To the Graduate Council:

I am submitting herewith a dissertation written by David L. Gerhold entitled "Host Regulation of Nodulation Genes in Rhizobium meliloti and Bradyrhizobium japonicum." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Microbiology.

Gary Stacey, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

HOST REGULATION OF NODULATION GENES IN *RHIZOBIUM MELILOTI* AND *BRADYRHIZOBIUM JAPONICUM*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

David L. Gerhold

May 1989

ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Gary Stacey, for his constructive criticisms and support, as well as the rest of my committee, Dr. Karl Sirotkin, Dr. Beth Mullin, Dr. Raymond Beck, and Dr. Gary Sayler. Special thanks also to my advisor at Michigan State University, Dr. Frank B. Dazzo, who taught me enthusiasm for Rhizobium and lectins; and my advisor, Dr. Adam Kondorosi, and colleague Dr. Beatrix Horvath, who taught me to be a more productive scientist during my stay in Szeged, Hungary. On a personal level, I thank Dr. Nirupama Deshmane and Dr. Jae So who suffered through graduate school along side me, and Dr. Velupillai Puvanesarajah and my many talented coworkers in Dr. Stacey's laboratory. Finally, I am grateful to my parents and family for their unfailing support.

ABSTRACT

Rhizobium meliloti and *Bradyrhizobium japonicum* were analyzed by biochemical and molecular genetic methods to identify genes which respond to signals from their host plants. *B. japonicum* was previously shown to respond to seed lectin from its soybean host to become "competent" to nodulate. *B. japonicum* strain USDA110 was thus treated with soybean lectin or with genistein which induces the known *nodulation* (*nod*) genes. The proteins from treated and untreated cells were separated by two-dimensional electrophoresis to identify proteins induced by these host molecules. Two relatively abundant proteins induced by soybean lectin (S55), and by genistein (G58) were identified.

In a genetic approach, cosmid pR32 which contains the common *nod* genes and surrounding DNA, was searched for host inducible genes by fusing the *lacZ* structural gene, encoding β-galactosidase, to promoters within pR32. Transposon Tn*3*-*lac* was allowed to transpose randomly within pR32, and ~ 100 such pR32::Tn*3*-*lac* constructs were restriction mapped and mated from *E. coli* into *B. japonicum*. By assaying the *B. japonicum* (pR32::Tn*3*-*lac*) strains with and without soybean root exudate, two genistein-inducible operons, called HIL3 and HIL4, were newly identified. The *B. japonicum* USDA 110 *nodD* gene was also subcloned and transferred into a *Rhizobium meliloti* $nodD_1$-mutant. This complementation experiment established the functionality of the *B. japonicum* *nodD* gene and indicated its broad specificity towards flavonoids.

Rhizobium meliloti was previously known to carry three plant flavonoid-inducible operons made up of seven genes (nodABC, nodFEG, and nodH) involved in alfalfa nodulation. DNA sequence analysis by Dr. Kondorosi's group in Szeged, Hungary, identified a promoter sequence termed the "nod box" upstream of all three inducible operons. Three additional loci were identified which bore sequence homology to a synthetic nod box oligonucleotide, only one of which was associated with known nodulation genes. The unstudied two promoters, termed n4 and n5, were fused to the promoterless lac operon using transposon Mud-lacPR13, and found to be induced by the alfalfa-excreted flavone, luteolin. By constructing site-directed Tn5 mutants downstream of n4 and n5 it was found that n5 plays a significant role in nodulation of two R. meliloti hosts, alfalfa and sweetclover, whereas n4 was important only for alfalfa nodulation. Hybridization data suggested that the n5 locus is conserved among species of Rhizobium, whereas n4 is probably unique to R. meliloti strains, in agreement with the host-specific phenotype of n4 locus mutants.

TABLE OF CONTENTS

CHAPTER		PAGE
1.	INTRODUCTION	1
2.	LITERATURE REVIEW	5
	Plant Lectins, a Role in Nodulation?	6
	Plant lectins	
	Lectins and nodulation	
	Genetics of Nodulation	13
	Common nodulation genes	
	Host-Specific Nodulation Genes	
	Regulation of nodulation genes	
3.	MATERIALS AND METHODS	22
	Microbiology	22
	Growth of bacteria	
	Bacterial strains plasmids and bacteriophages	
	Plant nodulation tests	
	Protein Analyses	26
	Protein sample preparation	
	Protein electrophoresis	
	β-Galactosidase assays	
	Genetics	28
	DNA cloning and manipulations	
	Transformation	
	Plasmid DNA isolation	
	Genomic DNA isolation	

CHAPTER PAGE

3. (continued)

Bacterial conjugations

Mini-Mud-lac mutagenesis

Transplacement/homogenotization

DNA blotting and hybridizations

Tn5 mutagenesis

Tn3-lac mutagenesis

4. RESULTS 37

Identification of B. japonicum Proteins Induced by Host Signals 37

Root exudate-induced proteins

Soybean lectin- and genistein-induced proteins

NodD complementation

Host-Inducible Genes in B. japonicum 50

Tn3-lac mutagenesis of pR32

Host-inducible locus 4 (HIL4)

Host-inducible locus 3 (HIL3)

Host-Inducible Genes in R. meliloti 60

Subcloning the n4 and n5 loci

Lac-fusions to n4 and n5 are luteolin inducible

Tn5 mutagenesis

Symbiotic phenotypes of mutants

Hybridization of n4 and n5 regions to other rhizobia

CHAPTER PAGE

5. DISCUSSION 81

Identification of *B. japonicum* Proteins

Induced by Host Signals 81

Root exudate-induced proteins

Soybean lectin-induced proteins

Genistein-induced proteins

NodD complementation

Host-Inducible Genes in *B. japonicum* 86

Tn*3-lac* Mutagenesis of pR32 87

Host-inducible locus 4 (HIL4)

Host-inducible locus 3 (HIL3)

Host-Inducible Genes in *R. meliloti* 93

Summary 97

BIBLIOGRAPHY 99

VITA 109

LIST OF FIGURES

FIGURE		PAGE
1.	Arrangement of nodulation genes in rhizobia	14
2.	Cointegrate formation and homogenote formation	34
3.	Two-dimensional seperations of [^{14}C]-proteins from B. japonicum USDA110 treated with plant nutrient solution, or soybean root exudate	39
4.	Two-dimensional seperation of [^{14}C]-proteins from B. japonicum USDA110 treated with soybean lectin in plant nutrient solution	43
5.	Two-dimensional separations of [^{35}S]-proteins from B. japonicum USDA110 treated with soybean lectin and genistein in yeast extract-mannitol medium	47
6.	Nodulation of alfalfa (Medicago sativa 'Buffalo') by wild-type R. meliloti AK631, $nodD_1$ mutant MG107, and MG107(pDG39) containing B. japonicum $nodD_1$	51
7.	Cosmid clone pR32 from B. japonicum USDA110 showing known nodulation genes and Tn3-lac insertions	53
8.	Colony Morphology of HIL4 mutants DG62, DG63, and control strain DG49	57
9.	EcoRI restriction map of the Rhizobium meliloti nodulation region	62
10.	Restriction analysis and nod box hybridization of R. meliloti cosmids (Kondorosi et al. 1984) from the pSym nodulation region	64
11.	Transcriptional and translational reporter gene fusions	68
12.	Nodulation of alfalfa, and Sweetclover by Tn5 mutants of R. meliloti AK631	72
13.	Hybridization of the n4 locus (the 2.3kb PstI-BamHI fragment from pDG60) to EcoRI digested genomic DNA from: R. meliloti AK631, R. meliloti L5-30, R. leguminosarum 'trifolii' ANU843, R. leguminosarum 897, R. leguminosarum species MPIK3030, R. fredii USDA193, Bradyrhizobium japonicum USDA110, and Agrobacterium tumefaciens 136	77

FIGURE PAGE

14. Hybridization of the n5 locus (2.1kb EcoRI fragment from pDG100) to EcoRI digested genomic DNA from R. meliloti AK631, R. meliloti L5-30, R. leguminosarum 'trifolii' ANU843, R. leguminosarum 897, R. fredii USDA 193, Agrobacterium tumefaciens 136, R. species MPIK3030, Bradyrhizobium parasponiae ANU 289, and B. japonicum 61A101C 79

CHAPTER 1

INTRODUCTION

The biggest single factor limiting world agriculture today is probably the depletion of available nitrogen in our soils. This problem is commonly countered by growing legumes--plants which can replenish soil nitrogen through symbiotic cooperation with nitrogen-fixing bacteria called rhizobia. Such legumes constitute both forage and food crops including clover, alfalfa, peas, beans, peanuts, and soybeans. Soybeans, the USA's leading cash crop, contribute several billion dollars annually to the nation's economy and support a thriving industry which sells Rhizobium for inoculating soybean and other legume seeds.

It is often suggested that extending Rhizobium's nitrogen fixing capabilities to major nonlegume crops such as corn, wheat, and rice, would be a major boon for agriculture. This long-term goal will require a detailed understanding of legume nodulation in order to determine why non-legumes are not nodulated by rhizobia. Improving inoculum strains is also of major importance since inoculated rhizobia rarely compete well with indigenous rhizobia for nodulation. These nearly ubiquitous native strains often fix nitrogen poorly. By learning how rhizobia strains compete to form plant nodules, we can learn how to improve nodulation by inoculum strains, or breed plants which "select" effective nitrogen-fixing rhizobia. Thus the key to exploiting symbiotic nitrogen fixation is

to understand how legumes and rhizobia cooperate to form nitrogen fixing nodules.

