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Generation and characterization of a novel Rhizobium NGR234 mutant

Virginia Inez Copley

To the Graduate Council:

I am submitting herewith a thesis written by Virginia Inez Copley entitled "Generation and characterization of a novel Rhizobium NGR234 mutant." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Gary Stacey, Major Professor

We have read this thesis and recommend its acceptance:

Beth Mullin, Neil Quigley

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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We have read this thesis and

recommend its acceptance:

Stuart Riggs (for Beth Mullin)

Accepted for the Council:

Vice Provost and

Dean of Graduate Studies

Generation and Characterization of a Novel *Rhizobium* NGR234 Mutant

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Virginia I. Copley

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Dedication

This thesis is dedicated to my parents for their enthusiastic encouragement and support of my education.

Acknowledgements

I would like to thank all the people who were a part of the Stacey lab during my time at the University of Tennessee for their enthusiastic pursuit of knowledge and excellence in research. I would especially like to thank Dr. John Loh for his guidance and patience during the past three years. I am also grateful for the leadership and insights of my major professor, Dr. Gary Stacey. I would like to thank my committee members, Dr. Beth Mullin and Dr. Neil Quigley, for their advice about my research and the writing of this thesis.

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I would like to thank my parents, Inez and Fred Copley, for their emotional and financial support of my academic pursuits. I would like to thank my brother for setting a challenging academic standard for me to follow. I am also grateful to my fiancé, Carl Bowen, for being so accepting of a fifteen-month engagement so I could finish school.

Abstract

This work focuses on the genetic regulation of the nodulation genes found in the symbiotic bacterium *Bradyrhizobium japonicum*. This work specifically addresses the question of what regulates the important transcriptional regulator gene *nolA*.

The first part of this work uses *nolA-luxCDABE* fusions to show *nolA* induction and that the *nolA* promoter region can drive the *lux* operon. The plasmid-encoded fusion in *Escherichia coli* was used to show that the NolA protein induces the *nolA* promoter to drive the *lux* operon. The level of *nolA* induction over the course of the *E. coli* growth curve was determined by measuring the amount of light produced by the protein products of the *lux* operon. Without NolA, there was an initial spike of light production which tapered off as the culture density increased. In the presence of NolA, there was a second peak of light production at an optical density of 0.4 (measured at A_{600}). A Tn5 with a *nolA-luxCDBE* transcriptional fusion was introduced into the *B. japonicum* chromosome, but the *B. japonicum* cells did not provide sufficient amounts of the fatty acid substrate (i.e., myristolate) for light production. The *B. japonicum* cells gave off light when an aldehyde substrate (decanal) was added to the culture. The need for exogenous substrate limited the usefulness of the *nolA-luxCDABE* fusion as we had intended to use it to view *nolA* induction in growing, intact host plant nodules.

The second part of this work focuses on the generation of a *Rhizobium* species NGR234 mutant unable to induce a plasmid-encoded *nolA-lacZ* fusion in response to chitin or high cell population density. The mutants were generated by mating a plasmid (pJQ15Sp) bearing a Tn5 with antibiotic resistance markers into JNR1 cells (*R.* NGR234

with a plasmid-encoded *nolA-lacZ* fusion, pBGAlac4). The transposase was encoded on pJQ15Sp outside the insertion sequences. Some mutants (JNR7-9) were selected based on their lack of *nolA* induction in response to higher culture densities, and other mutants (JNR1Sp1-45) were selected based on their lack of *nolA* induction in response to chitin. The lack of response was confirmed by β-galactosidase activity assays. The plasmids were isolated from the mutants and checked for Tn5 insertions before Southern blots were done to determine the number of Tn5 chromosomal insertions. Southern blot analysis revealed that these mutants were interrupted in the same gene. Since interrupting a single gene removes *R.* NGR234's ability to induce the *nolA* promoter in response to chitin or high culture density, it seems likely that the single interrupted gene's product passes both signals to *nolA*.

One mutant, JNR9, was chosen for further analysis and plant nodulation assays. The interrupted gene of JNR9 was cloned into the cosmid vector pHC79 using the Promega Packagene kit (results confirmed by Southern blot). The resulting cosmid (pJNR9A) was found to have multiple copies of pHC79. It underwent subcloning to make the cosmid pD32A, which has only one copy of pHC79. The cosmid pD32A will be sequenced at a later time.

R. NGR234, JNR1, and JNR9 were used in 28-day nodulation assays on soybean, cowpea, mungbean, and siratro plants. On soybean, *R.* NGR234 causes large lumpy growths on the roots. The mutant JNR9 caused twice as many growths as the *R.* NGR234 and JNR1. On cowpea and mungbean plants, JNR9 did not nodulate as well as the controls. On siratro plants, it did nodulate as well as the wild-type bacteria. The differences in JNR9's nodulation ability in different plant hosts may indicate differences in the importance of NolA in the different hosts.

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1. Introduction

Legumes and Bacteria

Leguminous plants are agriculturally significant because of their use as food sources for people and animals. Their symbiotic relationship with bacteria from the family *Rhizobiaceae* affects their nutritional value so it is of interest to researchers. The bacterial symbiont's level of nitrogen-fixing ability affects the amount of protein in the plant (every amino acid contains nitrogen)(Vance 1997). The growth of legumes also improves soil quality by making fixed nitrogen available to non-legumes (Vance 1997).

Members of the family *Rhizobiaceae* may be divided into two large groups based on growth rates. Fast- and slow-growing rhizobia differ in some of their metabolic pathways, their flagellar arrangements, and their G+C contents. Fast-growing strains tend to have their symbiotic genes on plasmids, while the symbiotic loci of slow-growing strains are primarily in their chromosomes. Fast- and slow-growing strains that nodulate the same host plant share conserved DNA sequences in their nodulation genes (Broughton et al. 1985). Because of the differences in the cells expressing the nodulation genes, both fast- and slow-growing rhizobia must be studied if nodulation genetics are to be thoroughly understood.

This work focuses on two members of the *Rhizobiaceae, Bradyrhizobium japonicum* and *Rhizobium* species NGR234. *B. japonicum* is a slow-growing (12-hour doubling time) bacterium with a restricted host range: it colonizes the roots of soybean, siratro, mungbean, and cowpea plants. Its symbiotic genes are located on its chromosome. *R.* Sp. NGR234 is a fast-growing, broad host-range symbiont known to colonize over 110 genera of legumes including cowpea, mungbean, and siratro.

Rhizobium NGR234 is a frequently studied member of the family *Rhizobiaceae*. In laboratory cultures, it has a doubling time of about 4 hours. Its symbiotic genes are located on a large plasmid, pNGR234a, that is native to *Rhizobium* (Broughton et al. 1984). This large (536KB) symbiotic plasmid encodes genes involved in nodulation, nitrogen fixation and a type III secretion system, as well as *nodD1* and *nodD2* (Freiberg et al. 1997).

Since *B. japonicum* grows so slowly, it is convenient to transfer its nodulation genes to the faster-growing *R.* NGR234 for laboratory study. Plasmids carrying transcriptional gene fusions of the *nolA* promoter and *lacZ* that have been mated into *R.* NGR234 are induced by chitin and high cell population density in a manner consistent with that of *nolA* in *Bradyrhizobium japonicum* (Loh and Stacey, in press; John Loh, personal communication). At this time, *R.* NGR234 has no known *nolA* gene.

Nodulation

Leguminous plants and nitrogen-fixing bacteria live together in a mutualistic symbiosis. Bacteria from the family *Rhizobiaceae* infect the root hair cells of various legumes and the plant responds by creating new organs on its roots that are called nodules. The bacteroids (bacteria inside a nodule) live inside a membrane-bound symbiosome within the cytoplasm of the plant cell, and the nodule forms around the infected plant cells. (book chapters by Layzell and Atkins 1997, Vance 1997)

The regulation of the legume symbiosis is complex. The plant and bacteria must communicate to insure that the right bacterial strain has found the right plant species. The plant roots release flavonoids and isoflavonoids into the soil that attract the bacteria. These flavonoids and isoflavonoids are phenolic compounds such as luteolin and

daidzein (Peters et al. 1986). The bacteria respond to the flavonoid signals by producing complex lipo-chitin oligosaccharide signal molecules called Nod factors. These Nod factors trigger the early developmental stages of nodule formation such as root hair curling and cortical cell division (Dénarié et al. 1992; Spaink 1992). After the initial deformation of the root hair, the wall of the root hair cell then is deformed to make an infection thread for the bacterial cells to follow into the cortical cells which will become the developing nodule (Garcia et al. 1996) as a nodule meristem grows out from the root meristem. The developed nodule has a central core of infected and uninfected plant cells surrounded by cortical cells. The nodule has its own vascular bundles for the transport of nutrients to and from the nodule (Layzell and Atkins 1997). After the nodule has formed, the bacteroids enlarge and begin to fix nitrogen (as NH_4^+) for the plant using the enzyme nitrogenase. In return, the plant provides C_4 dicarboxylic acids (e.g. malate and aspartate) as carbon sources for the bacteroids and makes oxygen available through the O_2 binding protein leghemoglobin. The bacteroids require large amounts of oxygen for respiration (to produce the high ATP levels needed for nitrogen fixation), but nitrogenase is irreversibly inhibited by O_2. By binding the available oxygen with leghemoglobin, the plant cells are able to provide a steady supply of oxygen while tightly controlling the O_2 concentration (Layzell and Atkins 1997). Leghemoglobin in functional nodules causes a characteristic pink color.

Regulation of bacterial symbiotic genes

The *nod* genes, *nodABC,* which are essential for nodulation, are found in all rhizobia and encode genes responsible for the formation of the basic N-acylated chitin backbone of Nod factor (Germia et al. 1994; John et al. 1993; Röhrig et al. 1994). Individual

species and strains of rhizobia have host-specific genes to modify the basic chitin oligosaccharide chain in order to signal their preferred host plants. Nod factor is an oligosaccharide of 3 to 6 N-acetylglucosamine residues in length assembled by the gene products of *nodA, B,* and *C.* Host specific *nod* genes make decorations for the basic Nod factor (i.e., acyl groups, fatty acid chains, and sulphates; Denarie et al. 1992).

The bacterial genetic regulation of the nodulation genes (*nod/nol/noe*) involves several regulatory pathways. These pathways are shown in Figure 1 for *B. japonicum.* The NodD proteins, which are transcription activators, were discovered first. The sensor/regulator proteins NodVW are also transcriptional activators. The *nod* genes are directly repressed by *nolR* in *Sinorhizobium meliloti,* and it down regulates NodD1 expression as well (Kondorosi et al. 1989). The *nod* genes in *B. japonicum* are indirectly repressed by NolA1 through induction of NodD2, which acts as a supresser (Dockendorff et al. 1994).

The *nodD* genes, which are in the LysR family of transcriptional regulators (Schell 1993), play a major role in symbiotic regulation. Different rhizobia may have a single *nodD* gene (i.e., *Rhizobium legumnosarum*) or several copies of the gene (i.e., *Bradyrhizobium japonicum*), each of which responds to different compounds (Dockendorff et al. 1994). The plant flavonoids released from the host legume roots interact with NodD, which is constitutively expressed in free-living *Rhizobium* and *Bradyrhizobium* (Vance 1997). The conservation of the DNA target site is a characteristic of the LysR family (Goethals et al. 1992). In *B. japonicum,* NodD1 transcriptionally regulates the nodulation genes *nodYABC* by binding the conserved sequences in the promoter region (Hong et al. 1987). In the case of *nod* operons, this conserved target sequence, the *nod* box, is a 47-base pair (bp) region on the 5' end of

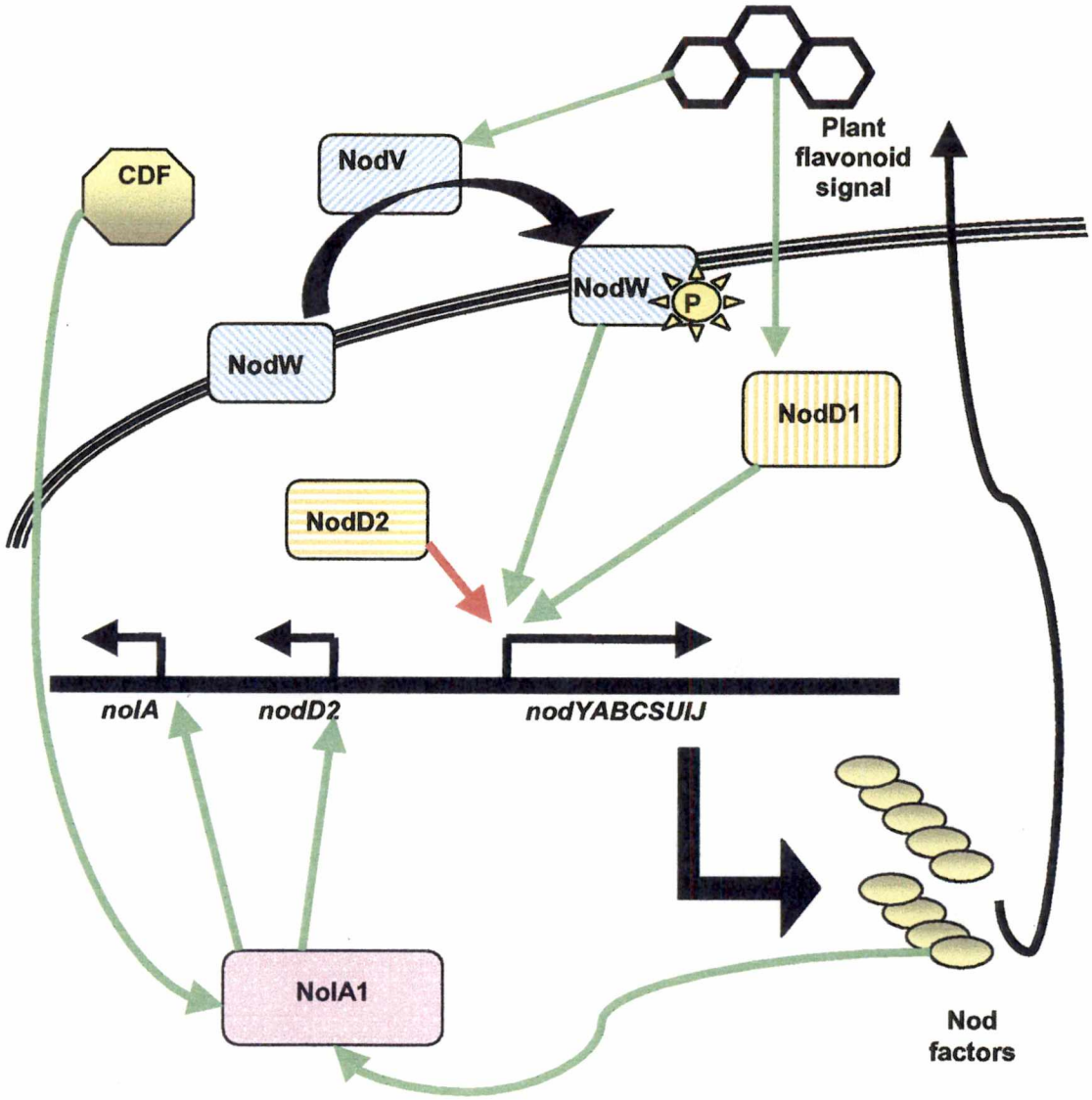


Figure 1. Regulation of the *nod* genes responsible for making Nod factor in *B. japonicum*, starting with the flavonoids signal from the plant. The green arrows represent activation or induction and the red arrow represents repression. CDF= cell population density factor, P=phosphorylation. This figure is adapted from a figure drawn by John Loh.

the operon (Fisher and Long 1989; Rostas et al. 1986). In *B. japonicum*, a homolog of NodD1, NodD2, binds and represses *nodYABC* in response to the transcriptional regulator NolA1 (Dockendorff et al. 1994).

