IDENTIFICATION OF RHIZOBIUM GENES THAT ARE INDUCED UNDER SYMBIOTIC GROWTH CONDITIONS IN THE BEAN SYMBIONT STRAIN *R. ETLI* CE144 WHICH LACKS THE SYMBIOTIC PLASMID

by

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(Under the Direction of RUSSELL W. CARLSON, PHD)

ABSTRACT

Rhizobium are a unique group of bacteria classified by their ability to initiate the formation of a novel organ, the nodule, on legume plants. The *Rhizobium*-legume symbiosis has been labeled as one of the most important plant-bacteria interactions (Puhler, 1998). The large symbiotic plasmids, pSyms, have been widely recognized as the carrier of essential symbiotic genes. Here, we sought to identify genes that are induced during simulated symbiotic conditions (low pH and flavonoids) that are not located on the pSym. Two methods, conventional vector cloning and transposon mutagenesis (containing a *gusA* reporter gene), were employed of which the latter was successful in obtaining insertion mutants in *R. etli* CE144 (pSym$^-$ derivative of *R. etli* CE3). Four mutants exhibited greater than 2-fold activity compared to normal in the β-glucuronidase assay. Normal was defined as β-glucuronidase activity under non-induced conditions. Sequence analysis revealed that insertions in the four mutants were a putative oxidoreductase, ABC transporter, hydrolase, and glutamine synthetase II (GSII). Due to high sequence similarity to GSII, this mutant was analyzed for a distinct phenotype and the observations are reported.

INDEX WORDS: *Rhizobium etli, gusA,* β-glucuronidase, symbiosis, pSym, transposon mutagenesis, glutamine synthetase

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DEDICATION

I dedicate this to…

My mother…a continuous pillar of strength, tough yet unconditional love, and symbol of faith I hope to exemplify.

My father… my #1 fan; an irreplaceable factor in my life; a reminder that we are perfect in our imperfections, and that I can only fail if I fall and don't get up.

My sister…you are priceless and I could not have received a more precious gift.

My best friend… truly a gift in my life; a perfect example of friendship. This journey would have been impossible without you there to share it with.

Together, you are my family, my sanity, my refuge. I love you and will always remember the encouragement and prayers you have provided me with along the way.

Tonna

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My most sincere thanks…

To my heavenly Father, I give ALL the glory and honor. I thank you for the trial this has been because I know you are preparing me for something greater.

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To my family who have patiently put up with my unpredictable attitudes in times of stress. I am grateful to have you to turn to and it is a comfort to know that I am not in this alone.

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LIST OF ABBREVIATIONS

A	Vincent's defined media with ammonium chloride only
BLAST	basic local alignment search tool
Bp	base pair(s)
Cfu	colonly forming unit
CIP	calf intestinal phosphatase
EtBr	ethidium bromide
EtOH	ethanol
G	Vincent's defined media with glutamate only
GA	Vincent's defined media with glutamate + ammonium chloride
GS	glutamine synthetase
Kb	kilo base pair(s)
Kan	kanamycin
LB	Luria-Bertani
LCO	lipochitinooligosaccharide
LPS	lipopolysaccharide
Mb	mega base pair(s)
mTn5	minitransposon derivative of Tn5
MU	Miller unit(s)
N	nitrogen
Nal	naladixic acid

NCBI	National Center for Bioinformatics
Neo	neomycin
NG	no growth
Nif	nitrogen fixation
Nod	nodulation
OD	optical density at the defined wavelength
PBC	*Bam*HI digested pRG970 with calf intestinal phosphatase
pNPG	p-nitrophenyl-β-D-glucuronide
pSym	symbiotic plasmid of rhizobia
p*Sau*144	pRG970 with a 1kb *Sau*3A1 insert from *R. etli* CE144
PCR	polymerase chain reaction
Spec	spectinomycin
Strep	streptomycin
TY	Tryptone-Yeast
Wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl galactose
X-glcA	5-bromo-4-chloro-3-indolyl glucuronide

CHAPTER 1

Introduction

The rhizobia

Rhizobia, or "root nodule bacteria", refer to a bacterial group that has nitrogen-fixing capabilities along with the ability to form nodules on most leguminous plants. Rhizobia are bacilli-shaped and range in sizes of 0.5 –0.9 µm x 1.2-3.0 µm. (Holt, 1994). These Gram negative rods do not form endospores and are motile via a single polar flagellum or two to six peritrichous flagella (Sadowsky, 1998). While they grow well in the presence of oxygen, rhizobia are also quite resilient in microaerophilic conditions at oxygen concentrations of less than 0.01 atm (Sadowsky, 1998). Optimal growth occurs at a pH of 6.0 – 7.0 and at a temperature between 25- 30ºC, with the maximum temperature being 35º C (Holt, 1994). Being chemoorganotrophs, they are able to use a variety of carbohydrates as carbon sources (Holt, 1994), making culture techniques relatively simple.

Ecologically, rhizobia reside in the soil as facultative microsymbionts (Somasegaran, 1994). When not in a symbiotic relationship with a host plant, they add to the diversity of the bacterial soil population. Outside of the nodule, rhizobia are most likely located on the rhizoplane (root surface), rhizosphere (soil surrounding the root surfaces) or even soil outside the vicinity of the root (Somasegaran, 1994.). The numbers and location of rhizobia in the soil are determined by a myriad of factors. Somasegaran (1994) determined the condition of the soil measured by pH, salinity, moisture content and temperature may lead to increases or decreases in rhizobial numbers. In addition, the presence of the host plant is significant. Rhizobia respond to

signals excreted by the host plant that will facilitate an increase in rhizobial numbers at the rhizosphere level. This response to plant signals is a crucial element in the development of symbiosis.

This symbiosis between the rhizobia and host plants is a distinguishing factor of this group. Within a symbiotic relationship, rhizobia are able to carry out the reaction of atmospheric N_2 to ammonia, that is essential for life on earth. With a few exceptions (Keister, 1975; McComb, 1975; Pagametal, 1975), this reaction is not known to occur with rhizobia in the free-living form (Somasegaran, 1994), making this rhizobia-host interaction of great significance.

The discovery of nitrogen-fixing symbioses

Although nitrogen fixation is an essential aspect of life, the symbioses responsible for it were not known until the 1500's. In the middle 1500's, various laboratories in Europe made discoveries that sparked interest in the field. During this time, Leonhard Fuchious published drawings of the first nodulated legumes (Perret, 2000). It was not until two centuries later that Woronin noted small bodies resembling bacteria inside the nodules (Perret, 2000). Frank supported this idea when he found that the nodulation of *Pisum sativum* was prevented by sterilizing the soil (Perret, 2000). However, Beyerinck provided the first proof that bacteria are responsible for nodules (Beyerinck, 1888). He prepared pure cultures localized in *V. faba* nodules and infected sterile *V. faba* with those cultures (Perret, 2000). Nodules were produced. It was a novel discovery in that bacteria were responsible for these novel organs. It undoubtedly created a wave of interest and shifted research attention towards elucidating the basis of this symbiotic relationship.

The rhizobia-legume symbiosis: general

Although rhizobia may participate in non-legume symbiosis with *Parasponia* (Trinick, 1973), the remainder of this work will simply refer to the rhizobia-legume symbiosis. The legume family Fabaceae contains 674 genera with an estimated 16,000 to 19,000 species (Allen and Allen, 1980). The Leguminosae are distributed worldwide and rank second in economic significance only to the grass family, the Poaceae (Spaink, 1998). As previously mentioned, members of the legume family may form nodules when associatied with members of the rhizobia. This unique characteristic of nodulation and nitrogen fixing symbioses is a result of an infection of the legume host with rhizobia. Figure 1.1 below schematically depicts this complex progression of interactions termed the infection process. The process of nodulation is initiated by a signal from the host plant that causes rhizobia to colonize the root surface (Hadri, 1998). The root hair responds to the presence of rhizobia by curling. Curling induces invagination of the root hair membrane containing the invading rhizobia. This structure is known as the infection thread (Hadri, 1998). The infection thread penetrates the host plant outer cell layers and spreads intercellularly through the root cortex. Bacterial cell division rapidly takes place within the infection thread and penetration continues until the nodule primordium is reached. The root hair membrane, of the infection thread surrounding the rhizobia, begins to bud off releasing vesicles containing rhizobia into the nodule cells. The plant cell membrane surrounding the rhizobia is termed the peribacteroid memebrane (Hadri, 1998). The rhizobia within the peribacteroid membrane differentiate into nitrogen-fixing bacteroids. Together, the bacteroid and peribacteroid comprise the peribacteroid membrane unit or symbiosome. As the bacteroids divide, the symbiosome does so concomitantly. The bacteroids develop the capacity of biological nitrogen fixation (Hadri, 1998).

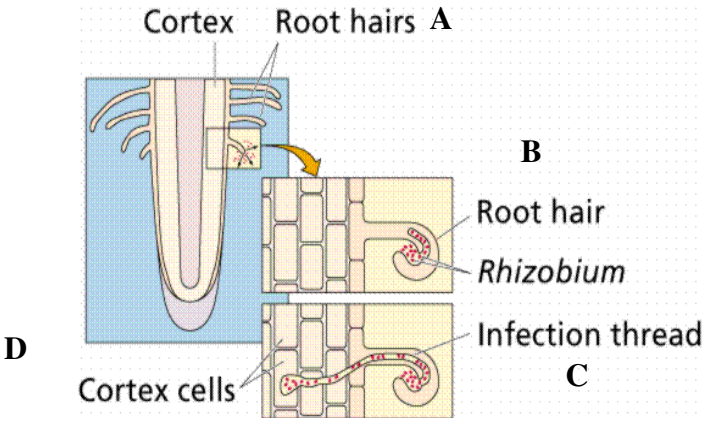

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Figure 1.1: Diagram of infection process: (A) Rhizobia attach to the root hair of a legume. (B) Rhizobia induce root hair curling. (C) Rhizobia begin to traverse into the cortex of the plant thru the infection thread where they rapidly multiply. (D) Rhizobia bud off from the end of the infection thread which is surrounded by host cell membrane and organogenesis of the nodule occurs.

Biological nitrogen fixation is possible via the enzyme nitrogenase. Nitrogenase is synthesized in the cytosol of the bacteroids (Scultze, 1998). The triple bond of molecular N_2 is extremely stable and requires a tremendous amount of reducing power (Schultze, 1998). Molecular N_2 is the natural substrate for nitrogenase and, in the presence of ATP, the triple bond strength can be overcome and N_2 is reduced to ammonia as shown in figure 1.2 (Schultze, 1998). Ammonia can then be used by the plant to convert precursor metabolites, such as α-ketoglutarate and phosphoenolpyruvate, into amino acids (Schultze, 1998). Animals may then ingest plant materials, and essential amino acids are used to synthesize proteins.

$$\mathbf{N_2 + 6e^- + 12\ ATP + 12\ H_2O \rightarrow 2NH_4^+ + 12\ ADP + 12\ Pi + 4\ H^+}$$

Figure 1.2: Nitrogenase reaction: reduction of dinitrogen (N_2) to ammonia (NH_4^+).

The capacity to induce the formation of nodules on most leguminous plants and fix atmospheric nitrogen into ammonia is a distinguishing trait of the rhizobia. It is this trait alone that has determined their classification into this separate group. Despite this commonality, the rhizobia are a highly heterogonous bacterial group.

The classification of the rhizobia

As previously mentioned, rhizobia are classified together for their ability to nodulate members of the Leguminosae. Rhizobia are members of the α-subdivision of Proteobacteria with the exception of *Burkolderia* and *Ralstonia* in the β-Proteobacteria subdivision (Moulin, 2001). Within the α-Proteobacteria subdivision, there are five general genera of rhizobia: *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium,* and *Mesorhizobium* (Weidner, 2003). These genera have been traditionally classified according to host specificity. The cross-inoculation groups formed the basis of species determination (Van Berkem, 1998). Cross-inoculation groups consist of plants mutually susceptible to nodulation by a particular kind of rhizobia (Burton, 1979). This method proved to be unreliable by inconsistencies with the rhizobia-legume interactions that were unexplainable. By 1944, speciation based on cross-inoculation was discontinued and the focus shifted to speed of growth, acid production ,serology, DNA base ratios, numerical taxonomy, DNA hybridization, and phage susceptibility (Burton, 1979). The analysis of genetic relatedness has undoubtedly created a more reliable mode of classification of the rhizobia.

The general genetics of rhizobia

Various methods to analyze genetic relatedness have been applied to the rhizobia. These include mutilocus enzyme electrophoresis, analysis of fingerprint patterns generated with the

polymerase chain reaction, analysis of fingerprint patterns generated by DNA restriction digestion, DNA-DNA reassociation and sequencing of the 16S rRNA and 23S rRNA genes (Van Berkem, 1998). Using these methods, several species have been extensively analyzed. These include *Sinorhizobium meliloti, Rhizobium leguminosarum, Bradyrhizobium japonicum*, and *Mesorhizobium loti* (Weidner, 2003). The genetics of these species will be reviewed below.

The chromosome

The chromosome has widely been accepted as the keeper of housekeeping genes in rhizobia (Brom, 2000). However, this view may be disputable. For example, *B. japonicum* lacks plasmids and consists of a single chromosome (9.1 Mb) (Puhler, 2004). After construction of a genetic map of the *B. japonicum* genome (Kundig, 1993), it was determined that genes required for symbiosis were clustered on a 410-kb symbiotic region of the chromosome (Gottfert, 2001). *Mesorhizobium loti* MAFF303099 contains one chromosome and two plasmids (Puhler, 2004). It has a 502-kb chromosomal element, known as the symbiosis island, that is required fo symbiosis. This integrated element is mobile and may convert nonsymbiotic mesorhizobia in the environment into symbionts, allowing it to be called a mobile symbiosis island (Sullivan, 2002). In contrast to the afore mentioned rhizobia, the genome of *Sinorhizobium loti* consists of one chromosome and two megaplasmids, pSymA and pSymB (Puhler, 2003). The chromosome and pSymB share genetic relatedness and *both* harbor genes essential for symbiosis (Puhler, 2003).

It is apparent that genome organization varies greatly among the various rhizobial species. Although, some of the just mentioned organisms represent exceptions, most of the bacterial genes required for symbiosis are normally present on one of the large symbiotic plasmids, or pSyms (Brom, 2000).

The plasmids

Rhizobia are noted for carrying a large amount of genetic information extrachromosomally. *R. melilot*iy contains some of the largest known bacterial plasmids termed megaplasmids (1200-1500 kb) (Campbell, 1993). Plasmid number varies from 2-10 (Garcia de los Santos, 1996) and may contribute to up to 40% of the total genome (Brom, 2000). These large plasmids carry many of the genes involved in symbiosis in most rhizboial species (Garcia de los Santos, 1996). For this reason, much of the research has concentrated on the genetics of the pSyms. Consequently, a pioneering effort of rhizobial genomics occurred with the sequencing of the symbiotic plasmid of *Rhizobium* sp. NGR234 (Freiberg, 1997). Sequencing revealed a 536-kb plasmid pNGR234a encoding most of the genes required for nitrogen fixation (Weidner, 2003).

Genes present on both the chromosome (Forrai, 1983; Noel 1984; Priefer, 1989) and extrachromosomal elements other than the pSym (Hynes, 1986; Hynes, 1989; Hynes, 1990) have been shown to contribute to the symbiotic process. Brom *et al.*, in 1992, showed that plasmids other than the pSym of *R. leguminosarum* bv. phaseoli are required for optimal symbiotic performance. Their results indicated a global participation of the rhizobial genome in symbiotic and free-living conditions (Brom, 1992).

The genetics of symbiosis

Symbiosis is a conversation between rhizobia and the host legume. A complex interaction of factors are required for symbiosis to be successful. The signals essential to this relationship are depicted in Figure 1.3. In response to nitrogen limiting conditions, the host plant

elicits compounds known as flavonoids. Flavonoids, such as naringinin and hesperitin, interact with the transcriptional regulator NodD in rhizobia, inducing a conformational change. NodD is able to bind to a conserved sequence upstream of the *nod* genes known as the nod box. Expression of the bacterial *nod* genes is then activated to produce Nod factors in the form of lipochitinooligosaccharides (LCOs) (Hadri, 1998). Nod factors interact with the host plant and the process of nodulation is initiated (Hadri, 1998). Of course, both plant and bacterial genes play crucial roles. However, for the purposes of this project, only the bacterial genes will be discussed. The best characterized genes involved in rhizobia-legume symbiosis are the *nod* genes, *nif/fix* genes, and *lps* genes. They will be briefly discussed.

The nod genes

The *nod* genes are key players in the initiation of the nodulation process. They are divided into three classes, common, host-specific and NodD (Schultze, 1998). Common *nod* genes (*nodABC*) are found in all rhizobial species and the products of these genes have similar interchangeable functions (Horvathetal, 1986). NodA, B and C are all involved in Nod factor biosynthesis, which is necessary for nodulation to occur. NodB and NodA work sequentially as a de-N-acetylase and acyl transferase, respectively, during the attachment of an N-linked fatty acyl group to the terminal glucosamine residue (Horvathetal, 1986). NodC is a processive glycosyl transferase and determines the chain length of the Nod factor produced (Horvathetal, 1986). Mutations in *nodABC* interfere with nodulation (Nod^-). The host-specific *nod* genes determine specificity for a legume host. For example, the *nodFE* genes encode enzymes involved in fatty acid synthesis and are required to produce the polyunsaturated acyl groups in the Nod factors of *R. leguminosarum bv. viciae* and *trifolii* and *S. meliloti* (Spaink, 1998).

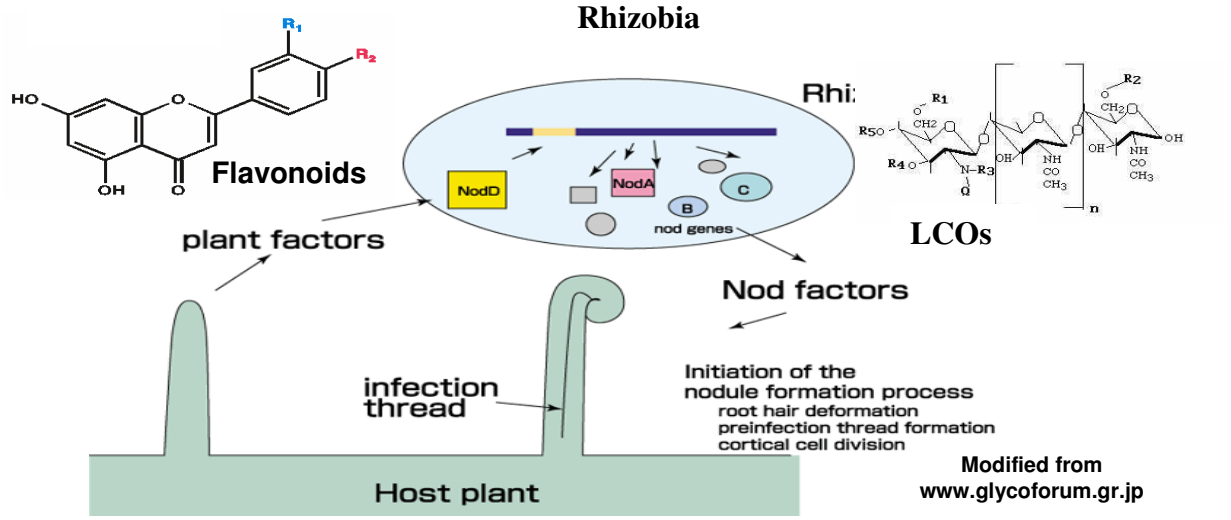

Figure 1.3 : Interaction between plant signal and bacterial genes that lead to nodulation. Plant factors, such as flavonoids interact with NodD in the rhizobia. NodD is able activate transcription of the *nod* genes. The gene products of the *nod* genes encode Nod factors,proteins that synthesize LCOs. Nod factors are able to interact back with the plant to initiate the process of nodulation.

