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Thomas G. McCabe
Major professor

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A GENETIC, MOLECULAR AND CYTOLOGICAL ANALYSIS OF THE *RHIZOBIUM FREDII* USDA 205 - *GLYCINE MAX* INTERACTION

By

Avraham Rasooly

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ABSTRACT

A GENETIC, MOLECULAR AND CYTOLOGICAL ANALYSIS OF
THE *RHIZOBIUM FREDII* USDA 205 - *GLYCINE MAX* INTERACTION

By

Avraham Rasooly

The interaction between soybean (Glycine max (L.) Merr.) and Rhizobium fredii USDA 205, a newly introduced soybean symbiont unable to nodulate North American soybean cultivars, was studied from several different perspectives. A 9.3 kb fragment containing R. fredii genes involved in nodulation was cloned by use of a heterologous probe. The nod genes from R. fredii appear to be organized differently from nod genes in other species. At least one nod gene is duplicated in R. fredii, and the nodulation genes are distributed on at least two large plasmids. The cloned genes were able to complement nodC- and nodD- mutants of R. meliloti. The allele permitting nodulation with R. fredii was introduced into a North American soybean genetic background by crossing a North American genotype with an Asian genotype. R. fredii was at least as good a symbiont as Bradyrhizobium japonicum with selected F_3 plants from the cross; however the ultrastructure of the nodules formed by R. fredii was unusual in several respects. The soybean gene ril which prevents nodulation by B. japonicum exhibited recessive epistasis to the allele which enables R. fredii to nodulate, and that the two traits are at different loci.

To my parents for their principles and example which have guided me throughout my life, and to my wife, my partner in life and in science for her support, advice and understanding.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Biological nitrogen fixation is a process by which free atmospheric dinitrogen is reduced to ammonia by the enzyme nitrogenase, and is a major source of fixed nitrogen. It has been estimated that 1.2×10^8 tons of atmospheric nitrogen are fixed by microorganisms annually, more than 60% of the global nitrogen fixed (Newton and Burgess, 1983). Fixed nitrogen fertilizer is a key to increased plant productivity. Leguminous plants have the ability to form symbiotic associations on their roots with nitrogen-fixing bacteria. While the plant supplies the bacteria with photosynthate and other nutrients, the bacterium supplies reduced nitrogen continuously, enabling legumes to grow without nitrogen fertilizers. Biological nitrogen fixation is especially important in developing countries unable to afford manufactured fertilizers (Bliss, 1985; Hodgson and Stacey, 1988).

Soybeans (Glycine max (L.) Merr.) are the second largest crop in both cash value and total acreage planted in the United States, with nearly 70 million acres planted. Another 70 million acres are planted worldwide (Stacey, 1984). Improving the nitrogen fixation ability of soybean might well increase soybean production (Morris and Weaver, 1983).

Any improvement in nitrogen fixation ability must be based on an understanding of the symbiosis between the plant host and the bacterial

symbiont. This comes from an interdisciplinary approach to study of the symbiosis, including cytology, metabolism, genetics and ecology. The main aspects of the symbiosis include:

-stages of the symbiosis

-the genetic basis of the plant-bacteria interaction

-metabolism of both plant and bacteria

-soil microbial ecology relating to nodulation

-factors which limit nitrogen fixation

-exploration of new sources of plant and bacterial genetic variability related to the symbiosis.

In this work the symbiosis of Rhizobium fredii USDA 205, a newly introduced soybean symbiont (Keyser et al, 1982), was studied from different perspectives in three sets of experiments:

1) Transfer of the ability to nodulate with R. fredii from genetically unimproved Asian cultivars to North American soybean cultivars.
   a. Evaluation of the nitrogen fixation ability of R. fredii as a symbiont with North American cultivar genetic background.
   b. Examination of the cytology of this interaction.
2) Analysis of interactions between soybean genes involved in symbiont selection.
3) Study of the molecular genetics of R. fredii, analyzing the genes involved in symbiosis.

We chose these questions because they represent topics of general interest in understanding the symbiosis, as well as enabling us to

evaluate the potential agronomic importance of R. fredii USDA 205. Our work is particularly relevant in the context of work done by others on many aspects of the plant-Rhizobium symbiosis. In particular, our work grows out of previous work on three areas which I will summarize in detail:

1) Developmental analysis of nodulation
2) Classical and molecular analysis of bacterial symbiosis genes
3) Plant symbiosis genes

DEVELOPMENTAL ANALYSIS - Rhizobium infection and nodule development

Rhizobium is a genus of Gram-negative soil bacteria with the ability to form nitrogen-fixing nodules on the root of leguminous plants. The symbiosis is very specific; each species of Rhizobium nodulates only a limited range of leguminous plants. Development of the symbiosis is a complex process requiring interaction between the plant host and the bacterial symbiont.

The sequence of events necessary for symbiotic nitrogen fixation has been summarized by Hodgson and Stacey (1984) using a naming system suggested by Vincent (1980). The process can be divided into three stages: preinfection, infection and nodule development, and nodule function. Each stage comprises a number of events (Table 1).

The first step in the establishment of symbiosis is the mutual recognition by the host and the symbiont, leading to specific attachment (Roa). Current theory suggests that lectins (carbohydrate binding proteins) found on the root surface bind to the bacterial cell surface (Dazzo and Truchet, 1983). Bacterial surface polysaccharides,

Table 1 - Events in the development of the plant-bacteria symbiosis

Stage	Event	Phenotypic code	Process
Preinfection	1 Root colonization	Roc	Competition
	2 Root adhesion	Roa	Competition
	3 Hair branching	Hab	Nodulation (Nod)
	4 Hair curling	Hac	Nod
Infection and nodule development	1 Infection	Inf	Nod
	2 Nodule initiation	Noi	Nod
	3 Bacterial release	Bar	Nod
	4 Bacteroid development	Bad	Nod
Nodule function	1 Nitrogen fixation	Nif	N-fixation (Nif)
	2 Complementary functions	Cof	Nodule physiology
	3 Nodule persistence	Nop	Not Examined

lipopolysaccharides (LPS), capsular polysaccharides (CPS), and extracellular polysaccharides (EPS) may all have a role in the recognition (Hodgson and Stacey, 1988). The plant also induces transcription of bacterial nodulation genes by secreting specific flavonoid compounds from the roots (Peters et al, 1986; Redmond et al, 1986; Firmin et al, 1986).

After the recognition and attachment, a newly formed root hair becomes deformed, curling and branching into a structure that resembles a shepherd's crook (Hab, Hac) which entraps the bacteria. An infection thread forms which transfers the bacteria from the surface into the root meristem (Inf). At the same time, the root cortical cells are dividing to form the nodule (Noi). Eventually, the bacteria are released from the infection thread (Bar) into a space surround by the peribacteroidal membrane, which appears to originate from the Golgi apparatus (Mellor and Werner, 1987). The bacteria divide and differentiate into bacteroids (Bad), specialized nitrogen-fixing forms of the bacteria which are unable to divide.

The enzyme nitrogenase is synthesized in the bacteroid. The nodule synthesizes leghemoglobin to reduce oxygen concentration allowing nitrogenase to form and function (Nif). Bacterial Nif genes are regulated positively in response to nitrogen limitation and symbiotic development, and regulated negatively in response to oxygen and higher levels of fixed nitrogen (Gray et al, 1986). The bacterial Nif genes are part of the Ntr global regulatory pathway which responds to enviromental stimuli (Gray et al, 1986). The ammonia formed is metabolized via the glutamine synthetase-glutamate synthase couple,

found in infected plant cells, with export of amides or ureides to other plant cells, while photosynthate is transferred to the bacteroids (Cof) to support nitrogen-fixation. Schubert (1986) has reviewed the specific metabolism of effective root nodules.

BACTERIAL GENETICS - genes involved in early nodulation stages

Nodulation and nitrogen fixation functions appear to be evolutionarily conserved (Djodjevic et al, 1985). The molecular genetics of three species have been studied in great detail: the alfalfa (Medicago sativum) symbiont R. meliloti, the clover (Trifolium spp.) symbiont R. trifolii, and the pea (Pisum sativum L.) symbiont R. leguminosarum. There appear to be many structural similarities in the symbiotic genes of these three species and of all other species which have been examined. In most cases the genes for symbiosis are found on very large (sym) plasmids (>100 kb) (Broughton et al, 1986). Another similarity is that the symbiosis genes are organized as clusters of several operons.

Rolfe and Gresshof (1988) recently summarized the genetics of nodulation and proposed that there are three main classes of genes involved in the early stages of nodulation:

1. NodABCD - The common nod genes which are interchangeable among the species and which are involved in early stages of nodulation.
2. NodI and NodJ - genes involved in infection thread formation.
3. Hsn genes - Host-specific nodulation genes defining the spectrum of plants the symbiont can nodulate (NodEFGHLMX).

Kondorosi et al (1986) have proposed a fourth group: Efn genes which are not essential for nodulation but increase its efficiency, including NodD2. Table 2 summarizes the bacterial genetics of nodulation (based on Rossen et al, 1987; Djordjevic et al, 1987).

Molecular organization and regulation of bacterial Nod genes

In all three Rhizobium species studied, the Nod genes are organized into two clusters on the sym plasmids (Kondorosi et al, 1984, 1985). One cluster is the common Nod genes which are organized into two transcriptional units: NodABC and NodD (Kondorosi et al, 1985; Mulligan and Long, 1985). These genes are highly conserved (at least 70% homology between any two species) and interchangeable among the various Rhizobium species. The other cluster is the host specificity genes.

A "nod box", a 26-47 base pair conserved promotor sequence, was found in all three species in front of the NodABC, NodFEL, and, in R. meliloti, the NodD2 trancription units (Egelhoff et al, 1985; Rostas et al, 1986).

The bacterial gene regulating nodulation appears to be NodD, which is responsible for activating transcription of other Nod genes in the presence of plant-secreted flavonoid compounds (Firmin et al, 1986; Redmond et al, 1986; Egelhof et al, 1985). In the model suggested by Kondorosi and Kondorosi (1986), NodD is transcribed continuously, and the NodD protein responds to a plant factor (plant flavonoid). The modified protein then interacts with the "nod box" sequences to initiate Nod gene transcription (Rosaras et al, 1986).

Table 2 - Bacterial genes involved in nodulation

Gene	Gene Product Function and location	Mutant Phenotype	Rhizobium species
COMMON GENES			
Nod A	Cytoplasmic	Hac^{-}[a]	R.m.,R.l.,R.t.[b]
Nod B	Not known	Hac^{-}	R.m,R.l.,R.t.
Nod C	Membrane-bound	Hac^{-}	R.m.,R.l.,R.t.
Nod D	Constitutive regulatory protein	Hac^{-}	R.l.,R.t.
NodD1/D2[c]	Regulatory proteins	delayed	R.m.
HOST-SPECIFICITY GENES			
Nod E	Not known	delayed	R.m., R.l.,R.t.
Nod F	Fatty acid biosynthesis[d]	delayed	R.m.,R.t.
Nod G	Ribitol dehydrogenase[d]	delayed	R.m.
Nod H	Not known	Hac^{-}	R.m.
Nod L	Not known	?	R.l.
Nod M	Aminophosphoribosyl tranferase[d]	?	R.l.
Nod X	Membrane-bound	?	R.l.[e]
INFECTION THREAD FORMATION GENES			
Nod I	Membrane-associated	delayed	R.l.
Nod J	Membrane-bound	delayed	R.l.

aabbreviations refer to stages as explained in Table 1. Delayed means that nodulation is normal but delayed in development. A "?" indicates that the stage at which nodulation is blocked is not known.

[b]abbreviations: R.l. - *R. leguminosarum*; R.m. - *R. meliloti*; R.t. - *R. trifolii*

[c]There are two *NodD* genes in *R. meliloti*. *NodD1* is constitutively expressed as in other species. *NodD2* is regulated by *NodD1*; both have a delayed nodulation mutant phenotype. *NodD2* is a "nodulation enhancer".

Table 2 (cont'd.).

[d]Gene product inferred by sequence homology

[e]*R. leguminosarum* cultivar Afghanistan only

PLANT GENETICS - Host factors involved in nodulation

Infection normally occurs in a distinct region behind the root tip (Bhuvaneswari and Bauer, 1980), in the region with newly emerging root hairs. This has been described as a "moving window of infectivity" (Rolfe and Gresshof, 1988). Experiments with split root systems (Matthews et al, 1987) showed that inoculation of one side suppresses nodulation of the other side of the root, indicating an additional systemic control of nodulation by the plant.

Plant nodulation and nitrogen fixation are regulated by the levels of combined reduced nitrogen, especially nitrate (reviewed in Fred et al, 1932). When these levels are high and nitrogen is available to the plant, the whole symbiosis is inhibited. "Nitrate tolerant" mutants of soybean were isolated (Carrol et al, 1985). These mutants have a supernodulated phenotype with up to 4000 nodules per plant, at least ten times as many as normal.

There are also plant nodulation mutants which do not nodulate (Matthews et al, 1987). With all of these mutants, a very high bacterial inoculum induces the formation of a small number of normal nodules. This result may indicate that these are also mutants in the autoregulation system which determines the number of nodules per plant, although there are other possible explanations.

Like *Rhizobium*, plants may have "host range" genes, which restrict the spectrum of bacteria with which they can form nodules. In soybean, the recessive allele *rj1* (Williams and Lynch 1954) restricts nodulation to specific rhizobitoxine-producing *Rhizobium* strains (Devine and Breithaupt, 1980), and is allelic to one of the *nod*$^-$ plant mutations

described above (Matthews et al, 1987). Other soybean mutants also restrict nodulation to specific Rhizobium serogroups. These include rj2 (Caldwell, 1966), rj3 (Vest, 1970), rj4 (Vest and Caldwell, 1972), and the allele which enables nodulation with R. fredii (Devine, 1984). Together, these results demonstrate the complexity of plant regulation of nodulation and suggest that the plant may play a major role in controlling the extent of the symbiosis.

