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Structure/function analysis of FegA and FhuA in bradyrhizobium sp.

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Structure/Function Analysis of FegA and FhuA in *Bradyrhizobium sp.*

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Abstract

Bradyrhizobium japonicum is a Gram-negative soil bacterium commonly known for its agriculturally significant mutualistic relationship with soybean. In this symbiosis, the bacteria and plant undergo complex molecular signaling characterized by sent and received signals resulting in the formation of infection threads and root nodules. This research aimed to compare two related bacterial outer membrane proteins, FegA and FhuA, associated with the molecular signaling between the bacteria and plant. Previous work has led to the hypothesis that the N-terminal domain (NTD) of FegA in *B. japonicum* is needed for a functional symbiosis to occur. Recombinant bacterial strains expressing altered FegA proteins were constructed, verified, and tested for their role in symbiosis. The recombinant FegA protein lacking the full-length NTD ($FegA_{\Delta NTD}$) was unable to complement a *fegAB* mutant's symbiotic defect. Future studies will investigate the stability and function of $FegA_{\Delta NTD}$.

Introduction

Bradyrhizobium japonicum and *Bradyrhizobium diazoefficiens* (formerly known as *B. japonicum)* are Gram-negative bacteria and members of the Rhizobium family of nitrogen-fixers (Menna, Barcellos, & Hingria, 2009). Members of this family is known for their mutualistic relationships with leguminous plants (Menna et al., 2009). In the symbiosis, bacteria gain a protected niche to occupy while the plants are fed nitrogen provided by the bacteria through nitrogen fixation (Menna et al., 2009). The interaction between the bacteria and the plant is reliant on a complicated signaling pathway between the two organisms (Gage, 2004). Host microbe pairing is highly specific. *Bradyrhizobium sp.* only forms a symbiosis with soybean.

For the initiation of symbiosis, soybean plants constitutively release isoflavonoids into the soil (Miadokova, 2009). *B. japonicum* responds by releasing Nod factor, which causes the root hairs of the plant to curl (Gage, 2004). A "shepherd's crook" structure surrounding the bacteria is formed (Gage, 2004). Next the bacteria degrade the cell wall of the plant and create an invagination in the root hair called an infection thread (Gage, 2004). Eventually, bacteria from the infection thread enter the root nodule cells. Once established in the plant, the bacteria differentiate into bacteroids within membrane-bound compartments called symbiosomes (Gage, 2004). The bacteriods convert nitrogen into ammonia, which is used by the plant (Gage, 2004). Because precise host/symbiont pairing is critical for plant health, it is hypothesized that there are "gatekeeper" proteins that send/receive signals to assist the bacteria with the critical transition from the (extracellular) infection thread to the (intracellular) nodule symbiosome.

Both *B. diazoefficiens* and *B. japonicum* can establish a symbiosis of this nature with soybean plants, perhaps through the use of similar "gatekeeper" proteins that propagate the

molecular dialogue (Gage, 2004). *B. japonicum's* FegA protein may be one such protein. FegA is an outer membrane protein encoded in the *fegAB* operon (Figure2). It is hypothesized to have a role in the molecular signaling pathway as previous work with a *fegAB* insertion mutant resulted in non-functional nodule production (Benson, Boncompagni, & Guerinot, 2005). More specifically, *fegAB* mutants induce shepherd's crooks, infection threads, and plant cortical cell proliferation, but fail to obtain the symbiotic niche required for N-fixation (Benson et al., 2005). These results imply that without FegA, a signal is not properly sent/received, and the nodule cell entry is blocked as a result. The exact mechanism in which FegA senses and propagates the hypothetical plant signal is still unknown (Benson et al., 2005).

FegA is a member of an outer membrane family that form beta barrels in bacteria cells (Figure 1). This beta barrel family is dependent on a TonB binding complex in the inner membrane to provide energy using proton motive force across the bacterial cell wall (O'Brian & Chatterjee, 2018). The purpose of the beta barrel is to allow the transport of substrates across the outer membrane by changing conformation in response to ligand-binding (O'Brian & Chatterjee, 2018). In keeping with this, the *fegAB* mutant is unable to utilize the fungal siderophore, ferrichrome, which is abundant in the soil but not found *in planta* (Benson et al., 2005). Siderophores are extracellular scavenging molecules with a specific affinity for iron. They are secreted by microbes into the local environment, and in some cases may be stolen by other microbes in the area. Iron-loaded siderophores are taken up by microbial cells, unloaded of their iron cargo, and either degraded or recycled back into the environment (O'Brian & Chatterjee, 2018). The iron uptake and symbiotic functions of FegA are thought to be distinct because ferrichrome is not an available iron source *in planta* and suppressor mutants of *fegAB*—which

induce functional nodules, but maintain the original *fegA* insertion and the ferrichrome update defect—are readily obtained from *fegAB*- infected plants (Benson et al., 2005).

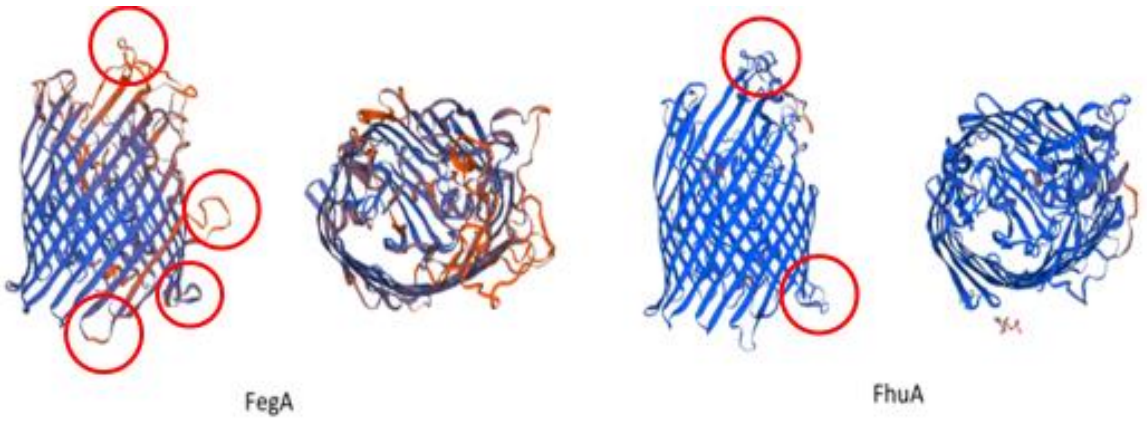


Figure 1. The Theoretical Crystal Structures of FegA and FhuA are Highly Similar, Yet Distinct. The structures above were created using a Swiss Model online program by entering the known amino acid sequences retrieved from the National Center for Biotechnology Information (NCBI) (Swiss model.2018). The beta-barrel regions found in both proteins are predicted to span the outer membrane. Regions of notable difference are circled in red. Both structures are missing the N-terminal domain in these predictions due to the uncertainty of the flexible structure.