Upon mutation of host-specific *nod* genes, nodulation is delayed, reduced, or host range alterations may occur (Kondorosi, 1984). Since host-specific *nod* genes are a factor in determining host-specificity, they are not interchangeable among different rhizobial species. The regulatory genes (*nodD, nodV, nodW, nolA, nolD, nolR and syrM)* are involved in the regulation of other *nod* genes (Spaink, 1998). For example, NodD is a LysR-*trans* activator protein that positively regulates expression of the *nod* genes (Henikoff, 1988). Upon a conformational change in response to plant-derived flavonoids (Figure 1.4), NodD can bind to the nod box to activate transcription of the *nod* genes. This activity results in the synthesis of the Nod factor that leads to nodulation.

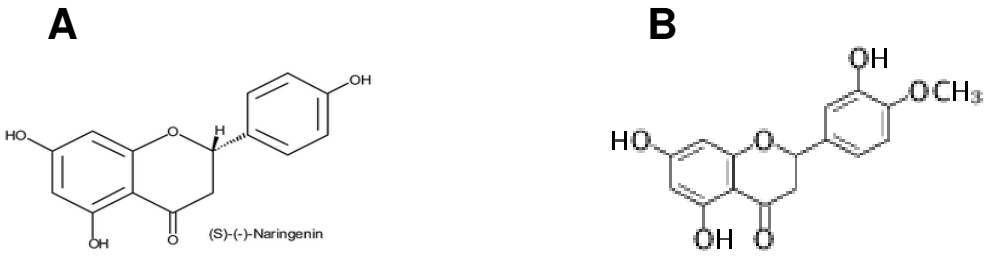

Figure 1.4: Structure of common flavonoids. The R1 and R2 positions of the flavone (as shown in Figure) may contain substiutents of the following groups; –H,–OH, -OCH3. The structures of (A) hesperitin and (B) naringenin contain common flavone compound with distinct substituents at the R1 and R2 positions.

Rhizobial species including, *R. leguminosarum* bvs. viviae and trifolii, *S. meliloti, R. etli, R. galegae, R.* tropici, and *R.* sp NGR234, contain the *nod* genes on a pSym (Hadri, 1998). In contrast, *B. japonicum, Azorhizobium caulinodans, S. fredii,* and *M. loti* harbor the nodulation genes on the chromosome (Hadri, 1998). The *nod ABCDIJ* genes are present in all rhizobia while others may be found in only a single species or strain (Hadri, 1998). In addition, certain *nod* genes are only present as single copies while others may have homologous sequences elsewhere in the genome (Hadri, 1998). For example, SyrM for symbiotic regulator, is a NodD homologue involved in enhancing exopolysaccharide synthesis and *nod* gene regulation (Mulligan and Long, 1989). In addition, a two-component regulatory system exists in *B. japonicum* that includes the flavonoid sensor NodV and the regulator NodW (Schultze, 1998).

The nif and fix genes

The genes that encode the subunits and assembly for the enzyme nitrogenase are known as the *nif* (nitrogen fixation) genes. The identification of the 20 *nif* genes in *Klebsiella pneumoniae* (Hadri, 1998) made way for their discovery in rhizobia. Ruvkum *et al.* (1982) was

able to use reverse genetics to discover a similar cluster in rhizobia essential for symbiosis. The *nif* genes were comprised in this cluster along with an unknown cluster of *fix* genes (Hadri, 1998). Functions for all of the *fix* genes have not yet been demonstrated. However, some resemble bacterial ferredoxins which may function in electron transfer to nitrogenase (Freiberg, 1997).

The lps genes

Lipopolysaccharides (LPS) are major components of the Gram-type negative cell wall and play an important role in bacterial-host interactions. The *lps* genes encode the biosynthesis of LPS which are involved in symbiosis. During the nodulation process, rhizobia must avoid plant defense mechanisms (Kannenberg, 1998). Although LPS has not been directly related to symbiotic development, possible roles have been proposed during the different stages. For instance, *Bradyrhizobium* LPS mutants fail to nodulate (Kannenberg, 1998) and *S. meliloti* LPS mutants induced a reduced number of infection events (Lagares, 1992). In additon, *R.* etli CFN42 LPS biosynthesis mutants have shown to be nonmotile, lack O-antigen and induce the formation of incompletely developed nodules (Cava, 1989). Various other findings provide indirect evidence that LPS are involved in symbiosis. However, the precise functions remain unclear.

Nevertheless, three separate genetic regions have been identified in *R. etli* that are involved in LPS biosynthesis. Regions α and γ are associated with the chromosome, while β is located on a non-symbiotic plasmid pb (Brom, 1992). In 1997, Garcia de los Santos *et al.* characterized the β region on plasmid pb and reported the sequence of two open reading frames,

lpsβ1 and lpsβ2, that are indispensable for LPS biosynthesis and symbiosis (Garcia de los Santos, 1997).

The diversity of rhizobia genetics

Despite the common genes involved in symbiosis, the genetic organization across the rhizobia varies greatly. Plasmids or megaplasmids may or may not be present. Symbiotic genes may be located on the chromosome, extrachromosomally, or on a mobile symbiosis island. A comparison of plasmids and genomes exhibit a lack of synteny and colinearity (Brom, 1996). This diversity is surprising within a single bacterial group. Of *M. loti,* 35% of all predicted proteins have no orthologues in *S. meliloti* (Weidner, 2003). Also, genes, located on the *S. meliloti* megaplasmid can be found dispersed within the chromosome of *M. loti* (Weidner, 2003). Additionally, the symbiosis island of *M. loti* and the symbiotic plasmid pNGR234a share genes that have no orthologues in *S. meliloti* (Weidner, 2003). There are only 20 symbiotic genes shared by all symbiotic genome compartments (SGCs) (Brom, 1996). The genes involved in nitrogen fixation (*nif* and *fix* genes) and nodulation (*nod* genes) are included (Brom , 1996). The randomness of organization is exhibited in the arrangements of the SGCs. For example, *R. etli fix* genes are distributed on two replicons p42a and p42d, reiterated three times in pSymA of *S. meliloti,* and present in two copies of operons in *M. loti* (Brom, 1996). In *B. japonicum,* these genes lie outside of the SGC, and are chromosomal in *R.* sp. NGR234 (Brom, 1996). Although *nod* and *nif/fix* genes are key determinants in symbiosis, it is probable that other loci influence the efficiency of interaction.

Genetic techniques

Several strategies have been used in the elucidaton of rhizobial genes involved in symbiosis. Central in this work is the use of broad host range mobilizable cosmid vectors and transposon mutagenesis (Hynes and Finan, 1998).

Plasmid pRK290 (20-kb) was one of the first vectors used as part of a cloning system for *Rhizobium.* Ditta *et al.* (1980), constructed a gene bank of *R. meliloti* using pRK290. This proved to be an efficient cloning vector because of two single cloning sites, conference of tetracycline resistance, and high frequency mobilization using a helper plasmid (Ditta, 1980). A gene bank was successfully constructed of the total cellular DNA of *R. meliloti* 102F34 as a collection of 15-20 kb *Bgl* II restriction enzyme fragments. A new vector, pLAFR1 (21.6-kb) (Friedman, 1982), was derived from pRK290 and used to construct a gene bank of *R. meliloti* 1021 with a mean insert size of 23.1 kb. This procedure was used to directly clone genes involved in nodulation by conjugation of the clone bank into *R. meliloti* Nod – mutants and selecting for transconjugants that regained the ability to nodulate plants (Long, 1982). Labes *et al.* (1990) were successful in the construction of lac fusion vectors for the study of gene function and expression in gram negative bacteria. These vectors were tested successfully in *R. meliloti* and *R. leguminosarum* (Labes, 1990). Continuing the use of cloning vectors, Oke *et al.* (1999) cloned 1-2kb genomic fragments into a vector. The vector was then integrated into the *R. meliloti* chromosome. The aim was to observe genes expressed at a specific time within the nodule. Within the nodule, 230 fusions were identified. Analysis of 23 revealed 3 genes already known to be involved in symbiosis, 6 with homology to proteins that had not been identified

with symbiosis and 14 with no significant similarity to proteins with known functions (Oke, 1999).

Transposon mutagenesis has proven to be an efficient method for obtaining mutants in *Rhizobium*. For symbiotic studies, it is pertinent to obtain stable, non-reverting mutations that can be easily traced (Hynes and Finan, 1998). These parameters are met with transposon mutagenesis. Several types of transposons have been employed for the generation of mutants. These include Tn*5* (Beringer, 1978), Tn*7* (Hernalsteens, 1978), Tn*1* (Casadesus, 1980), Tn*904* (Klapwijk, 1980), Tn*1831* (Pees, 1986), and Tn*10* (Hynes, 1986). Although all are functional, limitations make some less useful than others such as non-randomness of insertion, creation of deletions and unsuitable antibiotic resistance markers (Hynes and Finan, 1998). Because of seemingly random transposition in all hosts, provision of non-reverting mutants, and neomycin, streptomycin and bleomycin antiobiotic resistance markers, Tn5 is the most widely and successfully used transposon (Hynes and Finan, 1998).

The first recorded use of transposons in *Rhizobium* occurred in 1978 when Beringer reported a conveyance of Tn5-encoded drug resistance to *Rhizobium* (Beringer, 1978). The early eighties began with a sparked interest in using *Tn5* to detect rhizobial symbiotic genes. Forrai *et al*. (1983) reported symbiosis specific mutations. Five of the mutations were mapped to distinct chromosomal sites in *R. meliloti* and affected nodule development to some degree (Forrai, 1983). However, the results were not overtly described. In an attempt to not undertake a biased study in favor of the symbiotic plasmid, Noel *et al.* (1984) sought mutations in the entire genome of *R. phaseoli.* This article clearly noted the possible importance of chromosomal genes. Over one-half of the mutations obtained through Tn5-mutagenesis were chromosomal and elicited some affect on nodule development (Noel, 1984). Both studies of Forrai *et al*. and Noel *et al.*

attempted to step outside the trend of focusing on the symbiotic plasmids for elucidation of symbiotic genes. Both studies were successful in isolating chromosomal mutations that had a limiting effect on nodule development. The phenotypic results of *in planta* experiments were explicity described (Forrai, 1983; Noel, 1984) along with protein analysis of the mutants (Noel, 1984). Nevertheless, genetic analysis of the mutants was not employed most probably due to the technical limitations at the time.

With the advent of numerous genetic tools to analyze data, efficient constructs to accurately detect gene function and expression are a necessity. A bi-functional green fluorescent protein (GFP) and *gusA* gene Tn5 was developed (Xi, 1999). This construct allowed the efficient monitoring of gene expression and localization of *R. etli in planta.* In 2001, Xi *et al.* employed this bi-functional Tn5 in wild-type *R. etli* to identify genes required for symbiosis. Expression levels of different genes under imitated symbiotic conditions were determined by induction of the *gusA* gene. Wild-type *R. etli* was grown aerobically or microaerobically in the presence or absence of nodule extract (Xi, 2001). Partial sequence analysis revealed mutations in known nodulation genes, known nitrogen fixation genes, and others with homology to known proteins. Of the mutations, 50% were genes of already known function and involvement in symbiosis.

Rhizobium etli

Rhizobium etli is a species originally identified as a group within *R. leguminosarum* bv. phaseoli. The question of whether *R. leguminosarum* bv. phaseoli was a true taxonomic unit first arose in 1988 when Pinero *et al.* undertook the task of analyzing the genetic diversity among *R. leguminosarum* bv. phaseoli isolates. Fifty-one isolates were analyzed based on electrophoretic

mobilities of 15 metabolic enzymes and 46 distinctive multilocus genotypes were distinguished (Pinero, 1988). The mean diversity among the electrophoretic types was 0.691 and was the highest value yet recorded for any bacterial species (Pinero, 1988). Using 2-D polyacrylimide gel electrophoresis pattern of proteins, similar conclusions were attained (Robert, 1980). Also, more than 50 native Mexican strains had distinct genomic fingerprints and plasmid profiles (Flores, 1988). Pinero *et al.* proposed a need for reclassification among *R. leguminosarum* bv. phaseoli strains on the basis of chromosomal variation rather than phenotypic characteristics or plasmid-bourne host specificity.

By 1991, *R. leguminosarum* bv. phaseoli was classified into two groups, types I and II (Vasquez, 1991). Type I groups contained reiterated *nifHDK* repeats and a narrow host range. Type II groups contained *nifHDK* genes in single copies and a broad host range (Vasquez, 1991). Vasquez *et al.* found that Type I groups shared a unique organization of *nod* genes in that *nodA* was separated from *nodBC* by 20-kb. Type I strains eventually were characterized further and came to be known as *Rhizobium etli* (Segovia, 1993). Sequence analysis of the 16S rRNA gene revealed a significant difference from *R. leguminosarum* (Segovia, 1993). Also, *R. etli* strains were shown to have identical restriction pattern analysis (Segovia. 1993).

Rhizobium etli was established as a new species in 1993. This species shared phenotypic and growth requirements of the other rhizobia. All *R. etli* strains are naladixic acid resistant (Segovia, 1993). At the molecular level, they are distinguished from other species by DNA hybridization tests, multilocus enzyme eletrophoretic profiles and ribosomal gene sequences previously mentioned. Other distinctions include the presence of nitrogenase reductase gene reiterations and the separation of the *nodA* and *nodBC* genes (Segovia, 1993). *R. etli* strains nodulate and confer nitrogen fixation on *Phaseolis vulgaris,* exclusively.

***Rhizobium etli* genetics and current *Rhizobium* sequencing projects**

The *R. etli* genome consists of one chromosome and six plasmids (p42a-f). Gonzalez *et al* (2003) presented the complete sequence of *R. etli* CFN42 symbiotic plasmid, p42d (Figure 1.5). Sequencing revealed 371,255 bps with 359 coding sequences. The *nod* and *nif* genes were found clustered in 125 kb (Gonzalez, 2003). As a distinguishing characteristic of *R. etli* previously mentioned, *nodA* is 2kb away from *nodBC*. Sequencing also revealed the presence of three *nodD* genes (Gonzalez, 2003).

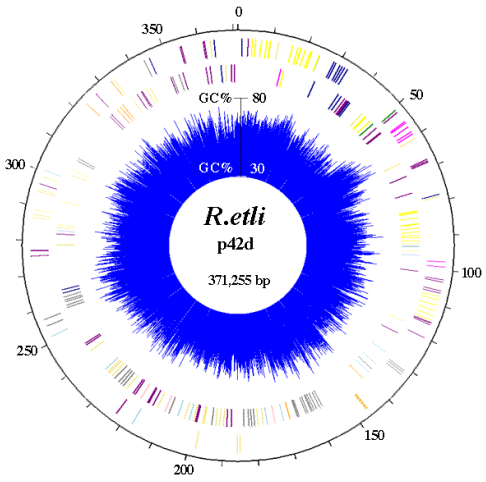

Figure 1.5: The Symbiotic Plasmid (p42d) of *R. etli* CFN42. The sequence and annotation were released by Gonzalez *et al* in 2003 (GenBank accession U80928). As the largest continuous sequence to ever be accomplished in Mexico, it contains 371,255 bps and 359 predicted and known genes. Image obtained from www.kazusa.or.jp/rhizobase/.

In general, rhizobia are known to contain a large amount of reiterated DNA sequences in plasmids and chromosomes. Complete operons, genes, regulatory sequences and insertion sequence elements can be found in these repeats. Mavingui *et al.* (1998) showed that repeated sequences can participate in recombination events that can lead to genomic rearrangments. An

amplicon is one of these repeats that may be amplified. The first amplicon was found in *R. etli* CFN42 and consisted of a 120-kb region bordered by *nifHDK* repeats (Mavingui, 1998).

R. etli genomics is following the footsteps of its predecessors. Presently, there are complete sequences available for three rhizobial species as seen in Table 1.1. The first to be completely sequenced was *Mesorhizobium loti* MAFF303099 disclosing one chromosome (7036-kb) and two plasmids (352 and 208-kb) (Kaneko, 2002). Following was the sequence of *Sinorhizobium meliloti* 1021 consisting of a single chromosome (3654-kb) and two megaplasmids, pSymA (1354-kb) and pSymB (1683-kb) (Galibert *et al.,* 2000; Capela *et al.,* 2001; Barnett *et al.,* 2001; and Finan *et al.,* 2001). The sequencing of *Bradyrhizbobium japonicum* uncovered a surprisingly large single chromosome (9105-kb) (Kaneko, 2002). Thirty-four percent of *B. japonicum* genes share significant sequence similarity to *M. loti* and *S. meliloti.* However, eighteen percent present no similarity (Kaneko, 2002).

Current information for sequencing progress of the *R. etli* genome is described and may be viewed in Table 1.2 (http://kinich.cifn.unam.mx/~retligen/). The approach is a combination of shotgun and BAC mapping. Two shot-gun libraries have been constructed

Table 1.1. Rhizobia with complete sequences available.

Organism	Genome Size (kb)	Genome Structure
Bradyrhizobium japonicum USDA110	9105	Chromosome (9105 kb)
Mesorhizobium loti MAFF303099	7596	Chromosome (7036 kb) Plamid pMLa (351 kb) Plasmid pMLb (208 kb)
Sinorhizobium meliloti 1021	6690	Chromosome (3654 kb) Megaplasmid pSymA (1354 kb) Megaplasmid pSymB (1683 kb)

from the wild-type strain *R. etli* CFN42 and a derivative strain DE42 (lacking the majority of the plasmids). In addition, a BAC library has allowed the identification of clones containing all plasmid sequences and 384 BAC clones exclusive to the chromosome. In addition, the sequences of the plasmids p42a-d have been determined. From the shotgun libraries, 3x coverage of the chromosome has been achieved. Despite active progress on the sequencing of the *R. etli* genome, the results are not readily available to the public.

Significance

Plants are undoubtedly essential to life on earth. They are responsible for converting solar energy to chemical energy that are used as food, fuel, feed and fiver (Gresshoff, 2003). This is only possible if nitrogen is available and nitrogen is the major limiting factor for plants (Vance, 1998). The availability of high-quality, protein-rich food positively correlates with the availability of nitrogen. The earth's population is expected to reach 10 billion by the year 2035 (Stacey, 1992). To maintain healthy levels of protein intake, the increase in crop production is crucial. The role of rhizobia in this process is critical. Rhizobia allow legumes to provide 25-35% of the world's protein intake (Vance, 1998). Worldwide, 250 million hectares of legumes are grown and they are responsible for the fixation of 90 tons of nitrogen per year (Stacey, 1992). The cost to replace nitrogen fixed by legumes with ammonia produced by the Haber-Bosch process would be extreme which increases the need for biological methods (Vance, 1998).

Agronomic significance of the *Rhizobium*-legume symbiosis is obvious. However, there are other uses for this relationship that will produce definite environmental benefits. Legumes have been shown to facilitate the degradation of pesticides and other soil contaminants, known as phytoremediation (Vance, 1998). In addition, legumes such as alfalfa, may be grown for generation of electrical energy. After separation into stems and leaves, stems may be gasified

and the leaves used for animal feed. Gasification of this biomass, can increase the amount of electricity by 50% (Vance, 1998).

