# CIRCADIAN ORCHESTRATION OF SYMBIOTIC GENES IN PHOTOSYNTHETIC NITROGEN-FIXING BACTERIA

by

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ABSTRACT

CIRCADIAN ORCHESTRATION OF SYMBIOTIC GENES IN

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Rhizobia have long been known for their symbiotic association with the roots of legumes where they perform nitrogen fixation in specialized organs called nodules. The photosynthetic strains of Bradyrhizobia are of particular interest because they possess both photosynthetic and nitrogen-fixing capabilities. Sequencing and analysis of the genomes of *Bradyrhizobium* sp. ORS278, *Bradyrhizobium* sp. BTAi1, and a novel strain, *Bradyrhizobium oligotrophicum* BO-1 have revealed that they do not contain the canonical *nod* genes which encode nodulation factors (NF) that initiate symbiosis. The NF-independent symbiotic pathway has been proposed for the relationship between the photosynthetic Bradyrhizobia and semi-aquatic *Aeschynomene* legumes. This symbiotic association could involve exchange of novel signaling molecules and mechanisms by which bacteria enter into the plant. To elucidate the relationship between photosynthesis and nitrogen fixation involved in the NF-independent pathway of the ORS278 strain, two genes involved in circadian regulation, BRADO4470 (*labA*) and BRADO3946 (*chk*), were mutated. Mutation of each gene hinders symbiotic efficiency and the nitrogen fixation process. Thus, we hypothesized that circadian regulation may optimize symbiotic processes since one aspect of the

mutualism, the nitrogenase enzyme responsible for nitrogen fixation, is inhibited by oxygen produced during daytime photosynthesis. Indeed, acetylene reduction assay not only showed that the two mutants produce a nitrogen fixation-deficient phenotype, but that the wild type only performed nitrogen fixation actively during the night period. Genome-wide transcriptional profiling of ORS278 revealed that approximately 50% of genes are differentially expressed between night and day in the root nodule, primarily involving different metabolic processes including nucleoside monophosphate, cofactor, and porphyrin-containing compound metabolisms. To confirm the differential gene expression in the root nodule, a set of hosukeeping genes were identitifed for qRT-PCR experiments. Various microarray-based gene expression datasets from *Bradyrhizobium japonicum* USDA110 were used and homologs of the identified housekeeping genes (HKGs) were determined and tested in ORS278 and BTAi1. These genes appeared to be reliable in new treatments, including studies measuring gene expression at different times during the day. Since certain symbiotic properties are managed in a circadian fashion, it makes sense that the NF-independent pathway could be regulated temporally. Comparative genome analysis between ORS278, BTAi1, and the novel BO-1 strain, showed high similarity between genes involved in photosynthesis, nitrogen fixation, and symbiosis indicating that they may share the same type of regulatory mechanism, including the temporal management of their relationship with *Aeschynomene indica*.

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# DEDICATION

I would like to dedicate this work to my wife, Stephanie, who means everything to me. She is the salt of the earth. I would not be the person I am today without her. I would also like to dedicate this work to my parents, Steve and Barbara, who would do anything for their children and who raised me to believe in doing what you love. To my brother, Stevie, who has always been my best friend and original teacher. To my grandparents, Tom and Janey, who have inspired and taught me since I was a young boy. To my friends, who were always there for laughs and a much-needed beer. To the rest of my family, friends, and pets not mentioned, and those who have impacted my life from near and far, I want to thank you for all the ways you have helped shape my life.

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# CHAPTER 1

## INTRODUCTION

Leguminous plants and rhizobia have long been associated with each other as they form a symbiotic mutualism that allows the plant to thrive in nitrogen deficient soil, and in turn the bacteria receive some photosynthetic products from the plant. Rhizobia are soil-dwelling microorganisms and establish a symbiotic relationship with their host legume, resulting in development or root nodules where they perform biological nitrogen fixation (BNF). However, some *Bradyrhizobium* sp. strains are unique, possessing photosynthetic abilities and using alternative pathways to form their symbiotic relationship, which may have larger implications for the role of BNF in ecological biodiversity and agriculture. BNF is of growing importance in the society of today, especially in regards to the rapidly increasing population and its dependence on a sustainable food supply as well as the preservation of biodiversity.

For rhizobia-legume mutualism, the associations between bacteria and host are very specific, so there must be some form of communication between plant and microbe that allows successful internalization and colonization. Model examples of nitrogen-fixing symbiotic interactions such as those between *Bradyrhizobium japonicum* and the soybean, *Glycine max*, or *Sinorhizobium meliloti* and the alfalfa plant, *Medicago sativa*, have been characterized extensively (Bhuvaneswari et al. 1980; Kosslak et al. 1987; Paffetti et al. 1998; Zuanazzi et al. 1998). For *B. japonicum* and the soybean plant, the initiation begins when the bacterium recognizes plant

released isoflavonoids and causes the expression of *nod* genes responsible for synthesizing lipochito-oligosaccharides (LCOs). These compounds, also known as nodulation factors (NFs), are slightly different among rhizobial species and specify host compatibility (Schultze and Kondorosi 1998). The NFs elicit root hair curling in which an infection thread is formed and elongated until it reaches the nodule primordium. The microbes multiply within the root nodule until they are eventually internalized into the plant where they differentiate into bacteroids (Schultze and Kondorosi 1998). Bacteroids, the nitrogen-fixing endosymbiotic form of rhizobia, have a major shift in gene expression as compared with free-living cultures (33%), of which most upregulated genes in bacteroids are related to efficient symbiosis and nitrogen fixation (Pessi et al. 2007). Elucidating the metabolic dynamics within a nodule is important for understanding nitrogen fixation. For instance, of particular interest is the mechanism by which the bacteroid mediates between respiration and nitrogen-fixation in an anoxic or microoxic environment. In addition, it is interesting to consider how the oxygen produced from plant photosynthesis in green stem nodules of *Aeschynomene* species is kept from inhibiting the nitrogenase enzyme.

Rhizobia have intimate links with specific hosts and many of these relationships between rhizobia and legume share common features. However, the photosynthetic bradyrhizobia and their hosts have some unique symbiotic properties. Not only do *Bradyrhizobium* sp. BTAi1 and *Bradyrhizobium* sp. ORS278, possess photosynthetic capabilities, but they also have a mode of symbiotic association with plants from the *Aeschynomene* genus. These two species of rhizobia do not follow the canonical symbiotic pathway because they lack the *nodABC* genes encoding enzymes to synthesize NFs. Nevertheless, they still successfully establish a mutualism and form nodules on their host (Giraud et al. 2007). Considering this, there must be a NF-independent

symbiotic pathway, and for strains such as ORS278 and BTAi1, the 'crack-entry' method of symbiosis has been proposed. In some tropical legumes, there exist gaps in the epidermal layer of the plant at the site of emergence of lateral roots where bacteria can enter and become internalized (Boogerd and van Rossum 1997; Sprent 1989). It is worth noting that all *Aeschynomene* species studied exhibited nodule formation at the emergence site of the lateral roots, showing that all photosynthetic *Bradyrhizobium* strains enter the plant via crack-entry. However, the exact invasion process concerning infection thread formation, the role of NFs, further colonization, and bacteroid differentiation is not completely understood for all *Aeschynomene* species (Bonaldi et al. 2011).

The differences in symbiotic pathways, while elusive, could rely on host and symbiont specificity. Photosynthetic bradyrhizobia strains do not exclusively utilize the NF-independent pathway, as there are strains that share similar properties (i.e., stem-nodulation) but do harbor the *nod* genes in their genome. These special symbionts are usually associated with specific hosts; however, certain *Aeschynomene* species are not exclusively nodulated by the photosynthetic strains of bradyrhizobia but can rather have a variety of symbionts (Miche et al. 2010). The *Aeschynomene* species can be organized into three different cross-inoculation (CI) groups based upon their associated symbionts. CI-group I includes those *Aeschynomene* species that are only root nodulated by non-photosynthetic bradyrhizobia as well as those with weak specificity. The CI-group II plants can be nodulated by either photosynthetic or non-photosynthetic strains, but the photosynthetic strains can form nodules on the stems and nodulate CI-group III plants whereas the other strains cannot. Finally, CI-group III plants are exclusively nodulated by photosynthetic strains lacking the *nod* genes (Alazard 1985; Molouba et al. 1999; Chaintreuil et al. 2001; Giraud and Fleischman 2004; Miche et al. 2010). By comparing and analyzing the strains of host and

symbiont from various categories, it may be possible to dissect the differences that account for the alternate methods of symbiotic engagement. Moreover, we could elucidate the roles and regulation of each process by studying specific genes across genomes in these specialized strains that are associated with both photosynthesis and nitrogen-fixation.

*Aims of this study*

This study aims to link photosynthesis with biological nitrogen fixation (BNF) to larger applications in evolutionary ecology (e.g., symbiotic pathways) and sustainable agricultural techniques. By designing an experiment to test the temporal regulation of nitrogen fixation in the photosynthetic strain, *Bradyrhizobium* sp. ORS278, we can better understand this process in NF-independent strains and uncover novel information concerning their symbiotic pathway. Using gene deletion mutagenesis to create mutant strains deficient in nitrogen fixation, we can provide direct evidence of their involvement in the symbiotic regulation. Based upon this, we can uncover more genes and pathways that are under circadian control using gene expression studies and transcriptomic analysis. When determining differential gene expression, normalization is paramount to provide accurate information, therefore constitutively expressed reference genes will be used as internal controls. Lastly, by isolating a similar novel photosynthetic nitrogen-fixing symbiont, we can perform a comparative genome analysis with related strains to see if there may be some conserved or shared characteristics that are required for successful symbiosis under cricardian regulation.

*Photosynthesis and nitrogen fixation*

In chapter 2, I investigate the symbiotic roles of photosynthesis and nitrogen fixation between the microsymbiont *Bradyrhizobium* sp. ORS278 and its host *Aeschynomene indica*. Photosynthesis and nitrogen fixation are a means to provide a source of fixed carbon or reduced nitrogen for biomass production; however, it is difficult for these processes to occur concurrently in the same place. The nitrogenase enzyme, responsible for fixing nitrogen, is inhibited by the presence of oxygen, which is a well-known by-product of photosynthesis (Gallon 1981). A few microorganisms perform both processes, but still it is not completely understood how the oxygen evolving organisms can perform nitrogen fixation. Cyanobacteria are usually candidate models for studying the relationship between photosynthesis and nitrogen fixation as they commonly perform both activities, although nitrogen fixation occurs in a special cell type, called the heterocyst. Spatial and temporal segregation are most commonly utilized but anomalies between photosynthesis and nitrogen fixation still produce enigmatic questions (Mitsui et al. 1986; Stal and Krumbein 1987; Berman-Frank et al. 2001). Do these organisms perform oxygen-evolving photosynthesis and nitrogen fixation at the same time? If the nitrogenase enzyme is not compartmentalized, how is it protected from oxygen? What other mechanisms are used to separate nitrogen fixation and photosynthesis besides temporal and spatial separation? By studying the role of each process in the *Bradyrhizobium* sp. ORS278-*A. indica* symbiosis, a better understanding of this interaction can be achieved in a symbiotic context.

*Normalizing gene expression in novel species*

In chapter 3, I examine the importance of normalization techniques in quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis used to validate other

gene expression studies. Gene normalization procedures are becoming more and more important in high-throughput expression profiling for answering biologically relevant questions (Vandesompele et al. 2002). RNA-seq and qRT-PCR are quickly becoming daily and common tools for biologists seeking information about a particular gene or set of genes in an experimental setting. To confidently display such information, gene expression has to be normalized by referencing it against an internal control. These reference genes are considered reliable for determining baseline expression values due to their constitutive expression throughout any condition and are often referred to as housekeeping genes (HKGs). The most commonly used internal controls are those that are necessary at all times for cell growth and survival, including genes encodings β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT), and ribosomal RNA were considered reliable controls (Huggett et al. 2005). However, multiple studies have revealed some HKGs typically considered reliable can indeed be affected by certain experimental treatments and therefore produce inaccurate data (Dheda et al. 2004; Jain et al. 2006). This demonstrates the need for validation of these internal controls within the species of interest for the particular experimental treatment to generate dependable data (Guenin et al. 2009). In this study, we hope to identify and validate a set of HKGs whose homologs can be applied and validated to similar gene expression studies in closely related organisms to generate reliable data in a consistent fashion. Identifying and validating normalization procedures for future gene expression studies with ORS278 and *A. indica* will allow us to produce more accurate data.

*Genomic analysis of photosynthetic nitrogen-fixing bacteria*

In chapter 4, I explore a novel strain of photosynthetic *Bradyrhizobia* to characterize important features involved the the circadian regulation of symbiotic genes. The omics revolution in the twenty-first century has set the framework for rapid advances in the fundamental understanding of many biological concepts. Defining a species, determining the role of a gene product, or teasing apart complex regulatory networks have all become more accessible because of the advances in high-throughput technologies. Comparative genome analysis among recently sequenced microorganisms can provide insights into common features of metabolisms, help define protein functions, or reveal intra-species diversity as well as serve as a foundation to discover even more about the novel strain or species (Pellegrini et al. 1999; Tettelin et al. 2008). These platforms have changed the way that we approach problems and have provided new avenues for biological exploration. Large-scale comparative analyses allow scientists to uncover trends across a wide range of microbes that share a few common traits, such as surveying plant-associated bacterial genomes to identify associated gene clusters (Levy et al. 2018). However, observing the genomes of a few select strains can provide useful information also, such as the analysis of novel marine tropical cyanobacteria from the *Moorea* genus helped identify unique metabolites (Leao et al. 2017). Comparative genomic analysis allows novel organisms to quickly find their place in the tree of life, building a better picture of evolutionary development. Analyzing the genome of novel species and strains closely related to *Bradyrhizobium* sp. ORS278 will help to characterize features important to the circadian regulation of symbiotic genes between ORS278 and *A. indica*.

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CHAPTER 2

# TEMPORAL REGULATION OF SYMBIOTIC GENES IN THE PHOTOSYNTHETIC NITROGEN-FIXING BACTERIUM, *BRADYRHIZOBIUM* SP. ORS278


## ABSTRACT

The photosynthetic soil bacterium *Bradyrhizobium* sp. strain ORS278 forms a mutualism with *Aeschynomene* plants, resulting in root and stem nodulation. In this symbiosis, the bacterium provides the plant with N source via nitrogen fixation, and it receives photosynthetic products from the plant. While oxygen is produced through photosynthesis, the nitrogenase enzyme responsible for fixing nitrogen, is inhibited by oxygen. To perform two processes without conflict, there must be a regulatory mechanism by which the bacterium avoids the deleterious effect of oxygen on the nitrogenase activity. Thus, we hypothesize that if nitrogenase activity is restricted to the night, the deleterious effect of oxygen produced during the day could be avoided. In this study, two genes, BRADO4470 (*labA*) encoding a putative protein that may function in circadian feedback and BRADO3946 (*chk*) encoding a putative sensor histidine kinase, thought to be involved in the temporal regulation of nitrogen fixation and possibly feedback control of circadian rhythm in photosynthesis, were independently mutagenized via double-homologous recombination. Then, their phenotype was compared with the wild type for nitrogen fixing ability in *Aeschynomene indica* plants. Acetylene reduction assays showed that the wild type fixed nitrogen during the night,

while the mutant strains did not. This result indicates that BRADO4470 and BRADO3946 are likely involved in the temporal regulation of nitrogen fixation through circadian feedback systems. RNA-seq analysis of ORS278 within the root nodules showed that approximately 50% of the genes were differentially expressed and were mostly involved in different metabolic processes. A better understanding of the symbiotic genes could allow optimization of nitrogen fixation (e.g., during the night) and photosynthesis (e.g., during the day), resulting in a more productive agricultural practice for members of symbiotic nitrogen-fixing rhizobia.

## INTRODUCTION

*Bradyrhizobium* sp. ORS278, belonging the Group II photosynthetic bradyrhizobia and nodulating *Aeschynomene* plants from CI-group III, is a model organism to study NF-independent symbiosis. A large-scale transposon mutagenesis of ORS278 produced multiple mutants that were either deficient in nodule production or unable to perform nitrogen fixation. Many of the nodule development-deficient mutants were found to have genes altered in purine biosynthesis, suggesting that some purine derivative may have an important role in NF-independent symbiosis. The nitrogen-fixing deficient mutants, on the other hand, had genes altered in a variety of cellular processes (Bonaldi et al. 2010).

Two genes of interest that resulted in nitrogen-fixing deficient phenotypes included BRADO3946 (*chk*), and BRADO4470 (*labA*). The former encodes a large putative sensory histidine kinase (2,117 amino acids), which was previously identified to regulate synthesis of the photosynthetic apparatus. Chk is likely a global regulator of other metabolic functions because of

the presence of a homolog in another non-photosynthetic strain (Jaubert et al. 2009; Bonaldi et al. 2010). This would make sense considering that this two-component regulator has input and output protein domains related to photosynthesis, nitrogen fixation, and regulation via light input. Although alteration of this gene results in a nitrogen-fixing deficiency, the exact mechanism of regulation is not yet understood.

BRADO4470 encodes a homolog of the *labA* gene found in cyanobacteria and has a role in regulating the circadian clock system through a negative feedback interaction with KaiC (Taniguchi et al. 2007). The *kaiABC* genes are responsible for the self-oscillating endogenous clock in many bacteria and in turn control the expression of many genes dependent on circadian regulation. Simply, the circadian clock can be considered in three parts: an input pathway that provides information from environmental stimuli to the oscillator, the oscillator which creates the timing loop, and the output pathway which distributes temporal information to regulate downstream cellular processes (Taniguchi et al. 2010). KaiC, an autokinase and autophosphatase, is the integral component of the circadian clock and can repress its own gene expression in concert with LabA in a negative feedback manner (Ishiura et al. 1998; Nakahira et al. 2004; Nishiwaka et al. 2004, Taniguchi et al. 2007). It is interesting to consider the implications of a circadian clock negative feedback control homolog in the symbiotic context with ORS278 and why its mutagenesis would result in a nitrogen-fixing deficient phenotype for *A. indica* upon inoculation. Perhaps circadian regulation of bacteroid nitrogen-fixation could temporally protect the oxygen sensitive nitrogenase enzyme from the photosynthetic byproduct of the plant (Gallon 1981; Bonaldi et al. 2010). Although circadian gene expression has not yet been described in photosynthetic bradyrhizobia, temporal separation of photosynthesis and nitrogen fixation has been observed in

certain strains of cyanobacteria (Berman-Frank et al. 2003; Mohr et al. 2010; Pennebaker et al. 2010; Zehr 2011).

There have been many evolutionary adaptations to protect the nitrogenase enzyme in cyanobacteria such as compartmentalization of the nitrogenase complex, temporal regulation between photosynthesis and nitrogen fixation, or even increasing respiration rates (Fay 1992; Berman-Frank et al. 2003). Typically, nitrogen fixation is performed in specialized compartments called heterocysts, which maintain an anaerobic environment (Fay 1992). Others, such as those from *Gloeocapsa*, *Cyanothece*, and *Crocosphaera* genera, perform nitrogen fixation in the dark, temporally segregating photosynthesis from the oxygen-sensitive nitrogenase (Millineaux et al. 1981; Stal 2009; Mohr et al. 2010; Pennebaker et al. 2010; Zehr 2011). However, in the marine cyanobacterium, *Trichodesmium*, nitrogen fixation is curiously performed during the day. Although the exact mechanism is not yet understood, the bacterium likely uses a combination of both a spatial and temporal separation of photosynthesis and nitrogen fixation (Berman-Frank et al. 2001; Zehr 2011). Regardless of the mechanism of separation of photosynthesis and nitrogen fixation, it is obvious that there have been evolutionary advances to accomplish this unique feature. It is also quite possible that one of these mechanisms could have either independently evolved in photosynthetic bradyrhizobia, or perhaps horizontally acquired from cyanobacteria since they share common environments (Dvornyk et al. 2003). In either case, a better understanding of the role of circadian regulation of nitrogen fixation and photosynthesis could provide novel insights into the complex NF-independent symbiotic pathway.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The wild type strain of *Bradyrhizobium* sp. ORS278 was cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of $Na_2HPO_4$, 250 mg of $Na_2SO_4$, 320 mg of $NH_4Cl$, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per L (Sadowsky *et al.*, 1987). ORS278 was incubated aerobically at 30°C with vigorous shaking at 200 rpm for 3 days. Appropriate antibiotics were supplied for each strain ($\mu g \cdot ml^{-1}$): nalidixic acid, 25 (all ORS278 strains); kanamycin, 150 (*labA* and *chk* mutant strains). *Escherichia coli* strains and plasmids were grown aerobically in Luria-Bertani (LB) medium at pH 7.0 which contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of nanopure water (Bertani 1951). All *Escherichia coli* cultures were grow at 37°*C* (except DH5α pKD78 incubated at 30°C), with vigorous shaking at 200 rpm overnight. Where applicable appropriate antibiotics were applied in $\mu g \cdot ml^{-1}$: gentamicin, 15 (pJQ200SK and pKD78); kanamycin, 50 (pRK2013). Additional 15 g per L of agar was added when making agar plates for each medium when necessary. All bacterial strains and plasmids in this study are listed in Table 1.

**Construction of mutant strains**. Two knock-out mutants were constructed using site-specific mutagenesis by means of deletion mutation for the genes BRADO3946 and BRADO4470. The *Bradyrhizobium* sp. ORS278 *labA* gene (BRADO4470) was amplified by PCR using the following primers: 5’ ATTAGGGCCCCGAATGCACCAGTTCTTCCT 3’ and 5’

ATATGGGCCCAGC AGTGGTAGCTGTCGTA 3', forward and reverse respectively. The ORS278 *chk* gene (BRADO3946) was amplified by PCR using the following primers: 5' ATATGGGCCCACTTGCCGGATCACTCTC 3' and 5' TATAGGGCCCTGTCTTTC AGCTCCAGGAT 3', forward and reverse respectively. *ApaI* restriction sites were added to both forward and reverse primers as indicated by the underlined sequence. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) and gel electrophoresis, a PowerPac$^{TM}$ Basic (Biorad, Hercules, CA), was used to confirm the correct size of amplified PCR products. Then, the amplified genes, 1.8 Kb for *labA* and 2 Kb for *chk*, were each inserted into a pJQ200SK-Gm$^{R}$ suicide vector (Quandt and Hynes 1993) using the heat shock method to construct a recombinant plasmid. The cloning plasmids were each transformed into the Lambda Red recombinase expression plasmid, DH5α pKD78-Cm$^{R}$, via electroporation with Micro Pulser$^{TM}$ (Biorad, Hercules, CA), at 1.8 kV to create electrocompetent cells for pKD78-pJQ200SK-*labA*-*λ* and pKD78-pJQ200SK-*chk*-*λ* (Datsenko and Wanner 2000). A 1.47kb kanamycin antibiotic cassette from pKD4-Km$^{R}$ plasmid was amplified by PCR using the following primers: 5' GTGTAGGCTGGAGCTGCTTC 3' and 5' CATATGAATATCCTCCTT AG 3', forward and reverse respectively. Kanamycin antibiotic cassette fragments then were amplified again with 60 bp primers which combined a kanamycin antibiotic cassette primer with an additional 40 bp *labA* and *chk* homology sequence. For *labA*: 5' ATGTCA TCTTCGACCAATAAGATTGCGCTCTTCATTGACGGTGTAGGCTGGAGTGCTTC 3' and 5' CTAGTCCTCGAAATCGTCATCGGGAGACGTCGTGGTGGGCCATATGAATATCC TCCTTAG 3', forward and reverse respectively. For *chk*: 5' ATGCCGCGACCTTTTGAGACTCGGATTCTTGTAGTCGTCCGTGTAGGCTGGAGCTGC

TTC 3' and 5' CGGCGATGTTGCGGACCTGTGCTGTCAGGTTGGACGCCATCATATGAA TATCCTCCTAG 3', forward and reverse respectively. The PCR fragments and the electrocompetent cells were ligated to construct a recombinant plasmid for each (pRK78-pJQ200SK-*labA*-Km and pRK78-pJQ200SK-*chk*-Km) by electroporation using Micro Pulser$^{TM}$, at 2.2 kV. The resulting constructs were transferred from *E. coli* to the wild type strain ORS278 by tri-parental mating with the helper strain pRK2013-$Km^R$. Transconjugants were selected for sucrose-induced lethality ($SacB^S$), and nalidixic acid and kanamycin resistances ($Na^R$ and $Km^R$). The mutant strains DNA were extracted and confirmed by PCR.

**Growth curve measurements.** Initial cultures of wild type, *labA* mutant, and *chk* mutant strains were grown in AG medium with appropriate antibiotics until they reached the mid exponential phase at $OD_{600}$ 0.8, and then subcultured into 125 ml flasks, 3 replicates of 30 ml for each strain, at $OD_{600}$ 0.005. All samples were incubated at 30 °C with shaking at 200 rpm. $OD_{600}$ of each culture was monitored every 12 h until each culture reached the stationary phase. This experiment was repeated 3 times.

***A. indica* growth and symbiotic phenotype observation.** *A. indica* seeds were surfaced sterilized by immersion in concentrated sulfuric acid (99 %) for 40 minutes. Seeds were then washed thoroughly in at least six changes of sterile water and left to soak in sterile water over night. Seeds were then aseptically transferred onto 0.8 % water agar plates, covered in foil, and incubated in the dark at 30°C. Germinated seeds that had root emergence of approximately 1.5 cm were selected and placed into sterilized plastic pouches (Mega International) with a total of three seeds placed in one pouch (Halverson and Stacey 1986). A straw was placed into either end of a pouch where it left the space between its bottom end and the bottom of a pouch approximately 5

cm. Two pouches (with the straw side facing out) were placed into a hanging paper folder using paper clips to attach them together, then this folder was placed into a folder rack. To prepare the inoculum for the pouch experiment, wild type *Bradyrhizobium* sp. ORS278 and mutant strains were grown in 20 ml of AG medium with the appropriate antibiotics at pH 6.8 and incubated aerobically at 30°C with vigorously shaking at 200 rpm until all cultures reached the mid-log phase (0.8 $OD_{600}$). Cells were harvested by centrifugation at 4,000 rpm for 10 min and washed with half-strength nitrogen-free Broughton and Dilworth (B&D) medium (pH 6.8) containing 500 µM $CaCl_2$, 250 µM $KH_2PO_4$, 250 µM $K_2HPO_4$, 5 µM Fe-citrate, 125 µM $MgSO_4 \cdot 7H_2O$, 125 µM $K_2SO_4$, 0.5 µM $MnSO_4 \cdot H_2O$, 1 µM $H_3BO_3$, 0.25 µM $ZnSO_4 \cdot 7H_2O$, 1 µM $CuSO_4 \cdot 5H_2O$, 0.05 µM $CoSO_4 \cdot 7H_2O$, and 0.05 µM $Na_2MoO_4 \cdot 2H_2O$ (Lee *et al.*, 2012). Strain suspensions in the half-strength B&D medium were all adjusted to have an $OD_{600}$ of 0.1 (ca. $1x10^8$ cells/ml). After pouches and all cultures were prepared, each pouch was nourished with 20 ml of half-strength B&D medium. For each bacterial strain, 1.0 ml of 0.1 OD600 of culture was inoculated on each seed. All pouches were incubated in a growth chamber with 16 h of day and 8 h of night at 27°C. All pouches were watered with 20 ml of half-strength B&D medium every 2 days. After 40 days incubation, the physical properties of seeds inoculated with different bacterial strains were measured, including average nodule number, nodule dry weight, and plant dry weight. In one pouch experiment, 6 pouches were used for each strain (18 seeds total), and this experiment was repeated three times.

**Acetylene reduction assay.** An acetylene reduction assay was used to quantify the nitrogen-fixing activity of ORS278 wild type, *labA* mutant, and *chk* mutant strains by detecting the ethylene production via gas chromatography (GC). A total of 6 *Aeschynomene indica* seeds

were sterilized, germinated, and inoculated with bacterial culture as described in the pouch experiment section. After 40 days, each intact root nodule was transferred into a 27 ml sterilized glass jar and sealed with a serum cap. 10% of the air inside the glass jar was replaced with an equal amount of pure acetylene gas, the air in the glass jar mixed via shaking. 250 µl of gas from the sample was drawn by using a gas-tight syringe (Hamilton Co., Reno, NV) and injected into the port of the gas chromatograph (GC-2014; Shimadzu) equipped with a hydrogen flame detector. Glass jars with no root served as a control for this experiment. The area of ethylene peak (3.6 min) and acetylene peak (4.75 min) were recorded. After acetylene reduction assay measurement, the nodules of each sample were dried in the oven (Chicago Surgical & Electrical Co.) at 65°C for 3 days. The nodule dry weight was measured by using an analytical balance (Denver Instruments APX-60). This experiment was repeated three times for each ORS278 strain.

**Bacteroid isolation.** *Bradyrhizobium* sp. ORS278 bacteroids were extracted from *Aeschynomene indica* nodules as described previously with modifications (Karr et al. 1984). Briefly, nodules were disrupted in MEP (5mM $MgCl_2$; 1mM EDTA; 50mM K-phosphate buffer, pH 7.0) containing 17% w/v sucrose (MEPS) and polyvinylpyrrolidone (PVP) (1g of nodules: 1/3g PVP: 10ml MEPS). The mixture was passed through four layers of cheesecloth and centrifuged at 400*g* for 10 minutes at 5°C. The resulting supernatant was centrifuged at 8000*g* for 15 minutes at 5°C. The pellet was resuspended in MEPS buffer (2ml/g original weight nodules) and was layered onto a gradient consisting of 30% (10ml), 40% (5ml), and 57% (6ml) (w/w) sucrose in MEP. The gradient tubes were centrifuged in a T-865 fixed angle rotor at 72,000*g* for 35 minutes at 5°C in a Beckman XL-90 ultracentrifuge. The bacteroid layer at the 40/57% sucrose interface was collected, diluted with two volumes of MEP, and applied to a second gradient consisting of 30%

(15ml), and 40% (10ml) (w/w) sucrose. The tubes were centrifuged as in a T-865 rotor as described above. The pellet was collected and resuspended in 30ml dd$H_2O$, then centrifuged 8000*g* for 15 minutes at 5°C. The bacteroid pellet was collected and prepped for RNA isolation.