LIMITING FACTORS - Energetic cost of symbiosis

Symbiosis has a high energy cost to the plant. Nitrogen fixation uses 13.5 moles of ATP per mole of ammonia formed, compared to 12 moles ATP needed to reduce one mole of nitrate (Hodgson and Stacey, 1988). In terms of the individual plant, therefore, exogenous nitrate is a more efficient source of reduced nitrogen, and this may explain why nodulation is inhibited by reduced nitrogen (Fred et al 1932). It has been estimated (Stacey, 1984) that 15-30% of the total carbon fixed by symbiotic plants photosynthetically simply sustains the process of nitrogen fixation by supplying energy to the bacteroids and plant cells in the nodules, and by providing the carbon skeletons to which the fixed nitrogen can be attached and transported in the plant.

The enzyme nitrogenase also evolves H_2, which is regarded as a significant waste of energy. Of 16 ATP molecules and eight electrons used to produce two molecules of NH_3, 4 ATP molecules and two electrons are used to reduce protons which are lost as H_2 released to the atmosphere. The enzyme which catalyzes hydrogen uptake, encoded by the Hup gene, is found in some Rhizobium species. It converts H_2 to water

and ATP (Schubert and Evans, 1976).

Schubert and Evans (1976) propose that the ability of these Rhizobium species to recycle hydrogen efficiently may increase the efficiency of nitrogen fixation. In fact, in some cases plants inoculated with hup^+ strains or isolines showed increased dry weight as compared to plants inoculated with hup^- strains, although this increase was not always seen in the seed weight (Hodgson and Stacey, 1988). Therefore, more studies are needed to determine the effect of hydrogen uptake on legume productivity.

Mobilization of Nitrogen

Nitrogen nutrition of soybean plants during pod development is important in determining seed yield (Morris and Weaver, 1983). During this stage, nitrogen is mobilized from leaves to developing pods and the rate of photosynthesis and nitrogen-fixation drops. Morris and Weaver (1983) suggest that B. japonicum strains with a high rate of nitrogen-fixation replace mobilized leaf nitrogen, because they continue to fix nitrogen. It has been suggested that bacterial storage carbohydrates provide the energy for this extended nitrogen-fixing capacity (Hodgson and Stacey, 1988).

Ecological factors limiting efficient nitrogen fixation

The main factor limiting efficient nitrogen fixation is competition for nodule induction sites on roots between Rhizobium strains in the soil. Often, the native strains are well adapted to the soil environment but have not been selected for effective nitrogen-

fixation. These native strains can outnumber and outcompete the bacteria in the inoculum so that inoculated strains occupy only 0-17% of the nodules (Caldwell, 1970; Ham et al, 1971; Ham, 1978). Restriction of nodulation by the plant so that only preferred Rhizobium strains nodulate (Devine, 1977), or development of desirable strains that can also compete successfully with the native strains, are two means of increasing the efficiency of nitrogen fixation.

Some of the bacterial genes regulating competition between two R. leguminosarum strains for a specific pea cultivar were mapped to a symbiotic plasmid and cloned (Dowling et al, 1988). From the plant side, some soybean genotypes which restrict nodulation have also been identified. For example, there is a group of genotypes which reduce nodulation with B. japonicum serogroups 123, the major native B. japonicum in the United States midwest (Keyser et al, 1988).

CONCLUSION

Nitrogen fixation is a complex process involving many plant and bacterial genes. From the plant perspective it appears that the plant restricts nodulation and nitrogen fixation to a minimum level essential for growth, inhibiting nodulation when reduced nitrogen is available and in the root region under the inoculated region.

The symbiosis is based on mutual recognition of both partners and the exchange of chemical recognition signals. The complexity of the nodulation process requires coordination of bacterial and plant genes. The initial events in nodulation and the bacterial genes involved therein are well known, but events subsequent to nodule initiation and

the plant genes affecting the symbiosis are not well characterized. There is a need for a system to study these aspects of the symbiosis.

From an agronomic perspective, competition between rhizobia for nodulation is important. The ideal situation is one in which the plant will only form nodules with a specific symbiont, chosen for its fixation ability. Soybean genes which restrict nodulation are known (*rj1*, *rj2* and *rj3*). The question is how to overcome these restrictions in order to create a non-competitive situation in which only desired symbionts form nodules. Through an understanding of the plant signals that are involved in nodulation as well as the bacterial gene regulation during nodulation it may be possible to understand why *Rj* genes restrict nodulation. In particular, it appears that the *rj1* system where only one group of bacterial strains is able to overcome the restriction may be a good model to study when trying to improve nitrogen-fixation ability of soybean.

In summary, important questions to be addressed in nitrogen-fixation research include regulation of the later stages of nodulation, the contribution of plant genes to the symbiosis, and finding the basis for successful competition between bacterial strains for a plant host. We have studied these questions using the symbiosis of *R. fredii* USDA 205 and soybean.

CHAPTER 1

THE NOD REGION OF RHIZOBIUM FREDII USDA 205

Abstract

DNA hybridization of total genomic DNA of from Rhizobium fredii USDA 205 with the cloned "common" NodABCD genes from R. meliloti revealed DNA homology to four EcoRI fragments. The major fragment at 9.3 kb was cloned and was able to complement R. meliloti nodC⁻ and nodD⁻ mutants, but not a R. meliloti nodB⁻ mutant. The region of Nod gene homology to the R. meliloti common Nod gene probe on the cloned fragment was found to be restricted to a 1.8 kb XhoI fragment. R. meliloti common Nod genes hybridize to two of the large endogenous plasmids of R. fredii. A mutant missing the smaller of these plasmids is able to carry out early steps of nodulation, including root hair curling, suggesting that the mutant still contains the common Nod genes. Hybridization with gene-specific probes suggests that at least NodD is duplicated in the USDA 205 genome, and is found on two megaplasmids.

Introduction

The Rhizobium-legume symbiosis is a complex interaction between the plant host and the symbiont. Root nodules form as a result of a multi-step developmental process in which many plant and bacterial

genes participate. Nodulation and nitrogen fixation functions appear to be evolutionarily conserved (Djordjevic et al, 1986). Generally, the genes for symbiosis are found in clusters on very large (sym) plasmids (Broughton, 1984; Kondorosi and Kondorosi, 1986; Djordjevic et al, 1986). Rolfe and Gresshof (1988) have summarized the evidence that there are three main classes of genes involved in the early stages of nodulation: NodABCD - the common nod genes which are interchangeable among the species and which are involved in early stages of nodulation; NodI and NodJ - genes involved in infection thread formation; and Hsn genes - host-specific nodulation genes which define the spectrum of plants the symbiont can nodulate (including NodEFGHLMX). Kondorosi and Kondorosi (1986) have proposed a fourth group: Efn genes which are not essential for nodulation but increase its efficiency, including NodD2. There are three NodD genes in R. meliloti (Honma and Ausubel, 1987).

The Nod genes are organized into two clusters on the sym plasmids. One cluster is the "common" Nod genes which are organized into two transcriptional units: NodABC and NodD (Kondorosi et al, 1985; Mulligan and Long, 1985). These highly conserved genes are involved in early stages of nodulation, and are interchangeable among the various Rhizobium species. Another cluster is the host specificity genes which are also organized into two transcriptional units in R. meliloti, NodGEF and NodH (for reviews see Denarie and Kahan, 1987; Rolfe and Gresshof, 1988). The bacterial gene regulating nodulation appears to be NodD, which is responsible for activating transcription of other Nod genes in the presence of plant-secreted flavonoid compounds (Peters et al, 1986; Redmond et al, 1986; Firmin et al, 1986).

R. fredii is a newly-introduced, fast-growing soybean symbiont isolated in the People's Republic of China (Keyser et al, 1982). The organization of the symbiosis genes in *R. fredii* may be different than in other rhizobia. It was shown (Ramakrishnan et al, 1986) that the *NodABC* and *NodD* transcription units which are linked in other Rhizobia are approximately 50 kilobases apart in *R. fredii* USDA 193, and the size of the entire *Nod* region may be as much as 100kb. Furthermore, when the "sym" plasmid (based on homology to the *R. meliloti* common *Nod* gene cluster) of *R. fredii* 206 was deleted, the resulting mutant was NOD^{+}, although it showed reduced nodulation (Mathis et al, 1985). Cloned *NodD* alone was able to complement a USDA 193 NOD^{-} mutant generated by deletion of the sym plasmid (Ramakrishnan et al, 1986).

R. fredii USDA 205 is the type strain of *R. fredii* (Scholla and Elkan, 1984). There have been various estimates of the number of plasmids in USDA 205 (Sadowsky and Bohlool, 1983; Plazinski et al, 1985; Broughton et al, 1984; Heron and Pueppke, 1984), however it appears to have four large plasmids (125 kb, 180 kb, 340 kb and >460 kb) as estimated by Heron and Pueppke (1984). Deletion of the larger plasmid (the authors only observed two plasmids) produces a NOD^{-} mutant strains (Sadowsky and Bohlool, 1983; Carlson and Yadav, 1985; this work) which had reduced extrapolysaccharide and lipopolysaccharide content (Carlson and Yadav, 1985). Although the authors do not state this explicitly, the plasmid that was deleted in all the mutants was probably the 180 kb plasmid.

We have identified and cloned a 9.3 kb EcoRI fragment from *R. fredii* USDA 205 with homology to the *R. meliloti* *NodABCD* region. The

clone complemented R. meliloti nodC⁻ and nodD⁻ mutants but not nodB⁻. There is homology to the common Nod genes on two plasmids, which suggests duplication or dispersal at least NodD in the USDA 205 genome. Although deletion of the 180 kb plasmid leads to a NOD⁻ phenotype, the mutant can still carry out early stages of nodulation including root hair curling and root branching, suggesting that it retains common Nod gene functions.

Materials and methods

Microbiological Techniques - Bacterial strains and plasmids are listed in Table 3. Triparental mating to introduce pRfIR1 into R. meliloti and R. fredii was performed with the helper plasmid, pRK2013 (Figurski and Helinski, 1979). To induce loss of plasmids by heat-curing, R. fredii 5A14 was grown in TY+rifampicin (20 μg/ml) broth with shaking at 34°C for two weeks. Single colonies were then screened for deletions of plasmids using the modified Eckhardt gel procedure described below.

Bacteria inoculation and test for nodulation ability - Plant seeds (alfalfa or soybean) were surface-sterilized in 20% commercial bleach for 3 minutes, washed briefly with sterile distilled water, then soaked in 3% H_2O_2 for five minutes and washed three times with sterile distilled water. The seeds were germinated on NF plates for 3 days. Seedlings were inoculated with 5 mls of a fresh bacterial culture (OD 0.5-0.8). Soybean and pea seedlings were transferred to 250 ml Ehrlenmeyer flasks and grown under sterile conditions, using a 1:1 vermiculite:perlite soil mix for soybean, and FP medium with 0.25% agar for peas. Alfalfa seedlings were transferred to 200 ml test tubes

Table 3 - Bacterial strains and plasmids

Strain	Relevant characteristics	Source and reference
E. coli		
DH5α	F⁻ lacZ M15 recA1	BRL
Rhizobium		
R. meliloti		
RCR2011	Wild type	
S9B8	Rm1021 nodD::Tn5	Jacobs et al, 1985
S2B2	Rm1021 nodB::Tn5	Jacobs et al, 1985
S170	Rm1021 nodC::Tn5	Jacobs et al, 1985
S8A2	Rm1021 nodC::Tn5	Jacobs et al, 1985
RR3	S9B8 (pRfIR2)	This work
RR4	S2B2 (pRfIR2)	This work
RR5	S170 (pRfIR2)	This work
RR6	S8A2 (pRfIR2)	This work
R. fredii		
5A14	USDA 205 Rif^R	Dr K.D. Nadler
RR1	5A14 *NOD-* (cured of 180 kb plasmid)	This work
RR2	RR1 With pRfIR2	This work
RR7	RR1 With pRmSL26	This work
RR8	RR1 With pJB5	This work

Table 3 (cont'd.).

Plasmids		
pRK2013	Kan^R, ColE1 replicon, RK2 transfer genes	Figurski & Helinski, 1979
pUC18	Amp^R, ColE1 replicon	Yanisch et al, 1985
pRfIR1	pUC18::*R. fredii* Nod homologous EcoRI fragment	This work
pRmJ1	Amp^R, ColE1 replicon:: *R. meliloti nodABCD*	Egelhoff et al, 1985
pPRL497	Kan^R, RSF1010 replicon, positive selection vector	Elhai and Wolk, 1988
pRfIR2	Kan^R, Amp^R pPRL497::pRfIR1	This work
pRmSL26	Tet^R, RK2 replicon:: *R. meliloti nod* region	Long et aη, ±98≥

containing FP medium with 0.25% agar. Pea and alfalfa seedlings were grown in continuous light at room temperature. Soybean seedlings were grown in continuous light at 25°C.

DNA purification - Plasmid isolation, restriction digestion, transformation and gel electrophoresis were done using standard techniques (Maniatis et al, 1982), with one modification: the phosphatase reaction of the vector was carried out with calf intenstinal alkaline phosphatase (Boehringer) at 55°C for 30 min. Total DNA was prepared from R. fredii 5A14 was isolated as described (Carlson et al, 1985). Large plasmids were visualized using a modified Eckhardt gel procedure (Plazinski et al, 1985).

DNA fragments for cloning were gel purified after electrophoresis by electroelution in 0.5X TBE (Maniatis et al, 1982). To prepare probes, DNA fragments were electroeluted from agarose gels into a low melting agarose (NuSieve GTG, FMC) patch in the gel, or onto DEAE-cellulose paper (Schleicher & Schuell). Labelling reactions were either carried out directly in the low-melting agarose or DNA was eluted from the paper using 2.5M NaCl at 62°C. Probes were labelled using the random priming procedure as described (Feinberg and Vogelstein, 1983).