Table 1.2: Overview of current progress in the *R. etli* CFN42 genome project.
Table information adapted from http://kinich.cifn.unam.mx/~retligen/

Replicon	**Size (bp)**	**Status**	**Genes and Functions/Commentary**
Plasmid p42a	194,541	Complete	Transporter system type IV, conjugation
Plasmid42b	184,240	Complete	Thiamine, lipopolysaccharide synthesis
Plasmid p42c	250,367	Complete	Transport systems ABC
Plasmid p42d	371,257	Complete	Symbiosis (nodulation and nitrogen fixation)
Plasmid p42e	~ 450,000	In process	Physical map complete
Plasmid p42f	~550,000	In process	Physical map complete
B1-chromosomal fragment	164,782	Finished	Diverse cellular functions

In addition to agronomic and economic importance, the *Rhizobium*-legume symbiosis is also a model system to observe the modes of which a Gram-negative bacterium infects and survives within a eukaryotic cell. *Rhizobium* is known to be phylogenically related to the intercellular human pathogen *Brucella* (Kannenberg, 1998) and plant pathogen *Agrobacterium.* The elucidation of the processes of the *Rhizobium*-legume relationship may provide insight into the infection process of *Brucella.* A number of events are of significance. These include: 1) cell-cell recognition between prokaryotic and eukaryotic cell, 2)regulation of plant defense mechanism for successful symbiotic infection, 3) bacterial and plant differentiation processes (Carlson, 2001).

Success in creating a highly efficient biological manipulation of nitrogen will be of excellent advantage both agriculturally and environmentally. Elucidation of the molecular basis of symbiosis will be insightful to pathogenesis and has definite health implications.

Specific Aims

Before the complete nucleotide sequence of *S. meliloti* became available, a cellular proteome reference map was established by a comparison between cells in early and late exponential phases (Guerreiro, 1999). Chen *et al.* (2000) undertook a proteome analysis of a pSymA cured strain of *S. meliloti.* This was particularly interesting since the vast majority of genes essential in symbiosis are located on this megaplasmid. The finding revealed that over 250 proteins were induced or up-regulated in the nodule compared to the root (Chen, 2000). The proteins were transcripts of genes not located on the pSymA. Garcia-de los Santos *et al.* (1996) proposed that rhizobial plasmids play a functional role in survival and replication. This refutes the long-held idea that plasmids are for the purpose of conferring additional traits that enhance the ability of bacteria to colonize or compete in certain environments. It is likely that the *Rhizobium* genome is a complex integration of both chromosome and plasmids that *must* function together for optimal survival and for optimal nodulation abilities. The role of the chromosome in this integration would seem to be one of necessity. Focusing on the *Rhizobium etli* pSym for the elucidation of symbiotic genes has left the *Rhizobium etli* chromosome open for speculation regarding the identification of genes required for symbiosis. Although specific chromosomal mutants have been isolated in *R. meliloti* (Forrai, 1983) and *R. phaseoli* (Noel, 1984), the genetic analyses were extremely limited. Mutants have also been successfully generated in *Rhizobium etli* throughout the entire genome. Results reveal redundancy in genes already known to be involved in symbiosis (Xi, 2001). In no case is there, however, a documented study focused solely on the chromosomal of *R. etli* that are induced or repressed during symbiosis.

Identification of genes in the chromosome that exhibit an increased expression when subjected to simulated symbiotic conditions (i.e. pH5 or flavonoids) would be significant. First, the use of a strain lacking the pSym would avoid the need to re-characterize genes that are already known to be involved in symbiosis. Secondly, there are aspects of the pSym that are believed to be essential for certain events in the infection process. For example, flavonoids are considered to only interact with NodD and affect transcription of the *nod* genes. Both of these components (NodD and *nod* genes) are located on the pSym. An increased gene expression due to flavonoid induction in a pSym- strain would raise questions about another mode of flavonoid sensing. Lastly, mutations in the chromosomal genes that exhibit increased expression when subjected to simulated symbiotic conditions would aid in identifying other genes (i.e. other than pSym genes) that are required for symbiosis.

The aim of this work was to observe gene expression solely in the chromosome of *Rhizobium etli* CE3. *Rhizobium etli* CE144 was used for this work since it is a derivative of the wild-type *R. etli* that lacks the symbiotic plasmid. Two methods were employed: conventional vector cloning, and transposon mutagenesis, of which the latter yielded more promising results.

The first method, Approach 1, involved cloning 1-kb fragments from *R. etli* CE144 into a large vector pRG970 (24.6-kb). Fragments were cloned into a unique *Bam*HI site bordered by the *lacZ* and *gusA* genes. The vector pRG970/insert was introduced back into wild type *R. etli* CE3. Due to the bordering reporter genes, promoter expression or lack of expression could be detected qualitatively by observance of a blue or white color on X-Gal or X-GlcA agar, respectively. Although with some success, this method contained huge drawbacks such as low efficiency, non-reproducibility, and instability. The drawbacks lead us to use another method that seemed more promising; transposon mutagenesis.

The second method, Approach 2, involved mutagenizing the *R. etli* CE144 genome with *mTn5gusAoriV* containing a promoterless *gusA* gene. Approximately, 10,400 mutants were picked from selective media and stored in -80ºC for future screening. Mutants were subjected to conditions mimicking aspects of symbiosis such as growth in pH5 media or in the presence of flavonoids. Gene expression was quantitatively assessed via the β-glucuronidase assay. Mutants exhibiting 2-fold or greater gene induction upon shifting to pH 5 in three independent experiments were selected for further analysis. A unique PCR method was used to verify the presence of the transposon and generate PCR products for sequencing of flanking transposon sequences. Four mutants were identified and selected for sequencing and partial sequence analysis.

Specific aims were the same for both approaches. They involved the generation of a bank of *R. etli* CE144 fragments or mutants in which gene expression may be observed or assayed via the presence of the *gus*A genes. The fragments/mutants were subjected to simulated symbiotic conditions such as low pH or flavonoids, and *gusA* activity observed. Mutants were finally selected that exhibited induction under such conditions and sequences were analyzed.

CHAPTER 2

Approach 1: Conventional cloning method

2.1 Method description

2.1.1 Vector pRG970

As mentioned in the previous chapter, research on the genetics of symbiosis has been highly concentrated on the pSyms. Our group aimed to focus on the non-pSym genes that are induced and may play a significant role in symbiosis. In order to do so, we chose to use *R. etli* CE144 (obtained as a gift from Dale Noel) which is a $pSym^-$ derivative of *R. etli* CE3. The distinguishing factor in *R. etli* CE3 as compared to wild-type *R. etli* CFN42 is the presence of streptomycin resistance. Therefore, *R. etli* CE144 confered streptomycin resistance.

The vector of choice was pRG970 (24.6-kb), (obtained as a gift from Marcelle Holsters at the University of Gent, Belgium), Figure 2.1. It was believed to be suitable for the selection of promoters inducible by simulated symbiotic conditions, which was the goal of this project. Vector pRG970 carries both *lacZ* and *gusA* reporter genes. A TAA stop codon immediately precedes the ATG initiation codon of *lacZ.* Therefore, only transcriptional *lacZ* fusions can be obtained. The Shine-Delgarno sequence GGAGG is located from position -10 to -6 of the *lacZ* initiation codon. A fragment cloned into the unique *BamHI* or *SmaI* sites would be flanked by the 5' end of the *lacZ* on one side and the 5' end of the *gusA* on the other as shown in Figure 2.1.

To test pRG970 as a promoter probe vector, the authors introduced a *nodA* promoter fragment from *A. caulinodans (*Van den eede *et al,* 1992). The *nod* operon of *A. caulinodans* has been shown to be induced in the presence of flavonoids such as naringenin or liquiritigenin.

Two constructs pRG970-31 and pRG970-32 contained the *nodA* promoter oriented towards the *lacZ* or *gusA,* respectively. In the presence of 10μM naringenin, the transconjugants carrying these respective constructs were assayed for β-galactosidase and β-glucuronidase activity. pRG970-32 exhibited induced β-glucuronidase activity upon addition of naringinin while pRG970-31 showed induced β-galactosidase activity. The authors concluded that the *gusA* and *lacZ* genes were correctly transcribed due to flavonoid induction by the *nodA* promoter.

2.1.2 The use of lacZ and gusA reporter assays

Vector pRG970 is unique in that it contains promoterless *lacZ* and *gusA* reporter genes encoding the *E.coli* β-galactosidase and β -glucuronidase, respectively. The use of the *lacZ* gene was due to the fact that it provides a sensitive assay with a broad range of available substrates. However, for studies dealing with plants, endogenous β-galactosidase activity could become an issue. It would be invaluable to have a system that would not become confused with endogenous activity of the plant or bacteria. The inclusion of the *gusA* gene abolishes such confusion. Some plants and many Gram-negative bacteria lack β-glucuronidase activity. There exists a wide variety of substrates available for quantitative and qualitative analysis of gene expression. Hence, the use of pRG970 for this project seemed a suitable method.

Figure 2.3 is schematically represents the approach used. Fragments (1-kb) of the *R. etli* CE144 genome were created via a partial *Sau*3AI digest. These fragments were ligated into the *Bam*HI cloning site of vector pRG970 and introduced into wt-*R. etli* CE3. Plating of transconjugants on X-Gal and X-GlcA allowed detection of *lacZ* and *gusA* expression which was visible by an indigo phenotype. A blue colony under pH 7.0 conditions was indicative of constitutive expression. A white colony at pH 7.0 indicated a promoter that could possibly be

inducible when subjected to simulated symbiotic conditions. The white colonies were subjected to pH 5.0 conditions with the aim at finding inducible promoters.

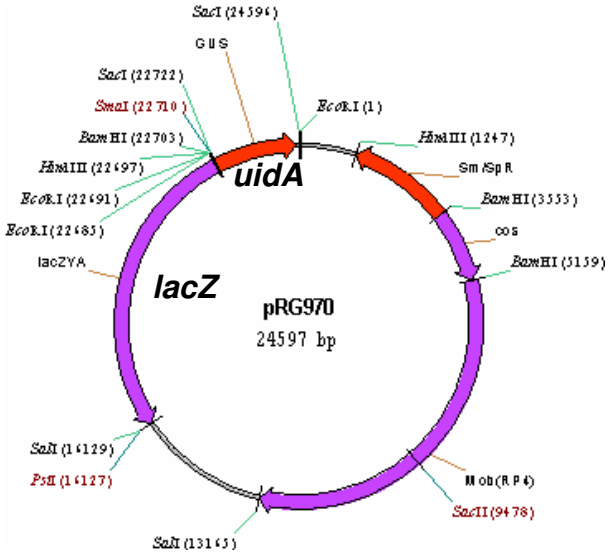

Figure 2.1: Schematic representation of pRG970 and restriction sites. The *uid*A gene encoding for β−glucuronidase is oriented in the forward direction. The *lac*Z gene, encoding for β−galactosidase is oriented in the reverse direction. A spectinomycin/streptomycin resistance cassette, *cos* sites, and mobility regions are visible in this diagram.

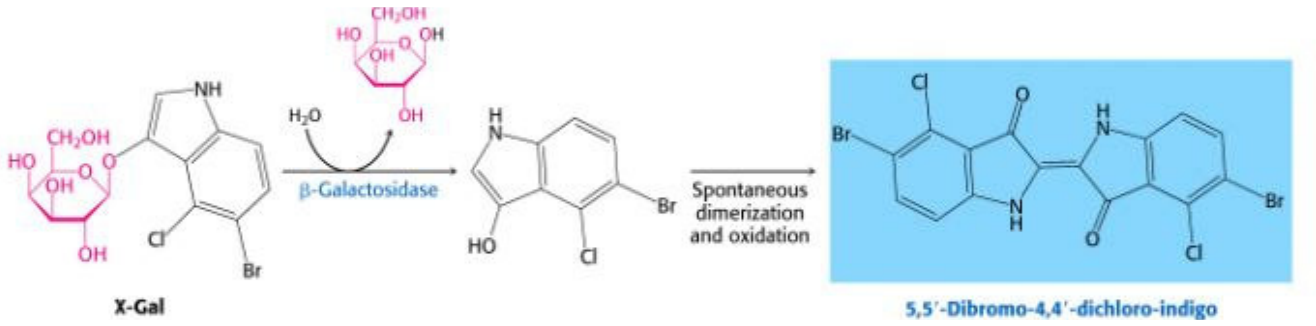

Figure 2.2: Reaction of β-galactosidase and chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). X-Gal is cleaved at the β-1,2 position between galactose and 5-bromo-4-chloro-3-indolyl by the product of the *lacZ* gene, β-galactosidase. A water insoluble blue dichloro-dibromo-indigo precipitate is produced. A similar results occurs in with the reaction of β-glucuronidase and chromogeneic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA). In contrast to β-D-galactose produced, β-D-glucuronidase is produced.

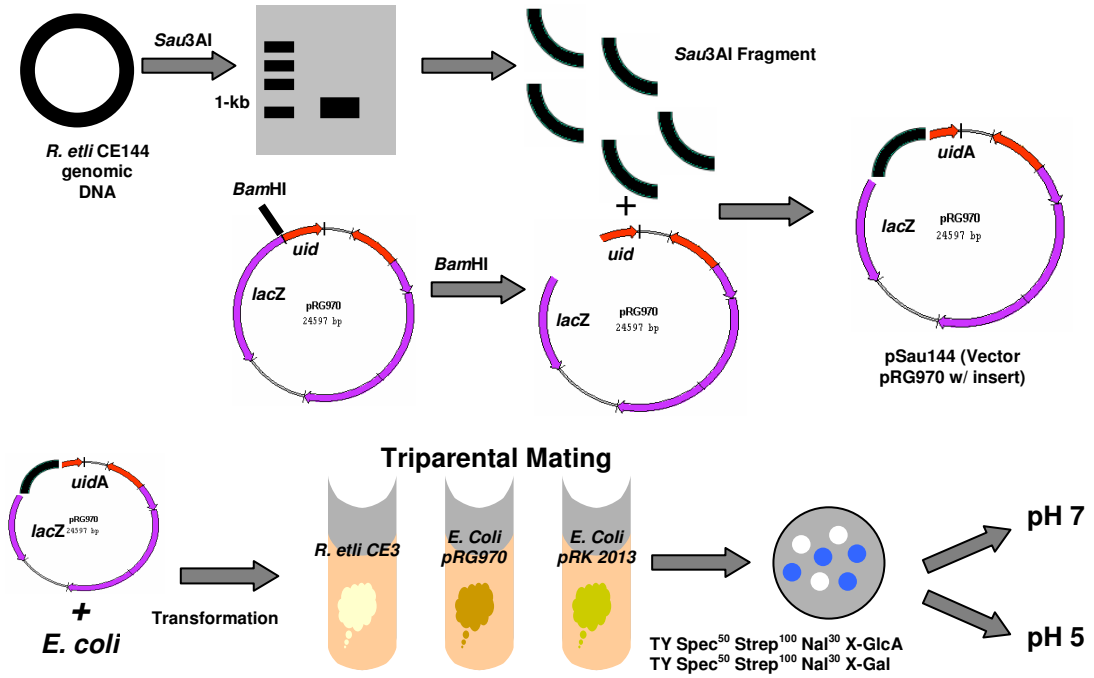

Figure 2.3: Schematic of Approach 1. *R.etli* CE144 genomic DNA was partially digested with *Sau*3AI to obtain a pool of 1-kb fragments. These fragments were ligated into the complementary *Bam*HI sites of pRG970. Vector pRG970 obtaining a *Sau*3AI insert is transformed into *E. coli.* A triparental mating allowed introduction of p*Sau*144 into wild-type *R. etli* CE3. The resulting transconjugants were plated on selective TY agar media containing X-Gal/X-GlcA. A blue colony under normal conditions indicated a constitutively expressed promoter. A white colony indicated a promoter that was not constitutively expressed and one that could be examined for induction by simulated symbiotic conditions.

2.2 Materials and Methods

2.2.1 Bacterial strains and cultivations used for Approach 1

Bacterial strains and culture methods are shown in Table 2.1. *Rhizobium* strains were routinely grown in TY media (Noel *et al,* 1984) and *E. coli* in LB media (Sambrook *et al,* 1989). If required, media were solidified by the addition of 1.5% (w/v) agar prior to autoclaving.

Table 2.1. Bacterial Strains and cultivation methods for Approach 1. Culture methods are for typical growth at respective temperatures and pH 7 conditions. This will be referred to as normal conditions.

Strain	Genotype	Culture method	Purpose	Source/Reference
***R. etli* CE3**	WT, $Strep^R$, Nal^R, Nod^+, Fix^+	TY/$CaCl_2$/$Strep^{200}$, 30°C, 3-5 days	Introduce final vector/insert to ensure wt background with ability to perform *in planta* studies	Obtained from Dale Noel, Marquette University, Milwaukee, WI
***R. etli* CE144**	$pSym^-$ derivative of CE3, Nal^R, Nod^-, Fix^-	TY/$CaCl_2$, 30°C, 3-5 days	Creation of 1-kb fragments to ensure absence of pSym genes	Obtained from Dale Noel, Marquette University, Milwaukee, WI
***E. coli* pRK2013**	Kan^R	LB, Kan^{40}, 37°C	Helper plasmid used for mobilization of pRG970 in triparental matings	Ditta *et al,* 1980
***E. coli* pRG970**	$Strep^R$, $Spec^R$, *lacZ, gusA*	LB, $Strep^{100}$, $Spec^{50}$, 30°C	Vector used for cloning of *R. etli* CE144 1kb fragments	Van den Eede *et al,* 1992
***E. coli* TOP10**		LB, 30°C	For transformations of vector pRG970 and triparental matings	Invitrogen, C4040-10

2.2.2 Verification of deficient nodulation of R. etli CE144

In order to verify that *R. etli* CE144 was deficient in nodulation (Nod^-), it was used to

inoculate *Phaesolis vulgaris* (common bean). As a positive control, *P. vulgaris* was inoculated with wild-type *R. etli* CE3. As a negative control, noninoculated *P. vulgaris* was subjected to identical conditions. This was repeated in duplicate with independent inoculants. Aseptic technique was critically followed to eliminate effects of cross-contamination.

Phaseolus vulgaris seedlings were surface sterilized with a wash in 95% EtOH followed by bleach (NaOCl) and a final wash with 1 liter H_20. A 0.8% agar solution was prepared with tap H_2O and layered in small sterile pots. Five seeds were placed with their scars to the center and incubated for 4 days at 30°C. Plant growth was performed in sterile plastic pots using a modified Leonard jar assembly (Somasegaran, 1998). Fifty milliliter cultures of *R. etli* CE3 and CE144 were prepared in TY. The plants were grown in sterile vermiculite:perlite (1:1 v/v) using an N-free nutrient solution (Werner *et al,* 1975). Two germinated seedlings were placed per Leonard jar unit. The 50 ml bacterial cultures were used for inoculation. Plants were grown for 4 weeks in a controlled environment chamber (15/9 hours light/darkness cycle with 25º/18ºC temperature regime and 70% relative humidity), periodically checking for sufficient nutrient solution. After harvest, plant phenotype was observed and roots were checked for the presence of nodules.

2.2.3 Determining a mode of selection via antibiotic tests

Antibiotic tests on the donor and recipient to be used in the tri-parental mating were necessary to determine the mode for selection of *R. etli* CE3 that obtained vector pRG970. In addition to the selectable markers of spectinomycin and streptomycin resistance on the vector, a resistance unique to *R. etli* CE3 for counterselection against *E. coli* containing vector pRG970 was needed.

Vector pRG970 encodes for streptomycin and spectinomycin resistance. *R. etli* CE3 is resistant to streptomycin (Segovia, 1993). In order to select for transconjugants, it was necessary to determine the sensitivity of *R. etli* CE3 susceptibility to spectinomycin This was done by subjecting *R. etli* CE3 to the following concentrations of spectinomycin: 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml. Plates were incubated at 30ºC and results recorded after 5 days.

