

CHARACTERIZATION OF MOCR, A GNTR TRANSCRIPTIONAL REGULATOR
IN *BRADYRHIZOBIUM JAPONICUM*

by

MAY NYAN TAW

Presented to the Faculty of the Graduate School of
The University of Texas at Arlington in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

MAY 2012

Copyright © by MAY NYAN TAW 2012

All Rights Reserved

ACKNOWLEDGEMENTS

First I would like to express my sincerest gratitude to my supervisor, Dr. Woo-Suk Chang, for giving me the opportunity to learn and work in his lab, and for his endless patience and guidance throughout my graduate experience at UTA. I would like to thank my committee members Dr. Jorge Rodrigues, for giving me confidence to do research especially when I first began grad school and for giving me advice, and Dr. Tom Chrzanowski, for instilling a sense of enthusiasm for microbiology in me. I'd like to thank Dr. Shawn Christensen for also advising and helping me on my protein work. I'd also like to also give a big hug and thanks to Dr. Michelle Badon, my GTA supervisor, who has constantly supported me, always kept my spirits up, and taught me many valuable lessons. I'd also like to thank Rawan Shishakly for helping to construct the *mocR* mutant strain and Blaine for all his advice on protein purification and EMSA.

My research and work in the lab would not have been possible without the constant encouragement and advice given by my lab members Hae-in, Jeong-min, Andrew, Yong-ha, Anchana, Dr. Yang, and James. I cannot begin to express how much all of you have helped me and supported me in the lab both professionally and personally.

I'd also like to express my deepest gratitude towards my parents, Dr. Nyan Taw and Indra Soe, and my brothers, Ko Junior and JJ, for always being there and believing in me.

Last, but not least, I would like to thank Sudeep – thank you for always pushing me and having faith in me when I had doubts -- I could not have done this without you.

April 20, 2012

ABSTRACT

CHARACTERIZATION OF MOCR, A GNTR TRANSCRIPTIONAL REGULATOR IN *BRADYRHIZOBIUM JAPONICUM*

MAY NYAN TAW M.S.

The University of Texas at Arlington, 2012

Supervising Professor: Woo-Suk Chang

The GntR family is one of the most widely distributed and prolific groups of the helix-turn-helix (HTH) transcription factors. In particular, microorganisms that live in complex, fast-changing environments such as soil tend to have a larger aggregate of the *gntR* regulatory genes. *Bradyrhizobium japonicum* is a Gram-negative soil bacterium capable of forming nodules and fixing nitrogen when in a symbiosis with the leguminous soybean plant (*Glycine max*). Although these metabolite-responsive *gntR* genes have been found to be involved in many cellular processes, little is known about their role in the *B. japonicum*-soybean symbiosis. The *blr6977* gene (*mocR*), one of 35 *gntR* genes in *B. japonicum*, was investigated by generating a knock-out mutant strain. The *mocR* mutant strain reached a higher saturation of optical density than the wild type only when grown in minimal media. Motility tests using 0.3% minimal medium agar also revealed enhanced motility by the *mocR* mutant compared to that of the wild type. Nodulation experiments were conducted in order to determine a nodulation phenotype of the mutant strain. The outcome indicated the *mocR* mutant was deficient in the number of nodules compared to the wild type, and it resulted in delayed nodulation. An electrophoretic mobility shift assay (EMSA) revealed protein-DNA interaction using purified

MocR protein and a probe containing the promoter region upstream of *blr6977*, indicating the possible autoregulation of the *mocR* gene.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
LIST OF ILLUSTRATIONS	viii
LIST OF TABLES	x
Chapter	Page
1. BACKGROUND AND SIGNIFICANCE.....	1
1.1 The U.S. Soybean Crop Industry and Environmental Impact.....	1
1.2 <i>Bradyrhizobium japonicum</i> and Symbiosis with the Soybean Plant.....	3
1.3 The GntR Superfamily of Transcriptional Regulators.....	5
1.4 The MocR Subfamily	6
2. MATERIALS AND METHODS	9
2.1 Rationale of Approach.....	9
2.2 Bacterial strains, plasmids, and growth conditions.....	9
2.3 Construction of <i>mocR</i> mutant strain	11
2.4 Construction of <i>mocR</i> complement strain	13
2.4.1 Insertion of <i>mocR</i> gene into the pGEM-T easy vector	13
2.4.2 Confirmation of orientation of <i>mocR</i> in pGEM-T easy vector	15
2.4.3 Transfer of <i>mocR</i> gene to pBBR1MCS-3 vector	15
2.4.4 Tri-parental mating of wild type <i>mocR</i> into <i>mocR</i> mutant.....	16
2.5 Phenotypic Tests.....	16
2.5.1 Growth curve	16
2.5.2 Colony morphology	17
2.5.3 Motility test	17

2.5.4 Pouch experiment	17
2.6 MocR Protein Purification	18
2.6.1 Construction of pQE2::MocR strain	18
2.6.2 Time-course analysis of protein expression	19
2.6.3 Determination of the MocR protein solubility	19
2.6.4 Overexpression of <i>B. japonicum</i> MocR protein in <i>E. coli</i>	20
2.7 Electrophoretic Mobility Shift Assay (EMSA)	21
2.7.1 Preparation of nucleic acid target	21
2.7.2 EMSA procedure	21
2.7.3 EMSA gel extraction	22
3. RESULTS AND DISCUSSION	23
3.1 Phenotypic comparisons of free-living <i>mocR</i> mutant	23
3.2 Symbiotic phenotype comparison of <i>mocR</i> mutant	27
3.3 MocR binds to its own promoter region	29
3.4 Discussion	32
3.5 Future Directions	35
APPENDIX	
A. QRT-PCR ANALYSIS OF GNTR GENES	37
REFERENCES	40
BIOGRAPHICAL INFORMATION	43

LIST OF ILLUSTRATIONS

Figure	Page
1. Rise soybean crop value in the U.S. from 1985 – 2010, USDA (2011).	1
2. Satellite image depicting effects of eutrophication of the northern Gulf of Mexico/Mississippi Delta, showing hypoxic coastal water, January 2003. (B) Monthly U.S. prices of natural gas and ammonia from 1985 – 2006.	3
3. Structural representation of the effector or oligomerizing C-terminal domain of the MocR subfamily.	7
4. Design of <i>mocR</i> mutant construction. The <i>mocR</i> gene was disrupted by inserting a kanamycin cassette gene. Consequent double homologous recombination then occurred into the genome of <i>B. japonicum</i>	12
5. Design of <i>mocR</i> complement strain construction. The <i>mocR</i> gene was cloned into the broad host range vector pBBR1MCS-3. Then triparental mating was carried out using the construct containing the wild type <i>mocR</i> gene with the <i>mocR</i> mutant and the <i>E. coli</i> pRK2073 helper strain.	14
6. Colony morphology growth of wild type <i>B. japonicum</i> (left half) and <i>mocR</i> mutant (right half) on AG media (A) and Minimal media (B). Plates were incubated at 30°C for 5 days on AG and 10 days on Minimal Media.	23
7. Growth rate of <i>B. japonicum</i> , the <i>mocR</i> mutant and its complement strain in AG media (A) and minimal media (B). The strains were incubated at 30°C and 200 rpm. Measurements of optical densities were taken over a period of 5 days at 600 nm. Values are the means of O.D. measurements of triplicate cultures and bars represent standard error.	25
8. (A) Motility tests comparing mutant, wild type and complement strains on 0.3% agar minimal media plates that were spotted with 15 µl of culture (OD ₆₀₀ =0.5) and incubated for 3-4 days at 30°C. Diameters of growth were then measured. Motility tests comparing the wildtype and mutant strains were also conducted on 1% AG agar (B), 0.3% AG agar (C), 1% MM agar (D), and 0.3% MM agar (E).	26
9. The <i>mocR</i> mutant strain had significantly larger diameter compared to the wild type, <i>P</i> < 0.001.	27
10. 3D Sigma plot of pouch experiment results. The graphs depict the distance of the root nodules from the RT (mm) and the average number of nodules counted at that position of soybean seeds that were treated with wild type USDA110 and <i>mocR</i> mutant using a (A) high inoculum (ca. 1x10 ⁸ cells/ml) and (B) low inoculum (ca. 1x10 ² cells/ml). Values are the means of 3 biological replicates each with 9 technical replicates.	28

11. Nodulation effect of the wild type, mutant, and complement strains on the soybean plant	29
12. 15% SDS-PAGE gel after running purified MocR protein (~53.79 kDa) (A), lysed protein extract before induction with IPTG (B), and lysed protein extract after induction (C).	30
13. EMSA gel with SYBR Green staining (A) and SYPRO Ruby staining (B). Lane 1, 1 kb ladder; Lane 2, empty vector with probe; Lanes 3-6, titration of protein with probe of the region indicated in the bottom panel. (A) Bottom arrow depicts the free probe; middle arrow depicts false shifts; top arrow depicts shift representing protein:DNA complex. (B) Top arrow indicates protein stained with SYPRO Ruby.	31
14. 15% SDS-PAGE gel after running EMSA gel extracted eluted protein.	32
A1. qRT-PCR analysis of the 15 <i>gntR</i> genes selected. Out of the 15 tested genes, 11 were annotated as a part of the FadR subfamily, 3 to the MocR subfamily, and 1 to the HutC/FarR subfamily. The histidyl-tRNA synthetase (<i>HisS</i>) gene was selected as a reference gene to normalize the expression data.	39

LIST OF TABLES

Table	Page
1. Table of bacterial strains and plasmids used in this study	11
A1. qRT-PCR primer sets	38

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 The U.S. Soybean Crop Industry and Environmental Impact

The leguminous soybean plant (*Glycine max*) is an extremely important crop to the U.S. agricultural economy, generating a total crop value of more than \$38 billion dollars in 2010, second only to corn (Figure 1) (7). It is the most abundant source of protein feed and the second largest source of vegetable oil in the world. In fact, the U.S. is the foremost producer soybean globally, exporting about half of U.S. soybean production to countries including China, European nations (EU), Mexico, and Japan (7). Soybean crops are the second most planted field in the U.S. with 77.4 million acres of acreage in 2010, mostly concentrated in the upper mid-west regions (7).

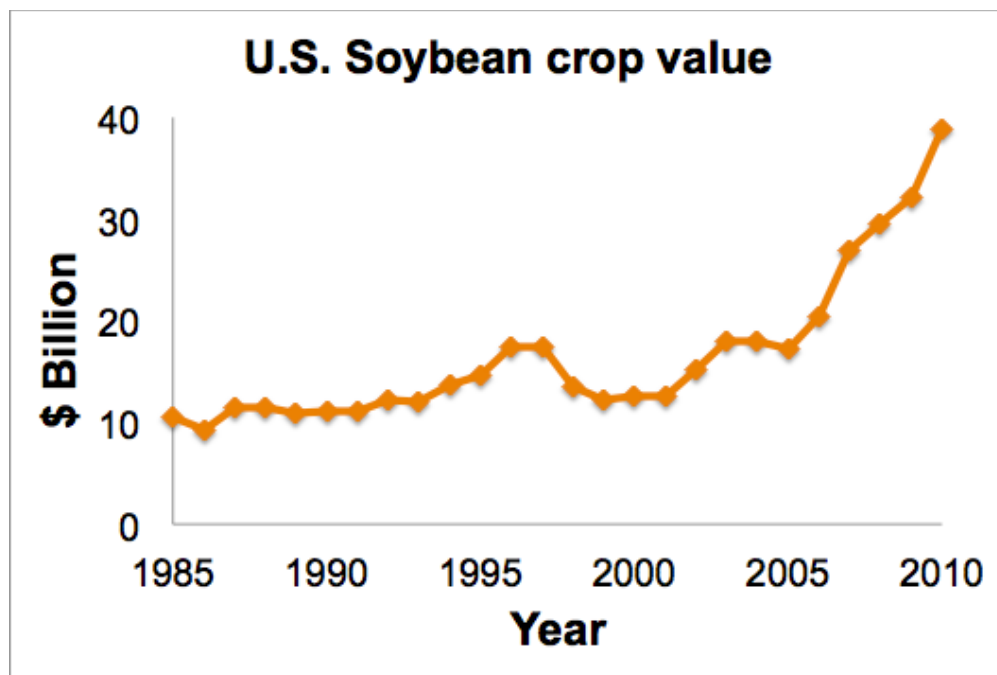


Figure 1. Rise of soybean crop value in the U.S. from 1985 – 2010, USDA (2011)

The approach to processing soybeans involves cleaning, cracking, dehulling, and grounding the seeds into flakes, allowing the separation and extraction of oil from meal components. The oil can then be used in many dietary products or for a component called lecithin, used for a variety of purposes including its coating properties and for pharmaceutical uses (7). Soybean meals are commonly used as a source of protein for feed manufacturers providing nutrients for livestock industries (19). Increases in soybean acreage along with improvements in seed choices, fertilizer, and the use of pesticides to produce higher crop yields, diminish per-bushel production, and increase profits.