Hybridization - Agarose gels were blotted onto nitrocellulose or nylon membranes using 0.6X SSC using standard techniques. Prehybridization (24 hours at 42°C) and hybridization (24 hours at 45°C) were carried out in 50% formamide. The filters were washed in 0.1X SSC at 45°C (4 washes for 30 minutes each).

For colony hybridization small colonies (10-12 h after

transformation) were transferred to nitrocellulose membranes. After baking (2 hours, 80°C), bacterial debris was scraped from the filters by rubbing the filters in two changes of 4X SET (0.75M NaCl, 0.1M Tris-HCl, 5mM EDTA; pH7.6). Prehybridization and hybridization were carried out as described above.

Light microscopy - Plants for the light microscopy analysis were grown in growth pouches as described by Russell et al (1985). Two days after inoculation the plants were examined with bright-field and phase optics.

Results

Genotype Of RR1 - RR1 is a NOD^- mutant of *R. fredii* generated by heat-induced deletion of a 180 kb plasmid. Microscopy of the early events in nodulation (two days after inoculation) showed that the mutant can still carry out early steps of nodulation. RR1 bacteria attach to the tip of the root hair normally (*roa+* phenotype), cause root hair deformation (Figure 1a) and root hair branching (Figure 1b), and induce root hair curling ($hac^{\pm}$)(Figure 1c).

Cloning of R. fredii Nod-gene homolgous region - A *R. meliloti* probe containing the common *Nod* genes was prepared by subcloning a 3.5 kb EcoRI-BamHI fragment containing the "common" *Nod* genes, *NodABC* and part of *NodD*, from pRmJ1, an 8.7 kb cloned EcoRI fragment in pBR325 (Jacobs et al, 1985). Total DNA from *R. fredii* 5A14 was digested with EcoR1, HindIII, and PstI, and probed with the *R. meliloti Nod* genes (Figure 2). In all the digests the probe hybridized to four bands, with one significantly more intense than the others.

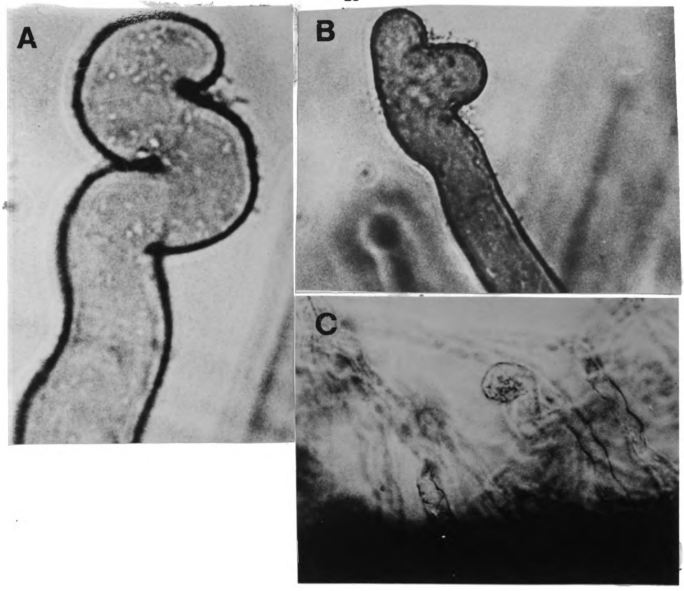

Figure 1 - Light microscopy of early events in nodulation with RR1

Sterilized Peking seeds were inoculated with RR1 and placed into growth pouches as described (Russell et al, 1985). Two days after inoculation, roots were examined.

A: Bacteria were attached to the roots hairs and root hair deformation was seen.

B: Root hairs with bacteria attached exhibited root hair branching.

C: Root hair curling was also seen.

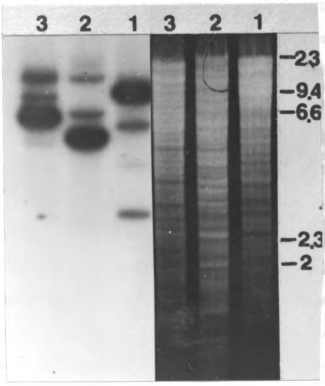

Figure 2 - Homology between R. meliloti Nod genes and R. fredii 5A14

Total genomic DNA from R. fredii 5A14 was digested with EcoRI (Lane 1), HindIII (Lane 2) and PstI (Lane 3) and separated on a 0.7% agarose gel, run in 1X TBE buffer. The gel was blotted onto a nitrocellulose membrane in 0.6X SSC and probed with a 3.5 kb EcoRI-BamHI fragment which contained cloned R. meliloti Nod genes (NodABC and a small part of NodD). A single major band is seen in all lanes (9.3 kb in EcoRI, 5.3 in HindIII and 6.0 kb in PstI). Minor bands are also seen in all lanes, later shown to be additional copies of NodD elsewhere in the genome (Figure 6). The probe was purified from a EcoRI-BamHI digest of pRmJ1.

Because the major homologous EcoRI band was at 9.3 kb, an R. fredii mini-library was constructed in pUC18 using 7-10 kb EcoRI fragments. The library was used to transform E. coli DH5α. Colony hybridization revealed five independent colonies with homology to the R. meliloti probe. The plasmids from these colonies all had identical restriction patterns, except that in two cases the insert was in the reverse orientation. A restriction map of the clone pRfR1 is shown in Figure 3. The region of homology to the R. meliloti Nod genes appears to be restricted to an internal 1.8 kb XhoI fragment (Figure 4).

The genes on the cloned region - The 9.3 kb R. fredii fragment was recloned into pPRL497, a wide host-range vector, to generate pRfIR2. This plasmid was then conjugated into various R. meliloti nod$^-$ mutants, and alfalfa seedlings were inoculated with the transconjugants. Nodulation was scored after five weeks. The results of these complementation tests are in Table 4. These data suggest that the cloned region contains NodC and NodD cistrons, but not NodB.

pRfIR2 was also conjugated into RR1, an R. fredii 5A14 NOD$^-$ mutant that is missing the 180 kb megaplasmid, one of the large endogenous plasmids (Figure 5). The transconjugant, RR2, was unable to nodulate soybean.

Genomic localization of the Nod region - The results of the microscopy showed that RR1 is able to induce root hair curling, which suggests that at least some nodulation genes are still present in RR1, even though an entire plasmid with some nodulation genes has been deleted. In other words, R. fredii symbiosis genes cannot all be on the one plasmid missing in RR1, but must be dispersed in at least two replicons

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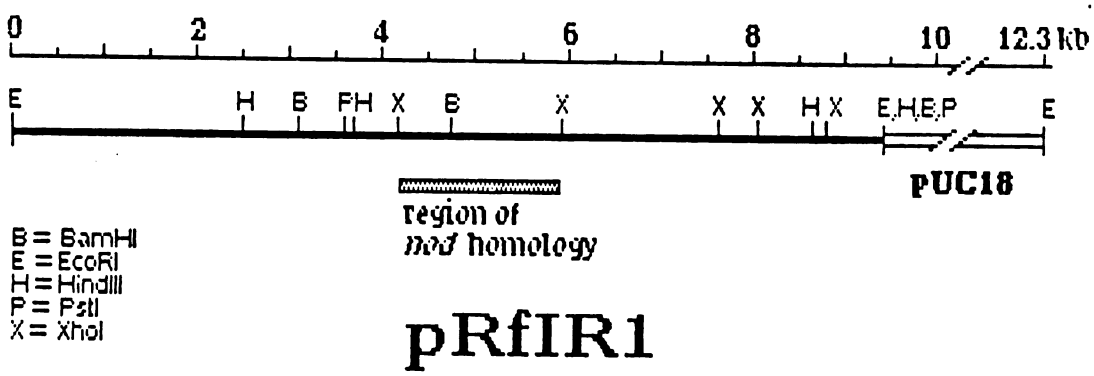

Figure 3 - Restriction map of pRfIR1

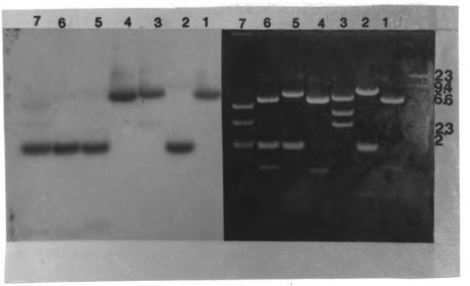

Figure 4 - Nod homologous region of pRfIR1

pRfIR1 was digested with HindIII (Lane 1), XhoI (Lane 2), PstI/EcoRI (Lane 3), PstI/HindIII (Lane 4), XhoI/PstI (Lane 5), XhoI/HindIII (Lane 6) and EcoRI/XhoI (Lane 7) and separated on a 0.8% agarose gel, and blotted onto a nylon membrane (Hybond-N, Amersham). The filter was probed with the 3.5 kb EcoRI-BamHI *Nod* gene fragment from *R. meliloti*. A single homologous band is seen at 1.8 kb in all lanes digested with XhoI. EcoRI separates the vector from the insert, generating a 2.7 kb vector fragment.

Table 4 - Complementation tests

strain	mutation	host plant	phenotype nod+	phenotype nod-
RR3 (S9B8 + pRfIR2)	nodD	alfalfa	11	17
RR4 (S2B2 + pRfIR2)	nodB	alfalfa	0	21
RR5 (S170 + pRfIR2)	nodC	alfalfa	12	20
RR6 (S8A2 + pRfIR2)	nodC	alfalfa	4	9
RR7 (RR1 + pRmSL26)	plasmid deletion	soybean	5	60
RR8 (RR1 + pJB5)	plasmid deletion	pea	6	22
RR8 (RR1 + pJB5)	plasmid deletion	soybean	0	20
RR2 (RR1 + pRfIR2)	plasmid deletion	soybean	0	20

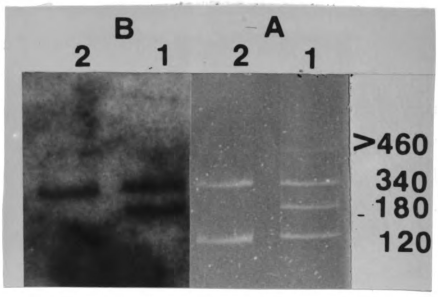

Figure 5 - Genomic localization of Nod homologous region in R. fredii

The large endogenous plasmids of R. fredii 5A14 were separated using a modified Eckhardt gel procedure (Plazinski et al, 1985) to gently lyse bacteria within the wells of the gel. The plasmids were separated on a 0.7% gel and blotted onto a nylon membrane and hybridized to the 3.5 kb R. meliloti Nod gene probe. The filter was stripped of probe by boiling in 0.1% SDS, as specified by the manufacturer, and then probed with pRfIR1 under the same conditions. pRfIR2 hybridized to the same plasmids as the R. meliloti Nod gene probe (data not shown).

A: Ethidium bromide stained gel. Lane 1 - R. fredii 5A14; Lane 2 - RR1. Approximate plasmid sizes are indicated.

B: Autoradiogram of blot probed with 3.5 kb R. meliloti Nod gene fragment.

in the R. fredii USDA 205 genome.

To pursue this point, the intact megaplasmids of 5A14 and RR1 were analyzed by preparing an Eckhardt blot, and probing with the 3.5 kb R. meliloti fragment. There were homologous regions on two megaplasmids in 5A14 (Figure 5); RR1 is deleted for the smaller of these two hybridizing plasmids, so only one band is seen in the autoradiogram. Both plasmids also had homology to pRfIR1 (data not shown).

In order to determine if genes are duplicated, we prepared Southern blots of total genomic DNA from 5A14 and RR1 digested with EcoR1. The blots were probed with several R. meliloti gene-specific probes prepared from pRmJ1: a 2.4 kb BglI fragment containing NodD region, a 0.6kb HindIII fragment containing NodC, and a 3.5 kb EcoR1-BamHI fragment containing NodABC and part of NodD (Figure 6). RR1 contains NodC but lacks several NodD-homologous fragments seen in wild-type. The major hybridizing fragment is retained in RR1.

We constructed the strain RR8, by conjugating into RR1 the R. leguminosarum sym plasmid pJB5. RR8 is able to nodulate peas but not soybeans, suggesting that the plasmid deleted in RR1 may contain host-specificity genes as well as nodulation genes. The pattern of nodulation induced by RR8 on peas is interesting. There are many, closely-spaced small nodules on secondary roots. When the Nod region from R. meliloti (on pRmSL26) was used to complement RR1, the resulting strain (RR7) nodulated soybeans at a low and variable frequency (Table 4).

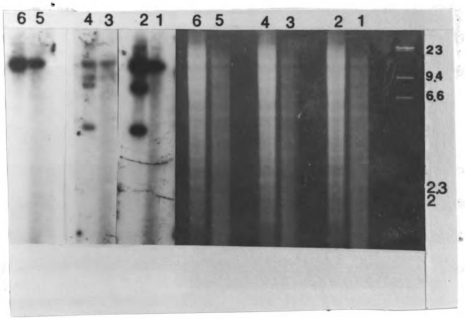

Figure 6 - Identification of specific Nod genes in 5A14

Total R. fredii 5A14 and RR1 genomic DNAs were digested with EcoRI, separated on a 0.7% agarose gel, and blotted onto a nylon membrane. The blot was probed with probes corresponding to individual R. meliloti Nod genes. The probes were prepared by digesting pRmJ1 with various enzymes, as described in the text, and separating fragments on a 1% agarose gel. Specific fragments were electroeluted onto DEAE paper (Schleicher & Schuell) using manufacturer's recommended procedures, except that elution from the paper was carried out with 2.5M NaCl at 62°C.

A: Ethidium bromide stained gel. Lane 1, 3, 5 - RR1; Lanes 2, 4, 6 - 5A14 DNA.

B: Autoradiogram of blot. Lanes 1 & 2 - probed with NodABCD; Lanes 3 & 4 - probed with NodD; Lanes 5 & 6 - probed with NodC.