**RNA isolation.** For all RNA isolation experiments in this study, RNA extraction was performed as previously described (Jeon et al. 2011). All cultures were condensed by centrifugation for 20 min at 4°C and 4,000 rpm in a fixed angle rotor. The cell pellet was then collected by decanting the supernatant. RNA isolation followed by using hot phenol method as described previously (Bittner *et al.*, 2003). The RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) was used to purify the isolated RNA according to manufacturer's protocol. RNA quantity was calculated using NanoDrop device (Thermo Scientific) and RNA quality was confirmed via gel electrophoresis.

**qRT-PCR gene expression analysis.** The qRT-PCR was performed with modification according to methods previously described (Chang et al. 2007). Briefly, cDNA was synthesized in 25-µl reaction solution containing 1.5 µl of M-MLV reverse transcriptase (200U/µl; Promega Corp.), 2 µl of random hexamers (250ng/µl; Invitrogen, Carlsbad, CA, U.S.A.), 5 µl of 2.5 mM dNTPs, and 1 µg of total RNA. Of the reaction solution, 1 µl of cDNA was used as a template for qRT-PCR. A total of 10 µl qRT-PCR reactions were prepared using 5 µl of 2 x All-in-One$^{TM}$ qPCR Mix and 0.1 µl ROX Reference Dye by GeneCopoeia (Rockville, MD, U.S.A.), 1 µl at 0.2 µM each of forward and reverse primers, and 1 µl of cDNA template. qRT-PCR was performed by using ABI PRISM 7300 instrument (Applied Biosystems, Foster City, CA, U.S.A.) and following the GeneCopoeia qPCR protocol. Negative control reactions which lacked reverse transcriptase were used to check for DNA contamination. Gene-specific primers for the selected candidate

reference genes were designed using Integrated DNA Technologies (Coralville, IA, U.S.A.) company software. The primers used for qRT-PCR are their amplicon length are listed in Table 2. The *parA* (bll0631) gene, a chromosomal partitioning protein, was used to normalize the expression of each gene in this study (Cytryn et al. 2007; Donati et al. 2011). All experiments were performed with three biological replicates, and three technical replicates for each biological replicate. Fold induction values were calculated in accordance with the method of Pfaffl (2001).

**RNA sequencing and transcriptomic analysis.** Genome-wide transcriptomic analysis was performed by submitting samples to the Department of Energy Joint Genome Institute (DOE-JGI), Walnut Creek, CA. All general aspects of library construction, sequencing, and analysis performed at the JGI can be found at http://www.jgi .doe.gov. Differential gene expression was determined from normalized read counts via DESeq2 (Love et al. 2014), and gene ontology enrichment analysis was assessed via adaptive clustering of GO categories and Mann-Whitney U tests (GO-MWU, https://github.com/z0on/GO_MWU) (Voolstra et al. 2011).

## RESULTS

**ΔlabA and Δchk mutant strains exhibit growth similar to wild type.** After performing growth curve analysis in AG medium, no significant difference was found between wild type and the mutants (Figure 2-1). Generation times for wild type ORS278, ΔlabA, and Δchk were 10.12 h, 10.48 h, and 10.97 h, respectively.

**ΔlabA and Δchk mutant strains show symbiotic deficiency concerning *A. indica* vitality.** *A. indica* plants were grown in pouches, initially inoculated with either wild type or mutant strains, then assessed for physiological characteristics. Plants inoculated with the wild type

showed a significant ($P < 0.05$ for *t*-test) increase in average dry weight compared to those inoculated with the mutant strains (Figure 2-2). After counting the number of nodules, the plants inoculated with the wild type produced a significant ($P < 0.05$ for *t*-test) amount more nodules than plants inoculated with either mutant strain (Figure 2-3). Average nodule dry weight exhibited no significant difference between inoculant treatments (Figure 2-4). Observation of plant phenotypes showed that plants inoculated with the wild type were healthier and more robust than those inoculated with the mutant strains (Figure 2-5).

**Wild type ORS278 primarily fixes nitrogen at night.** An acetylene reduction assay was used to assess nitrogenase activity for *A. indica* nodules. Plants were harvested and nitrogenase activity was measured during both night and day. In plants inoculated with wild type, nitrogenase activity during the night time exhibited a significant ($P < 0.05$ for *t*-test) increase in ethylene production compared to that measured during the day time (Figure 2-6).

**ΔlabA and Δchk mutant strains are nitrogen-fixing deficient.** The acetylene reduction assay shows that the mutant strains perform nitrogen fixation at a lower rate as compared with the wild time during both time measurements (Figure 2-6). Observation of the root nodules showed that nodules harboring the wild type were pink in color while mutant nodules were colorless/white (Figure 2-7).

**Half of the transcriptome is differentially expressed between day and night.** Transcriptome analysis of ORS278 isolated from *A. indica* root nodules during day and night showed that 3,233 of 6,746 genes are significantly differentially expressed. The top 50 most differentially expressed genes (DEGs), both upregulated and downregulated are displayed in Table 2-2 and 2-3, respectively. Functional distribution of DEGs are displayed in Table 2-4. Gene

ontology enrichment analysis of biological processes revealed that purine ribonucleoside monophosphate metabolism, nucleoside monophosphate biosynthesis, and ion transport were downregulated at night (Figure 2-8). Cofactor, and porphyrin-containing compound metabolisms were found to be upregulated at night (Figure 2-8). Molecular functions involved in ATPase, coupled to transmembrane movement of ions, and hydrogen ion membrane transport were downregulated, while 4 iron, 4 sulfur cluster binding was upregulated at night (Figure 2-8).

## DISCUSSION

Photosynthetic bradyrhizobia lacking the *nod* genes, such as ORS278, are a peculiar symbiotic anomaly in regard to the classic archetype equipped with nodulation factors and a well described mutualistic interaction. These microbes are an important variety that may hold the secrets to unlocking the ability to apply symbiotic nitrogen fixation to other non-leguminous hosts such as cereal crops. This study helps to define the role of circadian regulation of nitrogen-fixation and photosynthesis to better characterize the underlying mechanisms and inner workings of symbiosis between the photosynthetic nitrogen-fixing microbe, *Bradyrhizobium* sp. ORS278, and its tropical leguminous host, *Aeschynomene indica*, by observing temporal symbiotic phenotypes and the features of circadian-related genes.

Two genes, BRADO3946 and BRADO4470, *chk* and *labA*, respectively, were selected as candidates for study because of their implications in the circadian regulation network and known involvement in biological nitrogen fixation (Bonaldi et al. 2010). *labA* encodes a negative feedback protein in the circadian oscillator system and has been well characterized in the cyanobacteria

*Synechococcus elongatus* PCC 7942. This gene works as one part of a regulatory pathway for the internal clock and dictates downstream circadian gene expression as depicted in Figure 2-9 with possible connection to regulation of the nitrogenase complex (Taniguchi et al. 2007). *chk* encodes a putative sensor histidine kinase part of a two-component regulatory system, composed of over 2000 amino acids, and has input and output protein domains related to circadian-dictated processes. Two-component regulators are transmembrane proteins that consist of a receiver, histidine kinase (HK) which senses some environmental stimuli and a response regulator (RR) that relays information and initiates appropriate transcription (Dutta et al. 1999; West and Stock 2001). Typically, histidine kinases in bacteria have functions related to regulation of cell cycle components, stress responses, developmental pathways and even virulence, but they have also been implicated in blue-light detection and controlling transcription of photosystem and nitrogen fixation related genes (Elsen et al. 2000; Hoch 2000, Swartz et al. 2007; Laub 2011; Ibrahim et al. 2016). Interestingly, the HWE family of histidine kinases has been noted for its metabolic flexibility and are enriched in the *Rhizobiaceae* family, likely because of their interactions with eukaryotic hosts (Karniol and Vierstra 2003). In some rhizobia and purple photosynthetic bacteria there are homologous two-component regulators (RegSR, ActSR, RegBA, and PrrBA) that control the transcription of nitrogen fixation, $CO_2$ fixation, photosynthesis, and acid tolerance further demonstrating the versatility and wide applicability of these molecules (Emmerich et al. 2000).

When both genes were mutagenized the resultant strains showed no effect on free-living state growth but did have an impact on nitrogen-fixing abilities. It makes sense that the disruption of *chk* would result in a nitrogen-fixing deficient phenotype because of its domains related to nitrogen fixation and its probability as a global regulator. However, the role of *labA* and its deletion

resulting in a nitrogen-fixing deficient phenotype is a bit more multifaceted. It is possible that the components of the circadian clock control the transcription of the nitrogen-fixation regulon and perhaps works in tandem with a two-component sensor that signals diel cycling. Restricting metabolic activities such as nitrogen fixation, $CO_2$ assimilation, or respiration to certain temporal frames could benefit and optimize each process in terms of energy production and usage as well as create a conducive enzymatic environment.

*Aeschynomene indica* plants exhibited decreased physiological vitality when inoculated with Δ*labA* or Δ*chk* as compared with wild type ORS278. The mutant-inoculated plants' unhealthy appearances are likely a direct result of the nitrogen-fixation deficiency of the mutant strains. Without a steady nitrogen source some plants will mimic the drought stress response and suffer a decrease in biomass production due to a reduction in plant leaf area, photosynthesis rate, and chlorophyll content (Radin and Parker 1979; Zhao et al. 2005). Even when viewing the root nodules of *A. indica* under a dissecting microscope it is possible to see the different phenotypes produced from either mutant or wild type strains of *Bradyrhizobium* sp. ORS278. While the nodules harboring wild type ORS278 produced the characteristic pink color reflecting the presence of leghemoglobin, the nodules containing either Δ*labA* or Δ*chk* exhibited either a faintly pink or white color, which indirectly implies a nitrogen fixation deficiency. Leghemoglobins are hemoproteins produced by the plant and accumulated in the cytoplasm of infected plant cells associated with nodule development. The leghemoglobin sequesters the free oxygen to maintain a microoxic environment to help protect the oxygen sensitive nitrogenase enzyme and thus are required for symbiotic nitrogen fixation (Ott et al. 2005). The nitrogen fixation deficiency was also confirmed by performing an acetylene reduction assay using a gas chromatograph which measures

the nitrogenase enzyme's ability to reduce acetylene ($C_2H_2$) to ethylene ($C_2H_4$) (Dilworth 1966; Hardy and Knight 1967; Hardy et al. 1972). Ethylene production (i.e. nitrogenase activity) was greater during the night time period than during the day for nodules from plants inoculated wild type ORS278, however, lower levels were observed during both day and night for the plant nodules that were inoculated with either mutant strain. So, not only were nodule color phenotypes indicative of nitrogen-fixing deficiency, but the acetylene reduction assay confirmed the lack of nitrogenase activity for Δ*labA* and Δ*chk*. This not only implicates these two genes in the symbiotic efficiency between ORS278 and *A. indica*, but because the wild type fixes more nitrogen during the night, shows that there is circadian regulation of nitrogen fixing metabolism for this pair. By restricting nitrogen fixation to the night when the plant is respiring, photosynthetically produced oxygen is avoided, and microoxic conditions are likely more easily maintained in the symbiotic organs.

*Aeschynomene* legumes and other closely related species are able to be stem nodulated, so it makes sense that nitrogenase activity is limited to the night. At first, it may seem puzzling for nitrogen fixation in root nodules to exhibit the same temporal separation strategy assuming they are underground and protected from photosynthetic activity and byproducts. However, these tropical legumes are regularly flooded and can form floating mats to protect themselves and nodules from being completely submerged, and therefore both stem and root nodules can be exposed to photosynthetic activity (Loureiro et al. 1998; James et al. 2001). It is likely that no matter whether the rhizobia infect and form nodules in the stem or the root, they exhibit the same symbiotic nitrogen fixation tendencies. This was demonstrated by a proteomic analysis of ORS278 in both root and stem nodules that showed a similar proteome between both except that in stem

nodulated bacteroids there were more bacterial photosynthetic proteins, which makes sense because of their higher exposure to light and the previously shown role of ORS278's alternative photosynthetic pigments in successful stem nodulation (Giraud et al. 2000; Giraud et al. 2002; Delmotte et al. 2014).

The origin or acquisition of circadian regulation in *Bradyrhizobium* sp. ORS278 could be attributed to the environment in which they are typically found. Their associated hosts, *Aeschynomene* plants, thrive in inundated tropical environments and are usually always found near a water source if not constantly submerged. Freshwater nitrogen-fixing cyanobacteria share the same habitats as ORS278 and its host, and therefore it may be likely that adapted laterally transferred genes were utilized by the microsymbiont to create an efficient symbiosis (Howarth et al. 1988; Hirsch et al. 1995).

Genome-wide transcriptomic analysis of ORS278 bacteroids isolated from *A. indica* root nodules from day and night revealed that 3233 of 6746 genes were significantly differentially expressed demonstrating a major change in bacteroid activity temporally. Most genes were in the conserved hypothetical proteins (26%), no similarity (25%), or other (10%) functional categories but the next highest represented categories included transport and binding proteins (10%) and regulatory functions (5%). Though many of the highest up and down-regulated genes are annotated as hypothetical proteins, a few genes in each category reflect a change in metabolic pathways between light and dark conditions. One of the highly down-regulated genes at night include BRADO2008 encoding a bacteriophytochrome, likely involved in photosynthesis. The circadian clock gene, BRADO1479, encoding the KaiC protein was found to be down-regulated during the

night as well, which makes sense because the LabA protein, encoded by BRADO4470, operating in the negative feedback pathway is proposed to function at night (Taniguchi et al. 2007). Expression values for all circadian clock genes can be found in Figure 2-10. Interestingly, some photosynthetic related genes were found to be up-regulated at night, however, the two genes, BRADO1638 and BRADO1639, encoding 2-vinyl bacteriochlorophyllide hydratase and protochlorophyllide reductase subunit bchN, are part of light-independent reactions. Protochlorophyllide reductase has been documented that *Eubacteria* (anoxygenic photosynthetic bacteria) to be part of the system that allows organisms to synthesize chlorophyll and its derivatives in the absence of light. Even more striking is the fact that these light-independent genes have homology with the nitrogenase enzyme and likely function in a similar fashion due to oxygen lability (Schoefs 1999). Genes related to nitrogen-fixation were found equally up and down-regulated during the night time which might reflect the quick inactivation of the nitrogenase complex if exposed to adverse conditions. Though nodules were immediately flash frozen in liquid nitrogen when removed, oxygen exposure can inactivate nitrogenase in less than 1 minute (Kanemoto and Ludden 1984).

Gene ontology enrichment analysis showed that the biological processes of cofactor metabolism and porphyrin-containing compound metabolism were up-regulated during the night, while purine ribonucleoside monophosphate metabolism, nucleoside monophosphate biosynthesis, and ion transport were down-regulated at night. Both cofactor and porphyrin metabolic pathways are essential for nitrogenase biosynthesis, so if ORS278 is actively performing nitrogen fixation at night, then these precursors would indeed be up-regulated (Zhao et al. 2012). Nucleoside monophosphates have been proposed to serve as regulatory molecules in the

assimilation of ammonia for bacteroids, though its exact role is unclear (Upchurch and Elkan 1978). Molecular functions that were down-regulated include ATPase, coupled to transmembrane movement of ions, rotational mechanism and hydrogen ion transmembrane transporter, however $Fe_4S_4$ cluster binding was up-regulated at night. Similar to the biological processes, there is a down-regulation in ion transport during the night, likely to maintain optimal conditions for necessary metabolic processes. The up-regulation of $Fe_4S_4$ cluster binding during the night is intriguing because of their involved role in the formation of the nitrogenase complex and subsequent nitrogen fixation (Zheng and Dean 1994). $Fe_4S_4$ cluster binding, and porphyrin and cofactor metabolism up-regulation during the night period supports the hypothesis that symbiotic nitrogen fixation occurs during the night time for ORS278.

The observed changes in bacteroid phenotype and metabolism between night and day time contributes to the idea that symbiotic relationships are highly complex and regulated by a multitude of factors. Metabolic balance and to adaptation to environmental conditions must occur between host and microbe so that symbiotic efficiency can be optimized. To further understand temporal difference on the symbiotic state, more time points should be observed so that the dark-light boundaries can be better characterized. Further understanding of temporal changes during symbiosis is required to elucidate the intricacies of the rhizobia-legume relationship as NF-independent symbiosis could possibly be initiated in a circadian fashion similarly to the regulation of symbiotic nitrogen fixation.

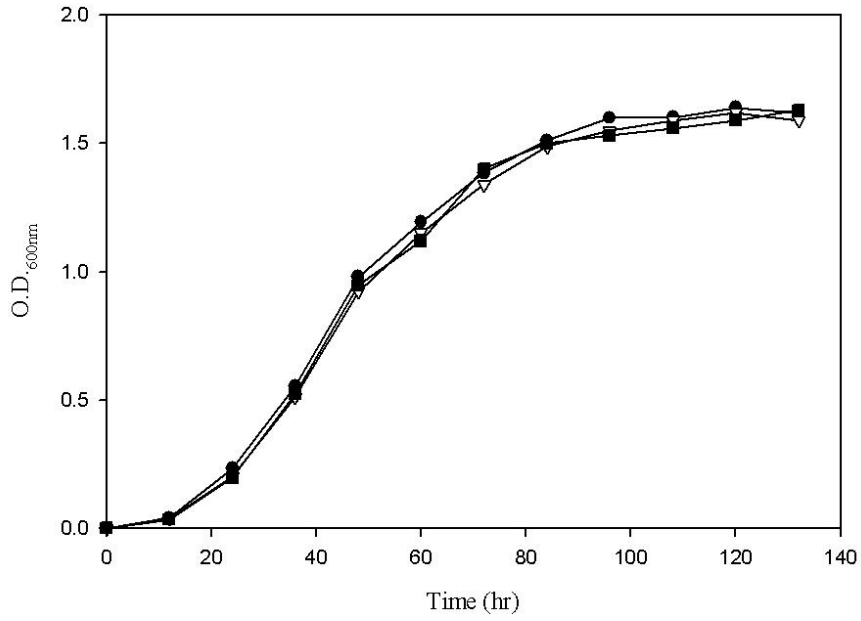


**Figure 2-1.** Growth of *Bradyrhizobium* sp. ORS278 (wild type), Δ*labA*, and Δ*chk* in AG media. Each time point represents average OD of three replicates. Symbols: ●, wild type; ■, Δ*labA;* ▽, Δ*chk.*

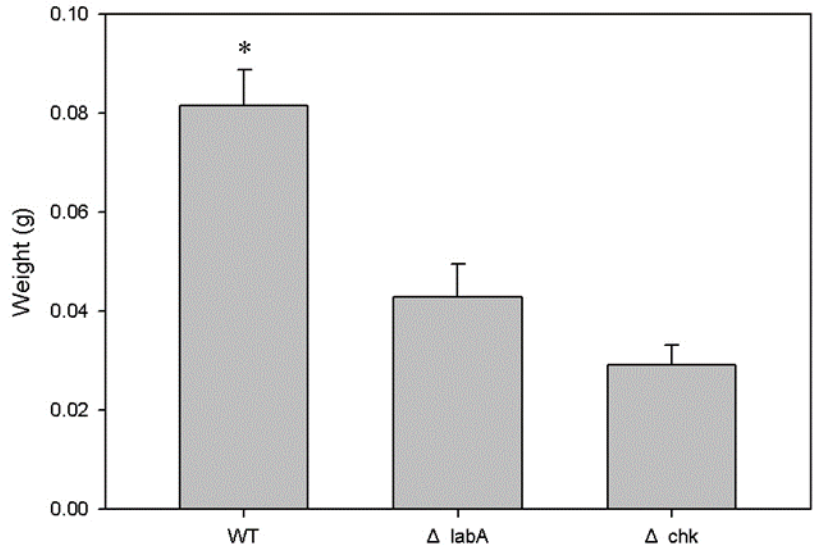


**Figure 2-2.** Average *A. indica* plant dry weight for each *Bradyrhizobium* sp. ORS278 strain inoculation. * indicates statistical difference ($P < 0.05$). Each bar represents average of 9 replicates with standard error.

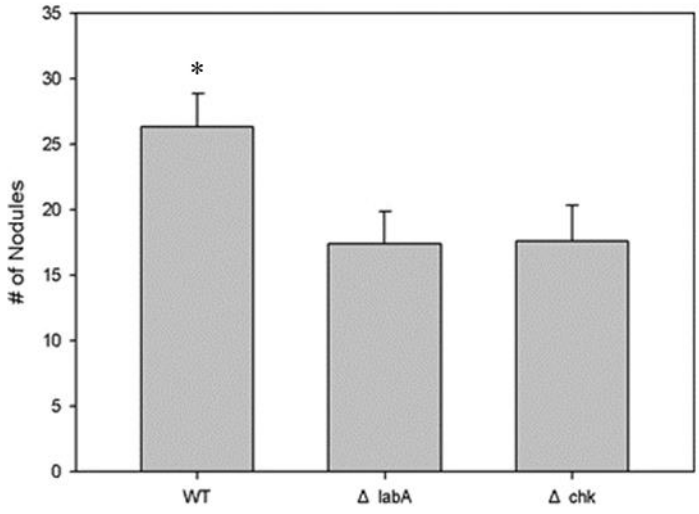

**Figure 2-3.** Average root nodule number for each *A. indica* plant inoculated with various *Bradyrhizobium* sp. ORS278 strains. * indicates statistical difference ($P < 0.05$). Each bar represents average of 9 replicates with standard error.

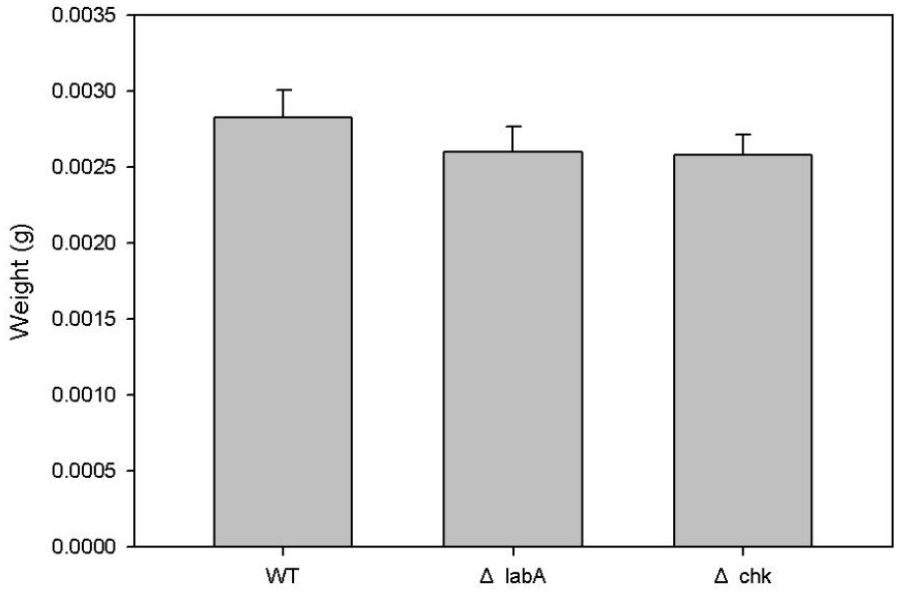


**Figure 2-4.** Average root nodule dry weight in *A. indica* plants inoculated with various *Bradyrhizobium* sp. ORS278 strains.

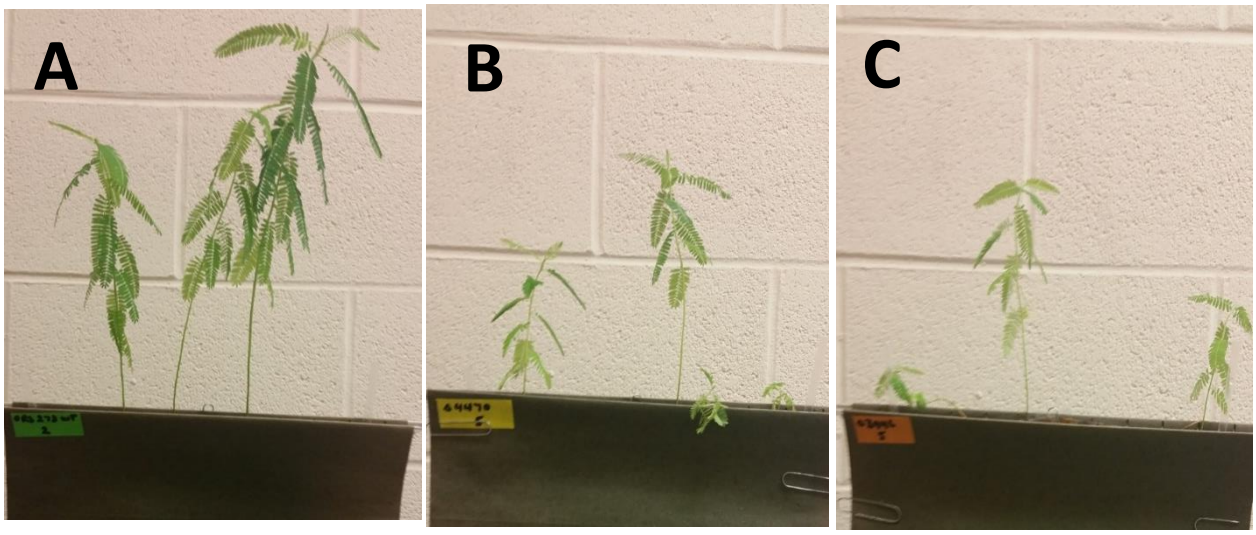


**Figure 2-5.** Typical *A. indica* plant phenotypes inoculated with **(A)** wild type *Bradyrhizobium* sp. ORS278; **(B)** ΔlabA mutant; **(C)** Δchk mutant.

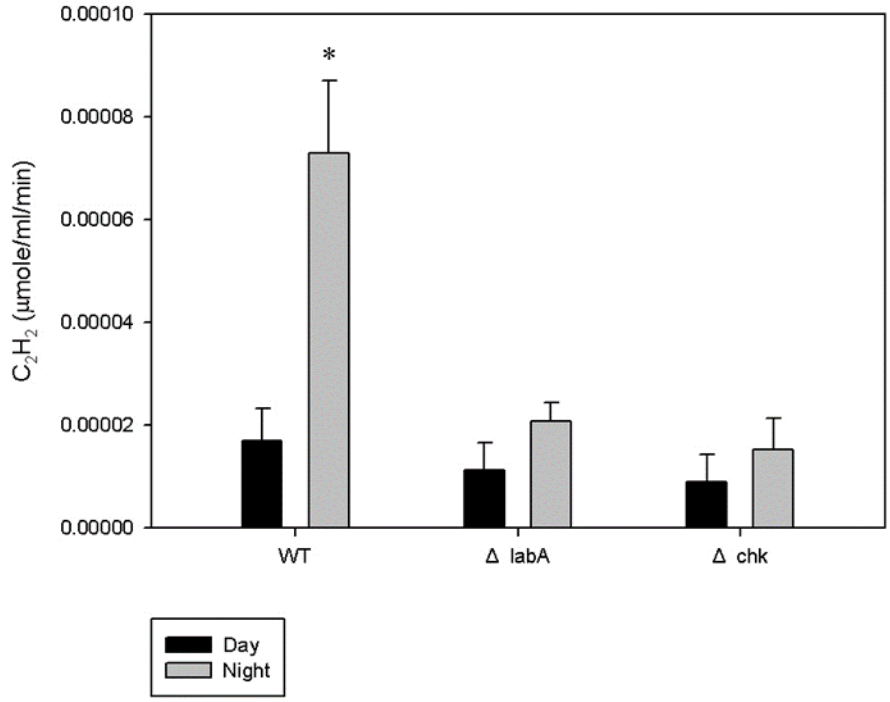


**Figure 2-6.** Average amount of ethylene production from *A. indica* root nodules inoculated with various *Bradyrhizobium* sp. ORS278 strains. Black bars indicate plants harvested during the daytime while gray bars represent plants harvest during the nighttime. * indicates statistical difference ($P < 0.05$).

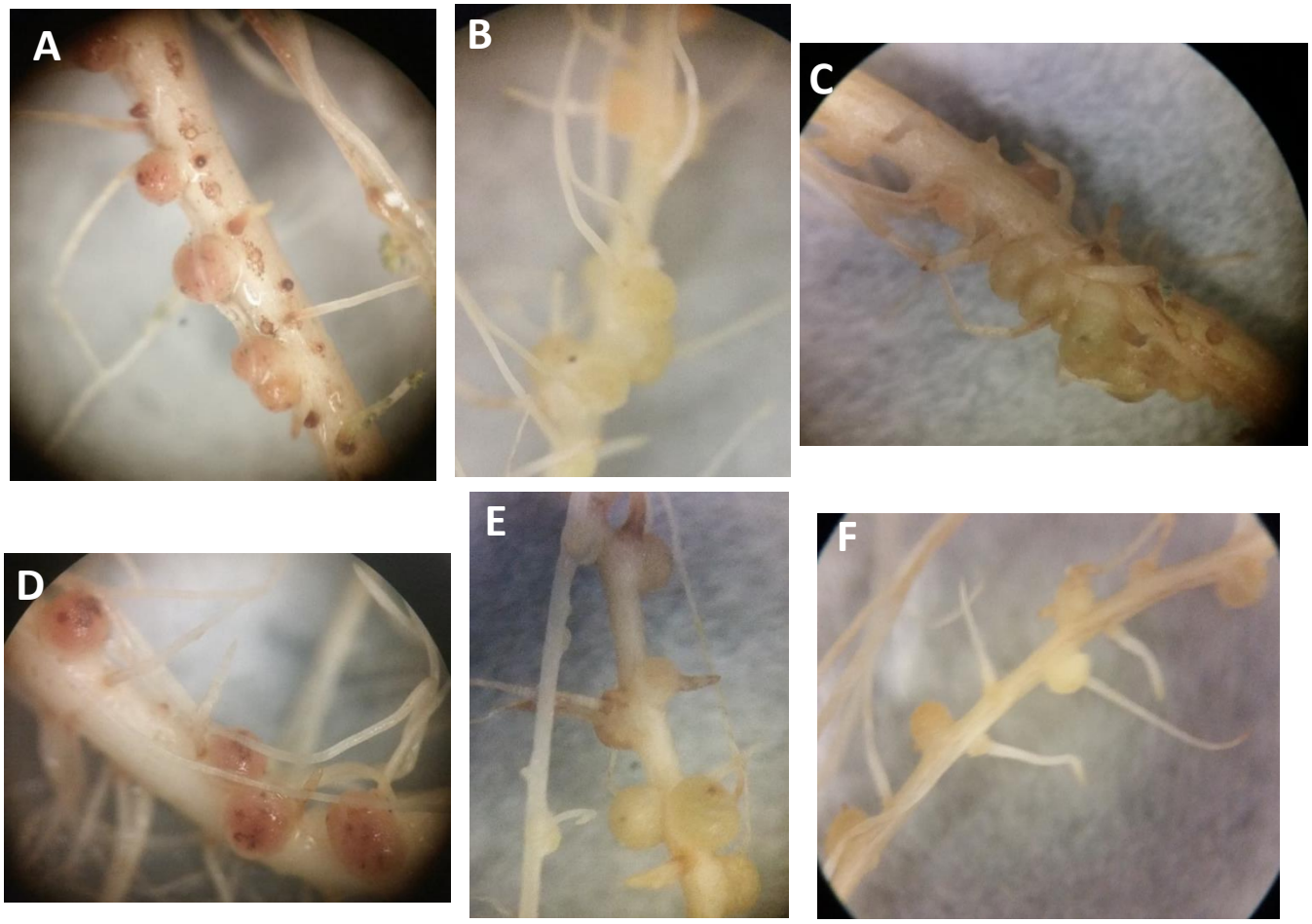


**Figure 2-7.** Root nodule phenotype for *Aeschynomene indica* plants inoculated with various *Bradyrhizobium* sp. ORS278 strains. **(A)** Wild type day; **(B)** Δchk day; **(C)** ΔlabA day **D**. wild type night; **(E)** Δchk night; **(F)** ΔlabA night.