Nitrogen is an important limiting nutrient for primary production in terrestrial ecosystems, and thus the use of synthetic nitrogen fertilizers have played an significant role in escalating agricultural crop yields and livestock production (1). However, due to the continued growing demand for soybean crop yields, the effects of using artificial fertilizers enriched with ammonium nitrate have had both economical and environmental effects.

The leaching of synthetic reactive nitrogen through over-use of synthetic fertilizers can have detrimental effects on the environment including eutrophication of marine ecosystems, soil and water acidification, and deprivation of biodiversity in aquatic ecosystems (12). It is even estimated that approximately 60% of the U.S. coasts and coastal rivers have been affected by nutrient pollution (29).

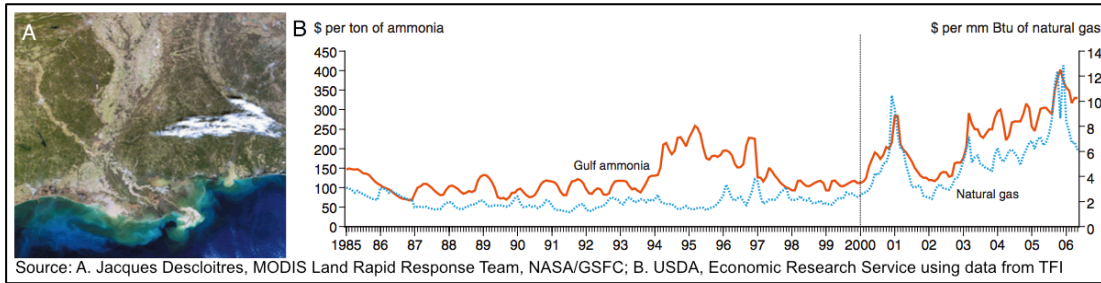


Figure 2. (A) Satellite image depicting effects of eutrophication of the northern Gulf of Mexico/Mississippi Delta, showing hypoxic coastal water, January 2003. (B) Monthly U.S. prices of natural gas and ammonia from 1985 – 2006.

Although soybean crops typically require less commercial fertilizer than other crops, due to their ability to fix nitrogen with the endosymbiont, *Bradyrhizobium japonicum*, over the past decade the cost of ammonium nitrate has nearly doubled impacting the cost of production of profitability to farmers (Figure 2) (7).

1.2 *Bradyrhizobium japonicum* and Symbiosis with the Soybean Plant

B. japonicum USDA110 is a Gram negative bacterium which inhabits soil environments. It is a member of the α -proteobacterial *Rhizobiaceae* family, which includes other nitrogen fixing rhizobia such as *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium* (11, 26, 34). It was first isolated from soybean nodule located in Florida, USA in 1957 and is considered one of the most agriculturally significant microbial species due to its ability to form nodules on the soybean from which it differentiates into nitrogen-fixing bacteroids (22). In fact, the relationship between *B. japonicum* and its host, *Glycine max*, is one of the most thoroughly investigated microbial symbioses; in particular the *B. japonicum* USDA110 strain has been well-studied in its molecular genetics, physiological, and ecological aspects (13, 22). The complete genome of *B. japonicum* USDA110 was sequenced in 2002 by whole-genome shotgun sequencing in tandem with the “bridging shotgun” method (22). The genome consisted of a single circular chromosome of 9,105,828 bp without plasmids, a GC content of 64.1%, and was found to

contain 8,314 open reading frames (22). Furthermore, 34% of the putative genes have sequence similarities to those present in *Mesorhizobium loti* and *Sinorhizobium meliloti*, whilst 23% of the genes remain unique to *B. japonicum* (22).

The process of nodulation between the soybean plant and *B. japonicum* involves a multi-step approach to establish a relationship. First, the leguminous plant must release root exudates, containing amino acids, dicarboxylic acids, and isoflavonoids, which act as a chemo-attractant for the symbiont in the rhizosphere (34). Isoflavonoids encompass a group of plant products based on a tricyclic C-15 unit and include compounds such as daidzein and genistein (28). In response to these compounds, *B. japonicum* undergoes a change in its transcriptional expression, most importantly with the activation of the *nod* genes, which regulate the production of Nod factors specifically with the LysR-family transcriptional regulator NodD and a classical two-component regulatory system, NodVW (26, 33). Nod factors are a lipooligosaccharide molecule with a chitin backbone, consisting of four or five N-acetylglucosamine molecules, and a lipid attachment (11, 26, 33). They are important in facilitating developmental changes in the host plant during early nodulation stages such as root hair adherence, root curling, and root cortex cell division (11).

Infection begins once *B. japonicum* cells are enclosed between root hair cell walls through a process called curling and an infection thread is formed into the epidermal cell (11). Within infection thread, the symbiont continues to divide and moves deeper inside the root until it reaches and colonizes the nodule cells where it differentiates into its bacteroid state and begins expression of nitrogen fixation genes (*nif* and *fix*) and other genes required for nodule formation and maintenance of the symbiosis (11). The *nif* and *fix* genes are essential in the process of nitrogen fixation, in which atmospheric nitrogen (N_2) is catalyzed to ammonium by the enzyme nitrogenase. The fixed nitrogen is then provided to the plant as a source of nitrogen, where in return the symbiont is reciprocated with photosynthates and amino acids as a source of carbon and energy (8, 16). The *nif* genes refer to nitrogen fixation related genes that

have homologies to those found in *Klebsiella pneumoniae*, whereas the *fix* genes refer to nitrogen fixation related genes specific to rhizobial species. One of the key components in regulating and activating the *nif* and *fix* genes in *B. japonicum* is the *nifA* gene which is a part of the *fixRnifA* operon (16). Under aerobic conditions, such as when the microbe is in free-living state, the operon is expressed minimally. However, inside the microaerobic conditions of the nodule, the expression of *fixRnifA* increases significantly and the NifA protein is synthesized. NifA, which is highly sensitive to high concentrations of oxygen, can then activate other *nif* and *fix* operons and begin nitrogen fixation (8).

1.3 The GntR Superfamily of Transcriptional Regulators

The GntR family, first identified and named after the gluconate repressor in the gluconate operon in *Bacillus subtilis*, is one of the most widely distributed helix-turn-helix transcription factors (10, 30). There are over 8,500 GntR regulators spread out among 764 bacterial taxa, most of which are found in the Proteobacteria, Firmicutes, and Actinobacteria (18). Members of this family contain a highly conserved DNA-binding N-terminal and a very diverse effector binding and/or oligomerization C-terminal. Due to its large number of members, the GntR family has been further divided into 6 subfamilies by their amino acid sequence alignment secondary structure predictions: AraR, DevA, FadR, HutC, MocR, PlmA, and YtrA (30).

GntR-like regulators have been found to control many fundamental processes such as motility, development, antibiotic production, and virulence (14, 17, 18, 20). Additionally, they can act as activators or repressors of gene expression in other parts of the genome. *B. japonicum* itself has 35 different genes encoding for putative GntR regulators. Despite the abundance of GntR family regulators, there have been relatively few studies that have extensively analyzed the protein and their operators to characterize protein function, especially in the MocR subfamily. The challenge in identifying the small molecules that bind to these regulators has

also proven difficult and is an important factor in linking the GntR regulators to different pathways. Thus, gene context and bioinformatics has been applied as one of the most important approaches to studies in this field. Furthermore, a trend suggesting that microorganisms inhabiting complex, fast-changing environments like soil tend to contain a greater number of GntR regulators in their genomes, makes an interesting facet to study the *gntR* family in *B. japonicum* and to determine its involvement in establishing the symbiosis and survival in the rhizosphere.

In a previous study two *gntR* mutants, *gtrA* (VanR) and *gtrB* (FadR), were generated in *S. meliloti* 1021 to determine the effects on nodulation (35). It was reported that the mutants had deficiencies in growth, motility, as well as nodulation with the alfalfa plant (35).

Another study, focusing on the *mocR* gene in *Sinorhizobium meliloti* L5-30 was also found to be involved in facilitating competition in rhizosphere to initiate nodulation with *Medicago sativa*. More specifically, the MocR protein was found to be a regulator of the *mocABC* operon, which is essential for the catabolism and utilization of rhizopine (L-3-O-methylscyllo-inosamine), a symbiosis-specific compound produced in the nodules and induced by *S. meliloti* (31).

1.4 The MocR Subfamily

Characterization of the *blr6977* gene, a MocR subfamily member, in *B. japonicum* was the focus of this study. Although the *blr6977* gene has been directly annotated as a transcriptional regulator of the GntR family, due to its high homology to MocR subfamily, we renamed the gene to *mocR*. The MocR subfamily is unique from other GntR subfamilies in that its members contain a highly conserved DNA-binding terminus along with a lengthy C-terminus averaging at 350 amino acids (Figure 3) (18). The C-terminus also has a high homology to class I aminotransferase enzymes involved in amino acid metabolism (5). Class I aminotransferases are dimeric proteins that work by catalyzing the transfer of an amino group to an acceptor

molecule such as an aldehyde or keto acid with the cofactor pyridoxal-5'-phosphate (PLP), a B₆ vitamin that is needed by many enzymes as a cofactor to become activated (5, 18). This suggests the possibility of catalytic activity in the C-terminal domain of the protein. Aminotransferases are usually involved in the process of synthesizing or degrading amino acids; more specifically, class I aminotransferases have been shown to be associated with the biosynthesis of aspartate, phenylalanine, tyrosine, histidine, and alanine (Kalinowski 2003).

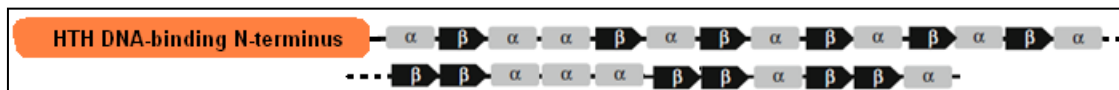


Figure 3. Structural representation of the effector or oligomerizing C-terminal domain of the MocR subfamily.

Only three MocR proteins have been characterized molecularly. The first of which was GabR from *Bacillus subtilis*, a positive transcriptional regulator of the *gabTD* operon. The proteins, GabT and GabD, are important for glutamate production from GABA, which can then be used for nitrogen metabolism (3). The GabR protein was also found to be capable of binding to its own promoter to negatively regulate transcription of *gabR* (3). Also, PLP was required to for proper induction of the *gabTD* operon, but unnecessary for its own repression (3).

This conflicted with TauR, another MocR-type protein, from *Rhodobacter capsulatus* which was not affected in the presence or absence of PLP as an activator of the *tpa-tauR-xsc* operon. The *tpa-tauR-xsc* along with *tauABC* are essential for taurine utilization as a sulfur source as well as its uptake respectively (36).

Finally, the *pdxR* gene regulates the synthesis of PLP in *Corynebacterium glutamicum*, by positively regulating the *pdxST* operon (21). Thus in this case, addition of PLP was indeed not required in order for PdxR to function (21).

Members of the GntR superfamily have been well associated with roles in modulating gene expression in response to the environment. The fact that *B. japonicum* contains a large

aggregate of them in the genome, makes it an interesting prospect to study their involvement in the context of both its free-living and bacteroid states. Previous studies have already associated certain GntR proteins with symbiosis, such as in the cases of GtrA and GtrB in *S. meliloti*. Since MocR in *S. meliloti* has already been associated in symbiosis with its involvement with the symbiosis-specific compound, rhizopine, and because very few MocR type genes have been characterized thus far, we decided to focus on one of the MocR members in *B. japonicum*.

HYPOTHESIS

The *blr6977* gene of *Bradyrhizobium japonicum* USDA110 encodes a MocR transcriptional regulator that plays a role in nodulation of the soybean plant.

CHAPTER 2

MATERIALS AND METHODS

2.1 Rationale of Approach

In order to characterize the role of the *mocR* gene, two strains were constructed: a *mocR* mutant strain of *B. japonicum* USDA110 and its complement strain. These strains were used along with wild type *B. japonicum* USDA110 in several phenotypic tests looking at the growth rate, colony morphology, and motility under rich and minimal media conditions, as well as a pouch experiment to determine the effects on nodulation. Furthermore, to molecularly characterize the protein encoded by *blr6977* the MocR protein was purified by cloning it into the pQE2 vectors (Qiagen Sciences, Maryland, MD) and over expressing the ~53.79 kDa protein by induction with IPTG in *E. coli* JM109 cells. Electrophoretic mobility shift assays (EMSA) were conducted using the purified protein and target nucleic acid probes containing the promoter region upstream of *blr6977* to determine if there was an interaction between the protein and target probes. EMSAs were performed using the EMSA Kit with SYBR Green and SYPRO Ruby Stains (Invitrogen, Carlsbad, CA). Gel shifts bands were then cut out and the protein eluted in 2X SDS-PAGE loading buffer and run through an SDS-PAGE gel to confirm the size of the protein in the complex.