In order to counterselect against *E. coli,* a resistance unique to *R. etli* CE3 was needed. *R. etli* CE3 is resistant to naladixic acid (Segovia, 1993). Therefore, *R. etli* CE3, *E. coli* DH5α and Top10 were grown on their respective media supplemented with the following concentrations of naladixic acid: 5 μg/ml, 10 μg/ml, 20 μg/ml, and 30 μg/ml. As a control, *R. etli* CE3 was grown on agar supplemented with 200 μg/ml streptomycin. In addition, both *E. coli* strains were grown on LB agar. *R. etli* and *E. coli* were incubated at 30ºC for 5 days and 37ºC for 2 days, respectively.

2.2.4 Preparation and manipulation of R. etli CE3 and plasmid DNA

-Preparation of DNA *R. etli* CE3 genomic DNA was extracted as described in Ausubel *et al,* 1999. This standard procedure involved removal of protein contaminants with phenol/chloroform/isoamyl alcohol (1:1:24) extraction, 100% EtOH precipitation, 70% EtOH precipitation to remove salts and small organic molecules, and a final resuspension in buffer.

Plasmid DNA was recovered using the alkaline lysis method for mini-preparations as described in Sambrook *et al*. For large scale preparation of plasmid DNA, maxipreparations were performed using the SDS alkaline lysis method as described (Sambrook *et al.,* 1998). In addition, some plasmid DNA preparations were obtained using the QIAgen spin mini- or maxi-preparation kits (Qiagen, Cat. Nos. 27104 or 12162).

-Determination of genomic DNA yield and quality The quantity of DNA was approximated by comparison of ethidium bromide staining with that of a known amount of DNA. For more accurate quantification, DNA was measured by its absorbance at 260 nm and calculated using the equation: 50 μg/ml x A_{260} x dilution factor.

-Enzymatic digestions The method to for partial digestion of *R. etli* CE144 genome with *Sau*3AI was adapted from Current Protocols in Molecular Biology (Ausubel *et al.,* 1999). It was performed in order to obtain *Sau*3AI (New England Biolabs (NEB), Cat. No. R0169S) ~1kb promoter fragments from *R. etli* CE144 genomic DNA to be ligated into pRG970. Digestion with Sau3AI was performed for up to 45 minutes and the samples loaded onto 0.8% agarose gel for gel electrophoresis.

To prepare pRG970 for ligations, pRG970 was digested with *Bam*HI (NEB, Cat. No. R0136S), cleaned with DNA Clean and Concentrator (Zymo Research, Cat. No. D4003), visualized on an agarose gel, and subjected to calf intestinal phosphatase (CIP). Vector pRG970, when digested with *Bam*HI, has the ability to self-ligate. This would produce problems because an intact plasmid resulting from self-ligation would be able to confer resistances on selective media. To eliminate the possibility of self-ligation and increase the chance of only pRG970 w/ insert being present on selective media, pRG970 was subjected to calf intestinal phosphatase (CIP) (NEB, Cat. No. M0201S) before ligation of the insert. CIP catalyzes the removal of 5' phosphate groups from the DNA. The 5' phosphoryl termini is required by ligases. The lack of it will prevent self-ligation.

-Gel extraction: "Freeze Squeeze"method Purification of pRG970 plasmid DNA and the *Sau*3A1 fragments from agarose gels was performed according to the "Freeze Squeeze"

method (Sambrook, 1998). DNA was also extracted using a Qiagen gel extraction kit (Qiagen, Cat. No. 20021) according to manufacturer's instructions.

-Choice of competent cells XL-10 Gold Ultracompetent cells (Strategene, Cat. No. 200315) were created for transformation of large DNA molecules with high efficiency. Since pRG970 is a rather large piece of DNA at 24.6-kb, these cells seemed to eliminate the chance of size bias in transformations. Cells were assayed for their ability to grow on 30 µg/ml naladixic acid and the combination of 30 µg/ml naladixic acid, 50 µg/ml streptomycin and 100 µg/ml spectinomycin. Introduction of DNA into cells were performed following the recommended conditions for heat shock transformation (Stratagene, California). pUC 18 or pUC 19 control transformations were included in each experiment so that transformation efficiency could be assayed.

-Ligations of pRG970 Vector pRG970 digested with *Bam*HI and subjected to CIP (PBC) and *Sau*3AI fragments were ligated in a 1:3 ratio (PBC:*Sau*3AI fragment) using the Quick T4 DNA ligase (NEB, Cat. No. M2200S) creating plasmids, **p*Sau*144**. The negative control included PBC alone subjected to ligation conditions. Two positive controls were included; undigested pRG970 and *Bam*HI digested pUC18. Five microliters of each ligation mixture were added to XL-10 Gold Ultracompetent cells, except in the case of pUC18 where 1 µl was added. DNA was introduced by the heat shock transformation as described (Stratagene, California) and cells plated on selective LB media containing 50 µg/ml spectinomycin and 100 µg/ml streptomycin. Of the colonies resulting from this experiment, ~10 were selected to check for the insert. The resulting colonies, containing p*Sau*144, were pooled, resuspended in λ buffer (1.211g Tris, 2.488 g $MgSO_4$), and triparental mated with *R. etli* CE3 and *E. coli* pRK2013. After collection of the overnight growth and resuspension in λ buffer, dilutions of 1, 100, and

1000 were performed on selective media (TY, 30 µg/ml naladixic acid, 50 µg/ml spectinomycin and 100 µg/ml streptomycin) to select for *R. etli* CE3 transconjugants.

2.2.8 Assay for promoter activity

In order to detect possible promoter activity of colonies containing p*Sau*144, colonies were plated on one of the following media.

(1) TY agar was prepared as normal (Noel *et al,* 1984). Stock solutions (20 mg/ml) of both X-Gal (Sigma, Cat. No. B4252) or X-GlcA (Sigma, Cat. No. B5285)were prepared in dimethylformamide (Sigma, Cat. No. D551). Of this, 2 ml was added for every 1 liter of media. Antibiotics, spectinomycin and streptomycin, were added to final concentrations of 50 µg/ml and 100 µg/ml, respectively.

(2) For pH5 conditions, TY preparation proceeded as described (Vedam *et al,* 2003) using a 40mM piperazine solution as a buffer.

Colonies resulting from the tri-parental mating were isolated by their blue or white phenotypes as individual colonies and replated on normal TY/X-GlcA supplemented with 30 µg/ml naladixic acid, 50 µg/ml spectinomycin and, 100µg/ml streptomycin. After confluent growth at 30ºC, colonies were plated again on pH7 TY/X-GlcA in parallel with pH5 TY/X-GlcA, both supplemented with selective antibiotics. Plates were incubated at 30ºC for 2 days and phenotypes recorded. These platings were performed twice and the results analyzed for stable changes in the phenotype of each colony when shifted from pH7 to pH5 media.

2.3 Results

2.3.1 R. etli CE144 is deficient in nodulation.

In order to ensure that promoter fragments obtained would be absent of the pSym, a pSym$^-$ strain was used; *R. etli* CE144. Such a strain is expected to be deficient in nodulation. *Phaseolus vulgaris* was inoculated with *R. etli* CE3 and *R. etli* CE144 as described in the Materials and Methods. Plants were examined for the presence of nodules after 4 weeks of controlled growth and the results are presented in Figure 2.4. Figure 2.4A represents *R. etli* CE3 inoculated *P. vulgaris.* Leaves were dark green in color and beans were visible. Nodules typical of *R. etli* infection occupied the root system as indicated in Figure 2.4C. In contrast, Figure 2.4B represents *R. etli* CE144 inoculated *P. vulgaris.* There was a notable dwarfing of leaf size as compared to the effects of *R. etli* CE3. In addition, leaves were light green, no beans were present, and nodules did not occupy the root system. The results were consistent with the absence of the pSym in *R. etli* CE144.

A **B** **C**

Figure 2.4. Observation of plant phenotype in differential inoculated *P. vulgaris*.
(A) *R. etli* CE3 inoculated *P. vulgaris.* (B) *R. etli* CE144 inoculated *P. vulgaris.* (C) Nodules typical of infection of *R. etli* CE3

2.3.2 Antibiotic markers as the mode of selection.

The mode of selection is critical in obtaining transconjugants of the desired strain that have obtained the vector. In this case, obtaining *R. etli* CE3 transconjugants that had acquired pRG970 with 1kb *Sau*3AI inserts from *R. etli* CE144 was the objective. Vector pRG970 harbors both spectinomycin and streptomycin resistance markers. *R. etli* CE3 is already resistant to streptomycin (200 µg/ml). The susceptibility to spectinomycin was undetermined and would be necessary to select for presence of p*Sau*144. The resistance of *R. etli* CE3 to various concentrations of spectinomycin was measured and the results are recorded in Table 2.2. No observable growth of *R. etli* CE3 occurred at any concentration of spectinomycin.

Table 2.2. *R. etli* CE3 susceptibility to spectinomycin. *R. etli* CE3 was cultured under normal growth conditions supplemented with various concentrations of spectinomycin. Plates were incubated for 3 days at 30°C and observed for growth. G=Growth, NG= No Growth

Spectinomycin concentration µg/ml	***R. etli* CE3**
25	NG
50	NG
100	NG
200	NG

In order to determine which *E. coli* strain to use for counterselection, the resistance of *R. etli* CE3 to naladixic acid (30µg/ml) was exploited. *R. etli* CE3, *E. coli* Dh5α, and *E. coli* TOP10 were subjected to several concentrations of naladixic acid. Plates were observed after 3 days of growth and the results are recorded in Table 2.3. As expected, *R. etli* CE3 was able to grow in the presence of naladixic acid, verifying its resistance. In addition, *E. coli* Dh5α was able to grow in the presence of naladixic acid due to a mutation it its gyrase gene. In contrast, *E. coli* TOP10 was not able to grow in the presence of naladixic acid.

Table 2.3. Effects of naladixic acid on *R. etli* CE3, *E. coli Dh5a,* and *E. coli* TOP10. *R. etli* CE3, *E. coli Dh5α*, and *E. coli* TOP10 were cultivated under normal growth conditions in media supplemented with the concentration of naladixic acid given in the table below. All plates were incubated for 3 days and results recorded. G=Growth, NG=No Growth

Naladixic Acid concentration µg/ml	***R. etli* CE3**	***E. coli* Dh5α**	***E. coli* Top10**
30	G	G	NG
20	G	G	NG
10	G	G	NG
5	G	G	NG
0	G	G	G

According to the results of Tables 2.2 and 2.3, the mode of selection of transconjugants was determined by use of the following antibiotics; spectinomycin 50µg/ml, streptomycin 100 µg/ml, naladixic acid 30 µg/m. The donor strain of vector pRG970 (which confers resistance to spectinomycin and streptomycin) was also determined to be *E. coli* TOP10 due to its susceptibility to naladixic acid.

2.3.3 Sau3AI efficiently digests R. etli CE144 genomic DNA

Fragments produced from a *Sau*3AI digest are complementary to sites created via a *Bam*HI digest of pRG970. Digestion conditions of *R. etli* CE144 genomic DNA with *Sau*3AI were optimized to obtain 1kb fragments. A partial digest of *R. etli* CE144 genomic DNA with *Sau*3AI was performed as described in the Materials and Methods and the results presented in Figure 2.5. Initially, undigested genomic DNA is localized at the top of the agarose gel. Digestion proceeds steadily over a period of 45 minutes. A 12-15 minute period was chosen to obtain the 1 kb *Sau*3AI fragments.

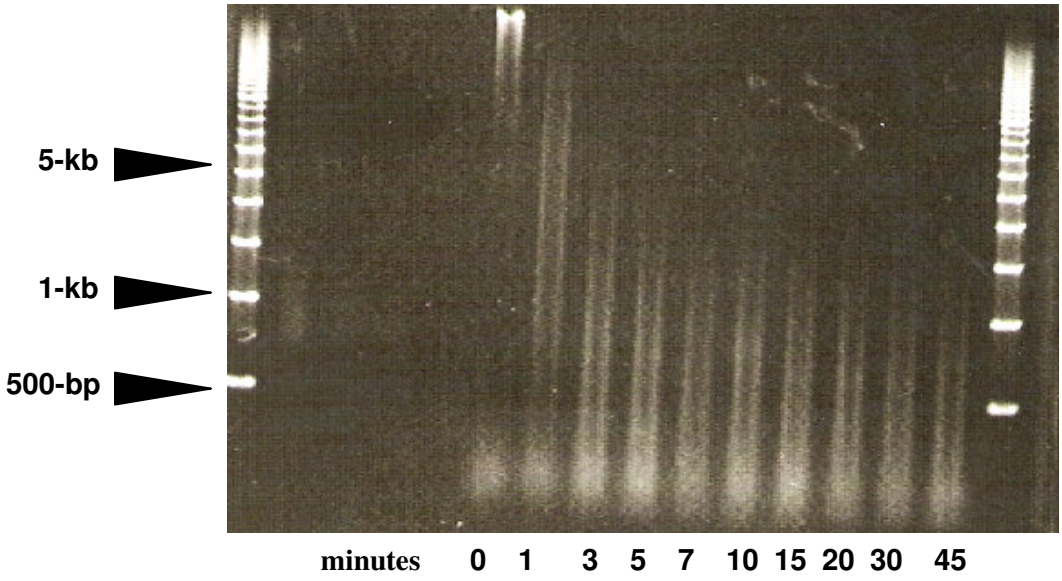

Figure 2.5. *Sau*3AI *p*artial digest of *R. etli* CE144 genomic DNA. Approximately 5µg of *R. etli* CE144 genomic DNA was partially digested with *Sau*3AI over a time span of 45 minutes. At time=0, 5µl of digest was added to 1.25µl stop solution. This was repeated for each time point. Afterwards, the samples were loaded unto a 0.8% agarose gel and electrophoresed.

2.3.4 Preparation of pSau144 and tri-parental mating into R. etli CE3

Before a tri-parental mating could be performed, p*Sau*144 needed to be successfully transformed into *E. coli* TOP10. As described in Materials and Methods, pRG970 was digested with *Bam*HI and subjected to CIP. This construct was then ligated with the 1kb *Sau*3AI genomic fragments from *R. etli* CE144, creating p*Sau*144, and transformed into competent *E. coli* TOP10 cells. *E. coli* transformants resulting from the introduction of p*Sau*144 were pooled and subjected to tri-parental mating with *R. etli* CE3 and *E. coli* pRK2013 as described in Materials and Methods. The tri-parental mating resulted in 210 colonies harboring p*Sau*144, Figure 2.6. Both positive controls (pRG970 vector only and pUC 19) worked efficiently which was evident by colonies too numerous to count (TNTC). The negative control (pRG970:CIP) also yielded 24 colonies which was contrary to what was expected. This may indicate that a slightly incomplete

CIP reaction occurred resulting in some self-ligation of pRG970. Therefore, one could estimate that approximately 10% of the 210 colonies may contain the pRG970 vector rather than p*Sau*144.

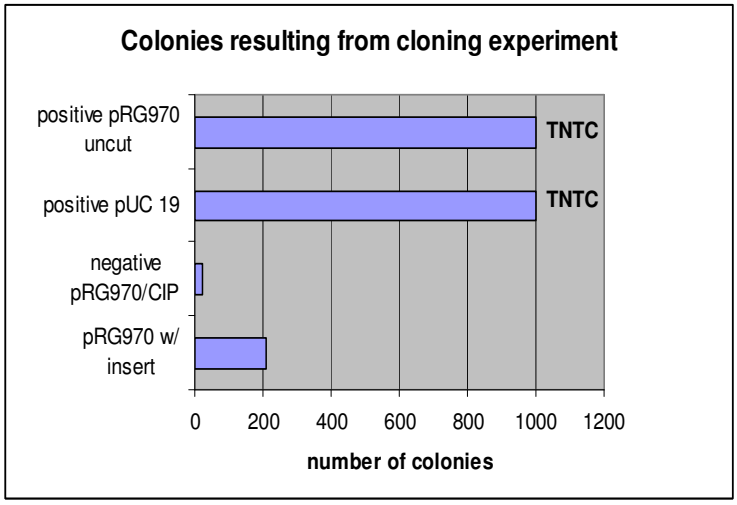

Figure 2.6. Colonies resulting from tri-parental mating. Triparental transconjugants were pooled and replated on selective media to obtain isolated colonies. Undigested pRG970 and pUC 19 served as positive controls. pRG970:CIP served as the negative control. p*Sau*144 is pRG970 harboring a 1kb *Sau*3AI fragment from *R. etli* CE144.

Of the 210 colonies, 10 were selected for verification of p*Sau*144. With the aim of releasing the insert, p*Sau*144 obtained from each of the 10 colonies was digested with *Bam*HI and electrophoresed on a 0.8% agarose gel as seen in Figure 2.7.

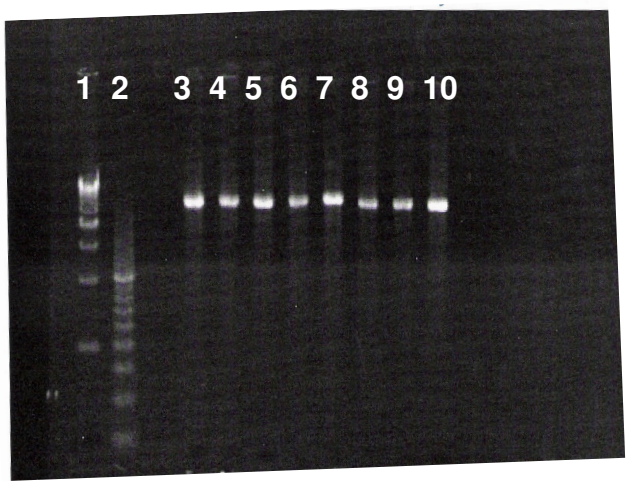

Figure 2.7. Verifying the presence of p*Sau*144 in *R. etli* CE3 transconjugants. Random tranconjugants were selected for minipreps. Minipreps (p*Sau*144) were digested with *Bam*HI. p*Sau*144 (~1-2 µg) was digested in a standard *Bam*HI digestion of 20 µl total volume. As a negative control, approximately 1 µg of purified pRG970 was digested in identical conditions. The resulting digest was loaded onto at 1% agarose gel and electrophoresed at 100V for 1.5 hours. Lanes 1 and 2 represent 2.5 kb and 500bp markers, respectively. The top band of lane 1 is ~35 kb.

Lanes 3-9 represent colonies from tri-parental mating. Lane 10 serves as a negative control being *Bam*HI digested pRG970 from a maxipreparation. *Bam*HI digestion did not enable identification by release of a 1-kb insert. It may be that the *Bam*HI site was compromised after ligation with the 1kb *Sau*3AI *R. etli* CE144 fragments and will be discussed. However, it is evident that each colony harbored either vector pRG970 or p*Sau*144.

2.3.6 Glucuronidase activity phenotype indicates inducibility and presence of p*Sau*144 *in the R. etli CE3::pSau144 transconjugants*

Vector pRG970 contains both *lacZ* and *uidA* reporter genes. The products of these genes are the colorimetric substrates X-Gal and X-GlcA, respectively. Upon induction of either enzyme with the appropriate substrate, a water insoluble blue dichloro-dibromo-indigo precipitate is produced as evidenced in Figure 2.8. Both genes are promoterless and would be under transcriptional regulation of the inserted genomic fragment (p*Sau*144) from *R. etli* CE144. A blue colony on normal media containing the substrate would indicate a constituitively-expressed promoter. A white colony would indicate a promoter that is not active under normal growth conditions, or the absence of an insert. We sought to select potential promoters that were not active under normal conditions and could possibly be induced by simulated symbiotic conditions.