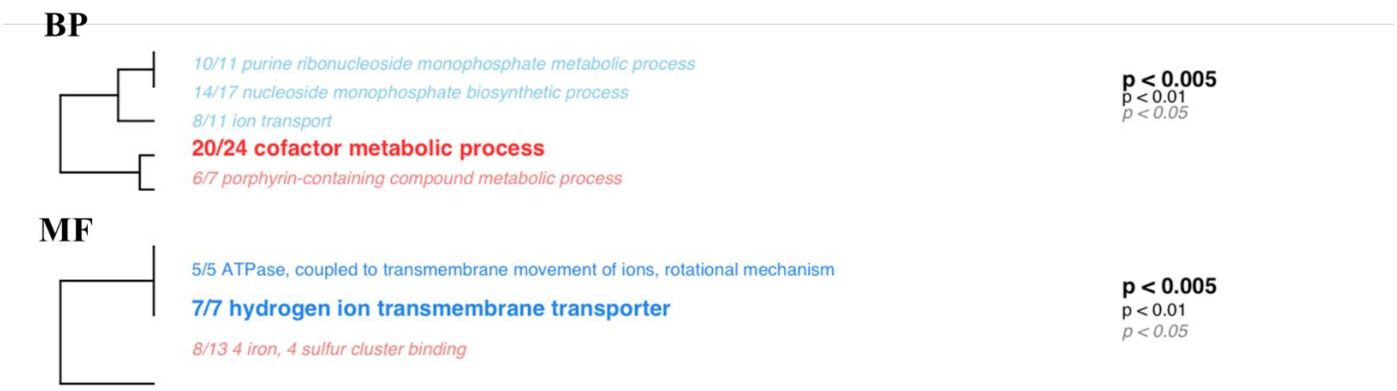


**Figure 2-8.** Gene ontology categories enriched by genes in ORS278 bacteroids extracted from *A. indica* root nodules during the night as compared to the day. Red text denote up-regulated genes and blue text denotes down-regulated genes. BP and MF denotes biological processes and molecular function, respectively. Font size indicates the significance of the term as indicated by the inset key displaying p-values. The fraction preceding the GO term indicates the number of genes annotated with the term (p-value < 0.05). The trees show distribution of genes among GO categories (the categories with no branch length between them are subsets of each other).

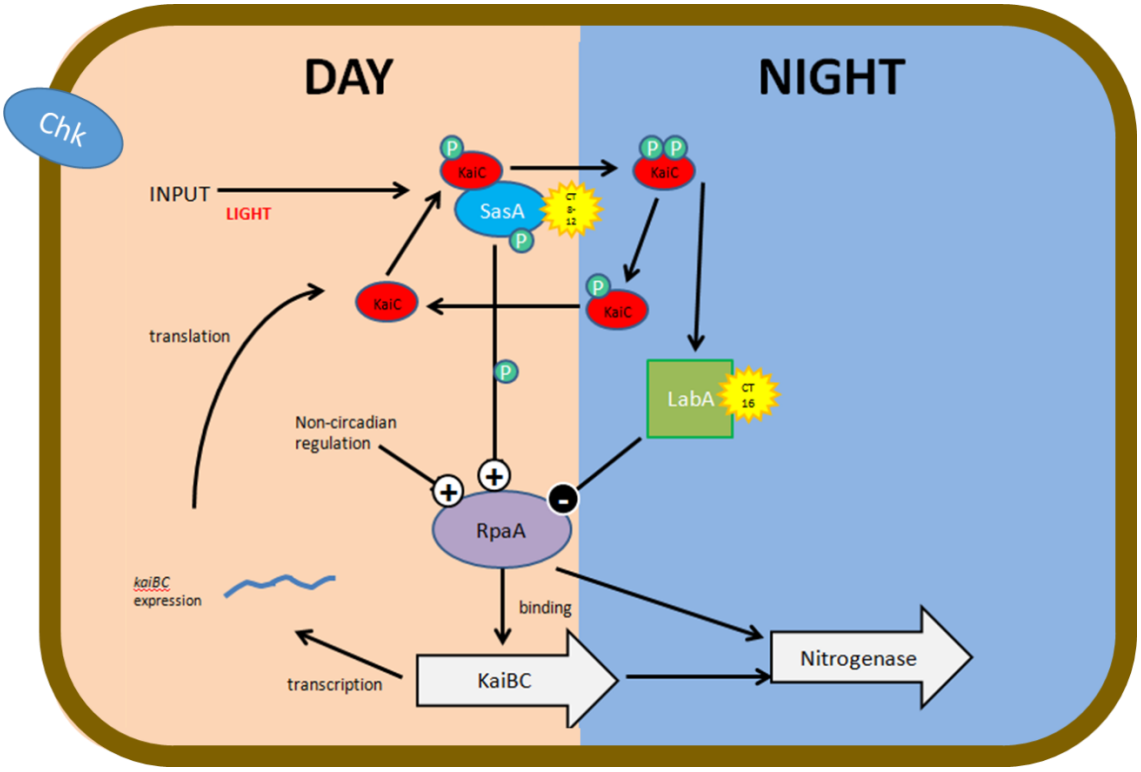


**Figure 2-9**. Modified figure from Taniguchi et al. 2007, showing the circadian clock proteins and possible pathway for nitrogen fixation regulation involving *labA* and *chk* genes.

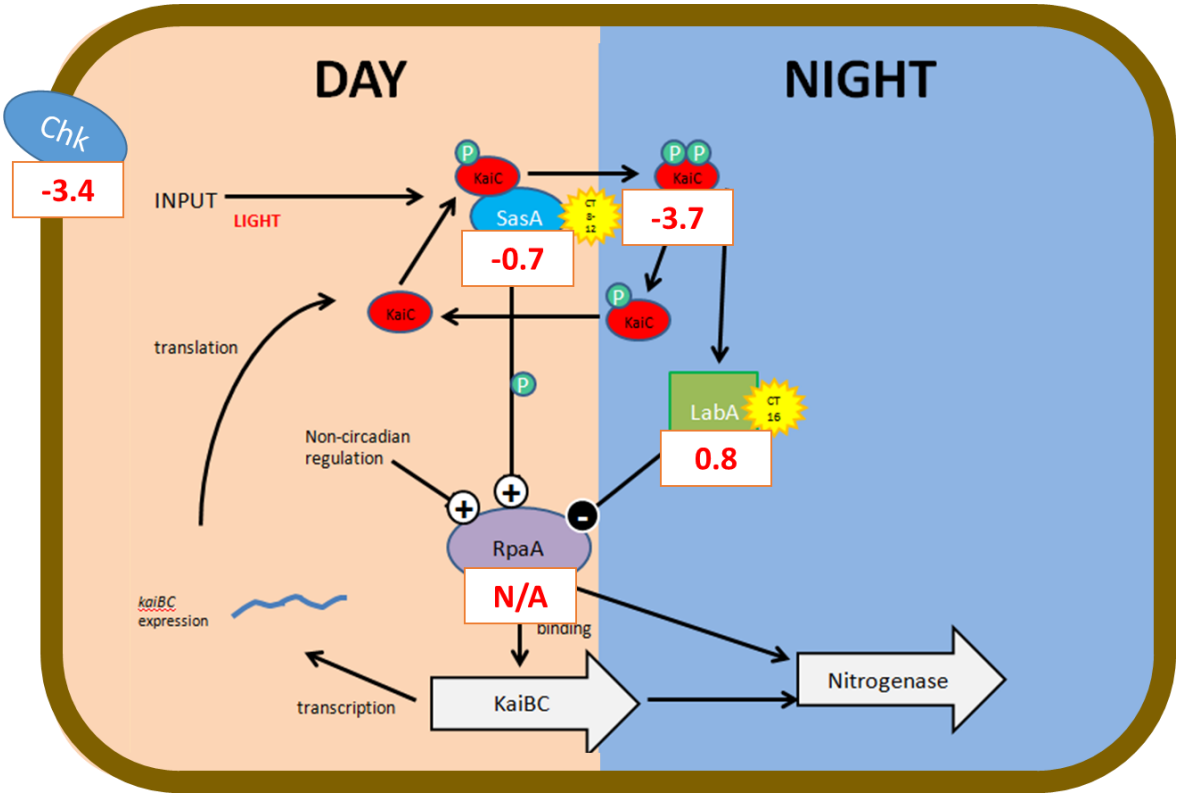


**Figure 2-10**. Modified figure from Taniguchi et al. 2007, showing the circadian clock proteins with gene expression values determined by RNA-seq. The white boxes with red numbers indicate $\log_2$ fold change gene expression values for each respective gene of the circadian clock.

**Table 2-1. List of bacterial strains and plasmids.**

| Strain or Plasmid | Relevant genotype or phenotype | Source or Reference |
|---|---|---|
| ***E.coli* strain** | | |
| DH5α | supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F' (traD36, proAB+ lacIq, Δ(lacZ)M15) endA1 recA1 hsdR17 mcrA supE44λ gyrA96 relA1 | *Bethesda Research Laboratories* |
| ***Bradyrhizobium* sp. ORS278 strains** | | |
| ORS278 | $Na^R$ Wild-type | (Giraud et al.) |
| Δ*labA* mutant | $Na^R$ $Km^R$ *labA*::Km | This work |
| Δchk mutant | $Na^R$ $Km^R$ *chk*::Km | This work |
| **Plasmids** | | |
| pKD4 | $Km^R$ expression vector, | (Datsenko and Wanner 2000) |
| pKD78 | Lambda Red recombinase expression plasmid | (Datsenko and Wanner 2000) |
| pRK2013 | $Km^R$ cloning vector | (Ditta et al.1980) |
| pJQ200SK | $Gm^R$ cloning vector | (Quandt and Hynes) |
| pJQ200SK-*labA* | $Gm^R$ cloning vector containing *labA* gene | This work |
| pJQ200SK-*chk* | $Gm^R$ cloning vector containing *chk* gene | This work |
| pKD78-pJQ200SK-*labA*-λ | $Gm^R$ cloning vector containing *labA* gene with Lambda Red recombinase expression plasmid | This work |
| pKD78-pJQ200SK-*labA*-λ | $Gm^R$ cloning vector containing *chk* gene with Lambda Red recombinase expression plasmid | This work |
| pKD78-pJQ200SK-*labA*-Km | $Gm^R$ cloning vector with suicide vector and internal *labA* gene substitute with kanamycin cassette | This work |
| pKD78-pJQ200SK-*chk*-Km | $Gm^R$ cloning vector with suicide vector and internal *chk* gene substitute with kanamycin cassette | This work |

*Na, Km, and Gm refer to nalidixic acid, kanamycin, and gentamicin, respectively.

**Table 2-2. List of top 50 upregulated genes in *Bradyrhizobium* sp. ORS278 bacteroid within *A. indica* root nodules during the night time determined via transcriptomic analysis.**

| Locus Tag | Description | Fold Change[1)] |
|---|---|---|
| BRADO4787 | putative ABC transport system, periplasmic substrate-binding protein | 11.2 |
| BRADO4350 | putative methane/phenol/toluene monooxygenase (A subunit, Alpha chain) | 10.2 |
| BRADO4351 | hypothetical protein | 9.4 |
| BRADO4352 | hypothetical protein | 9.4 |
| BRADO4353 | putative alcohol dehydrogenase (Zinc-containing)(AdhT-like) | 9.2 |
| BRADO4348 | putative methane/phenol/toluene monooxygenase (A subunit, beta chain) | 9.1 |
| BRADO6076 | conserved hypothetical protein; putative signal peptide | 8.9 |
| BRADO6883 | Putative ABC transporter, substrate-binding protein; putative branched-chain amino acid transporter | 8.7 |
| BRADO6077 | putative oxidoreductase, GMC family | 8.6 |
| BRADO4346 | putative amidohydrolase | 8.5 |
| BRADO4059 | putative monooxygenase | 8.4 |
| BRADO4349 | putative Ferredoxin--NAD(+) reductase; putative phenol hydroxylase (Phenol 2-monooxygenase P5 component) | 8.2 |
| BRADO6075 | Putative Lactoylglutathione lyase | 7.4 |
| BRADO4347 | putative methane/phenol/toluene monooxygenase (B subunit) | 7.3 |
| BRADO6882 | putative O-succinylbenzoate--CoA ligase | 7.3 |
| BRADO4780 | conserved hypothetical protein, putative membrane protein | 7.1 |
| BRADO0123 | putative Quinoprotein ethanol dehydrogenase family protein | 7.1 |
| BRADO4781 | conserved hypothetical protein, putative TIM-barrel signal transduction protein | 7.0 |
| BRADO3817 | ABC transporter substrate-binding protein, periplasmic component | 7.0 |

| | | |
|---|---|---|
| BRADO4786 | putative Transcriptional regulatory protein, AraC/XylS family | 7.0 |
| BRADO1707 | Putative Quinoprotein ethanol dehydrogenase | 6.9 |
| BRADO1758 | Putative fusion protein : FAD-binding monooxygenase (N-ter)/ alpha/beta-Hydrolase (C-ter) | 6.7 |
| BRADO4345 | conserved hypothetical protein | 6.7 |
| BRADO3816 | putative permease protein of sugar ABC transporter | 6.6 |
| BRADO4344 | 60 kDa chaperonin (Protein Cpn60) (groEL protein) | 6.6 |
| BRADO3439 | conserved hypothetical protein | 6.5 |
| BRADO1776 | Nitric-oxide reductase subunit C (Nitric oxide reductase cytochrome c subunit) (NOR small subunit) | 6.5 |
| BRADO0030 | putative beta-lactamase | 6.4 |
| BRADO4770 | conserved hypothetical protein; putative Zn-dependent hydrolases, including glyoxylases | 6.4 |
| BRADO0527 | putative branched-chain amino acid ABC transporter, periplasmic binding protein | 6.4 |
| BRADO1650 | conserved hypothetical protein; putative membrane protein | 6.3 |
| BRADO1927 | putative ribose periplasmic binding protein | 6.3 |
| BRADO6074 | putative cytochrome c | 6.3 |
| BRADO1647 | conserved hypothetical protein | 6.2 |
| BRADO2363 | putative sugar (D-ribose) ABC transporter (periplasmic binding protein) | 6.1 |
| BRADO1615 | Geranylgeranyl pyrophosphate synthetase (GGPP synthetase) (Farnesyltranstransferase) | 6.1 |
| BRADO4779 | conserved hypothetical protein | 6.1 |
| BRADO4785 | putative ABC transporter, permease protein | 6.1 |
| BRADO1648 | conserved hypothetical protein | 6.0 |
| BRADO1645 | Reaction center protein H chain (Photosynthetic reaction center H subunit) | 6.0 |
| BRADO4777 | putative oxidoreductase subunit; putative carbon-monoxide dehydrogenase (acceptor), middle subunit CoxM/CutM homologs | 6.0 |
| BRADO6736 | putative amino-acid ABC transport system, periplasmic binding protein | 6.0 |
| BRADO3748 | isocitrate lyase | 6.0 |
| BRADO4666 | putative HspC2 heat shock protein | 6.0 |
| BRADO4858 | Putative aliphatic amidase expression-regulating protein, amiC | 6.0 |
| BRADO4733 | putative periplasmic mannitol-binding protein; putative Trap-T transport system, dctP subunit | 6.0 |
| BRADO6877 | conserved hypothetical protein | 5.9 |
| BRADO0118 | putative TRAP-type C4-dicarboxylate transport system, binding periplasmic protein (DctP subunit) | 5.9 |
| BRADO4060 | conserved hypothetical protein | 5.9 |

| | | |
|---|---|---|
| BRADO3438 | conserved hypothetical protein | 5.9 |

1) $Log_2$ Fold Change values with adjusted *P*-value < 0.05.

**Table 2-3. List of top 50 downregulated genes in ORS278 bacteroid within *A. indica* root nodules during the night time determined via transcriptomic analysis.**

| Locus Tag | Description | Fold Change[1)] |
|---|---|---|
| BRADO0856 | conserved hypothetical protein; putative membrane protein | -7.3 |
| BRADO4730 | putative heavy metal efflux pump, HlyD family (CzcB-like) | -7.2 |
| BRADO1809 | sugar transport protein (ABC superfamily, membrane) | -6.7 |
| BRADO1808 | Putative ABC transporter ATP-binding protein | -6.2 |
| BRADO1908 | sn-glycerol-3-phosphate dehydrogenase FAD/NAD(P)-binding (aerobic) | -6.1 |
| BRADO5822 | conserved hypothetical protein; putative exported protein | -5.9 |
| BRADO6925 | Putative HlyD family secretion protein | -5.6 |
| BRADO7048 | Hypothetical protein, putative ubiquinone/menaquinone biosynthesis methyltransferase-related protein | -5.6 |
| BRADO4731 | putative cation efflux system protein; putative heavy metal efflux pump CzcA-like | -5.6 |
| BRADO1781 | hypothetical protein | -5.4 |
| BRADO5861 | Putative urease accessory protein ureD | -5.1 |
| BRADO2008 | putative bacteriophytochrome | -5.1 |
| BRADO1046 | putative signal transduction histidine kinase with Chase domain | -5.0 |
| BRADO0521 | putative diguanylate cyclase (GGDEF)/phosphodiesterase (EAL) with PAS and GAF domains | -4.9 |
| BRADO0294 | putative transposase IS66 family | -4.8 |
| BRADO5617 | conserved hypothetical protein | -4.8 |
| BRADO4147 | putative outer-membrane protein precursor | -4.8 |
| BRADO3458 | conserved hypothetical protein | -4.7 |
| BRADO2089 | putative sensor histidine kinase with multiple PAS domains | -4.7 |
| BRADO0465 | hypothetical protein | -4.7 |
| BRADO7059 | putative Polysaccharide export protein | -4.7 |
| BRADO1807 | ABC transporter periplasmic-binding protein ytfQ precursor | -4.6 |
| BRADO6908 | conserved hypothetical protein; putative Acyl-CoA N-acyltransferases | -4.6 |
| BRADO1657 | Transketolase (TK) | -4.6 |
| BRADO1909 | putative ATP-binding protein of sugar ABC transporter | -4.5 |
| BRADO0506 | conserved hypothetical protein | -4.5 |
| BRADO1910 | putative ATP-binding protein of sugar ABC transporter | -4.5 |
| BRADO5043 | Conserved hypothetical protein; putative two-component sensor histidine kinase with a methyl-accepting chemotaxis | -4.4 |

| | | |
|---|---|---|
| | protein (N-term), a PAS domain (central) and a histidine kinase (C-term) | |
| BRADO6917 | Putative methyl-accepting chemotaxis receptor/sensory transducer | -4.3 |
| BRADO3244 | putative response regulator in two-component reguatory system, sigma54 dependent transcriptional regulator. | -4.3 |
| BRADO3528 | putative metalloendopeptidase | -4.3 |
| BRADO5055 | Putative flagellin protein, C-terminus | -4.3 |
| BRADO1052 | conserved hypothetical protein; putative signal peptide | -4.3 |
| BRADO0855 | Putative Murein hydrolase export regulator, LrgB family protein | -4.3 |
| BRADO5953 | conserved hypothetical protein; putative histone acetyltransfersase (HAT) | -4.3 |
| BRADO1047 | RNA polymerase sigma-E factor (Sigma-24) protein | -4.3 |
| BRADO2007 | putative heme oxygenase | -4.2 |
| BRADO0142 | conserved hypothetical protein; putative membrane protein | -4.2 |
| BRADO1049 | Putative response regulator receiver (CheY-like protein) | -4.2 |
| BRADO0729 | Putative methyl-accepting chemotaxis protein (chemoreceptor) | -4.1 |
| BRADO1479 | circadian clock protein | -4.1 |
| BRADO1658 | Fructose-bisphosphate aldolase | -4.1 |
| BRADO1480 | putative signal transduction histidine kinase with PAS/PAC domains | -4.1 |
| BRADO5320 | putative sensor histidine kinase with multiple PAS and a response regulator receiver domain (modular protein) | -4.1 |
| BRADO0600 | Putative diguanylate cyclase signaling protein (with GGDEF, EAL and MHYT domains) | -4.1 |
| BRADO1911 | putative ABC transporter permease protein | -4.0 |
| BRADO1912 | putative sugar ABC transport system, permease component | -4.0 |
| BRADO1655 | D-fructose-1,6-bisphosphatase protein | -4.0 |
| BRADO4732 | putative outer membrane lipoprotein (NodT-like), RND efflux system | -4.0 |
| BRADO6926 | Toxin secretion ABC transporter (ATP-binding and membrane protein); hlyB-like protein | -4.0 |

1) $Log_2$ Fold Change values with adjusted *P*-value < 0.05.

**Table 2-4. Functional distribution of DEGs identified in ORS278 during night.**

| Functional Category | # of DEGs | % of Total |
|---|---|---|
| Adaptations and atypical conditions | 3 | 0.09 |
| Amino acids and amines | 10 | 0.31 |

| | | |
|---|---|---|
| Aminoacyl tRNA synthetases and tRNA modification | 11 | 0.34 |
| Aromatic amino acid family | 14 | 0.43 |
| Aspartate family | 17 | 0.53 |
| Biotin | 4 | 0.12 |
| Branched chain family | 12 | 0.37 |
| Carotenoid | 6 | 0.19 |
| Cell division | 15 | 0.46 |
| Chaperones | 14 | 0.43 |
| Chemotaxis | 69 | 2.13 |
| CO2 fixation | 4 | 0.12 |
| Cobalamin, heme, phycobilin and porphyrin | 28 | 0.87 |
| Conserved hypothetical protein | 824 | 25.49 |
| Degradation of proteins, peptides, and glycopeptides | 18 | 0.56 |
| Degradation of RNA | 2 | 0.06 |
| Detoxification | 21 | 0.65 |
| DNA replication, recombination, and repair | 42 | 1.30 |
| Drug and analog sensitivity | 20 | 0.62 |
| Fatty acid, phospholipid and sterol metabolism | 102 | 3.15 |
| Folic acid | 3 | 0.09 |
| Glutamate family / Nitrogen assimilation | 14 | 0.43 |
| Glycolate pathway | 3 | 0.09 |
| Glycolysis | 5 | 0.15 |
| Hydrogenase | 5 | 0.15 |
| Interconversions and salvage of nucleosides and nucleotides | 4 | 0.12 |
| Lipoate | 1 | 0.03 |
| Membranes, lipoproteins and porins | 22 | 0.68 |
| Menaquinone and ubiquinone | 3 | 0.09 |
| Molybdopterin | 1 | 0.03 |
| Murein sacculus and peptidoglycan | 10 | 0.31 |
| Nicotinate and nicotinamide | 3 | 0.09 |
| Nitrogen fixation | 31 | 0.96 |
| Nitrogen metabolism | 9 | 0.28 |
| No similarity | 808 | 24.99 |
| Nucleoproteins | 0 | 0.00 |
| Other | 326 | 10.08 |
| Others | 1 | 0.03 |
| Pantothenate | 0 | 0.00 |
| Pentose phosphate pathway | 1 | 0.03 |
| Phosphorus compounds | 2 | 0.06 |
| Polysaccharides and glycoproteins | 4 | 0.12 |
| Protein and peptide secretion | 7 | 0.22 |
| Protein modification and translation factors | 13 | 0.40 |

| | | |
|---|---|---|
| Purine ribonucleotide biosynthesis | 15 | 0.46 |
| Pyridoxine | 2 | 0.06 |
| Pyrimidine ribonucleotide biosynthesis | 8 | 0.25 |
| Pyruvate and acetyl-CoA metabolism | 20 | 0.62 |
| Pyruvate dehydrogenase | 5 | 0.15 |
| Quinolinate | 1 | 0.03 |
| Radiation sensitivity | 2 | 0.06 |
| Regulatory functions | 154 | 4.76 |
| Respiration | 36 | 1.11 |
| Riboflavin | 2 | 0.06 |
| Ribosomal proteins | 19 | 0.59 |
| RNA synthesis, modification, and DNA transcription | 12 | 0.37 |
| Serine family / Sulfur assimilation | 13 | 0.40 |
| Sugars | 18 | 0.56 |
| Surface polysaccharides, lipopolysaccharides and antigens | 21 | 0.65 |
| Surface structures | 3 | 0.09 |
| Symbiosis | 5 | 0.15 |
| TCA cycle | 10 | 0.31 |
| Thiamine | 4 | 0.12 |
| Thioredoxin, glutaredoxin, and glutathione | 22 | 0.68 |
| Transformation | 1 | 0.03 |
| Transport and binding proteins | 328 | 10.15 |
| Transposon-related functions | 5 | 0.15 |

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CHAPTER 3

# IDENTIFICATION AND VALIDATION OF REFERENCE GENES FOR NORMALIZATION OF EXPRESSION ANALYSIS IN NITROGEN-FIXING BACTERIA


## ABSTRACT

Reference genes, also sometimes referred to as housekeeping genes (HKGs), play an important role in gene expression analysis by serving as an internal control in quantitative reverse transcription-polymerase chain reaction (qRT-PCR) experiments, providing a baseline for measuring gene expression under a variety of conditions. These HKGs are usually involved in basic cellular functions and thus, their expression should remain at relatively constant levels. qRT-PCR is used to detect and monitor gene expression. Normalization of gene expression data depends on standard experimental conditions and baseline expression of HKGs, therefore it is important to identify and verify true HKGs for qRT-PCR analysis. The goal of this study is to identify and confirm HKGs in *Bradyrhizobium japonicum* USDA110, a nitrogen fixing bacterium which forms a symbiotic relationship with *Glycine max,* soybean, to provide the host with a source of nitrogen and receive photosynthetic products in return. By revealing such HKGs, novel insights into gene expression for this agriculturally important species could be provided. To identify potential HKGs, we analyzed previous DNA microarray-based gene expression data for *B. japonicum* under multiple treatments. As a result, we identified 7 constitutively expressed genes among 8,000 genes

across all conditions. Their fold change values were within a range of $-0.25 < x < 0.25$. To validate these potential HKGs, their expression in various experimental treatments was measured, including heat stess, pH stress, and heavy metal stress. The HKGs found in *B. japonicum* could also be applied in closely related species by identifying homologs in other nitrogen-fixing symbiotic bacteria as demonstrated in this study with two photosynthetic bradyrhizobia. Six homologs were found and measured in both *Bradyrhizobium* sp. ORS278 and BTAi1, and two and three genes, respectively, were validated under differing temporal conditions.

## INTRODUCTION

Rhizobia are soil dwelling, Gram-negative bacteria that form a symbiotic relationship with leguminous plants, forming specialized organs known as root nodules in which they fix nitrogen and in turn receive photosynthetic products. These bacteria are of great importance in the agricultural industry because when properly inoculated in farmland soil, they can improve crop yields and in turn, decrease reliance on expensive and harmful nitrogen fertilizers (Peoples et al. 1995). By performing biological nitrogen fixation (BNF), converting atmospheric nitrogen into ammonia, a form of N in which plants can readily use, these bacteria serve as essential components to successful farmland soil. These rhizobia could provide an efficient fertilization alternative to traditional chemical fertilizers which eventually runoff into nearby water sources resulting in ecological damage such as eutrophication events (Crews and Peoples 2004). To better understand nitrogen-fixing bacteria and their symbiotic partner, much effort has been put forth to optimize these relationships in terms of typical environmental stresses, such as oxidative, pH, heat, desiccation-induced, and osmotic stress (Zahran et al. 1994; Rodrigues et al. 2006; Cytryn et al.

2007; Thaweethawakorn et al. 2015). To better characterize the change in metabolic processes in response to different environmental conditions, the expression of the genes involved must be measured. One of the most common and reliable sources of gene expression analysis is qRT-PCR. In the qRT-PCR analysis, gene expression levels must be normalized against typical levels of expression found in the organism. By using a reference gene for quantifying baseline expression levels, a more accurate measurement can be provided for the true expression level of other genes. The reference genes used are typically known as HKGs, which are those that are constitutively expressed during any environmental or cellular condition (Thellin et al. 1999; Lee et al. 2007). Typical HKGs are usually involved in fundamental cell functions like the transport of molecules, various metabolic processes, or the cell cycle, and therefore tend to be ubiquitous in nature (Thellin et al. 1999).

HKGs have been routinely identified and used in other biological systems, both prokaryotic and eukaryotic, and are vital for normalization in gene expression analysis. These genes have been identified by a number of methods such as mathematic modeling, or a meta-analysis comparing expression from high-density oligonucleotide arrays (Warrington et al. 2000; Andersen et al. 2004). With the recent increase in databases harboring mass amounts of gene expression data, it has become easier to perform large-scale analysis to identify potential HKGs (Zhang and Li 2004). When testing new experimental conditions though, the expression of these reference genes may fluctuate slightly depending on the particular investigation and lead to bias or skewed data (Galisa et al. 2012). Therefore, the prospective candidates must then be validated in multiple different tissues or treatments and demonstrate some degree of expressional consistency to be considered a reliable internal control for downstream analysis (Jain et al. 2006).

qRT-PCR is not only a powerful technique to assess gene expression, but a popular method to validate candidate HKGs. This technique measures the amplification of complementary DNA (cDNA) from RNA transcripts via fluorescent probes and provides an accurate depiction of true gene expression due to its high sensitivity and dynamic range (Vandesompele et al. 2002). qRT-PCR is actually preferred over other methods of measuring gene expression such as Northern hybridization, not only because of its sensitivity and range, but precision and efficiency (Desroche et al. 2004; Dankai et al. 2015).