Nodulation is a complex process, requiring an intricate "courtship" between rhizobia and their legume hosts. This root nodulation process is characterized by several readily observable morphological steps (Vincent 1980). Upon encountering the root, rhizobia attach to root hairs and induce root hair curling. Bacteria enclosed by curled root hairs then pass through the plant cell wall to enter the root hair cells. As the rhizobia multiply and advance down the root hair to the cortical cells, they are enclosed by a tubular structure called an infection thread. Cortical cells are induced to divide in advance of this infection thread. Finally, rhizobia are released into these dividing cells where they fix atmospheric N2 into ammonia for plant use. A crucial aspect of the symbiosis is the biochemical communication by which legumes and rhizobia identify and signal one another to prepare ensuing steps in nodulation.

The biochemical events which accompany the infection process are difficult to elucidate because they occur on a microscopic scale between two intimately interacting organisms. Current nodulation research is focusing on identifying nodulation defective mutants of Rhizobium/Bradyrhizobium, and determining which step in nodulation is blocked. Biochemical analysis of these mutants may then reveal the function each mutant gene plays during nodulation.

Symbiotic genes in rhizobia have been classified as nodulation or host specific nodulation (nod or hsn, respectively) genes if they

play roles in plant nodulation, and nitrogen fixation or symbiotic nitrogen fixation (nif or fix, respectively) genes if they act to support nitrogen fixation. Nif genes in rhizobia are defined as those showing protein sequence homology to nitrogen fixation genes in the nonsymbiotic nitrogen fixer, Klebsiella pneumoniae. Genes designated fix are unique to symbiotic nitrogen fixers, implying plant-interactive functions.

Although "fast growers" (Rhizobium species) and "slow growers" (Bradyrhizobium species) conserve some nodulation genes and nodulate legumes in a morphologically similar process, they are vastly different genetically and biochemically (Dart 1974). Fast growers have maximal generation times of 3-5 hours, whereas slow growers require 8-12 hours. Slow growers tend to produce basic metabolic waste products, in contrast to acidic metabolites from fast growers. Genetically, fast growers tend to have smaller genomes, and carry their nodulation and nitrogen fixation genes on large symbiotic megaplasmids, designated "pSym." Slow growers seem to carry their symbiotic genes on the chromosome and tend to have broader host ranges than fast growers. For example: R. leguminosarum biovar 'trifolii' nodulates clovers (Trifolium), R. leguminosarium biovar 'phaseoli' nodulates beans (Phaseolus), and R. meliloti nodulates alfalfa (Medicago), and sweetclover (Melilotus). Bradyrhizobium japonicum can nodulate soybeans (Glycine), cowpeas (Vigna), and siratro (Macroptilium). One exceptional strain, called Rhizobium species MPIK3030 has a 6 hour generation time, megaplasmid-borne nod

genes, and can nodulate legumes from nine genera, including soybeans, siratro, and alfalfa (Lewin et al. 1987).

The long-range goal of the research described is to further our understanding of the nodulation process. This knowledge may be used in the future to improve the competitiveness of rhizobial inocula and possibly to extend the bacterium's host range. By identifying and studying those particular genes which respond to the host plant's presence, we can elucidate each step in the intricate "courtship" which occurs between rhizobia and legumes.

CHAPTER 2

LITERATURE REVIEW

Early observations and physiological experiments on the legume nodulation process showed the existence of host-symbiont specificity, and the temporal sequence of events occuring in nodulation. Since 1980 a clearer understanding of the molecular genetics of nodulation has been developing as well. Rhizobial genes required for nodulation have been identified using (Tn*5*) mutagenesis and complementation, and their regulation studied with *lac* gene fusions. Hypotheses about the general functions of some genes have been formulated by observing the behavior of mutants on plants. These genetic analyses will soon be nearly exhausted and biochemical analyses will have to be performed to assign enzyme functions to each gene. Genetic and biochemical technologies must be further developed to identify nodulation genes in the plant as well. In the final analysis, this complete understanding may be necessary in order to exploit symbiotic nitrogen fixation for Man's benefit.

Physiological evidence has shown the importance of plant-symbiont signaling, lectin-dependent and lectin-independent attachment, and has revealed vital functions for bacterial polysaccharides during nodulation. *Rhizobia* approaching a legume root probably first encounter nutrients and signal compounds which trigger the bacteria to begin symbiotic differentiation (Bauer 1981). Such nutrients include plant-excreted amino acids, mono- and polysaccharides, and a wide variety of organic compounds (Wright and

Northcote 1974). Plant-derived signal compounds include lectins and flavonoid compounds; secondary metabolic intermediates which transcriptionally induce rhizobial nodulation genes (i.e. Mulligan and Long 1985). Both plant lectins, which have different specificities for sugar ligands, and flavonoids, which are characteristic for particular legume genera, play roles in determining host-symbiont specificity.

Plant Lectins, a Role in Nodulation?

Plant lectins. Investigations of nodulation physiology have suggested a role for plant lectins as determinants of host-symbiont specificity. Lectins are defined as noncatalytic glycoproteins which bind to specific carbohydrate moieties. The binding specificity of each lectin is characterized by its hapten/s, individual sugars which can compete for the active (binding) site of each lectin. Lectins have been isolated from the seeds and occasionally, roots and leaves of a variety of plants, notably the legumes: jackbean, soybean, broadbean, pea and clover; as well as the nonlegumes: potato, tomato, rice, and wheat (Etzler 1983).

Despite the isolation and characterization of numerous plant lectins, their function/s remain unknown. Suggested functions include signals to N_2-fixing symbionts, recognition or defense against invading pathogens, cell wall growth, and transport of oligosaccharides or plant hormones (Strosberg et al. 1986). All known legume lectins share strong amino acid sequence homologies, including lectins from leaves, stems, and seeds (Strosberg et al.

1986). Although legume seed lectins have been studied most, it is apparent that one plant can contain several lectins with differing specificities (Etzler 1983).

Soybean seed lectin (SBL) has been well-characterized and is commercially available (E·Y Laboratories) in a highly purified form. SBL functions as a tetramer consisting of protomers of roughly 30-33kd. Each protomer bears one hapten binding site with specificity for galactose or N-acetyl-galactosamine. Binding affinities have been determined for N-acetyl-galactosamine ($K = 2.6 \times 10^4 M^{-1}$) and the best ligand known, N-diamine-napthalene sulfonyl-galactosamine ($K = 5.9 \times 10^5 M^{-1}$, DeBoeck et al. 1984). These moderate affinities may be misleading, in that the intended biological receptors for SBL are probably more complex and may have much higher binding affinities. SBL is coded for by a single gene called Le1 (Vodkin et al. 1983), however affinity column-purified SBL gives four closely spaced bands on SDS-PAGE gels, indicating some heterogeneity among protomers. The basis for this heterogeneity is unknown, but may reflect differences in the covalently attached oligosaccharide. Each protomer reportedly carries a single N-linked oligosaccharide $(Mannose)_9 - (GalNAc)_2$, and an N-terminal signal peptide which is removed post-translationally.

Lectins and nodulation. Wolpert and Albersheim (1976) have shown that the lectins from five legumes bind specifically to the lipopolysaccharides borne by their respective symbionts. Similarly, Tsien and Schmidt (1980, 1981) and Bhuvaneswari et al. (1977) have found that soybean seed lectin binds to the extracellular

polysaccharides and lipopolysaccharides of Bradyrhizobium japonicum. This binding occurs in a growth phase-dependent manner, with the greatest number of SBL-binding cells occurring in late log phase (Bhuvaneswari et al. 1977). It should be noted that some Rhizobium strains seem not to bind host lectins and that the vagaries of lectin-binding assays cause many scientists to doubt the importance of lectins in nodulation.

Whether lectin binding to the cell surface is important or not, the surface polysaccharides of Rhizobium and Bradyrhizobium appear to play vital roles during interaction with the plant. Mutants which specifically lack exopolysaccharides (Leigh et al. 1985), periplasmic B-1,2 glucans (Geremia et al. 1987), or lipopolysaccharide O-antigens (Carlson et al. 1988), form ineffective (non N_2-fixing) nodules. Mutations affecting exopolysaccharides or lipopolysaccharides can also disturb the nodulation process (Dazzo et al. 1988, and Puvanesarajah et al. 1987). Caution must be exercised, however, in that many mutations which affect polysaccharide biosynthesis are pleiotropic. Therefore, the symbiotic phenotypes of such mutants may stem from changes other than alterations in polysaccharides.

B. japonicum and R. leguminosarum have both been reported to elaborate proteins which mediate attachment to roots of their host plants. Vesper and Bauer (1985) suggest that those B. japonicum cells which can adhere to plastic via hydrophobic interactions are also more infective on soybean roots. These hydrophobic interactions

are reportedly mediated by pili. An "adhesin" which mediates attachment to Vicia or pea root hairs has been purified from R. leguminosarum and characterized (Smit, Tubbing, Kijne and Lugtenberg, submitted; Smit, Logman, Boerrigter, Kijne, and Lugtenberg, submitted; et al. submitted; Smit, Kijne and Lugtenberg, submitted. This 14kd acidic protein required calcium ions to mediate anchoring to the bacterial cell surface, and has thus been called "rhicadhesin." Smit et al. indicate that rhicadhesin can competitively inhibit attachment of B. japonicum to root hairs, and that B. japonicum elaborates a similar adhesin. It will be interesting to see whether rhicadhesin can be shown to be an important prerequisite for nodulation.

A research group studying clover nodulation has postulated that seed and root lectins play important roles in nodulation (Kamberger 1979, Dazzo and Hubbell 1975a, Bohlool and Schmidt 1974). Work with R. trifolii suggests that the clover seed and root lectin, Trifoliin A, mediates attachment of this bacterium to clover root hairs (Dazzo and Hubbell 1975b). This binding occurs polarly (end-on), following apparent digestion of R. trifolii exopolysaccharides by host enzymes. Irreversible adhesion follows after several hours, and is thought to be mediated by bacterial cellulose microfibrils (Dazzo and Gardiol 1984). This work remains controversial, however, since it has not been possible to demonstrate whether lectin-mediated attachment to root hairs is an important prerequisite to infection and nodulation.