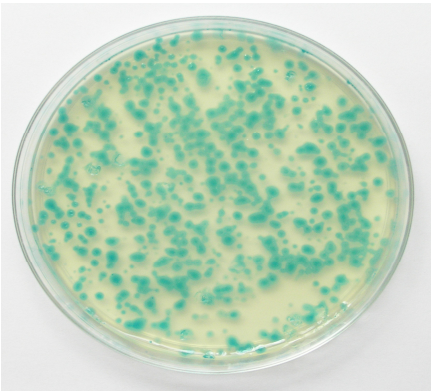

Figure 2.8. Phenotype of active β-glucuronidase colonies. Colonies resulting from the triparental mating were grown on selective media containing X-GlcA according to Material and Methods. The blue color is typical of a colony producing the water insoluble indigo precipitate. This precipitate can only be produced if the reporter gene, *gusA,* is expressed.

All colonies grown in the presence of X-Gal were white. The results suggested the likelihood that *lacZ* gene was not functioning properly. Consequently, only colonies grown in the presence of X-GlcA underwent further analysis. A total of 181 individual colonies were selected from the initial 210 tri-parental mating transconjugants and grown in the presence of X-GlcA. Of these 181 colonies, 51 exhibited an initial white phenotype (inducible) while 130 exhibited an initial blue phenotype (constitutive).

2.3.8 Stability of the glucuronidase activity

The 181 colonies grown under normal pH 7 conditions were individually selected and re-grown under normal conditions as shown in Figure 2.9 and the color noted.

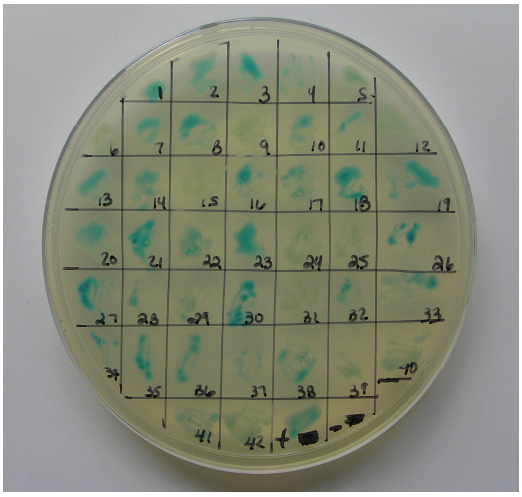

Figure 2.9. Phenotypic selection and separation of individual colonies. Colonies were individually selected and plated on selective media containing X-GlcA. This figure represents colonies that had an initial blue phenotype. Each square represents a single colony. *R. etli* CE3 was used as a negative control to ensure there was no *gusA* activity.

It was necessary to determine if the transconjugants, as reflected by a color change in the colony, would be stable after repeated plating of the colonies. Stability of the phenotypes was an important factor for the reliability of the method. An unstable phenotype would increase the chances of false positives. Of each of the initial 51 white colonies, 27 exhibited a color change to blue while 24 remained white when replated on normeal pH 7 media. No change in color should have been observed if p*Sau*144 was stable. When each of the 51 originally white

colonies were subjected to pH 5 conditions in the presence of X-GlcA, 34 colonies remained white, 5 failed to grow, and 12 turned blue. Of these 12 blue colonies, only two exhibited a stable change from white to blue phenotype indicating a stable induction of glucuronidase activity at pH 5 conditions.

An initial blue colony was considered to be constitutively expressed under normal conditions. If a blue colony turned white when exposed to low pH conditions, repression of glucuronidase activity by the 1kb insert would be implicated. Of the initial 130 blue colonies, 45 decreased in intensity of color, 15 remained blue, and 67 reverted to a white phenotype when replated on normal pH 7 media with X-GlcA. Of the initial 130 blue colonies, when grown under pH 5 conditions, 14 became less intense, 86 remained blue and 29 turned white. However, none of the 29 colonies exhibited a stable change from blue to white.

In summary, from a total of 181 (51 initial white and 130 initial blue) colonies observed, a total of two possible stable transconjugants existed. While this is a positive result for obtaining *R. etli* CE144 promoters up-regulated by pH 5 conditions, it was concluded that the overall stability of this approach was problematic and therefore a second approach (next chapter), was investigated.

2.4 Discussion

The efficient and reliable use of pRG970 to create a 1kb library of the *R. etli* CE144 genome would have allowed *in planta* studiesto identify non-pSym genes induced during symbiobis. However, this approach presented a number of problems.

First, verification of p*Sau*144 transfer to wild-type *R. etli* CE3 was uncertain. Since the *Bam*HI site of pRG970 is compromised after ligation with a *Sau*3AI fragment, and the release of

an insert did not occur. To overcome this, digestion with both *Hind*III and *Pst*I may have allowed detection of fragments containg the insert. In spite of this problem, plasmids in lanes 3-7 of Figure 2.7 seem to be slightly larger in size compared to those in lanes 8 and 9 which migrate similarly to the pRG970 vector control in lane 10. Since it is possible that approximately 10% of the colonies resulting from transformation with p*Sau*144 (Figure 2.6) did not contain an insert due to an incomplete CIP reaction, it may be that lanes 3-7 represent transconjugants that harbored p*Sau*144, while lanes 8 and 9 harbored the vector pRG970.

Second**,** when transconjugants expected to harbor p*Sau*144 were plated on agar supplemented with X-Gal, 100% of the colonies exhibited a white phenotype. The *lac*Z gene may have been disrupted with *Bam*HI digestion to yield a non-functional gene product.

Third, there was a great deal of instability with regard to the expression of the glucuronidase activity.Of a total of 181 colonies containing p*Sau*144, only 2 exhibited a stable phenotypic change from white to blue when going from pH 7 to pH 5 media. While this was a reasonable frequency of transconjugants, sorting through this instability to find stable transconjugants was a tedious process. Therefore, it was concluded that these problems required another approach which is described in the next chapter.

CHAPTER 3

Approach 2:Transposon Mutagenesis

3.1 Method description

3.1.1 Transposons

Barbara McClintock first observed transposition in the 1940's and 1950's in maize (McClintock, 1950). It was then that transposons were noted to greatly influence gene expression. Transposons are mobile genetic elements that are able to relocate themselves from one genomic location to another. They have proven to be useful genetic tools for both small and large-scale genomic studies in microorganisms. At the same time that transposons are highly efficient and effective, the simplicity of the mechanism deems them a highly attractive method for the identification of essential genes and genes necessary for optimal virulence of many microorganisms.

There are many different types of transposons that have advantages and disadvantages based on its desired usage. By far the most popular transposon is Tn5 (Simon, 1989). Its advantages include high frequency of transposition in many Gram-negative species, it generally inserts with little target sequence specificity, it exhibits a low probability of genome rearrangments upon transposition and a high stability once established in the genome, it encodes resistance to neomycin/kanamycin (*nptII),* and its complete genome is sequenced (5818 bp) (Simon, 1989).

3.1.2 Rhizobium and transposon mutagenesis

In 1984, Noel *et al* used Tn5 mutagenesis to mutagenize *R. phaseoli.* Instead of focusing solely on the plasmids, this group sought mutations in the entire genome that would result in altered nodulation or symbiotic nitrogen fixation (Noel, 1984). Only mutants suffering loss or deletion in the *nif* plasmid were completely unable to induce nodule tissue. However, over half of the symbiosis-specific mutations appeared to be chromosomal. All of them had an effect on nodule development to some degree. In support of chromosomal genes being essential after initiation of nodule development, *A. tumefaciens* carrying rhizobial plasmids still leads to incomplete nodule developent lacking nitrogen-fixing activity (Noel, 1984).

Xi *et al* was successful in the construction of a bi-functional *gfp*- and *gusA*-containing mini-Tn5 (*mTn5*) to study gene expression and bacterial localization (Xi, 2001). *Rhizobium, Azospirillum* and *Pseudomonas* mutants resulting from the introduction of this transposon were tested for effects on growth and for visual localization in their host plants. The bi-functional transposon was shown not to interfere with growth of any strain tested. In addition, bean seedlings were inoculated with a mutant carrying the *mTn5* and harvested after three weeks. Nodules were examined in 100 μm sections under a fluorescence microscope. Those nodules filled with bacteria carrying the bi-functional *mTn5* were strongly fluorescent while no fluorescence was observed for the wild-type strain within the nodule.

3.1.3 Using transposon mutagenesis to create mutants in R. etli CE144

Upon deciding that Approach 1 had problems that could not be resolved within a reasonable timeframe, a second approach was considered and implemented. *R. etli* CE144 remained as the background for generation of mutants so that identification of inducible non-

pSym genes could be made. A transposon (*mTn5gusAoriV)* with a promoterless *gusA* gene and neomycin/kanamycin resistance cassette was obtained as a gift (Jan Michiels at the Catholic University of Kulueven, Belgium*),* and was the mode of mutagenesis.

mTn5gusAoriV allowed the rapid generation of *R. etli* CE144 mutants that could be screened in inducing conditions (i.e. pH 5 and flavonoids) via the β-glucuronidase assay as shown in Figure 3.1. Mutants exhibiting increased activity under inducing conditions were selected for sequencing and further characterization.

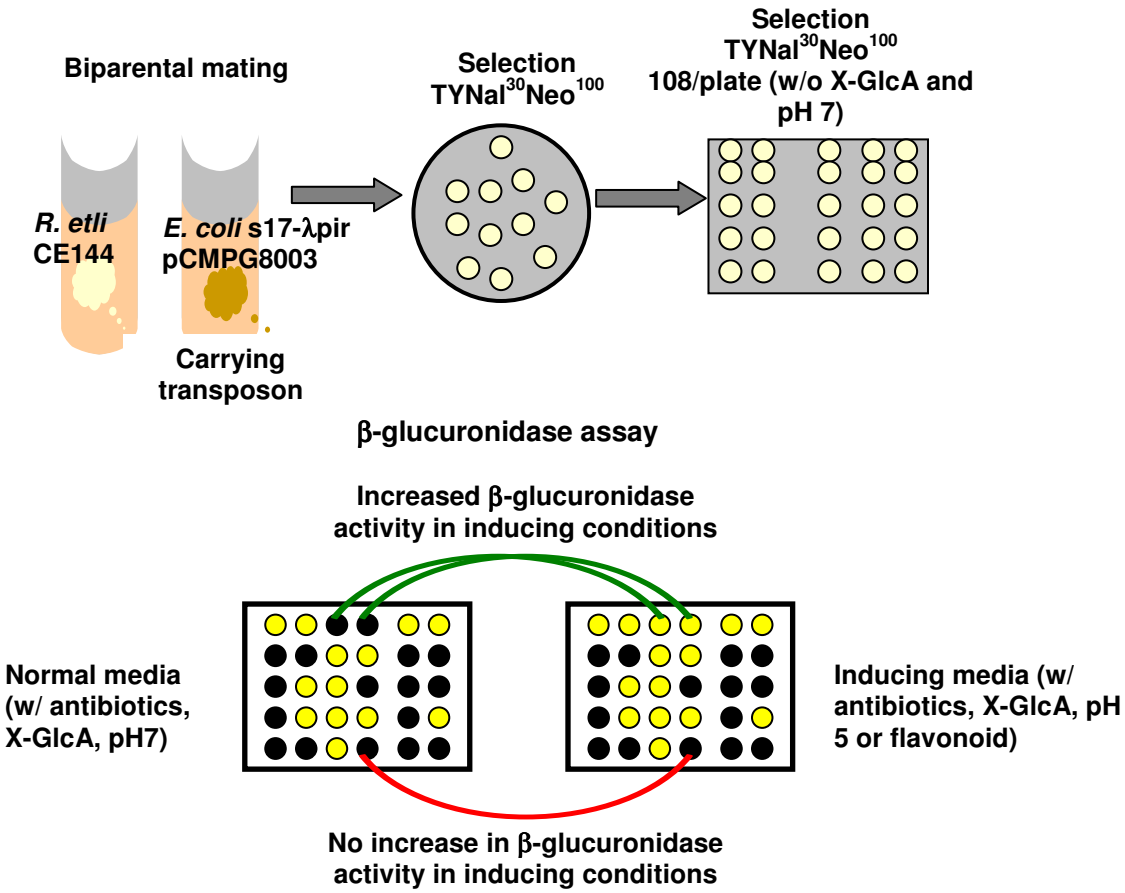

Figure 3.1: Schematic of microtiter plate procedures. *R. etli* CE144 and *E. coli* s17lpir pCMPG8003 (containing the *mTn5gusAoriV* transposon) were biparental mated to perform the mutagenesis and selection was done as described in the Materials and Methods. The β-glucuronidase assay was first done with each well of a 96-well microtiter plate representing a single colony. Each colony was assayed under inducing conditions in parallel with normal

conditions. Those colonies showing a significant level of upregulation (indicated by green lines) in inducing media were selected to assess reproducibility and for further analysis.

3.2 Materials and Methods

3.2.1 Bacterial strains and cultivations used for Approach 2.

The strains and culture conditions used for transposon mutagenesis are listed in Table 3.1.

Table 3.1. Bacterial strains and cultivations for transposon mutagenesis

Strain	Genotype	Culture conditions	Purpose	Source/Reference
R. etli CE3	WT, $Step^R$, Nal^R, Nod^+, Fix^+	TY, $Strep^{200}$, 30°C, 3-5 days	Wild type strain used as a control in assays	Obtained from Dale Noel, Marquette University, Milwaukee, WI
R.etli CE144	$pSym^-$ derivative of CE3, Nal^R, Nod^-, Fix^-	TY, 30°C, 3-5 days	Strain used to perform mutagenesis to ensure absence of pSym genes	Obtained from Dale Noel, Marquette University, Milwaukee, WI
E.coli s17λpir pCMPG8003	*λpir* lysogen of S17-1 *mTn5gusAoriV,* Kan/NeoR	LB, Kan^{40}, 30°C	Strain harboring the transposon used to mutagenize CE144	Obtained from Jan Micheils at K.U. Lueven, Haverlee, Belgium
E.coli TOP10		LB, 30°C when transformed with transposon	General cloning used in maintenance of mutant DNA library	Invitrogen, C4040-10

3.2.1 Transposon mTn5gusA-oriV

The mTn*5gusA-oriV* was (obtained as a gift from Jan Michiels at K.U. Leuven, in Belgium) adapted from mTn5*gusA-pgfp21* (pFAJ1819) by Xi *et al.*, 1999. Xi *et al* amplified the *oriV* sequence from pBR322 by PCR with primers OJM131 (5'-CTGA*CCTCAGG*AAAAGGCCAGCAAAAGGC-3') and OJM132 (5'-CTGA*CCTGAGG*TTCCACTGAGCGTCAGAC-3') carrying a *Sau*I recognition site at their 5'

end (in italics). After a digestion with *Sau*I, the *oriV* fragment was ligated into *Sau*I digested pFAJ1819. The resulting construct was named pFAJ1819b. The *gfp* genes of pFAJ1819b were deleted by an *Nde*I digestion followed by a partial *Xho*I digestion. This construct was ligated and named pCMPG8003 (schematically represented in Fig.3.2) and conjugated to *E. coli* S17-1λ*pir* to allow biparental conjugations (Xi, personal communication).

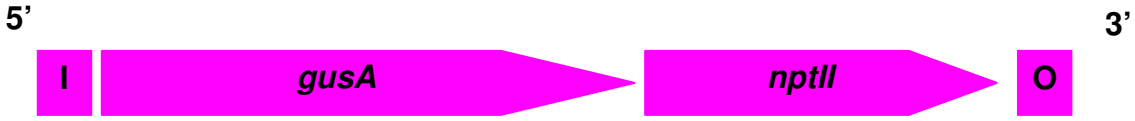

Figure 3.2: *mTn5gusAoriV*. The construct used for mutagenesis contains a promoterless *gusA* gene which encodes for β-glucuronidase that may be assayed by the cleavage of p-nitrophenyl-β-D-glucuronide into β-D-glucuronic acid and p-nitrophenol. It also contains the *nptII* genes which encodes kanamycin/neomycin resistance. The construct is approximately 4.6kb in size.

3.2.2 Antibiotic Tests

To determine media to use for selection and counterselection, antibiotic tests were performed on *R. etli* CE144 at 30°C for 3 days and the results are shown in Table 3.2.

Table 3.2 Antibiotic tests of *R. etli* CE144

Antibiotic	Concentration (μg/ml)	Growth (G) or No Growth (NG)
Streptomycin	200	NG
Naladixic acid	30	G
Neomycin	30	NG
"	50	NG
"	100	NG
"	150	NG
"	200	NG
None	N/A	G

The antibiotic tests shown in Table 3.3 were performed on *E. coli* s17-λpir pCMPG8003 in 1 ml liquid LB cultures and LB agar plates at 30 and 37°C for 2 days.

Table 3.3: Antibiotic tests of *E. coli* s17-λpir pCMPG8003

Antibiotic	Concentration (μg/ml)	Growth (G) or No Growth (NG)
Kanamycin	40	G
Naladixic acid	30	NG
None	N/A	G

3.2.3 Protocol to construct R. etli CE144:mTn5gusA-oriV mutants

To mutagenize *R. etli* CE144, a bi-parental mating between *R. etli* CE144 and *E. coli s17-λpir* pCMPG8003 was performed as described (Xi *et al,* 1999) Selection was done on TY agar plates supplemented with 100 µg/ml neomycin and 30 µg/ml naladixic acid ($TYNeo^{100}Nal^{30}$). The plates were incubated for 3-5 days at 30°C. The following controls were included: *E. coli* s17-λpir pCMPG8003 and *R. etli* CE144 plated on selective media. *E. coli* s17-λpir pCMPG8003 was grown at 30 and 37°C. A noninoculated plate was used to ensure no contamination. Control plates were allowed to incubate for 5 days.

3.2.4 Collection of mutants from mTn5gusA-oriV mutagenesis

The resultant mutant colonies were picked with sterile toothpicks and plated 108/plate on square plates of $TYNeo^{100}Nal^{30}$. The mutamts were incubated at 30°C for 2 days. Upon growth, each colony was inoculated into 100 µl liquid media of the same composition in 96-well microtiter plates. The plates were shaken (210-215 rpm) at 30°C for 2 days. A breathable seal was used to cover the plates to prevent evaporation and allow entrance of oxygen (USA Scientific, Cat. No. 9123-6100). An equal volume of 50% glycerol was added and plates were stored at -80°C.

3.2.5 Preparation of Inducing Media

-pH 5 media TY media at pH5 was prepared as described (Vedam *et al,* 2003). A 40 mM piperazine solution was prepared. The antibiotics neomycin (100 µg/ml) and naladixic acid (30µmg/ml) were added to media before use.

-Flavonoid media It is widely accepted that the presence of NodD is need for flavonoids to have an inducing affect on rhizobial genes. *R. etli* CE144 lacks the pSym and therefore lacks the NodD protein. We sought to seek genes that may be induced by flavonoids without the presence of NodD. For flavonoid induced media, a stock solution of (1mM) naringinin was prepared in methanol and stored at –20°C until use. Naringinin was added to sterile TY media at a final concentration of 1 µM immediately before use. The antibiotics neomycin (100 µg/ml) and naladixic acid (30 µg/ml) were added to media before use.

3.2.6 Expression test using the β-glucuronidase assay

Overnight cultures of select plates were prepared by defrosting the -80°C stock cultures on ice and inoculating 20 µl from each well into 180 µl of fresh medium ($TYNeo^{100}Nal^{30}$) in a microtiter plate and incubating at 30°C overnight. From the overnight cultures, 5 µl (1/40 dilution) of each well was inoculated into 200 µl each of normal media and inducing media. The plates were incubated for 40 hours.