The constitutive nature of the HKG is of importance since it is the standard for which all gene expression data are normalized against, and the improper selection could lead to inaccurate results (McMillan and Pereg 2014). Recently, some have advised against the use of traditional HKGs developed from other studies, as their expression could differ greatly between biological samples (Cheng et al. 2013). Others have claimed that HKGs which are constitutively expressed in all tissues or conditions for a given species do not exist and therefore should be identified and validated prior to each experiment. Here we provide data on various potential HKGs for *Bradyrhizobium japonicum* identified from multiple microarray analyses and validated under new conditions using qRT-PCR. Homologs for these newfound genes were then identified and tested in two photosynthetic bradyrhizobia strains, *Bradyrhizobia* sp. ORS278 and *Bradyrhizobium* sp. BTAi1. Having reliable reference genes in these other rhizobia strains would not only strengthen the notion that identifying internal references for each particular species and experiments is of paramount importance but would also help facilitate reliable gene expression data for studies concerning these organisms. Both ORS278 and BTAi1 are of great interest because of their implications in the nodulation-factor independent symbiotic pathway, which demonstrates a novel

symbiotic engagement that could be applied to hosts other than legumes (Giraud et al. 2007; Beatty and Good 2011).

## MATERIALS AND METHODS

**Bacterial strain growth conditions.** The wild type strains of *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium* sp. ORS278, and *Bradyrhizobium* sp. BTAi1 were cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of $Na_2HPO_4$, 250 mg of $Na_2SO_4$, 320 mg of $NH_4Cl$, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per L (Sadowsky *et al.*, 1987). All strains were incubated aerobically at 30°C with vigorous shaking at 200 rpm for 3 days. Appropriate amount of chloramphenicol antibiotic was added to the media for *B. japonicum* in each test ($\mu g \cdot ml^{-1}$) and nalidixic acid was used in media ORS278.

**Selection of candidate reference genes.** Candidate reference genes were selected by filtering microarray expression data from multiple different treatments in *B. japonicum* to obtain an output of genes with log2 fold change values within a window of $-0.25 < x < 0.25$ (Table 3-1). The program R studio (http://www.rstudio.com) was used to download thirteen *B. japonicum* microarray GEO files from NCBI which included the following conditions: bacteroid state v. free-living, chemoautotrophic v. arabinose supplemented chemoautotrophic growth, chemoautotrophic v. heterotrophic growth, heterotrophic v. arabinose supplemented autotrophic growth, DMSO v. Pterocarpan, $H_2O_2$ prolonged exposure, $H_2O_2$ fulminant shock, high nitrogen v. low nitrogen,

indole-acetic-acid 1mM exposure, minimal v. rich media, paraquat fulminant shock, paraquat prolonged exposure, and salt. The expression values for all genes were also converted to log2 fold change values, and satisfactory genes within the target window were selected for.

**HKG homolog identification.** Homologs from the candidate genes identified in *B. japonicum* were found in both ORS278 and *B.* BTAi1 by performing nucleotide BLAST analysis through NCBI and taking the best hit with the following parameters: e-value cutoff 1e-5, query coverage 50%, and percent identity 35%.

**Heat shock analysis.** *B. japonicum* subcultures were grown in AG media with selective antibiotics and incubated aerobically at 30°C with vigorous shaking at 200 rpm until they reached exponential phase with $OD_{600}$ of 0.8-1.0. Three replicates of 30 ml cultures were heat shocked by incubation in a water bath at 42°C for 30 minutes then RNA was immediately extracted.

**Heavy metal exposure.** *B. japonicum* cultures were grown in AG media with selective antibiotics and incubated aerobically at 30°C with vigorous shaking at 200 rpm. Nine subcultures of 30 ml each with proper selective antibiotics were incubated aerobically at 30°C with vigorous shaking at 200rpm until they reached mid exponential phase with $OD_{600}$ of 0.8. The cultures were then washed by first being centrifuged for 10 minutes at 4,000 rpm and resuspended in AG media. The cultures were centrifuged again to obtain a cell pellet and resuspended in 30 ml of modified AG media with a final concentration of 1mM of either Zn or Cu for each replicate. Three culture replicates of each 1mM metal solution were incubated for 12 hours at 30°C with vigorous shaking at 200rpm then RNA was extracted. Three control replicates were resuspended in normal AG solution and incubated for 12 h at 30°C with vigorous shaking at 200 rpm then RNA was extracted.

**pH stress analysis.** *B. japonicum* cultures were grown in AG media with selective antibiotics and incubated aerobically at 30°C with vigorous shaking at 200 rpm. Subcultures of 30 ml each with proper selective antibiotics were incubated aerobically at 30°C with vigorous shaking at 200rpm until they reached exponential phase with $OD_{600}$ of 0.8-1.0. The cultures were washed by first being centrifuged for 10 minutes at 4,000 rpm and resuspended in AG media. The cultures were centrifuged again to obtain a cell pellet and resuspended in 30 ml of AG media with modified pH of 4.0. Three replicates of cultures were incubated for 30 minutes at 30°C with vigorous shaking at 200rpm then RNA was extracted.

**Photosynthetic bradyrhizobia temporal analysis.** For both *Bradyrhizobium* sp. ORS278 and *Bradyrhizobium* sp. BTAi1, cultures were growth in AG media as described earlier, but under 16 hours light and 8 hours dark cycle. Cultures were harvested and RNA isolated during the middle of each temporal (day and night) point for qRT-PCR analysis.

**RNA isolation.** For heat stress, heavy metal exposure, and pH stress RNA extraction was performed as previously described (Jeon et al. 2011). All cultures were condensed by centrifugation for 20 min at 4°C and 4,000 rpm in a fixed angle rotor. The cell pellet was then collected by decanting the supernatant. RNA isolation followed by using hot phenol method as described previously (Bittner et al. 2003). The RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) was used to purify the isolated RNA according to manufacturer's protocol. RNA quantity was calculated using NanoDrop device (Thermo Scientific) and RNA quality was confirmed via gel electrophoresis.

**qRT-PCR gene expression analysis.** The qRT-PCR was performed with modification according to methods previously described (Chang et al. 2007). Briefly, cDNA was synthesized in

25-µl reaction solution containing 1.5 µl of M-MLV reverse transcriptase (200U/µl; Promega Corp.), 2 µl of random hexamers (250ng/µl; Invitrogen, Carlsbad, CA, U.S.A.), 5 µl of 2.5 mM dNTPs, and 1 µg of total RNA. Of the reaction solution, 1 µl of cDNA was used as a template for qRT-PCR. A total of 10 µl qRT-PCR reactions were prepared using 5 µl of 2 x All-in-One$^{TM}$ qPCR Mix and 0.1 µl ROX Reference Dye by GeneCopoeia (Rockville, MD, U.S.A.), 1 µl at 0.2 µM each of forward and reverse primers, and 1 µl of cDNA template. qRT-PCR was performed by using ABI PRISM 7300 instrument (Applied Biosystems, Foster City, CA, U.S.A.) and following the GeneCopoeia qPCR protocol. Negative control reactions which lacked reverse transcriptase were used to check for DNA contamination. Gene-specific primers for the selected candidate reference genes were designed using Integrated DNA Technologies (Coralville, IA, U.S.A.) company software. The primers used for qRT-PCR are their amplicon length are listed in Table 3-3. The *parA* (bll0631) gene, a chromosomal partitioning protein, was used to normalize the expression of each gene in this study (Cytryn et al. 2007; Donati et al. 2011). All experiments were performed with three biological replicates, and three technical replicates for each biological replicate. Fold induction values were calculated in accordance with the method of Pfaffl (2001).

**Gene expression stability analysis.** Candidate reference gene expression stability was obtained by importing raw expression values for all proposed internal control genes into *geNorm* software (Vandesompele et al. 2002) program and calculating the M-value, which provides the average pairwise variation for a specific gene compared against other reference genes. The *NormFinder* software (Andersen et al. 2004) was also used to further validate the expressional stability of potential HKGs under the new test conditions.

## RESULTS

**Candidate reference genes reflect typical HKGs.** The genes obtained by filtering the microarray expression data from 13 different treatments (Table 3-1) have similar functionality to previous genes categorized as HKGs (Table 3-2). Many of these genes encode for proteins involved in major cellular processes such metabolism, or molecule transport, however, a couple genes (bll3109, blr3561) did not fit typical housekeeping gene conformity.

Since electron transport is integral to common cellular metabolic processes, it makes sense that a few of these candidate HKGs belong to the oxioreductase protein family. blr6296 encodes a probable GDP-mannose 6-dehydrogenase which is an enzyme most likely involved in the breakdown of different sugars but has been characterized to encode for the biosynthesis of alginate (Waite et al. 2005). blr6258 is only annotated as a putative oxidoreductase protein, closer analysis of this protein is required to deduce exactly what cellular process this protein is involved in. bll6396 encodes a putative 2-dehydropantoate 2-reductase which may be responsible for pantothenate biosynthesis, a precursor to Coenzyme-A (Gaballa et al. 2010).

The remaining candidate HKGs detailed in this study are involved in the transferase class of enzymes. bll6306 encodes a probable glycosyltransferase, which belongs to a superfamily of transfer enzymes that are usually responsible for the transmission of a glycosyl group from a sugar to a carbohydrate chain (Saxena et al. 2005). bll8166 encodes a probable phospholipid N-methyltransferase which is involved in the biosynthesis of phosphatidylcholine, a methylated derivative of phosphatidylethanolamine, where both are essential phospholipids in cell membranes (de Rudder et al. 2000).

**blr6358 and bll8166 are constitutively expressed under new test conditions.** blr6358, encoding a putative oxioreductase protein, and bll8166, encoding a probable phospholipid N-methyltransferase maintained fold change expression levels in the approximate target window of $0.8 < x < 1.2$ (log2 fold change $-0.25 < x < 0.25$) under heat stress, Zn and Cu exposure, and pH stress (Table 3-4).

**bll8166 and blr3561 are the most stably expressed internal control genes.** Using both *geNorm* and *NormFinder* software to provide estimates of gene expression stability, bll8166 and blr3561 were found to have the lowest values (most stable). For *geNorm* software, all genes were below the cutoff value of 1.5 (Figure 3-1A & B) to accept them as reliably stably expressed gene (Vandesompele et al. 2002). *NormFinder* showed that most candidate genes had about the same value for average gene expression stability, with bll3109 and blr3561 being the lowest (Fig. 3-2). Though the two programs didn't completely agree as to which gene is most stably expressed, it is worth noting that all genes in the study provided consistent stability expression values.

**bll8166 and blr3561 serve as reliable reference genes when measuring gene expression under new test conditions.** When measuring the expression of 12 genes involved in either heat stress, heavy metal exposure, or pH stress via qRT-PCR, bll8166 and blr3561 both proved to be reliable reference genes showing constitutive expression while the other involved genes were upregulated.

**Six homologs found in ORS278 and BTAi1.** Homologs of for all HKGs in *B. japonicum* were found in both ORS278 and BTAi1 except bll6396 which encodes a putative 2-dehydropantoate 2-reductase (Table 3-4 and Table 3-6).

**Homologous genes are constitutively expressed in ORS278 and BTAi1 under temporal conditions.** For *Bradyrhizobium* sp. ORS278, the genes BRADO0162, encoding a phospholipid N-methyltransferase, and BRADO6616, encoding a putative oxidoreductase were constitutively expressed during both day and night conditions. For *Bradyrhizobium* sp. BTAi1, the genes BBta_1004, encoding a putative glycosyltransferase, BBta_0193, encoding a phospholipid N-methyltransferase, and BBta_2283, encoding a putative short-chain dehydrogenase/reductase were constitutively expressed under temporal conditions.

## DISCUSSION

Soil dwelling nitrogen-fixing bacteria are an integral component of not only microbial ecosystems in native topsoil but of strong agricultural communities as well. These organisms provide an avenue of opportunity to supply plant crops with a biological source of nitrogen instead of using chemically produced fertilizers (Crews and Peoples 2004). By enhancing and optimizing BNF capabilities in the symbiosis between microbes and plants, crop production could be vastly increased without diminishing ecological diversity in the surrounding environment or damaging surrounding ecosystems, all the while promoting a sustainable system for food production (Jensen and Hauggaard-Nielsen 2003).

To successfully apply and better understand this symbiosis, the exact mechanisms of interaction must be defined on a molecular level. That is, a reliable assessment of the expression of particular genes of interest could reveal insight into these microbial processes, and it can be argued that normalization of expression data is the most important aspect, especially when performing downstream analyses (Guenin et al. 2009). Selection of a suitable reference gene or

set of reference genes, as some argue that the use of multiple internal controls is better, is the most reliable way to accurately gauge the expression of a particular gene of interest in a given experiment (Vandesompele et al. 2002; Guenin et al. 2009). This technique has been used is a range of studies from microbes to humans and even in plants, proving its versatility and reliability in multiple systems (Libault et al. 2008; Mehta et al. 2010; Rivera et al. 2014).

In this study, seven candidate reference genes were proposed by taking expression values from a large bank of microarray expression data, then selecting for genes that displayed constitutive expression levels under all conditions. Most of these genes fit the typical conformity of previously described internal control genes as those that are necessary for cell survival and normally used by the organism (i.e. metabolism, transport of molecules, cell cycle, etc.). However, there were a couple of unexpected results, such as the gene bll3109, which is speculated as a calcium-binding protein, probably involved in signal transduction pathways, and blr3561, a hypothetical protein. Though blr3561 is characterized as a hypothetical protein, it does have a bacterial SH3 domain which has been predicted to be involved bacterial cell wall binding and recognition, and possibly even the binding of metals (Kamitori and Yoshida 2015). Still, it would make sense for a calcium-binding protein or a metal-binding protein to be constantly expressed if it plays a role in maintaining homeostatic conditions within the cell.

Though most of the genes proved to be normally expressed under the new conditions outlined is this study with no fold change greater than 2, with the exception of bll6306, a couple of the genes seemed to be more reliable than the others. bll8166 and blr6358 maintained steady expression under heat, pH, and metal stress. These new conditions were not only chosen because

of their absence from the original microarray dataset, but also because these are stressors that *B. japonicum* normally encounters in the environment.

To validate their use as internal controls for gene expression studies, bll8166 and blr6358 were used as reference genes to measure the expression of 12 genes either involved in heat stress, heavy metal exposure, or pH stress via qRT-PCR. Both bll8166 and blr6358 served as reliable reference genes as their expression remained constitutive while the respectively involved genes demonstrated upregulation as predicted. The 12 tested genes were either chosen based on homology to genes documented in other rhizobia species, or for their annotation as being involved in the regulation response of the particular treatment process. For heat stress, heat shock genes and the corresponding sigma factor in the heat shock complex operon were chosen for their role in the stress response. For heavy metal stress conditions, genes for copper tolerance, and zinc metallopeptidase were initially chosen for Cu and Zn heavy metal exposure stress response respectively. A gene encoding a putative monooxygenase is actually a homolog of a cobalt-zinc-cadmium resistance gene (*czc*) found in many soil bacteria including rhizobia, that is thought to mediate zinc resistance in heavy metal polluted environments (Abou-Shanab et al. 2007). The heavy-metal transporting P-type ATP transferase gene was chosen for its role in efflux systems to transport zinc ions across the cytoplasmic membrane in zinc resistant bacteria (Nies 1999). For pH stress two two-component response regulator genes were selected for gene expression analysis, blr0155 (*actR*) and bll0904 (*regR*). These genes are homologs to those found in *Rhizobium meliloti* where they were shown to be a part of a two-component regulatory system involved in acid tolerance response by possible pH sensing (Tiwari et al. 1996). The other two genes chosen for acid stress gene expression analysis are involved in glutathione metabolism. Glutathione

biosynthesis has been shown to be essential for bacterial response to acid tolerance in *Rhizobium tropici* species (Riccillo et al. 2000).

Heat tolerance is a major factor in the survivability of microbes and some will only proliferate within a specific temperature range. The optimum growth temperature for *Bradyrhizobium japonicum* is between 25°C to 30°C and will successfully nodulate and fix nitrogen between these temperatures (Jordan 1982). However, if the temperature rises above the optimum range there are complications in the symbiotic process (i.e. root hair formation, differentiation into bacteroid, nodule development, etc.) and a delay in nodulation, and if it surpasses 42 °C, nodulation of soybean is significantly inhibited (Zahran 1999). The gene *rpoE2*, encoding a putative ECF sigma factor, had been identified in *S. meliloti* and proposed to play a regulatory role in the bacterial response to heat stress, though it is speculated other genes are involved as well (Sauviac et al. 2007).

Metals such as nickel, zinc, copper, and cadmium have been shown to be toxic to rhizobia when exposed under long-term conditions (Chaudri et al. 1992). Much effort has been put forth to diagnose underlying mechanisms of microbial responses to toxic metals in the soil, as the lack of data in the past has caused dispute over regulation of heavy metal concentrations (McGrath et al. 1995; Chaudri et al. 2007; Giller et al. 2009). To maintain a healthy ecosystem, not only must regulations be set to protect the environment, but solutions must be found to the already existing problems of pollution. One study has identified a strain, *Bradyrhizobium* sp. (vigna) strain RM8, to not only be tolerant to toxic Ni and Zn but also reduce the amount of the respective metals in plant organs (Wani et al. 2007). Though it is difficult to compare and validate ecotoxicology

studies between the field and laboratory, more information is needed to consider the implications of heavy metal buildup in the soil (Giller et al. 1998).

Soil pH is an important aspect of crop fertility as it limits the nitrogen fixation process and survival of rhizobia (Graham et al. 1994). A proper range of pH must be maintained for the majority of rhizobia as both alkaline and acidic soils result in growth deficiencies, however, efforts have been made to identify certain strains which may be more tolerant to a wider range of pH and therefore of agricultural interest (Asanuma and Ayanaba 1990; Kulkarni and Nautiyal 2000; Nautiyal et al. 2000). One strain of *Rhizobium tropici* displays exceptional acid tolerance which is linked to the molecule glutathione, which is involved with the activation of potassium efflux pumps to cope with various environmental stresses (Riccillo et al. 2000). As with other types of stressors, bacterial cells also respond to pH stress via the participation of a particular sigma factor. In *S. meliloti*, the sigma factor RpoH1 played a vital role during low pH stress by regulating genes that code for heat shock and chaperone proteins (de Lucena et al. 2010).

Understanding the underlying mechanisms of the heat, heavy metal, and pH stress response and genes involved in overall stress response in these nitrogen-fixing organisms is of great interest as this knowledge could lead to an enhancement in the symbiotic process benefitting agricultural endeavors.

Since *B. japonicum* shares close homology with other nitrogen-fixing species that inhabit the soil and form symbiotic relationships with leguminous hosts, candidate HKGs were also found in the photosynthetic relatives, *Bradyrhizobium* sp. ORS278 and BTAi1 via BLAST. Homologs for six of the seven genes identified and validated in *B.* japonicum were found in ORS278 and BTAi1, with the exception of bll6396, which encodes a putative 2-dehydropantoate 2-reductase.

Finding and validating HKGs in both ORS278 and BTAi1 could be important concerning gene expressions for elucidating the nodulation factor-independent symbiotic pathway that both of these photosynthetic species use to nodulate *Aeschynomene* species (Giraud et al. 2007). Since current work is being performed involving circadian regulation of symbiotic genes on these photosynthetic strains, the candidate HKGs were validated under differing temporal conditions (i.e. night and day). Two genes in ORS278, and three genes in BTAi1 exhibited constitutive expression under these conditions. Interestingly, the two stable genes in ORS278, BRADO0612 encoding a phospholipid N-methyltransferase and BRADO6616 encoding a putative oxidoreductase, and two of the stable genes in BTAi1, BBta_0193 encoding a phospholipid N-methyltransferase and BBta_2283 encoding a putative short-chain dehydrogenase/reductase are both homologs for the validated genes blr6358 and bll8166 in *B. japonicum.* The third stable gene in BTAi1, BBta_1004 encodes a putative glycosyl transferase was also found to be a suitable HKG in *B. japonicum*. This further demonstrates the reliability of these particular genes to serve as internal controls for gene expression studies in diverse conditions. Other experiments focusing on important microbes such as *Sinorhizobium meliloti, Mesorhizobium loti,* or other members of the Bradyrhizobiaceae family could perhaps benefit from using homologs of the seven internal controls genes uncovered here to normalize their expression data.

Seven candidate HKGs were identified and validated within this study for the use of normalizing gene expression data in *Bradyrhizobium japonicum*, and homologs were then determined and tested in *Bradyrhizobium* sp. ORS278 and *Bradyrhizobium* sp. BTAi1. By analyzing mass amounts of microarray data, using qRT-PCR to monitor expression under new treatment conditions, and measuring gene stability with the programs *geNorm* and *NormFinder*,

the genes in *B. japonicum*, bll3109, blr3561, blr6296, bll6306, blr6358, bll6396, and bll8166 have proven to be prime candidate for use as internal controls to normalize expression data when performing qRT-PCR analysis. For ORS278 and BTAi1, the genes BRADO0162, BRADO6616, BBta_1004, BBta_0193, and BBta_2283 appear to serve as reliable controls for future temporal expression studies. Though all of these are suitable for normalization purposes, blr3561, blr6358, and bll8166 showed to have more stability and consistent expression throughout the study for *B. japonicum*. Further analysis of these genes under new conditions would provide support of their efficacy to be used as an internal control.

FIGURES & TABLES

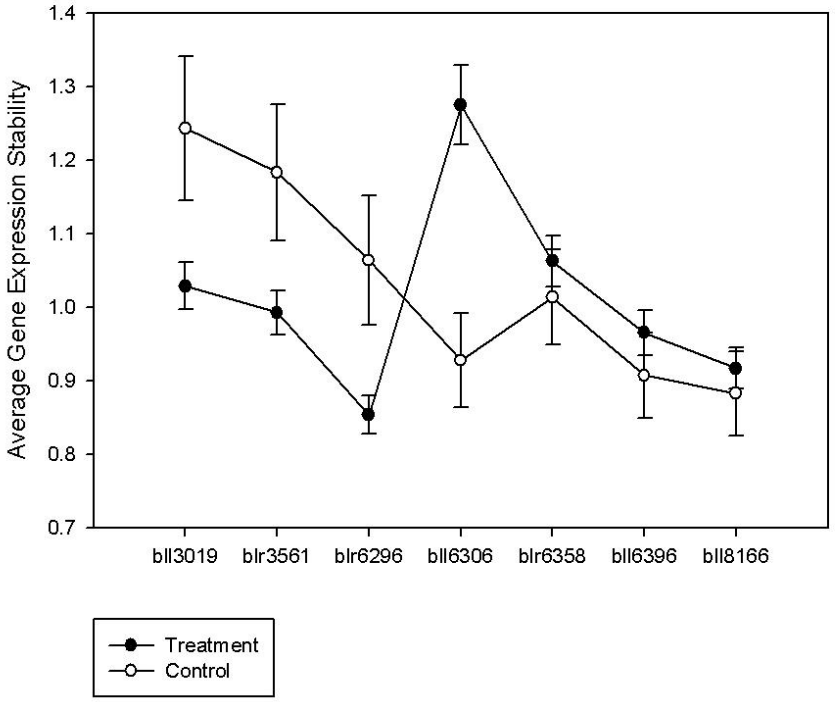

**Figure 3-1A.** M-values as estimated by *geNorm* software for gene expression stability of seven candidate genes in *B. japonicum* under both treatment (filled point) and control (open point) conditions. Vertical bars represent standard error, with n=3.

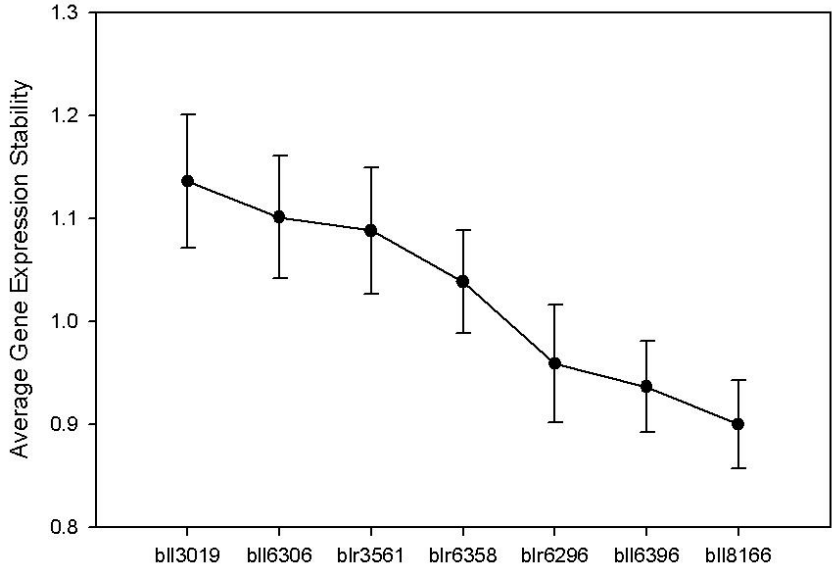


**Figure 3-1B.** M-values as estimate by *geNorm* software for gene expression stability of seven candidate genes in *B. japonicum* under all conditions. Vertical bars represent standard error, with n=3.

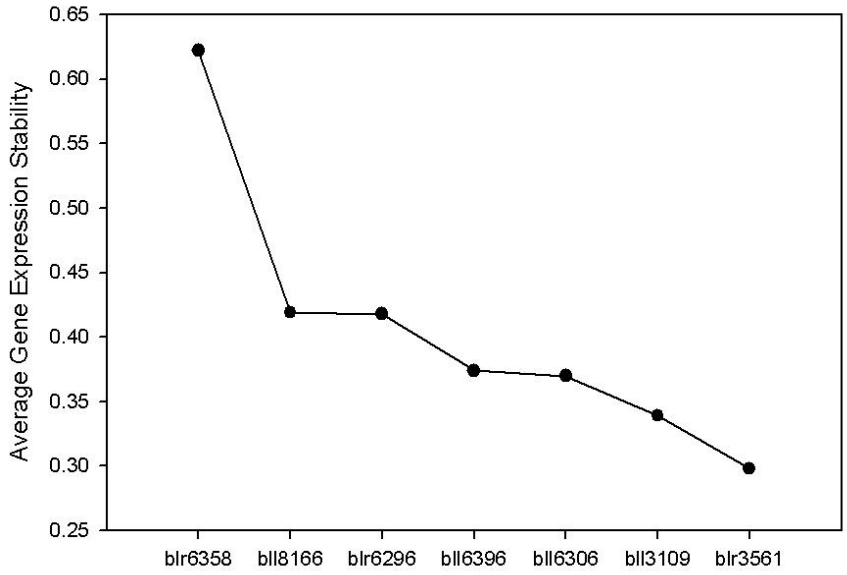


**Figure 3-2.** Average gene expression stability of seven candidate HKGs in *B. japonicum* using *NormFinder* software.

**Table 3-1. Microarray conditions and GEO dataset numbers.**

| **Treatment** | **GEODataset no** |
|---|---|
| Minimal v. rich media | GSE8033 |
| NaCl (50mM) | GSE8036 |
| Bacteroid | GSE8042 |
| Heterotrophy v. arabinose supplemented chemoautotrophy | GSE10295 |
| Chemoautotrophy v. heterotrophy | GSE10296 |
| Chemoautotrophy v. arabinose supplemented chemoautotrophy | GSE10298 |
| Paraquat fulminant shock (5mM for 10 minutes) | GSE26236 |
| Paraquat prolonged exposure (0.1mM) | GSE26252 |
| DMSO v. Pterocarpan 6 (20uM for 24h) | GSE26380 |

| | |
|---|---|
| $H_2O_2$ prolonged exposure (0.3mM) | GSE26960 |
| $H_2O_2$ shock (10mM for 10 minutes) | GSE26961 |
| Indole-3-acetic acid (1mM) | GSE36913 |
| High nitrogen v. low nitrogen | GSE66091 |

**Table 3-2. Candidate reference genes obtained from microarray expression data in *B. japonicum*.**

| Gene | Function |
|---|---|
| bll3109 | putative Ca binding protein |
| blr3561 | hypothetical protein |
| blr6296 | probable GDP-mannose 6-dehydrogenase |
| bll6306 | probable glycosyltransferase |
| blr6358 | putative oxidoreductase protein |
| bll6396 | putative 2-dehydropantoate 2-reductase |
| bll8166 | probable phospholipid N-methyltransferase |

**Table 3-3. List of primers used for candidate reference genes in *B. japonicum*.**

| Gene | Forward primer (5'-3') | Reverse Primer (5'-3') | Amplicon length |
|---|---|---|---|
| parA[a] | TCAACCTTCTGACGGTGAACGC | TGCAGCAATTGCGACAGACCTT | 100 |
| bll3109 | CCGATGTCAAACAGGTCAA | GCAATCCTGTGACCGAAAT | 144 |
| blr3561 | CGTTTAACCTCAGCAGCTAT | TGGTCATCATCCCAATTGTC | 136 |

| | | | |
|---|---|---|---|
| blr6296 | CGACCGTCCATCAGATATTC | GTCTTTCGGTAGGCATGAG | 101 |
| bll6306 | GTTCTGGGAGTCGTGTAATC | ACCAAGTCAGACCATACCT | 102 |
| blr6358 | TTGAAATGGGCCGAGCAA | CAAGAAACCCGCCATCCA | 235 |
| bll6396 | GCTCGTCAAATCCTACCATAC | CCGACTTCCTCCGATAGAA | 126 |
| bll8166 | CTGGATTCGCGATCCTTTG | TATTCGACCAGCGTCAGAT | 186 |

[a] The parA gene (bll0631) served as the reference gene for all qRT-PCR analyses in this study

**Table 3-4. Candidate reference genes in ORS278.**

| Gene | Function |
|---|---|
| BRADO6535 | hypothetical protein |
| BRADO7042 | putative glycosyl transferase, group 1 |
| BRADO0162 | phospholipid N-methyltransferase |
| BRADO3450 | hypothetical protein |
| BRADO7074 | putative virulence factor MviN-like protein |
| BRADO6616 | putative oxioreducatase; putative glucose/ribitol oxioreductase |

**Table 3-5. List of primers used for candidate reference genes in ORS278.**

| Gene | Forward primer (5'-3') | Reverse Primer (5'-3') | Amplicon length |
|---|---|---|---|
| parA[a] | AGGAGATCAAGAGCCTGTG | ATGATCGGGAATTCGACCT | 118 |
| BRADO6535 | GCAACGACGTCATCATCAA | CGGATTTGACGAACCAGAAA | 105 |
| BRADO7042 | CATCATTCACCGCTGCTATC | CAGTCAACGCAATGAACCT | 122 |
| BRADO0162 | TCGTGCAGTTCACCTATTC | GGAAGGTTCATCCAGATCC | 94 |
| BRADO3450 | CGCTCGCCTATCTCTATCG | AGAAGCTGCTCCAATAGGT | 102 |

| | | | |
|---|---|---|---|
| BRADO7074 | ACGCATTCTCTCGCTCTAT | GCGCATGCCAGGTATTATC | 101 |
| BRADO6616 | GCTCGACGTCAATCTCAAC | TTGTAGAACGACTGCTCCT | 144 |
| BRADO1624[b] | ATCATCACCCATCTCGACT | GTCGTGAAGAAGAACGTGAT | 101 |

[a] The parA gene (BRADO0071) served as the reference gene for all qRT-PCR analyses in this study.

