brasilense* has a functional ethylene receptor (AzoEtr1), allowing it to bind ethylene. Figure 1 is a sequence alignment of the first 150 amino acids of the ethylene binding domain in AzoEtr1 with the ethylene binding domains of the ethylene receptor in *Synechocystis* and the five isoforms from *Arabidopsis thaliana*. This highlights that many amino acids are conserved including the seven residues necessary for ethylene binding.

ETR2	MVKEIASWLLILSMVVFVSPVL--AINGGGYPRCNCEDEGNSFWSTENILETQRVS	FLI	58
EIN4	MLRSIGLGLLLFALLALVS-----GDNDYVSCNCDEG--FLSVHTILECQRVS	LLI	51
ERS2	MLKTLVQWLVFVFFFLIGSVVTAEDDGSLSLNCNDD--SLFSYETILNSQKVG	FLI	59
ETR1	M-----EVCNCIEPQ--WPADELLMKYQYIS	EFFI	28
ERS1	M-----ESDCFETH--VNQDDLLVKYQYIS	DALI	28
Azospirillum	MFGGVEAFFDTSA-----YLPHGVCLEFWR-----PEILTLHIVS	VLT	38
SLR1212	MAITAF-TLGDFFQANS-----YIPGHGCLWQ-----TPLVWLHVSA	FFT	41
		:: : . * :	
ETR2	AVAYFSLELLYFVSCSNVP--FKWVLEFIAFVLCGMTHLLHGWTYS-AHPFRLMMA		115
EIN4	AIAYFSLELLYFISFSNVP--FKWVLVQFIAFVLCGMTHLLNAWYYGPHSFQMLMW		109
ERS2	AIAYFSLELVYFVSRITNVPSPYNWVCEFAFVLCGMTHLLAGFTYG-PHWPWVMTA		118
ETR1	AIAYFSLELLYFVKKSAVFP-YRWVLVQFGAFVLCGATHLINLWTF-THSRTVAIV		86
ERS1	ALAYFSLELLYFVQKSAFVP-YKWVLMQFGAFVLCGATHLINLWTF-MHSAVAIV		86
Azospirillum	GLSYYSIFVALLYFVVKRRDVA-FTWIVLFAAFVLCGTHFFSLWTLW----	YDPYAV	93
SLR1212	AIAYFSLELLYFLRKRDIP-FPNIIFLSTFLCCTGTHFFDIITLW----	YPIYWI	96
	..*:*		
ETR2	FTVFKMLTALVSCATAITLITLIPLLKVKVREFM		150
EIN4	LTIFKFLTALVSCATAITLITLIPLLKWKVRELY		144
ERS2	VTVFKMLTGIVSFLTALSLVTLPLLLKAKVREFM		153
ETR1	MTAKVLTAVSCATAMLVHIIIPDLLSVKTRLEF		121
ERS1	MTIAKVSAAVSCATAMLVHIIIPDLLSVKNRELF		121
Azospirillum	EGIVKALTAMSVLTAVLWVQMPKALALPSATQL		128
SLR1212	SGTVKASMAIVSIITVFELIQIVPNALNLKSPTL		131
	* .:* * .. * :*		

Figure 1: *A. brasilense* contains a putative ethylene receptor, AzoEtr1.

Figure 1 shows the sequence alignment of the first 150 amino acids of the ethylene binding domain. The highlighted residues are necessary for ethylene binding in *A. thaliana*. These same residues are conserved in *A. brasilense*.

An ethylene binding assay previously performed in the lab showed that wild-type *A. brasilense* binds ethylene above the background. When AzoEtr1 is disrupted as an insertional mutant (AzoEtr1::Gm^R), ethylene binding is reduced, and when AzoEtr1 is expressed in yeast, yeast can bind ethylene. Just downstream of *AzoEtr1* in the genome is a gene encoding a putative response regulator suggesting that AzoEtr1 and this response regulator (RR^{Etr1}) form a signaling unit. Given that *A. brasilense* can bind ethylene, the lab is attempting to determine if the release of ethylene into the soil from the plant regulates *A. brasilense* attachment. My project examines three aspects that are essential to root attachment, which are the flagella, biofilm formation, and energy.

Flagella are organelles located on the cell surface of bacteria that aid in motility and attachment (Nakamura & Minamino, 2019). *A. brasilense* possess a dual flagellar system consisting of a singular polar flagellum and multiple lateral flagella (Alexandre et al., 1999). Different nutrient media conditions can trigger the synthesis of the different types of flagella systems. The polar flagellum forms when *A. brasilense* is grown in liquid media and mediates swimming by rotating the helical basal body of the flagellum (Alexandre et al., 1999; Joys, 1988). The polar flagellum is glycoprotein glycosylated with an O-linked polysaccharide chain (Belyakov et al., 2012). Biofilm initiation in *A. brasilense* is mediated by the polar flagellum (Ganusova et al., 2021). The polar flagellum allows the bacterium to swim to the appropriate surface and reversibly attach (Croes et al., 1993). By contrast, the lateral flagella form in viscous media, allowing the bacterium to swarm across surfaces (Moens et al., 1996). The viscosity of semi-solid media prevents the rotation of the polar flagellum. Lateral flagella are produced to allow the bacterium to remain motile (Partridge & Harshey, 2013). However, given that semi-solid media contains more friction, more force is required to propel the bacterium forward (Partridge & Harshey, 2013; Walker, 2002). In addition to more friction, semi-solid media also presents the challenges of a lack of water and increased surface tension (Partridge & Harshey, 2013). As a result, more lateral flagella are present on the bacterium compared to the single polar flagellum (Partridge & Harshey, 2013).

As mentioned previously, the polar flagellum helps mediate biofilm attachment. Biofilm formation is an important step in the colonization of roots by rhizobacteria (Bogino et al., 2013). Biofilms are multicellular communities held together through extracellular polymeric substances and cell-surface connections (Hickman et al., 2005; Wang et al., 2017). One of the key characteristics of biofilms is the ability to share nutrients between cells, resulting in enhanced microbial survival (Wang et al., 2017). The extracellular polysaccharides between the cells helps to facilitate nutrient transfer, horizontal gene transfer, cell-to-cell communication, and synergistic micro-consortia (Flemming & Wingender, 2010). Biofilms can adopt several different morphologies from compact clusters to flat and featureless. (Danhorn & Fuqua, 2007). Biofilm formation occurs in four main stages. The first stage is bacterial attachment, which is followed by microcolony formation. After microcolony formation, biofilm maturation occurs, which is then proceeded by detachment (Crouzet et al., 2014). The first phase of biofilm attachment involves the transition from free-swimming to sessile life, requiring a change in gene expression of the polar flagellum (Barahona et al., 2010). The transition from motile to sessile life is mediated by cyclic-dimeric guanosine monophosphate (c-di GMP) (Prüß et al., 2006). C-di

GMP is a universal secondary messenger in bacteria responsible for several bacterial processes such as biofilm formation, cell cycle progression, and surface adaptations (Jenal et al., 2017). C-di GMP is involved in the switch from motile to a sedentary lifestyle by reducing the expression of flagellar genes, causing reduced flagellar activity and loss of the ability to swim (Hengge, 2009). Additionally, high levels of c-di GMP correlate with high levels of surface attachment and cell aggregation (Hickman et al., 2005). According to a study conducted by Tischler and Camilli, vpsR transcription in *Vibrio cholerae* is modulated by c-di GMP, affecting EPS formation (Tischler & Camilli, 2004). Furthermore, another study conducted on *Pseudomonas aeruginosa* demonstrated that improved biofilm formation resulted from an increased level of c-di GMP, and defective biofilms were observed in cells depleted in c-di GMP (Hickman et al., 2005). The study also demonstrated that decreased levels of c-di GMP inhibited initial biofilm attachment, highlighting the role of c-di GMP on the polar flagellum and the transition into sedentary life (Hickman et al., 2005).