2.2. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used throughout this study are listed in Table 1. *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of deionized water and at 200 rpm when grown in broth media. All *B. japonicum* strains were grown in arabinose-gluconate (AG) medium containing 0.125 g of Na₂HPO₄, 0.25 g of Na₂SO₄, 0.32 g of NH₄Cl, 0.18 g of MgSO₄·7H₂O, 0.01 g of

CaCl₂, 0.004 g of FeCl₃, 1.3 g of HEPES, 1.1 g of MES, 1 g of yeast extract, 1 g of L-arabinose, and 1 g of Na-gluconate at 30°C and at 200 rpm when grown in broth media (32). For the growth curve assay, colony morphology, and motility tests *B. japonicum* was also grown in minimal media containing 0.3 g of K₂HPO₄, 0.3 g of KH₂PO₄, 0.5 g of NH₄NO₃, 0.1 g of MgSO₄·7H₂O, 0.05 g of NaCl, 4 ml of glycerol, 1 ml of trace elements, and 1 ml of vitamin stock per liter of deionized water. Trace elements contained 5 g of CaCl₂, 145 mg of H₃BO₃, 125 mg of FeSO₄·7H₂O, 70 mg of CoSO₄·7H₂O, 5 mg of CuSO₄·7H₂O, 4.3 mg of MnCl₂·7H₂O, 108 mg of ZnSO₄·7H₂O, 125 mg of NaMoO₄, and 7 g of nitrilotriacetate per liter of deionized water and adjusted to a pH of 5.0. The vitamin stock was prepared using 20 mg of riboflavin, 20 mg of p-amino benzoic acid, 20 mg of nicotinic acid, 20 mg of biotin, 20 mg of thiamine hydrochloric acid, 20 mg of pyridoxine hydrochloric acid, 20 mg of calcium pantothenate, 120 mg of inositol, and dissolved in 0.5 M disodium hydrophosphate. Antibiotics for strain or plasmid selection were ampicillin (50 µg/ml for *E. coli*, unless indicated otherwise), streptomycin (50 µg/ml for *E. coli*), kanamycin (50 µg/ml for *E. coli* and 150 µg/ml for *B. japonicum*), tetracycline (15 µg/ml for *E. coli* and 50 µg/ml for *B. japonicum*), and chloramphenicol (30 µg/ml for *B. japonicum*).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or characteristic	Source reference	or
<i>B. japonicum</i>			
strains			
USDA110	Wild-type	USDA, Beltsville, MD	
<i>mocR</i> mutant	<i>mocR</i> ::Km	This study	
<i>mocR</i> complement	<i>mocR</i> ::Km with pBBR1MCS3 vector with <i>mocR</i> insert	This study	
<i>E. coli</i> DH5a	<i>supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F' (traD36, proAB⁺ lacI^q, Δ(lacZ)M15) endA1</i>	Bethesda Research Laboratories	
<i>E. coli</i> JM109	<i>recA1 hsdR17 mcrA supE44λ⁻ gyrA96 relA1 Δ(lac⁻proAB) thi-1</i>	37	
Plasmids			
pRK2073	RK2, Tra ⁺ Sm ^r	25	
pGEM-Teasy (pGEMTE)	Amp ^r , Vector with MCS within α-peptide coding region of β-galactosidase	Promega, Madison, WI	
pBBR1MCS3	Broad host range expression vector, Tc ⁺	23	
pQE2	Protein expression vector with T5 promoter, 6xHis tag sequence, <i>lac</i> operator, <i>lacI^q</i> repressor, Amp ^r	2	
pGEMTE-MocR	pGEMTE vector containing 1.5-kb fragment including entire <i>mocR</i> gene	This study	
pBBR1MCS3-MocR	pBBR1MCS vector containing 1.5-kb fragment including entire <i>mocR</i> gene	This study	
pQE2-MocR	pQE2 vector containing entire coding region of <i>mocR</i> gene	This study	

2.3 Construction of the *B. japonicum mocR* mutant

A 2.5 kb fragment containing the *mocR* gene, *blr6977*, was amplified by PCR using whole genomic DNA extracted from *B. japonicum* and the primers RV 5'-AGT GGG TTT CCC TGA GTT TT-3' and FW 5'-CTG ATC AGC GTG CAG GCA GA-3'. The PCR reaction was run using a C1000 Thermo Cycler (Biorad, Hercules, CA) at the following conditions: (1) initial denaturation at 95°C for 5 minutes; (2) continued by 35 cycles of denaturation at 95°C for 90 seconds, annealing at 55°C for 90 seconds and extension at 72°C for 2 minutes, and a final

extension at 72°C for 5 minutes. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen Sciences, Maryland, MD). The PCR product was confirmed by loading onto a 1% agarose 1X Tris-EDTA (TAE) gel containing ethidium bromide and running the gel through a PowerPac™ Basic (Biorad, Hercules, CA) at 120 V for 35 minutes, and exposing the gel to UV light for visual confirmation of amplification of the correct band size. The fragment was then cloned into the pKO Ω suicide vector. The gene was then disrupted by the insertion of a 1.5 kb kanamycin cassette at the BsrGI site. The resulting plasmid was then introduced into the wild type *B. japonicum* by triparental mating with the pRK2073 helper strain. The intact *mocR* gene was substituted with the disrupted *mocR* gene by double homologous recombination. A diagram depicting the construction of the mutant strain is shown in Figure 4.

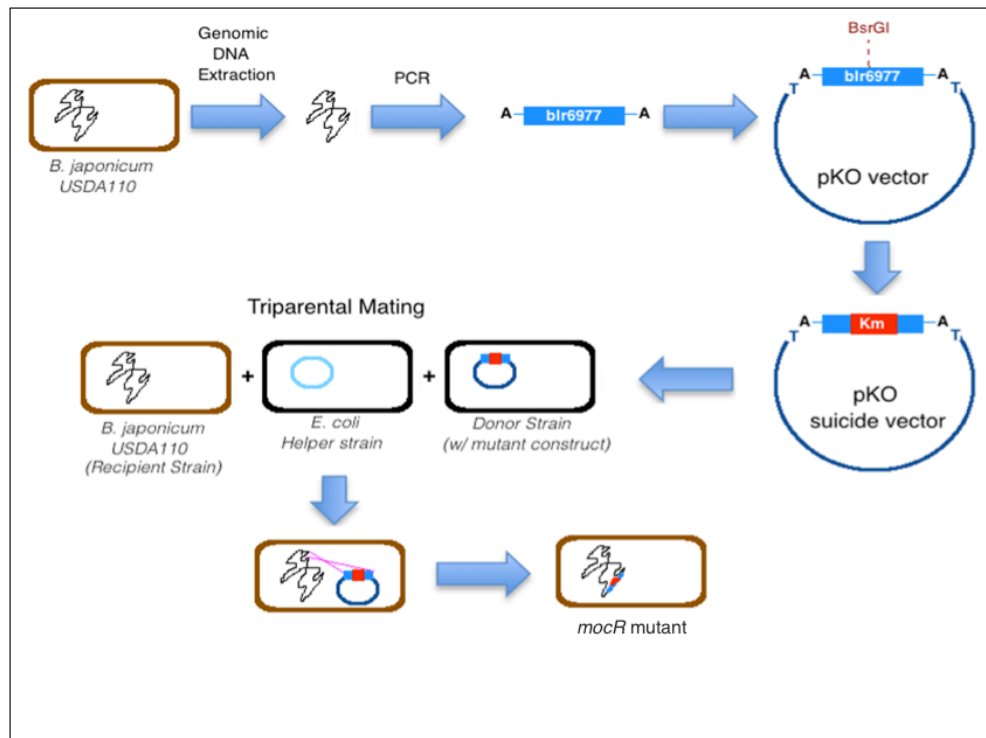


Figure 4. Design of *mocR* mutant construction. The *mocR* gene was disrupted by inserting a kanamycin cassette gene. Consequent double homologous recombination then occurred into the genome of *B. japonicum*.

2.4 Construction of the *B. japonicum mocR* complement strain

2.4.1 Insertion of *mocR* gene into pGEM-T easy vector

Genomic DNA from wild type *B. japonicum* USDA110 was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). The *mocR* gene was amplified using the genomic DNA as a template using the *blr6977* specific forward primer with an EcoRI enzyme site linker (MocR+EcoRI_FW: 5'—CCG GAA TTC AGT CGA GAC AAT ACG CG—3') and *blr6977* specific reverse primer with a HindIII enzyme site linker (MocR+HindIII_RV: 5'—GGG TTC GAA GAT GCA GCG GAA GAG G—3'). Two different restriction enzyme linker sites were added so that the orientation of the insert placed into the vector could be controlled. As recommended by the Promega GoTaq™ DNA polymerase system protocol, eight 50 µl PCR reactions were prepared using 5X Green GoTaq Reaction Buffer, 20-50 ng genomic DNA template, 1µM of the primer set, 10mM dNTP Mix, and 1.25u of GoTaq DNA Polymerase. The PCR reaction was run using a C1000 Thermo Cycler (Biorad, Hercules, CA) at the following conditions: (1) initial denaturation at 95°C for 5 minutes; (2) continued by 35 cycles of denaturation at 95°C for 90 seconds, annealing at 62°C for 90 seconds and extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen Sciences, Maryland, MD). The PCR product was confirmed by loading onto a 1% agarose 1X Tris-EDTA (TAE) gel containing ethidium bromide and running the gel through a PowerPac™ Basic (Biorad, Hercules, CA) at 120 V for 35 minutes, and exposing the gel to UV light for visual confirmation of amplification of the correct band size. Ligation was conducted using 110 ng of the purified PCR product, 50 ng of pGEM-T easy vector, 2X Rapid Ligation Buffer, 3 Weiss units of T4 DNA Ligase, and brought to a final volume of 10 µl with nano-pure water. The ligation reaction was incubated at 4°C overnight. Heat-shock transformation was conducted by adding the ligation reaction to 50 µl of thawed DH5α competent cells. The mixture was incubated on ice for 20 minutes, placed in a water bath

at 42°C for 90 seconds, and placed back on ice for another 90 seconds. Subsequently, 300 µl of LB medium was added to the mixture and incubated at 37°C at 200 rpm for 1 hour, after which 100 µl of the solution was spread onto an LB agar plate containing ampicillin and X-gal (50 µg/ml). The plates were incubated at 37°C for 24 hours, after which white colonies were screened for presence of the insert by colony PCR using the same primer set and conditions described previously. A diagram depicting the construction of the complement strain is shown in Figure 5.

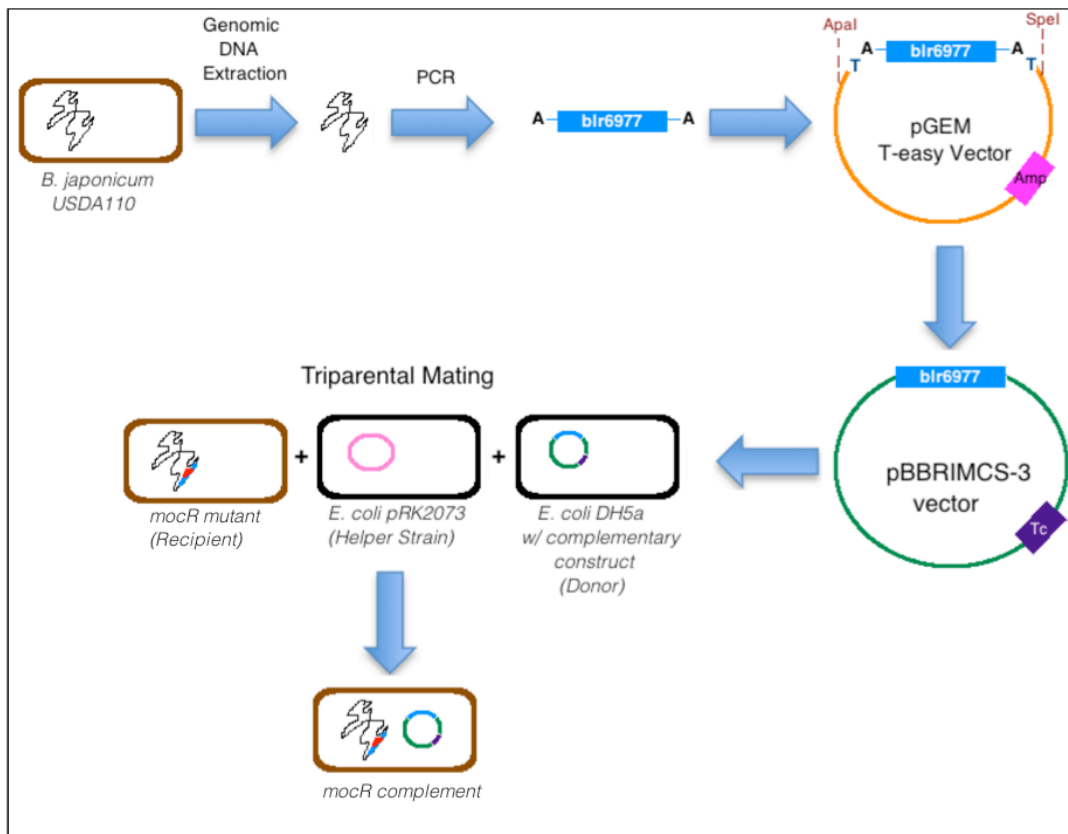


Figure 5. Design of *mocR* complement strain construction. The *mocR* gene was cloned into the broad host range vector pBBR1MCS-3. Then triparental mating was carried out using the construct containing the wild type *mocR* gene with the *mocR* mutant and the *E. coli* pRK2073 helper strain.