[b] The pufL gene (BRADO1624) served as a positive control for qRT-PCR analysis.

**Table 3-6. Candidate reference genes in BTAi1.**

| Gene | Function |
|---|---|
| BBta_4186 | hypothetical protein |
| BBta_1004 | putative glycosyltransferase, group 1 |
| BBta_0193 | phospholipid N-methyltransferase |
| BBta_7236 | hypothetical protein |
| BBta_7353 | UDP-glucose 6-dehydrogenase |
| BBta_2283 | putative short-chain dehydrogenase/reductase |

**Table 3-7. List of primers used for candidate reference genes in BTAi1.**

| Gene | Forward primer (5'-3') | Reverse Primer (5'-3') | Amplicon length |
|---|---|---|---|
| parA[a] | GATCAAGACGGCGGTAAAC | GTATCGATCAGCACCCATTC | 81 |
| BBta_4186 | GGCTCGTACATGAAGATCAG | TGGTAGAGCTGCAGATAGG | 113 |
| BBta_1004 | GGTGAACCAATATCCGAAGG | GAATGGAGATTCGCGTGAC | 95 |
| BBta_0193 | GCGCAATATGTCGATCCTC | GAAGCCCGGATTATATTCGAC | 135 |

| | | | |
|---|---|---|---|
| BBta_7236 | CAATCTGAACATCCGCTCTG | GCTGACATCGCACCATTTAT | 118 |
| BBta_7353 | CGATTTCAAATGGCCCGAT | CGACAACGGACGATAGATG | 88 |
| BBta_2283 | CGTGATCGGCATCAATCTG | CTTGGAGGCGATGTAGGAA | 151 |
| BBta_6431[b] | ATCATCACCCATCTCGACT | GTCGTGAAGAAGAACGTGAT | 101 |

[a] The parA gene (BBta_6807) served as the reference gene for all qRT-PCR analyses in this study

[b] The pufL gene (BBta_6431) served as a positive control for qRT-PCR analysis.

**Table 3-8. Fold change measurements for candidate genes during heat stress, heavy metal exposure, and pH stress in *B. japonicum.***

| Gene | Function | Fold Change[a] | | | |
|---|---|---|---|---|---|
| | | Heat Stress[b] | Cu Stress[c] | Zn Stress[d] | pH Stress[e] |
| bll3109 | putative Ca binding protein | 1.8 ± 0.1 | 0.9 ± 0.1 | 1.4 ± 0.1 | 1.6 ± 0.1 |
| blr3561 | hypothetical protein | 1.6 ± 0.1 | 1.1 ± 0.0 | 1.7 ± 0.1 | 1.1 ± 0.0 |
| blr6296 | probable GDP-mannose 6-dehydrogenase | 1.8 ± 0.1 | 1.3 ± 0.1 | 1.1 ± 0.1 | 1.3 ± 0.0 |
| bll6306 | probable glycosyl transferase | 2.1 ± 0.2 | 1.1 ± 0.1 | 2.3 ± 0.1 | 2.1 ± 0.2 |
| blr6358 | putative oxidoreductase protein | 0.8 ± 0.1 | 1.3 ± 0.2 | 1.4 ± 0.1 | 1.1 ± 0.1 |
| bll6396 | putative 2-dehydropantoate 2-reductase | 1.8 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.0 | 1.7 ± 0.2 |
| bll8166 | probable phospholipid N-methyltransferase | 0.9 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.1 |

[a] Fold change calculations were determined from gene expression values when compared to parA

[b] For heat stress conditions, cultures were incubated at 42°C for 30 min

[c] For Cu exposure conditions, cultures were resuspended in AG media containing 1mM Cu and incubated at 30°C for 12 h
[d] For Zn exposure conditions, cultures were resuspended in AG media containing 1mM Zn and incubated at 30°C for 12 h
[e] For pH stress conditions, cultures were resuspended in AG media with pH 4 and incubated at 30°C for 30 min

**Table 3-9. Fold change measurements for genes during heat stress, heavy metal exposure, and pH stress using blr6358 as a reference gene in *B. japonicum*.**

| Gene | Function | Fold Change[a] | | | |
|---|---|---|---|---|---|
| | | Heat Stress[b] | Cu Stress[c] | Zn Stress[d] | pH Stress[e] |
| blr0676 | heat shock protein | 23.9 ± 2.5 | N/A | N/A | N/A |
| blr5226 | heat shock protein | 23.6 ± 4.1 | N/A | N/A | N/A |
| blr5230 | heat shock protein | 130.5 ± 14.6 | N/A | N/A | N/A |
| blr5231 | sigma32-like factor | 58.1 ± 3.9 | N/A | N/A | N/A |
| bll0700 | heavy-metal transporting P-type ATP transferase | N/A | 7.7 ± 0.3 | 9.7 ± 0.2 | N/A |
| bll2211 | copper tolerance protein | N/A | 180.0 ± 15.7 | 11.5 ± 0.5 | N/A |
| blr3857 | Putative monooxygenase | N/A | 30.0 ± 1.8 | 4.1 ± 0.3 | N/A |
| bll4854 | zinc metallopeptidase | N/A | 136.1 ± 7.4 | 252.1 ± 18.9 | N/A |
| blr0155 | two-component response regulator | N/A | N/A | N/A | 30.6 ± 2.9 |
| bll0668 | glutathione synthetase | N/A | N/A | N/A | 35.0 ± 6.6 |
| bll0904 | Two-component response regulator | N/A | N/A | N/A | 180.4 ± 23.7 |
| bll1162 | Glutathione S-transferase | N/A | N/A | N/A | 9.1 ± 0.8 |

[a] Fold change calculations were determined from gene expression values when compared to blr6358

[b] For heat stress conditions, cultures were incubated at 42°C for 30 min

[c] For Cu exposure conditions, cultures were resuspended in AG media containing 1mM Cu and incubated at 30°C for 12 h

[d] For Zn exposure conditions, cultures were resuspended in AG media containing 1mM Zn and incubated at 30°C for 12 h

[e] For pH stress conditions, cultures were resuspended in AG media with pH 4 and incubated at 30°C for 30 min

*Genes not measured for certain experimental treatments are marked as N/A

**Table 3-10. Fold change measurements for genes during heat stress, heavy metal exposure, and pH stress using bll8166 as a reference gene in *B. japonicum*.**

| Gene | Function | Fold Change[a] | | | |
|---|---|---|---|---|---|
| | | Heat Stress[b] | Cu Stress[c] | Zn Stress[d] | pH Stress[e] |
| blr0676 | heat shock protein | 41.9 ± 6.1 | N/A | N/A | N/A |
| blr5226 | heat shock protein | 23.6 ± 3.6 | N/A | N/A | N/A |
| blr5230 | heat shock protein | 174.1 ± 17.6 | N/A | N/A | N/A |
| blr5231 | sigma32-like factor | 106.2 ± 12.5 | N/A | N/A | N/A |
| bll0700 | heavy-metal transporting P-type ATP transferase | N/A | 50.5 ± 3.7 | 61.9 ± 2.5 | N/A |
| bll2211 | copper tolerance protein | N/A | 127.1 ± 9.1 | 8.4 ± 0.4 | N/A |
| blr3857 | Putative monooxygenase | N/A | 22.6 ± 0.8 | 21.5 ± 0.9 | N/A |
| bll4854 | zinc metallopeptidase | N/A | 24.0 ± 1.2 | 140.0 ± 4.7 | N/A |
| blr0155 | two-component response regulator | N/A | N/A | N/A | 138.5 ± 30.3 |
| bll0668 | glutathione synthetase | N/A | N/A | N/A | 104.7 ± 21.7 |
| bll0904 | Two-component response regulator | N/A | N/A | N/A | 36.5 ± 5.2 |
| bll1162 | Glutathione S-transferase | N/A | N/A | N/A | 58.4 ± 7.8 |

[a] Fold change calculations were determined from gene expression values when compared to bll8166

[b] For heat stress conditions, cultures were incubated at 42°C for 30 min

[c] For Cu exposure conditions, cultures were resuspended in AG media containing 1mM Cu and incubated at 30°C for 12 h

[d] For Zn exposure conditions, cultures were resuspended in AG media containing 1mM Zn and incubated at 30°C for 12 h

[e] For pH stress conditions, cultures were resuspended in AG media with pH 4 and incubated at 30°C for 30 min

*Genes not measured for certain experimental treatments are marked as N/A

**Table 3-11. Fold change measurements for candidate HKGs during day and night in *Bradyrhizobium* sp. ORS278.**

| Gene | Function | Fold Change[a] |
|---|---|---|
| BRADO6535 | hypothetical protein | 0.81 ± 0.3 |
| BRADO7042 | putative glycosyl transferase, group 1 | 0.64 ± 0.2 |
| BRADO0162 | phospholipid N-methyltransferase | 0.98 ± 0.1 |
| BRADO3450 | hypothetical protein | 1.64 ± 0.1 |
| BRADO7074 | putative virulence factor MviN-like protein | 0.29 ± 0.8 |
| BRADO6616 | putative oxioreducatase; putative glucose/ribitol oxioreductase | 1.12 ± 0.0 |

[a] Fold change calculations were determined from gene expression values when compared to parA gene. Day replicates served as normal conditions while night replicates served at treatment conditions.

**Table 3-12. Fold change measurements for candidate HKGs during day and night in *Bradyrhizobium* sp. BTAi1.**

| Gene | Function | Fold Change[a] |
|---|---|---|
| BBta_4186 | hypothetical protein | 0.56 ± 0.2 |
| BBta_1004 | putative glycosyltransferase, group 1 | 1.24 ± 0.1 |
| BBta_0193 | phospholipid N-methyltransferase | 1.03 ± 0.1 |
| BBta_7236 | hypothetical protein | 1.57 ± 0.2 |
| BBta_7353 | UDP-glucose 6-dehydrogenase | 1.63 ± 0.3 |
| BBta_2283 | putative short-chain dehydrogenase/reductase | 0.95 ± 0.1 |

[a] Fold change calculations were determined from gene expression values when compared to parA gene. Day replicates served as normal conditions while night replicates served at treatment conditions.

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CHAPTER 4

# DRAFT SEQUENCE AND COMPARITIVE GENOME ANALYSIS OF A NOVEL *AESCHYNOMENE* LEGUME SYMBIONT, *BRADYRHIZOBIUM OLIGOTROPHICUM* BO-1

**ABSTRACT**

*Bradyrhizobium oligotrophicum*, previously known as *Agromonas oligotrophica*, is a soil dwelling, nitrogen-fixing bacterium. Typically, *B. oligotrophicum* strains are isolated from rice paddy soil. However, in this study we report a novel strain, *B. oligotrophicum* BO-1 isolated from the root nodules of *A. indica.* Using comparative genome analysis, the BO-1 strain was compared with the *B. oligotrohicum* S58 strain, and other closely related species. This novel strain shares similar interesting features with other photosynthetic bradyrhizobia (i.e., ORS278 and BTAi1),

including NF-independent symbiosis and the ability to nodulate both root and stems of *A. indica*. Compared to *Bradyrhizobium oligotrophicum* S58, BO-1 has different metabolic phenotypes as it is auxotrophic for L-alanine, but prototrophic for cysteine, proline, serine, threonine, and Coenzyme A. BO-1 also possesses glucose utilizing pathways which may have implications for its ecological niche. BO-1 genome, assembled into 25 contigs, has a size of 7,948,507 bp, with 65.27 % GC content, and 6,996 predicted protein-coding genes.

## INTRODUCTION

*B. oligotrophicum* S58, originally deemed *Agromonas oligotrophica,* is a budding soil microbe able to grow in extraoligotrophic environments (Ohta and Hattori 1983; Okubu et al. 2013). *B. oligotrophicum* S58 was isolated from paddy field soil and is thought to function in the cycling of nutrients and decomposition of organic matter because of its sensitivity to organic compounds (Ohta and Hattori 1983; Ryuda 2011; Okubu et al. 2013). It also serves a role in plant interactions as it can form both root and stem nodules on the semi-aquatic legume, *A. indica*, where it performs nitrogen fixation (Okubu et al. 2013). Interestingly it can also be found in high concentrations in the roots of rice, but it is unknown whether it supplies the plant with a nitrogen source (Kennedy 2005). The organism was recently reclassified as *B. oligotrophicum* based on DNA-DNA hybridization, phylogenetic organization, and phenotypic analysis, and reflects other photosynthetic bradyrhizobia due to its symbiotic interactions with *A. indica*, and close phylogenetic relation to other members of the clade such as *Bradyrhizobium* sp. ORS278 and *Bradyrhizobium* sp. BTAi1 (Ramirez-Bahena et al. 2013).

Photosynthetic bradyrhizobia are a novel anomaly in the symbiotic world as their mutualistic association with their host legumes have not been fully characterized. Typically, rhizobia genomes contain *nod* genes which encode lipochito-oligosaccharides, also known as nod factors (NF) and were once thought to be the required molecule to initiate the production of the symbiotic organ on plant roots known as a nodule (Geurts et al. 2005). However, the discovery of *nod* gene lacking strains that could still form nitrogen-fixing nodules such as *Bradyrhizobium* sp. ORS278 and BTAi1 lead researchers to believe that there must be an alternative symbiotic pathway, or NF-independent pathway (Giraud et al. 2007). Much work has been done to elucidate the mechanism for which these microbes form or initiate mutualistic nodule formation with their host *Aeschynomene* legumes as its understanding and characterization could perhaps have implications to moving this system to other crops (Charpentier and Oldroyd 2010; Bonaldi et al. 2010; Arrighi et al. 2012). *Bradyrhizobium oligotrophicum* may have a role to play in this enigma as it too also lacks *nodABC* genes but is still able to nodulate the stem and roots of *A. indica* (Saito et al. 1998; Okubu et al. 2013).

Rhizobia interactions with their host are very specific, as most strains can only form a symbiosis with a particular genus or species of legume (Bohlool and Schmidt 1974; Hirsch et al. 2001). Sorting out the differences and identifying conserved features between closely related species could reveal insight into not only what dictates host specificity, but also the underlying mechanisms of the symbiotic pathway. Genomic comparison between *Bradyrhizobium oligotrophicum* and its close relatives, ORS278 and BTAi1, revealed the presence in genes encoding type III secretion system in *B. oligotrophicum*, which aren't typically found in *nod* gene lacking strains (Mornico et al. 2012; Okubu et al. 2013). Though it is not known if the type III

secretion system is utilized in *B. oligotrophicum* and *A. indica* symbiosis, it has been shown to play a role in other plant-rhizobia symbiotic interactions (Marie et al. 2001; Okazaki et al. 2013). Genome analysis between all three strains (S58, ORS278, and BTAi1) did reveal highly conserved sequences for genes involved in nodulation, nitrogen fixation, and photosynthesis (Okubu et al. 2013). It is expected that genes involved in nitrogen fixation would be conserved among *Aeschynomene* associated symbionts, however, the conservation of the photosynthetic gene cluster in the nod factor lacking strains is interesting. Indeed, it has been shown that bacterial photosynthesis plays a role in *ex planta* cell survival and symbiotic efficiency, but it would be interesting to consider the regulation of symbiotic genes in a circadian fashion considering light input can regulate other cellular processes (Taylor and Zuhlin 1999; Giraud et al. 2000; Voigt 2006; Moglich et al. 2009; Bonaldi et al. 2010).

By sequencing and analyzing new strains of photosynthetic Bradyrhizobia, a better understanding of important symbiotic features, such as the circadian regulation of photosynthesis and nitrogen fixation, can be determined by comparison. In this study, the novel strain, *B. oligotrophicum* BO-1, is proposed based upon its origin of isolation, phenotypic characterization, and comparative genome analysis with *B. oligotrophicum* S58, *Bradyrhizobium* sp. ORS278 and BTAi1. Analyzing the genome of BO-1 and evaluating the conservation of genes involved in photosynthesis, nitrogen fixation, and the maintenance of the circadian clock will provide information as to whether other photosynthetic Bradyrhizobia similar to ORS278 share the features of circadian regulation of symbiotic genes.

## MATERIALS AND METHODS

**Isolation of BO-1 from *A. indica* root nodules.** *A. indica* root nodule samples (Figure 4-1) obtained from Suwon, South Korea (37.25859° N, 127.016° E) were initially washed with six changes of sterile water to remove residual soil and debris. Nodules were pooled in a sterile petri dish, rinsed with 100% EtOH, and immersed in 0.1% acidified $HgCl_2$ for 10 min. The nodules were then washed thoroughly in six changes of sterile water. Approximately 0.5 g of nodules were then placed in a micro-centrifuge (1.5 ml) tube and 500 µl of sterile water was added. Next the nodules were aseptically crushed with a sterilized wooden applicator stick, then vortexed to mix. Serial dilutions (1/10 and 1/100) of the crushed nodule mixture were made and 100 µl from each mixture (original included) was dispensed and spread on YEM agar plates. The YEM agar plates (pH 6.8) consisted of 0.5 g of $K_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 10 g of mannitol, 0.5 g of yeast extract, and 15 g of agar per liter with 0.002% actidione (cycloheximide) to inhibit fungal growth (Vincent 1970). Plates were incubated at 30°C and observed for colony formation and growth (about 3 days for rhizobial growth to appear). Selected colonies were re-streaked on arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of $Na_2HPO_4$, 250 mg of $Na_2SO_4$, 320 mg of $NH_4Cl$, 180 mg of $MgSO_4 \cdot 7H2O$, 10 mg of $CaCl_2$, 4 mg of FeCl3, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate, and 15 g of agar per L (Sadowsky et al. 1987).

**Bacterial strains and growth conditions.** Colonies isolated from *A. indica* nodules were transferred from the YEM agar plates and grown in AG media. All strains were incubated aerobically at 30°C with vigorously shaking at 200 rpm for 3 days.

**Pouch experiment and *A. indica* inoculation.** *Aeschynomene indica* seeds were surfaced sterilized by immersion in concentrated sulfuric acid (99 %) for 40 minutes. Seeds were then washed thoroughly in at least six changes of sterile water and left to soak in sterile water over night. Seeds were then aseptically transferred onto 0.8 % water agar plates, covered in foil, and incubated in the dark at 30°C. Germinated seeds that have their roots emerged approximately 1.5 cm were selected and placed into sterilized plastic pouches (Mega International) and a total of three seeds were placed in one pouch (Halverson and Stacey 1986). A straw was placed into either end of a pouch where it left the space between its bottom end and the bottom of a pouch approximately 5 cm. Two pouches (with the straw side facing out) were placed into a hanging paper folder using paper clips to attach them together, then this folder was placed into a folder rack. To prepare the inoculum for the pouch experiment, *Bradyrhizobium oligotrophicum* BO-1 strain was grown in 20 ml of AG medium at pH 6.8 and incubated aerobically at 30°C with vigorous shaking at 200 rpm until the culture reached the mid-log phase (0.8 $OD_{600}$). Cells were harvested by centrifugation at 4,000 rpm for 10 min and washed with half-strength nitrogen-free Broughton and Dilworth (B&D) medium (pH 6.8) containing 500 µM $CaCl_2$, 250 µM $KH_2PO_4$, 250 µM $K_2HPO_4$, 5 µM Fe-citrate, 125 µM $MgSO_4 \cdot 7H_2O$, 125 µM $K_2SO_4$, 0.5 µM $MnSO_4 \cdot H_2O$, 1 µM $H_3BO_3$, 0.25 µM $ZnSO_4 \cdot 7H_2O$, 1 µM $CuSO_4 \cdot 5H_2O$, 0.05 µM $CoSO_4 \cdot 7H_2O$, and 0.05 µM $Na_2MoO_4 \cdot 2H_2O$ (Lee et al. 2012). Strain suspension in the half-strength B&D medium was adjusted to have an $OD_{600}$ of 0.1 (ca. $1x10^8$ cells/ml). After pouches and culture were prepared, each pouch was nourished with 20 ml of half-strength B&D medium. For BO-1 culture, 1.0 ml of 0.1 OD600 of culture was inoculated on each seed. All pouches were incubated in a growth

chamber with 16 h of day and 8 h of night at 27°C. All pouches were watered with 20 ml of half-strength B&D medium every 2 days, and routinely observed for nodules.

**Sequencing.** Whole genome sequencing of *Bradyrhizobium oligotrophicum* BO-1 was performed The University of Texas - Austin Genome Sequencing and Analysis Facility (GSAF) on Illumina MiSeq platform with PE 2X300 bp run specifications. DNA was extracted from cultures grown on AG plates after the strain had been evaluated for ability to nodulate its host.

**Read quality filtering, assembly, and annotation.** Reads were quality filtered using BBDuk (Bushnell), removing Illumina artifacts and PhiX. Before trimming, reads with quality scores less than 10 were discarded, and after trimming reads shorter than 100bp were removed. Assembly of filtered reads was performed using SPAdes 3.10.1 and assembly quality assessed via QUAST 4.6.1 (Bankevich et al. 2012; Gurevich et al. 2013). Functional annotation and gene prediction was performed through Department of Energy Joint Genome Institute (DOE-JGI) Microbial Genome Annotation Pipeline (Huntemann et al. 2015).

**Genome analysis and comparison.** All genome analysis and functional comparison tools such as genome statistics, synteny, calculating average nucleotide identity, PCA analysis, identifying shared genes, and BLAST were performed through DOE-JGI's Integrated Microbial Genomes – Expert Review (IMG-ER) platform (Markowitz et al. 2009). The IMG-ER platform is an online database and workstation that allows users to review and curate both new and publicy available microbial genome annotations. The tools available through the Expert Review program allows for comparative analyses of genomes, which can improve previous annotations that have gaps. The IMG platform is also set up to support the inclusion of metadata for microbial genomes so that a better characterization and organization of organisms can occur (Markowitz et al. 2009).

In this study, default parameters were used for all genome analysis tools in IMG-ER. Phylogenetic analysis of 16S rDNA was performed using Bayesian phylogenetic software BEAST.

## RESULTS

**Plant nodulation phenotype.** *Bradyrhizobium oligotrophicum* BO-1 produced nodules on both stem and roots of *Aeschynomene indica* (Figure 4-2). Nodules produced exhibited pink internal color, indicating the presence of leghemoglobin (Figure 4-3).

**Sequence and assembly quality.** Illumina MiSeq generated 2,749,369 reads for a total of 824.8 Mbp and approximately 103X coverage. Assembly of *Bradyrhizobium oligotrophicum* BO-1 reads resulted in 25 contigs totaling over 7.95Mbp with an N50 value of 763,657 (Table 4-1).

**Genome structure and statistics.** After being annotated through JGI's Microbial Genome Annotation Pipeline, *Bradyrhizobium oligotrophicum* BO-1 genome has a total size of 7,948, 507 bp with 65.28 % GC content and is comprised of 6996 protein coding genes (4842 protein coding genes with COGs) with 1 rRNA gene, and 51 tRNA genes. Genome information for *Bradyrhizobium oligotrophicum* BO-1 and S58, and *Bradyrhizobium* sp. ORS278 and BTAi1 is listed in Table 4-2.

**Genome comparison of BO-1 with S58, ORS278, and BTAi1.** *Bradyrhizobium oligotrophicum* strains (BO-1 and S58) share 6685 genes and BO-1 contains 311 unique genes and 406 absent genes (Figure 4-4). Metabolic phenotypes found in BO-1 and not S58 include: L-alanine auxotroph, L-cysteine prototroph, L-proline prototroph, L-serine prototroph, L-threonine prototroph, Coenzyme A prototroph, and is also glucose utilizing (Table 4-3). The genomes of BO-1, S58, ORS278, and BTAi1 share 5587 genes, where BO-1 contains 203 unique genes (Figure

4-5). The photosynthetic gene cluster, nodulation, and nitrogen fixation genes were all found to be conserved in BO-1, however the second pair of circadian genes, *kaiBC*, were less conserved (<50% identity) in BO-1 (Table 4-7). Average nucleotide identity (ANI) between RDA-1 shows the highest similarity with S58 (98.7%), and approximately the same for ORS278 (87.8%) and BTAi1 (87.6%) (Table 4-8). Genome clustering via Principal Components Analysis (PCA) shows a separation between all four genomes, with ORS278 and BTAi1 being the closest together (Figure 4-6). Distance tree based on 16S rDNA alignments with several members from *Bradyrhizobia* genus was generated using Bayesian phylogenetic software, BEAST. The tree shows that BO-1 clusters with similar photosynthetic nitrogen-fixing strains (Figure 4-7). Genome synteny assessed via Dot Plot shows that BO-1 resembles S58 more than ORS278 as the comparison between BO-1 and ORS278 shows more inversions and translocations (Figure 4-8).

**DISCUSSION**

*Bradyrhizobium oligotrophicum* BO-1, though highly similar to *Bradyrhizobium oligotrophicum* S58, does have a few unique characteristics that provide some insight into the photosynthetic nitrogen-fixing *Aeschynomene* symbionts. These photosynthetic bradyrhizobia popularized for lacking the canonical *nod* genes, nodulate both stem and roots of *Aeschynomene indica* and belong to cross-inoculation group III (Alazard 1985; Giraud and Fleischman 2004). Based upon genomic analysis and comparison to related species and inoculation tests, *Bradyrhizobium oligotrophicum* BO-1 belongs to the same cross-inoculation group that includes its closest relative S58, as well *Bradyrhizobium* sp. ORS278 and BTAi1. Organisms from this

group are of great interest because of their role in elucidating the mechanism of nodulation factor independent symbiosis.

The four organisms in this study, BO-1, S58, ORS278, and BTAi1 though all have slightly different genome structure and sizes, show conservation in areas that are considered integral for symbiotic photosynthetic bradyrhizobia, such as photosynthesis, nitrogen fixation, and nodulation. *Bradyrhizobium oligotrophicum* BO-1 does differ from other strains of the same species because unlike the others which were isolated from paddy field soil, BO-1 was isolated from an *Aeschynomene indica* nodule. It would be interesting to isolate and sequence other *B. oligotrophicum* strains from host nodules to see if there is any observable differences. The metabolic phenotypic differences found in BO-1 as compared with S58 may help explain BO-1's survivability in particular habitats. For instance, BO-1 may not thrive in similar environments as its close relatives do and has adapted specific strategies for better survival where *A. indica* resides.

BO-1 was found to be auxotrophic for L-alanine which could have implications in the signaling pathway between microbe and host. The vitamin D-biotin is in part synthesized from L-alanine and has been shown to be a signal molecule between rhizobia and plant (Palacios et al. 2014). The need for biotin synthesis may be negated if the bacterium uptakes and stores plant produced biotin, which has been shown to promote growth and competition in the rhizosphere (Heinz et al. 1999; Hofmann et al. 2000). Other metabolic differences observed from the genome of BO-1 include prototrophy for L-cysteine, L-proline, L-serine, L-threonine, coenzyme A and glucose utilization via the standard Embden-Meyerhof pathway. L-cysteine is a precursor to glutathione which has shown to be important in detoxifying reactive oxygen species during the initiation of symbiosis between plant growth promoting bacteria and their host (Pauly et al. 2006;

Adediran et al. 2016). Though it is generally considered that amino acid biosynthesis is important for bacterial survival in diverse conditions, L-proline biosynthesis was specifically found to be necessary for *Sinorhizobium meliloti* to form healthy nitrogen fixing nodules with its host legumes, alfalfa and sweet white clover, likely due to its demand during bacteroid differentiation and subsequent proliferation (diCenzo et al. 2015). Furthermore, conservation of L-proline biosynthesis has been observed in *Bradyrhizobium japonicum* and it is likely necessary for efficient symbiosis between similar microbes and legumes (King et al. 2000).

Currently, there is no evidence for L-serine or L-threonine to be linked to successful symbiosis, however, it is possible the L-serine may play a role in regulating 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity which is associated with plant growth promoting microbes (Glick 2005). Coenzyme A has no documented link to rhizobia-legume symbiosis, but given its wide applicability likely serves a role in multiple pathways and provides the organism with metabolic versatility. BO-1 has glucose utilizing capabilities via the Embden-Meyerhof pathway which has been shown in other strains to be a more efficiency source of energy than those using other sugar metabolism pathways and also resulted in shorter generations and greater nitrogen-fixing rates (Mulongoy and Elkan 1977). Taken together, the different metabolic phenotypes of BO-1 suggest that it may be more suited for a symbiotic lifestyle than S58, which agrees with the origins of isolation of the respective strains. It would be interesting to co-inoculate *A. indica* with both BO-1 and S58, then isolate and grow the bacteria from the nodules to compare respective nodule occupancy. This may help to show whether BO-1 is truly more suited for host-association if it can outcompete similar strains.

Looking at the raw statistics between the genomes of *Bradyrhizobium oligotrophicum* BO-1 and S58, and *Bradrhizobium* sp. ORS278 and BTAi1, the genomes are all very similar. All four genomes are approximately the same size between 7.5Mbp and 8.5Mbp, and amount of protein coding genes around 6700-7700, where BTAi1 has the largest size and most protein coding genes and is also the only strain containing a plasmid. ORS278 has the smallest genome size and least amount of protein coding genes, and S58 and BO-1 fit in between which accurately reflects the phylogeny of the four species. Average nucleotide identity (ANI) comparison between strains shows approximately 87% between each two strains except BO-1 and S58 which are approximately 98%. ANI has been shown to be comparable to 16S rRNA and DNA-DNA hybridization for species differentiation between prokaryotes, where ANI values of 95-96% represent the threshold for species delineation (Kim et al. 2014). Between the genomes, more than 70% of the genes have homologs found in each other species, where BO-1 has the least number of unique genes and one less rRNA gene than the other genomes.