Biofilm formation is advantageous to *A. brasilense* in specific nutrient environments. Increased aggregation of *A. brasilense* is observed in high-carbon to nitrogen ratio and nitrogen-deprived media (Madi & Henis, 1989). In particular, biofilm formation is enhanced with the NaNO_3 as the nitrogen source compared to NH_4Cl or nitrogen-free media (Siuti et al., 2011; Zimmer et al., 1984). Due to this finding, the MMAB Low Nitrogen media used in the biofilm assay contains 0.01% NaNO_3 as the nitrogen source. As mentioned earlier, one of the ways *A. brasilense* improves plant growth is through nitrogen fixation. Due to the oxygen limiting environment created by biofilms, the formation of biofilms protects nitrogenase from oxygen, allowing for nitrogen fixation to occur ((Danhorn & Fuqua, 2007; Wang et al., 2017).

Another important aspect of root attachment of *A. brasilense* is carbon metabolism and energy storage. Unpublished data from the Binder lab contains RNA-sequencing (RNA-seq) on *A. brasilense* treated with 0.1 ppm ethylene and ethylene-free air. These data showed an upregulation in TCA cycle genes and carbon storage when treated with 0.1 ppm ethylene. To support this finding, the Binder lab performed metabolomics on *A. brasilense* treated with 0.1 ppm ethylene and ethylene-free air. The metabolomics data showed changes in TCA metabolites when treated with 0.1 ppm ethylene compared to ethylene-free air. Taken together, the RNA-seq and metabolomic data suggested changes in core carbon metabolism and carbon storage when *A. brasilense* is treated with 0.1 ppm ethylene. One method of carbon storage is through the synthesis of poly-B-hydroxybutyrate (PHB) granules. PHB is a polymer molecule that acts as a carbon and electron sink (Dawes & Senior, 1973). Under starvation conditions, PHB may serve as a carbon/energy source (Tal & Okon, 1985). Furthermore, Tal and Okon demonstrated that PHB levels increased with NaNO_3 in the media compared to NH_4Cl (Tal & Okon, 1985). Tal and Okon also discovered that in high carbon to nitrogen ratios, *A. brasilense* accumulated PHB granules. The MMAB media used in my PHB granule assay has a high carbon to nitrogen ratio. Tal and Okon also explored the inverse relationship between nitrogenase activity and hydroxybutyrate activity. Nitrogenase activity was only seen in bacteria cells rich in PHB accumulation and low levels of hydroxybutyrate activity. Cells with high hydroxybutyrate activity reported low levels of nitrogenase activity. The authors proposed that PHB supplied the reductive power necessary to power nitrogenase (Tal & Okon, 1985). PHB granule synthesis and nitrogen fixation are chemically linked because both processes rely on NADPH (Benemann et al., 1971; Uchino et al., 2007). During nitrogen fixation, NADPH provides the necessary reducing power for nitrogenase (Benemann et al., 1971). In PHB granule synthesis, NADPH aids in the synthesis of PHB from acetyl coenzyme A (CoA) (Uchino et al., 2007). Given the

chemical connection between the two processes, the lab currently hypothesizes that cells are most likely either increasing PHB accumulation or building a biofilm.

To better understand the effect of ethylene on root attachment in *A. brasilense*, several *in vitro* experiments were conducted on the effect of ethylene on the flagella, biofilm formation, and PHB granule synthesis. The insertional deletion mutant AzoEtr1::GmR knocks out the ethylene binding protein and probably RR^{Etr1} too. This disruption causes the AzoEtr1::GmR mutant to behave as if ethylene is binding. Preliminary data from the lab determined that ethylene inhibited biofilm formation. I hypothesized that ethylene is affecting the formation of biofilm by altering the morphology of the polar flagellum. To determine if ethylene is causing a dispersal of biofilm or halting the formation of new biofilm, a biofilm assay that transitions between ethylene-free air and 0.1 ppm ethylene was performed. Additionally, I performed SDS-PAGE western blots to discover if ethylene was altering the molecular weight of the flagella. The unpublished RNA sequencing data and metabolomics data showed an upregulation in carbon storage and carbon metabolites when treated with 0.1 ppm ethylene. I hypothesized that the addition of different TCA metabolites, specifically citrate and pyruvate, will affect biofilm formation. I also hypothesize that treating with 0.1 ppm ethylene will alter PHB accumulation in the cells. To detect the PHB levels, I captured microscopy images of *A. brasilense* cells treated with ethylene-free air and 0.1 ppm ethylene. The results from these experiments will add clarity to how ethylene controls root attachment of *A. brasilense*.

METHODS

A. brasilense was grown in accordance with the protocols presented in “*Azospirillum brasilense*: Laboratory Maintenance and Genetic Manipulation” (Gullett et al., 2017).

SDS-PAGE Western Blot of Polar Flagellum

A. brasilense cells are grown in TY overnight. The samples are washed in Che buffer three times. The OD₆₀₀ is adjusted to 1.0. 30 µL of the samples are placed in 5 mL of MMAB. The ethylene samples are treated with 0.1 ppm ethylene. After 16 hours, the samples are collected by centrifugation at 5K rpm for 3 minutes. 5 mL are collected and pelleted. The cells are washed once with PBS, and the OD₆₀₀ is adjusted to 1.0. 5 mL of the samples are spun down at 5K rpm for 3 minutes. The pellet is resuspended in 80 µL of 1x lamelli buffer in PBS. The samples are vortexed briefly and placed on ice for 5 minutes. The cells are then spun down at max rpm at 4°C for 18 minutes. The supernatant was collected into a fresh Eppendorf Tube. The samples are then heated for 5 minutes at 95°C. The cells are then vortexed. 20 µL of each sample are loaded into each well on 8% SDS-PAGE. The Western blot is run for 1 hour and 10 minutes at 120V on ice. While the western is running, the PVDF is activated by placing into methanol for 30 seconds, and then soaked in transfer buffer. A sandwich is established by soaking each component in transfer buffer. The sandwich is built in the following order: sponge, sponge, sponge, gel, PVDF, sponge, sponge, sponge. The sandwich is clamped tightly and placed into the transfer tank. A wet transfer is performed at 60V for 2 hours. After the transfer, the PVDF is carefully removed and stained with Ponceau S for protein visualization. After staining, the PVDF is washed with DI water and blocked in 5% milk in TBS-T for 1 hour. After blocking, the primary antibody As-Fla polyclonal antibody is added at a concentration of 1:1,000 in TBS-T and incubated overnight. After the overnight incubation, the PVDF is washed in a series of washes: 2x5 minutes in milk, 2x5 minutes in TBS-T, 2x5 minutes in TBS. The goat-anti-rabbit conjugated to horseradish peroxidase secondary antibody is added at a concentration of 1:10,000 in TBS-T and incubated

for 2 hours. After the incubation, a series of washes are performed: 3x5 minutes in milk, 3x5 minutes in TBS-T, 3x5 minutes in TBS. To image the PVDF, a 1:1 mixture of peroxide and luminol is added and incubated for 4 minutes. The PVDF is imaged using a Chemidoc and chemiluminescence. The polar flagellum forms a band at ~100 kDa.

SDS-PAGE Western Blot of Lateral Flagella

A. brasilense cells are grown on swarm plates (MMAB +0.5% Tween-20) for 5 days. The cells are then scraped off the plate and resuspended in PBS. The cells are collected by centrifugation at 5K rpm for 3 minutes. The cells are washed once with PBS, and the OD₆₀₀ is adjusted to 1.0. 5 mL of the samples are spun down at 5K rpm for 3 minutes. The pellet is resuspended in 150 µL of 1x lamelli buffer in PBS. 20 µL are loaded into each well on 8% SDS-PAGE. The Western blot is ran for 1 hour and 10 minutes at 120V on ice. While the western is running, the PVDF is activated by placing into methanol for 30 seconds, and then soaked in transfer buffer. A sandwich is established by soaking each component in transfer buffer. The sandwich is built in the following order: sponge, sponge, sponge, Gel, PVDF, sponge, sponge, sponge. The sandwich is clamp tightly and placed into the transfer tank. A wet transfer is performed at 60V for 2 hours. After the transfer, the PVDF is carefully removed and stained with Ponceau S for protein visualization. After staining, the PVDF is washed with DI water and blocked in 5% milk in TBS-T for 1 hour. After blocking, the primary As-Laf polyclonal antibody is added at a concentration of 1:1,000 in TBS-T and incubated overnight. After the overnight incubation, the PVDF is washed in a series of washes: 2x5 minutes in milk, 2x5 minutes in TBS-T, 2x5 minutes in TBS. The goat-anti-rabbit conjugated to horseradish peroxidase secondary antibody is added at a concentration of 1:10,000 in TBS-T and incubated for 2 hours. After the incubation, a series of washes are performed: 3x5 minutes in milk, 3x5 minutes in TBS-T, 3x5 minutes in TBS. To image the PVDF, a 1:1 mixture peroxide and luminol is added and incubated for 4 minutes. The PVDF is imaged using a Chemidoc and chemiluminescence. The lateral flagella form a band at ~45 kDa.