2.4.2 Confirmation of orientation of *mocR* gene in pGEM-T easy vector

To ensure proper expression of the *mocR* gene in the vector, it was important to determine the orientation of the insert so that transcription would run in the same direction as that of the promoter placed next to it. To determine the orientation of the insert in the pGEM-T easy vector, plasmid extraction was conducted using the AccuPrep Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea) and a double restriction enzyme digestion was carried out using *HindIII* and *SpeI* in NEBuffer 2 (New England Biolabs, Ipswich, MA). The digestion reaction was run through a 1% agarose 1X TAE gel containing ethidium bromide and exposed to UV light for confirmation of the correct band pattern.

2.4.3 Transfer of *mocR* gene to pBBRIMCS-3 vector

Plasmid extraction was repeated on colonies that contained vectors containing the correct orientation of the insert as well as for the *E. coli* pBBRIMCS-3 vector and digested with *ApaI* and *SpeI* in NEBuffer 4 (New England Biolabs, Ipswich, MA). The digestion reaction was run through 1% agarose 1X TAE gel containing ethidium bromide, and the band corresponding to the size of the insert was extracted and purified using the QIAquick Gel Extraction Kit (Qiagen Sciences, Maryland, MD). The purified products were concentrated for 30 to 45 min at the vacuum-aqueous setting at 30°C using the Eppendorf Vacufuge Plus (Eppendorf, Hauppauge, NY). Ligation was conducted using 1u T4 DNA Ligase and 1X T4 DNA Ligase Reaction Buffer (New England Biolabs, Ipswich, MA), as well as 50 ng of digested pBBRIMCS-3 and 10 ng of gel extracted insert. The ligation reaction was incubated overnight at 16°C. Heat-shock transformation was conducted as previously described using *E. coli* DH5 α competent cells and plated onto LB-agar plates with tetracycline and X-gal. White colonies were screened by plasmid extraction and digestion with *ApaI* restriction enzyme.

2.4.4 Tri-parental mating of wild type *mocR* gene into *mocR* mutant strain

Tri-parental mating was performed by harvesting the *E. coli* pBBRIMCS-3::MocR strain, *E. coli* pRK2073 helper strain, and *mocR* mutant strain at an OD of 0.5. The cell pellets of the recipient, helper, and donor strains were combined at ratios of 1:1:1, 2:1:1, and 3:1:1. The solutions were centrifuged at 7,000 rpm for 5 minutes at room temperature and the pellets were resuspended in 1 ml of AG media and centrifuged in the same conditions. The supernatant of each mixture was removed and resuspended again in 50 μ l of AG media. A membrane was placed in the center of an AG plate, and the donor, recipient, and helper solutions were combined into a single microcentrifuge tube and placed onto a membrane. The plates were incubated at 30°C for 48 hours. The membrane was removed using sterilized tweezers and placed in a 50 ml falcon tube containing 3 ml of AG media and vortexed. Serial dilutions were performed and 100 μ l of each dilution was plated with tetracycline, kanamycin, and chloramphenicol. The plates were incubated for 6 to 9 days. Colonies were screened for the presence of the wildtype and mutant *mocR* genes by colony PCR using the primer set described previously.

2.5 Phenotypic Tests

2.5.1 Growth curve

An isolated colony of the wild type USDA110 strain, the *mocR* mutant strain, and the *mocR* complement strain were inoculated into 25 mL AG medium starter cultures with chloramphenicol (30 μ g/ml), and incubated at 30°C to an OD₆₀₀ of 1. The cultures were subcultured (1:100) with the same medium and the cell density was measured at 12 hour intervals at 600 nm with a Spectronic Genesys™ 5 spectrophotometer. Replicates of three were used to calculate the mean OD. The growth curve assay was repeated using minimal medium.

2.5.2 Colony morphology

To isolate and compare colony morphologies, the wild type strain and the *mocR* mutant strain, were grown to an optical density of 0.5-1.0 at 30°C at 200 rpm in AG media with appropriate antibiotics. The strains were streaked side by side on AG agar and minimal media agar and incubated at 30°C for 7-10 days.

2.5.3 Motility test

The wild type, *mocR* mutant, and *mocR* complement strains were grown to an OD₆₀₀ of 0.8. Aliquots of 5 µl of the bacterial cultures were pipetted on the center of the surface of AG agar (0.3% and 1% agar) and Minimal Media agar (0.3% and 1% agar) and incubated at 30°C for 3-4 days. The diameters of the growth were recorded using a ruler and black background.

2.5.4 Pouch experiment

(i) Preparation of Inocula

A single colony from the wild type, *mocR* mutant, and *mocR* complement strains was used to inoculate 50 ml of AG media in 250-ml Erlenmeyer flasks for each strain. The cultures were grown to an OD₆₀₀ of 1 at 30°C at 200 rpm and diluted to an OD₆₀₀ of 0.1 and 1×10^{-7} by serial dilutions with half-strength B&D media which did not contain nitrogen (24).

(ii) Preparation of soybean seeds and pouches

Seeds were sterilized by stirring them in 30% Clorox for 10 minutes, washing 3 times with distilled water, stirred in 0.1M HCl solution for 10 minutes, and washed again in distilled water. The seeds were placed in petri dishes (7 seeds/plate) with sterile M3 filter paper soaked in 10 ml of sterile nano-pure water. The petri dishes were covered completely in aluminum foil and kept at room temperature for 2-3 days. The germinated seeds were placed in growth pouches (3 seeds per pouch) that had previously been soaked in distilled water and autoclaved.

Once placed inside the growth pouch, the root tip was marked on the pouch and placed in hanging folders. Straws were inserted into the side of growth pouches. The folders were transferred to a rack and 1 ml of the prepared culture was used to inoculate the seeds. Approximately 20 ml B&D solution was dispensed into the straws. The growth pouches were kept in a growth chamber with 12 hour light periods at 27°C. The pouches were treated with 10-20 ml of half-strength B&D solution for 3-4 weeks until the plants were harvested for nodule counting. During nodule counting, the pouches were removed from the growth chamber and nodules were counted, along with the distance of the location from the root tip.

2.6 MocR Protein Purification

2.6.1 Construction of pQE2::MocR strain

The *mocR* coding region was amplified using the *B. japonicum* USDA110 genomic DNA as a template and the *blr6977* coding region forward primer with an SphI enzyme site linker (SphI_*blr6977_FW*: 5'—ATA TGC ATG CCC GGC TGC TCT GG—3') and *blr6977* coding region reverse primer with a *HindIII* enzyme site linker (*HindIII_blr6977_RV*: 5'—CAG CAA GCT TTC ACA TAA GAC GA—3'). As recommended by the Sigma-Aldrich Taq DNA Polymerase system protocol, a 50 µl PCR reaction was prepared using 5X Taq DNA Polymerase Reaction Buffer, 100-200 ng genomic DNA template, 1µM of the primer set, 10mM dNTP Mix, and 1.25u of Taq DNA Polymerase. The PCR reaction was run using a C1000 Thermo Cycler (Biorad, Hercules, CA) at the following conditions: (1) initial denaturation at 95°C for 5 minutes; (2) continued by 35 cycles of denaturation at 95°C for 2 minutes, annealing at 59°C for 1 minute and extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen Sciences, Maryland, MD). A double restriction enzyme digestion was carried out on approximately 1 µg of pQE2 vector (Qiagen Sciences, Maryland, MD) and PCR product using SphI and *HindIII* restriction enzymes and NEBuffer 2 (New England Biolabs, Ipswich, MA) and

further purified using the QIAquick PCR Purification Kit (Qiagen Sciences, Maryland, MD). Ligation was conducted using 35 ng of digested pQE2 vector and 37 ng of digested PCR Product, transformed into *E. coli* JM109 competent cells, and spread onto LB agar plates with ampicillin (100 µg/ml). The *E. coli* JM109 strain was chosen as a host strain due its inclusion of an intrinsic *lacI^q* repressor to efficiently restrain transcription of the powerful phage T5 promoter. Colonies were screened by plasmid extraction and subsequent double restriction enzyme digestion using *SphI* and *HindIII* to look for the correct band pattern of sizes.

2.6.2 Time-course analysis of protein expression

To determine the optimal conditions for protein expression, a single colony of a pQE2::MocR strain was inoculated to 10 ml of LB media containing ampicillin and grown overnight. Subcultures were prepared by inoculating 50 ml of LB media with ampicillin with 2.5 ml of starter culture and grown to an OD₆₀₀ of 0.5-0.7, after which 1 ml of culture was harvested, re-suspended in 50 µl of 2X SDS-PAGE loading buffer, and stored at -20°C. The subcultures were induced with IPTG to a final concentration 0.01 mM and grown at 21°C, 30°C, and 37°C for 12 hours, 8 hours, and 4 hours respectively. From each induced culture, 1 ml of culture was harvested, re-suspended in 50 µl of 2X SDS-PAGE loading buffer, and stored at -20°C. The un-induced and induced samples were heated to 95°C for 5 minutes and spun down before loading the samples onto a 15% SDS-PAGE gel at 125 V for 45 minutes using the Mini-PROTEAN 3 Cell (Biorad, Hercules, CA). The SDS-PAGE gel was microwaved on the “high” setting for 45 seconds in coomassie stain and left for 15 minutes. The coomassie stain was removed and the gel was incubated in destain solution overnight at ~50 rpm. An image of the gel was recorded.

2.6.3 Determination of the MocR protein solubility

A single colony of pQE2::MocR strain was inoculated into 10 ml of LB medium with ampicillin and grown overnight. Approximately 2 ml of the overnight culture was used to

inoculate 50 ml of LB medium with ampicillin and grown to an OD₆₀₀ of 0.5-0.7. The culture was induced with IPTG to a final concentration of 0.01 mM and grown for 12 hours at 21°C and 200 rpm. The cells were harvested by centrifugation at 4000 x g for 20 minutes. The supernatant was removed and the pellet was resuspended in 5 ml of lysis buffer containing 6.90 g of NaH₂PO₄·H₂O, 17.54 g of NaCl, 0.68 g of imidazole, and adjusted to a pH of 8.0 with NaOH. The solution was frozen in liquid nitrogen and thawed in cold water. The sample was kept on ice and sonicated 10 times for 15 seconds with 15 second pauses using the XL-2000 sonicator (Misonix, Newtown, CT). The lysate was centrifuged at 10,000 x g at 4°C for 30 minutes and the supernatant was transferred to a new microcentrifuge tube and kept on ice, and the pellet was resuspended in 5 ml of lysis buffer and also kept on ice. Next 5 µl of 2X SDS-PAGE loading buffer was added to 5 µl of the supernatant and to 5 µl of the pellet. The samples were heated for 5 minutes at 95°C and loaded onto a 15% SDS-PAGE gel and run as previously described.

2.6.4 Overexpression of B. japonicum MocR protein in E. coli

A culture inoculated with the pQE2::MocR strain was grown to an OD₆₀₀ of 0.5-0.7. The culture was induced with IPTG to a final concentration of 0.01 mM and grown for 12 hours at 21°C and 200 rpm. The cells were harvested by centrifugation at 4,000 x g for 20 minutes. The supernatant was removed and the pellet was resuspended in 3 ml of lysis buffer. Approximately 3 mg of lysozyme from chicken was added to the mixture, incubated on ice for 30 minutes and sonicated on ice 10 times for 15 second intervals with 15 second pauses. The lysate was centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatant of the lysate was transferred to a new 50 ml tube and 1 ml of 50% Nickel-nitrotriacetic acid (Ni-NTA) agarose was added as per the TAGZyme Kit System (Qiagen Sciences, Maryland, MD). The mixture was incubated on ice for 1 hour at 200 rpm. The mixture was loaded onto a column and the flow-through collected. The resin was washed twice with 4 ml of wash buffer containing 6.90 g of NaH₂PO₄·H₂O, 17.54 g of NaCl, 1.36 g of imidazole, and adjusted to a pH of 8 with NaOH. The

protein was eluted in 2 ml of elution buffer containing 6.90 g of NaH₂PO₄·H₂O, 17.54 g of NaCl, 17 g of imidazole, and adjusted to a pH of 8 with NaOH. The eluate was then analyzed by SDS-PAGE.