Since these strains all share the unique ability to perform photosynthesis, and fix nitrogen in both root and stem nodules of *Aeschynomene indica*, their symbiotic pathway may involve some circadian regulation to optimize the process. The oxygen sensitivity of the nitrogenase enzyme, responsible for the microbe's nitrogen-fixing abilities, forces microoxic conditions on the nodule environment (Gallon 1981; David et al. 1988; Soupene et al. 1995). Plant produced leghemoglobin which gives the nodules interior the characteristic pink-red color, helps to sequester oxygen so that nitrogen fixation can be performed (Wittenberg 1974). Though nitrogen-fixation is tightly regulated by a generally conserved network including the *nif* and *fix* genes, the exact mechanisms can differ depending on the species and host (Dixon and Kahn 2004). This intricate management

ensures that the energetically expensive, and oxygen sensitive nitrogen-fixing process is performed efficiently. However, since the discovery of the photosynthetic clade of bradyrhizobia that can fix nitrogen in green-tissue nodules, it has not been fully understood how these microbes manage the excess oxygen produced as a photosynthetic byproduct during nitrogen-fixation.

After a large-scale Tn5 mutagenesis was performed in *Bradyrhizobium* sp. ORS278 to uncover genes involved in the nodulation-factor independent symbiotic pathway, a number of nodulation deficient or nitrogen-fixing deficient phenotypes were produced (Bonaldi et al. 2010). Of the mutagenized genes that produced nitrogen-fixing deficient phenotypes, two were found to possibly implicated in regulation of an internal circadian clock. BRADO4470 encodes a homolog of a negative feedback protein in a cyanobacterial species, and BRADO3946 is a putative histidine kinase with global regulatory roles and protein domains associated with photosynthesis, nitrogen fixation, and regulation via light input. Potentially, these genes may work in conjunction with the circadian clock to temporally regulate nitrogen fixation and photosynthesis so that the nitrogenase enzyme can function at night to avoid inactivation from oxygen produced via photosynthesis (Bonaldi et al. 2010). Homologs of these proteins were found in both *B. oligotrophicum* BO-1 and S58, with high identity (>95%) indicating a level of conservation in the photosynthetic bradyrhizobia. It is interesting to note however, that two homologs of both primary circadian clock genes, *kaiBC*, are found in both ORS278 and BTAi1, but only one copy of each is highly conserved in BO-1 and S58, with the second homolog having less than 50% similarity to the original in ORS278. Though, it is not known whether the conservation or loss of this second homolog could affect the efficacy of circadian regulation of nitrogen-fixing genes. Temporal mechanistic control of nitrogen fixation has been documented in certain cyanobacterial species but would be a novel

discovery in a symbiotic context for rhizobia (Berman-Frank et al. 2001; Rabouille et al. 2014). Since symbiotic initiation comprises a complex exchange of molecular information between microbe and host and is not yet fully understood in the nodulation factor independent symbiotic pathway, circadian regulation may play a role. Indeed, circadian regulation of bacterial entry into the plant could improve efficiency considering optimization of available energy and environmental conditions.

The sequencing and analysis of *Bradyrhizobium oligotrophicum* BO-1 has added information to the photosynthetic clade of bradyrhizobia and could influence the understanding of their unique symbiosis with *Aeschynomene* legumes in the future. Sequencing and analysis of more photosynthetic Bradyrhizobia strains could show conservation of important mechanisms related to the regulation of symbiotic genes. Further analysis of this particular strain needs to be conducted in order to better characterize its role in both the free-living state as well as in the symbiotic context. It would be interesting to see if BO-1 can undergo an endophytic life in rice roots as S58 does, and whether or not the metabolic phenotypic differences observed in the genome between the two strains results in different environmental survival and proliferation. Inoculating rice roots with BO-1 and physically confirming an endophytic presence would then allow the investigation into whether or not the microbe actively fixes nitrogen and provides it to the plant. Growing BO-1 in different types of media and/or environments, then evaluating their symbiotic efficiency could demonstrate the organism's survivability and inclination towards a host-associated niche.

FIGURES & TABLES

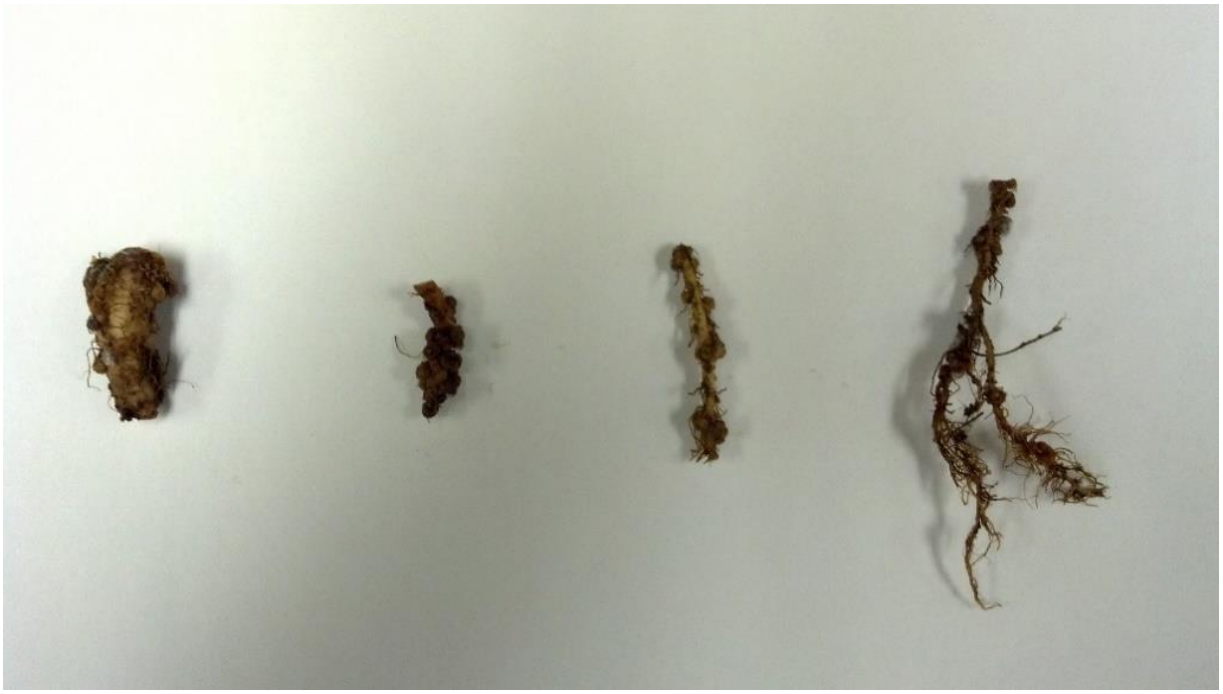

**Figure 4-1.** Root nodule samples of *A. indica* from which BO-1 strain was isolated.

**Figure 4-2.** *A. indica* inoculated with *B. oligotrophicum* BO-1.

**Figure 4-3.** *A. indica* inoculated with *B. oligotrophicum* BO-1 root nodule interior.

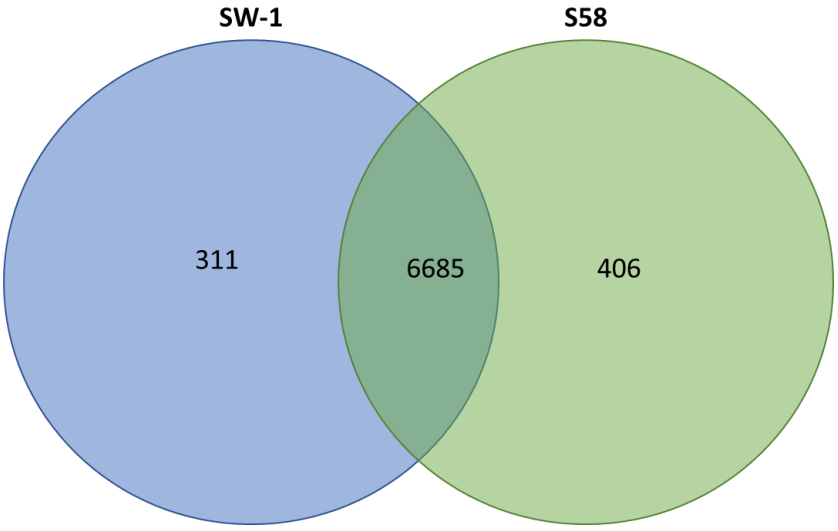


**Figure 4-4.** Venn diagram of shared genes between *Bradyrhizobium oligotrophicum* BO-1 and S58 genomes.

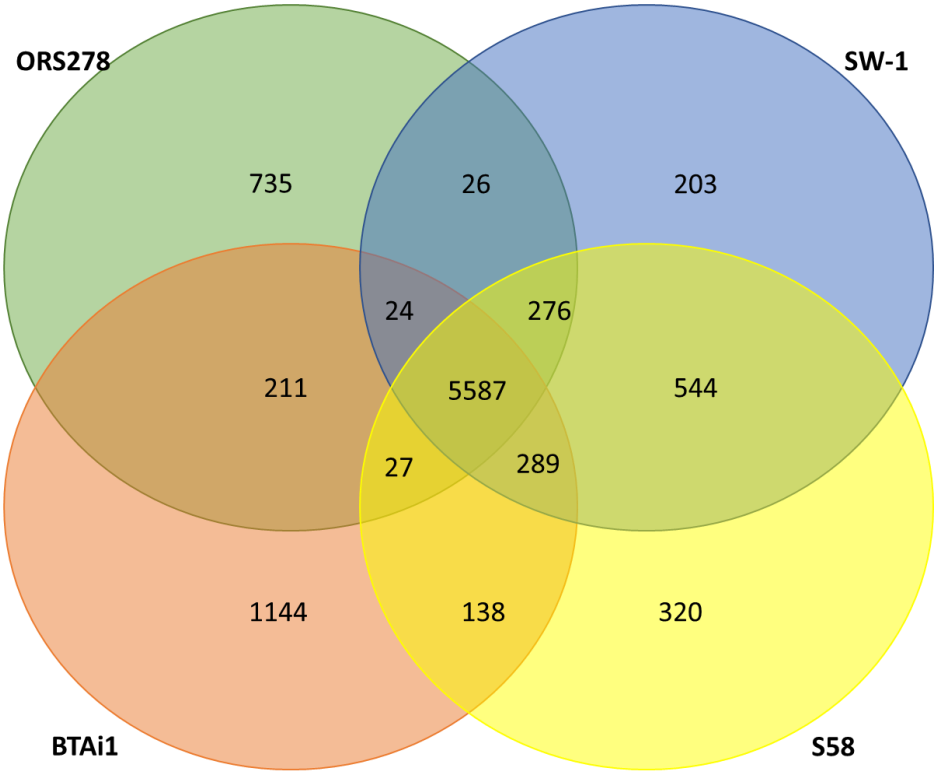


**Figure 4-5.** Venn diagram of shared genes between *Bradyrhizobium oligotrophicum* BO-1 and S58 and *Bradyrhizobium* sp. ORS278 and BTAi1 genomes.

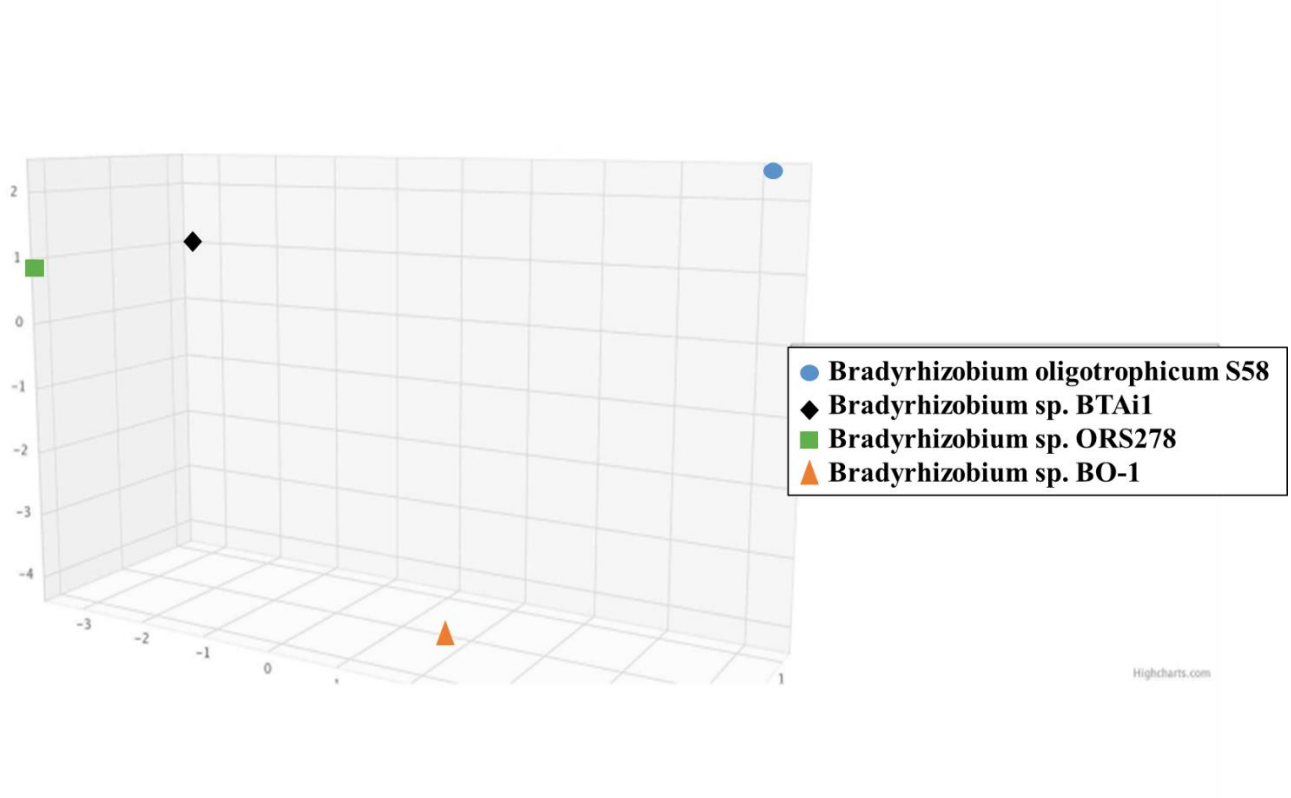


**Figure 4-6.** PCA analysis of genome clustering for *B. oligotrophicum* BO-1 and S58, and *Bradyrhizobium* sp. ORS278 and BTAi1. Note: stain RDA1-1 signifies *B. oligotrophicum* BO-1.

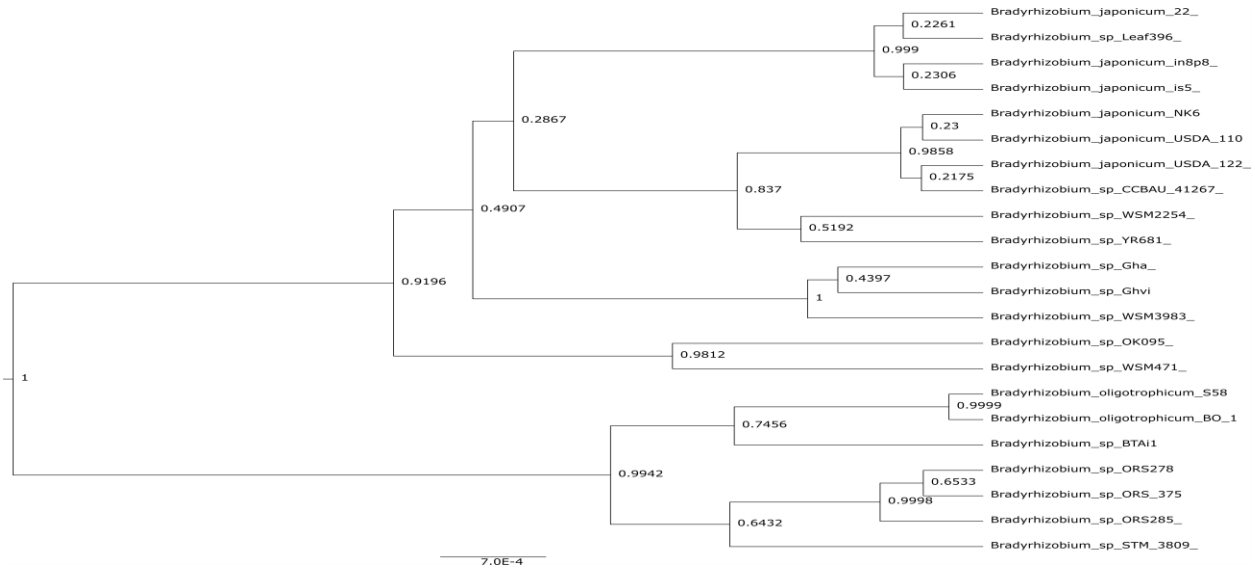


**Figure 4-7.** Phylogenetic tree of various *Bradyrhizobium* species based on 16S rDNA sequences using Bayesian phylogenetic software BEAST.

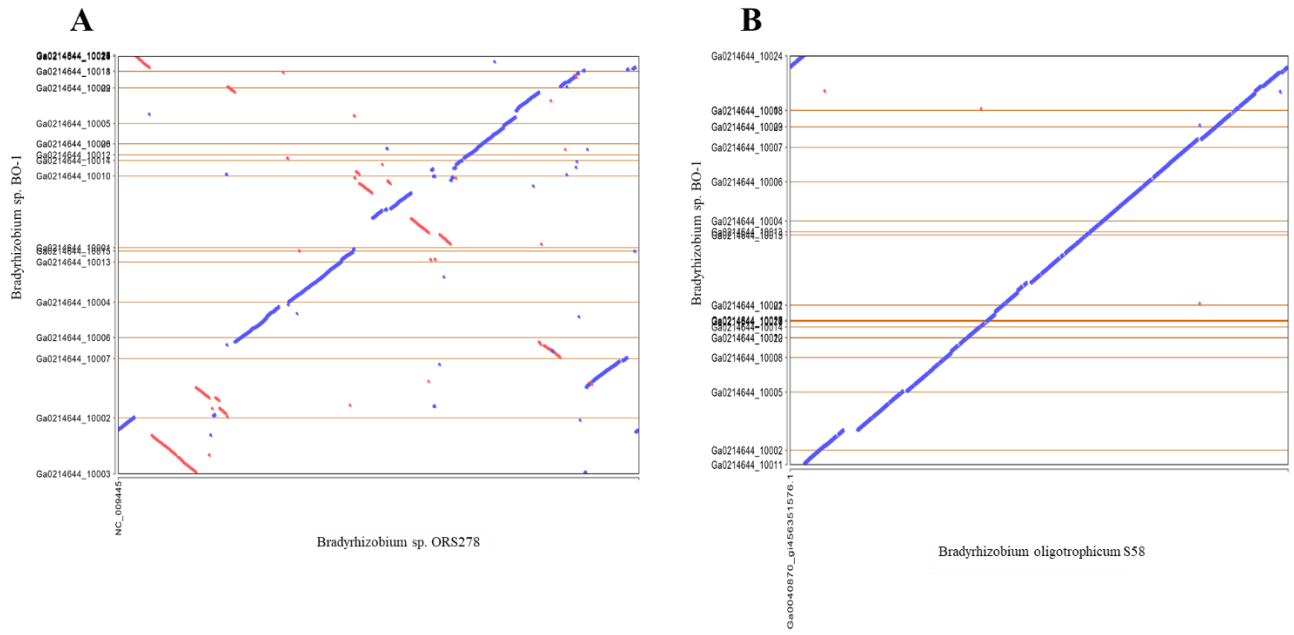


**Figure 4-8.** Syntenic Dot Plot between *Bradyrhizobium oligotrophicum* BO-1 with (A) *Bradyrhizobium* sp. ORS278 and (B) *Bradyrhizobium oligotrophicum* S58. The plot represents

the set of all maximal unique matches (MUMs) between the two genomes. Forward MUMs are plotted as blue lines/dots and reverse MUMs are plotted as red lines/dots.

**Table 4-1. Assembly information of *Bradyrhizobium oligotrophicum* BO-1 assessed via QUAST.**

| | **BO-1 assembly** |
|---|---|
| # of contigs (> = 0 bp) | 25 |
| # of contigs (> = 1000 bp) | 25 |
| # of contigs (> = 5000 bp) | 19 |
| # of contigs (> = 10000 bp) | 16 |
| # of contigs (> = 25000 bp) | 15 |
| # of contigs (> = 50000 bp) | 15 |
| Total length (> = 0 bp) | 7954020 |
| Total length (> = 1000 bp) | 7954020 |
| Total length (> = 5000 bp) | 7943185 |
| Total length (> = 10000 bp) | 7923660 |
| Total length (> = 25000 bp) | 7912816 |
| Total length (> = 50000 bp) | 7912816 |
| # of contigs | 25 |
| Largest contig | 1362850 |
| Total length | 7954020 |
| GC (%) | 65.27 |
| N50 | 763657 |
| N75 | 398819 |
| L50 | 4 |
| L75 | 7 |
| # N's per 100 kbp | 0 |

All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

**Table 4-2. Genome statistics for *Bradyrhizobium oligotrophicum* BO-1 and S58, and *Bradyrhizobium* sp. ORS278 and BTAi1.**

| | **BO-1** | **S58** | **ORS278** | **BTAi1** |
|---|---|---|---|---|
| **Genome Size (bp)** | 7,948,507 | 8,264,165 | 7,456,587 | 8,492,513 |
| **GC %** | 65.28 | 65.13 | 65.51 | 64.81 |
| **# of rRNA** | 1 | 2 | 2 | 2 |
| **# of tRNA** | 51 | 53 | 50 | 52 |
| **Protein coding genes** | 6996 | 7130 | 6752 | 7741 |

| | | | | |
|---|---|---|---|---|
| **Protein coding genes with COGs** | 4842 | 4906 | 4421 | 5070 |

**Table 4-3. Metabolic phenotypes found in *Bradyrhizobium oligotrophicum* BO-1 and not S58.**

| **BO-1 v S58 Metabolic Phenotype Differences** |
|---|
| Auxotroph (L-alanine auxotroph) (IMG_PIPELINE; 2017-10-11) |
| Prototrophic (L-cysteine prototroph) (IMG_PIPELINE; 2017-10-11) |
| Prototrophic (L-proline prototroph) (IMG_PIPELINE; 2017-10-11) |
| Prototrophic (L-serine prototroph) (IMG_PIPELINE; 2017-10-11) |
| Prototrophic (L-threonine prototroph) (IMG_PIPELINE; 2017-10-11) |
| Prototrophic (Coenzyme A prototroph) (IMG_PIPELINE; 2017-10-11) |
| (Glucose utilizing) (IMG_PIPELINE; 2017-10-11) |

**Table 4-4. Nodulation genes in *B. oligotrophicum* BO-1 genome.**

| Nod Protein | Description | Locus Tag[1)] | Similarity (%) | Query Coverage (%) | NCBI Accession No. |
|---|---|---|---|---|---|
| NodA | NodA family N-acyltransferase | absent | - | - | WP_050404916.1 |
| NodB | chitooligosaccharide deacetylase NodB | Ga0214644_1000122 | 30 | 70 | WP_011053465.1 |
| NodC | chitooligosaccharide synthase NodC | absent | - | - | WP_027562752.1 |
| NodD | LysR family transcriptional regulator | Ga0214644_10006308 | 62 | 98 | WP_050404918.1 |
| NodE | beta-ketoacyl-[acyl-carrier-protein] synthase family protein | Ga0214644_100011046 | 38 | 99 | WP_010912832 |
| NodF | nodulation protein NodF | absent | - | - | WP_033181209 |
| NodG | 3-oxoacyl-[acyl-carrier-protein] reductase | | 91 | 100 | WP_044536450 |
| NodH | nodulation protein NodH | absent | - | - | WP_040140406 |
| NodI | nodulation factor ABC transporter ATP-binding protein NodI | Ga0214644_10009189 | 38 | 70 | WP_007539201 |
| NodJ | nodulation protein J | absent | - | - | WP_050404912 |
| NodK | nodulation protein K | absent | - | - | AAA63599 |
| NodL | nodulation protein L | absent | - | - | 1914270A |
| NodM | nodulation protein M | Ga0214644_10001678 | 78 | 100 | BAC46897 |
| NodN | nodulation protein N | Ga0214644_1001359 | 86 | 100 | NP_770599 |
| NodO | nodulation protein O | absent | - | - | AFJ42517 |
| NodP | nodulation protein P | Ga0214644_10011145 | 70 | 91 | BAB55898 |
| NodQ | NodQ bifuctional enzyme | Ga0214644_10002456 | 80 | 100 | NP_768115 |
| NodV | NodV two-component regulator | Ga0214644_10004565 | 31 | 100 | AAG60698 |
| NodW | NodW two-component regulator | Ga0214644_10004566 | 37 | 96 | AAG60697 |

1) Absence indicates no hits with >30% amino acid identity and >50% query coverage.

**Table 4-5. Conservation of genes between ORS278 and *B. oligotrophicum* BO-1 involved in nitrogen fixation.**

| Locus Tag | | | Blastp Result | |
|---|---|---|---|---|
| ORS278[1,2)] | BO-1 | Gene Description | Similarity (%) | Query Coverage (%) |
| BRADO0045 | Ga0214644_10003186 | Apolipoprotein N-acyltransferase (ALP N-acyltransferase) | 90 | 100 |
| BRADO0113 | Ga0214644_10003115 | RNA polymerase sigma-54 factor | 95 | 100 |
| BRADO0134 | Ga0214644_1000390 | Translation initiation factor IF-3 | 96 | 100 |
| BRADO0157 | Ga0214644_1000369 | 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (Phosphoglyceromutase) (PGAM) (BPG-dependent PGAM) (dPGM) | 94 | 100 |
| BRADO0212 | Ga0214644_1000318 | imidazoleglycerol-phosphate dehydratase | 96 | 100 |
| BRADO0234 | Ga0214644_10011253 | Succinate dehydrogenase flavoprotein subunit | 98 | 100 |
| BRADO0340 | Ga0214644_10011148 | putative Ribosomal RNA small subunit methyltransferase B (rRNA (cytosine-C(5)-)-methyltransferase) (16S rRNA m5C967 methyltransferase)(rsmB) | 91 | 100 |
| BRADO0376 | Ga0214644_10011112 | tRNA (Guanine-N(1)-)-methyltransferase | 92 | 100 |
| BRADO0377 | Ga0214644_10011110 | 16S rRNA processing protein, putative function | 83 | 99 |
| BRADO0404 | Ga0214644_1001182 | succinyl-CoA synthetase, beta subunit | 98 | 100 |
| BRADO0405 | Ga0214644_1001181 | succinyl-CoA synthetase, alpha subunit, NAD(P)-binding | 99 | 100 |
| BRADO0409 | Ga0214644_1001177 | dihydrolipoamide dehydrogenase, FAD/NAD(P)-binding, component of the 2-oxoglutarate dehydrogenase and the pyruvate dehydrogenase complexes | 96 | 100 |
| BRADO0530 | Ga0214644_10003375 | [Protein-PII] uridylyltransferase (PII uridylyl-transferase) (Uridylyl-removing enzyme) (UTase) | 96 | 100 |
| BRADO0560 | Ga0214644_10003405 | putative diguanylate cyclase (GGDEF)/phosphodiesterase (EAL) | 94 | 99 |
| BRADO0571 | Ga0214644_10003415 | Putative protein methyltransferase hemK modifies release factors RF-1 and RF-2 | 78 | 100 |
| BRADO0575 | Ga0214644_10003419 | conserved hypothetical protein | 94 | 100 |
| BRADO0589 | Ga0214644_10003433 | putative Peptidylprolyl isomerase | 96 | 92 |
| BRADO0665 | Ga0214644_10003506 | hypothetical protein; putative serine protease/outer membrane autotransporter precursor/Membrane-associated phospholipid phosphatase | 89 | 100 |
| BRADO1049 | Ga0214644_10003903 | Putative response regulator receiver (CheY-like protein) | 98 | 100 |
| BRADO1122 | Ga0214644_10002399 | phosphoglycerate kinase | 95 | 99 |
| BRADO1205 | Ga0214644_10002316 | Phosphoribosylaminoimidazole carboxylase ATPase subunit (AIR carboxylase) (AIRC) | 94 | 100 |
| BRADO1364 | Ga0214644_10002241 | conserved hypothetical protein | 88 | 100 |
| BRADO1426 | Ga0214644_1000281 | hypothetical protein BRADO1426 | 59 | 100 |
| BRADO1477 | Ga0214644_100055 | chemotaxis protein methyltransferase | 93 | 100 |
| BRADO1675 | Ga0214644_10006586 | high-affinity phosphate transport protein (ABC superfamily, membrane) | 95 | 100 |
| BRADO1677 | Ga0214644_10006584 | high-affinity phosphate transport protein (ABC superfamily, atp_bind) | 95 | 100 |
| BRADO2020 | Ga0214644_10006229 | putative electron transfer flavoprotein dehydrogenases | 95 | 100 |
| BRADO2034 | Ga0214644_10006216 | pyruvate phosphate dikinase | 96 | 100 |
| BRADO2036 | Ga0214644_10006212 | L-aspartate oxidase | 80 | 99 |
| BRADO2133 | Ga0214644_10006110 | putative GTP cyclohydrolase II, riboflavin biosynthesis | 98 | 100 |
| BRADO2179 | Ga0214644_1000664 | putative acyl-CoA dehydrogenase | 95 | 98 |
| BRADO2274 | Ga0214644_1000713 | Ribulose bisphosphate carboxylase large chain (RuBisCO large subunit) | 57 | 98 |
| BRADO2671 | Ga0214644_10004348 | conserved hypothetical protein | 72 | 95 |
| BRADO2682 | Ga0214644_10004333 | Methylmalonyl-CoA mutase small subunit (MCM-beta) | 85 | 100 |