Glycoprotein Stain

A. brasilense cells are grown in TY overnight. The samples are washed in Che buffer three times. The OD₆₀₀ is adjusted to 1.0. 250 µL of the samples are placed in 25 mL of MMAB. The ethylene samples are treated with 0.1 ppm ethylene. After 16 hours, the samples are collected by centrifugation at 5K rpm for 3 minutes and resuspended in 15 mL of PBS. The cells are washed once with PBS, and the OD₆₀₀ is adjusted to 1.0. 15 mL of the samples are spun down at 5K rpm for 3 minutes. The pellet is resuspended in 80 µL of 1x lamelli buffer in PBS. The samples are vortexed briefly and placed on ice for 5 minutes. The cells are then spun down at 22K rpm 4°C for 90 minutes. The pellet is resuspended in 150 µL of 1x lamelli buffer in PBS. 50 µL of each sample are loaded into each well on 8% SDS-PAGE. The gel is run for 1 hour and 10 minutes at 120V on ice. The gel is stained using the Pierce™ Glycoprotein Staining Kit. After running the gel, the gel is fixed with 50% methanol for 30 minutes. The gel is then washed with 3% acetic acid twice for 10 minutes. The oxidization reagent is then added and allowed to incubate. After 15 minutes, the gel is washed with 3% acetic acid three times for five minutes. After the washed, the Pierce Glycoprotein Stain reagent is added and incubated for 15 minutes. After the incubation period, the reduction reagent is added. After 15 minutes, the gel is washed with 3% acetic acid and DI water. The gel is then imaged using a ChemiDoc.

Biofilm Assay with Gas Treatment Transition

A. brasilense cells are grown overnight in TY media. The overnight cultures are washed three times with Che buffer. 5 mL of the sample are collected, and the OD₆₀₀ is adjusted to 1.0. 12-well plates are prepared by heating a needle and poking a hole in the lid of each well to ensure equal gas flow. Each hole is covered with surgical tape to allow gas exchange. 1 mL of MMAB Low Nitrogen is added to each well. 30 µL of bacteria is added to each well. The plates are placed into the gas chamber. On the third day, the three day ethylene and 3 day ethylene-free air plates are removed. The 3 day ethylene-free air plate / 2 day ethylene plate is moved into the ethylene on the third day. The 3 day ethylene / 2 day air plate is moved into the air chamber on the third day. On the fifth day, all the remaining plates are removed. On the day of plate removal, all the liquid in the wells is dumped. 500 µL of 3% Crystal Violet is added to each well and incubated for 30 minutes. After 30 minutes, the crystal violet is dumped, and the plates are washed vigorously in DI water four times. In between washed, the plates are banged on a paper towel to remove residual water. 2 mL of 95% ethanol is added to each well for 2 minutes. After two minutes, the OD₆₀₀ is collected for each well. Once the OD₆₀₀ is collected from each well, the values are plotted. An analysis of variance (ANOVA) is performed to determine statistical significance.

Biofilm Assay with Different Metabolite Nutrient Medias

A. brasilense cells are grown overnight in TY media. The overnight cultures are washed three times with Che buffer. 5 mL of the sample are collected, and the OD₆₀₀ is adjusted to 1.0. 12-well plates are prepared by heating a needle and poking a hole in the lid of each well to ensure equal gas flow. Each hole is covered with surgical tape to allow gas exchange. 1 mL of MMAB Low Nitrogen media of interest is added to each well. 30 µL of bacteria is added to each well. The plates are placed into the gas chamber for three days. On the third day, all the liquid in the wells is dumped. 500 µL of 3% Crystal Violet is added to each well and incubated for 30 minutes. After 30 minutes, the crystal violet is dumped, and the plates are washed vigorously in DI water four times. In between washes, residual water is removed between washes. 2 mL of 95% ethanol is added to each well for 2 minutes. After two minutes, the OD₆₀₀ is collected for each well. Once the OD₆₀₀ is collected from each well, the values are plotted. An analysis of variance (ANOVA) is performed to determine statistical significance.

Biofilm Microscopy Imaging

A. brasilense cells are grown in TY overnight. The samples are washed in Che buffer three times. The OD₆₀₀ is adjusted to 1.0. 2 mL of cells are collected and spun down. The cells are resuspended in 1 mL of MMAB Low Nitrogen. The 10 µL of the solution are placed onto poly-L-lysine coated slides. The slides are then each placed into an airtight jar. Each jar contains a Kimtech kimwipe dampened with DI water. The jars are sealed with parafilm and an airtight lid. The ethylene treated samples are injected with 0.1 ppm ethylene. The slides are incubated overnight and imaged using a Zeiss Axio Observer Z1 inverted phase contrast fluorescence microscope.

Polyhydroxy Butyrate Granule Staining

A. brasilense cells are grown in TY overnight. The samples are washed in Che buffer three times. The OD₆₀₀ is adjusted to 1.0. Overnight batch cultures of *A. brasilense* are grown using 500 µL of the OD₆₀₀ 1.0 cell cultures to inoculate 500 mL of MMAB. The ethylene treated cells are

injected with 0.1 ppm ethylene. After growing overnight, 5 mL of cells are collected and the OD₆₀₀ is adjusted to 1.0. 2 mL OD₆₀₀ 1.0 cell cultures are collected and spun down. The cells are then incubated with 500 µL of 0.5% Nile Red stain for 30 minutes. After staining, the cells are washed 3 times with Che buffer and resuspended in 1 mL of Che buffer. 10 µL of each sample are placed on a coverslip and covered with an agar pad. The slides are then imaged with a Zeiss Axio Observer Z1 inverted phase contrast fluorescence microscope. Once images from each slide were obtained, a grid using ImageJ is generated on each slide. Ten squares are randomly selected, and the number of PHB granules is counted in each cell. The percentage of PHB granules in each cell is calculated from the total number of cells. The median values for each treatment are calculated, and a Mann-Whitney test of the median values is performed to determine statistical significance.

All reagents used are made in accordance to in accordance to the recipes presented in “*Azospirillum brasilense*: Laboratory Maintenance and Genetic Manipulation” (Gullett et al., 2017). The MMAB and MMAB Low N are made with NaNO₃ instead of NH₄Cl. The recipes not found in the text are stated below.

Che Buffer

- a. 1.3 g K₂PO₄
- b. 1.7g KH₂PO₄

MMAB Low N 0.4% Citrate

- a. 3 g K₂HPO₄
- b. 1 g NaH₂PO₄·H₂O
- c. 0.15 g KCl,
- d. A pinch of sodium molybdate dihydrate
- e. 0.1 g of NaNO₃
- f. 5.0 g C₄H₆O₅
- g. 3.5 g sodium citrate
- h. 1000 mL of DI water
- i. Adjust pH to 6.85 – 7.0
- j. After autoclave add 5 mL MgSO₄ stock solution and 0.50 mL CaCl stock solution

MMAB Low N 0.4% Pyruvate

- a. 3 g K₂HPO₄
- b. 1 g NaH₂PO₄·H₂O
- c. 0.15 g KCl,
- d. A pinch of sodium molybdate dihydrate
- e. 0.1 g of NaNO₃
- f. 5.0 g C₄H₆O₅
- g. 45.4 mM pyruvic acid
- h. 1000 mL of DI water
- i. Adjust pH to 6.85 - 7.0
- j. After autoclave add 5 mL MgSO₄ stock solution and 0.50 mL CaCl stock solution

RESULTS

Polar and Lateral Flagella Western Blots and Glycoprotein Stain

Figure 2 and Figure 3 show the results from a western blot performed using polar and lateral antibodies, respectively. As shown in Figure 2, the application of ethylene causes no measurable effect on the amount of polar flagella and there is no shift in the apparent molecular weight. Figure 3 shows a difference in the amount of lateral flagella between samples grown in liquid media and samples grown in semi-solid media. Lanes 1 – 6 are WT samples grown in liquid media, and no bands form in these lane, indicating there is no difference in the ethylene-free air and 0.1 ppm ethylene treated samples. Lane 7 is a WT sample grown in semi-solid TY swarm plates in ethylene-free air, and a band forms at 45 kDa. The formation of a band at 45 kDa indicates the presence of a lateral flagella.