A subfamily of TonB-dependent receptors, called TonB dependent transducers, can send and receive signals across the outer membrane in addition to their transport functions(Koebnik, 2005). TonB-dependent transducers generally mediate their signaling function via their unique N-terminal domains (Koebnik, 2005). Because of its dual functionality, FegA is hypothesized to be a TonB dependent transducer. Perhaps it receives a plant signal during the soybean infection process and transports iron in the soil. Careful examination revealed that FegA contains a unique N-terminal domain in comparison to the ferrichrome transporters in *Escherichia coli* and *B. diazoefficiencs* (i.e., FhuA) (Stockwell, S. B., 2008).

Figure 2. The *fegAB* operon in *B. japonicum* strain 61A152. The *fegA* gene is approximately 2200 bp and the promoter is approximately 200 bp. The gene to the right of *fegA* is *fegB* which is approximately 1100 bp. *fegB* is predicted to produce an inner membrane protein (Benson et al., 2005). Previous studies have concluded that FegB is not needed for functional symbiosis (Stockwell, S. B., 2008)

One way to study the role of FegA is to perform a structure/function analysis using a highly similar, yet non-symbiotic FegA homolog. FhuA, found in *B. diazoefficiens* strain USDA110, is 84% similar to the FegA protein in *B. japonicum* (Stockwell, S.B. Guerinot, Mary Lou, N/A). Like FegA, *B. diazoefficien's* FhuA is required for ferrichrome utilization (Stockwell, S. B., 2008). Despite these similarities, FhuA is *not* required for a functional symbiosis with soybean (Stockwell, S. B., 2008). The largest structural difference between these proteins is found in their N-terminal domains (Figure 3), which has further supported the hypothesis that the N-terminal domain of FegA protein plays a unique role in symbiosis signaling.

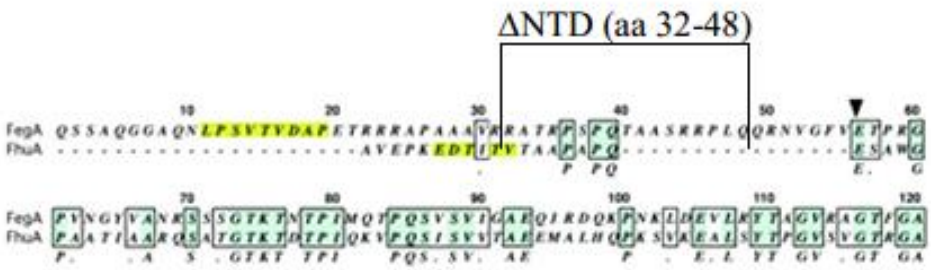


Figure 3. The N-Terminal Domains of FegA and FhuA are the Most Divergent. The two aligned proteins are matured and lack the N-terminal signal sequences (Stockwell, S.B. Guerinot, Mary Lou, N/A). Regions of similarity are boxed and shaded. The highlighted regions on both alignments are the TonB box required for transport function. The black arrow head shows the beginning of the strong sequence similarity between the two proteins. The ΔNTD in FegA found in one of the constructs (Figure 4) is labeled. This region was chosen because it did not overlap with the TonB box yet removed a significant portion of the non-conserved NTD.

The goal of this study was to investigate the function of the NTD of FegA by altering its sequence using recombinant DNA technologies, then testing its ability to complement the ferrichrome utilization and symbiotic defects of the *fegAB* mutant. Mutant constructs of *fegA* with the NTDs swapped between *fegA* and *fhuA* and one construct with a partial deletion of the *fegA* NTD were created. Mutant *fegA* alleles were made by gene cloning and introduced into the *fegAB* mutant using tri-parental mating and homologous recombination. Genotyping by PCR and gel electrophoresis was used to confirm the recombinant *B. japonicum* clones. Following genetic confirmation, *in planta* and ferrichrome utilization assays were performed. The evidence collected serves to support the hypothesis that the NTD is required for the symbiotic function of FegA, however follow-up experiments are warranted.

Methods

Maintaining Bacteria. Cultures of *Bradyrhizobium* were routinely grown using Arabinose Gluconate (AG) medium at 30°C. The *fegAB* mutant (i.e., the recipient for the mutant *fegA* alleles) was grown in the presence of streptomycin (100 µg/mL). *Escherichia coli* containing the *Bradyrhizobium* suicide plasmid, *pBR332,* were grown in Luria-Bertani (LB) medium with tetracycline (20 µg/mL) at 37°C. *E. coli* cells containing the helper plasmid, *pRK2013*, were cultivated using LB medium with kanamycin (30 µg/mL) at 37°C.

Creating Recombinant Clones. Mutant alleles of *fegA* were created within the cloning plasmid, *pGEMT-Easy* using overlap extension PCR with engineered primers and PFU-Ultra polymerase. Recombinant DNA was confirmed by sequencing (Stockwell, S.B. Guerinot, Mary Lou, N/A). These alleles were NTD swap constructs—i.e., FegA with the FhuA NTD or FhuA with the FegA NTD (Figure 4). Constructs were subcloned into *pBR332* suicide plasmid by Epoch Life Sciences. The constructs were added into the genomic DNA of the *fegAB* mutant through homologues recombination in a tri-parental mating, thus creating the recombinant *B. japonicum* clones.