NodV and NodW are a sensor and regulator pair of proteins that also regulate the *nod* genes in *B. japonicum*. NodV is the sensor that phosphorylates NodW the transcriptional regulator (Sanjuan et al. 1994). The phosphorylation of NodW is triggered by the presence of plant flavonoids that are known inducers of the *nod* genes (Loh et al. 1997). NodW can partially compensate for the lack of *nodD1* in the activation of the *nod* genes (Dockendorff et al. 1994). In some hosts (i.e., mungbean, cowpea, siratro), the *B. japonicum nodVW* genes are essential for nodulation, but in soybean they are not (Göttfert et al. 1990).

The transcriptional repressor, *nolR,* was originally discovered in *S. meliloti* (then called *Rhizobium meliloti*) (Kondorosi et al. 1989). Later, homologous sequences were found in other rhizobia and sinorhizobia and the *nolR* gene was identified in *R. leguminosarum* (Kiss et al. 1998). Kiss et al. (1998) did not find homologous sequences in *Bradyrhizobium, Mesorhizobium, Azorhizobium,* or *Agrobacterium*. In *S. meliloti*, Kondorosi et al. (1989) showed that NolR represses *nodD1* and the *nodABC* operon by binding at overlapping regions of their promoters.

NolA1 is the longest of the three proteins encoded by *nolA*. The mRNAs for these proteins are induced at two different promoter regions and transcribed from three different ATG start sites (Fig. 2). NolA1 has an N-terminal, helix-turn-helix DNA-binding motif similar to that of the MerR-type regulatory proteins (Sadowsky et al. 1991) and it induces its own expression from the first promoter region. This autoregulation is a characteristic of MerR-type regulatory proteins (Garcia et al. 1996; Lund et al. 1986;

Holmes et al. 1993). NolA1 induces the expression of NolA2 and NolA3, both of which lack the DNA-binding domain, from the second promoter. The functions of NolA2 and NolA3 are unclear, although there are some indications that they play some role in the nodulation of some hosts (Loh et al. 1999). In *B. japonicum*, NolA1 indirectly represses *nod* gene expression by inducing *nodD2*, a homolog of *nodD1* that represses *nodYABC*. NolA acts both during the early stages of infection and during bacteroid development and maintenance within the host plant cell (Garcia et al. 1996). It was first identified as a host-specific nodulation gene because of its impact on plant host range, but it is also involved in the genotype-specific nodulation of soybeans (Sadowsky et al. 1991). On soybean roots, *nolA* mutants produce normal nodules after a slight delay in nodulation, but on cowpea roots they are grossly defective in nodule formation, nitrogen fixation, and symbiosome structure (Garcia et al. 1996). *B. (Arachis)* species strain NC92, whose host range partially overlaps that of *B. japonicum* USDA110 but also includes peanuts (*Arachis hypogaea*), also has a copy of *nolA*. The *B.* NC92 *nolA* is necessary for efficient nodulation of host plants while the *B.* NC92 *nodD1* and *nodD2* are not (*nodD1* and *nodD2* mutants nodulate host plants after a slight delay) (Gillette and Elkan 1996).

The direct genetic regulation of *B. japonicum nolA* is unclear, but it is known to be induced at high cell population densities (i.e., A_{600}>0.4) and in the presence of low levels of chitin oligomers. It is also induced by Nod factors as a type of feedback regulation (Loh and Stacey, in press) so the chitin induction is not surprising. However, a chitin binding site has not been found on NolA. Recently, a cell density factor isolated from the used media of *B. japonicum* cultures was shown to induce *nolA* (Loh et al., in press).

Figure 2. The *B. japonicum nolA* promoter region. The two promoters (P1 and P2) are indicated by bent arrows. The three ATG start sites are boldfaced and numbered according to the NolA protein encoded. Each is preceded by a putative ribosomal binding site (underlined). The –10 and –35 consensus sequences for P2 are boxed and labeled. The boldfaced reversed arrows indicate a possible stem-loop region covering the second ATG start site. This figure is adapted from Figure 1 of Loh et al. 1999.

The Luciferase Method of Gene Expression Measurement

Luciferase is the protein that catalyzes the bioluminescent reaction in *Vibrio fischeri* and *Vibrio harveyi*. It is expressed at high cell population densities similar to the density of the bacterial population inside the light organs of squid, their symbiotic hosts. The phenomenon of sensing the population density based on the concentration of a signal molecule is termed quorum sensing. It allows bacteria in a given population to synchronize genetic events. An autoinducing molecule, a homoserine lactone, is produced at low levels by the protein product of *luxI* (an auto-inducer synthase). This homoserine lactone diffuses freely across the cell membrane, so a high intercellular concentration occurs at high population cell densities. The homoserine lactone binds and activates LuxR, which in turn induces the *lux* operon, including *luxI* (Fig.3). (Meighen 1991)

As shown in Figure 3, the *lux* operon encodes the two luciferase subunits (LuxA and LuxB) and a fatty acid reductase complex (LuxCDE) which synthesizes the fatty aldehyde substrate for luciferase (Meighen 1991). Luciferase gives off a blue-green light that is transient and quantifiable, and it can be measured without killing the cells or interrupting their growth (Simpson et al. 1998; Applegate et al. 1998). The net aldehyde oxidation reaction for the luciferase-catalyzed light production in the presence of molecular oxygen, reduced flavin mononucleotide (FMN), and a long-chain aldehyde (R-CHO) is as follows:

$$FMNH_2 + R\text{-}CHO + O_2 \rightarrow FMN + RCOOH + H_2O + \text{light (490nm)}$$

where R stands for a long-chain aliphatic carbon moiety (Karp 1989).

The *lux* operon can be used as a cassette on plasmids and transposons. In rapidly growing cells, the fatty acid reductase has access to a sufficient supply of fatty

acids such as myristolate from cell membrane biosynthesis. Smaller cassettes with only LuxA and LuxB require the addition of an aldehyde substrate (i.e., n-decanal). Previously, O'Kane et al. (1988) used *luxAB* fused with the nitrogenase *nifD* promoter to show the NifD expression pattern in *B. japonicum* bacteroid-infected cells of soybean root nodules. O'Kane et al. were able to view the luminescent bacteria within the living cells of bisected nodules still attached to the plant root. He provided exogenous substrate by applying decanal to paper wicks placed in the humidity chamber with the soybean plant. Luciferase requires oxygen for the bioluminescent reaction, so at higher cell population densities less light is produced due to oxygen limitation. The *lux* operon can be driven by a variety of promoters, including the *B. japonicum nolA* promoter that is induced by NolA1 (see below).

Rationale for current work

Although high cell population density and chitin induce *nolA*, it is not known how these signals interact with other genes or gene products to cause the induction of *nolA*. It is thought that there is an intermediary between the chitin or cell population density signals and *nolA* due to the lack of any obvious binding sites on the NolA protein. In this work, random transposon mutagenesis resulted in *R.* NGR234 mutants. These mutants have a functional *nolA-lacZ* transcriptional fusion on a plasmid, but chitin or high cell population density no longer induced the fusion.

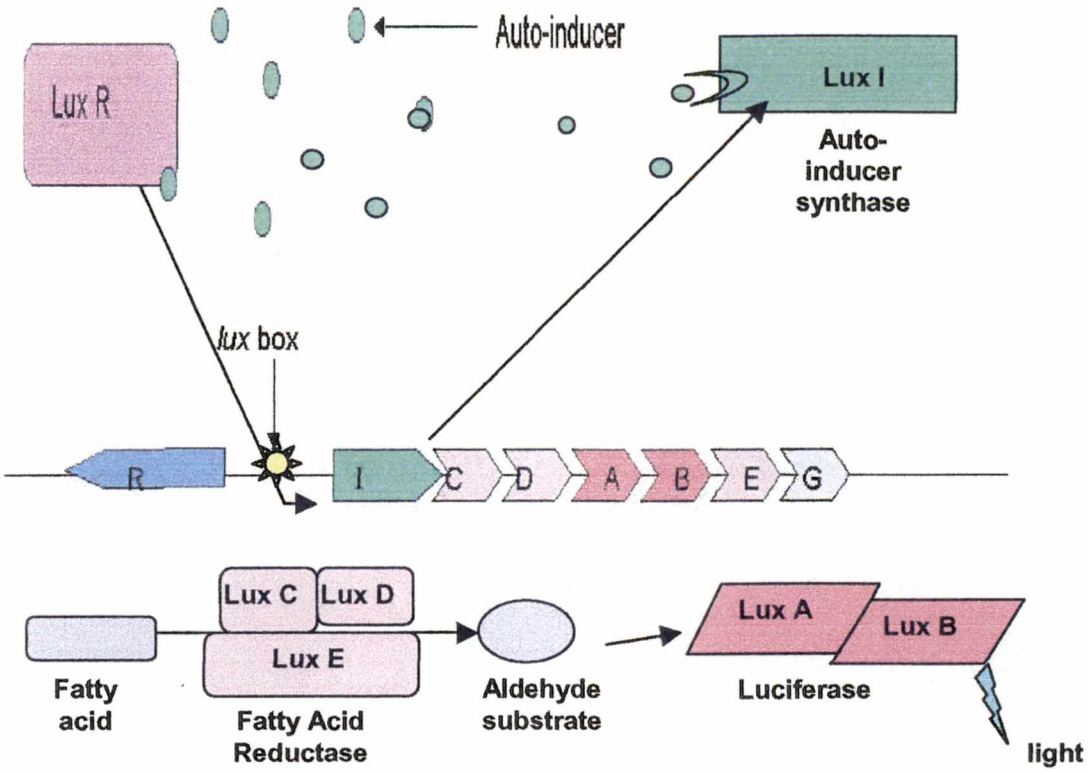


Figure 3. Regulation of the *lux* operon. The autoinducer molecules bind LuxR and cause it to bind the *lux* box and activate transcription of the *lux* operon.

2. Materials and Methods

Bacterial Strains, Plasmids and Cosmids

The bacterial strains, plasmids and cosmids used in this research for are listed in Table 1. Details on the strains generated during the course of this project are given in the text.

Culture Media and Growth Conditions

Bacteria

Bradyrhizobium japonicum strains grew in modified RDY medium (So et. al. 1987) for routine growth and luminosity assays. *B. japonicum* strains grew in HM medium (Cole and Elkan, 1973) for matings.

Escherichia coli strains grew in LB medium (Sambrook et al., 1989) for routine growth, plasmid isolation, and matings. *E. coli* strains grew in M9 medium (Sambrook et al., 1989) for luminosity assays.

Rhizobium NGR234 strains grew in TYC medium for routine growth, matings, chromosomal DNA isolation, and plant nodulation assays. For mutagenesis screening and β-galactosidase activity assays, *R.* NGR234 strains grew in supplemented minimal medium, +MM, (Bergersen, 1961) containing 0.3g K_2HPO_4, 0.3g KH_2PO_4, 0.5g NH_4NO_3, 0.1g $MgSO_4$ ($7H_20$), 0.05g NaCl, 4ml glycerol, and 1ml RDY trace elements. After autoclaving, 0.0002g d-biotin, 0.0001g thiamine, 0.0001g calcium pantothenate, 100µl 0.1M $CaCl_2$, and 2ml 20% glucose were added to 100ml aliquots of the cooled medium.

Table 1. Bacterial strains, plasmids and cosmids.

Strains and plasmids	Relevant characteristics	Source or Reference
Bacterial Strains		
E. coli		
JM109	*endA1, recA1, gyrA96, thi, hsdR17*($r_K^- m_K^+$),*relA2, supE44, (lacpro*AB) [F', *traD36, proAB, lacI*^{q}*Z* M15]	Messing, 1983; Promega
JLA10	JM109 harboring pJLA10	J. Loh
LUX1	JM109 harboring pLuxA1	J. Loh
LUX2	JM109 harboring pLuxA2	J. Loh
SV17	Lambda pir+	G. Sayler
DH1	F-, recA1, endA1, gyrA96, thi-1, hsdR17 (rk-, mk+), supE44, lambda	Low, 1968; Meselson and Yuen, 1968
***R.* NGR234**	Wild type, Rf-R	
JNR1	Wild type with pBGAlac4	J. Loh
JNR9	JNR1 w/ chromosomal Tn5 insertion, Sp/Sm-r	This work
JNR1Sp1 – JNR1Sp45	JNR1 w/ chromosomal Tn5 insertion, Sp/Sm-r	This work
B. japonicum		
pVIC1-1 to pVIC1-11	USDA110 with pVIC1	This work
USDA110	Wild type, Cm-R	USDA, Beltsville, MD
Plasmids		
pJLA10	pUC with *nolA*	J. Loh

Table 1. Continued

Plasmids	Relevant characteristics	Source or Reference
pUCD615	pUC19 with a Tn5 transposon containing the *Vibrio fischeri lux* operon and transposase outside the insertion sequences.	Rogowsky et al., 1987
pLuxA1	pUCD615 with the 1.5KB BamHI fragment of the *nolA* promoter cloned into the mcs before the *lux* operon.	J. Loh
pLuxA2	Sibling of pLUX1	J. Loh
pTE3	Tc-r	Egelhoff and Long 1985
pBgtrpA-88	1.2KB *SaliI-BglII* clone of *nolA* in pTE3, Tc-r	Garcia et al. 1996
pUTK82	Described in this work.	B. Applegate, unpublished
pVIC1	Described in this work.	This work
pJQ15Sp	8kb Tn5 transposon w/ Sp/Sm-r on omega cassette	M. Hynes, unpublished
pJNR9A	Described in this work.	This work
pJNR9D3	Described in this work.	This work
pD32A	Described in this work.	This work
pHC79	Cosmid vector	N. Quigley
pBGAlac4	*nolA-lacZ*, Tc-r, Ap-r	M.G. Stacey
pSUP1011	Has Tn5, used for positive control in Southern blots.	Simon et al. 1983

B. japonicum and *R.* NGR234 strains grew at 30°C, and *E. coli* strains grew at 37°C. The following antibiotics were used for plasmid and mating selection: with *E. coli* and *R.* NGR234, ampicillin (Ap, 100μg /ml), kanamycin (Km, 30μg /ml), tetracycline (Tc, 12μg /ml), streptomycin (Sm, 30μg /ml) spectinomycin (Sp, 100μg /ml), and rifampicin (Rf, 50μg /ml); with *B. japonicum*, ampicillin (100μg /ml), kanamycin (100μg /ml), streptomycin (100μg /ml), spectinomycin (100μg /ml), tetracycline (100μg /ml), and chloramphenicol (Cm, 24.9μg/ml).

Legumes

Nodulation assays used soybean (*Glycine max* cultivar Essex), cowpea (*Vigna unguiculata* cultivar Red Caloona), mungbean (*Vigna radiata* cultivar King), and siratro (*Macroptilium atropurpureum*). Seeds were sterilized by soaking in 20% bleach for 10 minutes, washing three times with sterile water, then soaking in 0.1N HCl for 10 minutes. After sterilization, the seeds were washed five times with sterile water, and then placed on sterile, wet paper towels in an autoclaved pan covered in aluminum foil. The seeds were incubated at 30°C for 2 days.

For growth pouch assays, CYG seed growth pouches (Mega International, Minneapolis, MN) were dampened with deionized water, wrapped in foil, and autoclaved. The sterile pouches were placed in manila folders using paper clips, and a plastic drinking straw was inserted into each pouch to facilitate watering. Four germinated seeds were placed in each pouch using sterile forceps. The folders of plants were placed in wire racks and incubated in a 30°C growth chamber (16-hour light/8 hour dark cycle). The pouches were watered daily (10-20 ml sterile water), and given Plant Nutrient Solution (Wacek and Brill 1976) weekly. Siratro plants required water every

other day. Plants were grown for 21 days post-inoculation.