2.7 Electrophoretic Mobility Shift Assay (EMSA)

2.7.1 Preparation of nucleic acid target

The fragment of the promoter region was amplified by PCR using *B. japonicum* USDA110 genomic DNA as a template and the promoter specific forward primer (MocR_Prom_A2: 5'—CTG GCA TTG CGG CTG ATG GA—3') and promoter specific reverse primer (MocR_Prom_A3: 5'—GAA GGC CCT GAT CGT CGA CT—3'). The PCR reaction was run at the following conditions: (1) initial denaturation at 95°C for 5 minutes; (2) continued by 25 cycles of denaturation at 95°C for 90 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen Sciences, Maryland, MD) and run through a gel electrophoresis. The probe was gel-purified using the QIAquick Gel Extraction Kit (Qiagen Sciences, Maryland, MD).

2.7.2 EMSA procedure

Different concentrations of the MocR protein was added to a solution containing 25 ng of the nucleic acid target, 5X binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM Tris, 25% glycerol, pH 7.4), and brought to a final volume of 10 µl with nano-pure water. The reaction was incubated at room temperature for 15 minutes and loaded onto a 5% 1X tris-borate-EDTA (TBE) non-denaturing gel containing 2 ml of 10X TBE buffer, 2.5 ml of 40% wt/vol acrylamide-bisacrylamide stock solution (37.5:1), 0.05 g of ammonium persulfate, 12 µl of *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 15.5 ml of nano-pure water. The gel was run at 200 V for 30 minutes at 4°C using the Mini-Protean II Electrophoresis Cell (Biorad,

Hercules, CA). To stain the DNA first, the gel was incubated in 20 ml of 1X TBE buffer containing 2 μ l of SYBR Green EMSA gel stain (10,000X) (Invitrogen, Carlsbad, CA) protected from light for 20 minutes. The gel was washed in ~100 ml of deionized water twice and visualized under 300 nm UV transillumination. To stain the protein, the gel was incubated in SYPRO Ruby EMSA protein gel stain (Invitrogen, Carlsbad, CA), protected from light for 3 hours and washed twice with distilled water. The gel was then incubated in destain solution containing 10% methanol and 7% acetic acid overnight. The gel was washed in distilled water and visualized 300 nm UV transillumination.

2.7.3 EMSA gel extraction

After visualizing the EMSA, the band shift was excised with a scalpel and incubated with 50 μ l of 2X SDS-PAGE loading buffer in a 1.5 ml microcentrifuge tube at room temperature for 48 hours at 200 rpm. After incubation, 20 μ l of the solution was transferred to a new microcentrifuge tube and boiled for 5 minutes at 95°C and spun down briefly. The sample was then loaded onto a 15% SDS-PAGE gel and run as previously described.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Phenotypic comparisons of free-living *mocR* mutant

Several phenotypic tests were conducted to compare the wild type and mutant strains. The colony morphologies of the wild type and mutant strains were compared side by side on different mediums including AG and minimal media (Figure 6). The appearance of individual colonies of the wild type and mutant strains showed no dissimilarities in morphology, size, or mucoidity.

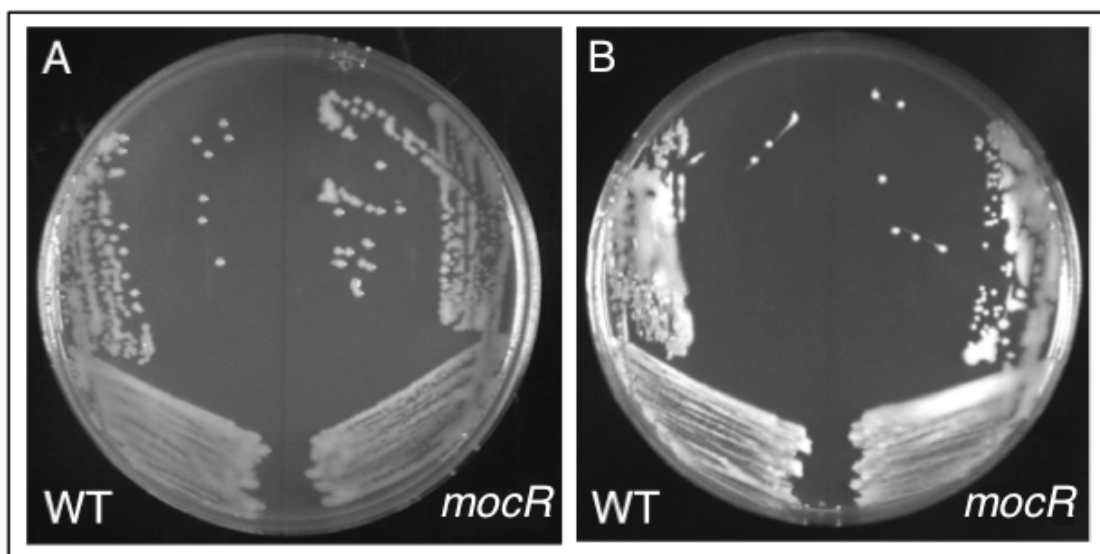


Figure 6. Colony morphology growth of wild type *B. japonicum* (left half) and *mocR* mutant (right half) on AG media (A) and Minimal media (B). Plates were incubated at 30°C for 5 days on AG and 10 days on Minimal Media.

The growth rates of the wild type and mutant strains were also compared in AG and minimal media. In AG media, the generation times for the wild type, mutant, and complement strains

were 6.7, 6.8, and 6.7 h, respectively, and did not have a statistical difference. There were also no differences between the strains in the lag, exponential, or stationary phase (Figure 7a). Similarly in minimal media, there was no significant difference in the generation times of the wild type, mutant, and complement strains which were 10.0, 10.1, and 10.0 h, respectively. However, when grown in minimal media, the *mocR* mutant yielded a higher optical density (O.D.) of saturation of approximately 1.01 compared to the wild type, which reached an O.D. of approximately 0.87 in the stationary phase (Figure 7b).

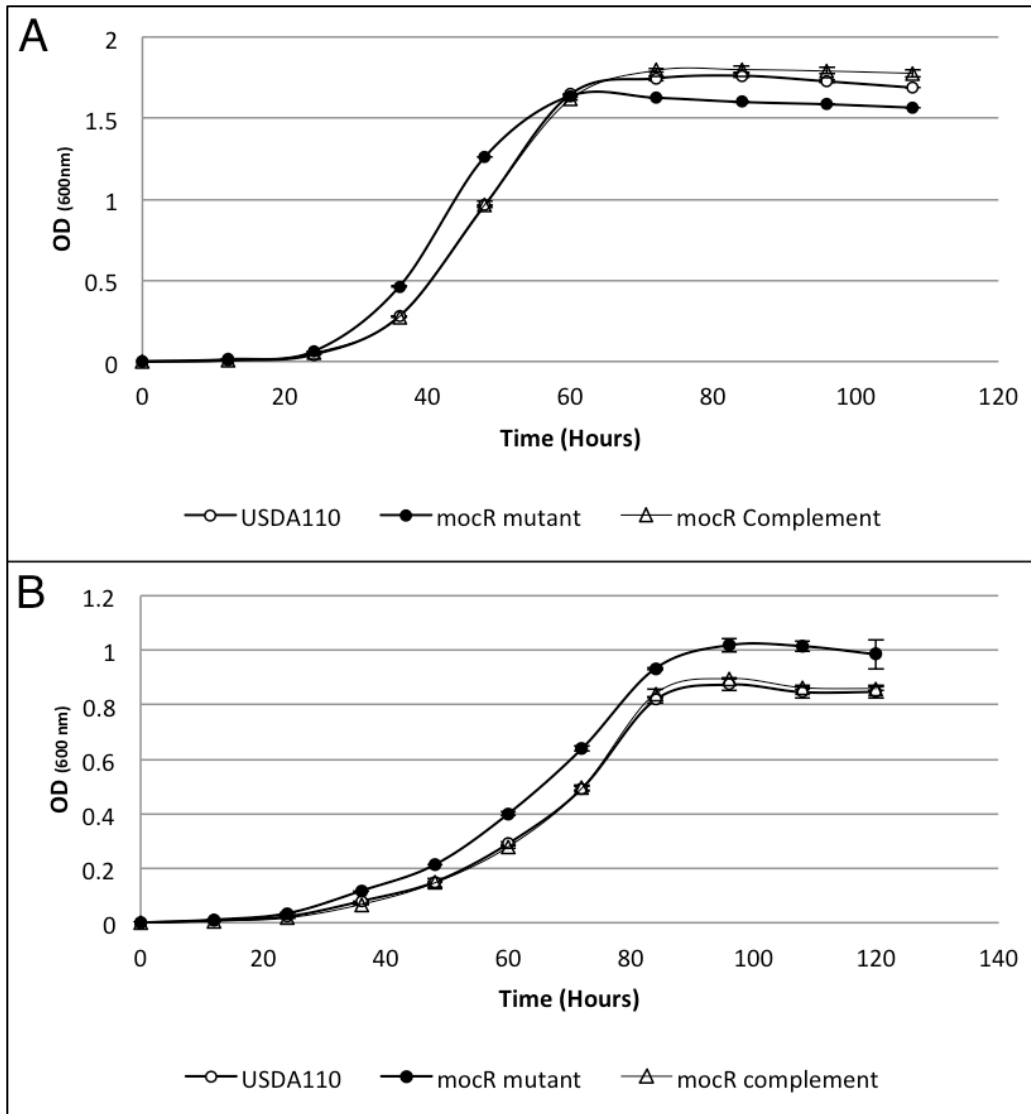


Figure 7. Growth rate of *B. japonicum*, the *mocR* mutant and its complement strain in AG media (A) and minimal media (B). The strains were incubated at 30°C and 200 rpm. Measurements of optical densities were taken over a period of 5 days at 600 nm. Values are the means of O.D. measurements of triplicate cultures and bars represent standard error.

Motility tests were done comparing the wild type, mutant, and complement strains on AG rich media and minimal media using 1% and 0.3% agar (Figure 8). In 1% agar AG media, the mean diameters of the wild type and mutant strains were 7.3 ± 0.2 and 7.3 ± 0.2 mm. The mean growth diameters in 0.3% agar AG media were 17.1 ± 0.2 and 14.8 ± 1.9 mm for the wild type

and mutant strains respectively. Neither condition in AG media showed significant differences between the two strains. However in minimal media, after 3 to 4 days of incubation, the *mocR* mutant had a significantly wider mean diameter of growth of 23.2 ± 0.2 mm compared to the wild type and complement strains which had mean diameters of 16.8 ± 0.2 mm and 12.2 ± 0.2 mm, respectively (Figure 9).

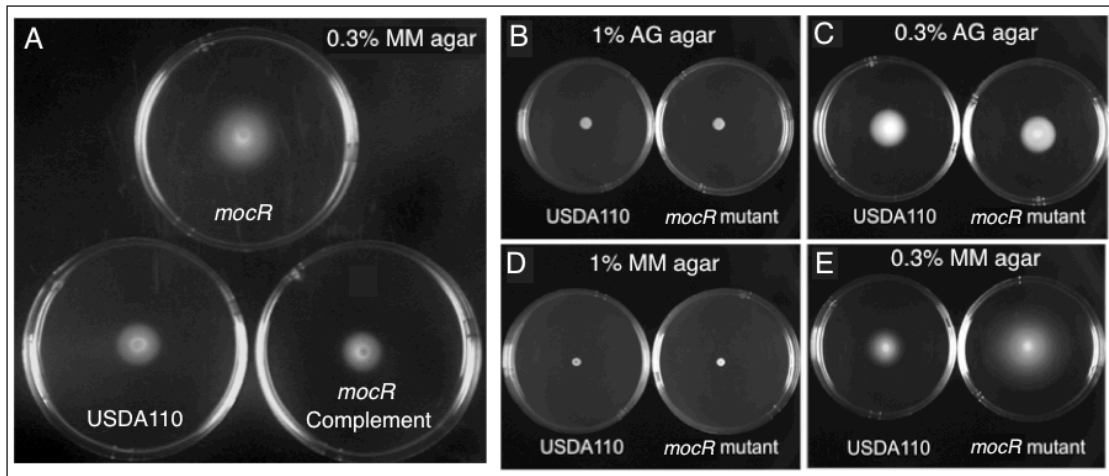


Figure 8. (A) Motility tests comparing mutant, wild type and complement strains on 0.3% agar minimal media plates that were spotted with 15 μ l of culture ($OD_{600}=0.5$) and incubated for 3-4 days at 30°C. Diameters of growth were then measured. Motility tests comparing the wildtype and mutant strains were also conducted on 1% AG agar (B), 0.3% AG agar (C), 1% MM agar (D), and 0.3% MM agar (E).