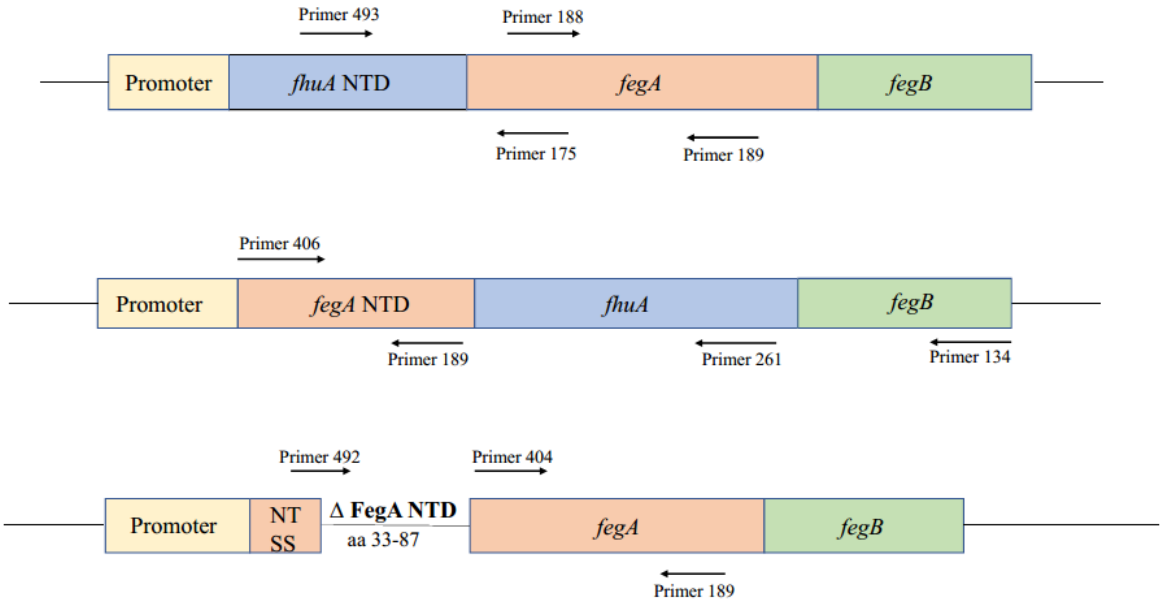


Figure 4. Schematic of Three Modified *fegA* Alleles with Altered NTDs *(i.e., $fegA_{NTDswap}$, $fhuA_{NTDswap}$, or $fegA_{\Delta NTD}$).* These illustrate the desired chromosomal knock-in of the respective pBR322 constructs. Note that all occur before the streptomycin cassette (not shown) in the *fegAB* mutant genomic DNA. A single homologous crossover event needed to occur in front of the streptomycin cassette for proper expression of the mutant *fegA* allele. To test for this crossover event, specific primers were designed to amplify the flanked region using PCR and indicate which crossover occurred. The first scenario has the *fhuA* NTD paired with the *fegA* gene. Primers 493 and 175 will amplify the recombinant region and indicate a successful crossover. The second scenario has the *fegA* NTD paired with the *fhuA* gene. Primers 406 and 261 will amplify the recombinant region and indicate a successful crossover. The third scenario has a 141 bp in frame deletion (amino acids 32-48) of the NTD of FegA. Primer 492 is engineered to amplify the shortened NTD while primer 404 amplifies the normal NTD.

Tri-Parental Mating. The tri-parental mating process is an effective method for transferring plasmid DNA from one bacterium into another. In this case, recombinant *fegA* alleles within recombinant pBR322 plasmids in *E. coli* were transferred into recipient *B. japonicum fegAB* mutant cells. The mating utilized three bacterial strains: the recipient, donor, and helper. The recipient was the *fegAB*::ΩStr mutant. *E. coli* with one of three recombinant *pBR322* suicide plasmids ($fegA_{NTDswap}$, $fhuA_{NTDswap}$, or $fegA_{\Delta NTD}$) served as the donor, while *E.*

coli carrying the *pRK2013* plasmid provided the transfer functions for the conjugation. The donor plasmids contained sequences that were identical to genomic DNA that flanked the *fegA* loci. These identical sequences allowed for homologous recombination resulting in the insertion of the entire plasmid DNA into the chromosome of the recipient, thereby creating a knock-in (KI) of the engineered allele.

Tri-parental matings were created with the three bacterial strains grown to optimal cell densities (i.e., early log phase of growth). The equations used for determining the correct volume needed of each strain were: x(OD) = 0.1 for *E. coli* and x(OD) = 0.18 for *B. japonicum* where OD is the measured absorbance at 600 nm. After determining the needed volumes, microcentrifuge tubes were filled with: 1) each unique strain, 2) unique parings of the recipient strain with each donor strain, 3) unique pairings of the helper strain with each donor strain, 4) and complete combinations of the recipient, helper, and a donor strain. Doing three matings in parallel, this approach created 15 different combinations. Combinations 1-3 were all negative controls. All mixtures were centrifuged at 10,000 rpm for 1 minute. The supernatant of each tube was removed, and 0.2 mL of liquid AG medium was added to each tube. The tubes were vortexed to resuspend the cells in solution, and the entire contents of the tubes were poured onto AG medium plates and incubated at 30°C for 5 days.

After the incubation period, 5 mL of 0.01% tween 20 was poured onto each plate. Using a sterile scraper, the bacteria cells were removed from the AG medium plate and transferred into a capped glass test tube using a pipette. The tubes were vortexed for 1 minute, then 250 µL of each combination was transferred onto AG medium plates containing tetracycline (200 µg/mL) and rifampicin (40 µg/mL). The mixtures containing all three bacterial types were plated in triplicate. The plates were incubated for 2 weeks at 30°C and single colonies were observed,

patched, an propagated. Only *B. japonicum* cells with the recombinant *pBR322* suicide plasmids integrated into their genomes at the *fegAB* locus were expected to grow on these plates.

Genomic DNA Extraction. Genomic DNA from *Bradyrhizobium* was needed to characterize the location of the homologous crossover event that presumably occurred during the mating. Prior to the extraction, 10 mL cultures of each strain were grown in AG broth and incubated at 30°C with shaking for one week. The cultures were centrifuged at 6,000 rpm for 10 minutes to produce a cell pellet and the supernatant was discarded. The pellet was resuspended in 5 mL of 0.85% NaCl and centrifuged at 10,000 rpm for 2 minutes. The supernatant was again discarded. The remaining pellet was resuspended in 2.5 mL of TES buffer and centrifuged at 10,000 rpm for 2 minutes. The supernatant was once again discarded. The final cell pellet was resuspended in 2.5 mL of Tris-EDTA buffer ($T_{10}E_{25}$) and 50 µL of Lysozyme (10 mg/L) was added. This solution was placed in a shaking incubator at 37°C for 15 minutes. Following this incubation, 300 µL of sarkosyl-proteinase k was added to the solution, which was then incubated for one hour at 37°C. (Ausubel, Brent, & Kingston, 1993)

Two milliliters of phenol:chloroform was added to the thawed solutions and divided into microcentrifuge tubes and centrifuged at 12,000 rpm for two minutes. The top aqueous phase of each tube was transferred to a new microcentrifuge tube and 250 µL of chloroform: isopropyl alcohol (24:1) was added. The mixtures were vortexed then centrifuged at 12,000 rpm for two minutes. The upper aqueous phase was transferred to a new tube. The chloroform step was repeated until phenol was no longer detected in the sample. At that point, 550 µL of cold 3M potassium acetate (KoAC) was added to each tube, followed by 300 µL of isopropanol. These solutions were stored overnight in the freezer to encourage the precipitation of genomic DNA. (Ausubel et al., 1993)

Next, DNA samples were centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded, and one milliliter of 70% ethanol was added to each tube. The tubes were centrifuged at 12,000 rpm for 15 minutes and the ethanol was poured out. The tubes containing only a pellet were placed in an incubator for one hour with the caps open. Following the drying period, 50 µL of sterile water was added to each tube to resuspend the DNA. Samples were stored at -20°C. (Ausubel et al., 1993)

Nanodrop. DNA samples were routinely assessed for quality and quantity using a Thermo Scientific NanoDrop One spectrophotometer. The machine was blanked with diH_2O and 1.5 µL of each sample was loaded onto the column pedestal. Outputs included DNA concentration and the absorbance ratios measured at 260:280 and 260:230 nm. These values were used to assess the quality of extracted DNA. To further optimize for PCR, the DNA was concentrated using a Zymo PCR Inhibitor Removal Kit using the manufacturer's instructions.

Genotyping Recombinant Clones using PCR. Purified genomic DNA was isolated from recombinant clones that were recovered from each tri-parental mating. Each clone's DNA was used as a template in a Polymerase Chain Reaction (PCR) containing; 10 µL of primers (20 ng/µL), 25 µL of One Taq Quick Load 2xMM (BioRad), 80 ng of template DNA and 10 µL of distilled water to achieve a final volume of 50 µL. The reaction entailed three phases (95°C for 60 seconds, 45°C for 60 seconds, and 72°C for 90 seconds) for 30 cycles. The product was separated on a 1% agarose gel in the presence of ethidium bromide and visualized using UV light.