Figure 4 is a glycoprotein stain that measures the amount of glycoprotein present on the polar flagella. No observable differences between the 0.1 ppm ethylene treated samples and the ethylene-free air treated samples are observed. The insertional disruption mutant AzoEtr1::GmR causes a shift in the glycoprotein-labeled band to a higher molecular weight than either the ethylene-free air samples or the 0.1ppm ethylene treated samples. Therefore, the knockout receptor mutant did not phenocopy the 0.1 ppm ethylene treated samples.

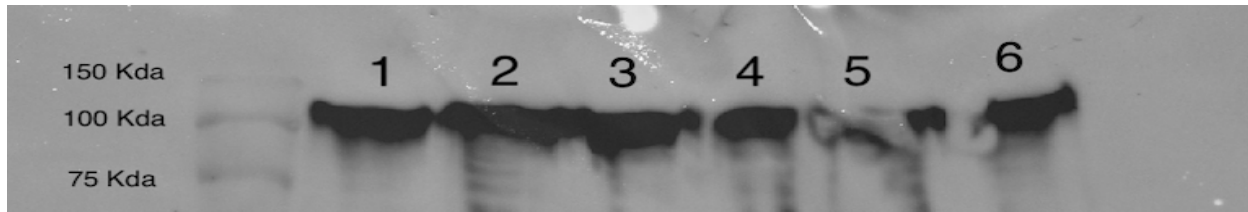


Figure 2: Ethylene causes no change in the amount of polar flagella

Lanes 1 – 3 are liquid MMAB WT samples treated with ethylene-free air. Lanes 4 – 6 are liquid MMAB WT samples treated with 0.1 ppm ethylene for 16 hours. The polar flagella form a band at ~100 kDa.

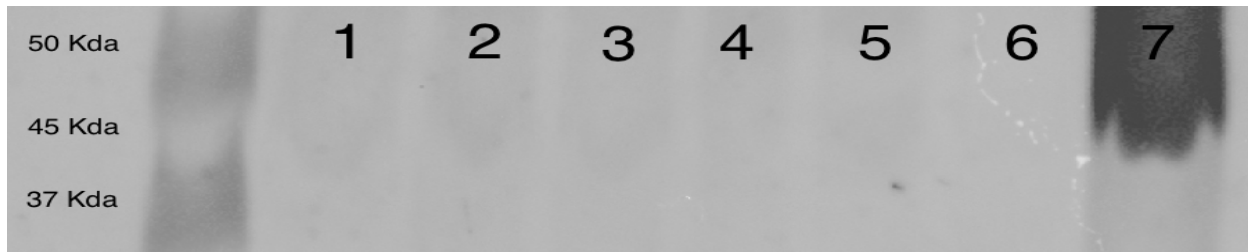


Figure 3: Liquid grown cultures do not form lateral flagella.

Lanes 1 – 3 are liquid MMAB WT samples treated with ethylene-free air. Lanes 4 – 6 are liquid MMAB WT samples treated with 0.1 ppm ethylene for 16 hours. Lane 7 is a swarm control from semi-solid TY swarm plates in ethylene-free air. The lateral flagella form a band at ~45 kDa. No lateral flagella are detected in Lanes 1 – 6. Lateral flagella are detected in Lane 7.

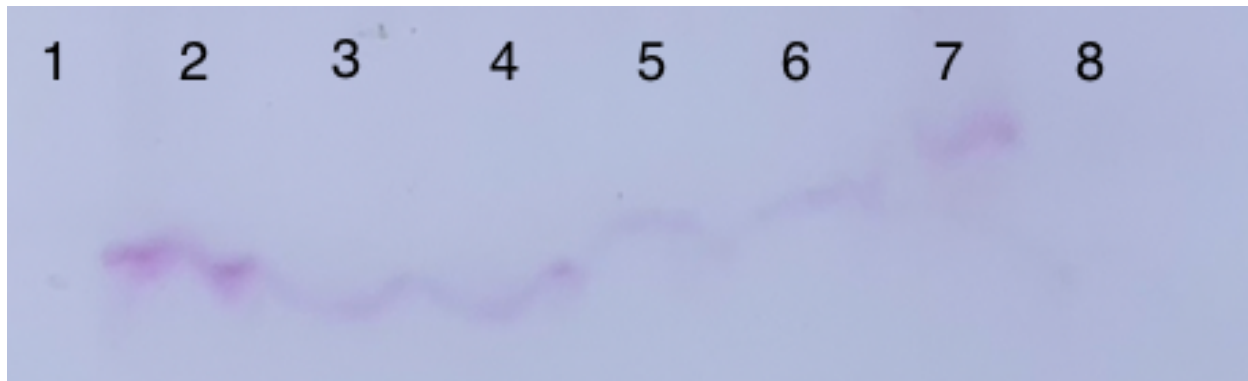


Figure 4: Glycostain reveals that ethylene does not cause a shift in staining pattern of the polar flagella.

Lanes 1 – 2 are liquid MMAB WT samples treated with ethylene-free air. Lanes 3 – 4 are liquid WT MMAB samples treated with 0.1 ppm ethylene for 16 hours. Lane 5 -6 are liquid cultures of AzoEtr1::GmR treated with ethylene-free air. Lane 7 is the positive control (horseradish peroxidase). Lane 8 is the negative control (soybean trypsin inhibitor).

Biofilm Assays and Visualization

Figure 5 shows the amount of biofilm formed at three and five days under different conditions. The OD₆₀₀ was collected to measure the amount of biofilm produced in each well. In the three and five day 0.1 ppm ethylene treated samples, there is a reduced biofilm formation compared to the ethylene-free air treated samples. The five day ethylene-free air samples had significantly more biofilm formation than the three day ethylene-free air treated plates. The samples treated for three days with ethylene-free air followed by two days with ethylene are not significantly different from the three day ethylene-free air treated samples, but they do show a higher amount of biofilm formation than samples treated with either three or five days with 0.1 ppm ethylene. Figure 6 further complements the data shown in Figure 5 by providing a visual representation of the reduced biofilm formation induced by a 0.1 ppm ethylene treatment. Panel A is ethylene-free treated samples and the image depicts a large biofilm. It is difficult to make out individual cells, but the image shows groups of cells held together with EPS and cell surface connections. Panel B is the 0.1 ppm ethylene treated samples. In Panel B, individual cells are visualized, and there is no biofilm formation occurring. Panel C is the AzoEtr1::GmR mutant. Like the ethylene treated samples, individual cells are visualized, and no biofilm formation is occurring.

Because unpublished results suggested that ethylene might be altering core metabolisms such as the citric acid cycle, we wished to know if metabolic intermediates altered the effects of ethylene on biofilm formation. Figure 7 shows the effects of 0.4% citrate on biofilm formation in the presence or absence of 0.1 ppm ethylene. The OD₆₀₀ was measured to determine the amount of biofilm produced in each well. In the absence of citrate, ethylene reduced biofilm formation as documented above. This reduction was blocked in the presence of citrate. A similar pattern was seen when 0.4% pyruvate was added (Figure 8). However, unlike citrate, pyruvate did not restore the control levels of biofilm.