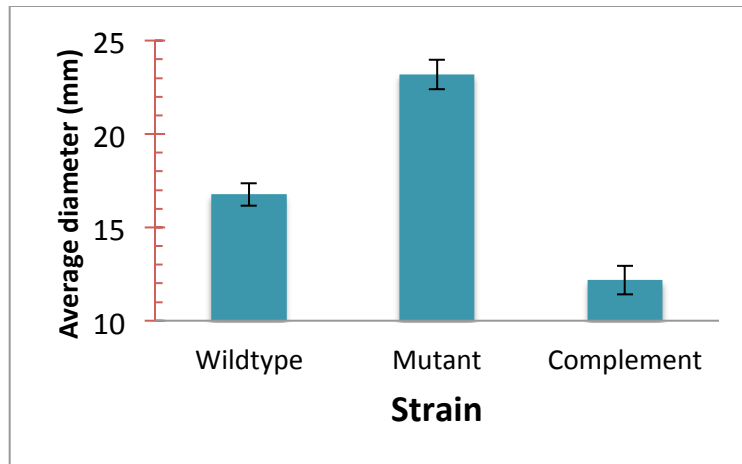


Figure 9. The *mocR* mutant strain had significantly larger diameter compared to the wild type, $P < 0.001$

3.2 Symbiotic phenotype comparison of *mocR* mutant

Pouch experiments were conducted to assay the nodulation efficiency of the *mocR* mutant. Initially, two triplicate sets of pouch experiments were conducted using the mutant and wild type strains – one using a high concentration of inoculum (ca. 1×10^8 cells/ml) and the other using a low concentration of inoculum (ca. 1×10^2 cells/ml). By varying concentrations of inocula relative efficiency of nodulation can be represented by comparing the number of nodules formed with respect to the concentration of the inoculum. At low concentrations, after pooling all the nodules counted there was not a significant difference in the number of nodules between the wild type and mutant strains. However, at high concentrations the wild type strain had a significant increase in the number of nodules compared to the mutant strain (Figure 10). In addition to counting the number of nodules after a period of 3-4 weeks, the distance of each nodule from the original root tip (RT) was measured to help determine the timing of nodulation. Nodules that are located close to the root tip indicate earlier formation of the nodule, whereas nodules positioned more distantly from the root tip points to delayed nodulation.

Results from pouch experiment revealed that the average number of nodules formed by the mutant strain was deficient compared to the wild type with 8.5 ± 0.08 and 9.7 ± 0.7 nodules

respectively. The *mocR* complement strain, like in the case of the motility test, was able to restore the wild type phenotype in the pouch experiment in terms of the efficiency of nodulation at high concentrations with an average of 9.6 ± 0.6 nodules (Figure 11). More nodules were located distantly from the root tip in the mutant strain compared to the wild type indicating a delay in nodulation at both high and low concentrations.

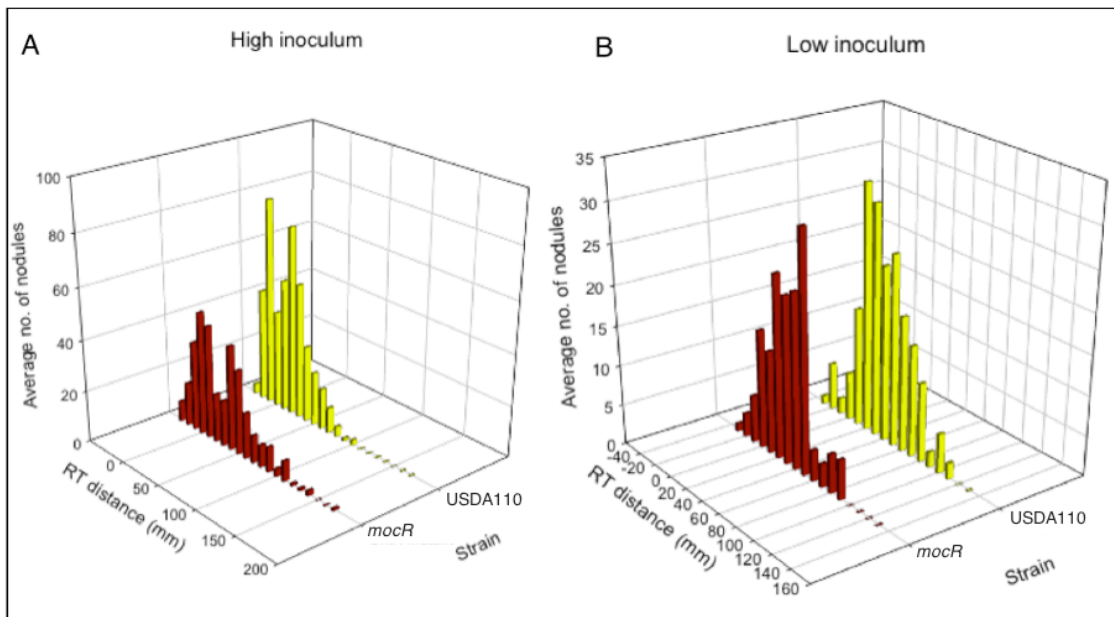


Figure 10. 3D Sigma plot of pouch experiment results. The graphs depict the distance of the root nodules from the RT (mm) and the average number of nodules counted at that position of soybean seeds that were treated with wild type USDA110 and *mocR* mutant using a (A) high inoculum (ca. 1×10^8 cells/ml) and (B) low inoculum (ca. 1×10^2 cells/ml). Values are the means of 3 biological replicates each with 9 technical replicates.

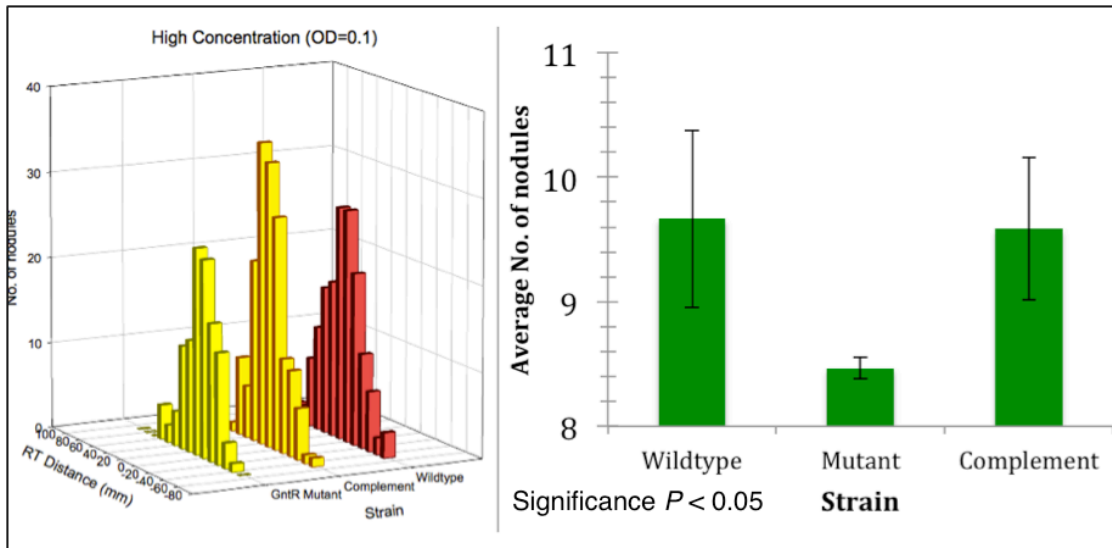


Figure 11. Nodulation effect of the wild type, mutant, and complement strains on soybean plant.

3.3 MocR binds to its own promoter region

Since many MocR proteins, particularly those in the MocR subfamily, have been found to autoregulate their expression by binding upstream in their promoter regions, an electrophoretic mobility shift assay (EMSA) was performed to determine if the MocR protein would bind upstream of the *mocR* gene. The MocR protein of *B. japonicum* USDA110 has a predicted size of 489 amino acids and a molecular mass of approximately 53.79 kDa. A MocR derivative with a His-tag attached to the C-terminus was expressed in *E. coli* JM109 cells and purified using a nickel-nitrotriacetic acid (Ni-NTA) column. Once the protein was purified, the size of the purified protein was confirmed by SDS-PAGE, which corresponded to the predicted molecular weight (Figure 12).

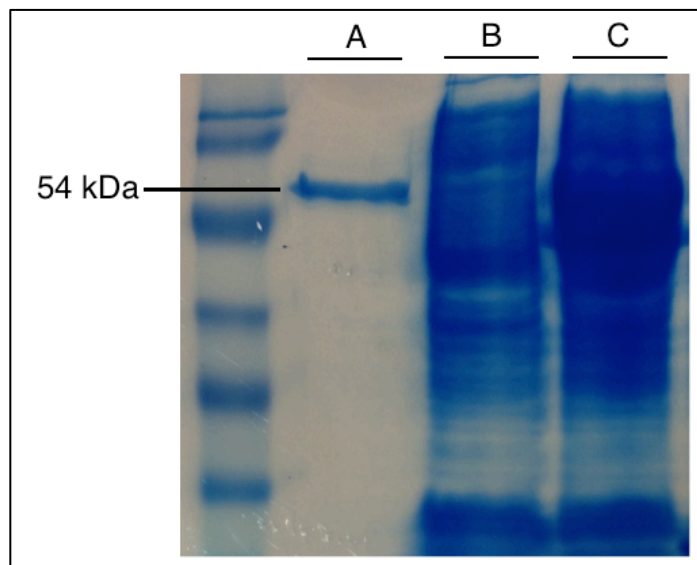


Figure 12. 15% SDS-PAGE gel after running purified MocR protein (~53.79 kDa) (A), lysed protein extract before induction with IPTG (B), and lysed protein extract after induction (C).

Four regions of the promoter upstream of the *mocR* gene were amplified using specific primers for each subset region. Each fragment was used as a nucleic acid target in an EMSA and tested against purified MocR protein by keeping the concentration of the DNA constant and titrating it with increasing gradient concentrations of protein ranging from approximately 11 to 55 pmol. Of the subset regions, a 198 bp DNA fragment located 168 to 366 bp upstream from the start codon of the *mocR* gene revealed a significant band shift after visualizing DNA with SYBR green (Figure 13). Furthermore, as the concentration of protein increased, the presence and intensity of the free probe decreased. Once this was established, the same gel was incubated in SYPRO Ruby to visualize the protein and to ensure that its position corresponded to the mobility of the band shift (Figure 13).

To ensure that shift was not due to the presence of contaminating protein from vector itself or from the host strain, protein from an empty pQE2 vector lacking the MocR insert was purified and run in the same conditions. Two distinct bands above the free probe were present

in the empty vector set run with the nucleic acid target, but lacked the protein-DNA complex band, indicating that the two bands were attributed to contaminating proteins.