***In planta* assays.** Confirmed recombinant knock-in clones were next grown on soybean plants to test if the symbiosis was restored by the introduced *fegA* allele. Liquid cultures of each test inoculant were grown in AG medium for 5-10 days. The soybean seeds were surface

sterilized with 1% bleach for 5 minutes and rinsed with sterile distilled water. Following the rinse, the seeds were soaked in 2% hydrogen peroxide for 15 minutes, then rinsed with sterile distilled water four separate times for 5 minutes each. Sterilized seeds were then incubated with a pure bacterial culture for 40 minutes to promote the infection of emerging roots. Immediately after the inoculation, the seeds were planted into sterile Leonard jars and placed in a growing chamber. The Leonard jars contained a 2:1 perlite to vermiculite mixture and a nitrogen-free nutrient solution. The N-free solution was made at 10x in 1 L (6.8 g K_2SO_4, 2.4 g KH_2PO_4, 0.2 g K_2HPO_4, 9.8 g $MgSO_4$, and 20 mL of 1000x micronutrient solution) then diluted to 1x (pH 6.5) for the Leonard jars. The growth chamber was kept at 28°C and cycled through 8 hours of light and 8 hours of darkness. Once the plants had broken through the soil and started to form shoots, cotton balls were placed over the soil to avoid cross-contamination between jars. Growing plants were top-watered with more N-free solution. After 4-6 weeks of growth, the roots were harvested, and the nodules were macroscopically examined. The nodules were counted and weighed along with the plant shoots. Representative leaf punches were used to determine chlorophyll production.

Chlorophyll Extraction. Chlorophyll was extracted from inoculated soybeans to assess the health of the plants. One tenth of a gram of leaf tissue was harvested from each plant for analysis. Two hundred microliters of ethanol was added to each sample and ground to break apart the tissue. The tissue slurry was then centrifuged at 10,000 rpm for 5 minutes and the supernatant was poured out and transferred to a new microcentrifuge tube. The remaining leaf tissue was resuspended in two hundred microliters of ethanol and centrifuged again at 10,000 rpm for 5 minutes. The supernatant of that spin was poured out and combined with the first collected supernatant. Next, three microliters of each final supernatant were mixed with 13 µL of

ethanol and 64 µL of acetone in a microtiter dish well. The absorbances were measured at 664 nm and 647 nm. These values were used to calculate the total chlorophyll content using the following equation: Total chlorophyll per gram of leaf $= 43 \times [(7.93 \times A_{664}) + (5.26 \times A_{647})]$

Results

Isolation and Genomic DNA Extraction from Recombinant *B. japonicum* Clones

As a result of the conjugations: 8 colonies were isolated from the $fegA_{NTDswap}$ combination, 11 colonies were isolated from the $fhuA_{NTDswap}$ combination, and 16 colonies were isolated from the $fegA_{\Delta NTD}$ combination. There were no colonies observed on the negative control plates. Five colonies from each mating were patched onto new selective plates and further genotyped. Cultures were grown to stationary phase and genomic DNA was extracted. The results of these extractions are shown in Table 1.

Table 1. Extraction of *fegAB*-/KI ($fegA_{\Delta NTD}$) Genomic DNA. Concentrations were measured via a Nanodrop. Clone 4 had an unusual A260/A230 reading so the sample was not used for genotyping of *fegAB*-/KI ($fegA_{\Delta NTD}$). The ideal ratio for A260/280 is 1.8, which indicates no contamination from salts (Nanodrop Product Manual). The ideal ratio for A260/A230 is 2.0 which indicates no contamination from organic products (Nanodrop Product Manual).

***fegAB*-/KI ($fegA_{\Delta NTD}$) clones**	**Nucleic Acid (ng/µL)**	**A260/A280**	**A260/A230**
1	32.8	2.001	1.825
2	49.7	1.758	2.313
3	82.4	1.935	1.938
4	17.1	1.907	3.400
5	32.2	2.034	1.893

Genotyping of *fegAB-*/KI *(fegA$_{\Delta NTD}$)*

To check the location of the potential crossover, a series of PCRs were performed using primers 492/189 and 40/189 to amplify the two crossover locations (See Figure 4 for primer locations). As shown in Figure 5, there was a band observed in the test lane one at the predicted size (i.e., 1402 bp) using the *fegAB-/*KI *(fegA$_{\Delta NTD}$)* genomic DNA as the template. There was no band observed in the test lane two, which also used the *fegAB-/*KI *(fegA$_{\Delta NTD}$)* genomic DNA as template (Figure 5). No bands were observed in the negative control lane 5 or positive control lanes (three and four). See supplemental tables 2 and 3 for details on the primers and PCR set up.

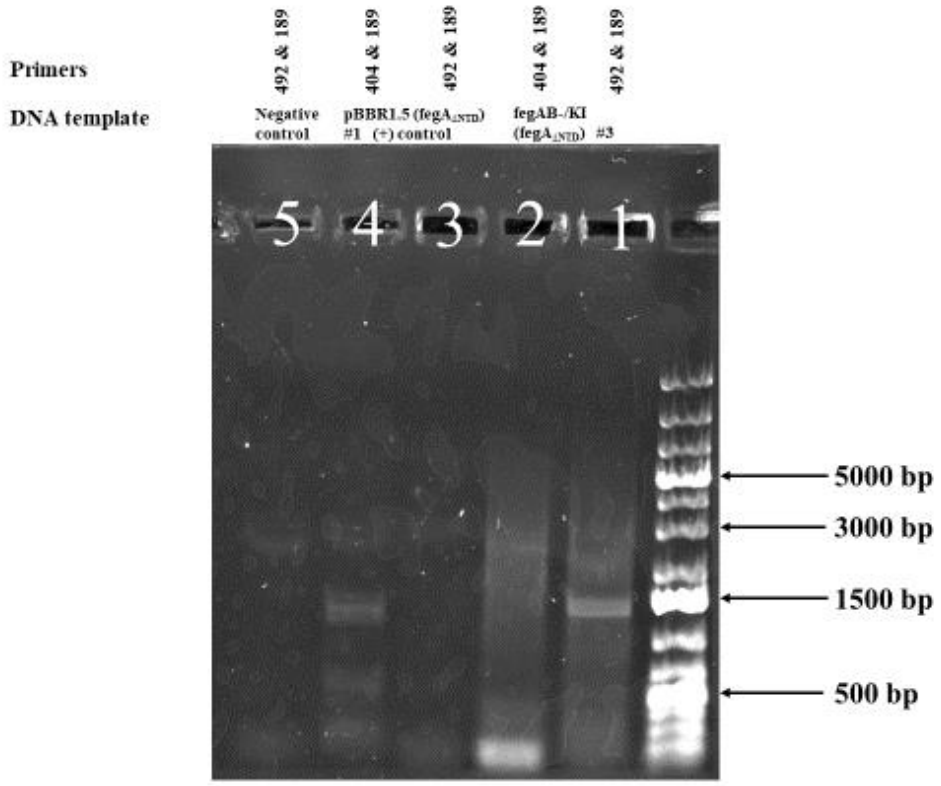


Figure 5. **Confirmed Crossover of *fegAB*-/KI *(fegAΔNTD).*** A standard molecular ladder is shown to the left, with relevant sizes labeled. Primer pairs and DNA templates are indicated above. Both positive controls for the test reactions did not produce a band at the expected size (lanes three and four). As expected, the negative control reaction, containing no template DNA, did not produce a product (lane 5).