Numerous investigations of a possible role for soybean lectin

in binding B. japonicum to soybean roots have yielded negative as well as positive evidence (Stacey et al. 1984, Pueppke 1984, L. Halverson, personal communication). These investigations are complicated by the fact that only binding to incipient root hairs is relevant to nodulation, whereas the binding assays measure specific and nonspecific binding to the entire root. Curiously, addition of 10-30 mM galactose to soybean nutrient solution prior to inoculation seems to diminish nodulation, however, galactose also severely stunts the growth of soybean roots (unpublished results). Recent work in this laboratory indicates that B. japonicum's response to soybean lectin is a physiological change requiring de novo protein synthesis, rather than a simple binding phenomenon (Halverson and Stacey 1986c).

Work from our laboratory indicates that soybean seed lectin (SBL) serves as a signal to B. japonicum, making the bacterium competent to infect soybean roots (Halverson and Stacey 1984, 1985, 1986c). This work is based on the observation by Bhuvaneswari et al. (1980) that only the short segment of a growing soybean root which bears emerging root hairs is available for initiation of nodulation at any one time. Thus, bradyrhizobia which are applied to an emerging root will form nodules at the very top of the root if they are immediately "competent" to nodulate. If the bradyrhizobia must respond to root exudate for 6 hours to become competent, however, they will initiate nodules further down the root at a position coincident with 6 hours of root growth.

The induction of nodulation competence by SBL was first

demonstrated using 10^8 cells per plant of the slow-nodulating B. japonicum mutant called HS111. Since only the soybean root segment bearing incipient root hairs is receptive to infection at any one time (Bhuvaneswari et al. 1981), it follows that the 56 mm grown by soybean roots before nodule initiation by HS111 corresponds to a time delay of 56 hours (roots grow 1.0 mm/hour under these conditions). Upon pretreatment with soybean root exudate or SBL, HS111 cells were able to nodulate at the position of the root tip at the time of inoculation. Thus, bacteria pretreated with SBL were able to initiate the nodulation process almost immediately, whereas untreated bacteria required several hours (presumably to respond to SBL in the rhizosphere) prior to initiating nodulation.

An equal number of wild-type USDA110 cells (10^8/plant) can nodulate around the original root tip position without SBL pretreatment. An inoculum of 10^4 USDA110 cells, however, nodulated several centimeters below the original root tip, whereas 10^4 cells pretreated with soybean lectin could nodulate at the original root tip position. This result can be interpreted to mean that a small subpopulation ($<10^{-4}$) of the 10^8 B. japonicum cells are competent to immediately initiate nodulation. The ability to make the 10^4 cell inoculum competent by SBL pretreatment demonstrates that the wild-type USDA110 cells undergo the same physiological change as the nodulation-impaired HS111 mutant.

Further work using HS111 provided hints as to how SBL induces nodulation competence. A time course experiment established that approximately six hours of SBL treatment is required for induction.

Addition of the SBL hapten galactose prevented induction, indicating that SBL binding is necessary. Interestingly, other galactose and N-acetyl-galactosamine-specific lectins were unable to mimic induction. Thus, there must be another property unique to SBL which is necessary to stimulate nodulation by B. japonicum. Work with inhibitors of transcription or translation (Halverson and Stacey 1986b) indicated that SBL stimulates nodulation competence by inducing de novo protein synthesis in B. japonicum rather than simply by mediating physical attachment to roots.

Halverson and Stacey's (1984, 1986a, 1986b) experiments firmly establish a role for SBL in nodulation. This evidence can be reconciled with two fundamental theories of SBL function. The simplest possibility is that SBL serves as a positive signal, initiating a positive change in B. japonicum that makes it more efficient for nodulation. It has often been suggested, however, that lectins may recognize invading pathogens by binding to their surfaces and triggering a defensive response by the plant host (Keen 1982). If this is the case, it is possible that B. japonicum has simply developed an inducible response for evading detection by SBL, either by inactivating SBL or by masking binding sites on the bacterial surface.

Despite this evidence, the lectin recognition hypothesis remains highly controversial. Proof that lectins play an important role in Rhizobium-legume symbioses will require the identification of

lectin-inducible genes and/or elucidation of pertinent physiological changes in lectin-treated rhizobia.

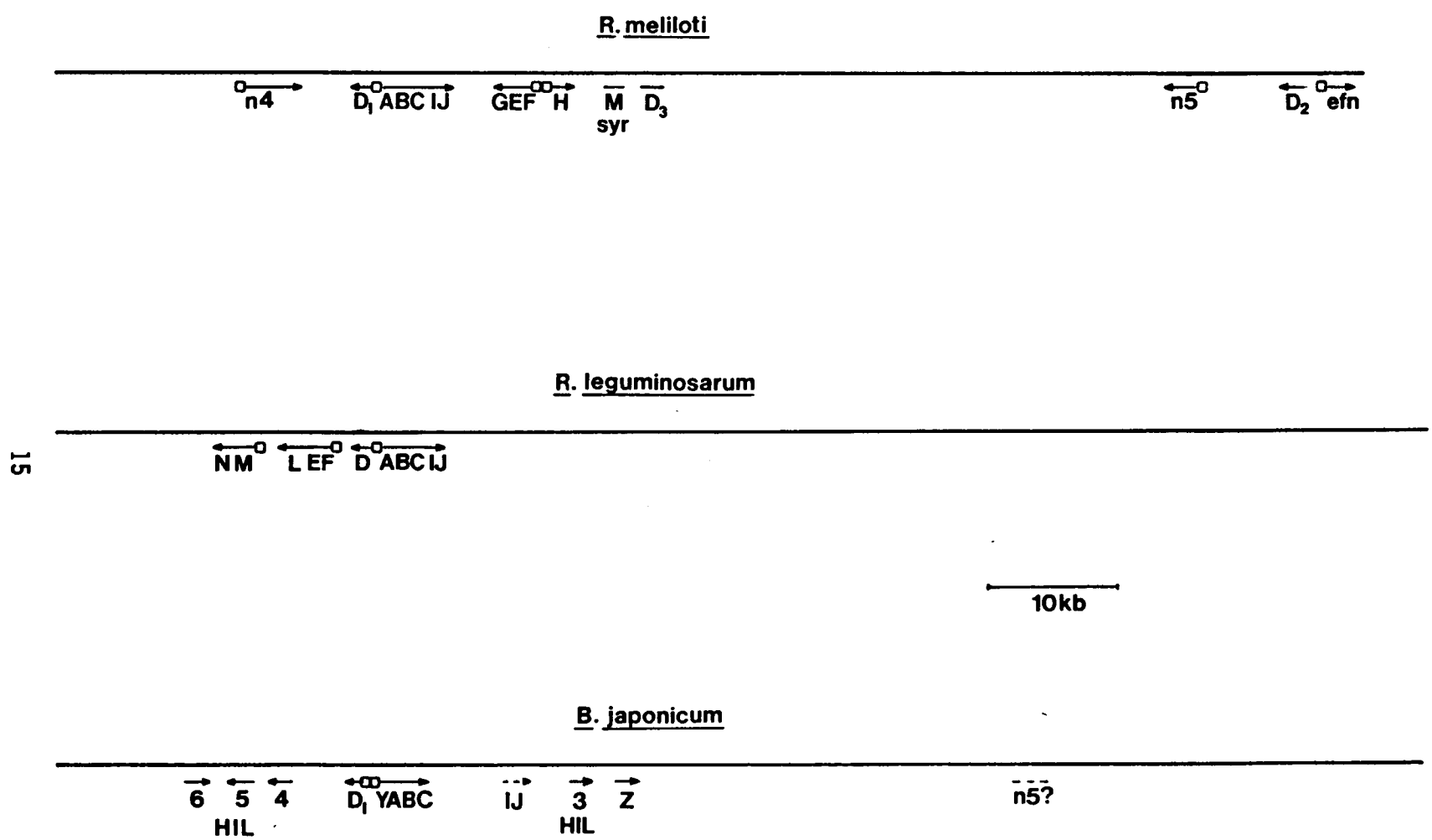
Genetics of Nodulation

Common nodulation genes. The study of legume nodulation has focused recently on genetic manipulations of Rhizobium species in order to identify those bacterial genes which participate in the infection process. Although the B. japonicum soybean symbiosis is the most important economically, study of nodulation molecular genetics was pioneered with fast growing species; therefore, more is known about R. meliloti, R. leguminosarum, and R. trifolii than Bradyrhizobium. The conservation of gene functions between species makes it convenient to consider a common legume-rhizobia system, and to point out differences in nodulation genes where they are known. Approximately 10-12 nodulation genes have been identified in well-studied Rhizobium species; these are called nodA-J (Figure 1).

NodABC and D genes are common to all Rhizobium and Bradyrhizobium species examined, sharing both DNA sequence homology and functionality (Innes et al. 1985, Quinones et al. 1986). Thus nodABC and D are referred to as "common nod" or hac (for root hair curling) genes (Mulligan and Long 1985). These genes are necessary for nodulation of any legume, and their transfer into Agrobacterium tumefaciens was sufficient to allow inefficient nodulation of legumes by this plant pathogen (Banfalvi et al. 1983; Putnoky and Kondorosi 1986).

Although the biochemical functions of the nodABC gene products

Figure 1. Arrangement of nodulation genes in rhizobia. Arrows indicate the direction of transcription of each operon and boxes indicate known nod box promoters. Nod gene letter designations are indicated. Other loci are designated: n4 and n5 for "nod box loci", syr for "symbiotic regions," efn for "efficiency of nodulation" region, and HIL for "host-inducible loci."