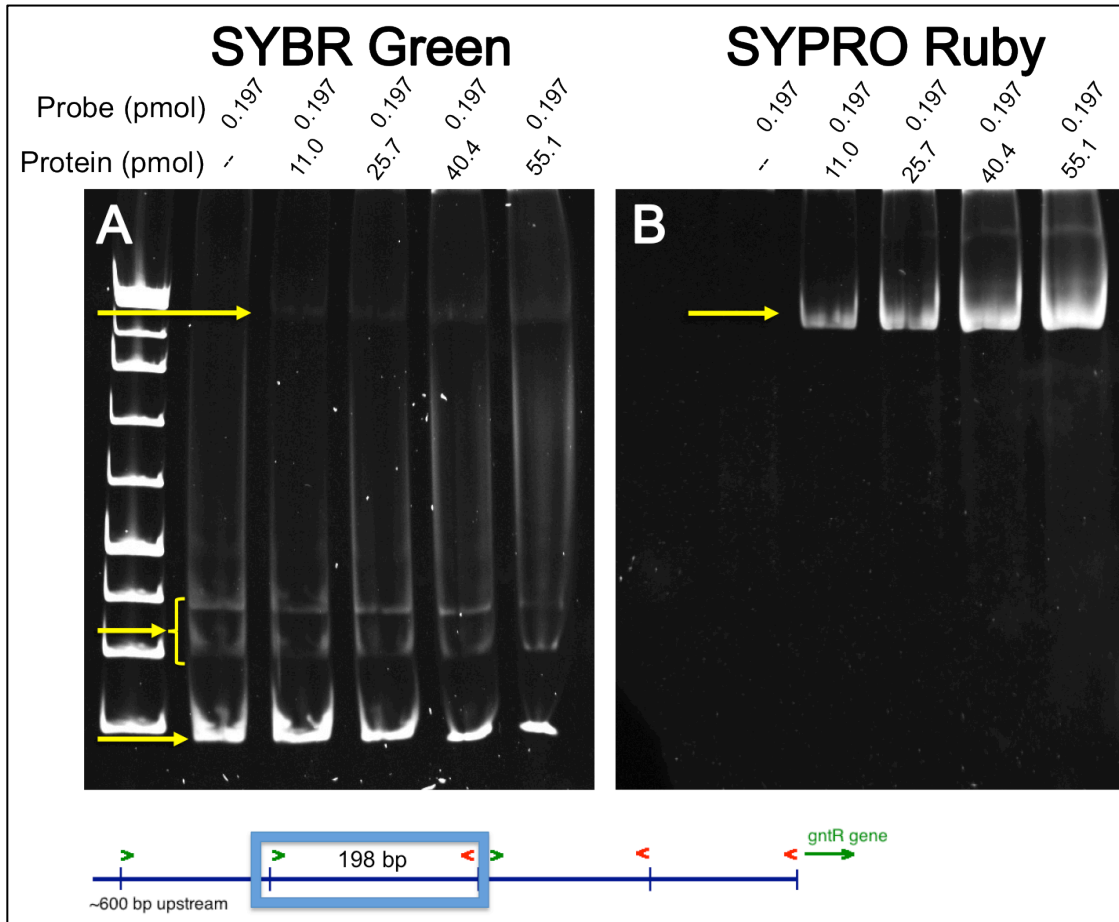


Figure 13. EMSA gel with SYBR Green staining (A) and SYPRO Ruby staining (B). Lane 1, 1 kb ladder; Lane 2, empty vector with probe; Lanes 3-6, titration of protein with probe of the region indicated in the bottom panel. (A) Bottom arrow depicts the free probe; middle arrow depicts false shifts; top arrow depicts shift representing protein:DNA complex. (B) Top arrow indicates protein stained with SYPRO Ruby.

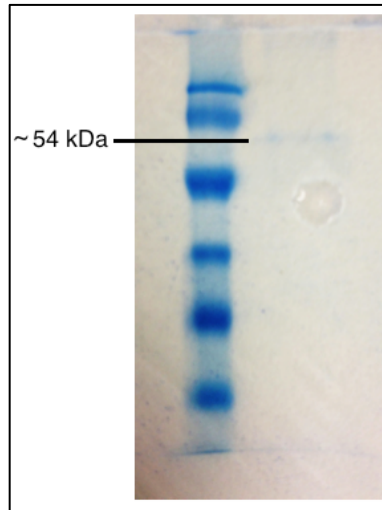


Figure 14. 15% SDS-PAGE gel after running EMSA gel extracted eluted protein.

Finally, to confirm that protein in the protein-DNA complex was the MocR protein, the shifted band was extracted and incubated in 2X SDS-PAGE loading buffer to elute the protein by passive diffusion, and the sample was run on an SDS-PAGE to determine the size of the eluted protein, which was consistent with the molecular weight of the MocR protein (Figure 14).

3.4 Discussion

Although the GntR family of transcriptional regulators are broadly distributed in the prokaryotic world, there have only been a few studies focused on the characterization of the MocR subfamily of proteins (18). In previous studies, *mocR* genes in other microorganisms such as *Sinorhizobium meliloti*, have been implicated in affecting a variety of biological processes such as growth, motility, and nodulation (35, 20). Thus the aim of this study was to determine if and how a MocR type protein encoded by *blr6977* can affect *B. japonicum* USDA110 in symbiotic and free-living conditions.

The phenotypic results generated by the *mocR* mutant revealed differences only under certain conditions, namely when minimal media was used. Although the colony morphology of the mutant did not show any distinct contrasts to the wild type under AG and minimal media conditions, there were differences in growth and motility when grown with minimal media. Growth of the mutant strain in minimal media was able to reach a higher level of saturation as the wild type strain. This contradicts the effects of the *gtrA* and *gtrB* mutants in *S. meliloti*, which fall under the FadR and VanR subfamilies respectively, where both mutants were deficient in growth compared to the wild type in rich media (35). Furthermore the *gtrA* and *gtrB* mutants, which were deficient in motility and produced diminutive swarming ring diameters in LB/MC with 0.3% agar, also differed from the *mocR* mutant in which the opposite effect occurred and produced larger swarming rings but only under minimal media conditions (35). This suggests that *blr6977* may influence motility or chemotaxis directly or indirectly through its role as a transcriptional regulator of other genes.

The fact that the mutant phenotypes could only be observed in minimal media suggests that the growth conditions have an important effect on the regulator. Perhaps in minimal media the MocR protein acts a transcriptional repressor, negatively regulating other genes that affect growth and motility. Thus in the *mocR* mutant, where the MocR protein is no longer functional, the genes that are normally repressed continue to be expressed. This may explain why a knock-out of the *mocR* gene resulted in enhanced motility and induced a higher cell mass. On the other hand, in rich AG medium perhaps some component of the medium enables a separate pathway to negatively regulate the MocR-regulated genes permitting normal growth and motility.

Since host-symbiont interactions and the process of nodulation are of a unique interest for *B. japonicum*, a pouch experiment was performed to test the efficiency of forming nodules on the roots of soybean plants with the mutant and complement strains. Successful recognition and adherence of *B. japonicum* cells to the root tips of the soybean plant is a complex and essential process in initiating nodulation, and mutants that are inefficient in doing so have a tendency for

delayed nodulation (15). The role of motility and chemotaxis in the efficiency of nodulation in other rhizobia, such as *S. meliloti*, have been found to confer significant advantages in promoting initial root tip contact adsorption and in facilitating the development of infection, although deficient or nonmotile mutants are able to induce formation of normal nodules (6, 34, 35). Therefore, we were interested in seeing the effects of enhanced motility of the *mocR* mutant strain on nodulation. The outcome revealed that whilst motility was enhanced, there was still a deficiency as well as a delayed-effect in terms of nodulation, indicating its inefficacy to establish nodulation promptly post-inoculation of the plant root perhaps due to its inability to properly adhere to the surface of the root tips. Similar results were observed in *S. meliloti*, in which the nodules produced by the *gtrA* and *gtrB* mutants were smaller and also had a delayed-nodulation (35).

To this date, only three MocR-type proteins have been molecularly characterized in other prokaryotes, the first of which was GabR from *Bacillus subtilis*, which negatively regulates the gene that encodes it and positively regulates the *gabTD* operon associated with γ -aminobutyrate aminotransferase (GABA) utilization as a sole nitrogen source (4, 21, 36). Another notable distinction of MocR subfamily proteins is its preference to bind to direct repeats, as opposed to inverted repeats like that of other MocR proteins. However, due to the dissimilarities between the consensus sequences as well as the distance between them, a composite could not be used to determine the binding site of MocR.

An EMSA was conducted using the promoter region of the *blr6977* gene to determine if the gene was capable of autoregulation and to narrow down the consensus sequence of the target DNA site. A shift in the mobility of the DNA compared to the free probe using a specific region of the promoter showed interaction between the probe and protein, and visualization of the protein and subsequent gel extraction of the band shift indicated the presence of the protein in complex with DNA.

The program REPFIND predicted two sets of direct repeats within the region of the target DNA, a 6 bp motif (CTTGGG) separated by 11 bp which is equivalent to a single helical turn and another 6 bp motif (AGCCGA) with a spacer of 6 bp (29). In other cases, GabR in *B. subtilis* also has a 6 bp motif (ATACCA) separated by 34 bp, TauR in *Rhodobacter capsulatus* binds to a 10 bp (CTGGAC[T/C]TAA) repeat with a 23 bp interval, and PdxR in *Corynebacterium glutamicum* has a 11 bp motif (AAAGTGW[-/T]CTA) with a 13 bp spacer (4, 21, 36).

3.5 Future Directions

It is important to be able to identify the consensus sequence to which the MocR protein binds to due to its role as a transcriptional regulator. Thus further experimentation, either through DNA footprinting assays or by site mutagenesis of the candidate direct repeats and further EMSA, can resolve which base pairs are essential for binding. This information can then be used to look at other genes that may be regulated by sifting through the promoter regions for that particular sequence, in order to pinpoint genes that are indeed controlled by the transcriptional regulator and how they are affected through qRT-PCR. Perhaps this can help elucidate how the MocR protein is involved in such processes such as nodulation and motility.

Furthermore, 5'-RACE can be a useful tool in specifying where the transcriptional start site of the *mocR* gene is. Identifying the transcriptional start site (TSS) of a gene by using bioinformatics is not particularly easy, however, utilization of 5'-RACE and subsequent sequencing of the RNA transcript can provide important information on how far upstream the essential binding sites are from the TSS.

In fact, there is a possible operon consisting of three genes that are divergently transcribed from *blr6977*. Although whether they were truly in an operon would need to be confirmed by RT-PCR. It would be very interesting to determine if these genes are also influenced by the MocR protein. One drawback of focusing on those genes lie in the lack of their

annotation. According to the annotations, their functions are still unknown and are annotated as hypothetical proteins.

Also, to shed light upon how the *blr6977* itself is regulated, such as whether it is capable of positive or negative autoregulation like other MocR proteins, a LacZ translational fusion using the promoter region of the *mocR* gene can determine whether it is responsible for its own expression or repression. As noted previously, for other MocR proteins both scenarios are possible.

Finally, aminotransferase-like C-terminus of the MocR protein has been found to be both necessary and unnecessary for proper functioning and binding. It would indeed be interesting to see if adding PLP to the EMSA reactions can affect its ability to bind to target sequences and if perhaps they do indeed have some function or activity to catalysis.

APPENDIX A

qRT-PCR ANALYSIS OF GNTR GENES

Materials and methods for RNA isolation and qRT-PCR analysis

B. japonicum wild type and *mocR* mutant strains were cultured and harvested in minimal media to an OD of 0.5-0.8. Isolation of total RNA was conducted by the hot phenol extraction method as described previously (27). Then DNase treatment and RNA purification were carried out using the RNase-free DNase set and RNeasy minikit respectively (Qiagen Sciences, Maryland, MD).

To compare the expression of several *gntR* genes in the *B. japonicum* wildtype and *mocR* mutant strain, quantitative reverse transcription-PCR (qRT-PCR) was conducted. Specific primers were designed for 15 of the *gntR* genes selected and are listed in Table 3. The qRT-PCR process was performed as previously described (9). The histidyl-tRNA synthetase (*HisS*) gene was selected as a reference gene to normalize the expression data.

Results and discussion for qRT-PCR analysis

The expressions of 15 *gntR* genes were compared between the wild type and *mocR* mutant using gene specific primers. Since the phenotypic differences were observed when grown in minimal media, cells were harvested from cultures grown in those conditions. Of the 15 genes, 11 were homologous to the FadR subfamily, 3 had homologies similar to the MocR subfamily, and 1 was linked to the HutC/FarR subfamily.

The expression of each gene was normalized relative to the expression of the *HisS*, which has a relatively constant expression in different stress conditions and has also been used as a reference gene in other studies performing similar analyses. In this study, the qRT-PCR analysis revealed that almost all of the *gntR* genes tested did not show a significant fold induction relative to *HisS* in both the wild type and mutant strains, including the *mocR* gene itself. The only gene that seemed to have a large fold induction in both the *mocR* mutant and wild type strains was the *bll3158* gene, a member of the FadR subfamily.