In Planta Assays with *fegAB*-/KI *(fegA$_{\Delta NTD}$)*

Multiple assays were done using the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* strain to investigate the role of the NTD of FegA in symbiosis. In the first experiment, the plants were grown for four weeks in the window-sill before harvesting. Non-functional nodules were observed on plants inoculated with the *fegAB*-/KI *(fegA$_{\Delta NTD}$)*, while none were seen on the mock-inoculated control plants.

In the second assay, plants were grown in both the window-sill and growth chamber (Appendix Figures 11 and 12). Once again, immature nodules formed on the roots of the *fegAB*-/KI *(fegA$_{\Delta NTD}$)*-inoculated plants. The observed nodules were smaller and smooth, unlike wild-

type nodules which typically appear larger and contain vertical ridges. No nodules were observed on the negative control plants.

For the third assay, plants remained in the growth chamber for the entire assay (Appendix Figure 10), and wild type and *fegAB* mutant strains were added to the experiment to serve as additional positive and negative controls. There was no mock-inoculant negative control for this assay. Wild-type nodules were observed on plants inoculated with the WT61A152 strain. The *fegAB* mutant samples had non-functional nodule formation in two out of the three jars. No nodules were observed on the roots of plants grown in the third jar. The *fegAB-/*KI *(fegA$_{\Delta NTD}$)* samples had a mixed growth of wild-type and immature nodules. One nodule was sectored (i.e., containing both functional and non-functional portions within the same nodule).

The fourth assay contained a full set of experimental, positive and negative control strains: WT61A152, *fegAB-/*KI *(fegA$_{\Delta NTD}$)*, the *fegAB* mutant and mock-inoculant (Figure 6). Plants were grown for four weeks in the growth chamber (Supplemental Figure 10). The shoots of the resulting plants were of similar fresh weight and chlorophyll content (Figures 7 and 8.

The average nodule number per plant was also similar (Figure 9), although the size (i.e., weight) differed (Figure 10). This was expected since the WT61A152 nodules are often larger in size than those produced from plants inoculated with *fegAB-*. This trend held true in this experiment, as WT61A152-inoculated plants produced large nodules with red interiors and pronounced ridges (Figure 6). In contrast, the *fegAB* mutant and *fegAB-/*KI *(fegA$_{\Delta NTD}$)* clone produced mostly small, non-functional nodules (Figure 6). Two plants from each set (i.e., inoculated with *fegAB-/*KI *(fegA$_{\Delta NTD}$)* or *fegAB-*) produced both non-functional nodules and a small number of wild-type nodules at the root crown. This was not unexpected, as suppressor nodules are known to routinely form on plants inoculated with *fegAB-* (Benson et al., 2005).

Bacteria found within these kinds of nodules have been shown to contain the original *fegAB* mutation and the inability to transport ferrichrome (Benson et al., 2005). The no-bacteria control inoculant did not produce nodules on any of the plant samples, as expected (Figure 6).

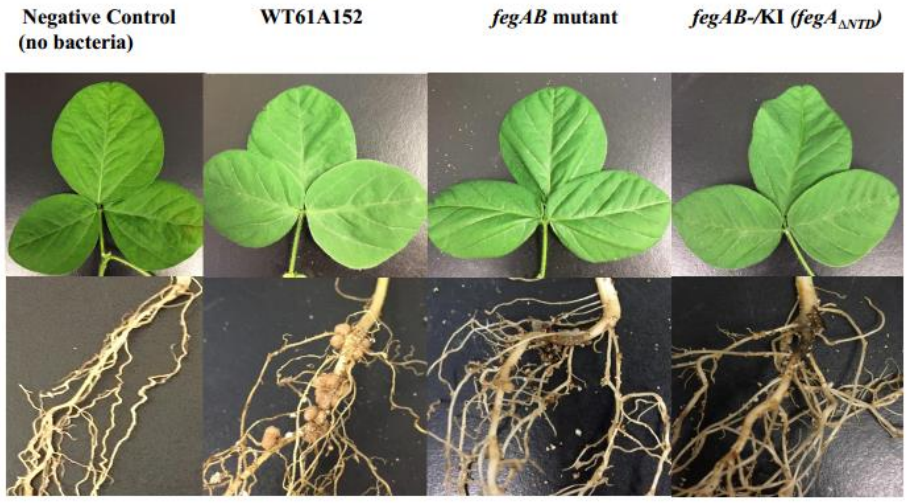


Figure 6. **Representative Root Crown and Leaf Pictures from Inoculated and Mock-Inoculated Plants.** Sterilized seeds were inoculated with water (negative control), WT61A152, *fegAB*-, or *fegAB*-/KI *(fegA*$_{\Delta NTD}$*)* and grown for 4 weeks. There were no nodules on the negative control plants. The WT61A152 inoculation produced wild-type nodules in clusters near the crown of the roots. Both the *fegAB* mutant and *fegAB*-/KI *(fegA*$_{\Delta NTD}$*)* produced small, immature nodules on the main and lateral roots. Chlorophyll content of the leaves was similar.

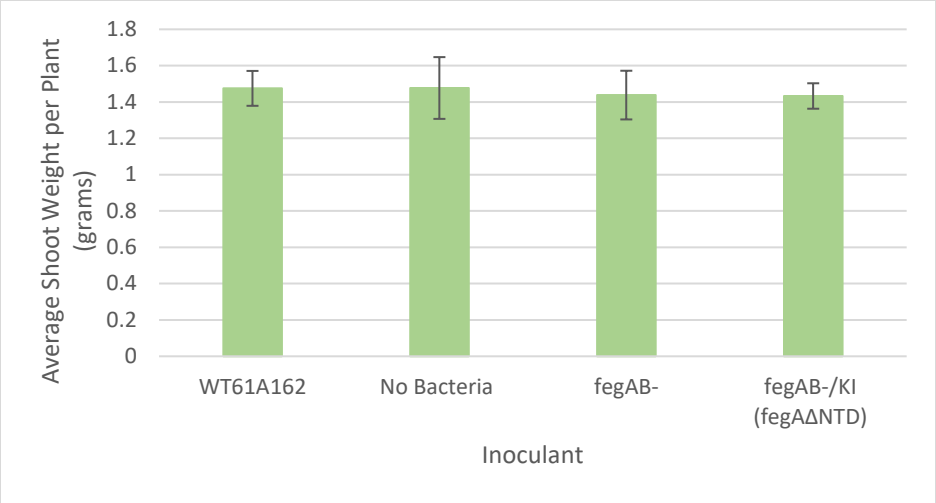


Figure 7. **Inoculated Plants were of Similar Weight.** Sterilized seeds were inoculated with WT61A152 (n = 8), *fegAB*-/KI *(fegA$_{\Delta NTD}$)* (n = 9), *fegAB*- (n = 9), and no bacteria (n = 7). Plants were grown for four weeks and harvested. Fresh shoot weight was measured for each plant. Standard errors from the means are shown as y-axis error bars. The calculated F value of the means is 0.0387 which implies the variance between the means is due to chance (One-way ANOVA calculator). The calculated p-value is 0.9896 which indicates no statistical significance between the averages (One-way ANOVA calculator).