Discussion

Nodulation genes in fast-growing Rhizobium species have been shown to be organized as clusters on a single, large "sym" plasmid. Our results suggest that in *R. fredii* 5A14, the *Nod* genes may be dispersed on more than one plasmid and there may be more than one copy of the *Nod* genes.

It has been shown that there is more than one copy of the *NodD* gene in some other *Rhizobium* strains (Herman et al, 1987) but it may be that additional *Nod* genes are duplicated in *R. fredii*, based on results from other studies. Barbour et al (1985) showed that deleting a sym plasmid from *R. fredii* USDA 206 did not produce a *NOD*$^-$ phenotype, suggesting that there may be another copy of the *Nod* genes, although the deletion mutant did induce fewer nodules (Mathis et al, 1986). Ramakrishnan et al (1986) deleted the sym plasmid of *R. fredii* USDA 193, generating a *NOD*$^-$ mutant which nevertheless must have retained some of the genes involved in early nodulation since it can attach to root hairs. This mutant strain was able to form nodule-like structures in the presence of *NodD* (but not other *Nod* genes). *NodD* is a regulatory gene that by itself cannot restore nodulation. Our interpretation of these data is that some of the *Nod* structural genes which *NodD* product acts on must still be present, and that, of course, these structural genes cannot be on the deleted sym plasmid.

In many respects, our data are in agreement with those of Barbour et al (1985) and with Ramakrishnan et al (1986). RR1, a plasmid deletion mutant, is *NOD*$^-$ yet is able to carry out some early events in nodulation, such as root hair curling which requires the common *Nod*

genes. However, RR1 cannot be complemented by any of the cloned R. fredii common Nod genes. Another mutant with a similar phenotype was described by Downie et al (1985) who generated a NOD$^-$ mutant of R. phaseoli 8401 (pLJ1216) by heat-induced loss of a plasmid. When the 6.6 kb Nod gene region from R. leguminosarum was introduced into this mutant, attachment and root deformation was observed. This phenotype, ability to carry out only early steps in nodulation, has not yet been mapped to a gene. The mutant phenotype of all bacterial genes identified so far is either completely defective for all nodulation steps or delayed normal nodulation.

The cloned region on pRfIR2 was able to complement R. meliloti nodC$^-$ and nodD$^-$ mutants, but not nodB$^-$ mutants. If the NodABC genes are linked in R. fredii 5A14, as in other rhizobia, then it is puzzling as to why the NodB gene cloned is non-functional (or not able to complement in another species). Another puzzling result is that the homology to the R. meliloti common Nod genes is restricted to a 1.8 kb XhoI fragment, which is too small to contain 4 genes. Perhaps this clone contains only part of the Nod gene region; the complementation data suggest that the only functional Nod genes on the clone are NodC and NodD. The cloned region was also not able to complement the deletion in RR1, so pRfIR1 obviously does not include all the essential Nod genes deleted in RR1.

We suspect, based on two results, that among the genes deleted in RR1 are host-specificity genes. First, pJB5 from R. leguminosarum can restore nodulation to RR1, but only on peas. Second, a small percentage of RR1 cells with the R. meliloti Nod gene clone, pRmSL26,

were able to nodulate soybean in a delayed pattern similar to that seen with host-specificity gene mutants (Downie et al, 1985).

Hybridization of gene-specific NodC and NodD probes to digested genomic DNA of 5A14 and RR1 showed that both had homologous fragments. Interestingly, hybridization with a NodD probe revealed multiple bands besides the 9.3 kb fragment cloned as pRfIR1. Since the NodD probe hybridized to the 9.3 kb band only in RR1 and not to other fragments as in 5A14, it appears that at least NodD is duplicated on the deleted plasmid. These hybridization data suggest that there are at least two, and probably three, copies of NodD in 5A14. Ramakrishnan et al (1986) showed that there are two copies of NodD in USDA 193. Three NodD genes have been identified in R. meliloti (Herman et al, 1987). It was shown (Herman et al, 1987) that the NodD products from the various fast-growing Rhizobium species differ from each another in that they confer different responsivness to different sets of flavonoids exuded by plants. R. fredii 5A14 has two known host plants: G. max and G. soja, and it may be interesting to see if the different 5A14 NodD genes respond to a different spectrum of host flavonoids.

Ramakrishnan et al (1986) showed that the common nod genes in R. fredii 193 are not linked but are on the same plasmid (within a 100 kb region). Our data show that the organization of sym genes in 5A14 may be more complex than other rhizobia studied. First of all, the common Nod genes are located on two megaplasmids. Secondly, the Nod genes cloned by homology to a probe from R. meliloti were unable to complement a nodB$^-$ mutant. Taken together, these results raise questions about the general assumption that the common Nod genes are

always clustered on a single megaplasmid, as seen in other rhizobia.

CHAPTER 2

NODULATION ABILITY OF *Rhizobium fredii* USDA 205

Abstract

Rhizobium fredii is a recently discovered fast-growing Rhizobium able to nodulate soybeans [*Glycine max* (L.) Merr.]. Most *R. fredii* strains cannot form nodules on North American cultivars. The purpose of this study was to integrate the allele which enables soybean to nodulate with *R. fredii* into a North American genetic background in order to study this symbiosis and the factors which influence its effectiveness. "Peking", a cultivar homozygous for the recessive allele conferring the ability to nodulate with *R. fredii*, was crossed with L61-5047, a subline of the North American cultivar "Harosoy". Parents and selected F_3 plants were inoculated with either *Bradyrhizobium japonicum* strain 110 or *R. fredii* USDA 205 and grown under sterile conditions. Dry weight, a general measure of plant growth, was correlated with nodule weight. On the basis of nodule weight, when Peking and F_3 plants nodulated with *B. japonicum* or *R. fredii* were compared, *R. fredii* was superior to *B. japonicum*. F_3 plants were inferior to Harosoy because Peking is a poor symbiont, regardless of the nodulating bacterium. Our results suggest that *R. fredii* may have agronomic value and it should be evaluated with isolines of North American cultivars able to nodulate with *R. fredii*.

Introduction

Rhizobium fredii was first isolated from *Glycine max* (L.) Merr and *Glycine soja* Sieb. and Zucc. nodules in the People's Republic of China in 1982 (Keyser et al, 1982). *R. fredii* was first shown to nodulate the soybean cultivar "Peking", a genetically unimproved soybean line. Later it was found that the ability to nodulate with *R. fredii* is widespread in introduced lines from all maturity groups, except for North American cultivars. Fifty-six percent of 285 introduction lines, and more than eighty percent of the soybean lines from Southeast Asia nodulated with *R. fredii* (Devine, 1985). Only one *R. fredii* strain, USDA 191, has been found to form effective nodules with any North American cultivars (Yelton et al, 1983)

R. fredii showed a high plant-bacteria interaction effect with two lines of *G. soja* for accumulation of nitrogen. However, no line of *G. max* able to nodulate with *R. fredii* demonstrated superior effectiveness in comparison to nodulation with *Bradyrhizobium japonicum* (Keyser and Cregan, 1984). Several later studies also concluded that *R. fredii* is a poor symbiont of soybean compared to *B. japonicum*, in terms of relative acetylene reduction (Yelton et al, 1983) and of nitrogen-fixing and nodulation ability (DuTeau et al, 1986). McLoughlin et al (1985) showed that USDA 191 competes poorly against *B. japonicum* in nodulating "Peking".

Our study used *R. fredii* USDA 205, which generally does not form nodules on North American cultivars. Analysis of plants from a cross of Peking by "Kent" showed that the ability of soybean to be nodulated by *R. fredii* 205 is controlled by a single recessive allele at a single

locus (Devine, 1984). The purpose of this study was to integrate this allele into a plant population adapted to the midwestern United States in order to compare the effectiveness of the North American soybean-*R. fredii* symbiosis to nodulation with *B. japonicum*, and to study the relative contributions of various nitrogen-fixation parameters to the symbiosis.

Materials and methods

Preparation of F_3 seed: Peking and Harosoy plants were crossed in the glasshouse. Because parents are of different maturity groups seeds were sown in three intervals of one week to synchronize flowering. The F_1 plants were selfed to obtain F_2 seeds. F_2 seeds were surface sterilized, inoculated with *R. fredii* 205, and sown individually in 200 ml sterile Ehrlenmyer flasks filled with a 1:1 mixture of vermiculite and perlite wetted with nitrogen-free nutrient solution (Johnson et al, 1966). The plants were grown in a growth chamber with 12 h illumination. After five weeks the F_2 plants which had formed nodules were selected and transferred to pots and grown to maturity in the greenhouse. F_3 seed was harvested separately from each of four F_2 plants. Two seeds from each F_2 plant were retested for the ability to form nodules with *R. fredii* as described above.

Seed sterilization: Seeds were soaked in 20% commercial bleach (1% NaOCl) for 3 minutes, washed briefly with sterile distilled water, then soaked in 3% H_2O_2 for five minutes and washed three times with sterile distilled water.

Inoculation: Sterilized seeds were placed in sterile vials filled with

5-10 ml of bacterial inoculum for 60 minutes, and then sown.

Plant growth: Inoculated sterile seeds were sown in a 1:1 vermiculite:perlite soil mix in sterile Leonard jars (Vincent, 1970) containing 3.5 liters of NF nutrient solution. Plants were grown in a greenhouse, with supplementary lights to provide 9 hr day length. To reduce plant evapotranspiration, high humidity was maintained by flooding greenhouse benches with water. Data were collected after eight weeks.

Acetylene reduction: The entire root system was placed in a 20 ml test tube sealed with a rubber stopper. Two milliliters of acetylene were injected. After one hour three 300 ul samples were removed from each test tube with one ml syringes. Ethylene accumulation was measured by injecting samples into a Varian gas chromatograph equipped with a Nickel Pronpak R column and a flame ionization detector.

Root measurements: The number of nodules on the crown of the root, the primary root and the secondary root were counted and the number of large nodules were counted. Large nodules were defined as nodules with stripes, a diameter of >0.6 cm, weight >.09 g, These nodules appeared to be fully mature, however this measurement was subjective.

Shoot measurements: Data on fresh weight, number of leaves, pods, and flowers and the height of each plant were collected. For dry weight, plants were placed into paper bags and dried in a 65^{o}C oven for 72 hours before weighing.

Bacterial strains: *R. fredii* USDA 205 Rif^{R} was obtained from Dr. K.D. Nadler, and was grown in TY medium. *B. japonicum* 110 was obtained from Dr. B. Chelm, and was grown in YM (Vincent, 1970). Both strains were

grown at 29 C with shaking to 0.7 O.D. for inoculation.

Identification of bacterial strains in the nodule: Nodules were surface sterilized by soaking for 30 seconds in 70% ethanol and then for 5 mintues in 3% NaOCl. Sterilized nodules were crushed onto agar plates. Colonies of *R. fredii* USDA 205 Rif^R grow on rifampicin (20 ug/ml) plates and blacken when mature. *B. japonicum* 110 colonies are rough and turn pinkish when old on YM plate.

Experimental design and analysis: A 3 x 3 factorial, randomized complete block design with four replications was used, with three bacterial treatments (no inoculum, *B. japonicum*, *R. fredii*) and three plant genotype treatments (Peking, Harosoy and F_3). In each block there were eight $F_{2,3}$ plants (two plants from each $F_{2,3}$ family) for the inoculum treatments (*R. fredii* and *B. japonicum*) and one $F_{2,3}$ plant for the control (no inoculum), eight Peking and eight Harosoy plants for the inoculum treatments, and four Peking and Harosoy plants for the control. Blocks were defined by glasshouse location and time of harvest.

Analysis of variance and correlation analysis were performed using SAS (SAS Institute 1985). Data from $F_{2,3}$ families were pooled (labelled F_3 plants in the tables) and analysis performed on blocks means. The analysis of the effect of plant-bacteria combinations on traits related to nitrogen fixation (Table 5) used data from all treatments. The correlation analysis (Table 6) excluded data from control plants (no inoculum) and from the Harosoy-*R. fredii* treatment (ineffective nodulation) because the analysis correlates factors in effective symbioses. ANOVA and the Duncan test of the factors (Table 7

and 4) used only data from Peking and F_3 plants inoculated by R. fredii or B. japonicum in a 2x2 analysis. Including Harosoy-B. japonicum leads to an unbalanced analysis because the ineffective Harosoy-R. fredii treatment cannot be included.

Results

The performance of the three genotypes (Harosoy, Peking, and Harosoy x Peking F_3) inoculated with either B. japonicum or R. fredii, is shown in Table 5. Harosoy showed a low level of nodulation with R. fredii that was not significantly different from the uninoculated Harosoy (data not shown). This treatment was not considered to be effective nodulation and these data were not used in the analysis of measurements related to nitrogen fixation. Harosoy nodulated with B. japonicum produced significantly higher values in all the variables measured and was the most effective combination. Peking nodulated with B. japonicum produced the lowest values among all the combinations with effective nodulation.

Correlations among traits (Table 6) were calculated using data only from treatments with effective nodulation. Dry weight was highly correlated with nodule weight (r=.89), but the correlation between dry weight and acetylene reduction was lower. It is also noteworthy that dry weight was more highly correlated with the number of large nodules than with total number of nodules.