Afterwards, 135 µl from each well was transferred to a new microtiter plate and the OD was measured at 595 nm with a microtiterplate reader. Of this, 10 µl was transferred to a new microtiter plate. β-glucuronidase activity was assessed using p-nitrophenyl- β-D-glucuronide (pNPG) (Sigma, Cat. No. N-1627) (8 mg/ml in substrate buffer: 50mM Na_2HPO_4/NaH_2PO_4 pH7, 10 mM β-mercapto-ethanol, 0.1% Triton X 100, 0.1% SDS, 1 mM Na_2 EDTA) as a substrate at 37°C. Upon observing a yellow color, the reaction was stopped by adding 35 µl of stop buffer (1M Na_2CO_3) and the time recorded. The reaction proceeded for 5-7 hours. Plates were then measured at 415 nm and 595 nm. The equation below was used to calculate the Miller Units (Miller, 1979):

$$\frac{((OD415Sample - OD415Blank) - 1.75*(OD595Sample - OD595Blank))*1000*13.5}{Time * (OD595Sample135\mu l - OD595Blank135\mu l)}$$

Miller units between mutants grown in normal media and inducing media were compared. Those showing expression of 2-fold or higher than that of normal media were selected as possibly interesting mutants. Each of these mutants was assayed 8 times to examine the reproducibility of increased β−glucuronidase activity.

3.2.7 Y-linker Method to determine presence of the mTn5gusA-oriV and random insertion

This method was performed as described (Kwon, 2000) for the efficient amplification of multiple transposon-flanking sequences. The oligonucleotides in Table 3.4 were used in this experiment and were synthesized by the Synthesis and Sequencing Facility at the University of Georgia. Formation of the Y-linker was done according to the procedure of Kwon (2000) and is presented in Figure 3.3.

Genomic DNA was isolated from *R. etli mTn5gusA-oriV* mutants using Qiagen cell and tissue kit (Qiagen, Cat. No. 69504) and completed digested with *Xho1* (Promega, Cat. No. R616A). Ligations of digested DNA to the Y-linker was performed as described (Kwon, 2000). Two microliter aliquots were used as templates in the PCR amplification. The PCR amplification is presented in Figure 3.3.

Table 3.4: Oligonucleotides used for Y-linker PCR. The portions of Y-linkers 1 and 2 in red portray the non-complemetary parts of the two oligonucleotides. The underlined portion in Y linker two is a 4 bp overhang that is complementary to overhangs produced from a *XhoI* digest. Therefore, ligations to *XhoI* digested DNA was expected to be straightforward and efficient. Primer Rhi 628 was designed to anneal to a sequence approximately 50bp from the 5' end of the transposon. Primer Gus 500 was designed to anneal to a sequence approximately 500 bp from the 5'end of the transposon.

Designation	DNA sequence (5' → 3')
Y linker 1	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACA
Y linker 2	TCGATGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT
Y linker primer	CTGCTCGAATTCAAGCTTCT
Gus500 primer	AGTCTGCCAGTTCAGTTCGTTG
Rhi628 primer	CTCCTTAGCTAGTCAGGTACCG

3.2.8. PCR amplification and gel electrophoresis of Y-linker products

All PCR reactions were performed using a BioRad iCycler®. Each mutant DNA extraction was amplified in duplicate using two separate sets of primers. One set used the Y linker primer and a primer specific to the transposon (Rhi628) near the 5' end of the *gusA* gene. The other set used the Y linker primer and a transposon specific primer (Gus500) located approximately 500 bp from the 5' end of the *gusA* gene. The reaction contained 2 µl of DNA template, 1 µl of Y linker primer and 1 µl primer Rhi628 or 1µl primer Gus500, 25 µl Master Mix (Promega, Cat. No. M7502) and 21 µl nuclease-free water. Activation of *Taq* DNA polymerase occurred with incubation at 95°C for 15 minutes. The target sequences were then amplified through 30 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5 minute, followed with a final extension at 72°C for 10 minutes. PCR products were analyzed on a 1.0% agarose and visualized with ethidium bromide.

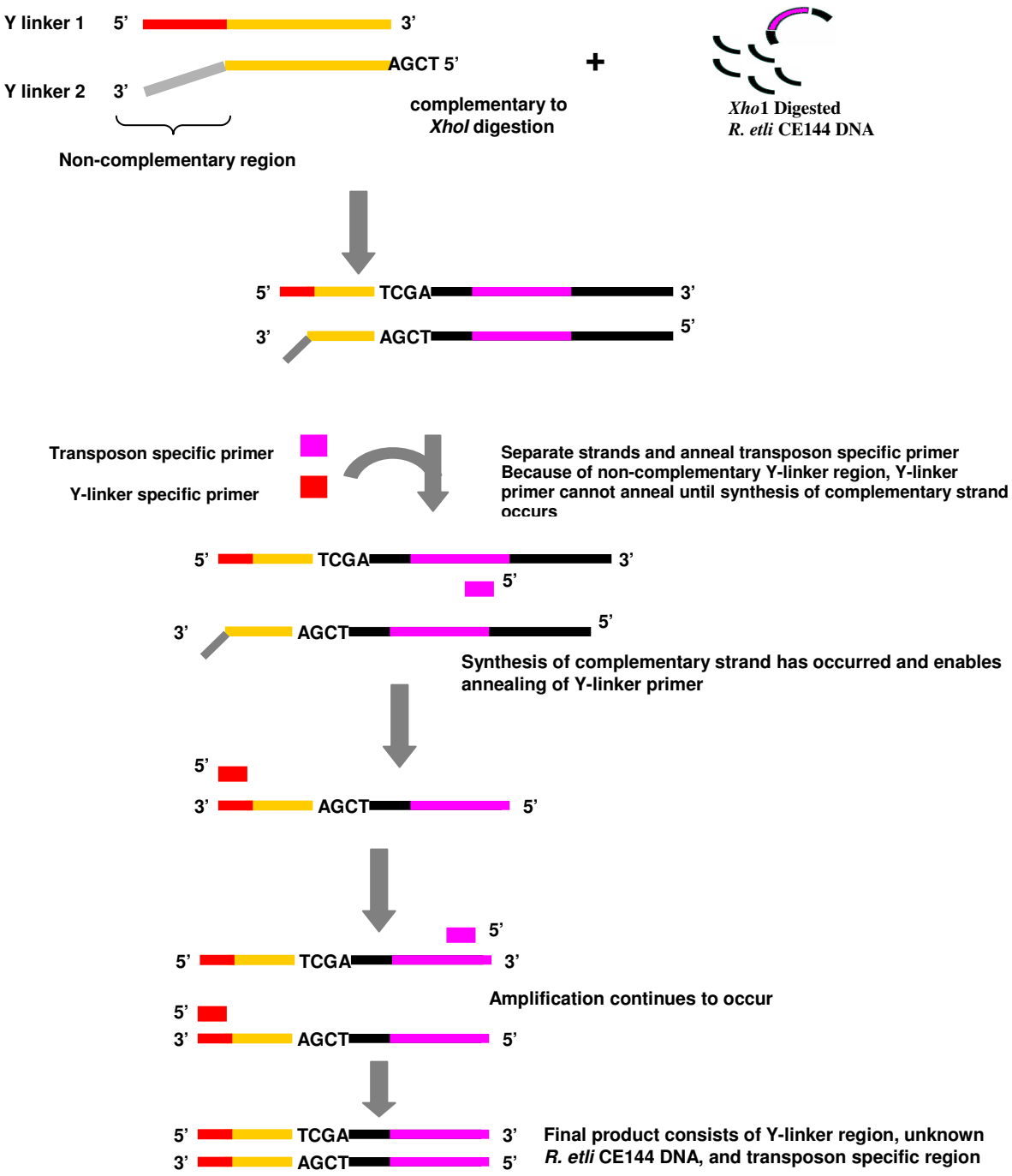

Figure 3.3. Schematic of Y linker method. The Y-linker was constructed as described in Materials and Methods. Two oligonucleotides, Y linker 1 and 2 were designed to have a noncomplementary region. Y-linker 2 was also designed to have a 4-bp overhang complementary to DNA subjected to a *XhoI* digest. Hence, when mixed with *XhoI* digested DNA and ligated, the Y-linker would attach to the digested fragments of DNA. Upon annealing of the transposon specific primer, synthesis produces the complement of Y-linker 1. The Y-linker specific primer is then able to anneal. Without the transposon present, no region of DNA would be produced for the Y-linker primer to anneal. Therefore, no amplification would occur.

3.2.9 Cloning interesting promoters into competent E. coli

R. etli CE144 *mTn5gusA-oriV* mutant DNA was extracted with a Qiagen cell and tissue kit (Qiagen, Cat. No. 69504), completely digested with *Xho1*(Promega, Cat. No. R616A), cleaned using DNA Clean and Concentrator-5 (Zymo Research, Cat. No. D-4003) and resuspended in 30 µl of nanopure water. The mixture was then ligated in the following reaction; 2 µl *Xho1* digested DNA, 10 µl T4 buffer, 1 µl T4 DNA ligase (Promega, Cat. No. M180A), 7 µl water for a final volume of 20 µl and incubated at room temperature overnight. After overnight incubation, 3 µl of ligation mixture were transformed into One Shot TOP10 Competent Cells (Invitrogen, Cat. No. C4040-10) according to manufacturer's instructions. Fifty microliters of each transformation mixture was plated on LB agar plates supplemented with 40 µg/ml kanamycin to select for presence of the transposon. The plates were incubated at 37°C overnight.

3.2.10 Miniprep on mTn5gusA-oriV transformants and preparation for sequencing

Two colonies from each of the four transposon mutants were selected for isolation of plasmid DNA to detect presence of *mTn5gusAoriV*. Plasmid DNA was isolated using a Qiagen miniprep kit (Qiagen, Cat. No. 27104) according to manufacturer's instructions. Isolated plasmid DNA was analyzed on a 1.0% agarose gel and visualized by ethidium bromide staining.

3.2.11 Preparation of plasmid DNA for sequencing

PCR products in the reaction mixture from the Y-linker PCR method were cleaned and resuspended to 30µl for sequencing. Products were sent to the UGA Synthesis and Sequencing Facility with two different primers; primer Rhi628 would anneal to the 5' end of the transposon and primer Gus500 would anneal 500bp from the 5' end of the transposon (see Table 3.4).

3.2.12 Analysis of sequence information

Sequences were submitted to several websites to search for significant similarities. The following websites were useful in determining sequence characteristics; National Center for Biotechnology Information (NCBI) in which each sequence was submitted as a BLAST (Basic Local Alignment Sequence Tool) inquiry (www.ncbi.nlm.nih.gov), the *Sinorhizobium meliloti* genome project (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/), the Rhizobase (www.kazusa.or.jp/rhizobase/index.html), and the Sanger Institute *Rhizobium leguminosarum* genome (www.sanger.ac.uk/Projects/R_leguminosarum/).

3.2.13 Phenotypic observations of mutant 87D8

Depending on the location of a mutation, a distinguishing phenotype may be observable. One of the four mutants showing inducible β-glucuronidase activity contained an insertion in a gene with high similarity to glutamine synthetase II. Here, we sought to determine any observable phenotypes that may support the evidence for effects of glutamine synthetase II on this mutant when grown at pH 5 conditions. Also, variations in growth under different conditions may suggest a role in symbiosis.

Growth in N-limiting and N-excess conditions. Vincent's defined media was prepared as described (Vincent, 1970). The pH of Vincent's media was adjusted to pH 5.0 as described (Vedam *et al,* 2003). Nitrogen-limiting conditions consisted of Vincent's media with 6.5mM glutamate serving as the sole nitrogen source. Nitrogen excess conditions consisted of 6.5 mM ammonium chloride only, or glutamate + ammonium chloride. The mutant 87D8, wild-type *R. etli*-CE3, and pSym$^-$ *R. etli* CE144 were plated on nitrogen-limiting and nitrogen-excess agar at pH 7 and pH 5 conditions and observations were noted.

β-glucuronidase activity in N-limiting and N-excess conditions. The β-glucuronidase assay was used to assess the activity of the putative glutamine synthetase II when subjected to nitrogen-limiting and nitrogen-excess conditions. The assay was performed as previously described in Materials and Methods.

Growth curves in N-limiting and N-excess conditions. To detect effects in the rate of growth, growth curves were done by growing mutant 87D8, wild-type *R. etli* CE3 and pSym$^-$ *R. etli* CE144 in nitrogen-limiting and nitrogen-excess conditions at pH 7 and pH 5 and measuring the OD at 595 nm over time.

Enzyme specific assay for glutamine synthetase II To detect the total glutamine synthetase present, the γGT assay was performed as described (Bender, 1977).

Phenol sulfuric acid assay. To detect levels of extracellular polysaccharide secreted from *R. etli* CE3, *R. etli* CE144, and 87D8 the phenol sulfuric acid assay was used. Cells were cultured in TY and glutamate + ammonium chloride medium at pH 7 and pH 5 conditions. Cells were pelleted and the supernatant removed. The assay was completed with the following components; 100 µl of supernatant, 100 µl 90% phenol, 1 ml concentrated sulfuric acid. The mixture was vortexed and allowed to sit at room temperature for 20 minutes. Optical density was measured at 490 nm.

3.3 Results

3.3.1 Naladixic acid and neomycin are suitable markers for selection

In developing a method to select for *R. etli* CE144 that had obtained a transposon insertion, it was necessary to use appropriate selectable markers. The construct *m*Tn*5gusAoriV* carries a kanamycin/neomycin resistance marker. Therefore, neomycin would be used in further

experiments. It was also necessary to have a unique marker that would select for *R. etli* CE144 and counterselect against any resilient donor *E. coli.* Naladixic acid was the first choice for use in counterselection since wild-type strain CE3 is resistant to the antibiotic.

R. etli CE144 exhibited no growth at any concentration of neomycin (30 µg/ml – 200 mg/ml), while being resistant to 30 µg/ml naladixic acid. *E. coli s17-lpir* pCMP8003 exhibited no growth on LB agar supplemented with 30 µg/ml naladixic acid at 30 and 37 degree Celcius, while exhibiting typical growth in the presence of 40 µg/ml kanamycin. It was concluded that the suitable mode of selecting for *R. etli* CE144 that had been subjected to transposon mutagenesis, was to use media containing 30 µg/ml naladixic acid and 100 µg/ml neomycin ($Neo^{100}Nal^{30}$).

3.3.2 β-glucuronidase activity leads to selection of four mutants up-regulated at pH 5

The β-glucuronidase assay involves the cleavage of pNPG into β-glucuronic acid and p-nitrophenol. The amount of p-nitrophenol produced may be measured at 417 nm and is an indirect measurement of activity of the gene in which the promoterless *gusA* gene has been inserted. From 10,000 mutants resulting from the transposon mutagenesis, 576 were assayed at pH5 conditions. Those mutants exhibiting greater than two-fold increase in β-glucuronidase activity as compared to gene expression during growth in normal pH 7 media were selected for further testing. This assay was then repeated to verify the stability of each mutation. Mutants showing a stable induction were considered to be inducible by low pH conditions and are shown below in Figure 3.4. A total of four mutants exhibited a notable and stable level of induction and were selected for further analysis. As an average of assays, the mutants 49F12, 50B5, 87A7, and

87D8 exhibited the following respective increases in *gusA* activity when grown at pH 5.0: 2.9-, 3.0-, 3.9-, and 2.2-fold.

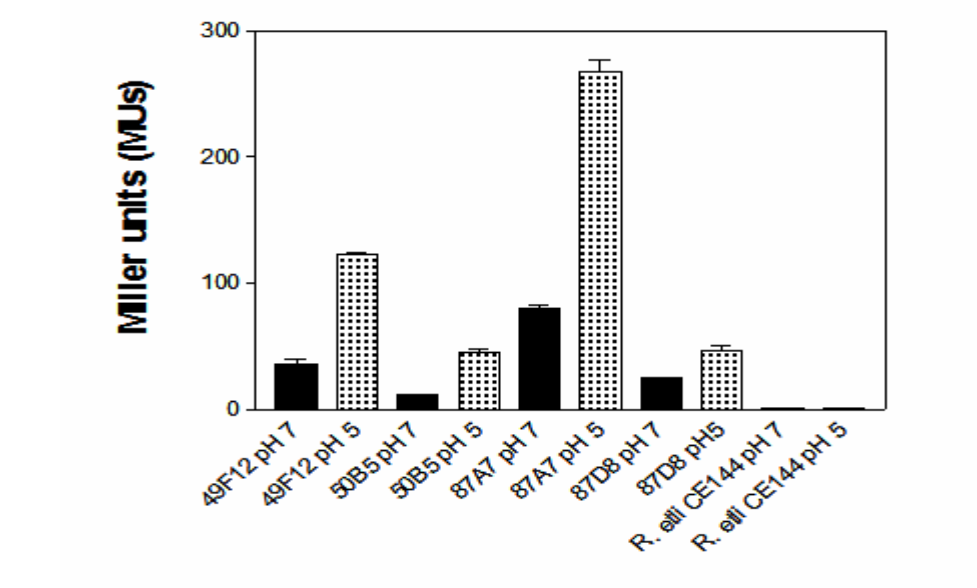

Figure 3.4 β-glucuronidase activity of 4 mutants showing induction at pH 5. Mutants 49F12, 50B5, 87A7, and 87D8, exhibited greater than 2-fold induction in the β-glucuronidase assay. As a negative control, *R. etli* CE144 was subjected to the same conditions to ensure no background β-glucuronidase activity was being produced.

3.3.3 Presence of >5kb band indicates recovery of mTn5gusAoriV

To maintain the mutant DNA in an *E. coli* background, mutagenized DNA was introduced into *E. coli* TOP10. For each mutant, genomic DNA was extracted and subjected to a complete digest with *Xho1*. *XhoI* does not cut within the transposon. Therefore, the intact transposon with flanking *R. etli* CE144 genomic DNA would result after a digest. The DNA was then self-ligated and transformed into competent *E. coli* TOP10 cells. Selection for the transposon on media supplemented with 40 µg/ml kanamycin produced colonies of which two were randomly selected from each plate. Minipreps were performed on selected colonies to determine the presence of the transposon. The minipreps were digested with *XhoI* and

electrophoresed on a 1% agarose gel as shown in Figure 3.5. The two colonies resulted in identical bands for 49F12 (lanes 3 & 4), along with 87A7 (lanes7 & 8). In the case of 50B5 (lanes 5 & 6) and the 87D8 (lanes 9 & 10), the randomly selected two colonies for each produced two distinct banding patterns. It is possible that two distinct colonies were present for 50B5. For 87D8, it may be that the mutant contains two transposon insertions. The exact reason for this is not known, but the presence of distinct insertions in 50B5 and 87D8 was verified with sequencing.

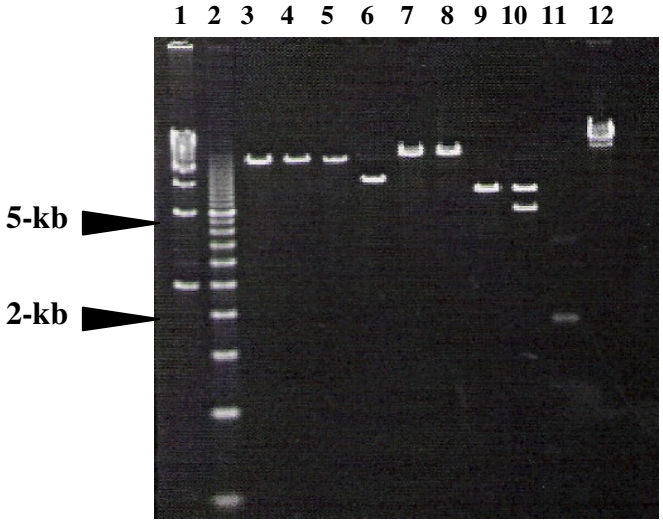

Figure 3.5. Recovery of plasmid containing *mTn5gusAoriV*. The four mutants in Figure 3.4 were subjected to a genomic DNA extraction. Genomic DNA was completely digested with *Xho*I and then religated with itself. Competent *E. coli* TOP10 cells were transformed with the self- ligated mutant DNA and selected for colonies containing the transpson. Two random colonies containing the transposon were selected for plasmid preparation. All lanes represent minipreps of the *E. coli* TOP10 transformants. Lanes were assigned as follows: Lane 1-2.5 kb marker; Lane 2- 500 bp marker; Lanes 3-4- 49F12; Lanes 5-6- 50B5; Lanes 7-8- 87A7; Lanes 9-10- 87D8. Lane 11 is a control pUC18 that should not be cut by *XhoI.* Lane 12 is lambda DNA and serves as a positive control for the digest indicating *XhoI* was functioning properly.