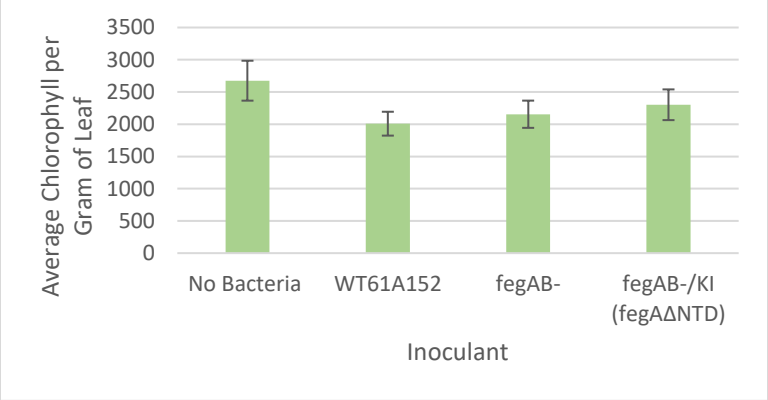


Figure 8. Harvest Plants had Similar Chlorophyll Content. A standard hole-punch was used to obtain 0.1g of leaf tissue from each plant inoculated with WT61A152 (n = 8), *fegAB-*/KI *(fegA$_{\Delta NTD}$)* (n = 9), *fegAB*- (n = 9), and no bacteria (n = 7). The tissue was ground and measured using a spectrophotometer at 664nm and 647nm. Standard errors from the means are shown as y-axis error bars. The calculated F value is 1.332, indicating that the variance between averages is not due to chance (One-way ANOVA calculator). The calculated p-value is 0.238 which indicates that the average chlorophyll variance was not statistically significant (One-way ANOVA calculator). An unpaired t-test between the plants inoculated with no bacteria or *fegAB*-/KI *(fegA$_{\Delta NTD}$)* produced a p-value of 0.3471, indicating no statistical significance between the two averages (GraphPad.2019).

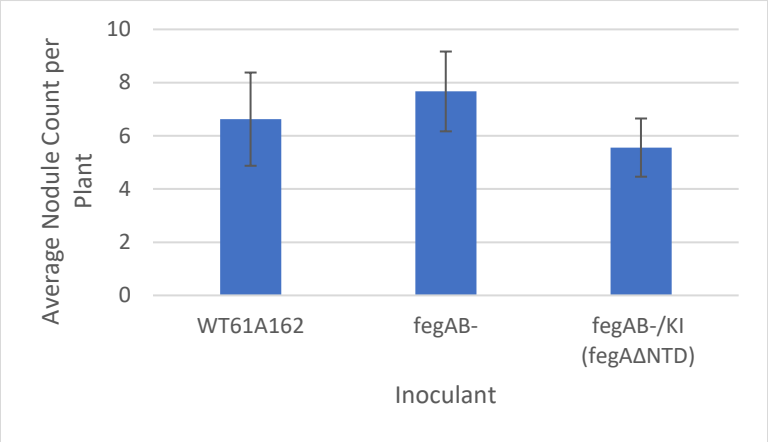


Figure 9. **There was no Observed Difference in Nodule Number per Plant.** Plants were inoculated with WT61A152 (n = 53), *fegAB*-/KI *(fegA$_{\Delta NTD}$)* (n = 50) and *fegAB*- (n = 69) and grown for four-weeks. Standard errors from the means are shown as y-axis error bars. The calculated F value is 0.549 which indicates that the variance is due to chance (One-way ANOVA calculator). The calculated p value is 0.585 which does not indicate that the variance in nodule count was statistically significant.

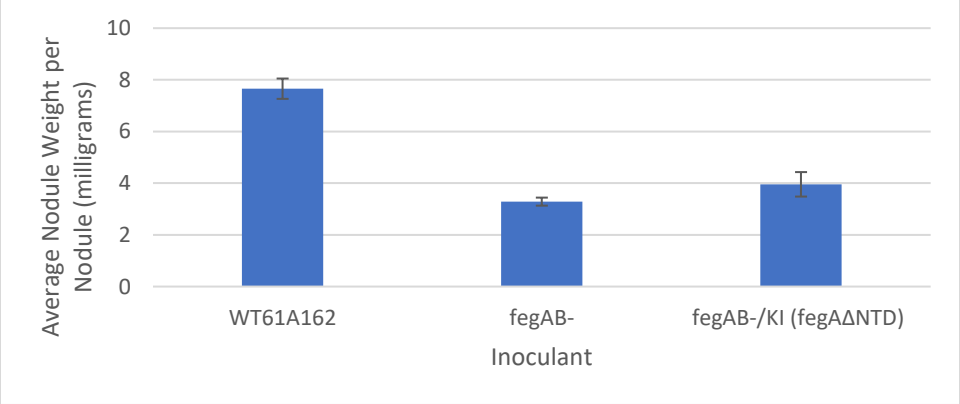


Figure 10. Collected Nodules Were of Different Weight. The plants inoculated with WT61A152 (n = 53), *fegAB*-/KI *(fegA$_{\Delta NTD}$)* (n = 50) and *fegAB*- (n = 69) produced different types of nodules during the four-week growth period. No nodules were observed on the mock-inoculated (i.e., "no bacteria") control plants. Standard errors from the means are shown as y-axis error bars. The calculated F value is 6.61 which indicates that the variance is not due to chance (Social science statistics: One-way ANOVA calculator). The calculated p-value is .0054 which indicates that the nodule weight variance is statistically significant (Social science statistics: One-way ANOVA calculator). An unpaired t-test between WT61A152 and *fegAB*-/KI *(fegA$_{\Delta NTD}$)* produced a p-value of 0.0278, indicating a statistical significance between the two averages (GraphPad.2019).

Discussion

A structure/function analysis of FegA was done to determine the significance of the NTD of FegA in the molecular signaling pathway with soybean plants. The highly similar *B. diazoefficiens* protein, FhuA, was used to inspire altered $FegA_{NTD}$ alleles. While the NTD swap constructs did not yield recombinant *B. japonicum fegAB*- strains that contained the suicide plasmid knocked into the appropriate locations to support *fegA* expression, the project did successfully create, confirm, and test a new *fegAB*-/KI($fegA_{\Delta NTD}$) strain.

The observation of colonies on the selection plates from the mating indicated that plasmid transfer and homologous recombination was successful (Table 1). The lack of growth on plates containing incomplete mixtures of bacteria indicated that cross-contamination was not an issue. Genomic DNA extracted from the transconjugants had lower than ideal concentrations but were enough for further genotyping. While ideal DNA concentrations are about 100 (ng/µL), the *fegAB*-/KI ($fegA_{\Delta NTD}$) concentrations ranged from 82 (ng/µL) to 17 (ng/µL) (Table 2). This variation in concentration is likely due to inefficiencies in the extended phenol/chloroform DNA extraction process. That said, the high level of exopolysaccharide found on *Bradyrhizobium* cells makes column-based DNA extraction difficult, which is why the phenol/chloroform extraction is still favorable. With A260/A280 ratios of the *fegAB*-/KI ($fegA_{\Delta NTD}$) samples around 1.8 (Table 2), it could be concluded that although our quantity may have been low, the quality was satisfactory.