An analysis of variance was performed on data excluding both Harosoy treatments (Table 7), because Harosoy nodulated with R. fredii is not effective and including Harosoy nodulated with B. japonicum

Table 5 - The effect of plant-bacteria combinations on nitrogen-fixation related traits

Plant	Bacterium	dry weight g/plant		nodule weight g/plant		number of nodules		number of large nodules		acetylene reduction u mole/hr	
		N	avg.	N	avg.	N	avg.	N	avg.	N	avg.
Harosoy	B.j.	19	1.69a	19	0.73a	17	17.71a	13	17.61a	19	82.97a
Peking	B.j.	26	0.62c	26	0.29c	26	4.61c	20	3.25c	26	33.00b
	R.f.	30	1.03b	30	0.51b	17	12.52b	21	10.05b	29	47.79b
F3	B.j.	17	0.76bc	16	0.40bc	17	11.69b	12	7.00b	16	49.66b
	R.f.	16	0.95bc	17	0.41bc	19	12.10b	12	10.00b	17	52.51b

Table 6 - Correlation matrix of measured parameters showing correlation cofficients, level of significance and the number of plants

	Nodule weight (g)	Number of Nodules	Number of Large nodules	Acetylene reduction (μmoles/hr)
Dry weight (g)	.89*** 105	.54*** 105	.72*** 78	.54*** 107
Nodule weight (g)		.58*** 105	.81*** 78	.60*** 107
Number of nodules			.82*** 76	.29** 104
Number of large nodules				.48*** 77

*** Probability < .0001
** Probability < .001

Table 7 - Summary of ANOVA showing MS and the levels of significance

		Factor				
		Dry weight (g)	Nodule weight (g)	Nodule number	No. large nodules	acetylene reduction (μmoles/hr)
Source	df	MS	MS	MS	MS	MS
Block	3	.0876	.06700	9.27	3.31	491.77
Plant	1	.0109	.00080	59.94**	11.47	431.14
Bacteria	1	.0420	.04370	64.85**	72.62**	381.79
Interaction	1	.3510*	.04650	37.55*	10.24	339.34
Error	9	.119	.02440	6.91	2.91	604.67
Among plants	a	.082	.01820	6.63	3.91	263.96
Pooled Error	b	.086	.01880	6.66	3.76	c

* Probability < .05
** Probability < .01

[a] The error degrees of freedom vary depending on the analysis performed 73 for nodule weight, 49 for large nodules number and 72 for the rest
[b] The error degrees of freedom vary depending on the analysis performed 82 for nodule weight, 58 for large nodules number and 81 for the rest
[c] can not be pooled because there are significant differences between the error terms

would lead to an unbalanced analysis. In the analysis of data from host genotypes compatible with R. fredii, plant-bacteria interaction was significant for dry weight and total nodule number. The main effect of bacteria was significant for number of nodules and number of large nodules. The main effect of genotype was significant only for number of nodules. F_3 plants had significantly more nodules than "Peking" (Table 8). R. fredii produced a significantly greater total number of nodules and more large nodules than B. japonicum. There was no significant difference in acetylene reduction between plant genotypes or bacterial strains despite the differences in nodule number and weight.

Discussion

The genetics of symbiosis between soybean and Rhizobium is complex. There are host genes with large effects: rj1 (Williams and Lynch, 1954), rj2 (Caldwell, 1966), rj3 (Vest, 1970) Rj4 (Vest and Caldwell, 1972) which restrict nodulation, and one locus enables R. fredii to nodulate North American cultivars (Devine, 1984). There is highly heritable, continuous variation for total N accumulation (Ronis et al, 1985) and nodule mass (Greder et al, 1986). Thus it appears that single plant genes control the recognition of and define the spectrum of bacteria able to establish the symbiosis, but that the development and efficiency of the symbiosis (measured by N accumulation and nodule mass) depends on quantitatively inherited factors, as can be seen in the large differences in nodulation efficiency between cultivars.

Table 8 - Main effect means for plant genotype and bacterial strain

	Plant				Bacteria			
	F_3		Peking		R. fredii		B. japonicum	
Trait	N	Mean	N	Mean	N	Mean	N	Mean
Dry weight (g)	33	.86[a]	56	.84[a]	47	.94[a]	42	.74[a]
Nodule weight (g)	33	.41[a]	56	.42[a]	47	.48[a]	42	.34[a]
Nodule number	33	12.12[a]	55	8.56[b]	46	12.26[a]	42	7.31[b]
No. large nodules	24	8.50[a]	41	6.73[a]	33	10.03[a]	32	4.65[b]
acetylene reduction (μmoles/hr)	33	51.13[a]	55	40.81[a]	46	49.53[a]	42	39.36[a]

In this study the F_2 plants were selected for the R. fredii recognition allele. The source of this allele was Peking, a poor nodulator. Peking was inferior to cultivars Bedford, Essex and Williams when nodulated by B. japonicum serogroup 122 in terms of total nitrogen content, shoot nitrogen and nodule dry weight (Keyser et al, 1984). Peking also compared poorly with cultivars Evans, Harosoy, Rampage, Williams and Hardee Rj2,Rj3 when nodulated by B. japonicum 61A76, as measured by top dry weight, nodule number and nodule weight (DuTeau et al, 1986). However, in both studies when bacterial strains were compared, R. fredii was equivalent to or better than B. japonicum in symbiosis with Peking, especially in terms of acetylene reduction and top dry weight (DuTeau et al, 1986).

These results are in agreement with ours, suggesting that Peking is a poor host. This would explain why the F_3 plants inoculated with B. japonicum were not as effective as Harosoy nodulated with B. japonicum. Although R. fredii is often described as a poor symbiont, our results suggest that the poor performances of R. fredii may be due to the host genotype with which it is tested. With the F_3 or with Peking, R. fredii performed as well as or better than B. japonicum (Table 8), although there were differences in the ultrastracture of the nodules formed by R. fredii (see following chapter). In these comparisons the Harosoy with B. japonicum symbiosis was not included although this was the best plant bacteria symbiosis. To best evaluate R. fredii with North American cultivars, isolines of Harosoy with the allele enabling it to nodulate with R. fredii should be developed.

There is also a question of how to evaluate the effectiveness of

the symbiosis. Nodule mass is highly correlated with seed yield (r=.85) (Greder et al, 1986). Similarly, in our study the trait most correlated with dry weight is nodule weight. Although this parameter is difficult to measure under field conditions, it appears to be a good measure of the symbiosis when effective nodulation (measured by acetylene reduction or ureides) is established. Nodule weight is less likely to be affected by transient conditions during measurement than aceytlene reduction, a measurement with a low precision of estimation (Bliss, 1985).

Widening the spectrum of bacteria able to nodulate North American cultivars may have agronomic advantages because it may improve the symbiosis. For example, R. fredii may have advantages in salty soils as it has been shown to be more salt tolerant (Yelton, 1983). Our study indicates that improvement of the plant genetic background may be pivotal in widening the spectrum of the bacteria able to nodulate North American cultivars effectively.

CHAPTER 3

THE ULTRASTRUCTURE OF THE SOYBEAN-*RHIZOBIUM FREDII* USDA 205 NODULES

Abstract

Rhizobium fredii USDA 205 forms effective root nodules with the *Glycine max* cultivar Peking but not with North American *G. max* (L) Merr cultivars. The recessive allele permitting nodulation was introduced into a North American cultivar (Harosoy) by crossing with Peking. Progeny of selected homozygous recessive F_2 plants and both parents were inoculated with either *Bradyrhizobium japonicum* or *R. fredii*. The cytology of the nodules showed that the bacterial symbiont may have a role in defining the ultrastructure of the nodule. While nodules of Peking inoculated by *B. japonicum* were normal, the nodules formed by *R. fredii* with Peking had several unusual features: a very small peribacteroid space, close proximity of the peribacteroid membrane and the bacteroidal membrane, and smaller than normal β-polyhydroxybutyrate granules. In the nodules of F_3 plants inoculated with *R. fredii* there were large vacuoles, and the bacteroids were in an electron-dense peribacteroid space. Light microscopy of these nodules revealed deformed cells in all nodule parts, including the region near the cortical tissue where young, newly-infected cells are usually found. A large proportion of the uninfected cells contained large starch

granules. Taken together, these findings suggest an incompatibility between the F_3 host and the R. fredii symbiont which leads to an unusual pattern of nodule development.

Introduction

The symbiosis between leguminous plants and Rhizobium is a complex process involving many plant and bacterial genes, requiring coordination between the plant host and the bacterial symbiont. The main stages of the symbiosis are recognition, growth and development of the nodule, nitrogen-fixation and transport of the fixed nitrogen.

Both bacteria and plant have recognition genes. Bacterial host-specificity genes have been cloned from several Rhizobium species, and it has been shown that transfer of these genes to a different species changes the host-range of the recipient (Beynon et al, 1980). Plant recognition genes which define the spectrum of bacteria able to form nodules, have been identified in soybean (Caldwell, 1966; Vest, 1970; Vest and Caldwell,, 1972; Devine, 1984).

There have been numerous reports about the initial recognition signals of the plant and bacteria (Rolfe and Gresshoff, 1988). However, coordination between the plant and bacterial partners continues beyond the initial recognition steps. Bacterial genes appear to modulate the expression of plant peribacteroid membrane nodulins (Werner et al, 1988). Infection of soybean with an ineffective strain of its symbiont, B. japonicum leads to premature senescence characterized by changes in the ultrastructure of the nodule, biochemical changes in the plant and in the bacteria and eventual lysis

of the bacteria (Werner et al, 1980, 1984; Regensburger et al, 1986). In another study of nodules formed by Rhizobium fredii USDA 193 with soybean cultivars "Peking" and "Virginia", cytological differences were observed when these same plants were inoculated with mutant bacteria defective in nitrogen-fixation (Shantharam et al, 1987). However, genetic interactions between host and symbiont in later nodule developmental stages are not well understood because it is difficult to screen for plant or bacterial mutants affecting these late stages.

The subject of this study is the ultrastructure of the interaction between soybean genotypes and R. fredii, a recently introduced fast-growing soybean symbiont isolated in the People's Republic of China (Keyser et al, 1982). Most R. fredii strains are unable to form nodules with North American soybean cultivars. The ability to nodulate with R. fredii is determined by a single recessive allele in the plant host (Devine, 1984). We transferred this allele into a North American genetic background by crossing Peking to "Harosoy" (formerly the dominant Midwestern cultivar in Maturity Group II) and selecting for homozygous recessive F_2 plants that could nodulate with R. fredii (see previous chapter). The two parents and progeny of the selected F_2 plants were inoculated with R. fredii or with the common soybean symbiont, Bradyrhizobium japonicum, and the ultrastructure of nodules in each treatment was examined. Although the F_3 individuals formed effective nodules with R. fredii, our data suggest that there may be some differences in the ultrastructure of these nodules compared with those formed by B. japonicum. It thus appears that the microsymbiont may play a major role in defining the ultrastructure of the nodule.

Materials and methods

Preparation of F_3 seed: Peking and Harosoy plants were crossed in the greenhouse. Because parents are of different maturity groups seeds were sown in three intervals of one week to synchronize flowering. The F1 plants were selfed to obtain F_2 seeds. F_2 seeds were surface sterilized, inoculated with *R. fredii* USDA 205, and sown individually in 200 ml sterile Ehrlenmyer flasks filled with a 1:1 mixture of vermiculite and perlite wetted with NF nutrient solution (Johnson et al, 1966). The plants were grown in a growth chamber with 12 h illumination. After five weeks the F_2 plants which had formed nodules were selected, transferred to pots, and grown to maturity in the greenhouse. F_3 seed was harvested from each of four F_2 plants separately. Two F_3 indididuals from each F_2 plant were retested for the ability to form nodules with *R. fredii* as described above.

Seed sterilization: Seeds were soaked in 20% commercial bleach (1% NaOCl) for 3 minutes, washed briefly with sterile distilled water, then soaked in 3% H_2O_2 for five minutes and washed three times with sterile distilled water.

Inoculation: Sterilized seeds were placed in 20 ml sterile scintillation vials filled with 5-10 ml of bacterial inoculum for 60 minutes, and then sown.

Plant growth: Inoculated sterile seeds were sown in a 1:1 vermiculite:perlite soil mix in sterile Leonard jars (Vincent, 1970) containing 3.5 liters of NF nutrient solution. Plants were grown for seven weeks in a greenhouse, with supplementary lights to provide 14 hr day length. To reduce plant evapotranspiration, high humidity was

maintained by flooding greenhouse benches with water.

Acetylene reduction: The entire root system was placed in a 20 ml test tube sealed with a rubber stopper. Two ml of acetylene were injected and after 1 h, three 300 µl samples were removed from each test tube with one ml syringes. Ethylene accumulation was measured by injecting samples into Varian gas chromatograph equipped with a Nickel Pronpak R column and a flame ionization detector.

Bacterial strains: R. fredii USDA 205 Rif^R was obtained from Dr. K.D. Nadler, and was grown in TY. B. japonicum 110 was obtained from Dr. B. Chelm, and was grown in YM (Vincent, 1970). Both strains were grown at 29°C with shaking to 0.7 O.D. for inoculation.

Identification of bacterial strains: Nodules were surface sterilized by soaking for 30 seconds in 70% ethanol and then for 5 minutes in 3% NaOCl. Sterilized nodules were crushed onto agar plates. Colonies of R. fredii USDA 205 grown on rifampicin (20 µg/ml) plates blacken when mature, while B. japonicum 110 grown on YM plates forms colonies with a rough cell morphology which turn pink when mature.

Electron and light microscopy: Electron microscopy of the nodules was conducted as described by Bal et al (1982). The same fixed nodule was used for light microscopy using magnification of x650 or x1000. A single plant from each of the three parent-bacteria treatments, and two F_3 plants inoculated with R. fredii or B. japonicum were examined using five samples from different parts of one nodule from each plant. Five specimens of each sample were examined by light microscopy, and two specimens from one sample was examined by electron microscopy.

Results

The general analysis of the nitrogen-fixing capacity of bacterial strains and plant genotypes and the effect of the plant-bacteria interaction is described in the previous chapter. The acetylene reduction data for the plants used in this study (μmoles ethylene/gr nodule) were: Harosoy-*B. japonicum* 24.3, Peking-*B. japonicum* 32.4, Peking-*R. fredii* 60.0, F_3-*B. japonicum* 86.5 (average of two plants), F_3-*R. fredii* 82.3 (average of two plants).