For leonard jar assays, the top jars of the assembled plastic leonard jars were filled with a moistened mixture of 1 part pearlite to 2 parts vermiculite. The jars were autoclaved on the fluid setting for 1 hour and allowed to cool overnight. The lower jars were filled with PNS, and 3 germinated seeds were planted in each jar using sterile forceps. The plants were incubated in a 30°C growth chamber (16-hour light/8 hour dark cycle). The lower jars were alternately refilled with either sterile water or PNS every 5 to 7 days, as needed. Plants were grown for 21 days post-inoculation.

Enzymes, Isotopes, and DNA primers

Unless stated otherwise, the enzymes used in this study were purchased from Promega (Madison, WI) or Takara Biomedical (supplied by Panvera Corporation, Madison, WI), and were used according to the manufacturers' protocols. ICN Biomedical (Irvine, CA) supplied radioisotopes. Primers Not1NolA and XbaiNolA were synthesized by Operon Technologies, Inc. (Alameda, CA). The Not1NolA primer sequence is 5'-GCGTTTACGTTGCAGCGGCCGCG-3' (the NotI site is underlined). The XbaiNolA primer sequence is 5'-CCGGTCGCCTCTAGAAGTTCGCC-3' (the XbaI site is underlined). I synthesized the primers LuxC and NolApr on a Beckman Oligo 1000 DNA synthesizer, courtesy of Dr. Gary Sayler. The LuxC primer has a melting point of 50°C and its sequence is 5'-GCATAATTATCAAAATCTTG-3'. The NolApr primer sequence is 5'GAAATTGAACAACATGAAC-3' and it also has a melting point of 50°C.

Genetic Techniques

Transformation, Conjugation, and Transduction

E. coli cells were transformed with plasmids using the heat-shock protocol described by Sambrook et al. (1989). Cells were made competent using either the protocol by Sambrook et al. (1989), or a modified procedure based on the protocol by Inoue et al. (1990). Cells were also transformed by electroporation (Sambrook et al. 1989). The electrocompetent *E. coli* SV17 cells were a gift from Gary Sayler's lab personnel.

The mating procedure was as follows: cells grew in liquid culture to an OD_{600} of 0.5 to 1.0. 1ml of donor cells were pelleted in an eppendorf tube in a microcentrifuge at 13,000rpm for one minute, the supernatant was decanted, and 1 ml of recipient cells was pelleted on top of the donor cells. The cells were resuspended and pelleted three times in fresh liquid medium corresponding to the solid medium to be used for the mating (HM for *B. japonicum*, TYC for *R. NGR234*, LB for *E. coli*). After the last wash, the cells were resuspended in 50 to 100 µl of liquid medium and placed in a small area of a sterile 0.2µM nitrocellulose filter disc on top of a plate of solid medium. The plates were incubated at the optimum growth temperature for the recipient overnight, and then the area of the filter with the mated bacteria was excised using a hot metal spatula to cut through the filter. The cells were resuspended in 500µl of liquid medium and plated in 100µl aliquots on the appropriate solid medium (RDY for *B. japonicum*, TYC for *R. NGR234*, LB for *E. coli*) with antibiotics to select for the transconjugants and against the donors and non-conjugated recipients. The same procedure was used for triparental matings except that the *E. coli* helper strain (containing pRK2013) was pelleted with the donor and recipient. As negative controls, recipients and donors were also treated and

plated separately.

Cosmid transductions were done with the Packagene kit from Promega. *E. coli* LE392 was provided in the kit for infection by the packaging positive control phage. The recombinant phage was allowed to infect *E. coli* DH1.

DNA Isolation and Manipulation

Plasmids were isolated from *E. coli* in small amounts (mini-prep) using the alkaline lysis method as described in Sambrook et al. (1989) for digests, agarose gel visualization, ligations, and transformations. For fluorescent sequencing, plasmids were isolated using the Wizard Plus SV Miniprep DNA Purification System from Promega. Plasmids were isolated in larger amounts (midi-prep) using the Qiagen Plasmid Midi kit (Qiagen, Inc., Valencia, CA).

Bacterial chromosomal DNA was isolated in small amounts using a method modified from Marmur's protocol (1969). Large amounts of bacterial chromosomal DNA were isolated using a cesium chloride gradient as described by Sambrook et al. (1989).

DNA fragments for ligation were isolated through electrophoresis in agarose gels (Sambrook et al., 1989), excision with minimal UV light exposure, and purification with the Qiaquick Gel Extraction kit from Qiagen.

DNA concentrations were measured with a Hoefer DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). The manufacturer's protocol was followed for the concentration measurements.

Transposon Mutagenesis

The plasmid pJQ15Sp was mated into JNR1 following the standard protocol described above. The transposase for the Tn5 transposon is outside the insertion sequences, so the Tn5 can only transpose once. The desired transconjugants were selected on TYC agar plates with Tc/Sp/Sm. Mutants unable to induce the *nolA-lacZ* fusion in a density dependent manner were found by plating the transconjugants on +MM-Tc/Sp/Sm plates supplemented with X-gal and leaving the plates for 4 to 5 days. Desired mutants remained white. Mutants unable to induce the *nolA-lacZ* fusion in response to chitin were replica plated onto +MM media-Tc/Sp/Sm/X-gal with and without chitin (0.004g/ml solution). Desired mutants appeared white on both plates.

Southern Blotting and Hybridization

Southern blots were done using chromosomal and plasmid DNA. The DNA was cut with the appropriate restriction enzymes, and similar amounts of digested DNA were electrophoresed on a 0.5% agarose gel with 0.5μg/ml ethidium bromide for visualization under ultraviolet light. The gel was documented next to a fluorescent ruler using the Alpha Imager program (Alpha Innotech Corp., San Leandro, CA). The DNA was transferred from the gel to a nylon filter using a model 785 Bio-Rad vacuum blotter (Hercules, CA) and the standard protocol in the instruction manual. After the transfer, the membrane was air-dried, then baked at 80°C for 30 minutes in a GCA model 19 vacuum oven (Precision Scientific Group, Chicago, IL).

The membrane was prehybridized in a glass tube with 25ml of hybridization buffer (7% SDS, 0.25M $NaPO_4$) in a 60°C hybridization oven (Fisher Biotech

Hybridization Incubator model FBHI10, Fisher Scientific, Pittsburgh, PA). Approximately 100ng of the DNA probe was labeled with [α-^{32}P]dATP using the Klenow DNA polymerase protocol from Promega. The labeled probe was cleaned with a G-50 fine sephadex (Sigma Chemical Co., St. Louis, MO) column made using a 1ml plastic syringe with glass wool in the bottom to hold back the sephadex beads. The used buffer was poured off the membrane, and then 25ml of fresh hybridization buffer was added to the tube. The probe was added to the tube, and the membrane was hybridized at 60°C overnight. The radioactive hybridization buffer was poured out, and the membrane was incubated at 65°C in wash buffer (5% SDS, 20 mM $NaPO_4$) for 30 minutes. The membrane was washed twice before being checked with a Geiger counter. Some membranes were washed three or four times before the number of counts per minute was below 2,000 directly over the positive control. Washing was terminated when the filter showed a gradient of radioactivity (lowest on the edges, highest over the expected hybridized bands and positive control band) and the counts per minute were below 2000cpm. The membrane was heat-sealed in a plastic bag, and then placed inside a radiation-blocking exposure cassette (Eastman Kodak Co., Rochester, NY) with two pieces of x-ray film. The cassette was stored at -80°C until the film was sufficiently exposed (30 minutes to 7 days). One piece of film was developed and compared to the original picture of the agarose gel. If the first piece of film was not sufficiently exposed, the second piece of film was developed later.

Luminosity measurement

Luminosity was calculated using the formula: (photons/second)/optical density. I used the DeltaTox PSI (Azur Environmental, Carlsbad, CA) to measure photons of light

given off per second from 1 ml of bacterial culture in a round glass cuvette. The optical density was measured at A600 using a spectrophotometer, and then the culture was normalized to 0.1 OD to prevent oxygen limitation in the light-emitting reaction.

Myristolate was limited in *B. japonicum*. Therefore, 40µl of a 1% decanal emulsion (in sterile water) was added to the glass cuvette immediately before measuring the photons/second. For growth curve measurements, 1ml samples were taken at regular intervals from 100ml cultures growing in shaking incubators.

Plasmid/Cosmid Construction

pVIC1

PCR techniques were used to amplify the *nolA* promoter from pJLA10 and to add restriction sites at either end of the promoter for directional cloning. A Not I site was added upstream of the promoter, and an Xba I site was added following the promoter sequence using the primers Not1NolA and XbaiNolA. The modified *nolA* promoter was ligated directionally into pUTK82 upstream of the eight stop codons before the Lux cassette on the transposon. The resulting plasmid, pVIC1, was electroporated into electrocompetent *E. coli* SV17 cells provided by Gary Sayler's lab.

pJNR9A

Eco RI fragments of chromosomal DNA from JNR9 were ligated into dephosphorylated pHC79 (also cut with Eco RI) and the resulting cosmids were packaged into lambda phage particles using the Promega Packagene kit. The recombinant phage infected *E. coli* DH1 cells, and the cells grew in LB broth for 30

minutes before streptomycin and spectinomycin were added to enrich the culture for cells carrying the cosmid with the desired Eco RI fragment. The bacteria grew in the liquid culture over night before being plated on LB- Tc/Sp/Sm agar plates. Cosmids from 10 colonies were isolated and digested with Eco RI. They all had an 18kb fragment, a 3kb fragment, and the 6.4kb pHC79. Although the 6.4kb fragment was smaller than the 18kb fragment, the 6.4kb band on the 0.8% agarose gel was as bright as, or brighter than, the 18kb fragment. Cosmids must be about 50kb to be packaged into the phage head, so pJNR9A has an 18kb insert, a 3kb insert, and approximately 4 copies of pHC79. The 18kb insert hybridizes to the Pst I Tn5 probe.

pD32A

The cosmid pJNR9A was digested completely with Cla I, then extracted with phenol: chloroform and ethanol precipitated to remove the enzyme. The resuspended pJNR9A was religated to remove the extra copies of pHC79. The resulting plasmid, pJNR9D3, still had extra copies of pHC79. It was digested with Cla I and religated to leave a single copy of pHC79 in the new plasmid.

β-galactosidase Activity Assays

The β-galactosidase activity of the *nolA-lacZ* fusion in the putative mutants of JNR1 was measured under conditions of low cell density (less than 0.1 OD), high cell density (above 0.2 OD), and at low cell density in the presence and absence of chitin (0.01mg/ml) (Sigma). The assays used 0.5ml of culture grown in +MM and followed the protocol of Yuen and Stacey (1996). JNR1 was the positive control and water was the negative control. The cultures were induced with chitin 4 hours before measurement.

3. Results

nolA-luxCDABE Fusions

Two *nolA* promoter *luxCDABE* fusions were made to be used in the study of NolA expression. The first was on a plasmid and was used in *E. coli* to study the expression of NolA over the course of the bacterial growth curve. The second was on a plasmid-borne mini-Tn5 and was used to make chromosomal insertions in *B. japonicum* in order to avoid problems with plasmid instability when measuring NolA expression *in planta*.

Plasmid-encoded fusion in *Escherichia coli*

In *E. coli*, NolA induced the *nolA-luxCDABE* fusion on the plasmid pLuxA1, a descendant of pUCD615 (Fig. 4). The plasmid pLuxA1 was co-transformed into *E. coli* with either pTE3 or pBGtrpA-88 (pTE3 with a *trp-nolA* transcriptional fusion). In the absence of tryptophan, the *E. coli trp* promoter is induced (Yanofsky and Horn, 1994). The plasmid pBGtrpA-88 contains a promoterless *nolA* fused to the *trp* promoter, and a functional NolA protein is expressed in the absence of tryptophan (Garcia et al. 1996). NolA1 induces the *nolA* promoter and LuxCDABE is expressed. LuxCDABE is also expressed in *E. coli* without pBGtrpA-88, but at lower levels (Fig. 5). The presence or absence of NolA affects the expression pattern of the luciferase. When normalized to an optical density of 0.1 at A_{600}, the light level peaks as expected at 0.2 OD (Applegate et al., 1998) in the absence of NolA. In the presence of NolA, a second peak appears at around 0.4 OD and the levels are higher over all (Fig. 6). Regardless of the plasmid cotransformed with pLuxA1, the luciferase light production (luminosity) levels vary over

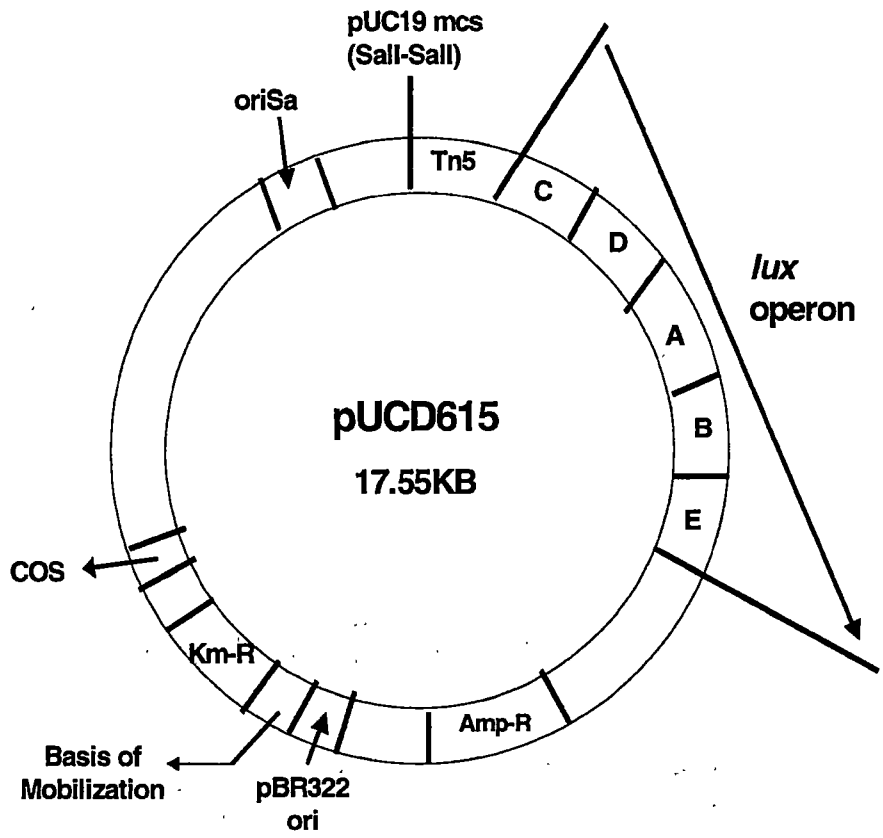


Figure 4. Map of pUCD615. pUCD615 is the ancestor of pLuxA1 and pLuxA2. The *nolA* promoter region was ligated into the multicloning site (mcs) by Dr. John Loh.