The genotyping of the *fegAB*-/KI ($fegA_{\Delta NTD}$) mutant supported the conclusion that the crossover of the suicide plasmid and *B. japonicum* genomic DNA occurred in the mating at the correct location. The gel image (Figure 5) has a faint band in the first test lane that would

indicate the crossover occurred properly. The specific size of the band (1400 bp) matching with the expected size is evidence that a mutant NTD of *fegA* is present in the bacteria strain. There was no band in the test lane two which confirms that the crossover did not occur after the streptomycin cassette. Even though the positive control for both test lanes (three and four) was unsuccessful in producing a band on the gel, the band in lane one was encouraging enough to proceed with the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* mutant testing. It is possible that the template DNA for the positive control was at an insufficiently low concentration and could not be amplified in PCR. The negative control for the PCR produced no bands (lane 5), indicating that cross-contamination of DNA between the samples or from an external source is unlikely.

The results of the four *in planta* assays suggest that the *fegA*$_{\Delta NTD}$ construct fails to complement the *fegAB* mutant defect. Plants inoculated with *fegAB*-/KI *(fegA$_{\Delta NTD}$)* produced small, immature nodules similar to those observed on *fegAB*- inoculated plants (Figure 6). These results support a hypothesis that the NTD is required for proper symbiosis in *B. japonicum*. Linking the symbiotic function to the NTD of the protein further supports the notion that FegA is a TonB-dependent transducer (Braun & Mahren, 2005). As mentioned previously, TonB-dependent transducers have been shown to upregulate genes after interaction with an outside signal or ligand at the outer membrane (Braun & Mahren, 2005). Based on the work of others, we propose that the NTD of FegA propagates a plant signal through the interaction of an anti-sigma factor found in the inner membrane and a sigma factor found in the cytoplasm (Koebnik, 2005). Future work could focus on identifying these interacting protein partners. Of particular interest is a third gene that was recently recognized within the *fegAB* operon (Figure 1). This gene occurs downstream of *fegB* and may provide a symbiosis-specific function. This has yet to be investigated.

As noted previously, small numbers of nodules with wild-type morphologies were observed on a small sample of *fegAB*- and *fegAB*-/KI *(fegA$_{\Delta NTD}$)* -inoculated plants. The presence of these nodules at the root crown indicates that the bacteria that induced them were present at the onset of the infection and did not appear later in the growth/watering process as a result of cross- jar contamination. Benson et al. (2005) noted a similar phenomenon. Their studies indicate that bacteria obtained from these "suppressor" nodules contain the original *fegA* streptomycin cassette insertion (Benson et al., 2005). They hypothesize that this may happen when a non-symbiotic clone obtains a second mutation to suppress the mutant phenotype (Benson et al., 2005). The nature of this mutation is still unknown. The presence of these suppressors indicates that the existence of the truncated FegA protein in *fegAB*-/KI *(fegA$_{\Delta NTD}$)* does not interfere with the suppression.

The chlorophyll extraction results contradict what was expected to be observed with the inoculated plants. That is, the plants without bacteria had the highest levels of chlorophyll per gram of leaf (Figure 10), although it was predicted that the plants inoculated with WT61A152 would have the highest level of chlorophyll per gram of leaf. It is possible that since the fourth *in planta* assay allowed the plants to grow for only 4 weeks, instead of 6, a marked difference was not observed. If the plants had grown for longer, the lack of available nitrogen in the non-symbiotic control plants may have been more apparent. It should be noted that the *fegAB*-inoculated plants also had higher chlorophyll levels than the WT61A152 inoculated plants which was surprising. Benson et al. (2005) collected the opposite results in their *in planta* assays. As predicted, Benson et al. (2005) observed that the WT61A152 inoculated plants received the benefit of the symbiosis with the bacteria and had higher chlorophyll levels than the *fegAB*-

inoculated plants. Again, it is possible that the shorter 4-week growth period is responsible for this observed difference.

In addition to the *in planta* assay, the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* strain was tested for its ability to utilize ferric ferrichrome (see Appendix). It was theorized that since the beta-barrel of the FegA protein was still contained in the mutant allele, ferrichrome would be able to cross through the outer membrane. Unfortunately, the plate iron utilization assays did not produce consistent results, thus making it difficult to make any conclusions about the role of FegA's NTD in iron utilization. Not only did the positive control iron sources fail to support growth, the wild type cells inconsistently grew using ferric ferrichrome. Similarly, *fegAB*-/KI *(fegA$_{\Delta NTD}$)* did not grow using ferric ferrichrome as a sole iron source.

Technical issues aside, perhaps the *fegA$_{\Delta NTD}$* construct is not properly expressed in the *fegAB*-/KI *(fegA$_{\Delta NTD}$)*. This could be tested using RT-PCR to confirm that *fegA* mRNA is present in cells grown under low iron conditions. Inadequate expression is unlikely, given the fact that full-length *fegA*, driven by its endogenous promoter as in the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* strain, has been shown to complement the *fegAB* mutant in the iron assay (Stockwell, personal communication). More likely, the FegA$_{\Delta NTD}$ protein is unstable and/or mislocalized in the cell. Western blots to detect the 5xHis tag that has been engineered into the *fegA$_{\Delta NTD}$* construct will help to investigate this problem. Stockwell et al. (unpublished) created a mutant *B. japonicum* clone similar to the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* clone. They found that the produced protein from the mutant clone was unstable using western blot. The production of an unstable protein would explain why the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* clone was unable to uptake ferric ferrichrome.

For the near future, the results of the iron utilization assay and *fegA$_{NTDswap}$* clones need to be revisited to confirm the role of the NTD of FegA. The observed behavior of *fegAB*-/KI