3.3.4 Y-linker method is successful in indicating presence of transposon

According to Kwon *et al*, the Y-linker PCR method may be used to determine randomness of transposon insertion and to efficiently amplify flanking DNA of transposon

insertions. The principle of this method was illustrated in Figure 3.3. In short, a synthesized known sequence (Y-linker) is ligated to the *XhoI* transposon fragment. A primer specific to the transposon was used along with a primer specific to the Y-linker. Therefore, no amplification is expected to occur without the presence of the transposon. Two transposon-specific primers were used in this case. Primer Rhi628 anneals to the *gusA* specific 5' end of the transpson and is represented by lanes 2-5 in Figure 3.6.

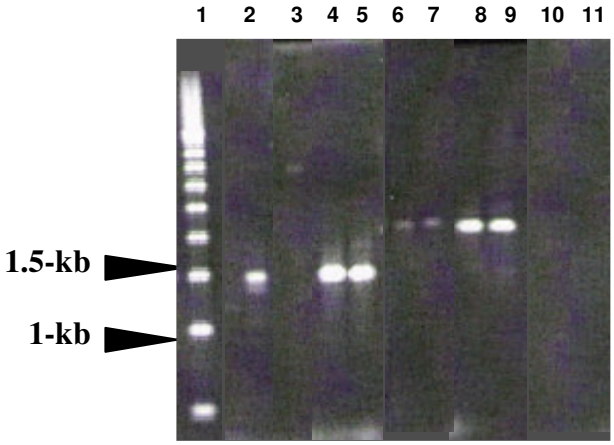

Figure 3.6. PCR method using Y-linker to determine presence of *mTn5gusAoriV*. Genomic DNA extracted from each mutant was subjected to a complete digest with *XhoI.* After self-ligation, transformation into competent *E.coli,* selection on kanamycin, and preparation of plasmid DNA, the resulting DNA was subjected to the Y-linker PCR as described in the Materials and Methods. Two primer sets were used, Y linker primer and Rhi628 (lanes 2-5) and Y linker primer and Gus500 (lanes 6-9). Lane designations are as follows; Lane 1-500bp marker; Lanes 2-5; 49F12, 50B5, 87A7, and 87D8 with primer Rhi628; Lanes 6-9- 49F12, 50B5, 87A7 and 87D8 with primer Gus500; Lanes 10-11- *R. etli* CE144 serving as negative control. This experiment was independently repeated three times.

Primer Gus500 was designed to anneal approximately 500 bp from the 5' end of the transposon and is represented by lanes 6-9 in Figure 3.6. Mutant 49F12 reveals a band at ~1.5kb with primer Rhi628 (lane 2) and one approximately 500 bp larger with primer Gus500 as expected (lane 6). Similar results were visible with mutants 87A7 and 87D8. However, mutant 50B5 revealed a seemingly large band at ~5kb with primer Rhi628 (lane 3). Instead of a larger

band being produced from the use of primer Gus500, mutant 50B5 exhibited a band similar in size to the other mutants at 2.0 kb (lane 7). The reason for this is not known. A negative control of *R. etli* CE144 with no transposon insertion revealed no bands as expected (lane 10 & 11). This was necessary to ensure that the PCR products produced were not artifacts.

3.3.5 Sequencing information supports independent insertion in four distinct genes

Sequence information is necessary to search databases for significant alignments. It was also necessary to verify that each of the four mutants were distinct in their makeup. For sequencing of the four mutants induced at low pH conditions, PCR products from the Y-linker method were cleaned and sequenced by the Synthesis and Sequencing Facility at the University of Georgia. Sequencing runs were done using the following primers: Rhi628 and Gus500 (Table 3.4). Each sequence was analyzed for the presence of the primer, transposon specific and Y-linker regions. The results may be viewed below in Figure 3.7. In the case of mutant 49F12, only portions of the Y-linker could be detected at the 3' end of in the sequence resulting from primer Rhi628. The sequence resulting from primer Gus500 had too many errors to be useful. The 50B5 sequence showed distinct portions of both the transposon and of the Y-linker. The results from both sequences overlapped and shared 578 bp. Similar results were obtained for 87A7 and 87D8. Mutant 87A7 shared approximately 656 bp between the two sequence sets, while mutant 87D8 shared 593 bp.

3.3.6 Submission to the BLAST database reveals significant homologies

Sequence information obtained for each mutant was submitted to the NCBI database (www.ncbi.nlm.nih.gov) as a BLASTx inquiry. BLAST (Basic Local Alignment Search Tool) is

based on the theory of local alignments. The concept of local alignment is to find isolated regions in sequence pairs that have high levels of similarity. It is important to note that the

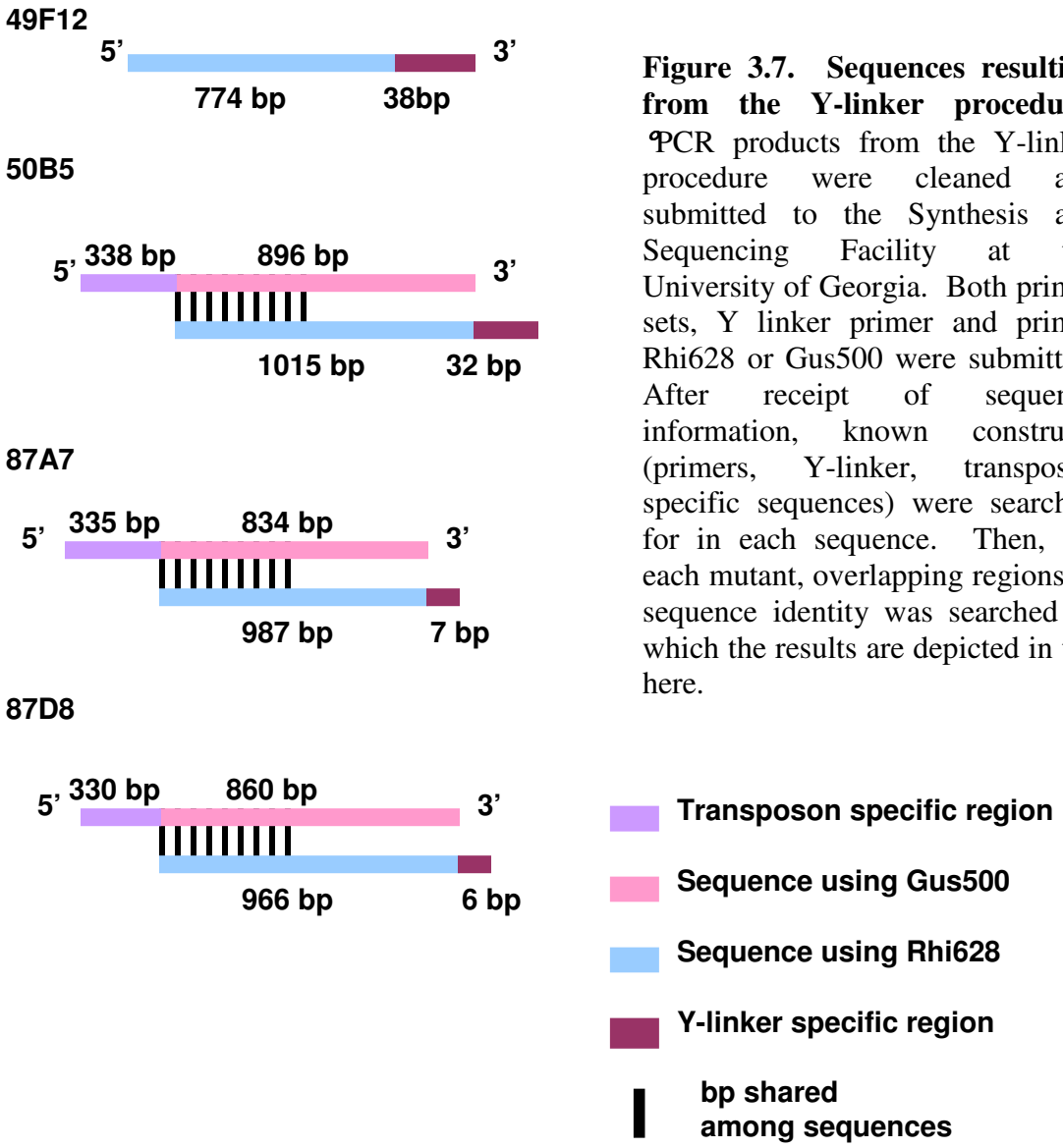

Figure 3.7. Sequences resulting from the Y-linker procedure. PCR products from the Y-linker procedure were cleaned and submitted to the Synthesis and Sequencing Facility at the University of Georgia. Both primer sets, Y linker primer and primer Rhi628 or Gus500 were submitted. After receipt of sequence information, known constructs (primers, Y-linker, transposon specific sequences) were searched for in each sequence. Then, for each mutant, overlapping regions of sequence identity was searched of which the results are depicted in the here.

significant hits for the four sequences were either other species of *Rhizobium* an other phylogenetically related genus. The top four hits were recorded along with their respective E values and percent identities. The E-value is similar to the traditional P-value of statistical

hypothesis tests. The lower the E-value, the less likelihood that the similarities are the result of random chance.

Table 3.5. 49F12 exhibits similarity to an oxidoreductase

Gene	Organism	E value	Identity
D-threo-aldose 1-dehydrogenase	*M. loti MAFF303099*	2e-35	63%
Predicted oxidoreductase (aryl-alcohol dehydrogenase)	*Rhodobacter sphaeroides*	7e-30	54%
Predicted oxidoreductase (aryl-alcohol dehydrogenase)	*Burkholderia fungorum LB400*	2e-25	47%
Predicted oxidoreductase (aryl-alcohol dehydrogenase)	*Mesorhizobium sp.* BNC1	1e-21	42%

Table 3.6. 50B5 exhibits similarity to an ABC transporter

Gene	Organism	E value	Identity
Probable ATP-binding/permease fusion ABC transporter	*A. tumefaciens*	6e-32	77%
ABC Superfamily, ATP binding	*Acinetobacter sp. ADP1*	4e-27	62%
ATP-binding protein of ABC transporter	*M. loti MAFF303099*	9e-26	63%
Putative ABC transporter	*N. meningitidis Z2491*	1e-25	63%

Table 3.7. 87A7 exhibits similarity to a hydrolase

Gene	Organism	E value	Identity
Hypothetical protein, probable hydrolase	*A.tumefaciens*	8e-19	51%
Hydrolase	*A. tumafaciens*	8e-19	62%
Conserved hypothetical protein	*S. meliloti*	3e-17	49%
Hydrolase, haloacid dehalognase family	*Brucella abortus*	4e-13	41%

Table 3.8. 87D8 exhibits high similarity to glutamine synthetase II

Gene	Organism	E value	Identity
Glutamine synthetase II	*R. etli*	4e-79	99%
Glutamine synthetase II	*R. leguminosarum bv. viciae*	5e-79	98%
Glutamine synthetase II	*R. leguminosarum*	6e-78	95%
Glutamine synthetase II	*M. loti MAFF303099*	8e-78	95%

3.3.7 Phenotype characterization of 87D8

87D8 was chosen for further phenotypic analysis because of the extremely high similarity to glutamine synthetase II, a known and assayable enzyme. 87D8 was first subjected to a β-glucuronidase assay to once more verify an increased level of activity at low pH, (data not shown). The results confirmed the increased level of induction at pH5 conditions to be 1.88-fold greater than activity at pH7. This assay was then repeated with growth of mutant 87D8 under nitrogen limiting and nitrogen excess conditions at pH7 or pH5. Growth under these same conditions was also monitored on agar plates for any observations of visual phenotypic

variations. Finally, to detect any differences in enzyme activity when subjected to different conditions, an enzyme specific test for glutamine synthetase was performed.

Growthof 87D8 in nitrogen limiting and nitrogen excess conditions on solid media. In attempts to detect any phenotypic variance in the mutant, we subjected mutant 87D8 to several growth conditions in which nitrogen was limited (glutamate only) and nitrogen was in excess (ammonium chloride only or glutamate + ammonium chloride). Growth in each of these conditions was also done at pH7 or pH5.

Growth of wild-type *R. etli* CE3 was typical at pH 7 conditions in the presence of glutamate or glutamate +ammonium chloride. There was a notable difference in colony size with glutamate allowing production of very large colonies. However, in the presence of only ammonium chloride at pH 7 conditions, growth was retarded with production of smaller than normal colonies. At all pH 5 conditions, *R. etli* CE3 formed very small colonies

Growth of pSym$^-$ *R. etli* CE144 on ammonium chloride at pH 5 revealed little growth and tiny colonies, while at pH 7 growth was normal. Growth at pH 7 or pH5 in the presence of glutamate produced small colonies. In fact, the colonies resulting from pH 5 growth were extremely small, perhaps due to cessation of growth. In the presence of glutamate + ammonium chloride in pH 7 conditions, growth was typical producing large colonies; however, no growth was evident at pH 5.

87D8 exhibited apparent confluent growth at all conditions and at both pH 7 and pH 5. The 87D8 colonies were also abnormally large, amorphic, watery nature and milky white color (at pH 7 and pH 5) as seen in the left portion of Figure 3.8 compared to *R. etli* CE144 as seen in the right portion of Figure 3.8. It was not possible to distinguish individual colonies with this

mutant since they seemed to run together. This was a notable phenotype in that 87D8 produced colonies typical of *R. etli* CE144 on TY agar at pH 7 or pH 5 conditions.

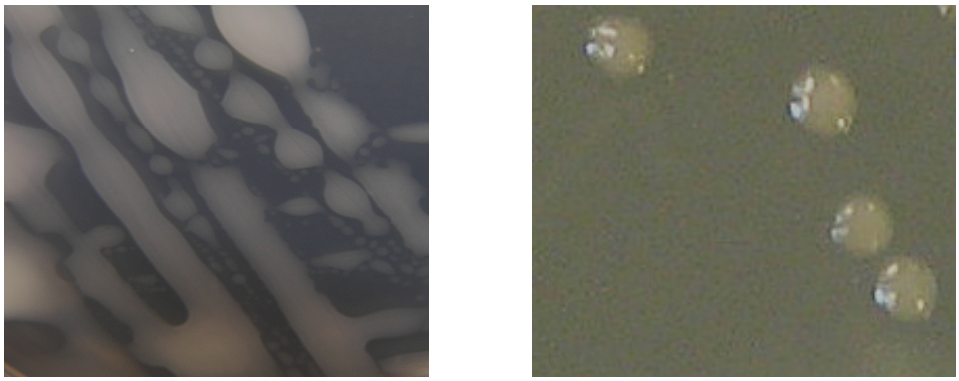

Figure 3.8. Phenotype on Vincent's defined minimal media. Cultures were plated on Vincent's defined minimal media with glutamate as the nitrogen source. To the left is 87D8 and to the left is the parent strain *R. etli* CE144.

Doubling times of 87D8 in nitrogen limiting and nitrogen excess conditions in liquid media.

While growth differences were indicated on solid media, it is difficult to detect an observable difference in the rate of growth on solid media. In order to obtain more accurate measurements of growth, growth curves were performed using liquid media. 87D8 was cultured in TY at pH 7 or pH 5 conditions and Vincent's minimal medium (Vincent, 1970) with the following supplements; glutamate only, ammonium chloride only, or glutamate + ammonium at pH7 or pH5 conditions. Optical density (595 nm) was measured over time and doubling time calculated. The results are shown in Table 3.9.

R. etli CE3 exhibited a faster doubling time in all pH 7 conditions as compared to pH 5 conditions, with the exception of media supplemented with glutamate + ammonium chloride. Under these conditions, *R. etli* CE3 showed a 2.7-fold increase in doubling time at pH 5 compared to pH 7. Doubling times were comparable in the presence of ammonium chloride or

glutamate at pH 7 conditions. However, there is a notable lag in doubling time in the presence of ammonium chloride at pH 5 conditions. As expected, doubling times in TY media at both pH 7 and 5 were faster than all other conditions with the pH 5 condition being 2.6-fold slower than pH 7.

R. etli CE144 exhibited a faster doubling time at pH 7.0 conditions in the presence of ammonium chloride compared to growth at low pH. Doubling times in the presence of glutamate only at both pH 7 and 5 were comparable. In contrast, in the presence of glutamate + ammonium chloride, the doubling time of the pH 7 condition was twice as fast as the pH 5 condition. This finding was consistent with growth differences observed on agar plates in which colonies were extremely small on pH 5 vs pH 7 agar.

87D8 exhibited doubling times comparable to the parent strain *R. etli* CE144 when grown on TY media. Doubling time on glutamate at both pH conditions were similar to each other and were extremely slow. No growth was detectable under pH 7 ammonium chloride or pH 5 glutamate + ammonium chloride conditions.

Table 3.9. Doubling times of *R. etli* CE3, *R. etli* CE144, and 87D8 in N-limiting and N-excess conditions. Cultures were grown and optical density measured over time as described in the Materials and Methods. Doubling time was caculated by determining the time (in hours) needed for the culture to double in turbidity. The quantities given are shown in hours. NG= no growth

	A7	**A5**	**G7**	**G5**	**GA7**	**GA5**	**TY7**	**TY5**
***R. etli* CE3**	**6.5**	**20**	**7.5**	**11**	**17.5**	**6.5**	**2.5**	**6.5**
***R. etli* CE144**	**7.5**	**18.5**	**17.5**	**20**	**9**	**18.5**	**6.5**	**11**
87D8	**NG**	**35.5**	**46.5**	**46.5**	**18.5**	**NG**	**6.0**	**9.0**

β-glucuronidase assay in nitrogen limiting and nitrogen excess conditions of 87D8. β-glucuronidase activity was measured to detect possible differences under the varying growth conditions. Mutant 87D8 was cultured similarly to the initial microtiter plate cultures, except media included the following compositions at pH7 and pH5: TY, glutamate only (**G**), ammonium chloride only (**A**), and, glutamate + ammonium chloride (**GA**). The production of p-nitrophenol is expected to be proportional to the activity of glutamine synthetase II. If expression of the putative glutamine synthetase II was affected in some way in response to different conditions, there should be a corresponding increase or decrease in β-glucuronidase activity. The results are shown in Figure 3.9. As expected, in TY media, the pH5 condition was significantly induced, approximately 2-fold, as compared to the pH7 condition. Growth under conditions of nitrogen excess (ammonium chloride only or glutamate + ammonium chloride) produced slight levels of induction at 1.4-fold and 1.2-fold respectively. Growth in glutamate only medium (nitrogen limiting) produced a large 3.7x increase in β-glucuronidase activity at pH5 conditions.