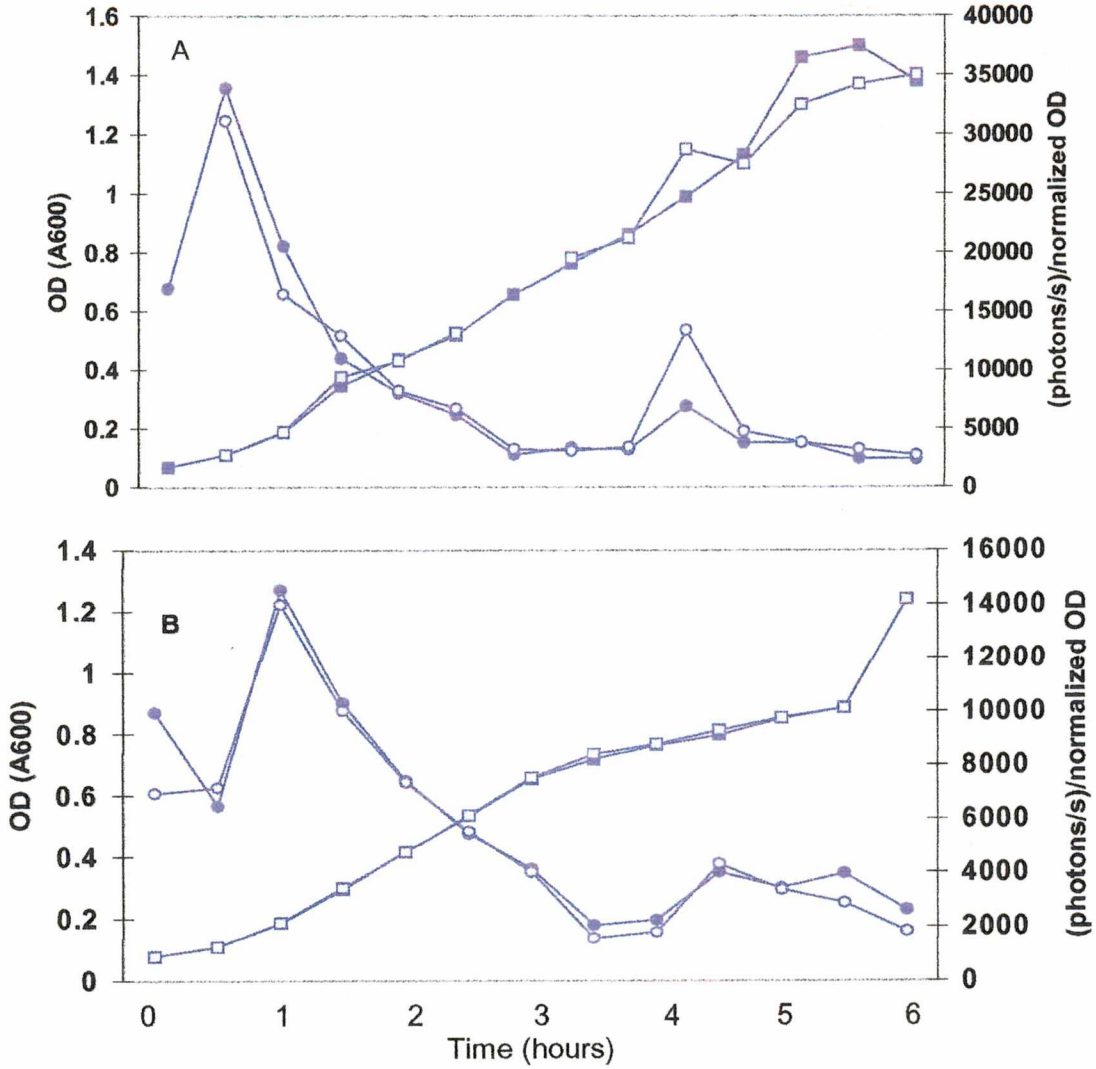


Figure 5. Changes in luminosity over the course of the *E. coli* growth curve for co-transformants carrying pTE3 and pLuxA1. Each graph is a separate experiment with two replicates in each experiment. The shapes are light and dark-filled to differentiate the two data sets. The squares are the optical density measured at A600 at half-hour intervals. The circles are the luminosity, measured as (photons/second)/normalized OD. The OD was normalized to 0.1.

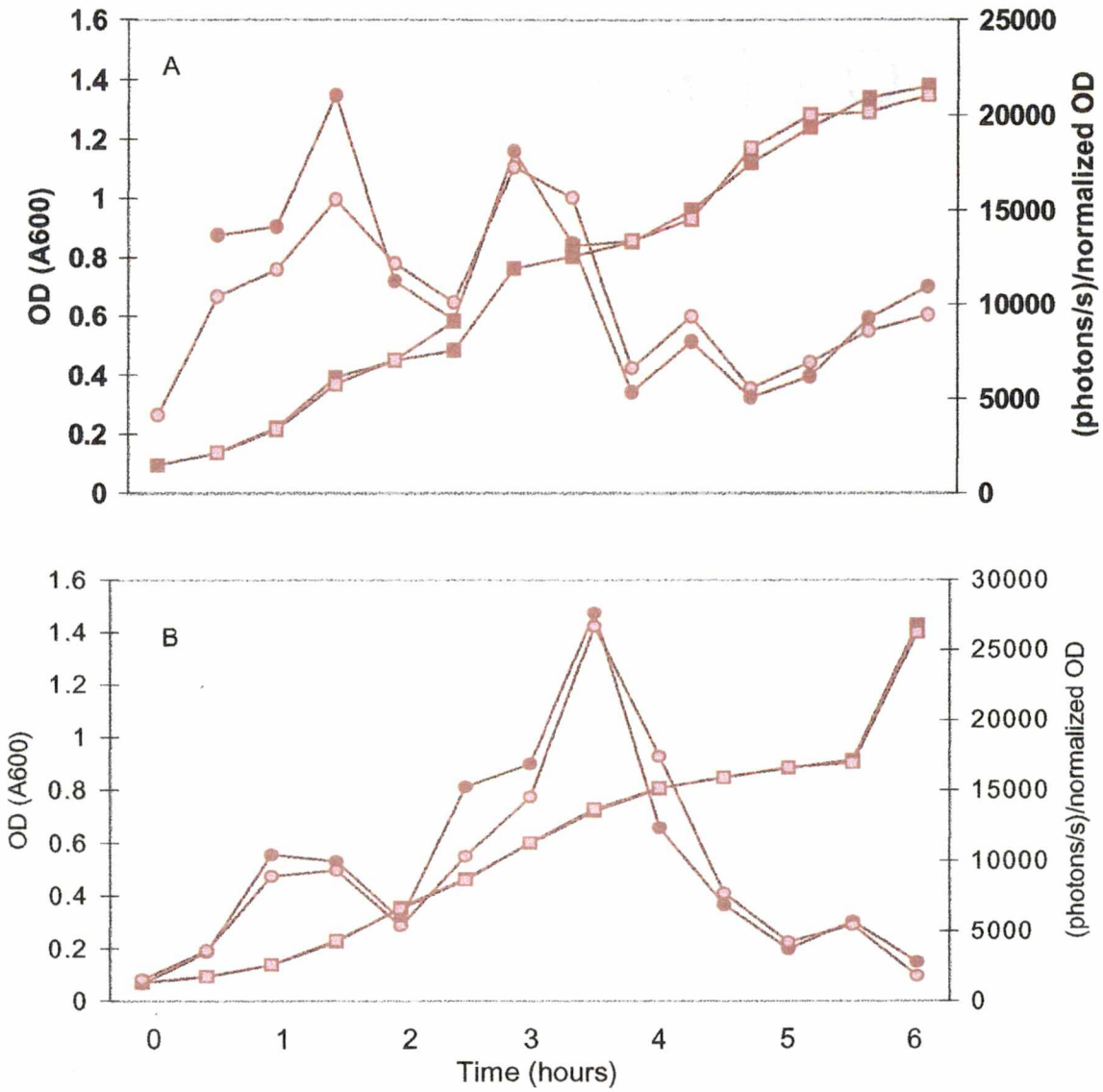


Figure 6. These graphs show the changes in luminosity over the course of the *E. coli* growth curve for co-transformants carrying both pBGtrpNolA-88 and pLuxA1. Each graph is a separate experiment, with two replicates in each experiment. The shapes are light or dark-filled to differentiate the two data sets. The squares are the optical density measured at A600 at half-hour intervals. The circles are the luminosity, measured as (photons/second)/normalized OD. The OD was normalized to 0.1.

the growth curve of the *E. coli* (Fig. 7).

Chromosomal Insertion in *Bradyrhizobium japonicum*

Bradyrhizobium japonicum was mated with *E. coli* SV17 carrying the plasmid pVIC1. This plasmid, based on pUTK82 (Fig. 8), has a Tn5 transposon with kanamycin resistance and the *nolA* promoter directionally cloned between the NotI and XbaI sites to drive the promoterless *luxCDABE* operon. Since the transposase is outside the insertion sequences the mating provides a stable chromosomal insertion of the Tn5 in the Km-R transconjugants.

Eleven of the resulting kanamycin resistant strains were tested for luminescence and four of them responded with an increase in luminosity when aldehyde substrate (decanal) was added to the culture immediately before measuring the light levels (Fig 9). Chromosomal insertions of the four responsive strains were made by mating in pTE3 to cure them of pVIC1 (both plasmids are in incompatibility group P). All of the resistant strains had extremely low light levels without the added decanal, possibly due to low levels of myristolate, the natural substrate of the aldehyde synthase. Myristolate is a fatty acid commonly found in bacterial cell membranes. This project was stopped due to the necessity of added substrate. If the *B. japonicum* VIC1 strains were used in nodulation assays on soybean plants, the nodules would have to be cut in order to expose the bacteroids to sufficient substrate and oxygen concentrations.

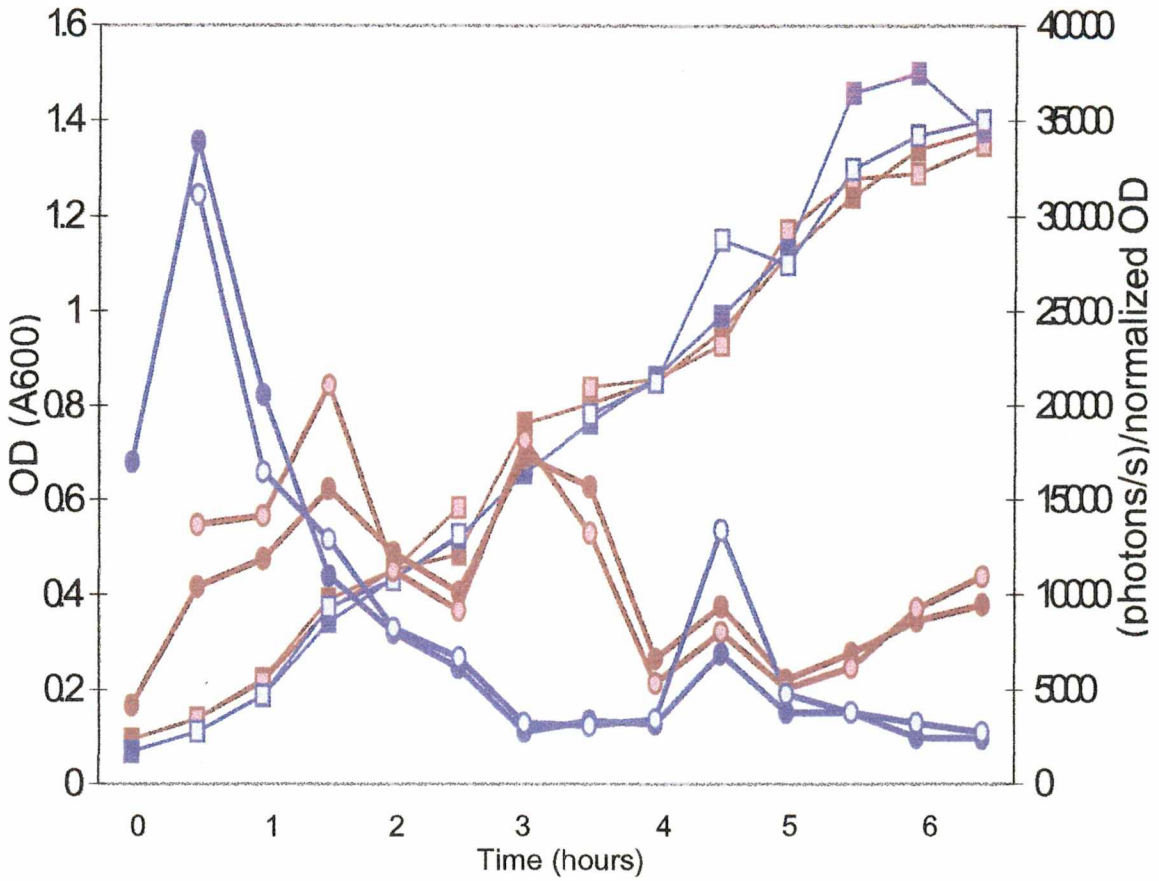


Figure 7. Changes in luminosity over the course of the *E. coli* growth curve for co-transformants carrying pLUXA1 and either pBGtrpNolA-88 or pTE3. The optical density (squares) was measured at A600 at half-hour intervals. The luminosity (circles) was measured as (photons/second)/normalized OD. The OD was normalized to 0.1 by diluting or concentrating the samples at each time point. The burgundy shapes show data for the pBGtrpNolA-88 co-transformant duplicates in the experiment shown in figure 4A. The blue shapes show data for the pTE3 co-transformant duplicates in the experiment shown in figure 3A. The shapes are light and dark-filled to differentiate the two data sets for each co-transformant.

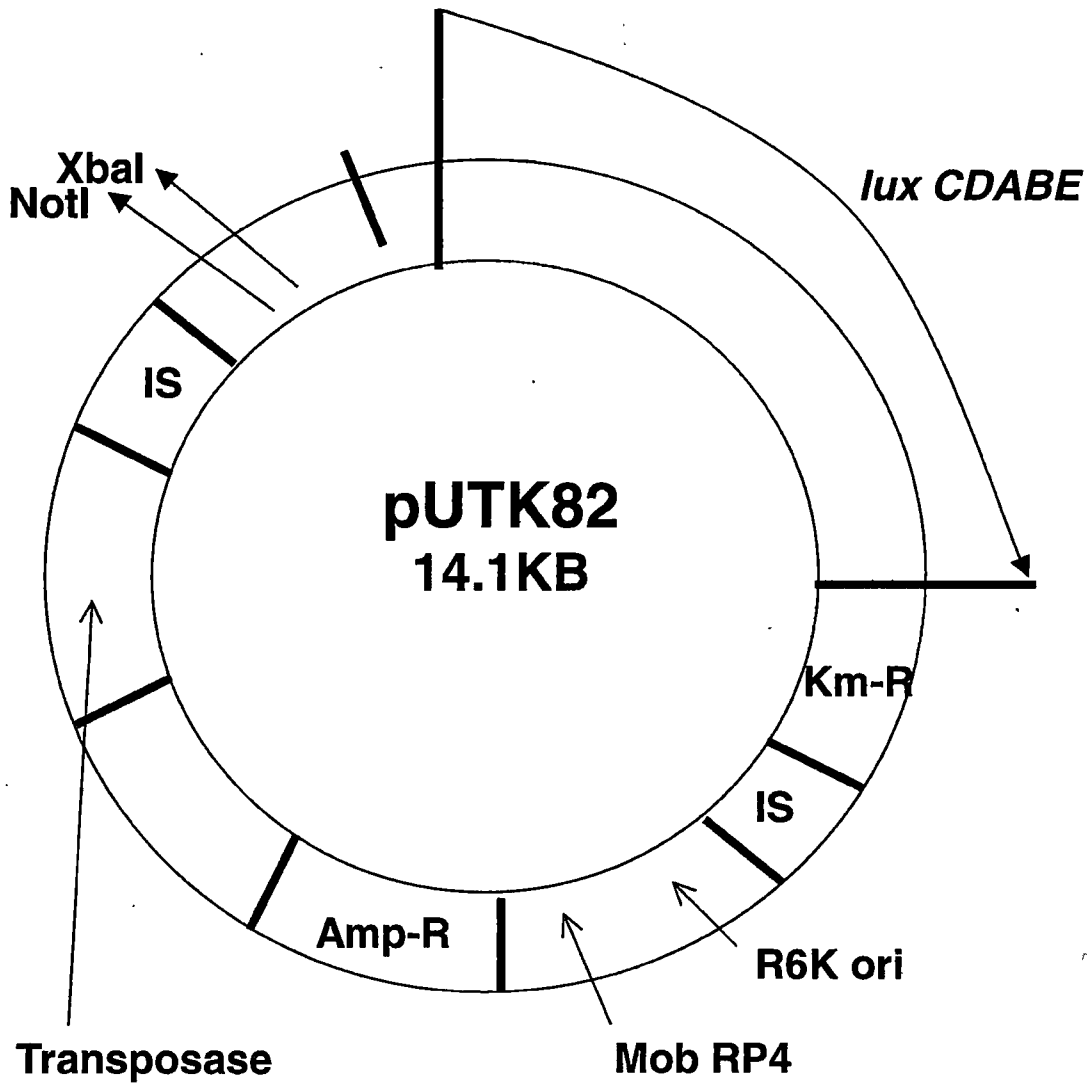


Figure 8. Map of pUTK82. pUTK82 is the ancestor of pVIC1. The *nolA* promoter region was added between the Not I and Xba I sites to make pVIC1. A series of eight stop codons are encoded between the Xba I site and the promoterless *lux* operon to prevent a translational fusion (Bruce Applegate, personal communication).