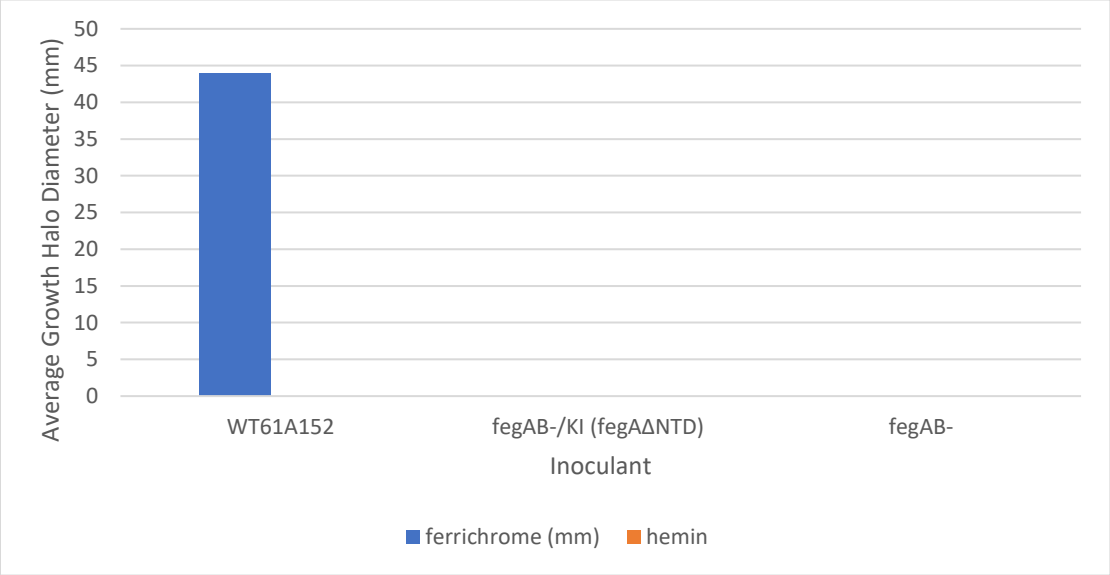
(fegA$_{\Delta NTD}$) being unable to utilize ferrichrome indicates some issues with the mutated *B. japonicum* strain. Western blots and RT-PCR will need to be done to confirm the stability and expression of the altered NTD in *fegAB*-/KI *(fegA$_{\Delta NTD}$).* The genotyping of the *fegA$_{NTDswap}$* clones also needs to be revisited to observe the behavior of other mutated FegA proteins in iron utilization and symbiosis. The *fegA$_{NTDswap}$* clones will need to be further genotyped with different primer pairings or recreated. In conclusion, although future work is needed to better understand the ferrichrome utilization of the altered NTD, the results of this study support the hypothesis that the NTD of FegA is needed for symbiosis.

Appendix

Iron Utilization Assay. Liquid cultures of *fegAB*-/KI *(fegA$_{\Delta NTD}$)*, *fegAB*- (negative control), and WT61A152 (positive control) were grown in AG broth within acid-washed flasks a week before the assay. Seventy milliliters of Yeast Extract (YE) broth (pH 5.2) was poured into sterile flasks—one flask per test strain. The flasks were placed in a hybridization oven at 50°C to cool the molten agar. Once in the cooled, but liquid state, 1.4 mL of 10% mannitol was added to each flask to create YEM. Five hundred sixty microliters of 100 mM HBED (i.e., the iron chelator) was added to each flask followed by 3.5 mL of the bacterial test culture. Each mixture was then poured into three plates and allowed to solidify. Once solid, a sterile toothpick was used to drill a hole in the center of each plate. Into this hole, 7 µL of 5 mM 3:1 ferric ferrichrome, 3.5 µL of 5 mM Hemin was deposited. In other positive control set ups: ferric citrate or ferrioxamine B was deposited in four holes on a single plate, (1, 2, 2, 5 µL of 5 mM solutions). The plates grew for 5-7 days and the resulting growth halo diameters were measured.

Three iron utilization assays were done to determine if iron could be utilized by the *fegAB*-/KI ***(fegA$_{\Delta NTD}$)*** clone. *fegAB*- and WT61A152 were used as negative and positive controls for ferrichrome utilization. Hemin served as a positive control iron source. It was expected that the *fegAB*-/KI ***(fegA$_{\Delta NTD}$)*** clone would be able to uptake ferrichrome, like the WT61A152 strain, because it is hypothesized that the NTD of FegA is specialized for symbiosis. Our results did not support this, as it was observed that *fegAB*-/KI ***(fegA$_{\Delta NTD}$)*** was unable to growth using ferric ferrichrome as its sole iron source (Supplemental Figure 11). None of the strains were able to grow using the positive control iron sources (hemin, ferric citrate, and ferrioxamine B), which

indicates that there was a problem with the assay. Follow-up studies will be used to troubleshoot the issue.



Supplemental Figure 8. **Iron Utilization of Inoculants.** Bacterial strains were seeded into YEM containing the iron chelator, HBED. Iron sources (ferric ferrichrome or hemin) were point-deposited and plates were incubated for 5-7 days. Each bacterial strain had two plates with ferric ferrichrome and one plate with hemin. The positive controls for the assays did not produce expected growth halos in the low iron medium. The *fegAB* mutant was not predicted to produce any growth halos.

***In planta* growth conditions.** The four *in planta* assays used two different growth conditions to provide light for the soybean plants. The growth chamber was the preferred choice of set up, but the window sill was used while the chamber was out of operation for the earlier assays (Supplemental Figure 12).

Supplemental Figure 9. ***In planta*** **Assay Setting**. Both the window sill and growth chamber were used to provide light for the plants during the assays. The growth chamber has a large container of water under the bottom shelf to provide humidity for the plants.

PCR Genotyping. The genotyping of *fegAB-/KI (fegA$_{\Delta NTD}$)* (Figure 5) had specific primer pairings to determine which potential crossover occurred in the tri-paternal mating. Each primer pairing was matched with a template DNA to produce an expected size based off which crossover was present in the genomic DNA (Supplemental Table 2). There were several primer pairings that were used in genotyping the other two recombinant clones (Supplemental Table 3).

Supplemental Table 2. Details of the PCR to Confirm the *fegAB*-/KI *(fegA$_{\Delta NTD}$)* Crossover Event. Contents of each PCR reaction and the expected fragment sizes of the PCR products (Figure 6). The primer pairings are listed with the stock numbers that are used in Table 4.

Tube #	DNA sample	Primer Pairing	Expected Size
1	*fegAB*-/KI *(fegA$_{\Delta NTD}$)* #3	492 & 189	1402 bp
2	*fegAB*-/KI *(fegA$_{\Delta NTD}$)* #3	404 & 189	1433 bp
3	pBBR1.5 (*fegA* Δ NTD) #1	492 & 189	1400 bp
4	pBBR1.5 (*fegA* Δ NTD) #1	404 & 189	1693 bp
5	None	492 & 189	0 bp

Supplemental Table 3. PCR Primer Information. List of primers used during the genotyping of each recombinant clone produced during the tri-parental mating. Each primer has a given stock number for personal organization purposes.

Primer	Stock #	Sequence (5' – 3')	Tm (degrees Celsius)
4920 midseq F	493	ATGCGAGCTGGAAGCTCC	57.9
fegA stop HindIII R	175	TTTAAGCTTCCACTTGTAGGAGACGCTGGC	63.2
mid*fegA* seq F	188	TCTACACTGCGTATGCGAG	54.3
mid*fegA* seq R	189	AGATGTAGGACGCTATGCC	54.0
fegA @49AA F	406	TCAAGCTCTTCACAACGCAATGTCGGCTTCGTC	65.9
4920 midseq R	261	TCGTCGCCACATTTTGAC	53.3
fegA@39AA(SapI/CAH). F	404	TCAAGCTCTTCACAGACCGCCGCCAGCCGCAGA	72.1
Δ32-48 junction F	492	GCCGCCGTGCGCCAACGCAATGTC	69.7
seq*fegB* (226p + ATG) R	134	CGGGATCCCATGGTCCACACCTGGACCAGCCTG	71.1

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