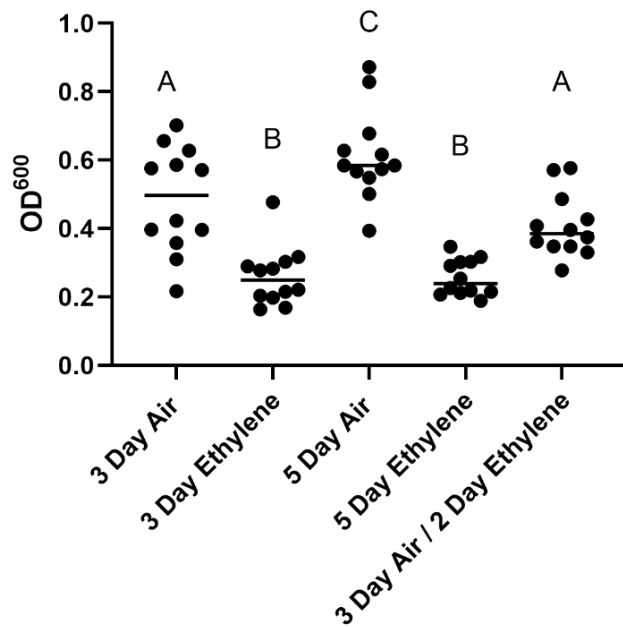


Figure 5: Biofilm formation in *A. brasilense* with air to ethylene transition

Wild-type SP7 *A. brasilense* cells were grown in MMAB-Low N for either three days or five days. The ethylene treated samples were treated with 0.1 ppm ethylene, and the air-treated samples were treated in ethylene-free air. The experimental plate was grown in ethylene-free air for three days and transitioned into 0.1 ppm ethylene for two days. The transition plate shows a decrease in biofilm formation compared to the four day ethylene-free air treatment.

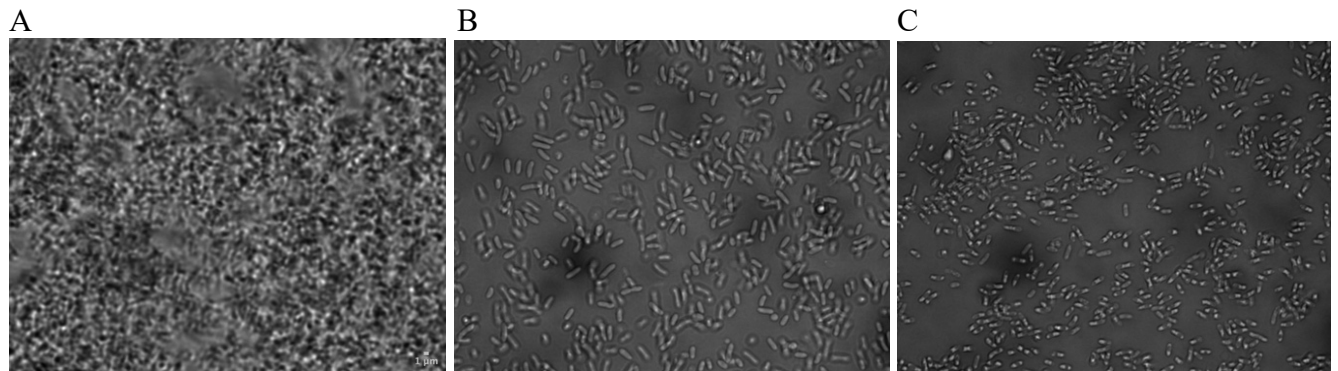


Figure 6: Images of biofilm in WT SP7 and 1-2B *A. brasilense*.

A. brasilense samples were grown overnight in MMAB-Low N on poly-L-lysine coated slides. Panel A depicts SP7 cells' biofilm formation after 24 hours of ethylene-free air treatment. Panel B depicts SP7 cells' biofilm formation after 24 hours of 0.1 ppm ethylene treatment. Panel C depicts 1-2B biofilm formation after 24 hours of ethylene-free air treatments. Ethylene treated samples and 1-2B samples depict less biofilm formation compared to the ethylene-free air treated samples.

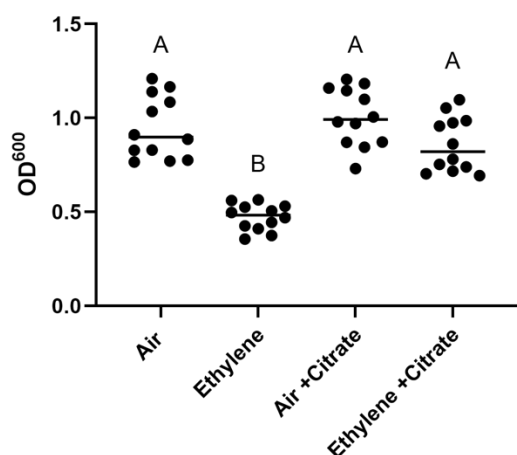


Figure 7: Biofilm formation in *A. brasilense* with 0.4% citrate

Wild-type SP7 *A. brasilense* cells were grown in MMAB-Low N or MMAB-Low N + 0.4% citrate for five days. The ethylene treated samples were treated with 0.1 ppm ethylene, and the air-treated samples were treated with ethylene-free air. The 0.1 ppm ethylene treated samples showed reduced biofilm formation compared to the ethylene-free +/- citrate samples and the ethylene treated samples with citrate added. There is no significant difference between the ethylene-free air +/- citrate samples and the 0.1 ppm ethylene + citrate treated samples.

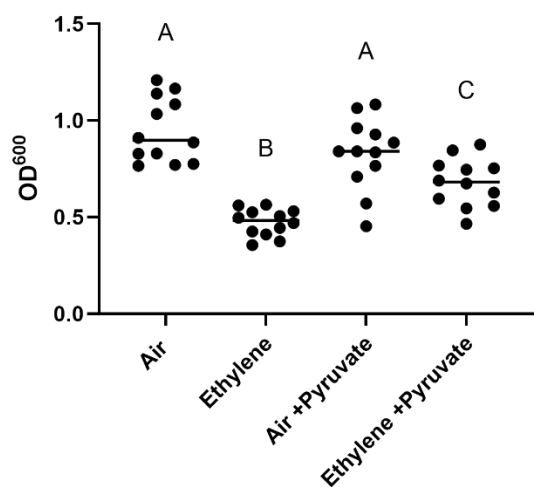


Figure 8: Biofilm formation in *A. brasilense* with 0.4% pyruvate

Wild-type SP7 *A. brasilense* cells were grown in MMAB-Low N or MMAB-Low N + 0.4% citrate for five days. The ethylene treated samples were treated with 0.1 ppm ethylene, and the air-treated samples were treated with ethylene-free air. The 0.1 ppm ethylene treated samples showed reduced biofilm formation compared to the ethylene-free +/- pyruvate samples and the ethylene treated samples with pyruvate added. The ethylene-free air treated samples with pyruvate added showed increase biofilm formation compared to the 0.1 ppm ethylene treated samples with pyruvate added. The 0.1 ppm ethylene treated samples with pyruvate added showed increased biofilm formation compared to the ethylene treated samples with no pyruvate.

PHB Granule Synthesis

We used light microscopy to examine the effects of ethylene on the number of PHB granules in cells. Figure 9 shows the percentage of total cells with zero to five PHB granules. In the 0.1 ppm ethylene treated samples, there is a higher accumulation of PHB with a higher percentage of total cells having between three and five PHB granules compared to the ethylene-free air treated samples. The ethylene-free air treated samples have a lower number of PHB granules per cell with many having zero or only one PHB granule. Figure 10 features a sample image from each treatment. PHB granules can be visualized as puncta using DIC imaging. To confirm the puncta are PHB granules, the cells are stained with Nile Red. Nile Red is a lipophilic dye that binds and fluoresces when bound to PHB granules. The first column of panels (A and D) are differential contrast images taken at 100x. The second column (B and E) are the fluorescent images. The third column (C and F) are the fluorescent images overlayed on the DIC images. In the presence of PHB, there will be puncta, and the signal will be diffuse in the absence of granules. Examining the images, the glowing puncta are PHB granules. In the ethylene-free air treated samples, fewer puncta are visible in the individual cells. In the 0.1 ppm ethylene treated samples, more puncta are visible in individual cells.