Table A1. qRT-PCR primers used in this study

Gene Locus	Primer	Sequence (5' to 3')	Amplicon size (bp)
blr1043	Forward	GAAGAGCTGCGTGCCTCA	81
	Reverse	ATAGATCGCGTTGTGGAAGC	
blr1279	Forward	GAGGCCAATGACGTGATAAC	90
	Reverse	CTCTGCCAGAAACGAGTTGTG	
blr2901	Forward	GAGTTCATCTCGGAGGTCCTT	110
	Reverse	TCTCTTCTGAAACTGGCACCT	
bll3158	Forward	AGGAGATTCGTGCTGCACTG	107
	Reverse	TTGTTCTTGAAGCTGCTCTCC	
blr3319	Forward	GCGTCGAAATCTCTTAGCAAC	106
	Reverse	GATATCCACCTGCCAACTCG	
bll4018	Forward	CCTTCCACGACGAACTCTTT	110
	Reverse	GCTGTATTGCCGCATCTTCT	
bll4154	Forward	GATCCGATGGTGTGCTGTC	154
	Reverse	CGTGTGCGTGAATAGGTGTC	
blr4427	Forward	TCGTCTCGGAACTGTCACTG	138
	Reverse	CGCCTTCTCGAAACTCTTGT	
blr0382	Forward	ACTTCCACGCCAGCTCTAC	130
	Reverse	ACGATATTCTGGGCCTTGC	
blr0404	Forward	TCCGTGCTACTTCAATTTTCG	150
	Reverse	AGCGCTGAATTGGTGATGTAG	
blr3325	Forward	CAAGAACGATGTCTGGGTGAT	124
	Reverse	CGAGAAGGTGCCGAAATAAAT	
blr3551	Forward	TTCTCGACGATATCTGGATGC	84
	Reverse	GATGCTCGTTCGGATAAATTG	
bll3877	Forward	CGCATCATCAAGGCTCATATC	93
	Reverse	TCATGATGACCTCTCCTCGAC	
bll4368	Forward	TGGAACCTGCATATTTGGACTG	102
	Reverse	ACGACCTCCTCATGAAGCAT	
blr7971	Forward	AAGCTCGATACGCCAAAATC	212
	Reverse	AAGACAAAACCTGCCGACCTG	

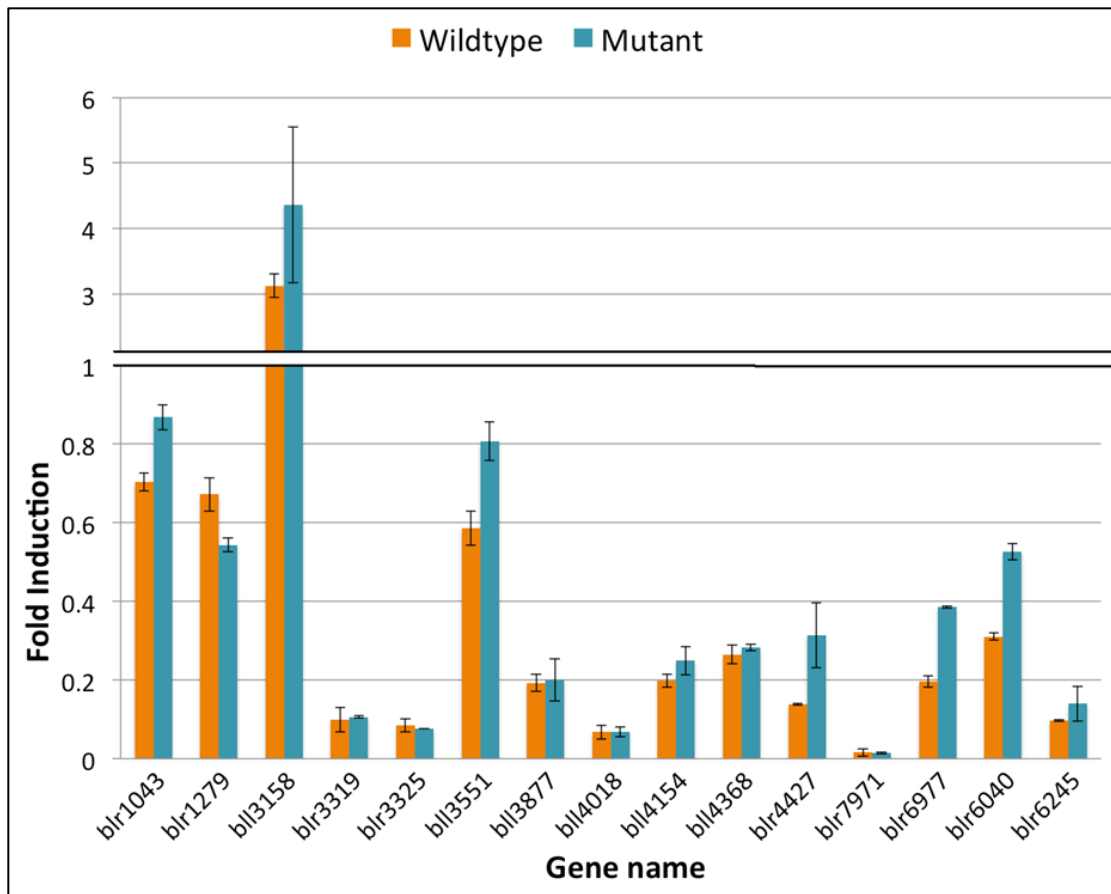


Figure A1. qRT-PCR analysis of the 15 *gntR* genes selected. Out of the 15 tested genes, 11 were annotated as a part of the FadR subfamily, 3 to the MocR subfamily, and 1 to the HutC/FarR subfamily. The histidyl-tRNA synthetase (*HisS*) gene was selected as a reference gene to normalize the expression data.

REFERENCES

1. **A.R., M., J. K. Syers, and J. R. Freney.** 2004. Agriculture and the Nitrogen Cycle: Assessing the impacts of fertilizer use on food production and the environment. Island Press, Washington DC, USA.
2. **Arnau, J., C. Lauritzen, G. E. Petersen, and J. Pedersen.** 2006. Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expr Purif* **48**:1-13.
3. **Belitsky, B. R.** 2004. *Bacillus subtilis* GabR, a protein with DNA-binding and aminotransferase domains, is a PLP-dependent transcriptional regulator. *J Mol Biol* **340**:655-64.
4. **Belitsky, B. R., and A. L. Sonenshein.** 2002. GabR, a member of a novel protein family, regulates the utilization of gamma-aminobutyrate in *Bacillus subtilis*. *Mol Microbiol* **45**:569-83.
5. **Bramucci, E., T. Milano, and S. Pascarella.** 2011. Genomic distribution and heterogeneity of MocR-like transcriptional factors containing a domain belonging to the superfamily of the pyridoxal-5'-phosphate dependent enzymes of fold type I. *Biochem Biophys Res Commun* **415**:88-93.
6. **Caetano-Anolles, G., L. G. Wall, A. T. De Micheli, E. M. Macchi, W. D. Bauer, and G. Favelukes.** 1988. Role of Motility and Chemotaxis in Efficiency of Nodulation by *Rhizobium meliloti*. *Plant Physiol* **86**:1228-35.
7. **Economic Research Service, U.** May 7, 2010 2010, posting date. Soybean and Oil Crops. [Online.]
8. **Fischer, H. M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. *Microbiol Rev* **58**:352-86.
9. **Franck, W. L., W. S. Chang, J. Qiu, M. Sugawara, M. J. Sadowsky, S. A. Smith, and G. Stacey.** 2008. Whole-genome transcriptional profiling of *Bradyrhizobium japonicum* during chemoautotrophic growth. *J Bacteriol* **190**:6697-705.
10. **Fujita, Y., and T. Fujita.** 1987. The gluconate operon gnt of *Bacillus subtilis* encodes its own transcriptional negative regulator. *Proc Natl Acad Sci U S A* **84**:4524-8.
11. **Gage, D. J.** 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev* **68**:280-300.
12. **Galloway, J. N., and E. B. Cowling.** 2002. Reactive nitrogen and the world: 200 years of change. *Ambio* **31**:64-71.

13. **Giraud, E., L. Moulin, D. Vallenet, V. Barbe, E. Cytryn, J. C. Avarre, M. Jaubert, D. Simon, F. Cartieaux, Y. Prin, G. Bena, L. Hannibal, J. Fardoux, M. Kojadinovic, L. Vuillet, A. Lajus, S. Cruveiller, Z. Rouy, S. Mangenot, B. Segurens, C. Dossat, W. L. Franck, W. S. Chang, E. Saunders, D. Bruce, P. Richardson, P. Normand, B. Dreyfus, D. Pignol, G. Stacey, D. Emerich, A. Vermeglio, C. Medigue, and M. Sadowsky.** 2007. Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia. *Science* **316**:1307-12.
14. **Haine, V., A. Sinon, F. Van Steen, S. Rousseau, M. Dozot, P. Lestrade, C. Lambert, J. J. Letesson, and X. De Bolle.** 2005. Systematic targeted mutagenesis of *Brucella melitensis* 16M reveals a major role for GntR regulators in the control of virulence. *Infect Immun* **73**:5578-86.
15. **Halverson, L. J., and G. Stacey.** 1986. Effect of lectin on nodulation by wild-type *Bradyrhizobium japonicum* and a nodulation-defective mutant. *Appl Environ Microbiol* **51**:753-60.
16. **Hennecke, H.** 1990. Nitrogen fixation genes involved in the *Bradyrhizobium japonicum*-soybean symbiosis. *FEBS Lett* **268**:422-6.
17. **Hillerich, B., and J. Westpheling.** 2006. A new GntR family transcriptional regulator in *Streptomyces coelicolor* is required for morphogenesis and antibiotic production and controls transcription of an ABC transporter in response to carbon source. *J Bacteriol* **188**:7477-87.
18. **Hoskisson, P. A., and S. Rigali.** 2009. Chapter 1: Variation in form and function the helix-turn-helix regulators of the GntR superfamily. *Adv Appl Microbiol* **69**:1-22.
19. **Hymowitz, T.** 1990. *Soybeans: The success story*. Timber Press, Portland, OR.
20. **Jaques, S., and L. L. McCarter.** 2006. Three new regulators of swarming in *Vibrio parahaemolyticus*. *J Bacteriol* **188**:2625-35.
21. **Jochmann, N., S. Gotker, and A. Tauch.** 2011. Positive transcriptional control of the pyridoxal phosphate biosynthesis genes *pdxST* by the MocR-type regulator PdxR of *Corynebacterium glutamicum* ATCC 13032. *Microbiology* **157**:77-88.
22. **Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110 (supplement). *DNA Res* **9**:225-56.
23. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop, 2nd, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**:800-2.
24. **Lee, H. I., J. H. Lee, K. H. Park, D. Sangurdekar, and W. S. Chang.** 2012. Effect of Soybean Coumestrol on *Bradyrhizobium japonicum* Nodulation Ability, Biofilm Formation, and Transcriptional Profile. *Appl Environ Microbiol* **78**:2896-903.

25. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in Rhizobium. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from Rhizobium meliloti. *J Biol Chem* **257**:8724-30.
26. **Loh, J., and G. Stacey.** 2003. Nodulation gene regulation in Bradyrhizobium japonicum: a unique integration of global regulatory circuits. *Appl Environ Microbiol* **69**:10-7.
27. **M, Bittner.** 2003. Expression analysis of RNA, p. 101-288. *In* B. D and S. J (ed.), DNA microarrays: a molecular cloning manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. **Phillips, D. A., and Y. Kapulnik.** 1995. Plant isoflavonoids, pathogens and symbionts. *Trends Microbiol* **3**:58-64.
29. **R.W., H., S. A., and W. D.** 2002. Sources of nutrient pollution to coastal waters in the United States: implications for achieving coastal water quality goals. *Estuaries* **25**:656-676.
30. **Rigali, S., A. Derouaux, F. Giannotta, and J. Dusart.** 2002. Subdivision of the helix-turn-helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies. *J Biol Chem* **277**:12507-15.
31. **Rossbach, S., D. A. Kulpa, U. Rossbach, and F. J. de Bruijn.** 1994. Molecular and genetic characterization of the rhizopine catabolism (mocABRC) genes of Rhizobium meliloti L5-30. *Mol Gen Genet* **245**:11-24.
32. **Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. H. Keyser.** 1987. Genetic Diversity in Bradyrhizobium japonicum Serogroup 123 and Its Relation to Genotype-Specific Nodulation of Soybean. *Appl Environ Microbiol* **53**:2624-30.
33. **Spaink, H. P.** 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol* **54**:257-88.
34. **van Rhijn, P., and J. Vanderleyden.** 1995. The Rhizobium-plant symbiosis. *Microbiol Rev* **59**:124-42.
35. **Wang, Y., A. M. Chen, A. Y. Yu, L. Luo, G. Q. Yu, J. B. Zhu, and Y. Z. Wang.** 2008. The GntR-type regulators gtrA and gtrB affect cell growth and nodulation of Sinorhizobium meliloti. *J Microbiol* **46**:137-45.
36. **Wiethaus, J., B. Schubert, Y. Pfander, F. Narberhaus, and B. Masepohl.** 2008. The GntR-like regulator TauR activates expression of taurine utilization genes in Rhodobacter capsulatus. *J Bacteriol* **190**:487-93.
37. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-19.

BIOGRAPHICAL INFORMATION

May Nyan Taw is a native of Burma and completed her Bachelor of Science degree in Biochemistry in 2009 from Fairleigh Dickinson University at Teaneck, New Jersey. She then joined the lab of Dr. Woo-Suk Chang to study microbiology at the University of Texas at Arlington in the 2010. After graduating from her Master of Science in spring of 2012, she plans to attend the Doctoral program for Microbiology at Cornell University.