R. meliloti
n4
D1 ABC IJ
GEF H
M
syr
D3
n5
D2 efn
R. leguminosarum
NM
L EF
D ABC IJ
10kb
B. japonicum
6
5
4
HIL
D1 YABC
IJ
3
HIL
Z
n5?

are unknown, some clues are available. NodC seems to encode a membrane protein (John et al. 1988). It has been suggested that the nodABC products make a plant hormone responsible for root hair curling and nodule initiation (Lugtenburg 1988). B. parasponiae and B. japonicum each carry an additional gene just upstream of nodA (nodK and nodY, respectively). No functions are as yet ascribed to either gene, however, nodY is cotranscribed with nodABC (Banfalvi et al. 1988) and produces a ~16Kd protein (Nieuwkoop et al. 1987).

R. trifolii, R. leguminosarum, R. meliloti, and Bradyrhizobium japonicum carry nodulation genes downstream of nodABC designated nodI and nodJ (Figure 1), which show homology between species (Downie and Johnson 1986, our unpublished results). NodI and nodJ are probably cotranscribed with nodABC in fast-growing rhizobia, but are seperated from nodYABC in B. japonicum by ~ 3 kb (Nieuwkoop et al. 1987). Mutations in nodIJ are believed to delay nodulation by resulting in abortion of growing infection threads (Innes et al. 1985). NodI shows sequence homology with several E. coli ATP-binding proteins involved in active transport, however, the functions of nodIJ are unknown.

Host-specific nodulation genes. Nodulation genes which are necessary or important for one host plant, but not for another are termed hsn genes. Such genes were first found in R. meliloti as operons nodFEG and nodH, respectively, (Horvath et al. 1986) (Figure 1). Mutations in these genes impaired alfafa (Medicago sativa) nodulation, but not sweetclover (Melilotus alba) nodulation. A clue

to nodF function comes from sequence comparisons, which suggest the gene product is an acyl-carrier protein with the pantothenate binding site found also in the E. coli and barley proteins (Shearman et al. 1986).

Nodulation genes homologous to nodFE are present in R. leguminosarum biovars 'trifolii' and 'Viciae', and R. species NGR234, however these genes have apparently adapted for invasion of specific hosts as they are not functionally interchangeable (Horvath et al. 1986, Downie and Johnston 1986, Bachem et al. 1986). A Tn5 insertion into nodFE of R. leguminosarum 'trifolii' abolishes its ability to nodulate white clovers (Trifolium repens), while still allowing it to nodulate subclover (Trifolium subterraneum), and extending its host range to include a new host, peas (Pisum sativum--Innes et al. 1985). Host specificity hsn genes from the pea symbiont R. leguminosarum 'Viciae' can confer the ability to nodulate peas on the bean symbiont R. luguminosarum 'phaseoli' (Hombrecher et al. 1984).

Rhizobium species NGR234 has an exceptionally broad host range and reportedly carries three clusters of host specificity genes. HsnI allows nodulation of soybeans, siratro, and the tropical legume Psophocarpus tetrogonolobus (winged bean), by virtue of a "promiscuous" nodD gene and possibly several closely linked genes (Lewin et al. 1987, Horvath et al. 1987). Hsn regions II and III are each able to confer nodulation of Vigna unguiculata (cowpea) on R. meliloti. Interestingly, Hsn region III can complement a R. meliloti nodH mutant (weakly) suggesting that these two loci are functionally related (Lewin et al. 1987).

Host specificity in Bradyrhizobium japonicum is determined in part by the "siratro host specificity," or nodZ gene which is homologous to the region I locus of R. sp. NGR234. Mutations in this locus abolish USDA110's ability to nodulate siratro but soybean nodulation is unaffected (Nieuwkoop et al. 1987). The homologous region I in R. sp. NGR234 also appears to facilitate nodulation of siratro, however its function has not been well characterized (Bassam et al. 1986). Another region operationally designated HIL6 is necessary for nodulation of American cultivars of the commercial soybean (Glycine max), cowpea (Vigna unguicalata), and pigeon pea (Cajanus cajun Deshmane, et al. submitted). A mutation in HIL6 does not, however, block nodulation of the ancestral soybean (Glycine soja), and nodulation of siratro (Macroptilium atropurpureum) and a Chinese soybean cultivar (Glycine max cv. 'Peking') are only delayed in nodulation. Two more loci have been identified just downstream of HIL6, and provisionally designated HIL5 and HIL4 (Figure 1). Mutations in HIL5 or HIL4 delay Glycine max nodulation, but have not been examined on other hosts. The HIL4 gene/s appear to confer a competitive advantage during nodulation of a particular soybean genotype (see Discussion). By analogy to plant pathogens, host specificity genes can act as virulence genes (by extending host range), and as avirulence genes (by restricting host range), simultaneously. Although it has been postulated that hsn genes affect recognition by the plant by altering the bacterial cell surface, their specific functions remain a mystery.

Regulation of nodulation genes. The R. meliloti operons nodABC(IJ), nodFEG, and nodH are transcriptionally induced by flavonoid compounds excreted by legume roots (Mulligan and Long 1985), Kondorosi, et al. submitted). A conserved 60-79bp sequence called the "nod box" occurs about 200bp upstream of each of these operons (Rostas et al. 1986). Deletion of part of this sequence from either end destroys inducibility of the downstream operon (Rostas et al. 1986, Spaink et al. 1987), indicating that the nod box promoter functions in regulation. The correlation between this sequence and the (unidirectional) inducibility of associated genes indicates that the nod box operates polarly. The nodABC and hsn genes from other Rhizobium and Bradyrhizobium species have also been shown to be flavonoid inducible (Innes et al. 1985, Rossen et al. 1985, Kosslak et al. 1988) except for the siratro hsn gene of B. japonicum which apparently lacks a nod box and is regulated differently (M. Schell, unpublished).

The induction of nod genes requires a functional nodD gene product (Mulligan and Long 1985) and may require other proteins as well. It is known that the nodD gene product binds to the nod box, and the current hypothesis suggests that the flavonoids bind to the nodD product to facilitate transcription of the downstream operon (Fisher et al. 1988). The products of different nodD genes exhibit specificity for flavonoids/ isoflavonoids particular to different host plants, thus contributing to host specificity (Horvath et al 1987). Thus, a nodA-lacZ fusion in NGR234 is inducible 40-50 fold by

siratro root exudate or cumestrin and somewhat less by alfalfa root exudate or luteolin (see appendix for structures). The same fusion in R. meliloti is induced maximally by luteolin or alfalfa root exudate and does not respond at all to cumestrin or siratro root exudate (Horvath et al. 1987).

It has also become apparent that some flavonoids can inhibit induction of nod genes by other flavonoids (Firmin et al. 1986). It is not known whether this inhibition occurs at the level of uptake or recognition by nodD, however the passive nature of flavonoid uptake (Recourt et al. 1988) suggests the latter. All of the known nodulation genes in a given Rhizobium/Bradyrhizobium strain respond to the same flavonoid inducer, with 4',7'dihydroxyflavone being optimal for R. leguminosarum 'trifolii', naringenin for R. leguminosarum 'Viciae', and genistein for B. japonicum (Redmond et al. 1986, Firmin et al. 1986, Kosslak et al. 1988, and Banfalvi et al. 1988).

Transfer of the broad host range nodD from NGR234 to R. meliloti not only allows induction of nodA by siratro root exudate, it also allows this strain to nodulate siratro, indicating that the flavonoid-specificity of nodD genes determines, in part, the bacterium's host range for nodulation (Horvath et al. 1987).

Despite the common function of nodD genes among rhizobia, different species regulate their nodD genes in diverse ways. R. meliloti strain AK631 bears three nodD genes with varying flavonoid specificities which accommodate the host plants alfalfa, sweetclover, and fenugreek (Trigonella foenum-graecum), which is ineffectively

nodulated (no N_2 fixation). These three nodD genes are apparently constitutively expressed. R. leguminosarum 'Viceae' carries only a single nodD gene which is autoregulatory (Firmin et al 1986). Bradyrhizobium japonicum reportedly carries several nodD copies (H. Hennecke, personal communication). The $nodD_1$ gene opposite nodYABC is genistein-inducible in B. japonicum, and mutations in $nodD_1$ abolish induction of nodYABC and HIL6 (Banfalvi et al., in press; Deshamane, and Stacey, submitted). Thus, the functional significance of other nodD-homologies in B. japonicum is still unresolved.

Clearly, host-induction of the known nodulation genes requires at least nodD, a flavonoid compatible with that nodD, and a nod box promoter. In R. meliloti strain AK631 six nod box sequences were detected by hybridization with a synthetic nod box oligomer (Rostas et al. 1986). The first three, n1, n2 and n3, are associated with nodABC(IJ), nodFEG, and nodH, respectively, and n6 is present in an ill-defined "efficiency of nodulation" (efn) region which includes $nodD_2$. Nod boxes n4 and n5 have no known function and are reportedly unnecessary for nodulation of alfalfa (Putnoky et al. 1986). Since these two highly conserved nod boxes are surrounded by sequences which are divergent, it seems likely that n4 and n5 are not pseudogenes or duplicated genes but may have "cryptic" functions.

CHAPTER 3

MATERIALS AND METHODS

Microbiology

Growth of bacteria. Escherichia coli was grown on LB medium (Luvia Broth) at 37°C. R. meliloti and R. species NGR234 were grown on TY medium or GTS defined medium at 30°C (Kondorosi et al. 1984). B. japonicum was cultured at 30°C on YEM (Wacek and Brill 1976), RDM (Bishop et al. 1976), YS (RDM sans gluconate and glutamate), and MM defined medium (Mort and Bauer 1982). Media chemicals were from Sigma Chemical Co., St. Louis, MO, except for tryptone and yeast extract (Difco Laboratories, Detroit, MI).

Antibiotics were used in the following concentrations (micrograms/milliliter) for E. coli: ampicillin 75, kanamycin 50, tetracycline 25, chloramphenicol 15, gentamycin 60, streptomycin 50, spectinomycin 100, trimethoprim 60; for Rhizobium: kanamycin 250, tetracycline 25, chloramphenicol 30, gentamycin 150, streptomycin 100, spectinomycin 150; for Bradyrhizobium: kanamycin 150, tetracycline 150, streptomycin 100.