| | | | | |
|---|---|---|---|---|
| BRADO2734 | Ga0214644_10004283 | Cytochrome c-type biogenesis protein cycK | 96 | 100 |
| BRADO2776 | Ga0214644_10004239 | phosphoribosylaminoimidazole-succinocarboxamide synthetase (SAICAR synthetase) | 99 | 100 |
| BRADO2842 | Ga0214644_10004164 | Ribulose-phosphate 3-epimerase (Pentose-5-phosphate 3-epimerase) (PPE) (R5P3E) | 97 | 100 |
| BRADO2937 | Ga0214644_1000476 | conserved hypothetical protein; putative signal peptide | 83 | 99 |
| BRADO2938 | Ga0214644_1000475 | putative hydrolase | 91 | 99 |
| BRADO3096 | absent | putative ABC transporter periplasmic substrate-binding protein | - | - |
| BRADO3225 | Ga0214644_1001046 | putative methyl-accepting chemotaxis protein | 92 | 99 |
| BRADO3306 | Ga0214644_100011053 | 50S ribosomal protein L9 | 93 | 100 |
| BRADO3308 | Ga0214644_100011051 | 30S ribosomal subunit protein S18 | 99 | 100 |
| BRADO3359 | Ga0214644_100011003 | Aspartyl-tRNA synthetase / asparaginyl-tRNA synthetase (Aspartate-/asparagine-tRNA ligase) (AspRS) | 95 | 100 |
| BRADO3367 | Ga0214644_10001995 | putative bifunctional enzyme: malic oxidoreductase (N-terminal); phosphotransacetylase (C-terminal) | 97 | 100 |
| BRADO3673 | Ga0214644_10001634 | Putative Cold shock protein, DNA binding, CspA-like | 96 | 100 |
| BRADO3770 | Ga0214644_10001686 | conserved hypothetical protein | 95 | 100 |
| BRADO3794 | Ga0214644_10001707 | putative sulfite reductase (NADPH) | 93 | 100 |
| BRADO3866 | Ga0214644_10001784 | Nitrogen assimilation regulatory protein | 99 | 100 |
| BRADO3867 | Ga0214644_10001785 | Nitrogen regulation protein | 96 | 100 |
| BRADO3868 | Ga0214644_10001786 | putative tRNA-dihydrouridine synthase (Nitrogen regulation protein nifR3) | 95 | 100 |
| BRADO3881 | Ga0214644_10001795 | Valyl-tRNA synthetase (Valine--tRNA ligase) | 95 | 97 |
| BRADO4083 | Ga0214644_10001421 | Dihydrolipoyl dehydrogenase (E3 component of pyruvate and 2-oxoglutarate dehydrogenases complexes) (Dihydrolipoamide dehydrogenase) | 96 | 100 |
| BRADO4085 | Ga0214644_10001419 | conserved hypothetical protein | 98 | 100 |
| BRADO4086 | Ga0214644_10001418 | Pyruvate dehydrogenase E1 component, beta subunit | 96 | 100 |
| BRADO4087 | Ga0214644_10001417 | Pyruvate dehydrogenase E1 component, alpha subunit | 98 | 100 |
| BRADO4094 | Ga0214644_10001409 | Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase) | 98 | 100 |
| BRADO4317 | Ga0214644_1001086 | Putative pyruvate ferredoxin/flavodoxin oxidoreductase | 96 | 100 |
| BRADO4549 | Ga0214644_1000137 | putative D-alanyl-D-alanine carboxypeptidase (Peptidase S11)(DD-carboxypeptidase) | 91 | 100 |
| BRADO4636 | Ga0214644_10010112 | putative transcriptional regulatory protein, related to MarR family | 90 | 100 |
| BRADO4683 | Ga0214644_10010208 | conserved hypothetical protein; putative Rhodanese family protein | 91 | 100 |
| BRADO4701 | Ga0214644_10010227 | conserved hypothetical protein; putative secreted protein | 84 | 100 |
| BRADO4782 | Ga0214644_1001426 | putative modular protein: ATP-binding protein (ABC transporter component)(N-terminal); uncharacterised protein of unknown function (C-terminal) | 91 | 100 |
| BRADO5016 | Ga0214644_10012160 | fumarase A (fumarate hydratase class I) | 97 | 100 |
| BRADO5022 | Ga0214644_10012168 | conserved hypothetical protein; putative signal peptide | 89 | 100 |
| BRADO5117 | Ga0214644_10008294 | putative Permease of the major facilitator superfamily | 91 | 100 |
| BRADO5368 | Ga0214644_1000820 | putative Metal-dependent hydrolase involved in phosphonate metabolism; PhnM protein | 86 | 97 |
| BRADO5394 | Ga0214644_10005568 | nitrogenase iron protein, nifH; dinitrogenase reductase | 98 | 100 |
| BRADO5436 | Ga0214644_10005572 | nitrogenase reductase-associated ferredoxin, nifN | 93 | 100 |
| BRADO5437 | Ga0214644_10005571 | nitrogenase molybdenum-cofactor synthesis protein nifE | 98 | 100 |
| BRADO5438 | Ga0214644_10005570 | nitrogenase molybdenum-iron protein beta chain, nifK | 94 | 100 |
| BRADO5449 | Ga0214644_10005560 | Mo/Fe nitrogenase specific transcriptional regulator, NifA | 95 | 100 |
| BRADO5546 | Ga0214644_10005367 | conserved hypothetical protein | 84 | 97 |

| | | | | |
|---|---|---|---|---|
| BRADO5734 | absent | conserved hypothetical protein; putative membrane protein | | |
| BRADO5803 | Ga0214644_10005108 | hypothetical protein BRADO5803 | 88 | 96 |
| BRADO5815 | Ga0214644_1000597 | putative 4-alpha-glucanotransferase (malQ-like) | 88 | 100 |
| BRADO5973 | Ga0214644_10007231 | aspartate aminotransferase A (AspAT) | 97 | 100 |
| BRADO6079 | Ga0214644_10007350 | DNA mismatch repair protein MutL | 93 | 100 |
| BRADO6119 | Ga0214644_100093 | putative ABC transporter (fused ATP-binding and permease components) | 88 | 98 |
| BRADO6137 | Ga0214644_1000921 | 50S ribosomal protein L36 | 100 | 100 |
| BRADO6491 | Ga0214644_10002503 | Electron transfer flavoprotein alpha-subunit (Alpha-ETF) (Electron transfer flavoprotein large subunit) (ETFLS) | 98 | 100 |
| BRADO6492 | Ga0214644_10002504 | Electron transfer flavoprotein beta-subunit (Beta-ETF) (Electron transfer flavoprotein small subunit) (ETFSS) | 97 | 100 |
| BRADO6832 | Ga0214644_10002837 | two-component system regulator: response regulator receiver (N-terminal) and histidine kinase (C-terminal) | 96 | 100 |
| BRADO6852 | Ga0214644_10002858 | Conserved hypothetical protein; putative bifunctional protein: Zn-dependent hydrolase including glyoxylases (N-terminal) and Rhodanese-related sulfurtransferase (C-terminal) | 95 | 99 |
| BRADO6923 | absent | hypothetical protein; putative RTX family exoprotein | - | - |

1) Giraud et al. 2007
2) Okubu et al. 2013

## Table 4-6. Conservation of genes between ORS278 and *B. oligotrophicum* BO-1 involved in photosynthesis.

| Locus Tag | | | Blastp Result | |
|---|---|---|---|---|
| ORS278[1,2)] | BO-1 | Gene Description | Similarity (%) | Query Coverage (%) |
| BRADO0154 | Ga0214644_1000371 | putative methylated-DNA--protein-cysteine methyltransferase (O-6-methylguanine-DNA alkyltransferase); putative ADA regulatory protein | 97 | 100 |
| BRADO0208 | Ga0214644_1000321 | ATPase component of the HslUV protease, also functions as molecular chaperone; heat shock protein | 97 | 100 |
| BRADO0211 | Ga0214644_1000319 | ATP-dependent protease hslV (protease subunit of a proteasome-like degradation complex) | 98 | 100 |
| BRADO0392 | Ga0214644_1001194 | heme ABC transporter (heme exporter protein A), ATP-binding protein (cytochrome c-type biogenesis) | 92 | 100 |
| BRADO0561 | Ga0214644_10003406 | putative polyhydroxyalkanoate synthesis repressor PhaR-like | 94 | 100 |
| BRADO1034 | Ga0214644_10003891 | putative branched-chain amino acid ABC transporter (ATP-binding protein) | 98 | 100 |
| BRADO1324 | Ga0214644_10002277 | Transcription elongation factor greA | 97 | 84 |
| BRADO1607 | Ga0214644_1000763 | Magnesium-chelatase 60 kDa subunit (Mg-protoporphyrin IX chelatase) (Mg-chelatase subunit D) | 86 | 98 |
| BRADO1610 | Ga0214644_1000760 | Phytoene dehydrogenase (Phytoene desaturase) | 93 | 100 |
| BRADO1614 | Ga0214644_1000756 | hydroxyneurosporene and rhodopin dehydrogenase | 89 | 99 |
| BRADO1617 | Ga0214644_1000753 | 2-desacetyl-2-hydroxyethyl bacteriochlorophyllide A dehydrogenase | 91 | 100 |
| BRADO1618 | Ga0214644_1000752 | bacteriochlorophyllide reductase subunit, 35.5 kDa chain | 96 | 100 |
| BRADO1622 | Ga0214644_1000748 | Light harvesting 1 beta subunit | 94 | 96 |
| BRADO1623 | Ga0214644_1000747 | Light harvesting 1 alpha subunit | 100 | 100 |
| BRADO1624 | Ga0214644_1000746 | photosynthetic reaction center L subunit | 96 | 100 |
| BRADO1625 | Ga0214644_1000745 | photosynthetic reaction center M protein | 98 | 100 |

| | | | | |
|---|---|---|---|---|
| BRADO1626 | Ga0214644_1000744 | heme oxygenase | 84 | 100 |
| BRADO1628 | Ga0214644_1000743 | Bacteriophytochrome | 95 | 100 |
| BRADO1629 | Ga0214644_1000742 | transcriptional regulator PpsR2; Fis family | 93 | 100 |
| BRADO1630 | Ga0214644_1000741 | cytochrome c2 | 92 | 100 |
| BRADO1632 | Ga0214644_1000739 | geranylgeranyl reductase | 87 | 100 |
| BRADO1633 | Ga0214644_1000738 | light harvesting pigment Major Facilitator Family (MFS) transporter, Bch2-like | 93 | 100 |
| BRADO1634 | Ga0214644_1000737 | Bacteriochlorophyll synthase 33 kDa chain (geranylgeranyl bacteriochlorophyll synthase 33 kDa chain) | 89 | 100 |
| BRADO1635 | Ga0214644_1000736 | transcriptional regulatory protein, HTH Fis-type family | 93 | 96 |
| BRADO1636 | Ga0214644_1000735 | putative regulator with a cobalamin (vitamin B12)-binding domain, AerR-like | 93 | 100 |
| BRADO1638 | Ga0214644_1000734 | 2-vinyl bacteriochlorophyllide hydratase | 93 | 100 |
| BRADO1641 | Ga0214644_1000731 | magnesium-protoporphyrin O-methyltransferase BchH subunit | 95 | 100 |
| BRADO1642 | Ga0214644_1000730 | protochlorophyllide reductase iron-sulfur ATP-binding protein BchL | 95 | 100 |
| BRADO1644 | Ga0214644_1000728 | photosynthetic complex (LH1) assembly protein LhaA, Major Facilitator Superfamily (MFS) transporter | 97 | 100 |
| BRADO1649 | Ga0214644_1000723 | Aerobic magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase (Aerobic Mg-protoporphyrin IX monomethyl ester oxidative cyclase) | 93 | 100 |
| BRADO1976 | Ga0214644_10006296 | Ubiquinol-cytochrome c reductase iron-sulfur subunit (Rieske iron-sulfur protein) (RISP | 96 | 100 |
| BRADO1977 | Ga0214644_10006295 | Cytochrome b/c1 [Contains: Cytochrome b; Cytochrome c1] | 94 | 100 |
| BRADO2467 | Ga0214644_10004543 | putative transmembrane cytochrome C biogenesis protein | 95 | 100 |
| BRADO2671 | Ga0214644_10004348 | conserved hypothetical protein | 72 | 95 |
| BRADO3556 | Ga0214644_10001588 | putative transcriptional regulatory protein, LacI family | 91 | 100 |
| BRADO3946 | Ga0214644_10001845 | Putative Sensor histidine kinase with a GAF domain and multiple HAMP and Response regulator receiver domains (modular protein) | 96 | 99 |
| BRADO3970 | Ga0214644_10001868 | hypothetical protein BRADO3970 | 94 | 93 |
| BRADO4041 | Ga0214644_10001466 | putative response regulator receiver (CheY-like protein) | 94 | 100 |
| BRADO4435 | Ga0214644_10001165 | conserved hypothetical protein; putative membrane protein | 94 | 100 |
| BRADO6289 | Ga0214644_10009153 | Putative Cation-transporting ATPase | 92 | 100 |

1) Giraud et al. 2007
2) Jaubert et al. 2009

## Table 4-7. Conservation of genes between ORS278 and *B. oligotrophicum* BO-1 involved in regulation of the circadian clock.

| Locus Tag | | | Blastp Result | |
|---|---|---|---|---|
| ORS278[1,2)] | BO-1 | Gene Description | Similarity (%) | Query Coverage (%) |
| BRADO1478 | Ga0214644_100056 | Circadian clock protein kinase kaiC | 89 | 100 |
| BRADO1479 | Ga0214644_100057 | circadian clock protein | 96 | 100 |
| BRADO3946 | Ga0214644_10001845 | Putative Sensor histidine kinase with a GAF domain and multiple HAMP and Response regulator receiver domains (modular protein) | 96 | 99 |
| BRADO3982 | Ga0214644_100056 | Circadian clock protein kinase KaiC | 39 | 99 |

| | | | | |
|---|---|---|---|---|
| BRADO3983 | Ga0214644_100057 | Circadian clock protein kaiB | 48 | 78 |
| BRADO4470 | Ga0214644_10001127 | conserved hypothetical protein with DNA-binding domain (DUF88) | 97 | 100 |
| BRADO6832 | Ga0214644_10002837 | two-component system regulator: response regulator receiver (N-terminal) and histidine kinase (C-terminal) | 96 | 100 |

1) Giraud et al. 2007

**Table 4-8. Average nucleotide identity (ANI) for *Bradyrhizobium oligotrophicum* BO-1 and S58, and *Bradyrhizobium* sp. ORS278 and BTAi1.**

| Genome 1 | Genome 2 | ANI 1->2 | ANI 2->1 | AF 1->2 | AF 2->1 |
|---|---|---|---|---|---|
| BTAi1 | S58 | 87.57 | 87.53 | 0.700 | 0.730 |
| BTAi1 | BO-1 | 87.64 | 87.64 | 0.710 | 0.750 |
| S58 | BO-1 | 98.67 | 98.67 | 0.920 | 0.950 |
| ORS278 | S58 | 87.73 | 87.74 | 0.790 | 0.710 |
| ORS278 | BO-1 | 87.79 | 87.79 | 0.790 | 0.740 |
| ORS278 | BTAi1 | 87.46 | 87.46 | 0.780 | 0.680 |

*AF designates alignment fraction.

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# CHAPTER 5

# CONCLUSIONS

Photosynthetic bradyrhizobia represent an avenue into which not only molecular work on symbiotic processes can be advanced through empirical experiments, but also an element contributing to the big picture that is sustainable agriculture. Change on the global scale in regard to both the human population and plant biodiversity is occurring more than ever. Rapid population increase has created a scenario in which we need to access and redefine the living practices of our species in order to continue inhabiting our planet. It has been projected that we will need to double our food production within the next few decades in order to accommodate this surging increase of people. Diets relying heavily on meat are now being favored which is increasing the demand for cereal crop production to feed livestock (Tilman 2002; Godfray 2012; Tscharntke 2012). In conjunction with the population increase and the world's demand for food, there comes a variety of complex dilemmas. For example, one of the most prominent of these problems arises from the impact of increased agricultural practices on wildlife and biodiversity, which accelerates climate change because of the production of excess greenhouse gases and use of synthetic fertilizers in order to meet the world's increasing demands (Tubiello 2007). Maintaining environmentally integrity is a major issue with some proposed agricultural practices. Disagreements upon what are the correct farming techniques and the turning of a blind eye to detrimental ecological impacts can have serious consequences for both the wild-life and domesticated crops. Another problem is that

of providing equitability and stability for food production and land usage to all people regardless of socio-economic status (Godfray 2012). The world is a big place but it is quickly being filled up. There is a finite amount of land on this planet, and unfortunately, much of is unavailable or unusable for sustainable agricultural practices (Tubiello 2007). Finding a way to share and equally distribute the land and the fruits of its labor is a challenging task. A balance between all of these factors must be obtained and held in fully-functioning operation to maintain sustainability of biodiversity and everyday life. In order to maintain a solid system around the globe, there must be communication between nations and an agreement upon all of these issues. With the ever-increasing technological advancements and as globalization continues to snowball, solutions to problems associated with global change are not science fiction. Answers to these problems and more need to be harnessed and brought forth in harmony in order to produce a sustainable and working model so that we are able to stay ahead of the curve. In this, there is still hope to preserve our species and those we share the planet with for all of our futures.

There can be serious consequences to global change if left unchecked. In particular, plant species can suffer tremendously from human malpractices, which will in turn result in harm to those who use or consume those plants. The most obvious threats are derived from climate change. It is known that even a few degrees of change in temperature can throw a whole ecosystem off balance, but the earth's temperature is not the only factor affected by climate change. Atmospheric composition, the amount of rainfall, the frequency of extreme events, and carbon sinks, are just a few other things that are influenced by climate change that can result in major environmental repercussions. In many cases, these environmental changes are apt to increase frequency of plant and animal disease. Depending on latitudinal location, different species of plants have evolved to

thrive in certain environmental conditions. If any of these conditions are altered, the organism will struggle not only in aspects of growth but even basic functionalities (i.e. stomatal operation, root densities, seed production, etc.) (Tubiello 2007). As with climate change, different methods of agricultural practice have resulted in changes in the surrounding environment, especially with the agricultural intensification in recent years. Functional biodiversity on farmlands is poorly understood and has likely resulted in large amounts of environmental degradation. Many studies suggest that large-scale farming practices actually have less product efficiency per area, but at the same time, unregulated small farms tend to encroach on the surrounding wilderness (Tscharntke 2012). The heavy addition of synthetic nitrogen and phosphorous fertilizers to crops has been shown to contribute to ecologically harmful eutrophication events in nearby lake, rivers, and streams due to runoff (Tilman 1999). Not only do the synthetic fertilizers harm the surrounding plants and animals, but many times these toxic chemicals seep into local drinking water sources. Public health especially becomes an issue when harmful pesticides and air pollutants enter the picture. Some pesticides become necessary to keep crop yields at maximum but often times result in crop contamination and adverse effects on function agrobiodiversity (Tscharntke 2012). Air pollution has been better regulated lately for some countries but many others are still pumping harmful compounds into the ozone, like nitric oxide and its derivatives. The buildup of compounds in the ozone can have hostile effects on not only overall plant life but ecological composition and biodiversity. These effects have been poorly studied and require much more critical attention involving plant and animal interactions in association with other global changes (Tubiello 2007). Global change reflects the dynamic nature of life, of its ability to survive or eliminate those that cannot cope. Many changes in environmental structure can be measured and directly attributed to

human lifestyles and some of these changes represent threats to both domesticated and wild life. A general consensus and a plan of action for these matters is necessary to ameliorate these perils.

Finding an array of sustainable living solutions to resolve the world's inevitable agricultural, conservational, population, and climate problems will require much coordination and effort from around the world. If these troubles are solved amicably and intelligently, then it would be possible to appease most, if not all, of the global changes threatening life as we know it. Several ideas have been proposed for what needs to be done in order to handle these problems efficiently. Some believe that long-term agroecosystem experiments (LTAEs) will be required seeing as how most aspects of global change cannot happen overnight. Data collection over long periods of time is often portrayed as a daunting task, but this knowledge of the past will provide a better representation for what the future might hold. A systematic analysis for LTAEs and new experiments that are coordinated through an international network are necessary to develop a precise estimate for what to expect in the future (Ramussen et al. 1998). LTAEs can provide useful data for the cause, but the world is growing quickly and action must not be delayed. It is possible to provide enough food for the growing population in the upcoming decades while avoiding biodiversity abandonment but there must be multiple strategies put together, like closing the yield gap, sustainable intensification, increasing production limits, reducing waste, changing diet, expanding aquaculture, and land change science (Godfray et al. 2010; Turner et al. 2007). In regards to climate change, there must be comprehensive studies to prepare for agricultural and societal corrections necessary in the time ahead. New and improved irrigation techniques as well as crop models must be constructed in order to respond to the changing atmospheric conditions and occurrence of extreme climatic events (Tubiello et al. 2007). All of these ideas and more must

be held in the utmost regard when considering the legacy of humanity. While keeping the population alive and well is always at the forefront, maintaining what biodiversity is left in the world is equally imperative. The world is dynamic and is a system that requires a certain amount of diverse richness to preserve its very essence. The tools and ideas exist to mold the future, and even though there is an increase in innovative technology and a self-awareness of present-day problems, immediate planning as well as a preemptive strike is required to remedy these complications before they reach a tipping point.

In order to reduce the detrimental impact that synthetic nitrogen fertilizers have on not only humans, but the surrounding plants and animals, biological nitrogen fixation must be fully understood, optimized, and utilized. Starting small and working together can help to realize big ideas and attribute to major accomplishments. The regulation between nitrogen fixation and photosynthesis in photosynthetic bradyrhizobia may help to provide insight into the large question that is the NF-independent symbiotic pathway. The Tn5 mass mutagenesis of *Bradyrhizobium* sp. ORS 278 resulted multiple genes showing either a nodulation or nitrogen fixation deficient phenotype. Of these genes, BRADO4470 and BRADO3946 could be responsible for regulating symbiotic properties in a temporal fashion. BRADO 3946, *chk,* encodes a putative sensor histidine kinase, which as a part of a two-component regulatory system likely controls many cellular processes, specifically nitrogen-fixation, and photosynthesis because of its domains. BRADO4470, *labA* homolog for a negative feedback protein found in the circadian clock of cyanobacteria (Taniguchi et al. 2007; Bonaldi et al. 2010). Nitrogenase, the enzyme responsible for nitrogen fixation, is inhibited by oxygen, a well-known byproduct of photosynthesis. This can be an issue for the bacterial process in the nodules where the bacteroid can be directly exposed to

oxygen. However, if this symbiotic process can be regulated to where the nitrogen fixation process only occurs during the nighttime, the deleterious effect of oxygen could be avoided. In *Bradyrhizobium* sp. ORS278, it was found that nitrogen-fixation primarily occurred during the nighttime and if *labA* and *chk* were knocked out, that nitrogen fixation did not occur at wild type levels. This temporal regulation could be under the control of the KaiABC circadian clock system and may have evolved in these bacteria to allow an efficient means to reside within the plant nodule and fix nitrogen in a habitable environment. Furthermore, approximately 50% of the transcriptome contained differentially expressed genes between day and night for ORS278 bacteroids within *A. indica* root nodules. Of which, many of the DEGs have roles in metabolisms such as photosynthesis and respiration, and likely implicated in maintaining cellular conditions conducive to an efficient symbiotic environment. Cellular metabolisms must be finely balanced in order to maintain energy expenditure for various biological processes such as nitrogen fixation. If these processes could come to be understood on a molecular level, then the operation could be modified or optimized and perhaps applied to related species as was demonstrated by the identification and validation of HKGs in *Bradyrhizobium japonicum* USDA110. Using homologs of the reference genes found in *B. japonicum* for the photosynthetic bradyrhizobium allowed for accurate normalization of gene expression data gathered in this study. Using information gathered in closely related species and subsequently verifying is a solid foundation to build larger transfer studies upon, such as moving symbiotic processes from just rhizobia-legumes to other agriculturally important crops.

Engineering bacterial species may indeed prove useful when bioinoculants are used as a source of fertilization and need to be applied to different locations around the globe. Developing

nitrogen-fixing cereals and other crops poses many challenges, however. Different approaches may have to be taken in order to accomplish the end goal, whether it directly engineering nitrogen fixing plants, or moving an engineered microbe-host system to another plant completely (Mus et al. 2016). Since most studies have been performed in rhizobia-legume scenarios, it is more likely that nitrogen-fixing cereals will arise from genetically engineered bacteria. The well-studied symbiotic pathway and signal exchange found in rhizobia-legume symbiosis could theoretically be transferred to cereals since some parts of the signaling pathway are present in them (Charpentier and Oldroyd 2010). Engineering the bacteria itself has already begun in some aspects, as some scientists are considering combining genetic parts of microbes to create more complex systems and eventually programmed to complete specific tasks (Voight 2006; Hays et al. 2015). If temporal regulation could be adjusted to create an organism that has optimum nitrogen fixing capabilities in different zones other than the tropics, then profitable agriculture could conceivably be expanded to areas once thought impracticable. This is especially so if other components of symbiotic interaction were improved upon as well, such as heat/cold stress, oxidative stress, pH stress, etc. By understanding the role of circadian regulation of symbiotic genes in bradyrhizobia and attempting to apply new-found knowledge to other species, a database of useful information can be created and drawn for years to come. A solid foundation of novel research must be maintained so that further data can be established, thus allowing new ideas to accumulate, always gravitating towards something that can make a significant difference in the future.

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# APPENDIX

## EVALUATING NOVEL DESICCATION-TOLERANT *BRADYRHIZOBIUM* INOCULANTS FOR SYMBIOTIC EFFICIENCY AND SOYBEAN PRODUCTION


**ABSTRACT**

The soil bacterium *Bradyrhizobium japonicum* is an agriculturally important microbe because of its symbiotic association with the soybean plant, *Glycine max.* Soybean, the second most valuable crop in the U.S., is not only used for human and livestock consumption, but for a wide array of products such as biodegradable plastics, rubber, biodiesel, detergents, etc. As the demand for this crop continues to rise, breeders and farmers have been developing various ways to enhance its production. One such method involves inoculating soybeans with *B. japonicum,* which forms specialized organs known as nodules on the roots of the soybean plant, where the microbe converts atmospheric nitrogen ($N_2$) into ammonia ($NH_3$), a bioavailable form that can be readily used by the plant. Optimizing this process has the potential to reduce or even eliminate the use of chemical nitrogen fertilizers which have negative impacts on the surrounding environment. However, heat and drought could be the major constraints for survival of inoculants and the maintenance of their symbiotic activity in soybean fields. The objectives of this research are to i) isolate native rhizobial strains which will be selected for tolerance to heat and drought using a new molecular marker

system, and ii) compare their performance to commercial strains to develop a suitable inoculant that will be ultimately used in Texas soybean fields. Native Texas *Bradyrhizobia* strains were isolated and tested for their desiccation tolerance. Selected strains were then used to inoculate soybeans at an experimental plot in south Texas where plant vitality, nodulation, and production were measured. Soybeans inoculated with the desiccation-tolerant strains produced more numerous and bigger root nodules, have a higher plant dry-weight, and produce larger quantities of seeds per plant. This demonstrates that by using native inoculants for particular climates, farmers could enhance their crop production.

## INTRODUCTION

*Bradyrhizobium japonicum* is a soil-dwelling, Gram-negative bacterium that is known to engage in symbiotic association with soybean plants. This small organism could have a big impact on the future of food production and sustainable agriculture. It's mutualism with *Glycine max* results in the formation of symbiotic organs, known as nodules, which are located on the roots of the plant. Within the nodules, the microsymbiont performs nitrogen fixation to provide its host with a source of nitrogen, and in return receives some photosynthetic products. This process allows the plant to grow better in nutrient poor soils and other areas where it normally wouldn't thrive. Though biological nitrogen fixation (BNF) is restricted to a variety of prokaryotes, both free-living and symbiotic, their ability to provide a nitrogen source to the biosphere has major implications to global nitrogen and carbon cycling (Vitousek et al. 2013). BNF in a symbiotic context has been of interest due to its potential role in sustainable agriculture. Typically, synthetic nitrogen fertilizers are added in copious amounts to farmland soil for enrichment that will increase product yield,

however, this type of practice has devastating effects. Excess fertilizers eventually run into nearby water sources, resulting eutrophication events such as environmentally damaging algal blooms. This event leads to destruction of fishery habitats and creates hypoxic waters, or dead zones, which kill massive amounts of fish (Paerl 2009). The leached nutrients can also make their way into drinking water supplies, posing as a risk for public health (Kramer et al. 2006). BNF could decrease reliance upon synthetic fertilizers since the microbes are an eco-friendly alternative to provide nutrients to crops. However, the current rhizobia-associated mutualisms are primarily restricted to legumes. If BNF could be adapted to other crops such as cereals, whether inserting the nitrogen-fixing complex itself into the plant, or modifying microbe host specificity, some food productivity and environmental conservation issues could be remedied (Charpentier and Oldroyd 2010; Beatty and Good 2011). With the population continually rising, current meat and dairy dietary habits, and growing biofuel usage, agricultural production will need to be increased up to 110% by 2050 (Ray et al. 2013). It is also projected that up to 170 million people will be at risk for hunger by 2080 (Schmidhuber and Tubiello 2007). Sustainable agriculture and BNF could provide relief to these problems but public investment and flexible strategies must by developed.

The concept of sustainable agriculture extends far beyond just simply meeting food demand for current and future generations. The idea encompasses all aspects of living sustainably, providing resolution to not only food production, but how and where it is produced, transported, marketed, etc., all while considering its impact on the surrounding environment's biodiversity. Current conventional agriculture is primarily concerned with output and revenue turnover. This short-sightedness can lead to unhealthy and ecologically detrimental practices. Sustainable agriculture addresses all facets of science to produce a safe and over-arching agricultural plan that

remedies the problems of past, present, and future food production (Lichtfouse et al. 2009; Mulvaney et al. 2009). Genetically modified crops and mixed crop-livestock systems will have to be optimized and utilized to mitigate developing country hunger issues (Herrero et al. 2010). Improving resource-use efficiency for both high-input and low-input systems will be necessary for all farming systems on different scales (Spiertz 2009). Conservation agriculture will have to be considered to promote soil vitality so that more agricultural production can occur with less land (Hobb et al. 2008). All of these ideas and more will need to be implemented to begin building a model of lasting sustainable agriculture.