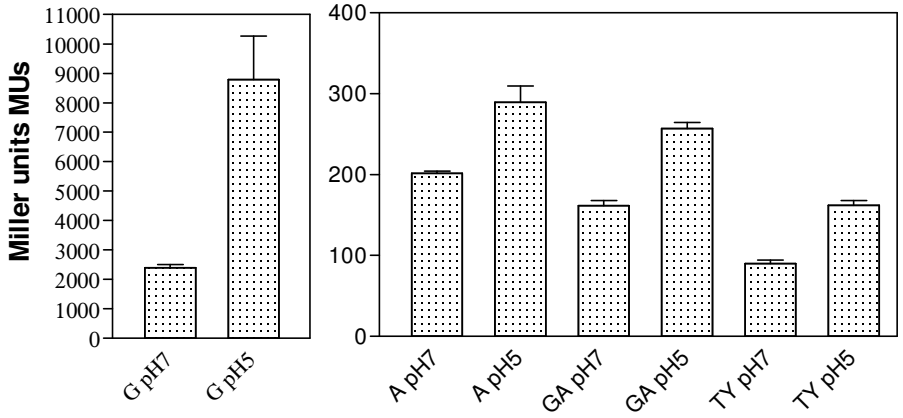

Figure 3.9. Effects of glutamine synthetase II mutant under various growth conditions. Mutant 87D8 was subjected to nitrogen limiting (glutamate only, **G**), nitrogen excess (ammonium chloride, **A** only, or glutamate + ammonium chloride, **GA**) conditions at pH7 and

pH5, and Tryptone-Yeast (**TY**) at pH 7 and pH 5. Activity of the mutant was measured via the β-glucuronidase assay as described previously in the Materials and Methods and the results expressed in Miller units.

Glutamine synthetase enzyme assay. Glutamine synthetase (GS) in *Rhizobium* is a major enzyme in the assimilation of ammonia. *Rhizobium* are known to contain at least two forms of glutamine synthetase, I and II and may contain a GSIII (Somerville and Kahn, 1983). GS I is heat stable and GS II is heat labile. Glutamine synthetase I and II (GSI and GSII) catalyze the conversion of ammonium and glutamate to glutamine (Somerville and Kahn, 1983). The γGT assay is used to measure the total amount of GS (both GSI and GS II) and was performed as described (Bender, 1977) and the results shown in Figure 3.10.

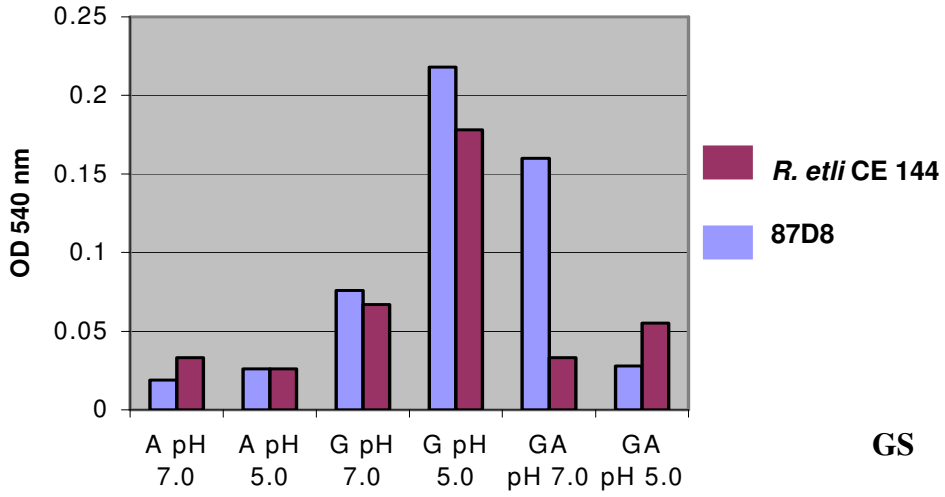

Figure 3.10. GS levels of *R. etli* CE144 and 87D8 in N-limiting and –excess conditions.

Both *R. etli* CE144 and 87D8 exhibited greater than 2-fold increase in GS levels in the presence of glutamate at pH 5.0 conditions compared to pH 7.0. GS levels of *R. etli* CE144 and 87D8 in the presence of ammonium chloride at both pH's were significantly decreased in comparison to glutamate only conditions. In the presence of glutamate + ammonium chloride at pH 7, the GS level of 87D8 was greater than 3-fold higher than that of *R. etli* CE144. GS leves for *R. etli* CE3

were also measured (data not shown). For *R. etli* CE3 the highest GS levels were evident in the presence of glutamate at pH 7. This was comparable to the level due to glutamate + ammonium chloride at pH 7. GS levels at all other conditions were slightly decreased.

Phenol sulfuric acid assay to detect extracellular polysaccharide levels. 87D8 and parent strain *R.etli* CE144 exibited a comparable increase in levels of extracellular polysaccharide at pH 5 conditions compared to pH 7 conditions when grown in TY media as shown in Figure 3.11 However, in the presence of glutamate + ammonium chloride, *R. etli* CE144 shows a dramatic decrease in levels at pH 7 and pH 5. In contrast, the levels of extracellular polysaccharide for 87D8 in the presence of glutamate + ammonium chloride remained relatively high at pH 7 and pH 5 conditions. This is consistent with the observation of slimy cells on glutamate + ammonium chloride agar medium.

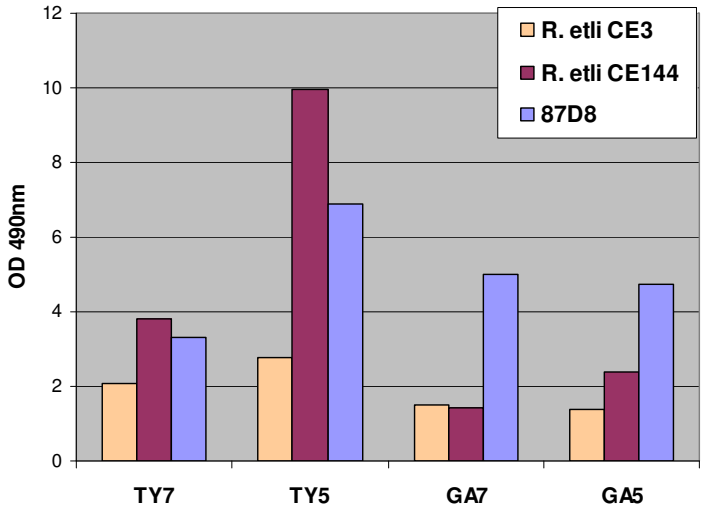

Figure 3.11. Extracellular polysaccharide levels of *R. etli* CE3, *R. etli* CE144, and 87D8. Extracellular polysaccharide levels were measure by the phenol sulfuric acid assay as described in the Materials and Methods. Results were normalized so that OD 490nm measurements represent the same number of bacterial cells for each respective strain.

3.3.8 Subjection to flavonoid conditions reveals no significant levels of increased activity

Flavonoids are plant-derived and are produced in response to signals from the *Rhizobium.* Common flavonoids, such as naringinin and hesperitin, are able to interact with a LysR-type activator within the bacterium known as NodD. Upon interaction with NodD, a conformational change occurs which allows binding to the *nod* box. *Nod* boxes are conserved sequences located upstream of the *nod* genes. When bound by NodD, transcription of the *nod* genes may occur and the result is the production of Nod factors in the form of lipochitinooligosaccharides (LCOs). Nod factors interact with the host plant and induce root hair curling and cortical cell division. It has been a widely accepted idea that NodD needs to be present in order for flavonoids to have an inducing affect on *Rhizobium.* We sought to test this theory in *R. etli* CE144 which lacks the NodD protein. Mutants resulting from the transposon mutagenesis were subjected to 1μM naringinin. The β-glucuronidase assay was used to detect any observable increases in activity. Mutants exhibiting increased activity after the first assay were selected for to determine reproducibility. There were no reproducible increases in activity in the presence of 1 μM naringinin (data not shown). This supports the widely held idea that NodD is needed for the inductive effects of flavonoids. However, only 197 of the 10,400 colonies were screened and further testing needs to be completed.

3.4 Discussion

Tn5 mutagenesis is a suitable strategy for the generation of mutants in the pSym$^-$ *R. etli* CE144.

The genome of *R. etli* is approximately 5000 kb (http://kinich.cifn.unam.mx/~retligen/). This project involved the detection of mutants on a genome-wide scale. The goal was to obtain the number of mutants that would theoretically represent the entire genome (approximately 5,000 mutants). Transposon mutagenesis was used to mutagenize the genome of *R. etli* CE144. This mutagenesis, coupled with the selection process was successful in obtaining approximately 10,400 *R. etli* CE144::*mTn5gusA* insertion mutants. A total of 576 were subjected to pH 5 conditions and a stable 2-fold induction of β-glucuronidase activity occurred for 4 (49F12, 50B5, 87A7, and 87D8) of the 576 mutants.

Sequencing of the *Tn5* insertion mutants indicates disruptions of several genes that may be important in symbiosis.

The Y-linker PCR method was employed not only to determine insertion of the transposon, but to generate PCR products to obtain sequence information. Analysis of sequences revealed insertion mutants in a predicted oxidoreductase, ABC transporter, hydrolase and glutamine synthetase II.

49F12 is a predicted oxidoreductase. The NCBI BLAST submission for the 49F12 sequence revealed similarity to predicted oxidoreductases (specifically alcohol dehydrogenases) with the top hit being in *M. loti* (see Table 3.5). Levels of alcohol

dehydrogenase have been shown to parallel levels of nitrogenase activity (Spaink., 1998). This may suggest an importance for alcohol dehydrogenase within the nodule.

The Sanger institute database for *R.leguminosarum* showed a similarity on the chromosome to PssT (rl3665) transmembrane component of the type I exopolysaccharide (EPS) transport system. Neighboring genes were found to be the remaining cluster involved in EPS biosynthesis and transport; PssN (rl3664) involved in EPS export, PssO (rl3663), PssP 9rl3662) involved in EPS polymerization. Exopolysaccharide productions by *R. leguminosarum* bv. trifolii is required for successful establishment of nitrogen-fixing symbiosis with clover (Wielbo, 2004). A mutant in PssT producing increased amounts of total EPS increased the number of nitrogen fixing nodule on clover (Mazur, 2003).

50B5 is a predicted ABC-transporter. The 50B5 sequence was suggested to contain an insertion in a putative ABC transporter. These results are consistent with all databases used. The ABC transporters make up a large superfamily of proteins which share a common function and common ATP-binding domain (Fath and Kolter, 1993). Gene identification numbers using Rhizobase includes, mlr4641 for *M. loti*, smc03900 for *S. meliloti,* and blr3795 for *B. japonicum*. Neighboring genes included a glucokinase in *M. loti* and putative saccharide exporting or lipid A/LPS core export proteins in *S. meliloti* (*Sinorhizobium* genome project). The Sanger Institute revealed an ABC ATPase involved in glucan export. In all cases, the involvement in sugar transport is suggested and may be important in synthesis of lipopolysaccharide, capsular polysaccharide or exopolysaccharide which are thought to be essential in the symbiotic process (Cava, 1989). A mutation into a protein secretion gene, showing significant homology to ABC transporters, of *R. leguminosarum* bv. trifolii allowed a

normal production of EPS but was not effective in nodulation (Krol and Skorupska, 1997). Mutations in ATP-binding cassettes and transporters in *R. leguminosarum* bv. trifolii may be responsible for defective nodulation (Krol, 1997).

87A7 is a predicted hydrolase. NCBI BLAST submission of 87A7 sequence revealed high similarity to a hydrolase in *A. tumefaciens.* A crucial event of the *Rhizobium*-legume infection process is the breaching of the host cell wall for bacterial entry. This is overcome by the use of cell wall degrading enzymes such as hydrolases. A combination of cell wall degrading enzymes allows *Azorhizobium caulinodans* to produce more nodules (Buvanna, 2002) implying a necessary role for hydrolases in optimal symbioses.

87D8 is a predicted glutamine synthetase II. Results of the 87D8 sequence submission were unanimous in confirming a high similarity to glutamine synthetase II. In all cases, the E-values and similarities were remarkable as shown in Table 3.8. In *M. loti* (according to Rhizobase), the putative glutamine synthetase (mlr0339) is located on the chromosome and is adjacent to a probable integral membrane sugar transporter (mlr0338). The gene for glutamine synthetase I is adjacent to the opposite side and is oriented in the opposite direction of glutamine synthetase II. The *S. meliloti* glutamine synthetase II predicted gene (smb20745) is located on the pSymB which is consistent with previous findings (Somerville and Kahn, 1983). The location of smb20745 is two genes upstream of a putative glycosyltransferase protein (smb20748). Using Kazusa, the predicted glutamine synthetase II was found within the vicinity of an exopolysaccharide production negative regulator. Rhizobia assimilate ammonia via glutamine synthetase and is important in the regulation of nitrogen. Plants are preferentially

nodulated by rhizobia capable of ammonia assimilation via glutamine synthetase, suggesting a role for glutamine synthetase in facilitating optimal symbiosis (Bravo, 1988).

No similarities to *R. etli* pSym. In the submission of all sequences, the symbiotic plasmid of *R. etli* was also searched for similarity. In all cases, no similarities were produced to pSym genes. This confirms the pSym$^-$ nature of *R. etli* CE144 and that all current mutant sequences do not include pSym genes.

Phenotypic characterization of mutant 87D8.

Activity and levels of glutamine synthetase. The reaction of glutamine synthetase (GS) involves the assimilation of ammonia and glutamate to form glutamine (Somerville and Kahn, 1983). Glutamine synthetase, along with glutamate synthase, is believed to play a role in the cycling of glutamine. Operation of this glutamine cycle is thought to be necessary for carbon utilization in *Rhizobium* (Duran, 1996). When the levels of glutamate are high, then the levels of glutamine synthetase should be proportional in order to cycle glutamate into the glutamine cycle. The levels are expected to be intermediate in the presence of ammonium chloride and low in the presence of glutamine (Stacey, 1992). Using the β-glucuronidase assay, the results, as shown in Figure 3.10, revealed slight levels of induction at pH 5 when subjected to nitrogen excess, A and GA, conditions. However, a high level of induction was noted in the presence of nitrogen-limiting, G, with a 3.7-fold increase over pH 7. These results seem to follow this pattern with a higher level of induction in the presence of glutamate only (nitrogen limiting) and lower levels in the presence of ammonium chloride and ammonium chloride/glutamate (nitrogen excess).

Ammonia assimilation requires GS activity and GS is the key enzyme that regulates the nitrogen flux in the cell. Biosynthesis of GS is repressed by high ammonia concentrations. GS is induced under conditions of nitrogen starvation and aid in the survival when the environment is low in nitrogen (Stacey, 1992). This may indirectly indicate that slight increases in pH may decrease the levels of GS activity. This also helps to support the induction of β-glucuronidase activity in response to a low pH environment. If the ammonia concentrations are high, due to its weak alkaline nature, the pH may increase slightly. Also, in nitrogen excess conditions (A and GA), there is no need for the induction of GS because assimilation of ammonia for the means of survival is not an issue.

As mentioned previously, GS levels are repressed in the presence of ammonium and induced in the presence of glutamate. Both *R. etli* CE144 and 87D8 exhibited greater than 2-fold increase in GS levels in the presence of glutamate at pH 5 conditions compared to pH 7 conditions. Both were severely depressed with ammonium chloride as the nitrogen source. The mutation in GSII should reveal a decrease in GS levels compared to the parent strain *R. etli* CE144. However, levels seemed comparable. What is notable is the drastic decrease in the GS level of 87D8 in the presence of glutamate + ammonium chloride when shifting from pH 7 to pH 5 (Figure 3.10) This may indicate that GSII is important in N-excess conditions at pH 5. As mentioned previously, rhizobia contain at least two glutamine synthetases (GS I and II) and may contain a third (GSIII). It is likely that in the presence of a GSII mutation, a second GS may compensate for the deficiency.

GSII was shown to be induced in N-limiting conditions at pH 5 and repressed in N-excess conditions at pH 5. These results may imply a role for GSII in symbiosis. Previous findings support this theory; *B. japonicum glnAglnII* mutants were severely affected in nodule

infection and nitrogen fixation (Carlson, 1987). A *glnA* mutant of *R. etli* was impaired in nitrogen fixation by 50% (Moreno, 1991). In addition, a GS^- mutant of *A. caulinodans* was also Fix^- (Donald and Ludwig, 1984).

Growth of 87D8 on solid and liguid media. 87D8 produced colonies exhibiting an extremely slimy, pleomorphic appearance distinct from wild type *R. etli* CE3 and parent strain *R. etli* CE144 on nitrogen-limiting or nitrogen-excess defined agar medium. This may be an indication of an overproduction of a cell surface component. This is supported by results of the phenol sulfuric acid assay, in Figure 3.11, in that 87D8 appears to produce a higher level of extracellular polysaccharide compared to the wild-type and parent strains, when grown in GA at pH 7 or pH 5. It is possible that the regulation of neighboring genes (i.e. regulator of polysaccharide export) is affected by a mutation in GSII. As mentioned previously, GS in other *Rhizobium* species are located in the vicinity of integral membrane sugar transporter, glycosyltransferases and exopolysaccharide negative regulators. GS or GS regulation may play a role in the transcription of these genes.

87D8 exhibited an overall increase in doubling times in nitrogen-limiting and nitrogen-excess conditions compared to wild-type *R. etli* CE3 and *R. etli* CE144. This is a drastic phenotype compared to growth in TY media at pH 7 or pH 5 conditions, which was comparable to the wild type and parent strains. From the observations, it seems that growth in N-limiting and N-excess defined conditions reveals an auxotrophic nature of 87D8. The auxotrophy is overcome with some factor provided in complex TY media. It would be expected that since GS is up-regulated at nitrogen limiting conditions, that 87D8 would suffer in growth. The doubling time seems to follow this pattern. In the presence of glutamate only (when GS levels are

expected to be high), 87D8 exhibits an extremely slow doubling time. This may be attributed to the mutation in GSII. It appears that the growth deficiency is not due to a shift in pH. However, the reason is not yet known.

Conclusions

Two methods were employed to discover genes involved in symbiosis that are not located on the pSym. Approach 1, conventional cloning, proved to be labor intensive and yielded unreliable results. Approach 2, transposon mutagenesis, was promising and lead to the isolation of four insertion mutants that were induced by low pH conditions. These insertion mutants represent a probable oxidoreductase, hydrolase, ABC transport and glutamine synthetase II. No conclusions are evident about the role they may have in symbiosis. However, their importance in symbiosis was postulated. The glutamine synthetase II insertion mutant was subjected to phenotypic characterization to detect alterations from the parent strain *R. etli* CE144 and wild-type *R. etli* CE3.

The advantages of this method include the ability to rapidly and efficiently generate a large bank of *R. etli* CE144 mutants. These mutants are able to be effectively and reproducibly screened via the β-glucuronidase assay to get an accurate assessment of induction under simulated symbiotic conditions.

In spite of the advantages, *Tn5* insertion mutants in *R. etli* CE144 would not enable *in planta* studies. This is due to the $pSym^-$ nature of *R. etli* CE144 which is required for symbiosis to occur. In contrast to Approach 1, the pSym would need to be introduced into each *R. etli* CE144 insertion mutant needing to undergo symbiotic assessment.

As just mentioned, introduction of pSym into *R. etli* CE144 to perform *in planta* studies is essential. This may be possible by phage transduction and its possibilities are being assayed (Elmar Kannenberg, personal communication). Also, since the sequence surrounding the *Tn5* insertion is now known, a mutation may be generated in the wild-type *R. etli* CE3.

In addition, further screening of remaining insertion mutants needs to be complete. A total of 576/10,400 insertion mutants were screened on pH 5 conditions. However, only 196 were screened for the affects of flavonoids. Screening of the entire bank may elucidate more genes affected by low pH conditions or genes induced by flavonoids. According to the current progress of this project, it is predicted that approximately 80 non-pSym genes may be inducible by symbiotic conditions.

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