Bacterial strains plasmids and bacteriophages. The plasmids, bacterial strains and bacteriophages used in these studies are listed in Table 1.

Plant nodulation tests. Alfalfa (Medicago sativa 'buffalo') or sweetclover (Melilotus alba 'white blossom') seeds were surface sterilized in 95% ethanol for 30 seconds, followed by 0.1% $HgCl_2$ for

Table 1. Bacterial Strains, Plasmids, and Bacteriophages

Strain	Relevant Characteristics	Source or Reference
E. coli		
HB101	*thr*, *leu*, *thi*, *hsdR*$^-$, *hsdM*$^-$ *lacZ4*, *recA*, *rpsL*	Davis et al. 1980
JM101	*hsdR*$^-$, thi, *rpsL*, *endA*, *sbcB*, *lac* proAB/F', *traD*, proAB *lacI*$^\alpha$	Yanisch-Perron et al. 1985
SF800	*polA*$^-$, *nal*^{R}	Stachel et al. 1985
M8820	*araD*, *rpsL*, (proAB, *argF*-*lacIPOZYA*) XIII	Casadaban 1975
MC4100	*araD*, (*araF*-*lac*) U169, *rpsL*, *relA*, *fla*	Casadaban 1975
R. meliloti		
AK631	wild-type, nod$^+$, fix$^+$	A. Kondorosi
Rm1021	wild-type, nod$^+$, fix$^+$	S. Long
n4-53	Tn5 homogenote, nod^D	This study
n4-54	Tn5 homogenote, nod$^+$	This study
n4-81	Tn5 homogenote, nod^D	This study
n4-84	Tn5 homogenote, nod^D	This study
n4-90	Tn5 homogenote, nod^D	This study
n4-9	Tn5 homogenote, nod$^+$	This study
n4-56	Tn5 homogenote, nod^D	This study
n4-64	Tn5 homogenote, nod^D	This study
n4-68	Tn5 homogenote, nod^D	This study
B. japonicum USDA110	Wild-type, nod$^+$, fix$^+$	H. Keyser
DG59	USDA110(pR32-HIL4::Tn3-*lac*)	This study

Strain	Relevant Characteristics	Source or Reference
DG62	USDA110(pR32-HIL4::Tn3-*lac*)	This study
DG63	as DG62, sibling?	This study
DG69	USDA110(pR32-HIL3::Tn3-*lac*)	N. Deshmane
NAD27	USDA110 HIL4::Tn*5*	N. Deshmane
R. species MPIK3030	Wild-type, nod^+, fix^+	A. Kondorosi
R. trifolii ANU843	Wild-type, nod^+, fix^+	B. Rolfe
R. leguminosarum 697	Wild-type, nod^+, fix^+	N. Brewin
R. fredii USDA193	Wild-type, nod^+, fix^+	A. Atherly
Agrobacterium tumefaciens A6	Wild-type, nod^+, fix^+	E. Nester
Plasmids		
pRK2073	tra^+ (RK2:ColE1), tmp^R	Ditta et al. 1980
pSF6	Str^R, Sp^R, mob^+, WHR	Selvaraj et al. 1984
pPR33	Tc^R, Mob^+, WHR	P. Ratet
pVK100	Tc^R, Km^R, WHR	Knauf & Nester 1982
pDG52	pSF6::5.2kb *Pst* I fragment bearing n5	This study
pDG60	pPR33::6.0kb *Kpn* I fragment bearing n4	This study
pDG100	pVK100::10kb *Hin*dIII fragment bearing n5 region	This study
pPP369	Cosmid clone from *R. meliloti* including n4 region	Putnoky & Kondorosi 1986

Strain	Relevant Characteristics	Source or Reference
pDG60::n4-351	Plant-inducible MudII PR13 n4-*lac* fusion	This study
pDG60::n4-584	Plant-inducible MudII PR13 n4-*lac* fusion	This study
pDG52::n5-132	Plant-inducible MudII PR13 n5-*lac* fusion	This study
pDG52::n5-597	Plant-inducible MudII PR13 n5-*lac* fusion	This study
pR32	Cosmid clone from *B. japonicum* including common *nod* genes	Russel et al. 1985
pDLG83	pR32::Tn3-*lac* in HIL4 region	This study
pDLG87	pR32::Tn3-*lac* in HIL4 region	This study
pDLG99	pR32::Tn3-*lac* in HIL3 region	This study
pHoHo1	Tn3::*lac*'ZYA, $tnpA^-$, Ap^R	Stachel et al. 1985
pSShe	Cm^R, *tnpA*$^+$	Stachel et al. 1985
Bacteriophages		
P1 cm, clr^{100}	Cm^R, temperature-sensitive repressor	
P1::Tn*5*	Tn^5, temperature-sensitive repressor	
Mu+	Wild-type phage/transposon	Ratet et al. 1988
Mu *cts*	Heat inducible *c* repressor gene-mutant of Mu+	Ratet et al. 1988
MudII PR13	Translational *lac*-fusion vehicle, Cm^R	Ratet et al. 1988

5 minutes. Following 6 washes in sterile dH_2O, seeds were allowed to imbibe in ~ 3 volumes of dH_2O overnight, treated for 3 minutes in 20% chlorox, and germinated on sterile filter paper-lined petri dishes following 5 more sterile dH_2O washes. One to two day-old seedlings were placed onto 6 ml 0.8% agar slants of Gibson's medium (Kondorosi et al. 1977) in 17 x 160 mm test tubes, and innoculated with ~ 10^7 R. meliloti cells/tube. Nodule numbers were counted every other day. Wild-type and mutant nodule numbers were compared using Student's t-test, or (if a large proportion of plants were unnodulated) by chi-square analysis of the number of plants with above and below the mean number of nodules.

Protein Analyses

Protein sample preparation. B. japonicum cultures (OD_{600} = 0.1-0.2) in YEM medium were treated for 16 hours with genistein 2 μM, SBL 10 μg/ml, genistein and SBL, or neither as controls. These cells were simultaneously labelled with 15 μCi/ml [^{35}S]-cysteine/methionine, harvested by centrifugation, and resuspended in 1/10 volume TES buffer (Tris-Cl 20 mM pH8, EDTA 5mM, Sucrose 5% w/v) plus 250 μg/ml lysozyme. After 30 minutes on ice, cells were broken by French press (16,000 psi, twice), or sonication (20 seconds at 40 mA, four times on ice). Resultant extracts were adjusted to 10 mM $MgCl_2$, treated for 20 minutes with 50 μg/ml DNAse + RNAse H, and boiled in SDS-Triton X100 sample buffer (Ames and Nikaido 1976) for 3 minutes.

Protein electrophoresis. Samples were mixed with urea, (550

mg/ml) loaded onto prerun isoelectric focusing (IEF) tube gels, and run overnight at 1,000 volts. IEF gels were then extruded into equilibration buffer and loaded onto denaturing electrophoresis gels (SDS-PAGE) as described (O'Farrell 1975, Duncan and Hershey 1984). SDS-PAGE gels were run for 5 hours at 35 mA per gel, impregnated with EN^3Hance^{TM} (New England Nuclear Corp.), dried under vacuum, and exposed to film (Kodak AR). Gel Fluorographs were compared by superimposing them over a light box. Proteins responding to soybean lectin or genistein were confirmed by comparing fluorograms from several independent samples.

β-Galactosidase assays. Rhizobia were grown to an OD_{600} of 0.1-0.2 in GTS (R. meliloti or R. species MPIK3030) or RDM (B. japonicum) at 30°C prior to induction. Flavonoids in ethanol were added to sterile test tubes and the solvent was allowed to evaporate. Two milliliters of culture was added, and each tube was shaken for 16-18 hours at 30°C. 0.5 ml of culture was added to 0.5 ml of Z-buffer (Miller 1972), followed by 1 drop of 0.1% SDS and 2 drops of chloroform. Samples were vortexed for 10 seconds and 0.2 ml of 3 mg/ml chlorophenol-red-β-galactoside (CPRG) substrate was added. The reactions were stopped after 5-60 minutes of color development by addition of 0.25 ml of 2M sodium carbonate, and pelleting of cells for 30 seconds at 15,000 g. Assays were read in a spectrophotometer at 574 nm and adjusted for reaction time (Miller 1972), and the 8-fold increased sensitivity of CPRG over ortho-dinitrophenyl-β galactoside (ONPG).

Genetics

DNA cloning and manipulations. DNA samples were digested with restriction endonucleases according to manufacturer's instructions. Agarose gel electrophoresis was performed in TAE buffer (Maniatis et al. 1982) plus 0.5 μg/ml ethidium bromide, and visualized on an ultraviolet light box (Fotodyne Foto System, New Berlin, WI). Photographs were taken using Polaroid 667 high speed positive film or type 55 positive/negative film. Ligations were performed using T4 DNA ligase and alkaline phosphatase from Bethesda Research Laboratories.

Transformation. One hundred ml of E. coli cells were grown to an OD_{550} of 0.2-0.3 in LB at 37°C and harvested by centrifugation at 5,000g. Cells were resuspended in 20 ml of 0.1M $CaCl_2$ at 4°C and after 20 minutes were pelleted, and resuspended in 1 ml of 0.1M $CaCl_2$. After incubation on ice for 24 hours, cells were used for transformation, or adjusted to 16% glycerol and frozen at -70°C. Approximately 50 ng of DNA was added to 0.1 ml competent cells for 15-30 minutes on ice. These cells were heat shocked at 42°C for 3 minutes and shaken with 1 ml of LB for 60 minutes at 37°C prior to plating on selective medium.