Soybeans are one of the most agriculturally important crops and could potentially serve as an initial model of sustainable farming practices. According to the USDA, soybeans make up 90% of oilseed production in the United States and are the world's largest source of animal protein feed, and second largest source of vegetable oil. Soybeans are also of high economic importance in the U.S. as they are the second highest valued cash crop behind corn and have the highest price per bushel. The U.S. is also the world's greatest producer of soybean (about 35%) and approximately half of it is exported (www.usda.gov). Soybean and its oil can be used for multiple purposes including human/livestock consumption, biofuel production, biodegradable plastics, rubber, etc. (Liu et al. 2007; John and Thomas 2008; Singh et al. 2008). The mutualistic association with *Bradyrhizobium japonicum* makes soybean a valuable agricultural commodity and candidate model for sustainable crops because it doesn't require excessive amounts of added synthetic nitrogen fertilizers.

Rhizobial inoculums and application techniques have been developed and used to increase soybean productivity and yields (Sogut 2006; Fatima et al. 2007). Survivability of rhizobial strains

in the soil can be monitored years after inoculation to determine its persistence (Albareda et al. 2009). Inoculums typically consist of one or more strains of microorganisms mixed with adhesive agents and other additives that protect the cells before use (Xavier et al. 2004). The bacterial strains must be robust and able to handle not only storage conditions, but new environmental conditions and also be able to outcompete native bacterial populations for resources (Kloepper et al. 1989; Bunemann et al. 2006; Lupwayi et al. 2006). Many factors can affect the quality of inoculants, starting from when the bacteria are mass produced, to formulation of the inoculum, through storage and transport, and finally to inoculation in the field (Herrmann and Lesueur 2013). One of the primary limiting factors to rhizobia survivability when applying inoculant onto the soybean seed coat and in the field is bacterial desiccation. Therefore, it would be beneficial to use strains that are resistant to drying out (Chao and Alexander 1984; Streeter 2003). For rhizobia to endure anhydrobiotic conditions, they must manage different stresses such as reactive oxygen species (ROS), high temperatures, various salts, and radiation (Vriezen et al. 2007). So, by isolating and evaluating heat and desiccation-tolerant rhizobia strains, their application to the field with environments exhibiting those climate conditions could substantially improve yield compared to typical commercial inoculants which aren't equipped to handle those stresses as readily.

In this study, native desiccation-tolerant rhizobial strains were isolated from various locations in Texas and applied as an inoculant to southern soybean crops at Rio Farms Inc., Monte Alto, TX. Their symbiotic efficiency and effect on yield was compared against commercial inoculants, and laboratory strains to determine efficacy.

## MATERIALS AND METHODS

**Isolation of native rhizobia strains.** Soybean root samples were gathered from multiple locations around Texas and brought back to the lab for rhizobia isolation from their nodules (Table A1-1). *Glycine max* root nodules were initially washed with at least six changes of sterile water to residual soil and debris. Nodules were pooled in a sterile petri dish and rinsed with 100% EtOH and immersed in 0.1% acidified $HgCl_2$ for 10 minutes. The nodules were the washed thoroughly in at least six changes of sterile water. Nodules were the placed in a micro-centrifuge (1.5 ml) tube and 500 µl of sterile water was added. Next the nodules were aseptically crushed and vortexed to mix. Serial dilutions of the mixture were made (1/10 and 1/100) and 100 µl from each mixture (original included) was dispensed and spread on YEM agar plates at pH 6.8 which contains 0.5 g of $K_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 10 g of mannitol, 0.5 g of yeast extract, and 15 g of agar per liter with 0.002% actidione (cycloheximide) to inhibit fungal growth (Vincent 1970). Plates were incubated at 30°C and observed for growth. Selected colonies were re-streaked on arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of $Na_2HPO_4$, 250 mg of $Na_2SO_4$, 320 mg of $NH_4Cl$, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate, and 15 g of agar per L (Sadowsky et al. 1987).

**Bacterial growth conditions.** The wild type strain *B. japonicum* USDA110, and all Texas rhizobial isolates (TX-CP, TX-EA, TX-VA, RF-SP5, RF-T61, RF-BB2) were cultured in arabinose-gluconate (AG) medium at pH 6.8. All strains were incubated aerobically at 30°C with vigorous shaking at 200 rpm for 3 days. Appropriate amount of chloramphenicol antibiotic was added to media for each *B. japonicum* strain ($\mu g \cdot ml^{-1}$). For inoculum preparation, all *B. japonicum*

strains, both wild-type and Texas isolates, were prepared in YEM broth and grown to exponential phase with $OD_{600}$ of 1.0 – 1.2, Cell Tech® was prepared as per manufacturer's instructions.

**RNA isolation.** For desiccation conditions, RNA extraction was performed as previously described (Jeon et al. 2011). All cultures were condensed by centrifugation for 20 min at 4°C and 4,000 rpm in a fixed angle rotor. The cell pellet was then collected by decanting the supernatant. RNA isolation followed by using hot phenol method as described previously (Bittner *et al.*, 2003). The RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) was used to purify the isolated RNA according to manufacturer's protocol. RNA quantity was calculated using NanoDrop device (Thermo Scientific) and RNA quality was confirmed via gel electrophoresis.

**qRT-PCR gene expression analysis.** The qRT-PCR was performed with modification according to methods previously described (Chang et al. 2007). Briefly, cDNA was synthesized in 25-µl reaction solution containing 1.5 µl of M-MLV reverse transcriptase (200U/µl; Promega Corp.), 2 µl of random hexamers (250ng/µl; Invitrogen, Carlsbad, CA, U.S.A.), 5 µl of 2.5 mM dNTPs, and 1 µg of total RNA. Of the reaction solution, 1 µl of cDNA was used as a template for qRT-PCR. A total of 10 µl qRT-PCR reactions were prepared using 5 µl of 2 x All-in-One™ qPCR Mix and 0.1 µl ROX Reference Dye by GeneCopoeia (Rockville, MD, U.S.A.), 1 µl at 0.2 µM each of forward and reverse primers, and 1 µl of cDNA template. qRT-PCR was performed by using ABI PRISM 7300 instrument (Applied Biosystems, Foster City, CA, U.S.A.) and following the GeneCopoeia qPCR protocol. Negative control reactions which lacked reverse transcriptase were used to check for DNA contamination. Gene-specific primers for the selected candidate reference genes were designed using Integrated DNA Technologies (Coralville, IA, U.S.A.) company software. Genes used to detect desiccation-tolerance are listed in Table A1-2. *parA*

(bll0631) gene, a chromosomal partitioning protein, was used to normalize the expression of each gene in this study (Cytryn *et al.*, 2007; Donati *et al.*, 2011). All experiments were performed with three biological replicates, and three technical replicates for each biological replicate. Fold induction values were calculated in accordance with the method of Pfaffl (2001).

**Desiccation experiment.** Each *B. japonicum* strain, including wild type and Texas native isolates, was grown in 5 ml AG medium with selective antibiotics and incubated aerobically at 30°C with vigorous shaking at 200 rpm for 2 days. All strains were subcultured in 200 ml AG media with chloramphenicol and incubated aerobically at 30°C with vigorous shaking at 200 rpm until the strains reached mid exponential phase at $OD_{600}$ of 0.8. For each strain, subcultures were divided into 8 of 20 ml cultures in 50 ml Eppendorf tubes. Each 20 ml culture was transferred to a 0.4 µm sterilized MF$^{TM}$ polycarbonate membrane filter (0.45 mm HA, 47 mm diameter; Millipore) and placed into a 60 x 15 mm sterilized Petri dish. Four of the membrane filters were incubated in the desiccator containing sterilized water to generate the 100% relative humidity (RH) to induce a hydrating condition, whereas another four membrane filters were incubated in the desiccator filled with a saturated potassium acetate solution to give a 27% relative humidity in order to induce a severe desiccating condition (Cytryn et al. 2007). The desiccators were incubated in the dark at 30°C and survival was monitored at 0, 4, 72 h by resuspending cells from membrane filters with Bergersen's minimal salts medium (MM) and spread onto AG agar plates at dilution factors ranging from 1:10,000 to 1:1,000,000. Percent survivals were determined after cells were incubated at 30°C for 4 days. This experiment was repeated three times.

**Field layout and design.** For planting design, one soybean cultivar was used, Vernal, and six different treatments/inoculants; Cell Tech®, no treatment, and *Bradyrhizobium japonicum* strains USDA 110, TXCP-01, TXVA-1107-3003, TXEA-1109-2000 (Figure A1-1). Rows for each subplot were 30 ft. long and 1.5 ft apart, where each subplot was treated with a different inoculant. An unplanted row was left in between each subplot to avoid cross-contamination of strains. Three biological replicate subplots were planted for each strain with 10 ft. alleys in between each biological replicate subplot row to avoid cross-contamination.

**Planting techniques.** Seeds were coated with respective inoculant and mixed until seed coat appeared glistening and completely covered with inoculum. Seeds were hand planted mid-March by placing three seeds in each hole approximately two inches deep. Each hole was approximately one foot apart and one milliliter of respective inoculant applied directly to the soil. For both years at Rio Farms Inc., planting technique was performed the same.

**Harvesting and physiology measurements.** Plants were harvest two times each year, once in early June for plant dry weight, and root nodules, and a second time in mid-August to harvest seeds. During the first harvest, plants were selected randomly for each treatment/cultivar, and root samples removed and immediately placed in 30% glycerol for cold storage. Plants were brought back to the lab and laid out to dry. Once completely dry, plant dry weight was measured. Root nodules were counted for each sample and categorized into size categories based upon nodule diameter. Random nodules were dissected for each treatment to observe nodule interior color for leghemoglobin presense. For the second harvest, all remaining viable plants were harvested and seed yield was counted for each plant.

**RESULTS**

**Native Texas *Bradyrhizobium japonicum* strains have better survivability under desiccation conditions.** Various strains identified to be desiccation-tolerant via qRT-PCR isolated from Delta, Victoria, and Lubbock counties in Texas showed better survivability than laboratory strain *B. japonicum* USDA110 under desiccation conditions (Table A1-3.) After both 4 and 72 hours, the native Texas strains showed a significant difference in survivability and were therefore selected as candidates for field trials.

**Plants inoculated with native Texas *Bradyrhizobia* strains has greater dry weight.** For 2016, native Texas strains TXCP-01, TXVA-1107-3003, and TXEA-1109-2000 when inoculated on Vernal seeds produced plants with a greater dry weight (Figure A1-3).

**Plants inoculated with native Texas *Bradyrhizobia* strains produced more root nodules in 2016.** For 2016, native Texas strains TXVA-1107-3003, and TXEA-1109-2000 when inoculated on Vernal seeds produced plants with a greater dry weight (Figure A1-5). TXCP-01 did not produce more nodules, but based upon nodule size, lacked smaller nodule production (>1mm and 1mm diameter) (Figure A1-6).

**Plants inoculated with native Texas *Bradyrhizobia* strains produced greater seed yields.** For 2016, native Texas strains TXCP-01, TXVA-1107-3003, and TXEA-1109-2000 when inoculated on Vernal seeds produced plants with higher soybean yield, though soybeans did not have different dry weights (Figure A1-11 & A1-12).

**DISCUSSION**

*Bradyrhizobium japonicum* native desiccation-tolerant Texas isolates proved to be more efficient in the symbiotic context which improved crop yield when used as an inoculant on soybean fields. Soybean plants inoculated with native strains produced more nodules, had a greater plant

dry weight, and produced more seeds as compared with commercial inoculants. The increased desiccation-tolerance likely allowed better survivability in the soil and on the seed coat when the soybeans were planted. The establishment and subsequent proliferation of the native *Bradyrhizobia* increased nodulation which directly influenced vitality and therefore production. The TXCP-01 strain did not produce as many nodules as the other two native strains and based upon nodules size, produced less of the smaller (>1mm and 1mm diameter) nodules. Indeed, smaller nodules may not be as effective at producing fixed nitrogen based upon surface area to volume ratios of nodules that determine nodule bacteroid capacity. Nonetheless, the native Texas inoculums produced greater soybean yields, which likely the most important agricultural statistic for soybean farmers.

Nodulation promoting rhizobia increase plant growth, so it makes sense that rhizobia better adapted to survive particular environments would have the same effect (Zhang et al. 1997). The rhizobia-legume symbiosis is ideal for arid and extreme environments as they can withstand a number of different stresses and at the same time, serve as a system to improve soil quality in eco-friendly manner (Zahran 1999). The development of commercial inoculants has made it easier for farmers to access bio-fertilizers and improve their soybean crop yields, where in the past, native strains have struggled (Thuita et al. 2012). However, other studies have shown that native strains out-compete commercial or foreign strains for nodule formation (Fabiano and Arias 1991; Blanco et al. 2010). There are many factors that can decide the symbiotic efficacy and competition of one strain versus another, including method of inoculation, application density, placement of inoculation, competition of inoculants during storage, rhizosphere responses, etc. (Moawad et al. 1984; McDermott and Graham 1989; Fabiano and Arias 1991; Lopez-Garcia et al. 2002; Blanco

et al. 2010). To increase nitrogen fixation rates and crop yields, both inoculation procedures and strains need to be evaluated an optimized before large-scale replication.

Improving inoculants and increasing BNF is not only necessary to meet the demands of increased soybean production, but also vital to develop sustainable agricultural techniques (Keyser and Li 1992; Zahran 1999). Over the past few decades, agriculture has become dependent on synthetic fertilizers which are becoming increasingly rare and expensive, and also cause environmental hazards. Therefore, it is of interest to find and utilize new avenues such as microbial biofertilizers for agricultural improvement (Bohlool et al. 1992; Bhardwaj et al. 2014). Biofertilizers include microorganisms such as nitrogen-fixers, potassium or phosphorus solubilizers, or certain molds and fungi. These organisms could prove as a solution to help produce more agricultural products which are projected to be in higher demands for developing countries in the next few decades (Mia and Shamsuddin 2010; Mohammadi and Sohrabi 2012). Using multiple different microorganisms to promote plant growth could be beneficial to all members involved by providing a rich community for biological proliferation. Co-inoculating soybeans with both *Bradyrhizobium japonicum* and plant growth-promoting rhizobacteria (PGPR) increased nodulation and nitrogen fixation in field trials (Dashti et al. 1998). PGPR and cyanobacteria can potentially improve crop production and soil quality while also serving as great models for both molecular study and sustainable agricultural practices (Singh et al. 2011). Evaluating multiple different combinations of biofertilizers in various environments for agricultural production and their influence on native ecology is a necessary project to help develop sustainable agricultural techniques.

Long-term agroecology experiments (LTAEs) are another aspect of creating a system of sustainability. LTAEs are large-scale field tests that can last multiple decades measuring different aspects of agricultural sustainability such as nutrient turnover, crop yields, land biogeochemistry, and ecological effects (Rasmussen et al. 1998). Extensive studies have shown that synthetic fertilizers have a negative impact of both above and below-ground biota, and therefore have a negative influence on agriculture (Birkhofer et al. 2008). Other long-term experiments can show how symbiotic organisms, such as arbuscular mycorrhizal fungi in the soil, can impact carbon and nitrogen cycling over time (Wilson et al. 2009). Analyzing the evolution and change of microbial communities when exposed to different fertilizers over long periods reveals insight into the importance of sustainable practices on land health (Bohme et al. 2005). More long-term experiments must be conducted in order to accurately assess how land, plants, and microbial communities can change over time. Combining both molecular microbiological techniques with long-term agroecology experiments could provide solid empirical data on how farming techniques, climate, and land ecology are interconnected.

It would be interesting to analyze other aspects of rhizobia-legume symbiotic field tests such as the metagenomes of field soil, or evaluating the oil and protein content of soybean seeds from various inoculant treatments. Studying all aspects of sustainable agricultural techniques, developing novel long-term agroecosystem experiments, and continuing to build upon previous knowledge will help in developing systems that can put into place to generate food stocks for current and future generations. Microbiology and the study of plant-microbe interactions, whether rhizobia-legume, arbuscular mycorrhizae, or study of the rhizosphere and microbial communities is considered one of the best ways to ensure sustainable food production while limiting deleterious

environmental impact (Tikhonovich and Provorov 2011). Beginning at the molecular level and testing in larger field experiments can provide insight into how nitrogen-fixing bacteria can influence the future of farming.

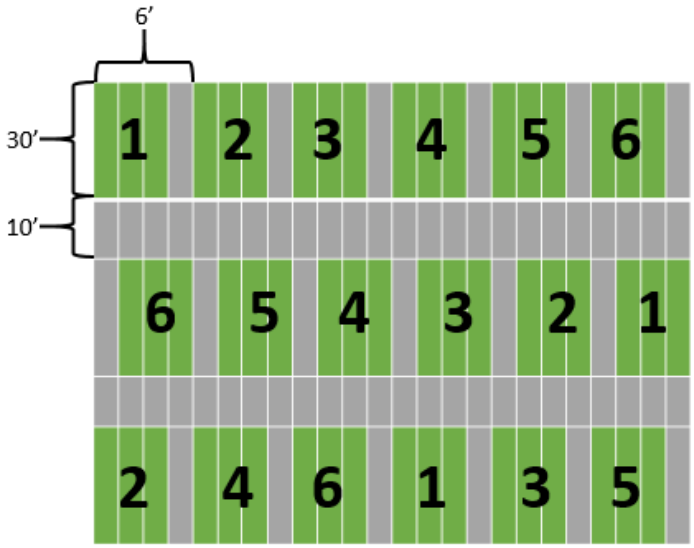


**Figure A1-1.** Plot layout and inoculum treatment for 2016 at Rio Farms Inc. Numbers correspond to inoculum: (1) Cell Tech®, (2) *B. japonicum* USDA 110, (3) *B. japonicum* TXCP-01, (4) *B. japonicum* TXVA-1107-3003, (5) *B. japonicum* TXEA-1109-2000, (6) no treatment.

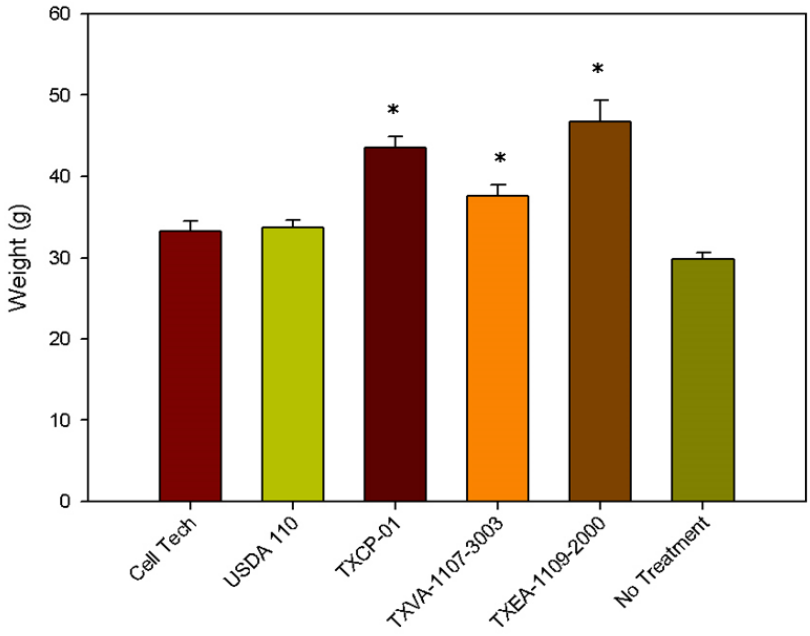


**Figure A1-2.** Average plant dry weight for Vernal soybean plants inoculated with various strains in 2016. * indicates statistical difference ($P < 0.05$) as compared to no treatment.

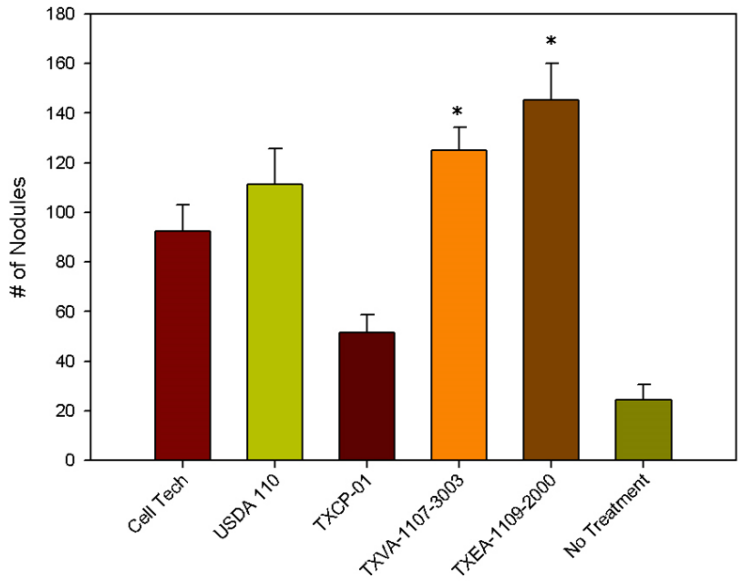


**Figure A1-3.** Average number of root nodules for Vernal cultivar inoculated with various strains in 2016. * indicates statistical difference ($P < 0.05$) as compared to no treatment.

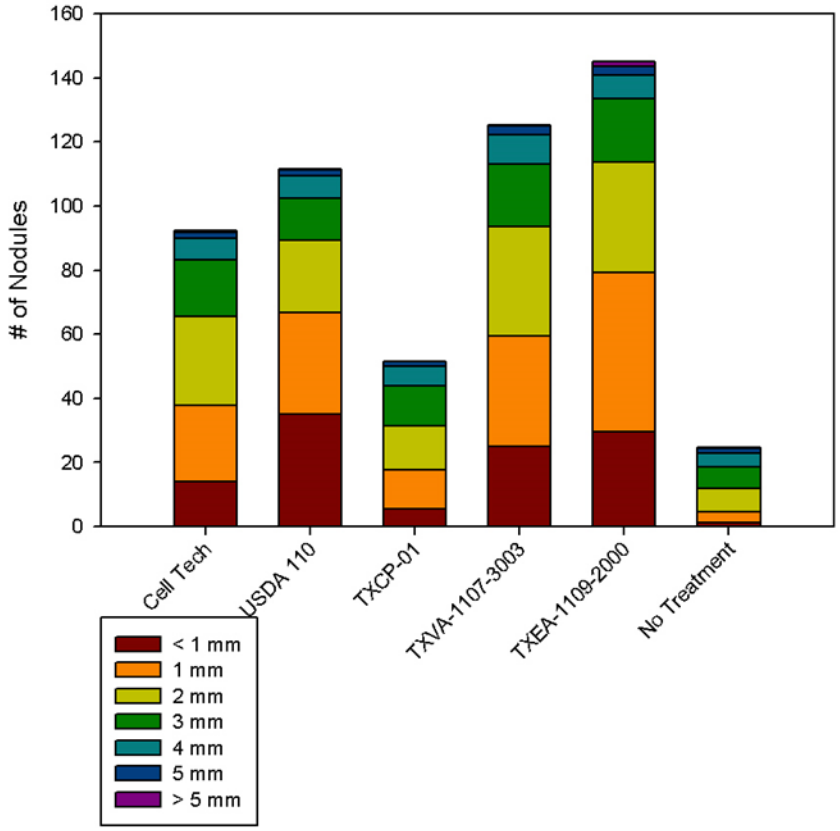


**Figure A1-4.** Average number of nodules for each size category for Vernal cultivar inoculated with various strains in 2016.

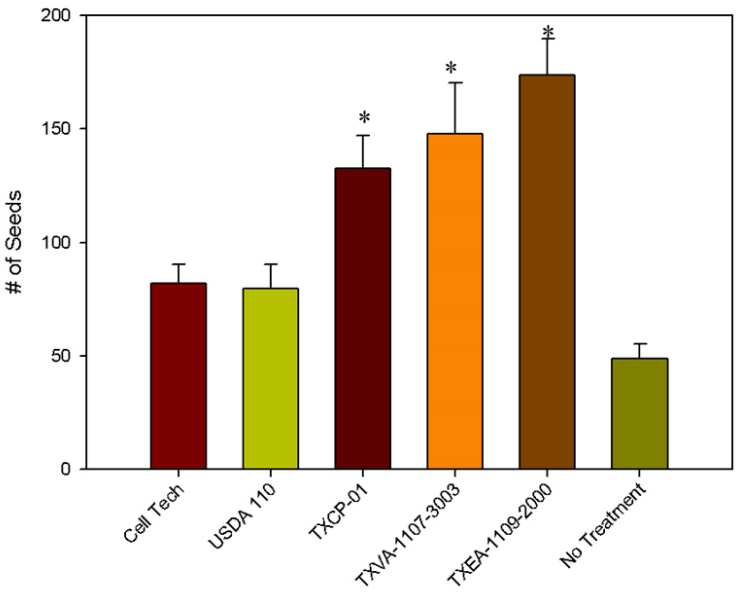


**Figure A1-5.** Average number of seeds produced per Vernal soybean plant inoculated with various strains in 2016. * indicates statistical difference ($P<0.05$) as compared with no treatment.

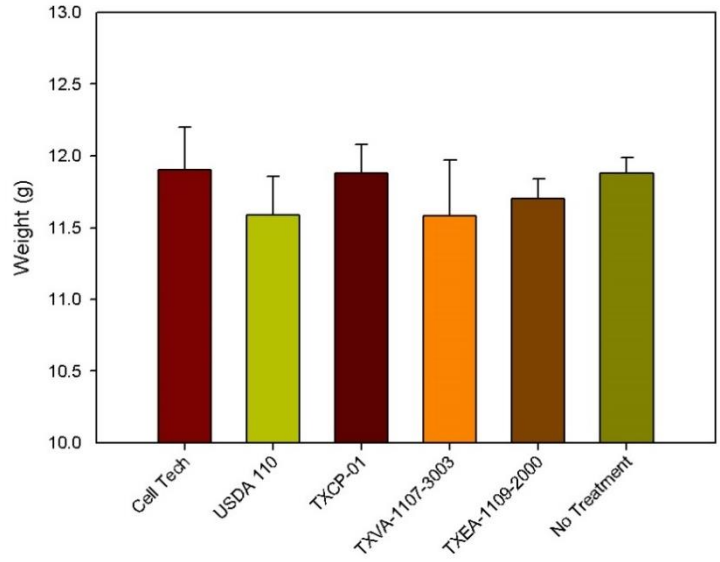


**Figure A1-6.** Average seed dry weight per 100 seeds for Vernal soybean plants inoculated with various strains in 2016.

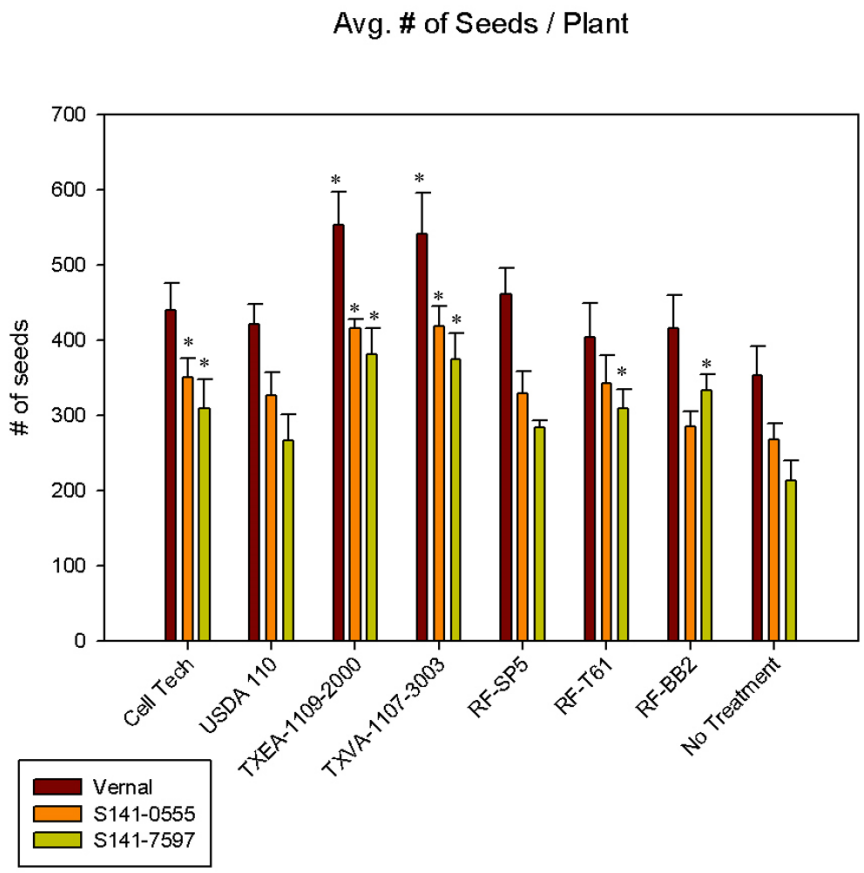


**Figure A1-7.** Average number of seeds produced per Vernal soybean plant inoculated with various strains in 2016. * indicates statistical difference ($P<0.05$) as compared with no treatment.

**Table A1-1. List of bacterial strains.**

| ***B. japonicum* Strain** | **Relevant genotype or phenotype** | **Source or Reference** |
|---|---|---|
| USDA110 | $Cm^R$ wild-type | USDA, Beltsville, MD |
| TXCP-01 | $Cm^R$ native isolate | Delta, Co., Tx |
| TXVA-1107-3003 | $Cm^R$ native isolate | Victoria Co., Tx |
| TXEA-1109-2009 | $Cm^R$ native isolate | Lubbock Co., Tx |
| Cell-Tech® | Liquid nitrogen-fixing inoculant | Monsanto BioAg |

*Cm refers to chloramphenicol

**Table A1-2. List of genes used to detect desiccation-tolerance via qRT-PCR analysis.**

| Gene | Marker ID | Function |
|---|---|---|
| bll0322 | otsA | probable trehalose-6-phosphate synthase |
| bll0323 | otsB | probable trehalose-phosphatase |
| blr6767 | treS | trehalose synthase |
| blr2455 | aceA | isocitrate lyase |

**Table A1-3. Survivability of *Bradyrhizobium japonicum* strains under desiccation conditions.**

| Strain | % Survival (4 hr) | % Survival (72 hr) |
|---|---|---|
| USDA 110 | 94.00 ± 0.16% (B) | 80.00 ± 0.98% (B) |
| TXCP-01 | 96.61 ± 0.01% (A) | 98.55 ± 0.27% (A) |
| TXVA-1107-3003 | 96.72 ± 0.40% (A) | 87.1 ± 0.61% (A) |
| TXEA-1109-2000 | 100.0 ± 0.27% (A) | 93.9 ± 0.63% (A) |

*Note: For each survivability category, letters (A) and (B) indicate a statistical difference from one another ($P < 0.05$).

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