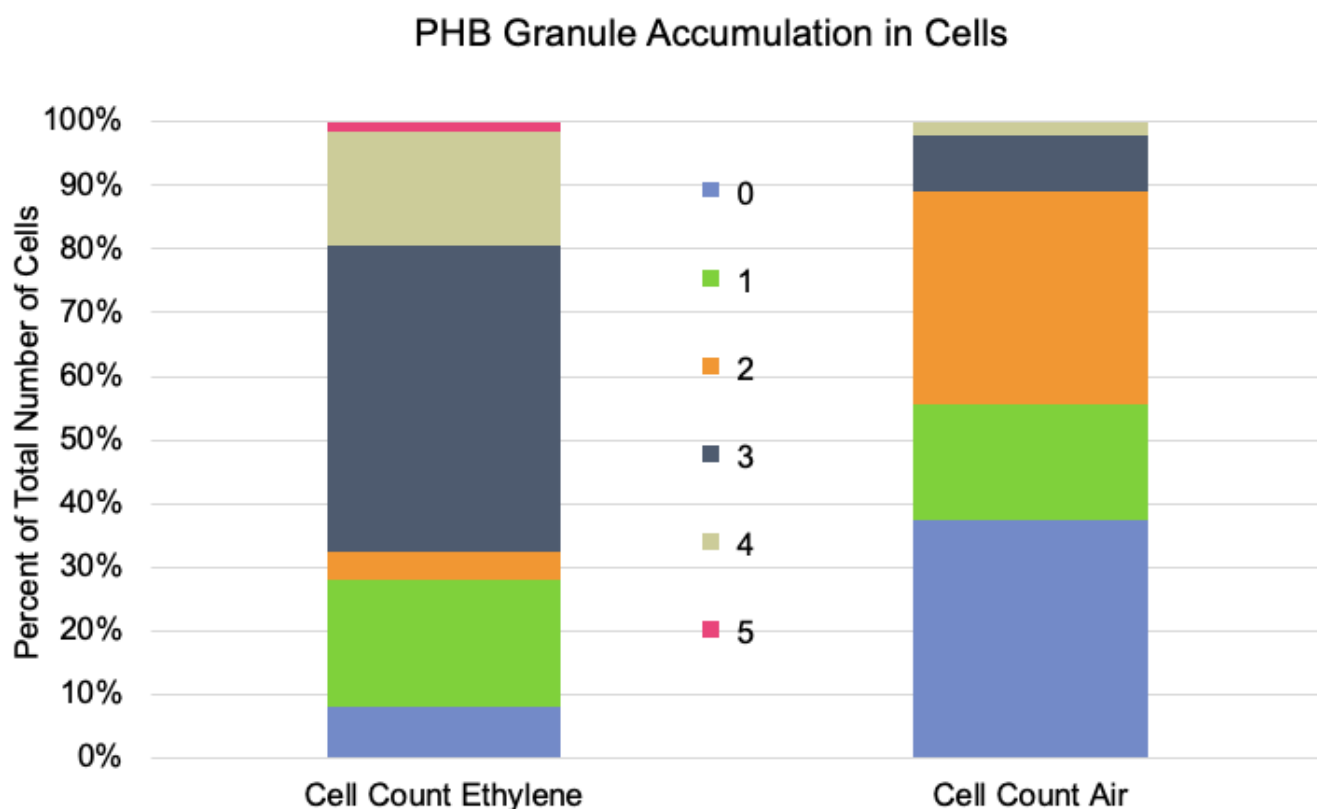


Figure 9: PHB granule accumulation in cells.

Wild-type SP7 *A. brasilense* cells were grown overnight in MMAB media under an ethylene-free air treatment or a 0.1 ppm ethylene treatment. Images of WT SP7 *A. brasilense* cells treated with ethylene-free air and 0.1 ppm ethylene were taken at 100x. The percentage of PHB granules in each cell was calculated from the total number of cells. In the 0.1 ppm ethylene treated samples, there were 20 cells with zero PHB granules, 50 cells with one PHB granule, 111 cells with two PHB granules, 121 cells with three PHB granules, 45 cells with four PHB granules, and 4 cells with five PHB granules. In the ethylene-free air treated samples, there were 144 cells with zero PHB granules, 71 cells with one PHB granule, 129 with two PHB granules, 34 with three granules, 8 cells with four PHB granules, and 0 with five PHB granules. The median PHB granule amount for ethylene and air is 2 and 1, respectively. A Mann-Whitney test of the median values determined a p-value of <0.001.

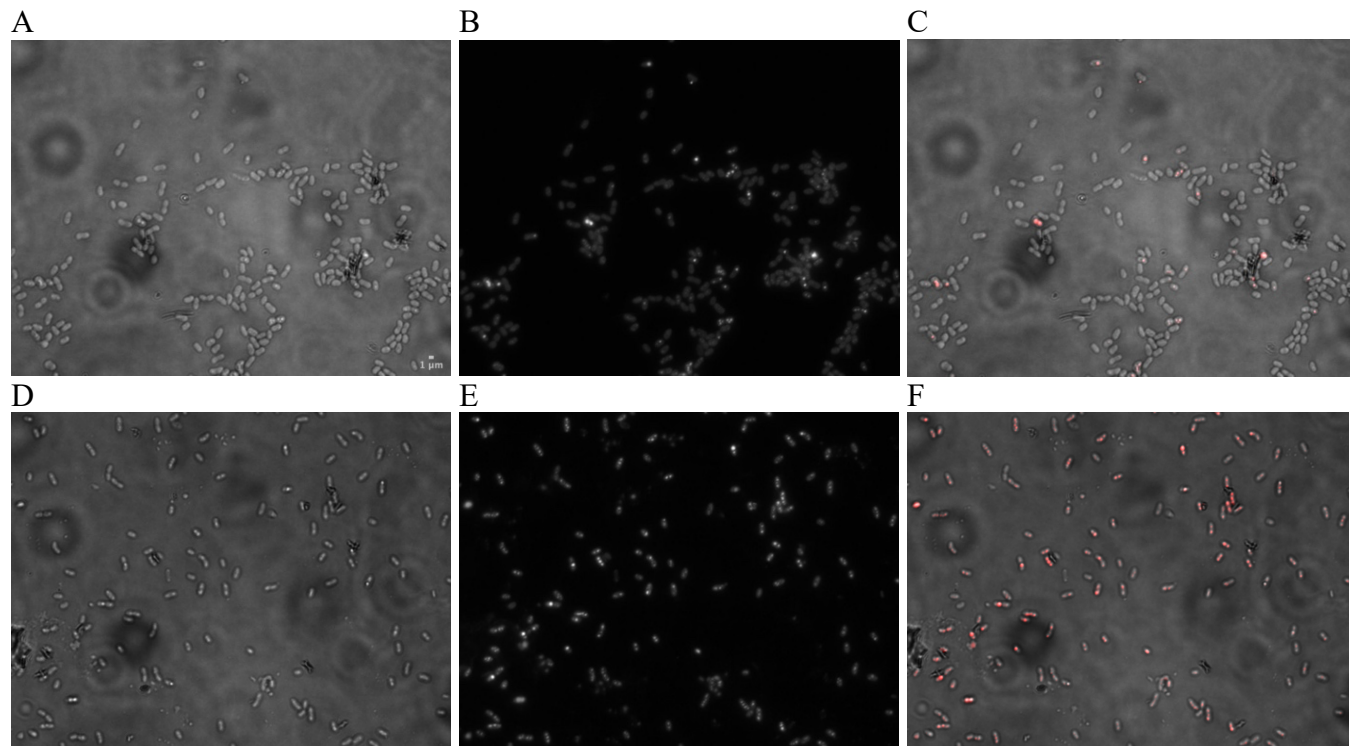


Figure 10: Images of PHB granules in WT SP7 *A. brasilense* cells.

WT SP7 *A. brasilense* cells were grown overnight in MMAB media under an ethylene-free air treatment or a 0.1 ppm ethylene treatment. Images of WT SP7 *A. brasilense* cells treated with ethylene-free air and 0.1 ppm ethylene were taken at 100x. . Panels A – C are ethylene-free air treated samples. Panels D – F are 0.1 ethylene treated samples. Panels A and D are DIC images. Panels B and E are fluorescent images. Panels C and F are the fluorescent images overlaid on the DIC image.