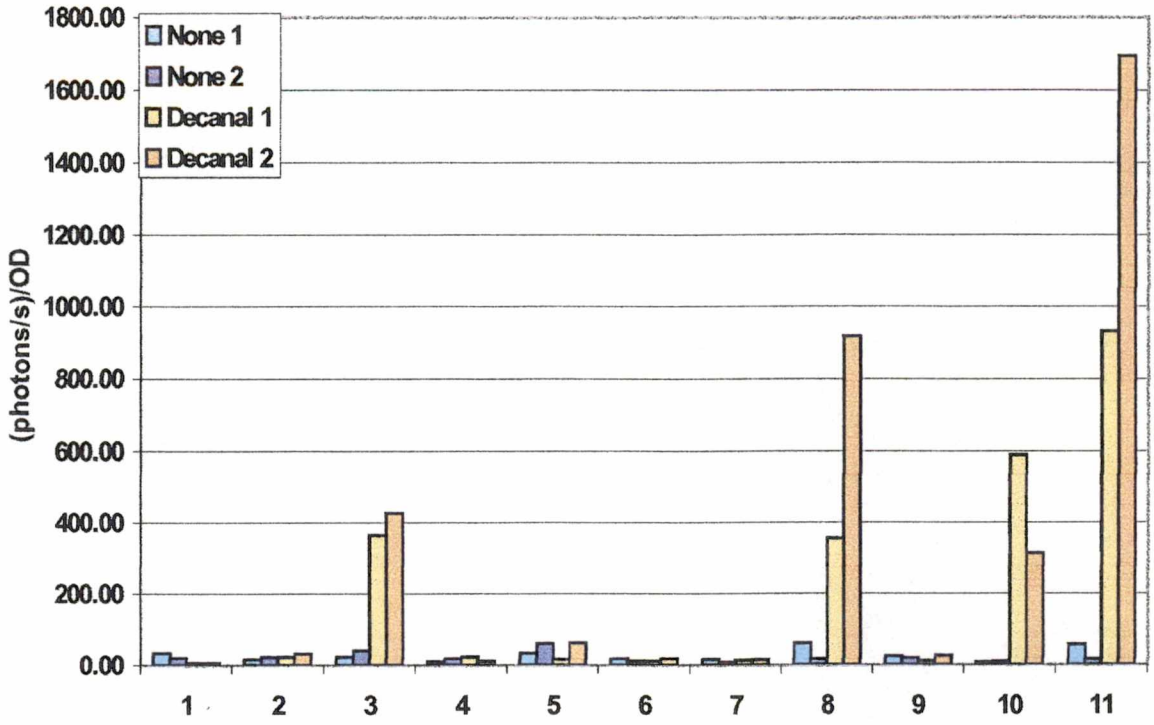


Figure 9. Luminosity of 11 *B. japonicum* transconjugants (pVIC1-1 to pVIC1-11) with the plasmid pVIC1. This experiment was done in duplicate. The blue bars are without decanal, and the orange bars are with decanal. Responsive strains are pVIC1-3, pVIC1-8, pVIC1-10, and pVIC1-11.

Rhizobium NGR234 Tn5 Mutagenesis and Mutant Confirmation

Transposon Mutagenesis

Transposon mutagenesis is now a common method of interrupting bacterial genes. Transposons are mobile pieces of double-stranded DNA that encode a transposase and have inverted repeating insertion sequences on each end of the transposon (Kleckner et al. 1975). The transposase recognizes and cuts the insertion sequences. Under natural conditions, transposons may move around the chromosome frequently and are a source of natural genetic variation. For mutagenic purposes, typically the transposon is on a plasmid and the transposase is on the plasmid somewhere outside the insertion sequences. The transposon may have added multicloning sites and antibiotic resistance genes. (See review by Kleckner 1981.)

Tn5 Mutagenesis

The plasmid pJQ15Sp (Fig. 10), which encodes the transposase outside the Tn5 (Fig. 11) insertion sequences, was mated into JNR1 (*R.* NG234 with pBGAlac4). The streptomycin/ spectinomycin resistant transconjugants were screened for either chitin or cell population density insensitivity (no *nolA-lacZ* induction at 0.4 OD). I generated and screened the putative mutants JNR1Sp1-45. JNR1Sp1-45 were identified by their failure to turn blue in the presence of 0.04μg/ml chitin on +MM plates with X-gal and antibiotics. April Wellborn, an undergraduate researcher, generated three putative mutants (JNR7-9). She screened them for a lack of response to high population density from the *nolA-lacZ* fusion on pBGAlac4 after five days growth on +MM with X-gal and antibiotics. She then conducted a β-galactosidase activity assay at higher and lower cell population densities with JNR1 and the putative mutant JNR9 (Fig. 12, upper graph). Unlike JNR1,

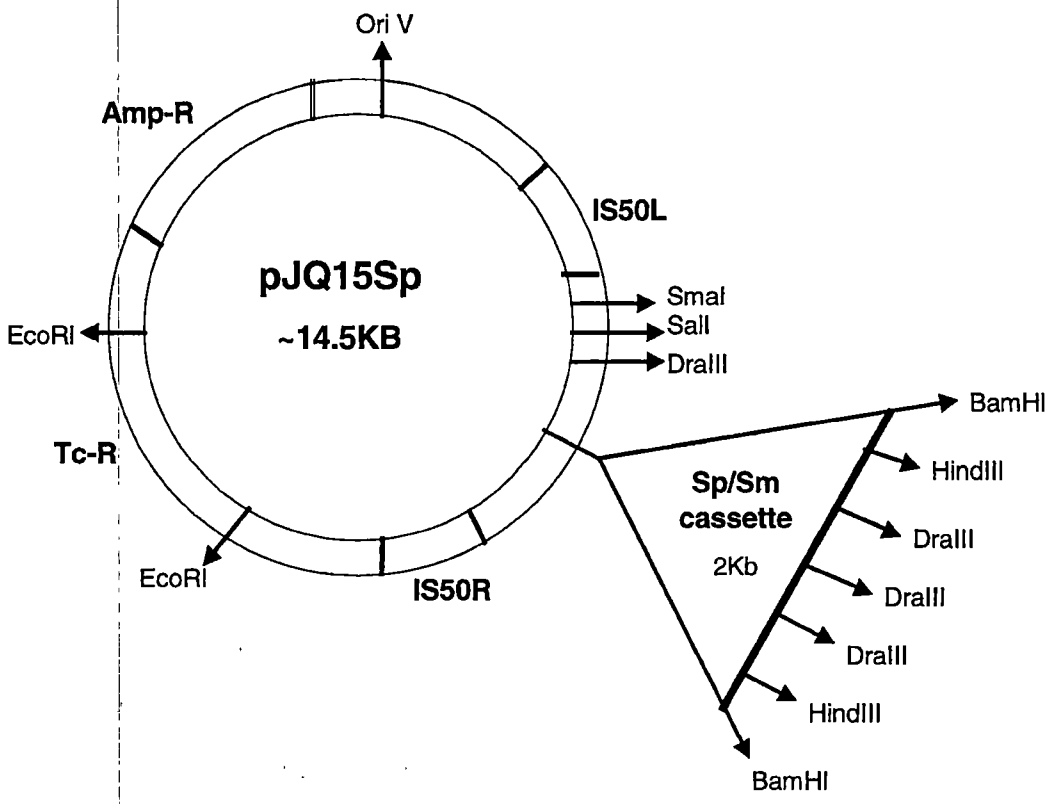


Figure 10. Map of pJQ15Sp. pJQ15Sp was used to mutagenize JNR1 cells. The Omega cassette (Sp/Sm resistant) was inserted into the unique BamHI site on the Tn5. (M. Hynes, personal communication about unpublished work)

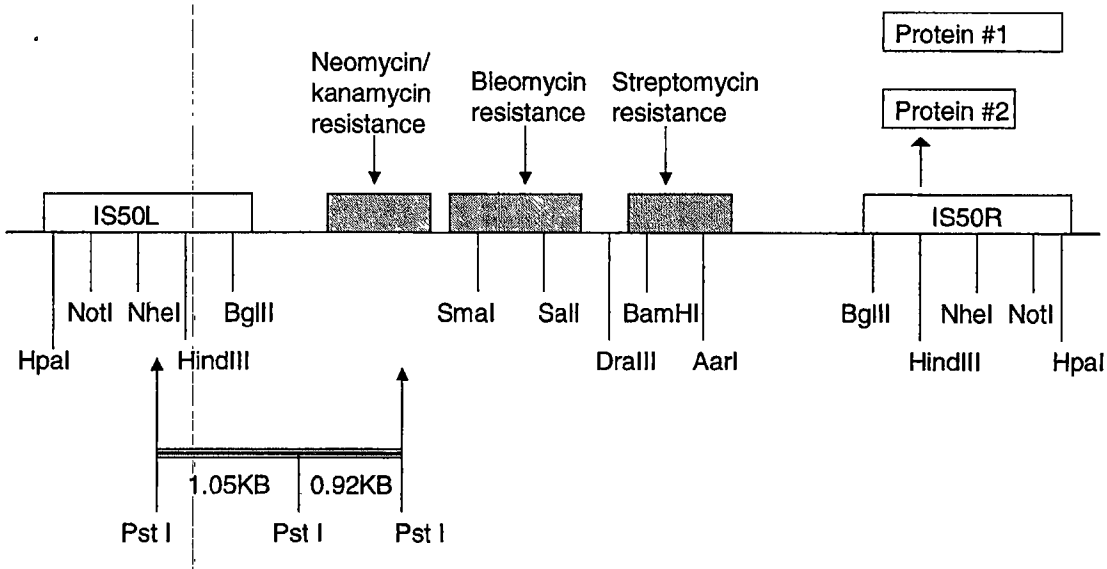


Figure 11. The 5.8KB Tn5 transposon. A single point mutation in the left insertion sequence prevents its protein expression (Rothstein 1981). The double line below the transposon indicates the locations of the Pst I fragments used as Southern blot probes for the Tn5.

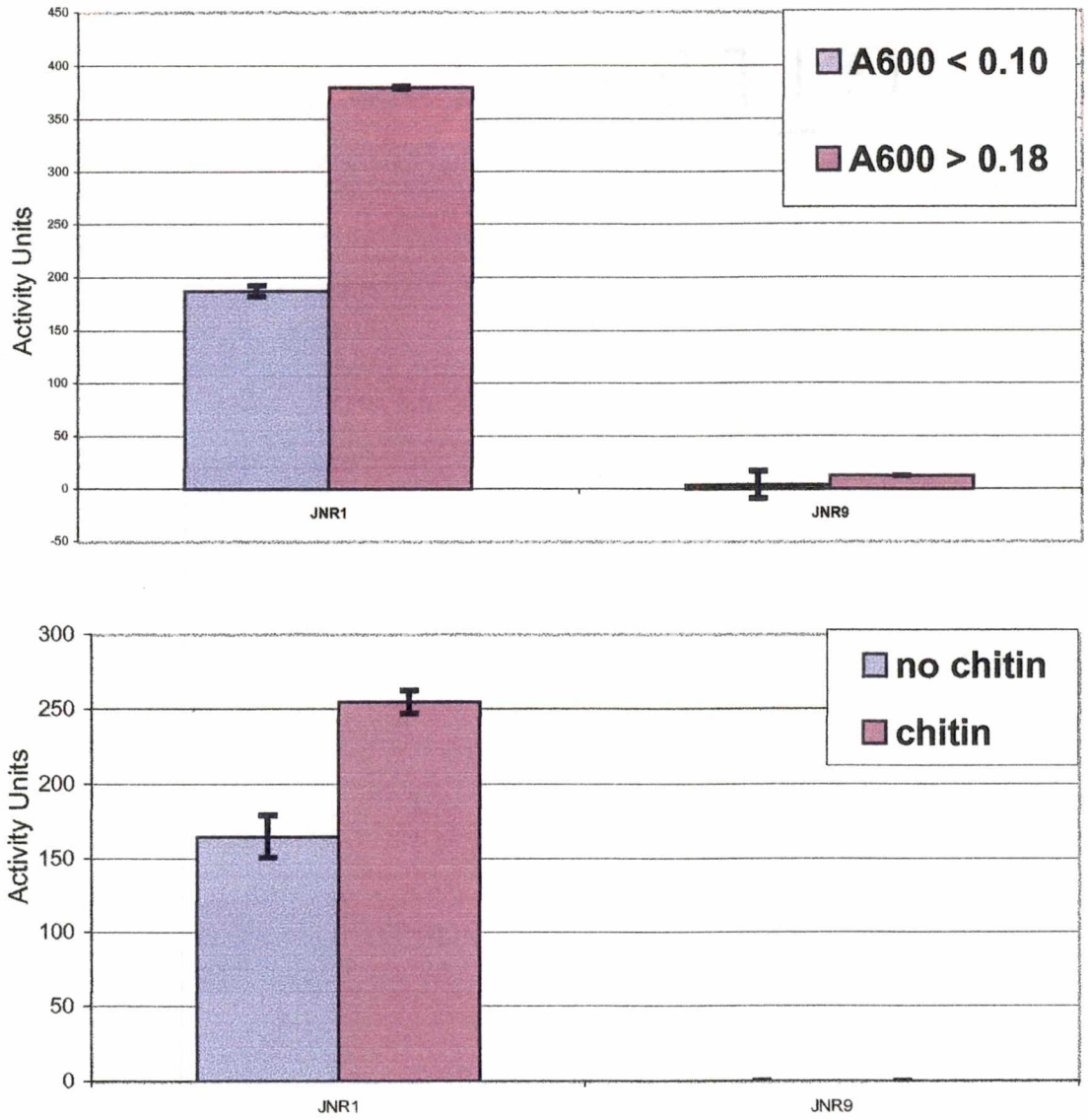


Figure 12. JNR9 β-galactosidase activity assay results. Each graph shows the average (3 replicates) β-galactosidase activities for JNR1 and JNR9 in liquid culture under *nolA*-inducing and non-inducing conditions. The upper graph represents higher (red) and lower (blue) cell population densities. The lower graph shows the effect of adding chitin (0.01mg/ml, 4 hour induction) to the cultures. In each case, mutant JNR9 fails to respond. The error bars show the 95% confidence intervals.

JNR9 failed to respond to the higher cell population density by inducing the *nolA-lacZ* fusion. Later, a β-galactosidase activity assay was conducted to confirm the suspicion that JNR9 would not respond to chitin induction either. JNR9 failed to induce the *nolA-lacZ* fusion in response to chitin (Fig. 12, lower graph).

Mutant confirmation

In order to confirm single chromosomal insertions of the Tn5, plasmids were isolated from the putative mutants. An insertion into the plasmid, pBGAlac4, would also cause an unresponsive phenotype. The plasmids were transformed into *E. coli* JM109 cells and the transformants were screened for streptomycin and spectinomycin resistance. The transformed *E. coli* cells were streptomycin and spectinomycin sensitive, which meant that the Tn5 had not transposed itself into the plasmid.

Next, chromosomal DNA was isolated from JNR9, JNR1Sp1, JNR1Sp5, and JNR1Sp7 for Southern blots. These four mutants came from different matings. At first, the 2KB omega cassette (a Bam HI fragment) from the Tn5 transposon was the probe for the Southern blots, but it also hybridized to JNR1 alone so it caused a double band in the mutants (Fig. 13). JNR1Sp1, JNR1Sp5, JNR1Sp7 and JNR9 all had the same banding patterns on the Southern blots when cut with Cla I, Sal I, Bgl II, Kpn I, and Sma I (Fig. 14). Later, the probe was changed to the 0.92KB Pst I fragment of the Tn5 (Fig. 11; also note the single bands in Fig. 16).