Plasmid DNA isolation. Plasmid DNA was isolated by the alkaline lysis method (Maniatis 1982). Three ml of cells were pelleted at 15,000g for 2 minutes in a 1.5 ml Eppendorf tube. Rhizobia were then resuspended by violent vortexing in TNE buffer (10

mM Tris-Cl pH 8.0, 1mM EDTA, 100mM NaCl) and recentrifuged, to remove exopolysaccharides. Pellets were resuspended in 0.1 ml of TEG buffer (25mM Tris-Cl pH 8.0, 10mM EDTA, 50mM Glucose) plus 4 mg/ml lysozyme. After 5 minutes at 20°C, 0.2 ml of 0.2N NaOH, 1% SDS was added and mixed in by inversion. Following 5 minutes on ice, 0.15 ml of ice cold potassium acetate solution (3M potassium acetate, 2M acetic acid pH = 4.8) was added with gentle vortexing. After 5 minutes on ice, chromosomal DNA and cell debris were pelleted for 5 minutes at 15,000g. The supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then twice with an equal volume of chloroform. Plasmid DNA was precipitated using 2 volumes of ethanol, and the pellet was rinsed with 70% ethanol and dried under vacuum. Pellets were resuspended in 0.05 ml of 1/10 TE (1mM Tris-Cl pH 8.0, 100nM EDTA) plus 50 μg/ml bovine pancreatic RNAse (BRL).

Large scale plasmid purification was achieved by scaling-up the alkaline lysis procedure for use on one liter of cell culture. Following ethanol precipitation the pellet was redissolved in 25 ml TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA). Twenty-five grams of CsCl were dissolved in the DNA solution and 2 ml of a 10 mg/ml ethidium bromide stock solution was added. This solution was ultracentrifuged at 45,000 rpm in a Beckman Ti60 rotor for 48 hours at 20°C. The plasmid DNA was visualized with a long-wavelength ultraviolet lamp, and removed using a 10 ml syringe with 18-gauge needle. Ethidium was removed by 6 successive butanol extractions, and the solution was diluted with 2 volumes water, and ethanol precipitated.

Genomic DNA isolation. Genomic DNA was isolated from 3 ml of early stationary bacterial cultures as follows. Bacteria were pelleted, resuspended in 1 ml TNE buffer (see above) + 0.1% sarcosyl by vigorous vortexing, and pelleted, to remove exopolysaccharides. Cells were resuspended in 0.5 ml TE buffer + 2% SDS and 0.5 mg/ml proteinase K and held at 37°C for one hour. This lysate was sheared 5-10 times using a Pasteur pipette and extracted twice with phenol/chloroform and twice with chloroform. The remaining solution was adjusted to 0.25M with sodium acetate pH 5.2, and precipitated with 2 volumes of ethanol.

Bacterial conjugations. Conjugal transfer of plasmids from E. coli to other E. coli strains or rhizobia was accomplished using triparental matings as described (Ditta et al. 1980). 0.1 ml of overnight donor and helper strain (pRK2073) cultures were mixed with 0.2 ml early stationary phase recipient cultures and the cells were collected on a 0.45 μM filter (Millipore). The filter was placed on YS medium for 2-4 days, and the resulting growth was resuspended in YS salts + 0.1% Tween-20. The mixture was plated on RDY with selection for the plasmid, plus 30 μg/ml chloramphenicol to counterselect E. coli (matings into B. japonicum). For mating plasmids into Rhizobium species, GTS minimal medium was used for plating out the mating mixture to counterselect auxotrophic E. coli strains. When pR32::Tn3-lac fusions were mated into E. coli SF800, the transconjugants were plated on LB containing naladixie acid, tetracycline, and ampicillin to select for the recipient strain, the

plasmid, and the transposon, respectively. Donor, helpers, and recipient strains were plated separately on selective media as controls, and selective plates were grown for 1 day (E. coli), 3 days (R. meliloti), 4 days (R. species MPlK3030), or 13 days (B. japonicum).

Mini-Mud-lac mutagenesis. Mud-lac PR13 mutagenesis was performed as described by Castilho et al. (1984). The target plasmid was transformed into MC4100/Mucts/Mud-lac IIPR13 using a 33°C heat shock to preserve the temperature sensitive "helper" Mucts. This transformant was grown to 0.2-0.5 OD_{600}, adjusted to 2.5mM $MgSO_4$, and heat shocked at 44°C for 25 min. After 30-120 min. shaking at 37°, cell lysis occurred. Resulting phage stocks were sterilized by adding chloroform to 1% and vortexing, followed by centrifuging 30 seconds at 15,000g.

A 0.1 ml aliquot of the resulting phage stock was added to 0.2 ml of M8820/Mu^+ cells in LB, 2.5mM $MgSO_4$, 1mM $CaCl_2$, and adsorption was allowed to occur for 20 min. at 20°C. After addition of 2 mL LB the transductants were shaken for 75-120 min. at 30°C to allow gene expression to occur. M8820/Mu^+ (target plasmid::Mud-lac PR13) constructs were then selected by plating on LB + chloramphenicol.

Transplacement/homogenotization. Following conjugation of plasmid::Tn5 constructs from E. coli into R. meliloti AK631, Tn5 mutations were recombined into the homologous site in the symbiotic megaplasmid by double homologous recombination. This transplacement,

or homogenotization, event was selected by mating pPH1 into the AK631 (plasmid::Tn5) strain and selecting for Tn5 and pPH1. The loss of the original plasmid occurs because it is incompatible with pPH1 (IncP). The integrity of each transplacement was verified by probing EcoRI digests of putative homogenote genomic DNA with the original target plasmid as described below.

DNA blotting and hybridizations. Restriction enzyme digests of DNA were resolved by electrophoresis through 1% agarose in 40mM Tris-acetate buffer plus 1mM EDTA pH 8.0. Gels were soaked for 1 hour in 0.5N NaOH plus 1.5M NaCl to denature DNA, and neutralized in 1M Tris-Cl plus 1.5M NaCl pH 8.0 for 1 hour. DNA was transferred onto Hybond-N paper (Amersham Corp.) overnight using 10 x SSC (1.5 M NaCl, 0.3 M Na-citrate) as transfer buffer, and immobilized by exposure to ultraviolet light for 3-5 minutes.

DNA filters were prehybridized for 4-16 hours at 65^{o}C in 6x SSC, 0.5% SDS, 0.2% Ficoll, 0.2% polyvinyl pyrrolidone, 0.2% bovine serum albumin, 50 µg/ml denatured salmon sperm DNA. Probe DNA was ^{32}P-labelled by nick translation using ~ 100 micro Curies ^{32}P-CTP as recommended by the manufacturer (New England Nuclear, Boston, MA).

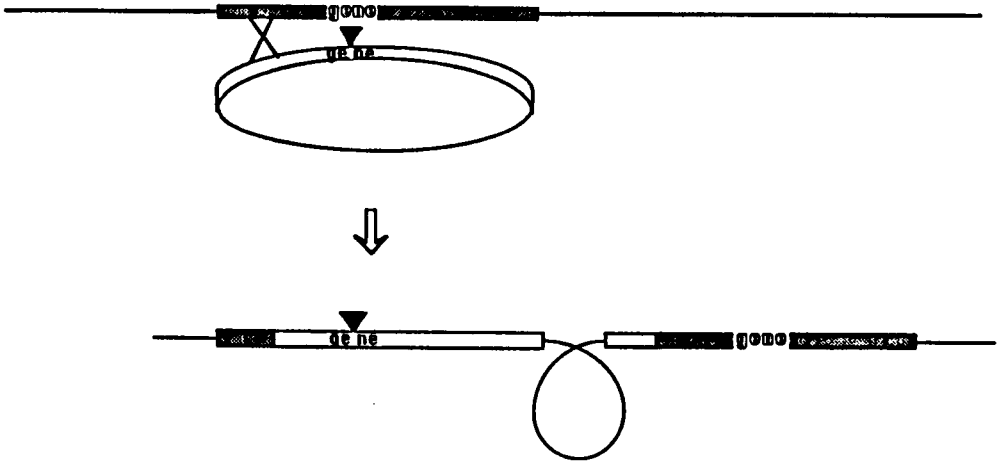
The ^{32}P-probe DNA was boiled for 3 minutes, added to the filter in prehybridization solution, and allowed to hybridize for 12-18 hours shaking at 65^{o}C. Filters were washed at 65^{o}C for 3-4 hours in 1x SSC, 0.5% SDS (high stringency), or 3x SSC, 0.5% SDS (low stringency). Filters were exposed to Kodak X-OMat AR film at -70^{o}C with intensifying screens (Southern 1975).

Tn5 mutagenesis. E. coli HB101 containing the plasmid of interest was lysogenized with phage P1 Cm^R, by incubating 0.1 ml of phage with 0.1 ml bacteria for 30 minutes at 30°C and selecting for the phage and plasmid on plates overnight at 30°C. These lysogens were then superinfected at a 1-10 multiplicity of infection with P1 clr^{100}::Tn5 (Quinto and Bender 1984). After 30-60 minutes in LB + 5mM calcium chloride at 30°C, cells were spread onto plates containing choramphenicol, and kanamycin. Since only about 2% of the resulting colonies carried Tn5 in the plasmid, plasmid::Tn5 constructs were selected by conjugation into R. meliloti AK631. Tn5 was then selected for recombination into the AK631 genome (see Figure 2) by mating incompatible plasmid pPH1JI into the R. meliloti (plasmid::Tn5) strains, and selecting for pPH1JI, and Tn5. These putative "homogenotes" were confirmed by probing genomic digests with the ^{32}P-labeled plasmid.