Nodules formed by Harosoy and Peking with *B. japonicum* showed a typical soybean nodule ultrastructure (Figures 7,8) with a well-defined, intact peribacteroid membrane surrounding the peribacteroid space and a small deposition of fibrillar material inside the peribacteroid space. There were one to four bacteroids in each peribacteroid space. The only difference between the two cultivars was that the peribacteroid spaces were more dispersed throughout the cytoplasm in Peking than in Harosoy (in three different observations; data not shown).

Nodules formed by *R. fredii* on Peking were distinct (Figure 9). The peribacteroid space was very small, and the peribacteroid membrane surrounding the bacteroids was so close to them that often the peribacteroid and bacteroid membranes were indistinguishable. In most cases there was one bacteroid per peribacteroid space. The β-polyhydroxybutyrate granules (PHB) were significantly smaller in *R. fredii* nodules than in *B. japonicum* nodules on Peking and no fibrillar material was seen. Some *R. fredii* bacteroids appeared to be in an electron dense matrix (A) that was seen clearly at higher

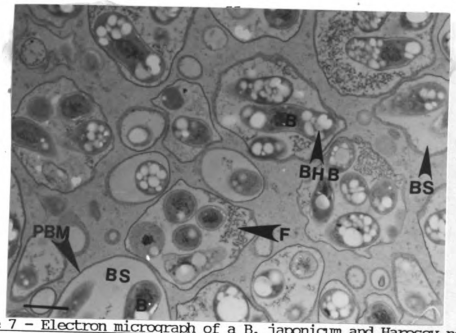

Figure 7 - Electron micrograph of a B. japonicum and Harosoy nodule

Magnification is 9400X. The length of the bar is 1 μ. Abbreviations: B - bacteroid; BHB - β-hydroxybutyrate; BS - peribacteroidal space; F - fibrillar material; PBM - peribacteroid membrane.

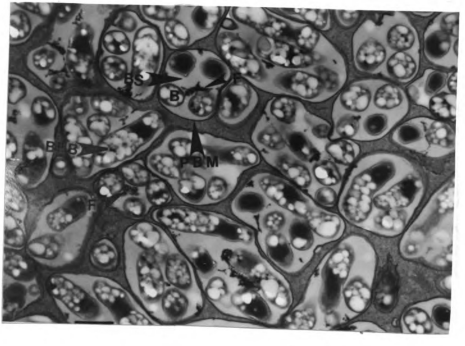

Figure 8 - Electron micrograph of a B. japonicum and Peking nodule

Magnification is 9400X. The length of the bar is 1 μ. Abbreviations are as in Figure 7.

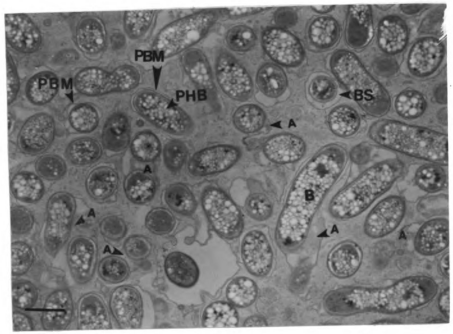

Figure 9 - Electron micrograph of a R. fredii and Peking nodule

Magnification is 9400X. The length of the bar is 1 μ. A - amorphic high electron dense matrix; other abbreviations as in Figure 7.

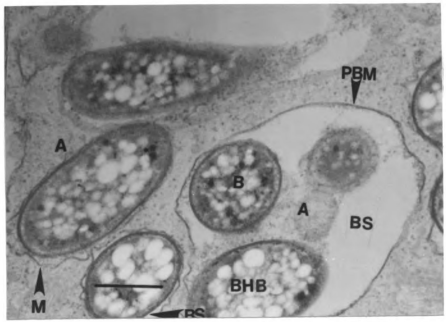

Figure 10 - High magnification electron micrograph of a R. fredii and Peking nodule

Magnification is 29,000X. The length of the bar is 0.3 μ.
M - membrane; other abbreviations as in Figures 7 and 9.

magnifications (Figure 10) compared to the translucent space in B. japonicum nodules (Figure 11).

The ultrastructure of B. japonicum nodules on the F_3 plants is shown in Figure 12. Some of the bacteroids were similar in structure to B. japonicum nodules on Harosoy with a generally well-defined peribacteroid space and fibrillar material (Figure 13). Others seemed to have the very small peribacteroid space characteristic of Peking-R. fredii nodules.

The ultrastructure of R. fredii nodules on the F_3 plants was distinctive (Figures 13,14,15,16). There were vacuole-like structures near the bacteroids surrounded by membrane. The bacteroids were in an amorphous electron-dense matrix (A) (Figures 14,15,16), and the peribacteroid membrane and peribacteroid space were generally not well defined (Figure 14), although in some cases both were clear and intact (Figure 15). The β-polyhydroxybutyrate granules were fine as in R. fredii nodules on Peking. Although the bacteroids appeared to be intact, with no signs of plasmolysis or cell degradation, in many cases it was not clear whether the bacteroids were inside or outside the vacuoles (Figure 14).

Light microscopy revealed details of the general organization of the nodule. Structures which look like holes were seen in a cell of an R. fredii nodule on an F_3 plant (Figure 17), and appeared to correspond to the vacuoles seen with the electron microscope. No such structures were seen in R. fredii nodules on Peking (Figure 18). Furthermore in the R. fredii nodules on F_3 plants, many of the uninfected cells had large starch granules (Figures 19,20), and cells with "holes" were seen

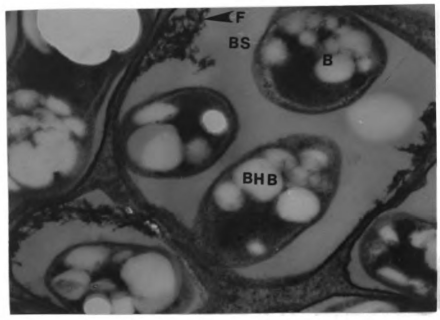

Figure 11 - High magnification electron micrograph of a B. japonicum and Peking nodule

Magnification is 38,000X. The length of the bar is 0.25 μ. Abbreviations as in Figure 7.

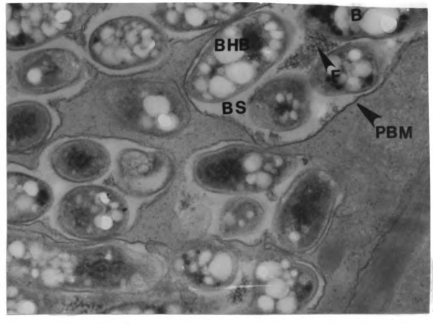

Figure 12 - Electron micrograph of a B. japonicum and F_3 nodule

Magnification is 9400X. The length of the bar is 1 μ. Abbreviations as in Figure 7.

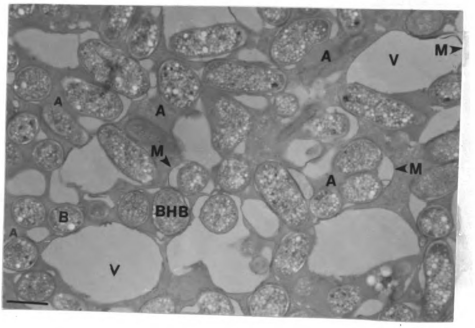

Figure 13 - Electron micrograph of a R. fredii and F_3 nodule

Magnification is 18,000X. The length of the bar is 0.5 μ.
V - vacuole; other abbreviations as in Figures 7 and 9.

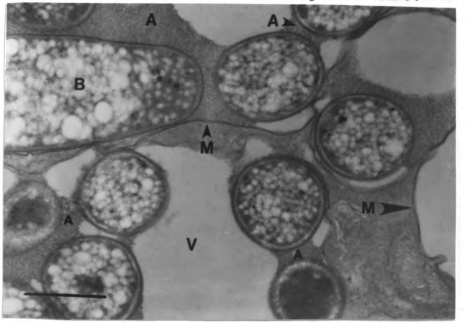

Figure 14 - High magnification electron micrograph of a R. fredii and F_3 nodule

Magnification is 25,000X. The length of the bar is 0.7 μ.
Abbreviations as in previous Figures.

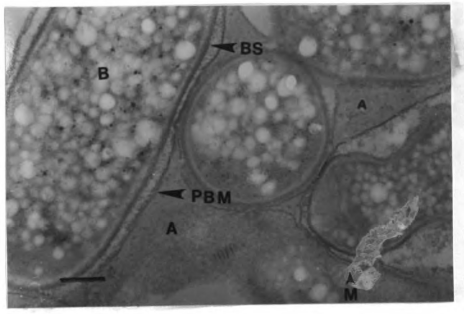

Figure 15 - R. fredii and F_3 nodule, bacteroid with well-defined peribacteroid membrane and peribacteroid space

Magnification is 38,000X. The length of the bar is 0.25 μ.
Abbreviations as in previous Figures.

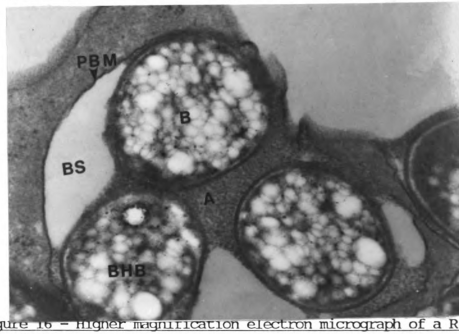

Figure 16 - Higher magnification electron micrograph of a R. fredii and F_3 nodule

Magnification is 38,000X. The length of the bar is 0.25 μ.
Abbreviations as in previous Figures.

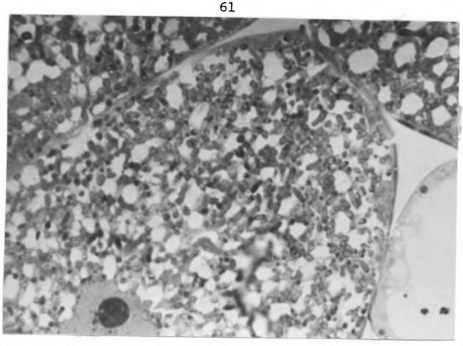

Figure 17 - Light micrograph of a R. fredii and F_3 nodule (near the center of the nodule)

Magnification is 650X.

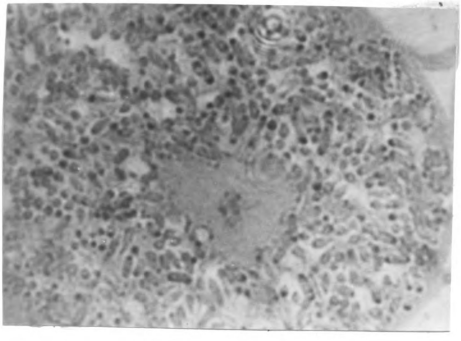

Figure 18 - Light micrograph of a R. fredii and Peking nodule

Magnification is 650X.

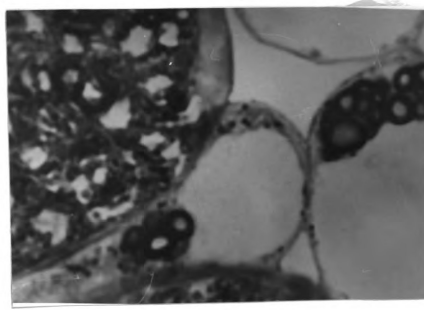

Figure 19 - Starch grains in uninfected cells of an R. fredii and F_3 nodules

The uninfected cells containing the starch grains are under the cortical cells. Magnification is 1000X.

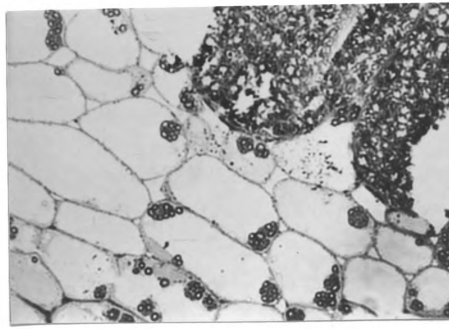

Figure 20 - Lower magnification view of uninfected cells in an R. fredii and F_3 nodule and young newly-infected cells

Magnification is 250X.

on the inner side of the cortical cells (Figure 20).

Discussion

We observed major differences between the ultrastracture of nodules formed by B. japonicum and R. fredii on Peking, including the extent of the peribacteroid space and the size of the β-hydroxybutyrate granules. Because we used a small sample size of two randomly selected plants from each treatment and one nodule from each plant, we have limited the generality of the observations and the results can only be examined from a qualitative perspective.

These differences suggest that the bacterial symbiont affects the development of the nodule ultrastructure. This observation contrasts with Sutton's (1983) suggestion that the size and the shape of the bacteroid and the number enclosed in each membrane are characteristics largely defined by the plant host. In fact, our results with Peking nodulated by B. japonicum or R. fredii showed clearly that the peribacteroid space and the number of bacteroids enclosed are directly related to the microsymbiont. Interestingly, Werner et al (1988) showed that the protein composition of the peribacteroid membrane was defined or controlled by the symbiont in an experiment comparing the protein composition of peribacteroid membranes from nodules formed by mutant and wild-type B. japonicum.

Another difference between the two bacterial strains was the size of the β-polyhydroxybutyrate granules. In B. japonicum nodules the β-polyhydroxybutyrate granules were large and occupied a major part of the bacteria. In R. fredii nodules the granules were much finer.

β-polyhydroxybutyrate content as well the activity of β-hydroxybutyrate dehydrogenase should be measured, along with a time course study of polyhydroxybutyrate synthesis during the plant growth. These studies may elucidate the biochemical basis and relevance of the different patterns of granule formation that we observed.

In all the nodules formed with B. japonicum (Figures 7,8,13) we observed a small deposition of fibrillar material (F), similar to that observed by Bergerson and Goodchild (1973). This fibrillar material was not seen in R. fredii nodules.