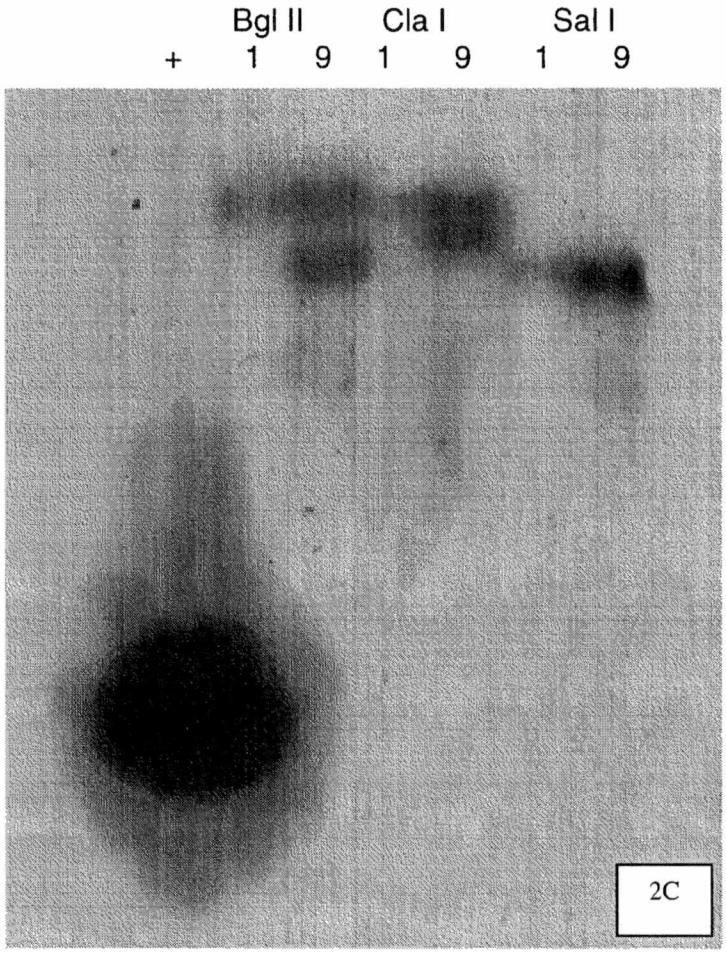
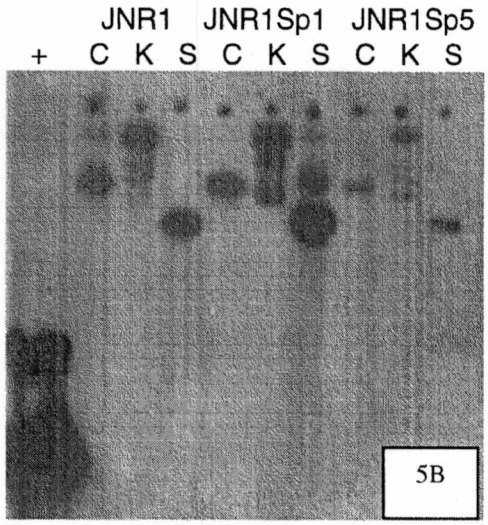


Figure 13. Southern blot 2C. This blot shows JNR1 and JNR9 chromosomal DNA cut with Bgl II, Cla I, and Sal I. The lower bands for JNR9 show the position of the Tn5 insertion are 15KB (Bgl II), 18KB (Cla I), and 12KB (Sal I). The upper bands coincide with the top of the chromosomal DNA smears at 20KB for Bgl II, 19KB for Cla I, and 15KB for Sal I. += positive control pSUP1011, 1= JNR1 and 9= JNR9.The common band in these blots is from hybridization of the probe (2kb omega cassette) to JNR1. Changing the probe to the 0.92 KB Pst I fragment from the Tn5 removes the common band (compare to fig. 14).



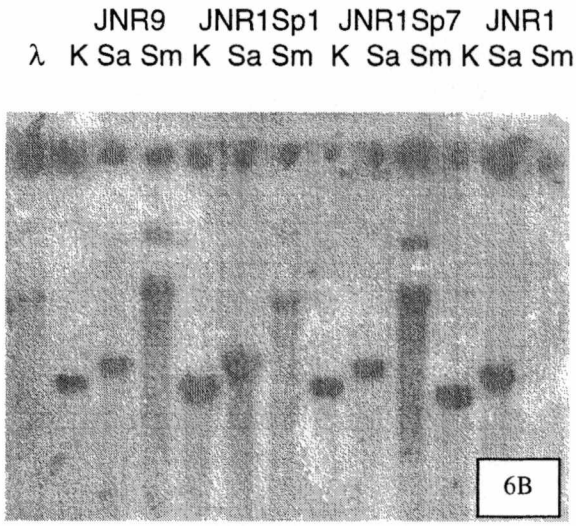


Figure 14. Southern blots showing identical banding patterns for 3 different Tn5 mutants.
Southern blot 5B shows JNR1, JNR1Sp1, and JNR1Sp5 chromosomal DNA cut with Cla I, Kpn I, and Sal I. The mutants have bands not present in JNR1. JNR1Sp1 and JNR1Sp5 have identical band patterns.
Southern blot 6B shows JNR1, JNR9, JNR1Sp1, and JNR1Sp7 chromosomal DNA cut with Kpn I, Sal I, and Sma I. JNR9, JNR1Sp1, and JNR1Sp7 have identical band patterns.
The common band in these blots is from hybridization of the probe (2kb omega cassette) to JNR1. Changing the probe to the 0.92 KB Pst I fragment from the Tn5 removes the common band (compare to fig. 16).
+= positive control pSUP1011, K= Kpn I, C= Cla I. In blot 5B, S= Sal I. In blot 6B, Sa= Sal I and Sm= Sma I.

JNR9 Cloning, Subcloning, and Sequencing Preparation

Due to the identical Southern blot banding patterns, only one mutant, JNR9, was selected to be cloned into the cosmid vector pHC79 in *E. coli* (Fig. 15). The new cosmid, pJNR9A, underwent subcloning for ease of digestion for sequencing. The interrupted gene was sequenced for further analysis.

Cloning with Phage

JNR9 chromosomal DNA was completely digested with Eco RI and ligated overnight to Eco RI digested, dephosphorylated pHC79 in a 1:1 ratio. Suitably sized recombinant cosmids were packaged into lambda phage particles using the Promega Packagene kit, and *E. coli* DH1 cells were infected. The infected cells were allowed to grow overnight in the presence of streptomycin and spectinomycin in order to enrich the culture for the desired transfectant. Cosmids from ten isolated Tc/Sp/Sm-resistant colonies were checked for the desired 18KB Eco RI fragment insert. All of the cosmids appeared to be identical on an electrophoretic agarose gel, so two (pJNR9A and pJNR9B) were selected for confirmation via Southern blot. Both pJNR9A and pJNR9B had a band matching the 18KB Eco RI band from JNR9 when the 0.92KB Pst I fragment from the Tn5 was used as a probe (Fig. 16). Due to the closeness of fragment size, the 1.05KB Pst I fragment was accidentally included with the intended 0.92KB Pst I fragment during the labeling of the probe. The 1.05KB band overlaps the Bgl II site on the IS50L region of the Tn5, so there are two bands in the positive control lane of the Southern blot in Figure 16.

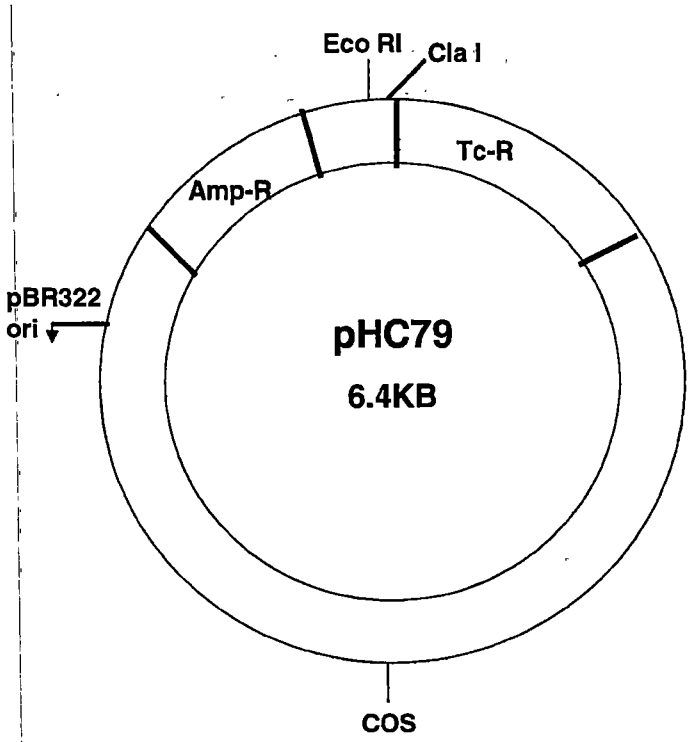


Figure 15. Map of pHC79. pHC79 is the cosmid vector used to clone the Tn5-interrupted gene from JNR9. 18Kb and 3KB EcoRI fragments were ligated into the EcoRI site, then λ phage packaged the recombinant DNA into phage particles and injected the resulting cosmid, pJNR9A, into *E. coli* DH1 host cells.

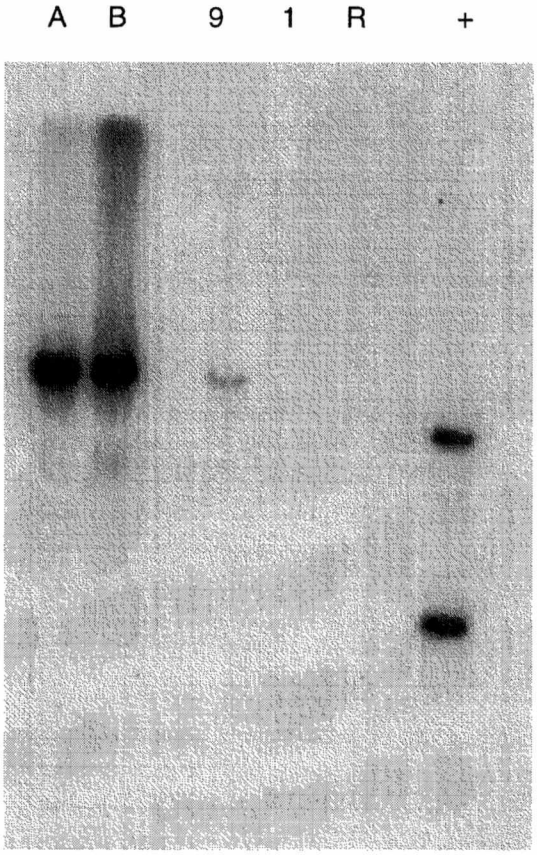


Figure 16. Southern blot of JNR9 clones. This Southern blot shows matching bands for JNR9 chromosomal DNA and the cloned cosmids pJNR9A and pJNR9B when digested with Eco RI. The positive signals for JNR9, pJNR9A, and pJNR9B are at approximately 18KB. A=pJNR9A, B=pJNR9B, 9=JNR9 chromosomal DNA, 1=JNR1 chromosomal DNA, R= *R.* NGR234 chromosomal DNA, and +=positive control pSUP1011 cut with Bgl II. The probe is a combination of the 1.01KB and 0.92KB Pst I fragments from the Tn5 (see Fig. 11). The lower band in the positive control lane is an internal Bgl II fragment from the Tn5.

Subcloning

A cosmid must be at least 50 KB in order to be packaged into lambda phage particles. Since pJNR9A (Fig. 17) contains an 18KB Eco RI fragment, a 3KB Eco RI fragment, and pHC79 (6.4KB), it must have 4 to 6 copies of pHC79. Cla I cuts pHC79 once, and cuts infrequently in *Rhizobium*. A Cla I digest of pJNR9A revealed a large band of the 2 inserted fragments and 1 copy of pHC79 (~28KB) and a 6.4KB band made by the extra copies of pHC79. In order to remove the extra copies of pHC79, pJNR9A was restricted with Cla I, phenol:chloroform extracted and ethanol precipitated to remove the enzyme, and then religated. The 6.4KB band was fainter in the resulting plasmids, but still present. One of those resulting plasmids, pJNR9D3, was again restricted with Cla I and religated to have only 1 copy of pHC79 (results were determined by digesting the plasmids of several transformants with ClaI and viewing the digests on a 0.8% agarose gel shown in Figure 18). The resulting plasmid, pD32A, can be used to sequence the interrupted gene.

Sequencing

When digested with Sal I, the plasmid pD32A yields several fragments, three of which are large enough (2.8KB, 8KB, and 15KB) to contain one side of the Tn5 and part of the interrupted gene (data not shown). Primers specific to the IS50 regions of the Tn5 are available at the UTK Molecular Biology Resource Facility. Sequencing was attempted several times, but was unsuccessful due to the difficulty of purifying adequate amounts of DNA template.

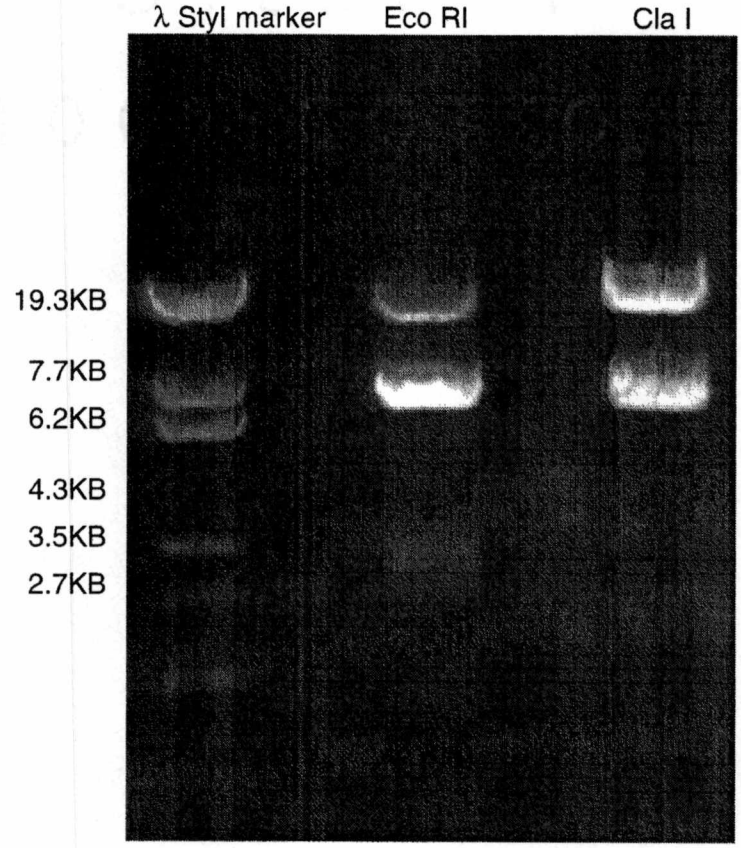


Figure 17. pJNR9A on an agarose gel. This 0.8% agarose gel shows Eco RI and Cla I digests of pJNR9A. The Cla I digest shows the extra copies of pHC79 as a separate band (6.4KB). The Eco RI digest shows pHC79 and two JNR9 chromosomal DNA fragments. The highest band contains the interrupted gene. Note the relative intensities of the 6.4KB band and the upper band.