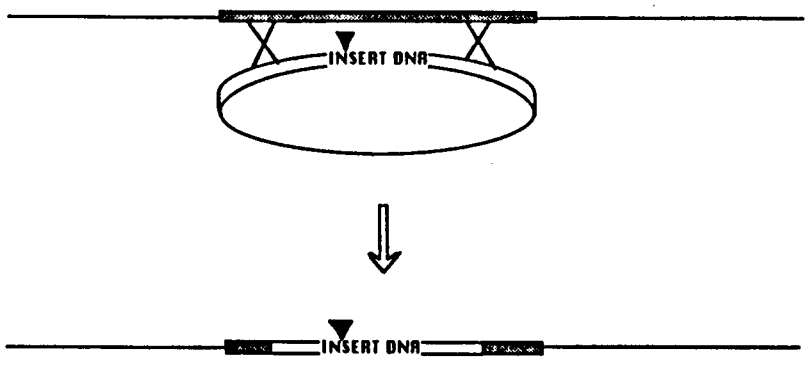
Tn3-lac mutagenesis. Tn3-lac (Tn3-HoHo1) mutagenesis was performed according to Stachel et al. 1985. Target plasmid pR32 was transformed into HB101 (pHoHo1, pSShe) and the transformant was mated with SF800 polA, nal^R. The defective transposase gene on Tn3-lac (pHoHo1) was complemented by pSShe TnpA to allow transposition. Tn3-lac insertions into pR32 were selected following the mating by selecting for the plasmid, transposon, and SF800 recipient strain (Tc^R, Ap^R, and Nal^R, respectively). The resulting pR32::Tn3-lac constructs were restriction mapped and transferred into B. japonicum

Figure 2. Cointegrate formation (A) and homogenote formation (B). A single crossover cointegrates the plasmid into the chromosome (A) whereas two corssovers flanking the mutation (triangle, B) replaces the chromosomal gene with the mutant gene.

A



B



USDA110 by conjugation. These strains were tested for induction by soybean root exudate, flavonoids, and soybean lectin, using β-galactosidase assays.

CHAPTER 4

RESULTS

Identification of B. japonicum Proteins Induced by Host Signals

In order to clarify the role of the plant in regulating nodulation genes in rhizobia, three approaches will be taken to identify new symbiotic genes. By studying how these genes are regulated and how mutational inactivation of each gene affects nodulation, our knowledge of communication between plant and symbiont can be increased.

To clarify the response of B. japonicum to host signals USDA110 will be treated with SBL or genistein, its proteins will be separated by two-dimensional electrophoresis, and proteins responding to these signal molecules will be identified by comparing gels from treated and untreated cells. Identification of host-inducible proteins may lead to isolation of host-inducible genes by molecular cloning techniques.

The common nodulation DNA region of B. japonicum will be directly searched for host-inducible genes using reporter gene fusions. By randomly inserting the promoterless lacZ gene into pR32, and assaying resulting constructs for soybean root exudate-inducible β-galactosidase activity, the DNA surrounding nodYABC will be examined for host-inducible genes.

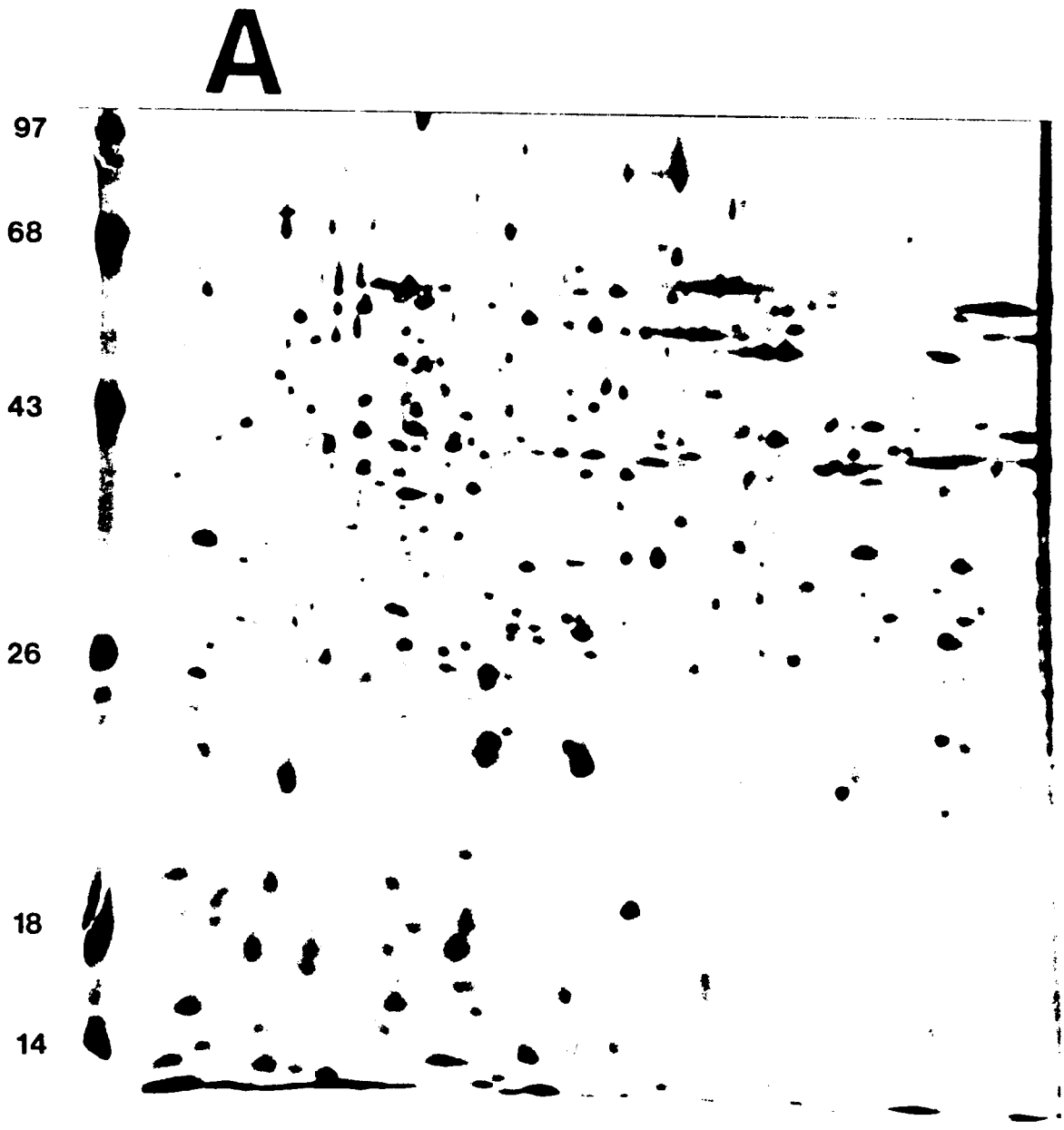
A genetic approach will also be taken to find symbiotic genes in R. meliloti using a promoter-specific oligonucleotide probe

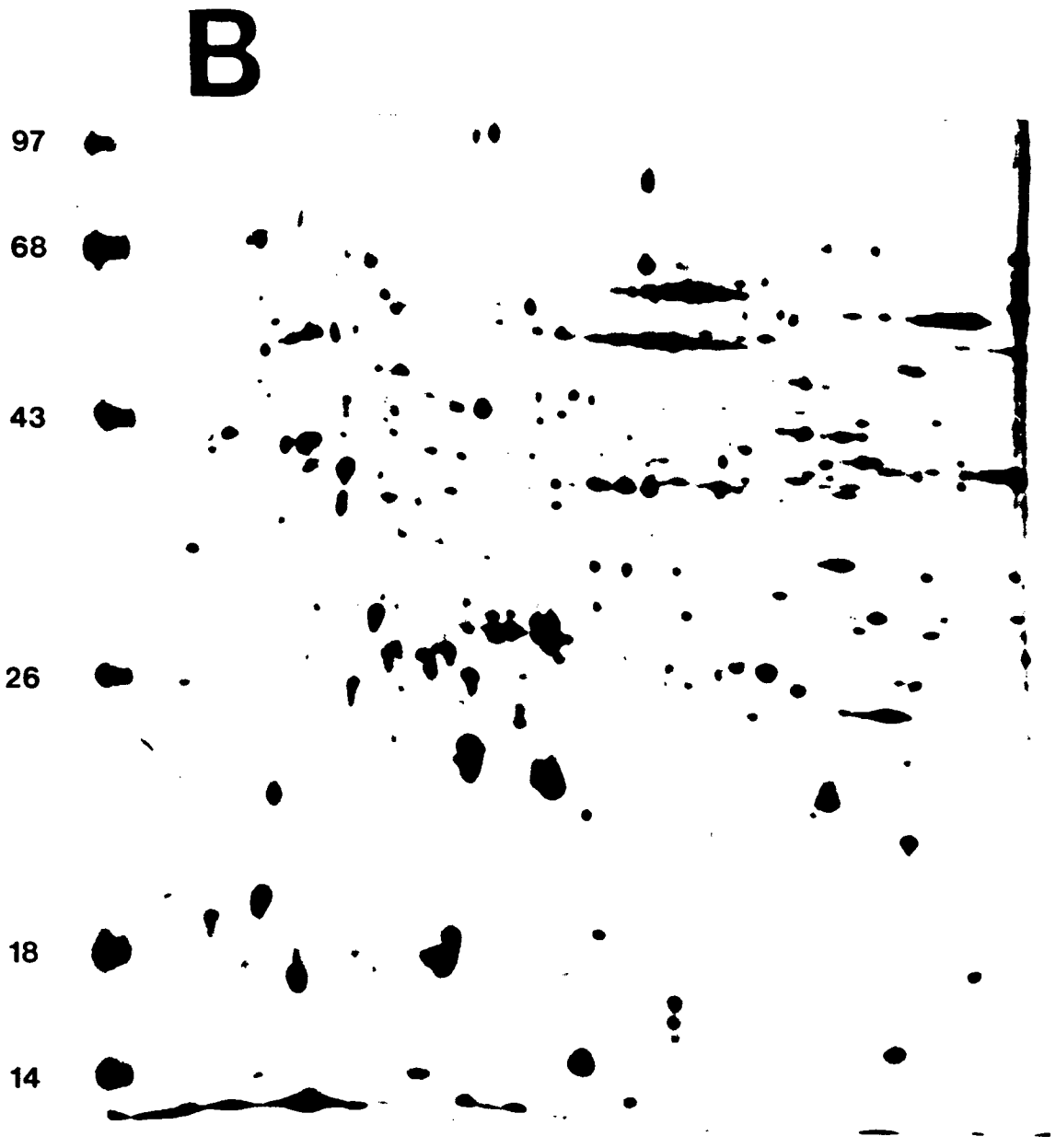
complementary to the nod box sequence. Two uncharacterized nod box loci will be examined for the presence of associated host-inducible operons by fusing these nod boxes to lacZ and testing for host induction of β-galactosidase. The functions of these nod box loci will be evaluated by testing Tn5 insertion mutants on alfalfa and sweet clover plants. By identifying new nodulation genes and characterizing them with respect to regulation and function our understanding of the nodulation process can be improved.