We observed an unusual ultrastructure of the nodules formed on F_3 plants by R. fredii, although the nodules were functional. Some of the observed characteristics were similar to those observed in senescing nodules, particularly the development of vacuoles (Kijne, 1975), the disappearance of the peribacteroid membrane (Werner et al, 1980; Tu, 1975), reduction in cytoplasm and vacuole-like structures (Newcomb, 1976). The main difference between these and senescent nodules is that we observed no structural changes in the bacteroid, while senescing nodules show bacteroid degradation (Werner et al, 1980) or Y-shaped bacteroid structures (Newcomb, 1976). Furthermore, we observed the bacteroids in an electron-dense matrix clearly unlike the translucent space seen in senescent nodules (Newcomb, 1976; Tu, 1975). In any case we are definetely not observing a typical senescence pattern since only 15% of nodule cells are senescent even at 10 weeks of growth (Tu, 1975), while we took our samples considerably earlier in plant development.

Light microscopy of samples from all parts of the nodules revealed

that the "holes" are not localized in one part of the nodule. It was especially interesting to see the structure of the cells near the cortical tissue (Figure 20) because in normal nodules, mature cells are located near the center of the nodule while young, newly-infected cells are located nearer the cortical cells (Tu, 1975). We observe that both young (Figure 20) and mature cells (Figure 19) have "holes."

The effect of various Rhizobium strains on nodule longevity has not been characterized (Sutton, 1983). However, when mutant bacteria unable to fix nitrogen are used to inoculate early senescence is observed (Werner et al, 1980,1984; Regensburger et al, 1986; Shantharam et al, 1987). Perhaps the senescence-like characteristics observed in F_3-R. fredii nodules suggest incompatibility between the bacteria and host. Although the allele enabling the F_3 plants to nodulate with R. fredii was selected for in the Peking-Harosoy cross, there may be additional genetic incompatibility factors in the F_3 plants from the Harosoy parent, leading to early senescence of the nodule. Along these lines the observation that the uninfected cells of the nodule contain large starch granules is also very interesting. Werner et al (1980) reported the same result when a nitrogen-fixation mutant bacterium was used to inoculate soybeans, and suggest that the granules indicate host-symbiont incompatibility. However, the starch granules we observe are smaller. Kathryn et al (1985) reported accumulation of starch granules in nodules formed by R. phaseoli Tn5 mutants.

Another possible explanation of our observations is that this was not senescence, but that the interaction between the F_3 plants and R. fredii leads to a different pattern of nodule development. For

example, there may be lack of coordination between the host and the symbiont, since it has been shown that nodule development is affected by both host and bacteria. However, there are no parallels reported in the literature. To identify the cause of this unusual nodule development, a time course study of nodule development must be done. The point at which aberrant development begins can be identified using this system to study nodule development and the role of the bacterial genotype in nodule development.

CHAPTER 4

HOST AND SYMBIONT REGULATION OF *rj1*-RESTRICTED NODULATION

Abstract

Although the efficiency of nitrogen fixation has a strong effect on soybean yield, using selected *B. japonicum* strains in an inoculum is often ineffective under field conditions because indigenous rhizobia outcompete the inoculated bacteria. One soybean gene, *rj1*, restricts nodulation to a few specific *B. japonicum* strains. These strains also induce chlorosis of the plant.

R. fredii is a newly introduced soybean symbiont. Most *R. fredii* strains are unable to nodulate North American cultivars. The ability of soybean plants to form nodules with *R. fredii* is controlled by one recessive allele. In this work, we studied whether *R. fredii* can overcome the nodulation block in *rj1/rj1* plants. If so, then *rj1* could be used to control nodulation in field conditions.

The allele allowing nodulation with *R. fredii* was introduced into a North American cultivar *rj1* background by crossing Peking (able to nodulate with *R. fredii*) with an isoline of Harosoy bearing *rj1*. F_2 plants homozygous for *rj1* were selected and selfed. F_3 progeny were inoculated with *R. fredii*. None of the forty F_3 plants tested were able to form nodules with *R. fredii*. However, progeny of F_2 plants selected for the ability to nodulate with *R. fredii* did segregate for

rj1, showing that the two alleles are at different loci. Our results suggest that rj1 restricts nodulation by R. fredii, and cannot be used in conjunction with an R. fredii inoculum to overcome the problem of bacterial competition for nodulation in the soil.

Introduction

The strong effect of nitrogen fixation on growth, yield and protein content of soybean is well known (Abel and Erdman, 1964). In many areas where soybean has been grown previously, inoculation with Bradyrhizobium japonicum cultures selected for nitrogen-fixing ability has little or no effect due to competition from native rhizobia. The native strains are well adapted to the soil and outnumber the bacteria in the inoculum, so that inoculated strains occupy only 0-17% of the nodules depending on strains, location and other ecological parameters (Caldwell, 1970; Ham et al, 1971; Ham, 1978).

One of the main elements controlling nodulation is the plant host. If plants can be modified to nodulate only with a particular Rhizobium strain, then perhaps these plants can improve the competitiveness of superior nitrogen-fixing rhizobia.

Four soybean genes restricting nodulation are known: rj1 (Williams and Lynch, 1954), rj2 (Caldwell, 1966), rj3 (Vest, 1970), and Rj4 (Vest and Caldwell, 1972). These genes restrict nodulation by specific B. japonicum strains or groups of related strains. The phenotype of rj1/rj1 plants is more general: these plants cannot be nodulated by most B. japonicum strains. The only strains that can nodulate rj1/rj1 plants are also phytotoxic to all soybean cultivars,

inducing chlorosis of the leaves (Owens and Wright, 1965). Another soybean allele which restricts nodulation is the dominant allele which prevents soybean from nodulating with R. fredii (Devine, 1984).

Serocluster 123 of B. japonicum dominates other B. japonicum strains in competition for the nodulation of soybean in the northern United States. Recently, Keyser and coworkers (Keyser et al, 1988; Cregan and Keyser, 1986) isolated some plant genotypes able to restrict nodulation by some strains of serogroup 123. The inheritance of this trait is still not known.

Only one group of bacterial genes involved in competition has been studied. A specific pea cultivar is host for two strains of R. leguminosarum but one strain is always able to outcompete the other. The genes involved in this competition have been cloned recently (Dowling et al, 1988). We attempted to control competition for nodulation by using rj1 to prevent nodulation by B. japonicum in the presence of the allele enabling nodulation with R. fredii. We wanted to know if this is a plant genetic background in which only R. fredii can nodulate. In the course of this study, we had to determine whether rj1 is allelic to or exhibits recessive epistasis to the allele which permits R. fredii nodulation.

Materials and methods

Preparation of seed: Peking and L66-2470, a subline of Harosoy rj1/rj1 plants were crossed. The F1 plants were selfed to obtain F_2 seeds. F_2 seeds were surface sterilized, inoculated with R. fredii USDA 205 or with B. japonicum 110. The seeds were sown individually in 200 ml

sterile Ehrlenmyer flasks filled with a 1:1 mixture of vermiculite and perlite, wetted with NF nutrient solution (Johnson et al, 1966). The plants were grown in a growth chamber with 12 h illumination. After five weeks the F_2 plants which formed nodules with 205, and F_2 plants which failed to form nodules with 110 were selected, transferred to pots and grown to maturity in the greenhouse. F_3 seed was harvested from each of the plants separately.

F_3 from each F_2 plant selected for the ability to nodulate with R. fredii were inoculated with either 205 or 110. The progeny of F_2 plants unable to nodulate with 110 were inoculated with 205.

Seed sterilization: Seeds were soaked in 20% commercial bleach (1% NaOCl) for 3 minutes, washed briefly with sterile distilled water, then soaked in 3% H_2O_2 for five minutes and washed three times with sterile distilled water.

Bacterial strains: R. fredii USDA 205 Rif^R (5A14) was obtained from Dr. K.D. Nadler, and was grown in TY medium. B. japonicum 110 was obtained from Dr. B. Chelm, and was grown in YM medium (Vincent, 1970). Both strains were grown at 29°C with shaking to 0.7 O.D. for inoculation.

Results

Allelism test of rj1 and the allele permitting R. fredii nodulation - The results of this test are summarized in Table 9. Thirty seeds from three of the seven F_2 plants able to nodulate with R. fredii (the remainder of the plants did not survive to produce seed) were inoculated with both 5A14, to confirm the selected phenotype, and with

Table 9 - Allelism test and segregation of rjl and the allele permitting nodulation with R. fredii

	Plants selected for allelism test			Plants selected for epistasis test		
	inoculum	Nod^+	Nod^-	inoculum	Nod+	Nod-
F_2	5A14	7/39	32/39	110	40/50	10/50
F_3	5A14/110	21/30	9/30	5A14	0/40	40/40

strain 110. Nine plants did not form any nodules, suggesting that these plants are rjl/rjl homozygous segregants from a heterozygous F_2 parent. The two phenotypes (rjl restriction and R. fredii nodulation) must be the result of separate genes and not alleles of a single locus.

Interaction between the two genes - Four F_2 plants unable to nodulate with strain 110 (rjl/rjl) (the remainder of the plants did not survive to produce seed) were grown and allowed to self. None of the forty progeny inoculated with 5A14 formed nodules. There are two possible explanations: one is that none of the F_2 plants carried the allele allowing R. fredii nodulation, and the other that rjl exhibits recessive epistasis to the allele permitting R. fredii nodulation. A X^2 analysis of this ratio may not be meaningful because some of the plants which might have been able to nodulate did not form nodules because of incomplete penetrance of the gene permitting R. fredii nodulation (observed in other experiments, data not shown).

Discussion

Our data suggest that rjl and the allele enabling nodulation with R. fredii are not alleles of the same gene and are not closely linked. Our data also shows that rjl exhibits recessive epistasis to this allele. The probability that among the plants selected in F_2 none would carry the allele enabling R. fredii to nodulate is .0039, if the genes are independent (as suggested by the segregation of the plants from the allelism test). Because of these findings, it seems that it will not be feasible to control competition for nodulation by using a

combination of rj1 hosts and inoculating with R. fredii.

Selecting plant genotypes that restrict nodulation of some of the native rhizobia in the soil (Keyser, 1988 Cregan and Keyser 1986) may be a solution in some regions, but because of very large regional differences between native rhizobial populations, it cannot be a general solution to this problem. In this respect, rj1 is unusual because it controls nodulation regardless of regional soil microbial ecology. Recently, some of the R. leguminosarum genes involved in the competition between bacteria for nodulation have been identified (Dowling 1988). A similar approach might be used to identify the genes which enable the rhizobotoxine-producing B. japonicum strains to nodulate rj1 plants. Transfer of these genes to selected rhizobia might generate strains which are specifically selected by the host, solving the problem of competition for nodule formation between agronomically useful bacterial symbionts and indigenous rhizobia in the soil.

APPENDIX

DATA FOR GENERATING RESTRICTION MAP OF *R. FREDII Nod* GENES

Table 10 - Restriction fragment lengths used to generate restriction map

The restriction digests were done using a plasmid with the cloned *R. fredii Nod* gene region inserted in the opposite orientation from pRfIR1 (Figure 3).

Enzyme		Fragment lengths							
BamHI		6.8	3.0	1.7					
BamHI	PstI	6.8	3.0	1.0	0.5				
BamHI	HindIII	4.0	3.0	2.6	1.0	0.6	0.5		
BamHI	XhoI	3.0	3.0	1.7	1.2	1.1	0.7	0.5	0.4
XhoI		6.9	1.9	1.7	0.7	0.4			
XhoI	PstI	3.8	3.2	1.9	1.7	0.5	0.4		
XhoI	HindIII	3.2	2.5	1.9	1.7	0.6	0.5	0.4	
XhoI	EcoRI	4.4	2.7	1.9	1.7	0.7	0.4		
HindIII		5.4	3.3	2.5	1.2				
HindIII	PstI	5.4	3.3	2.5	1.1				
PstI		8.5	3.8						
PstI	EcoRI	5.9	3.8	2.7					

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BamHI	XhoI	3.0	3.0	1.7	1.2	1.1	0.7	0.5	0.4
XhoI		6.9	1.9	1.7	0.7	0.4			
XhoI	PstI	3.8	3.2	1.9	1.7	0.5	0.4		
XhoI	HindIII	3.2	2.5	1.9	1.7	0.6	0.5	0.4	
XhoI	EcoRI	4.4	2.7	1.9	1.7	0.7	0.4		
HindIII		5.4	3.3	2.5	1.2				
HindIII	PstI	5.4	3.3	2.5	1.1				
PstI		8.5	3.8						
PstI	EcoRI	5.9	3.8	2.7					

BIBLIOGRAPHY

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Able, G.H. and L.W. Erdman 1964. Response of "Lee" soybean to different strains of R. japonicum. Agron.J. 6:423-424.

Bal, A.K., S. Shantharam and P.P. Wong 1982. Nodulation of pole bean (Phaseolus vulgaris L.) by Rhizobium species of two cross-inoculation groups. Appl. Environ. Microbiol. 44:965-71.

Barbour, W.M., J.N. Mathis and G.H. Elkan 1985. Evidence for plasmid and chromosome-borne multiple Nif Genes in R. fredii. Appl. Environ. Microbiol. 50:41-4.

Beynon, J.L., J.E. Beringer and W.A. Johanston 1980. Plasmid and host-range in R. leguminosarum and R. phaseoli. J. Gen. Microbiol. 120:421-429.

Bergersen, F.J. and D.J. Goodchild 1972. Cellular location of leghaemoglobin in soybean root nodule. Aust. J. Biol. Sci. 26:741-56.

Bhuvaneswari, T.V., B.G. Turgeon and W.D. Bauer 1980. Early events in the infection of soybean (G. max (L) Merr) by R. japonicum. Plant Physiol. 66: 1027-1031.

Bliss, A.F 1985. Breeding for enhanced dinitrogen fixation of common bean. In W. Paul (ed.), Nitrogen Fixation and CO_2 metabolism. Elsevier Science Publishing, Amsterdam, pp. 303-310.