DISCUSSION

The purpose of these experiments was to help solve the overarching question of the role of ethylene in *A. brasilense* root attachment. In particular, the experiments explored how ethylene affected the flagella, biofilm formation, and carbon storage. Preliminary data from the Binder lab showed that ethylene reduced biofilm formation in *A. brasilense*. Figure 5 shows that when SP7 cells are treated with 0.1 ppm ethylene for three and five days, there is reduced biofilm formation compared to the ethylene-free air treated samples, supporting the preliminary data. Two possibilities for the reduced biofilm formation when treated with 0.1 ppm ethylene are that ethylene is triggering a dispersal in biofilm or halting the formation of new biofilm. Analysis of samples first kept in ethylene-free air followed by transition to ethylene adds clarity to the role of ethylene in biofilm formation and regulation. There is no statistical significance between the three day ethylene-free air treatment and the transition plate, indicating that ethylene is halting the formation of new biofilm. This data suggest that ethylene may be playing a role in the formation of the biofilm but is not likely reversing the formation of biofilm. The microscopy from Figure 6 provides qualitative data on the physical appearance of a biofilm in ethylene-free air treated samples and 0.1 ppm ethylene treated samples. The ethylene-free air treated samples formed a biofilm which is characterized by extracellular polymeric substances and cell-surface connections. The 0.1 ppm ethylene treated samples do not show a formation of biofilm. Instead, individual rod-like cells are observed. AzoEtr1::GmR samples also do not form a biofilm, similar to the 0.1 ppm ethylene treated samples. The occurrence of the same phenotype indicates that ethylene is responsible for the lack of biofilm formation, which further confirms the preliminary biofilm data.

The unpublished RNA-seq and metabolomics data showed that when cells are treated with 0.1 ppm ethylene, there is an increase in TCA metabolites, and transcripts. With this in mind, I wanted to explore if the addition of citrate or pyruvate would alter biofilm formation. The addition of citrate had no measurable effect on biofilm formation in the absence of ethylene. However, the addition of pyruvate caused a small increase. Both citrate and pyruvate blocked the effects of ethylene on biofilm formation suggesting that these TCA metabolites may be playing a role in biofilm formation response to ethylene.

As mentioned previously, the polar flagellum mediates the initial attachment in biofilm formation (Croes et al., 1993). Differences in the polar flagella could explain the different phenotypes observed in biofilm formation. However, the western blot of the polar flagellum, Figure 2, showed no change in molecular weight between the ethylene-free air treated samples and the 0.1 ppm ethylene treated samples. The lateral flagella western blot, Figure 3, shows no bands in lanes 1 – 6. The samples in these lanes were grown in liquid MMAB, so the lack of lateral flagella formation is consistent with the literature (Partridge & Harshey, 2013; Walker, 2002). In lane 7, there is a dark, overloaded band. The cells in this lane were grown on semi-solid swarm plates. Semi-solid media plates are the nutrient condition that induces lateral flagella formation. The band is overloaded due to the mass amounts of lateral flagella that are produced in semi-solid media to help overcome the force of friction present in the media. Examining both western blots together, no molecular weight difference in the polar flagellum and the lateral flagella when treated with ethylene-free air and 0.1 ppm ethylene are observed. Although the actual molecular weight is not changing, there could be changes in the amount of glycoprotein present on the polar flagellum, which is explored in a glycoprotein staining assay. The glycoprotein stain of the polar flagellum, shown in Figure 4, did not show a difference in the amount of glycoprotein on the polar flagellum between the ethylene-free air treated samples and

the 0.1 ppm ethylene treated samples. However, the AzoEtr1::GmR mutant has a higher molecular weight of glycoprotein compared to the SP7 samples. The AzoEtr1::GmR mutant does not copy the ethylene SP7 phenotype which indicates that ethylene is not responsible for the observed difference. Although no difference in molecular weight is observed between ethylene-free air and 0.1 ppm ethylene treated samples, there could be differences in the type of glycoproteins present on the flagella or the glycosylation patterns. Further experimentation needs to be performed to determine if differences in glycosylation patterns could contribute to the differences observed in biofilm formation in ethylene-free and 0.1 ppm ethylene treatments.

The unpublished metabolomic data showed that in the presence of 0.1 ppm ethylene, there is increase in carbon-containing metabolites. In the PHB granules experiment cells treated with 0.1 ppm ethylene had a higher number of PHB granules in the cell compared to ethylene-free treated samples, which is consistent with the metabolomics data. PHB granules can be used as an energy source during starvation or act as a stress response. At this time, no conclusion on the function of PHB granules accumulation in response to ethylene can be made. As mentioned previously, nitrogen fixation and PHB granule synthesis both rely on the reducing power of NADPH (Benemann et al., 1971; Uchino et al., 2007). Because both processes rely on NADPH, it is hypothesized that the cell will either build a biofilm or synthesize PHB granules. In 0.1 ppm ethylene treated cells, we observe a reduced formation of biofilm, but an increase in PHB production in cells. In ethylene-free air treated samples, we observed an increase in biofilm formation and a reduction of PHB granules in individual cells. Both of these experiments were conducted under different nutrient conditions, so further experimentation on the number of PHB granules in each cell could be conducted in MMAB Low N. However, both mediums had a high carbon to nitrogen ratio. The biofilm assays were conducted in a low nitrogen media with a high carbon to nitrogen ratio. Low nitrogen media conditions promote biofilm formation (Madi & Henis, 1989). The PHB granule experiment was conducted in a nutrient media with more nitrogen than the biofilm media, but the media still had a high carbon to nitrogen ratio. Current literature states that high carbon to nitrogen ratios in nutrient media can induce biofilm formation and PHB granule accumulation. In high carbon to low nitrogen ratio environments, *A. brasilense* performs nitrogen fixation. Increased nitrogenase activity is observed in bacteria cells rich in PHB accumulation and biofilms (Danhorn & Fuqua, 2007; (Tal & Okon, 1985; Wang et al., 2017). Both PHB synthesis and biofilm formation create environments with increased nitrogenase activity, which leads to increased nitrogen fixation. One of the main methods *A. brasilense* beneficially improves plant growth is through nitrogen fixation. As a result, this data suggests that ethylene secretion from the plant may play a role in biofilm formation and PHB granule synthesis.

In conclusion, ethylene reduces biofilm formation and halts the formation of new biofilm. However, the addition of either pyruvate or citrate blocks this effect of ethylene. Ethylene does not alter the molecular weight of the polar and lateral flagella or the glycoprotein amount of the polar flagellum. Ethylene treatments induce a higher number of PHB granules in each cell. The observed phenotypic changes due to ethylene indicate that ethylene may have a role in regulating the surface attachment of *A. brasilense* to the plant root.

FUTURE DIRECTIONS

Future experiments could explore how the inhibition of biofilm formation due to ethylene binding affects root colonization. Additionally, further experimentation could explore other polar flagellum modifications to help explain the biofilm phenotypic differences observed when treated

with ethylene-free air and 0.1 ppm ethylene. More experimentation is needed to determine the effect of a higher amount of PHB granule in the cells when treated with 0.1 ppm ethylene. Further experimentation could also explore the effect of an ethylene pre-treatment on biofilm formation in SP7 *A. brasilense* cells.

Acknowledgements

This research was supported by an NSF grant (MCB-1517032).