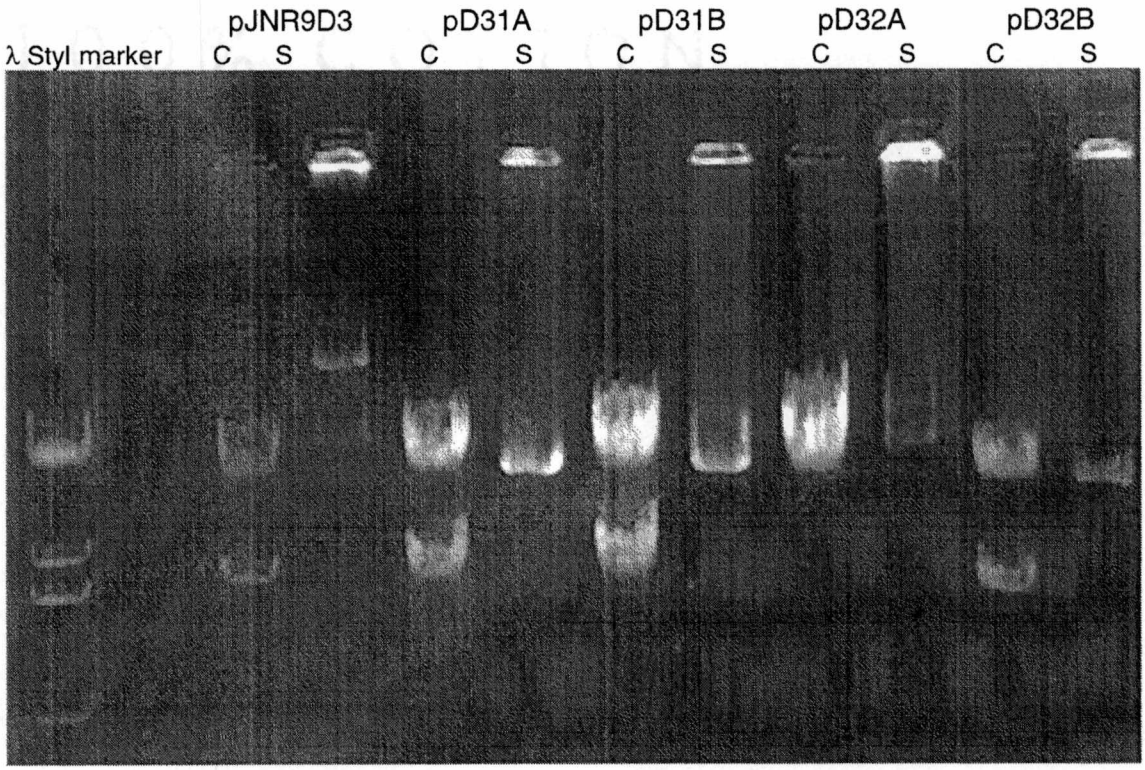


Figure 18. pJNR9A subclones on an agarose gel. This is a 0.8% agarose gel of subclone pJNR9D3 and its subclones cut with Cla I and Sma I. pJNR9A was digested with Cla I and religated to remove extra copies of pHC79. Two rounds of digestion and religation were required to remove all the extra copies. The first round resulted in pJNR9D3, and the second resulted in pD32A. The Marker bands, from top to bottom, are 19.3KB, 7.7 KB, 6.2KB, 4.3KB(faint), and 3.5KB.

JNR9 Plant Nodulation Assays

Four different legumes were inoculated with *R.* NGR234, JNR1, and JNR9 in order to determine the nodulation phenotype of JNR9. Cowpea (cultivar Red Caloona), mungbean (cultivar King), and siratro are natural hosts of *R.* NGR234. Soybean is not (Balatti et al. 1995). Uninoculated plants served as negative controls in each experiment. With 95% confidence, there were significant differences in nodulation for cowpea and mungbean, but not for siratro (Table 2). Plants grown in leonard jars were healthier and larger, with more nodules than plants grown in plastic growth pouches.

Soybean

Although *R.* NGR234 is not a natural symbiont of soybean, it did cause abnormal root growths to develop on plants grown in leonard jars 21 days post-inoculation. These root growths were uniformly white and firm, and protruded from both lateral roots and the taproot (a "popcorn" phenotype). *R.* NGR234 and JNR1 produced these growths in equal amounts. JNR9 produced twice as many root growths (Fig. 19). There were no root growths on any of the plants grown in plastic growth pouches.

Cowpea

R. NGR234 is a natural symbiont of cowpea. *R.* NGR234 and JNR1 produced similar amounts of nodules on plants in pouches and jars after 21 days. JNR9 produced significantly fewer nodules than *R.* NGR234 in pouches, and significantly fewer nodules than JNR1 in jars (See Table 3, Fig. 20.) Upon dissection and 10x magnification, the JNR9 nodules appeared to be functional (pink) and structurally identical to wild type nodules. The inoculated plants were green, and the uninoculated plants were pale

Table 2. Nodulation assay T-test results. These are the T-test results for 2 samples assuming equal variances at a 95% confidence level. The average number of nodules per inoculated plant in each inoculation group is compared to the average number of nodules per inoculated plant in the other inoculation groups. Values of 0.05 or less are significant (boldface). The soybean T-test used the average number of abnormal root growths.

Plant Host	**NGR234 and JNR1**	**NGR234 and JNR9**	**JNR1 and JNR9**
Cowpea pouches	0.230484	**0.008929**	0.051938
Cowpea jars	0.296556	0.056939	**0.002479**
Mungbean pouches	0.329736	**0.006005**	**0.009879**
Mungbean jars	0.087327	**0.000496**	**0.012578**
Siratro pouches	**0.04253**	0.056594	0.443278
Siratro jars	0.147777	0.10717	0.444682
Soybean jars	0.462259	**0.003484**	**0.008042**

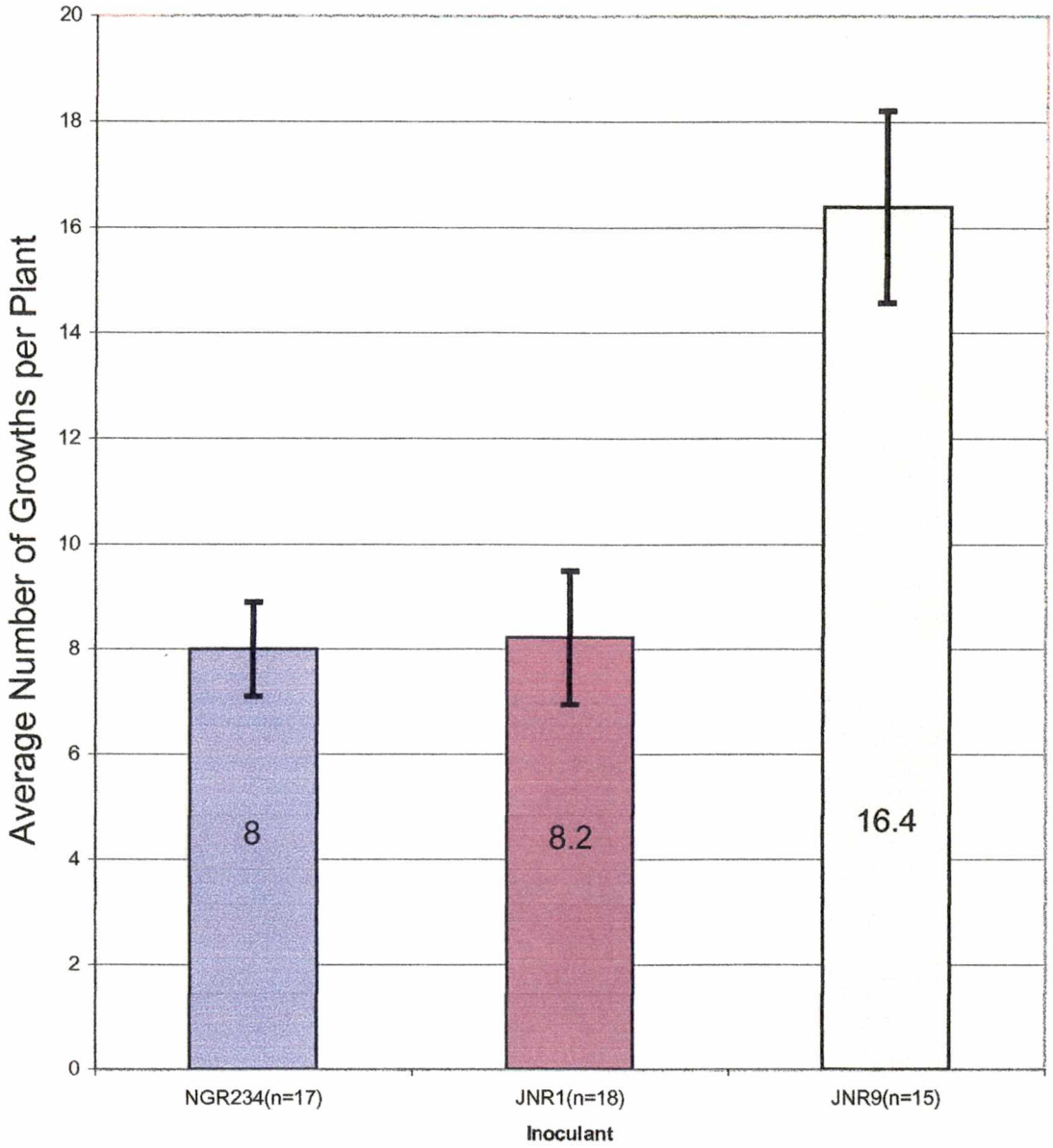


Figure 19. Soybean nodulation assay results. This graph shows the average number of lumpy, abnormal root growths on soybean plants 28 days post inoculation. The uninoculated plants (18 plants) had no abnormal root growths. The plants were grown in leonard jars. The error bars show the 95% confidence interval.

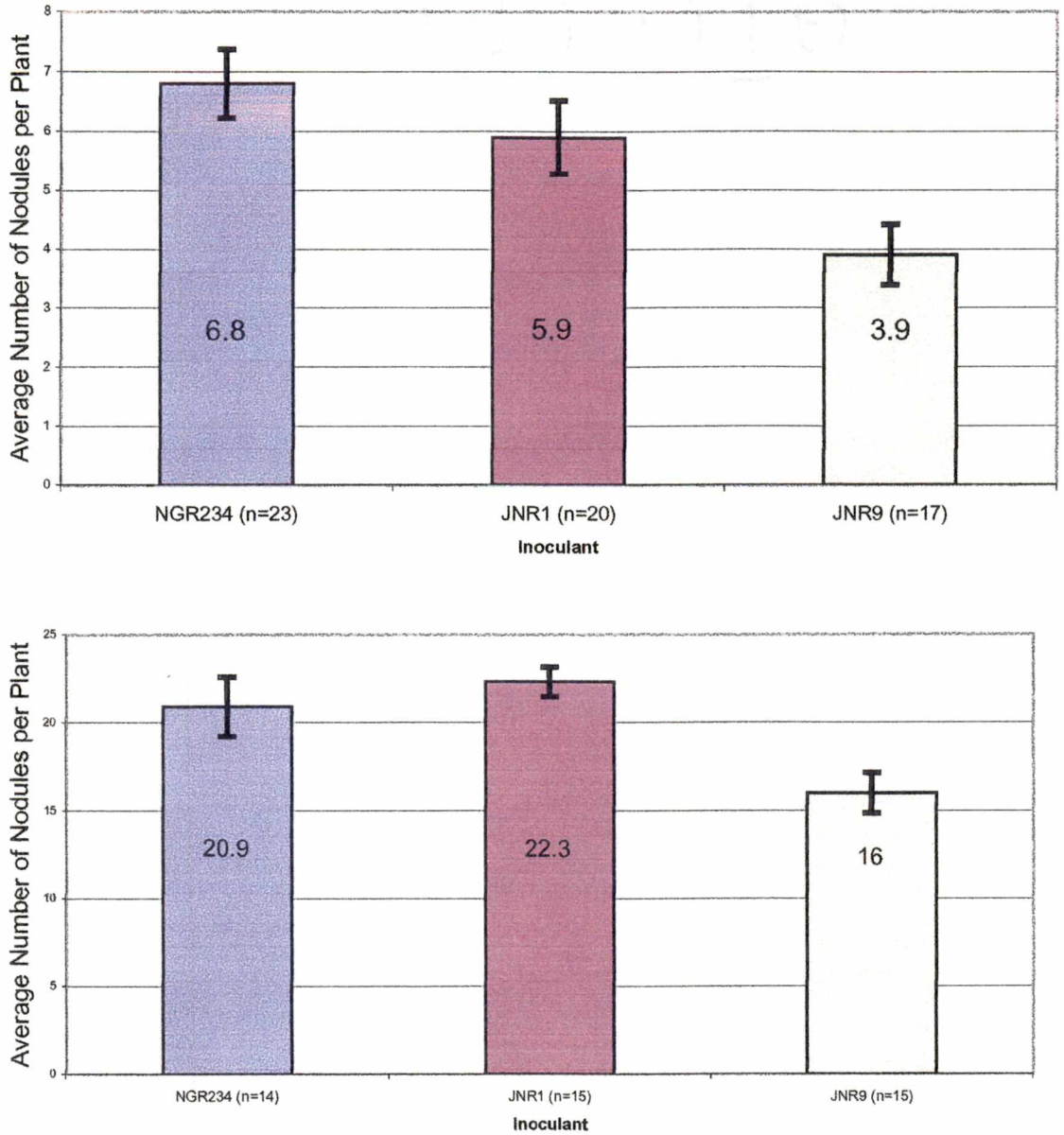


Figure 20. Cowpea nodulation assay results. These graphs show the average number of nodules per nodulated cowpea plant in leonard jars (lower graph) and growth pouches (upper graph) 28 days post inoculation. On the x-axis, n= number of plants in that inoculant group. Uninoculated plants had no nodules. The error bars show a 95% confidence interval.

Table 3. Cowpea nodulation data. These tables show the nodulation data for nodulated cowpea plants 28 days post inoculation. Table A plants were grown in pouches and table B plants were grown in leonard jars.

Table A

Inoculant Strain	**NGR234**	**JNR1**	**JNR9**
Fresh weight	545 mg	426 mg	308 mg
Weight / nodule	3.47 mg	3.61 mg	4.67 mg
% Plants nodulated	92%	84%	72%
Nodules/ plant	7±4	6±4	4±3

Table B

Inoculant Strain	**NGR234**	**JNR1**	**JNR9**
Fresh weight	630 mg	746 mg	648 mg
Weight / nodule	2.16 mg	2.40 mg	2.70 mg
% Plants nodulated	93%	100%	100%
Nodules/ plant	21±9	22±5	16±6

(chlorotic) after 3 weeks of growth.

Mungbean

R. NGR234 is a natural symbiont of mungbean. *R.* NGR234 and JNR1 produced similar amounts of nodules on plants in pouches and jars after 21 days. JNR9 produced significantly fewer nodules on plants in pouches and jars. (See Table 4, Fig. 21.) Upon dissection and 10x magnification, the JNR9 nodules appeared to be functional and structurally identical to the wild type nodules. The inoculated plants were green, and the uninoculated plants were pale (chlorotic) after 3 weeks of growth.

Siratro

R. NGR234 is a natural symbiont of siratro plants. There was no significant difference in nodulation between *R.* NGR234, JNR1, and JNR9 in jars after 21 days (Table 5, Fig. 22). In jars, all the nodules on each plant were clustered around the top of the root ball. In pouches, there was a significant difference between JNR1 and *R.* NGR234, but not between either of them and JNR9. Nodules from each inoculation group appeared red inside when cut open. The inoculated plants were green, and the uninoculated plants were pale (chlorotic) after 3 weeks of growth.