Broughton, W.J., N. Heycke, H. Meyer and C.E. Parkhurst 1984. Plasmid-linked nif and nod genes in fast growing rhizobia that nodulate G. max. P.N.A.S. 81:3093-97.

Caldwell, B.E. 1966. Inheritance of a strain-specific ineffective nodulating in soybeans. Crop Sci. 6:427-428.

---- 1970. Effect of R. japonicum strains on soybean yield. Crop Sci. 10:19-21.

Carlson, R.W. and M. Yadav 1985. Isolation and partial characterization of the extracellular polysaccharides and lipopolysaccharides from fast-growing R. japonicum USDA 205 and its nod$^-$ mutant, HC205, which lacks the symbiotic plasmid. Appl. Environ. Microbiol. 50:1219-24.

Carroll, B.J., D.L. McNeil and P.M. Gresshoff 1985. Isolation and properties of a soybean (G. max) mutant that nodulates in the presence of high nitrate concentration. P.N.A.S 82:4162-66.

Dazzo, F.B. and G.L. Truchet 1983. Interaction of lectins and their saccharide receptors in the Rhizobium-legume symbiosis. J. Membr. Biol. 73:1-10.

Denarie J. and D. Kahan 1987. R. meliloti genes controlling relationship with plants: a survey. Abstract, 11th North American Rhizobium Conference (Montreal, Canada).

Devine, T.E. 1984. Inheritance of soybean nodulation response with a fast-growing strain of Rhizobium. J.Hered. 75:359-361.

—— 1985. Nodulation of soybean plant introduction line with the fast-growing rhizobial strain USDA 205. Crop Sci. 25:354-356.

—— and B.H. Breithaupt 1980. Phenotypic thermal stability of rhizobiotoxine-induced chlorosis and the nodulation controlling gene, ril. Crop Sci. 20:394-396.

Djordjevic, M.A., P.R. Scholfied, R.W. Ridge, N.A. Morrison, B.J Bassam, J.Plazinski, J.M. Watson and B.G. Rolfe 1986. Rhizobium nodulation genes involved in root hair curling (Hac) are functionally conserved. Plant Molecular Biology 4:147-60.

Dowling, D.N., J. Stanley, C.H. Wong and W.J. Broughton 1988. Molecular genetics of competitive nodulation blocking of pea by R. leguminosarum. Abstract, 7th International Congress on Nitrogen Fixation (Cologne, Germany).

Downie, J.A., C.D. Knight, A.W.B. Johnston and L.Rossen 1985. Identification of genes and gene products involved in the nodulation of peas by R. leguminosarum. Mol. Gen. Genet. 198:255-62.

DuTeau, N.M., R.G. Palmer and A.G. Atherly 1986. Fast-growing R. fredii are poor nitrogen-fixing symbionts of soybean. Crop Sci.26:884-889.

Egelhof, T.T. and S.R. Long 1985. R. meliloti nodulation genes: identification of nodDABC gene products, purification of nodA protein,and expression of nodA in R. meliloti. J. Bacteriol. 164:591-99.

Elhai, J. and C.P. Wolk 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene 68:119-138.

Feinberg, A.P. and B. Vogelstein 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Figurski, D.H., and D.R. Helinski 1979. Replication of an origin-containing derivative of plasmid RK2 is dependent on a function provided in trans. P.N.A.S. 76:168-52.

Firmin, J.L., K.E. Wilson, L. Rossen and A.W. Johnston 1986. Flavonoid activation of nodulation genes in Rhizobium reversed by other compounds present in plants. Nature 324:90-92.

Fred, E.B., I.L. Baldwin and E. McCoy 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science, Madison, Wisconsin, p. 343.

Greder, R.R., J.H. Orf and J.W. Lambert 1986. Heritabilities and associations of nodule mass and recovery of Bradyrhizobium japonicum serogroup USDA 110 in soybean. Crop Sci. 26:33-37.

Gray N.G., W.R. Ronson, and F.M. Ausubel 1986. Regulation of nitrogen fixation genes. Ann. Rev. Genet. 20:567-91.

Ham, G.E., V.B. Cardwell and H.W. Johanson 1971. Evaluation of R. japonicum inoculants in soils containing naturalized population of rhizobia. Agron. J. 63:301-303.

—— 1978. Inoculation of legumes with Rhizobium in competition with naturalized strains, In W.E. Newton and W.H. Orme-Johnson (eds.), Nitrogen Fixation II. University Park Press, Baltimore, pp. 131-139.

Heron, D.S. and S.G. Pueppke 1984. Mode of infection, nodulation specificity, and indigenous plasmids of 11 fast growing R. japonicum strains. J. Bact. 160:1061-66.

Herman, P.S., C.A. Wijffelman, E. Pees, R.J.H. Okker and B.J.J. Lugtenberg 1987. Rhizobium nodulation gene nodD as a determinant of host specificity. Nature 328:337-40.

Hodgson, A.L.M. and G. Stacey 1985. Potential for Rhizobium improvement. CRC Critical Reviews in Biotechnology 4:1-75

Honma, M.A. and F.M. Ausubel 1987. R. meliloti has three functional copies of the nodD symbiotic regulatory gene. P.N.A.S. 84:8558-8562.

Hong, G.F., J.E. Burn and A.W.B. Johnston 1987. Evidence that DNA involved in the expression of nodulation genes in Rhizobium binds to the product of the regulatory gene nodD. Nuc. Acids Res. 15:9677-89.

Jacobs, T.W., T.T. Egelhoff and S.R. Long 1985. Physical and genetic map of R. meliloti nodulation gene region and nucleotide sequence of nodC. J. Bact. 162:469-76.

Johanson, G.V., H.J. Evans, and T.M. Ching 1966. Enzymes of the glyoxylate cycle in rhizobia and nodules of legumes. Plant Physiol. 41:1330-1336.

Kathryn, A.V., K.D. Noel, Y. Kaneko, and E.D. Newcomb 1985. Nodule initiation elicited by noninfective mutants of R. phaseoli. J. Bact. 162:950-9.

Keyser, H.H., B.B. Bohlool, T.S. Hu, and D.F. Weber 1982. Fast-growing rhizobia isolated from root nodules of soybeans. Science 215:1631-1632.

---- and P.B. Cregan 1984. Interactions of selected Glycine soja Sieb.& Zucc. genotypes with fast- and slow-growing Rhizobia. Crop Sci. 24:1059-1062.

----, P.B. Cregan and M.J. Sadowsky 1988. Soybean genotypes which restrict nodulation of strains of B. japonicum serocluster 123. Abstract, 7th International Congress on Nitrogen Fixation (Cologne, Germany).

Kijne, J.W. 1975. The fine structure of pea root nodules. 2. Senescence and disintegration of the bacteroid tissue. Physiol. Plant Path. 7:17-21.

Kondorosi, E., Z. Banfalvi and A. Kondorosi 1984. Physical and genetic analysis of a symbiotic region of R. meliloti: identification of nodulation genes. Mol. Gen. Genet. 193:445-5252.

---- and A. Kondorosi 1986. Nodule induction on plant roots by Rhizobium. T.I.B.S. 11:296-9.

Long, S.R., W.J. Buikema and F.M. Ausubel 1982. Cloning of R. meliloti nodulation genes by direct complementation of nod- mutants. Nature 298:485-488.

Maniatis, T.E., E.F. Fritsch and J. Sambrook 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mathis, J.N., W.M. Barbour and G.H.Elkan 1985. Effect of sym plasmid curing on symbiotic effectiveness in R. fredii. Appl. Environ. Microbiol 49:1385-1388.

Matthews, A., B.J. Carrol and P.M. Gresshoff 1987. Characterization of non-nodulation mutant of soybean (G. max (L) Merr): effects and absence of root hair curling. J. Plant Physiol. 131:349-61.

McLoughlin, T.J., P.A. Owens and S.G. Alt 1985. Competition studies with fast-growing Rhizobium japonicum strains. Can. J. Microbiol. 31:220-223.

Mellor, R.B. and D. Werner 1987. Peribacteroid membrane biogenesis in mature legume root nodules. Symbiosis 3:87-114.

Morris, D.R. and R.W. Weaver 1983. Mobilization of ^{15}N from soybean leaves as influenced by Rhizobial strains. Crop Science 23:1111-1114.

Mulligan, J.T. and S.R. Long 1985. Induction of R. meliloti nodC expression by plant exudate requires nodD. P.N.A.S. 82:6609-13.

Newcomb, W. 1976, A correlated light and electron microscopic study of symbiotic growth and differentiation in Pisum sativum root nodules. Can. J. Bot. 55:2163-86.

Peters, N.K., J.K. Frost and S.L. Long 1986. A plant flavone, luteolin, induces expression of R. meliloti nodulation genes. Science 233: 977-80.

Owens, L.D. and D.A. Wright 1965. Production of the soybean-chlorosis toxin by R. japonicum in pure culture. Plant Physiol. 40:931-933.

Ramakrishnan, N.E., R.K. Prakash, S. Shanthar, N.E. Duteau and G. Atherly 1986. Molecular cloning and expression of R. fredii USDA 193 nodulation genes: Extension of host range for nodulation. J. Bact. 168:1087-95.

Redmond, J.W., M. Bately, M.A. Djordjevic, R.W. Innes, P.L. Kuempel and B.G. Rolfe 1986. Flavones induce the expression of the nodulation genes in Rhizobium. Nature 323:632-35.

Regensburger, B., L. Meyer, M. Filser, J. Weber, D. Studer J.W. Lamb, H.M. Hahn and H. Henneeke 1986. B. japonicum mutants defective in root nodule bacteroid development and nitrogen fixation. Arch. Microbiol. 144:355-366.

Rolfe, B.G. and P.M. Gresshoff 1988. Genetic analysis of legume nodule initiation. Ann. Rev. Plant Mol. Biol. 39:297-319.

Ronis, D.H., D.J. Sammons, W.J. Kenworthy and J.J Meisinger 1985. Heritability of total and fixed nitrogen of the seed in two soybean populations. Crop Sci 25:1-4.

Rosatas, K., E. Kondorosi, B. Horvath, A. Simoncsits and A. Kondorosi 1985. Conservation of extended promotor region of nodulation genes in Rhizobium. P.N.A.S. 83:757-61.

Rossen, L., E.O. Davis and A.W.B. Johnston 1987. Plant-induced expression of Rhizobium genes involved in host specificity and early stages of nodulation. T.I.B.S. 12:430-3.

Russell, P., M.G. Schell, K.K. Nelson, L.J. Halverson, K.M. Sirotkin & G. Stacey 1985. Isolation and characterization of the DNA region encoding nodulation functions in B. japoniucm. J. Bact. 164:1301-8.

Sadowsky, M.J. and B.B Bohlool 1983. Possible involvment of megaplasmid in nodulation of soybeans by fast-growing rhizobia from China. Appl. Environ. Microbiol. 46:906-11.

SAS Institute 1985. SAS Procedures Guide for Personal Computers. SAS Institute, Cary, N.C.

Scholla, M.H. and G.H. Alkan 1984. R. fredii sp. nov., a fast-growing species that effectively nodulates soybeans. Int. J. Syst. Bacteriol. 34:484-86.

Schubert, K.R. 1986. Products of biologecal nitrogen fixation in higher plants. Ann. Rev. Plant Physiol. 37:359-74.

---- and H.J. Evans 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. P.N.A.S. 73:1207-11.

Shantharam, S., N.M. DuTeau, R.K. Prakash and A.G. Atherly 1987. Comparative cytology of effective and ineffective root nodules of North American cultivars of Glycine max L. formed by two Rhizobium fredii strains. Cytobios 50:181-190.

Stacey, G. and R.J. Upchurch 1984. Rhizobium inoculation of legumes. Trends in Biotechnology 2:65-70.

Sutton, W.D. 1983. Nodule development and senescence. In (ed.), Nitrogen Fixation, vol. 3. Clarendon Press.

Tu, J.C. 1975. Rhizobial root nodules of soybean as revealed by scanning and transmission electron microscopy. Phytopathology 65:447-454.

Werner, D., E. Morschel, R. Stripf, B. Winchenbach 1980. Development of nodules of Glycine max infected with an ineffective strain of Rhizobium japonicum. Planta 147:320-329.

----, E.Morschel, R. Kort, R.B. Mellor, S.Bassarab 1984. Lysis of bacteroids in the vicinity of the host cell nucleus in an ineffective (fix-) root nodule of soybean. Planta 162:8-16.

----, E. Morscel, C. Garbers, S. Bassarb, and R.B. Mellor 1988. Particle density and protein composition of the peribacteroid membrane from soybean root nodules is affected by mutation in the microsymbiont B. japonicum. Planta 174:263-270.

Vest, G. 1970. Rj3-a gene conditioning ineffective nodulation in soybean. Crop Sci. 10:34-35.

---- and B.E. Caldwell. 1972. Rj4-a gene conditioning ineffective nodulation in soybean. Crop Sci. 12:692-694.

Vincent, J.M. 1970. A manual for the practical study of root-nodule bacteria, IBP Hnd. 15. Blackwell Scientific Publications, Oxford, U.K.

---- 1980. In W.E. Newton and W.H. Orme-Johnson (eds.), Nitrogen Fixation II. University Park Press, Baltimore, pp. 101-131.

Wildworth, D. and A. Glenn 1984. How does a legume nodule work? T.I.B.S. 9:519-523.

Williams, L.F. and D.L. Lynch 1954. Inheritance of a non-nodulating character in soybean. Agron. J. 46:28-29.

Yanisch, P.C. and J.V. Messing 1985. Improved M13 phage cloning vectors. Gene 33:103.

Yelton, M.M., S.S. Yang, S.A. Edie, and S.T. Lim 1983. Characterization of an effective salt-tolerant, fast-growing strain of Rhizobium japonicum. J. Gen. Microbiol. 129:1537-1547.