REFERENCES

- Alexandre, G., Rohr, R., & Bally, R. (1999). A Phase Variant of *Azospirillum lipoferum* Lacks a Polar Flagellum and Constitutively Expresses Mechanosensing Lateral Flagella. *Applied and Environmental Microbiology*, 65(10), 4701-4704. <https://doi.org/doi:10.1128/AEM.65.10.4701-4704.1999>
- Arruebarrena Di Palma, A., Pereyra, C. M., Moreno Ramirez, L., Xiqui Vázquez, M. L., Baca, B. E., Pereyra, M. A., Lamattina, L., & Creus, C. M. (2013). Denitrification-derived nitric oxide modulates biofilm formation in *Azospirillum brasilense*. *FEMS Microbiol Lett*, 338(1), 77-85. <https://doi.org/10.1111/1574-6968.12030>
- Arshad, M., & Frankenberger, W. T. (2002). The Plant Hormone, Ethylene. In M. Arshad & W. T. Frankenberger (Eds.), *Ethylene: Agricultural Sources and Applications* (pp. 1-9). Springer US. https://doi.org/10.1007/978-1-4615-0675-1_1
- Barahona, E., Navazo, A., Yousef-Coronado, F., Aguirre de Cárcer, D., Martínez-Granero, F., Espinosa-Urgel, M., Martín, M., & Rivilla, R. (2010). Efficient rhizosphere colonization by *Pseudomonas fluorescens* fl13 mutants unable to form biofilms on abiotic surfaces [<https://doi.org/10.1111/j.1462-2920.2010.02291.x>]. *Environmental Microbiology*, 12(12), 3185-3195. <https://doi.org/https://doi.org/10.1111/j.1462-2920.2010.02291.x>
- Bashan, Y., & Holguin, G. (1993). Anchoring of *Azospirillum brasilense* to hydrophobic polystyrene and wheat roots. *Microbiology*, 139(2), 379-385. <https://doi.org/https://doi.org/10.1099/00221287-139-2-379>
- Belyakov, A. Y., Burygin, G. L., Arbatsky, N. P., Shashkov, A. S., Selivanov, N. Y., Matora, L. Y., Knirel, Y. A., & Shchyogolev, S. Y. (2012). Identification of an O-linked repetitive glycan chain of the polar flagellum flagellin of *Azospirillum brasilense* Sp7. *Carbohydrate Research*, 361, 127-132. <https://doi.org/https://doi.org/10.1016/j.carres.2012.08.019>
- Benemann, J. R., Yoch, D. C., Valentine, R. C., & Arnon, D. I. (1971). The electron transport system in nitrogen fixation by *Azotobacter*. III. Requirements for NADPH-supported nitrogenase activity. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 226(2), 205-212. [https://doi.org/https://doi.org/10.1016/0005-2728\(71\)90087-9](https://doi.org/https://doi.org/10.1016/0005-2728(71)90087-9)
- Binder, B. M. (2020). Ethylene signaling in plants. *J Biol Chem*, 295(22), 7710-7725. <https://doi.org/10.1074/jbc.REV120.010854>
- Bogino, P. C., Oliva, M. d. I. M., Sorroche, F. G., & Giordano, W. (2013). The role of bacterial biofilms and surface components in plant-bacterial associations. *International journal of molecular sciences*, 14(8), 15838-15859. <https://doi.org/10.3390/ijms140815838>
- Burdman, S., Okon, Y., & Jurkevitch, E. (2000). Surface Characteristics of *Azospirillum brasilense* in Relation to Cell Aggregation and Attachment to Plant Roots. *Critical Reviews in Microbiology*, 26(2), 91-110. <https://doi.org/10.1080/10408410091154200>

- Croes, C. L., Moens, S., van Bastelaere, E., Vanderleyden, J., & Michiels, K. W. (1993). The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. *Microbiology*, 139(9), 2261-2269. <https://doi.org/https://doi.org/10.1099/00221287-139-9-2261>
- Crouzet, M., Le Senechal, C., Brözel, V. S., Costaglioli, P., Barthe, C., Bonneau, M., Garbay, B., & Vilain, S. (2014). Exploring early steps in biofilm formation: set-up of an experimental system for molecular studies. *BMC microbiology*, 14, 253-253. <https://doi.org/10.1186/s12866-014-0253-z>
- Danhorn, T., & Fuqua, C. (2007). Biofilm Formation by Plant-Associated Bacteria. *Annual Review of Microbiology*, 61(1), 401-422. <https://doi.org/10.1146/annurev.micro.61.080706.093316>
- Dawes, E. A., & Senior, P. J. (1973). The role and regulation of energy reserve polymers in micro-organisms. *Adv Microb Physiol*, 10, 135-266. [https://doi.org/10.1016/s0065-2911\(08\)60088-0](https://doi.org/10.1016/s0065-2911(08)60088-0)
- Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, 8(9), 623-633. <https://doi.org/10.1038/nrmicro2415>
- Ganusova, E. E., Vo, L. T., Mukherjee, T., & Alexandre, G. (2021). Multiple CheY Proteins Control Surface-Associated Lifestyles of *Azospirillum brasilense* [Original Research]. *Frontiers in Microbiology*, 12. <https://doi.org/10.3389/fmicb.2021.664826>
- Gullett, J., O'Neal, L., Mukherjee, T., & Alexandre, G. (2017). *Azospirillum brasilense*: Laboratory Maintenance and Genetic Manipulation. *Curr Protoc Microbiol*, 47, 3e.2.1-3e.2.17. <https://doi.org/10.1002/cpmc.39>
- Hengge, R. (2009). Principles of c-di-GMP signaling in bacteria. *Nat Rev Microbiol*, 7(4), 263-273. <https://doi.org/10.1038/nrmicro2109>
- Hickman, J. W., Tifrea, D. F., & Harwood, C. S. (2005). A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proceedings of the National Academy of Sciences of the United States of America*, 102(40), 14422-14427. <https://doi.org/10.1073/pnas.0507170102>
- Jenal, U., Reinders, A., & Lori, C. (2017). Cyclic di-GMP: second messenger extraordinaire. *Nature Reviews Microbiology*, 15(5), 271-284. <https://doi.org/10.1038/nrmicro.2016.190>
- Joys, T. M. (1988). The flagellar filament protein. *Canadian Journal of Microbiology*, 34(4), 452-458.
- Madi, L. E. A., & Henis, Y. (1989). Aggregation in *Azospirillum brasilense* Cd: Conditions and factors involved in cell-to-cell adhesion. *Plant and Soil*, 115(1), 89-98. <http://www.jstor.org/stable/42937829>

- Michiels, K. W., Croes, C. L., & Vanderleyden, J. (1991). Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. *Microbiology*, 137(9), 2241-2246. <https://doi.org/https://doi.org/10.1099/00221287-137-9-2241>
- Moens, S., Michiels, K., & Vanderleyden, J. (1995). Glycosylation of the flagellin of the polar flagellum of *Azospirillum brasilense*, a Gram-negative nitrogen-fixing bacterium. *Microbiology*, 141(10), 2651-2657. <https://doi.org/https://doi.org/10.1099/13500872-141-10-2651>
- Moens, S., Schlöter, M., & Vanderleyden, J. (1996). Expression of the structural gene, *lafI*, encoding the flagellin of the lateral flagella in *Azospirillum brasilense* Sp7. *Journal of Bacteriology*, 178(16), 5017-5019. <https://doi.org/doi:10.1128/jb.178.16.5017-5019.1996>
- Nakamura, S., & Minamino, T. (2019). Flagella-Driven Motility of Bacteria. *Biomolecules*, 9(7), 279. <https://doi.org/10.3390/biom9070279>
- Partridge, J. D., & Harshey, R. M. (2013). Swarming: Flexible Roaming Plans. *Journal of Bacteriology*, 195(5), 909-918. <https://doi.org/doi:10.1128/JB.02063-12>
- Prüß, B. M., Besemann, C., Denton, A., & Wolfe, A. J. (2006). A Complex Transcription Network Controls the Early Stages of Biofilm Development by *Escherichia coli*. *Journal of Bacteriology*, 188(11), 3731-3739. <https://doi.org/doi:10.1128/JB.01780-05>
- Tal, S., & Okon, Y. (1985). Production of the reserve material poly- β -hydroxybutyrate and its function in *Azospirillum brasilense* Cd. *Canadian Journal of Microbiology*, 31(7), 608-613. <https://doi.org/10.1139/m85-115>
- Tischler, A. D., & Camilli, A. (2004). Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Molecular microbiology*, 53(3), 857-869. <https://doi.org/10.1111/j.1365-2958.2004.04155.x>
- Turner, L., Zhang, R., Darnton Nicholas, C., & Berg Howard, C. (2010). Visualization of Flagella during Bacterial Swarming. *Journal of Bacteriology*, 192(13), 3259-3267. <https://doi.org/10.1128/JB.00083-10>
- Uchino, K., Saito, T., Gebauer, B., & Jendrossek, D. (2007). Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. *Journal of Bacteriology*, 189(22), 8250-8256. <https://doi.org/10.1128/JB.00752-07>
- Walker, J. S. (2002). *Physics: An Introduction, Volume II*. Prentice Hall.

- Wang, D., Xu, A., Elmerich, C., & Ma, L. Z. (2017). Biofilm formation enables free-living nitrogen-fixing rhizobacteria to fix nitrogen under aerobic conditions. *The ISME Journal*, 11(7), 1602-1613. <https://doi.org/10.1038/ismej.2017.30>
- Zimmer, W., Roeben, K., & Bothe, H. (1988). An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum*. *Planta*, 176(3), 333-342. <https://doi.org/10.1007/BF00395413>
- Zimmer, W., Stephan, M. P., & Bothe, H. (1984). Denitrification by *Azospirillum brasilense* Sp 7. *Archives of Microbiology*, 138(3), 206-211. <https://doi.org/10.1007/BF00402121>