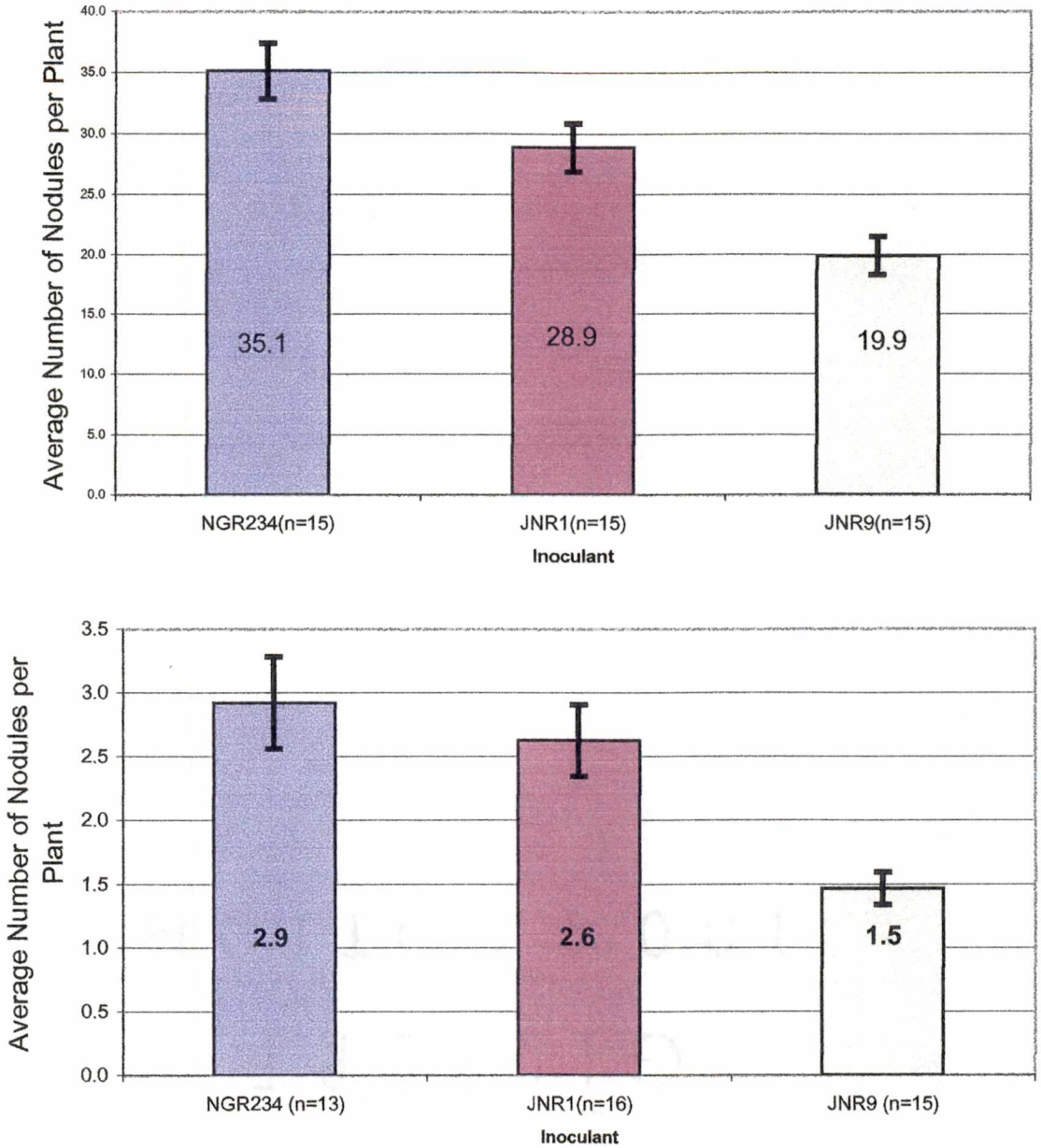


Figure 21. Mungbean nodulation assay results. These graphs show the average number of nodules per nodulated mungbean plant in leonard jars (lower graph) and growth pouches (upper graph) 28 days post inoculation. On the x-axis, n= number of plants in that inoculant group. Uninoculated plants had no nodules. The error bars show a 95% confidence interval.

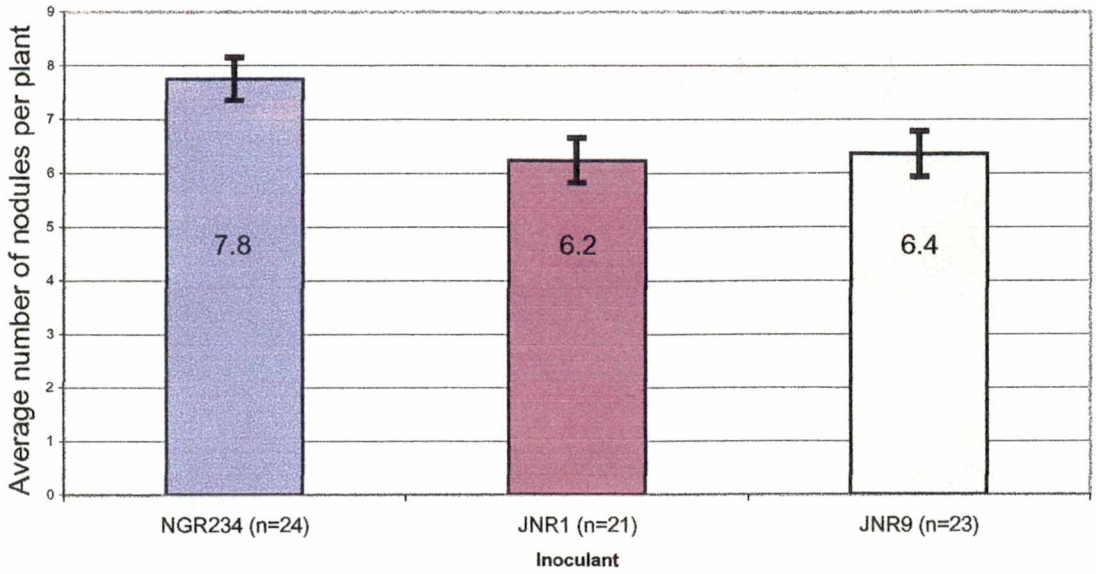
Table 4. Mungbean nodulation data. These tables show the nodulation data for nodulated mungbean plants 28 days post inoculation. Table A plants were grown in pouches and table B plants were grown in leonard jars.

Table A

Inoculant Strain	**NGR234**	**JNR1**	**JNR9**
Fresh weight	220 mg	280 mg	117 mg
Weight / nodule	5.5 mg	6.7 mg	5.3 mg
% Plants nodulated	67%	73%	65%
Nodules/ plant	3±2	3±2	2±1

Table B

Inoculant Strain	**NGR234**	**JNR1**	**JNR9**
Fresh weight	300 mg	380 mg	462 mg
Weight / nodule	0.57 mg	0.88 mg	1.55 mg
% Plants nodulated	100%	100%	100%
Nodules/ plant	35±13	29±11	20±9



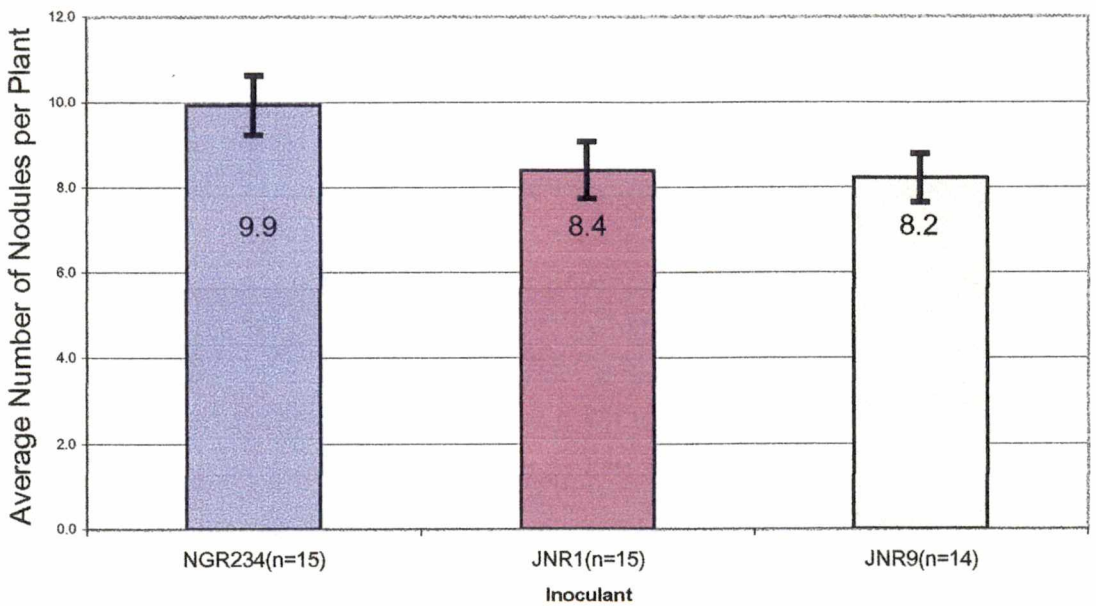


Figure 22. Siratro nodulation assay results. These graphs show the average number of nodules per nodulated Siratro plant in leonard jars (lower graph) and growth pouches (upper graph) 28 days post inoculation. On the x-axis, n= number of plants in that inoculant group. Uninoculated plants had no nodules. The error bars show a 95% confidence interval.

Table 5. Siratro nodulation data. These tables show the nodulation data for nodulated siratro plants 28 days post inoculation. Table A plants were grown in pouches and table B plants were grown in leonard jars.

Table A

Inoculant Strain	**NGR234**	**JNR1**	**JNR9**
Fresh weight	321 mg	296 mg	303 mg
Weight / nodule	1.72 mg	2.26 mg	2.16 mg
% Plants nodulated	100%	92%	100%
Nodules/ plant	8±3	6±3	6±3

Table B

Inoculant Strain	**NGR234**	**JNR1**	**JNR9**
Fresh weight	362 mg	404 mg	358 mg
Weight / nodule	2.43 mg	3.21 mg	3.11 mg
% Plants nodulated	100%	100%	100%
Nodules/ plant	10±4	8±4	8±3

4. Discussion

nolA-luxCDABE Fusions

The *nolA-luxCDABE* plasmid-encoded fusion pLuxA1 provided confirmatory data about *nolA* cell population density dependent autoinduction. Although it was in *E. coli* instead of its native *B. japonicum,* the *nolA* promoter was induced at 0.4 OD just as it is in *B. japonicum.* The similarity of expression in a different bacterium may be useful in future NolA expression experiments because *E. coli* is very easy to work with and grows very quickly.

In this work, NolA1 made from the *trp-nolA* fusion on a separate plasmid induced the *nolA* promoter. The *trp* promoter should have been expressing NolA1 steadily because of the absence of tryptophan in the medium. Perhaps the levels of NolA1 reached a sufficiently high concentration at 0.4 OD to induce the *nolA-lacZ* fusion, or perhaps another signal from the *E. coli* host cell itself was inducing the *nolA* promoter.

The *nolA-luxCDABE* transposon experiment showed that luciferase is not suitable for viewing the expression of NolA *in planta* due to lack of substrate. *B. japonicum* was unable to provide enough fatty aldehyde substrate for the reaction catalyzed by luciferase. Perhaps the plant host could be altered to deliver the substrate into the symbiosome, but this method would be time-consuming and impractical. Even if the substrate limitation could be overcome, the luciferase reaction becomes oxygen-limited at a level of cell density well below that of *nolA* autoinduction in liquid cultures.

The *nolA-luxCDABE* fusion could have commercial value as a detector of chitin (indicating a fungal source) in foods. The fusion could be inserted via a transposon into

a fast-growing bacterium such as *Seratia marcesens*. The newly bioluminescent bacterium could be used in a bioluminescent-bioreporter integrated circuit similar to that described by Simpson et al. (1998). Many strains of *S. marcesens* are pigmented at room temperature, so it should be relatively easy to check the color of the bacteria on the circuit, and then expose it to a sample of a food product such as yogurt. Any light given off by the bioluminescent-bioreporter would be interpreted as a sign of fungal contamination in the food product.

Rhizobium NGR234 mutant JNR9

Several independent chitin insensitive or cell population density independent mutants were isolated which all gave identical hybridizing band patterns on Southern blots. The identical banding patterns could mean that either the Tn5 insertion is in the same gene for all the mutants or the insertions are in closely linked genes (e.g., an operon). Given that several restriction enzymes (Cla I, Kpn I, Sal I, etc.) were used for the Southern blots, it is unlikely that the insertions are at different sites that coincidentally give similar banding patterns. Even very closely linked genes would have had different restriction fragment sizes with some of the restriction enzymes used.

The generation of a single mutant from many mutagenic matings was surprising because the mutants were selected using different criteria. Putative mutants JNR7-9 were selected for an inability to induce the *nolA-lacZ* fusion in response to high cell population densities, and putative mutants JNR1Sp1-45 were selected for an inability to induce the same fusion in response to chitin. Since putative mutants from both selection groups had the same interrupted gene, that interrupted gene must be involved in the

response to both chitin and high cell population density. The β-galactosidase activity assay results shown in figure 12 revealed that the mutant JNR9 is unable to induce the *nolA-lacZ* fusion in response to chitin or higher cell population density.

Based on the Southern blot analysis of the mutants and the β-galactosidase activity assay results of JNR9, both chitin and culture density sensitivity can be affected by mutating the same gene. This coincidence suggests that the signaling pathways for the cell population density factor and the Nod factor signals run together at some point before reaching *nolA*. Since there was only one interrupted gene, its gene product may be the intermediate step between the induction signals and *nolA*. The single interrupted gene's product may be activated by Nod factors and cell density factors to bind the *nolA nod* box and start transcription. Even a weak activation would be sufficient because any NolA1 made would then autoinduce *nolA*.

In plant nodulation assays, JNR9 nodulated cowpea and mungbean poorly in comparison to wild-type bacteria, but the nodules that were established by JNR9 were identical to those of wild-type bacteria and their host plants appeared to be equally healthy. The plants with fewer nodules simply allowed those nodules to grow larger in order to meet the plant's nitrogen needs. Since healthy, effective nodules are established by JNR9, it seems likely that the interrupted gene affects *nolA* regulation during infection but not during bacteroid maturation (the two points during nodulation when *nolA* must be induced).

The role of the interrupted gene during the early stages of infection should be studied further and in greater detail. For example, the number of infection threads plus developed nodules may be higher for JNR9 because of its difficulty in signaling *nolA* to

repress the *nod* genes. More infection threads might be needed to establish the required levels of symbiotic nitrogen fixation for healthy plant growth. JNR9 cells may continue to expend their energy and resources making Nod factors past the point where it is necessary to signal the plant.

On siratro plants, there was no difference in nodulation success between JNR9 and the wild-type bacteria. There were no significant differences in nodule number or size, and all the nodulated plants seemed equally healthy. JNR9's lack of effect on siratro plant nodulation could have two possible meanings. First, as is the case of the *B. japonicum nolA* in soybean, *nolA* (and therefore the interrupted gene) may not play a major role in siratro plant nodulation (but may be more important in other hosts). Second, the protein from the interrupted gene may only be used to signal *nolA* in certain hosts, or under certain conditions that are not met in siratro. If the second theory were true, the mutagenesis should have created mutants with Tn5 insertions in two different genes (one gene for siratro-generated signals and another for cowpea/mungbean-generated signals). Also, the chitin-insensitive mutants were screened for induction by chitin using a raw mixture of chitin from crab shells so the preferred chitin chain length for the Nod signal to siratro plants would have been present during the screening.

In the future after the interrupted gene is sequenced, the *B. japonicum* genome should be searched for homologs. The poorer nodulation in some plant hosts but not others is similar to that of *B. japonicum nolA* mutants (Garcia et al. 1996), so there may be a similar gene affecting *nolA* expression in *B. japonicum* as well. The search should also continue for the *R.* NGR234 copy of *nolA*. The interrupted gene from JNR9 affects the *B. japonicum nolA*, so it is reasonable to think there must be a native target in *R.*

NGR234. A version of *nolA* has not yet been found in *R.* NGR234, but it must have a *nolA*-like gene to repress Nod factor production if it has a gene to regulate *nolA* expression.

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Vita

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