Since several soybean root-derived molecules can serve as signals to promote nodulation, B. japonicum USDA110 cells were treated with soybean root exudate and analyzed electrophoretically to identify induced proteins. This study revealed the complexity of B. japonicum's response to host root exudate, and suggested use of purified SBL and genistein to help define responses to these molecules.

Root exudate-induced proteins. In early experiments USDA110 cells were treated with SBL or root exudate in plant nutrient solution according to Halverson and Stacey (1986d). Under these conditions, approximately 35% of all proteins visible on 2-D protein gels drastically increase or decrease in response to soybean root exudate (Figure 3). This dramatic response was interpreted, in large part, as a nutritional response as root exudates likely contain amino acids, peptides, mono- and polysaccharides, etc; which provide carbon sources unavailable to the control (PNS-treated) cells (Wright and Northcote, 1974). The complexity of the response to root exudate

Figure 3. Two-dimensional seperations of [^{14}C]-proteins from B. japonicum USDA110 treated with plant nutrient solution (A), or soybean root exudate (B).


A
97
68
43
26
18
14


B
97
68
43
26
18
14

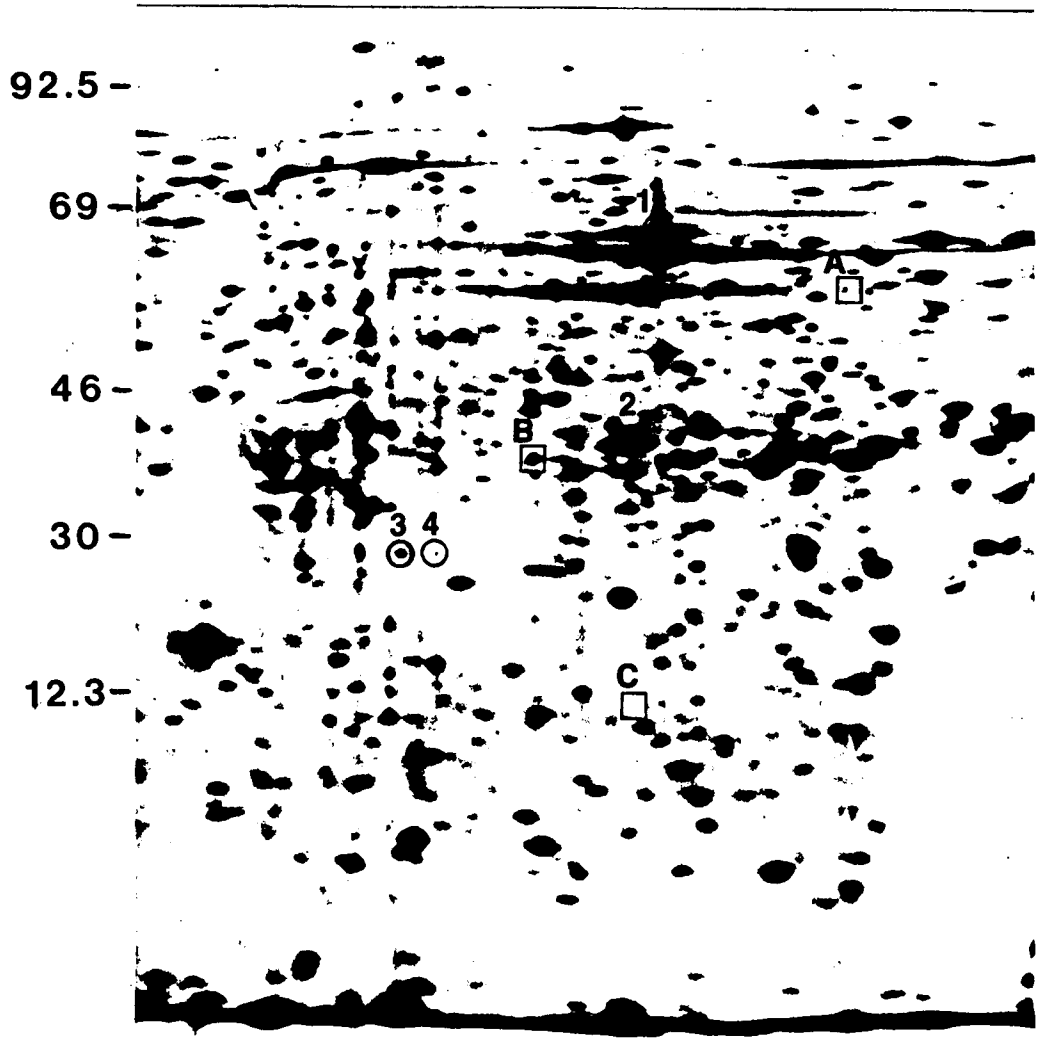
prohibited the corroboration of SBL- or genistein-induced proteins with root exudate-induced proteins, and the identification of protein responses pertinent to nodulation. Thus, purified molecules from soybean plants known to elicit nodulation-stimulating responses were used in further experiments.

Soybean lectin-induced proteins. In subsequent experiments USDA110 cells were treated with purified genistein or SBL, host molecules known to induce nodulation genes and cause nodulation competence, respectively (Kosslak et al. 1987, Halverson and Stacey 1986a, 1986c). Experiments in which USDA110 was treated with SBL in plant nutrient solution led to the identification of four SBL-inducible proteins (Figure 4, numbers 1-4) and three SBL-repressible proteins (Figure 4, A-C). Since none of these proteins appear to be abundant enough to attempt to isolate, these experiments were repeated using USDA110 cells treated with SBL during growth in rich media.

Treatment of USDA110 with SBL in yeast extract mannitol medium revealed a protein which occurs at markedly increased levels in response to SBL (Figure 5). This protein, called "S55," has an apparent molecular weight of 55kd, and a neutral isoelectric point (pI ~ 6.5 - 7.0). This protein may be identical to protein #1 in Figure 4. Three additional spots comigrate at 55Kd in the SDS-PAGE dimension, but occur at more acidic pI's, suggesting they may be charge-modified isoforms of S55.

Genistein-induced proteins. Genistein is known to induce

Figure 4. Two-dimensional seperation of [^{14}C]-proteins from B. japonicum USDA110 treated with soybean lectin in plant nutrient solution. Letters (A-C) indicate proteins which decrease in response to lectin whereas numbers (1-4) indicate proteins which increased in response to lectin. Portions of gels from control and lectin-treated samples are shown to substantiate the lectin-induced and lectin-repressed spots.


92.5
69
46
30
12.3
1
2
3
4
A
B
C

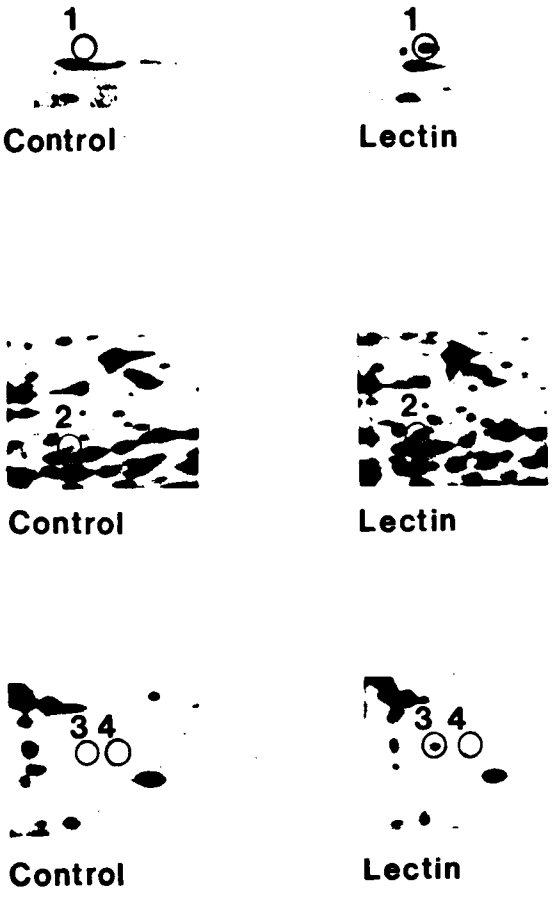
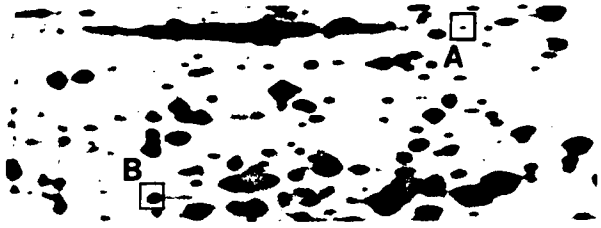


Figure 3 (continued)



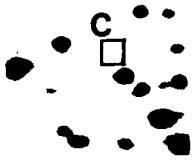
Control



Lectin



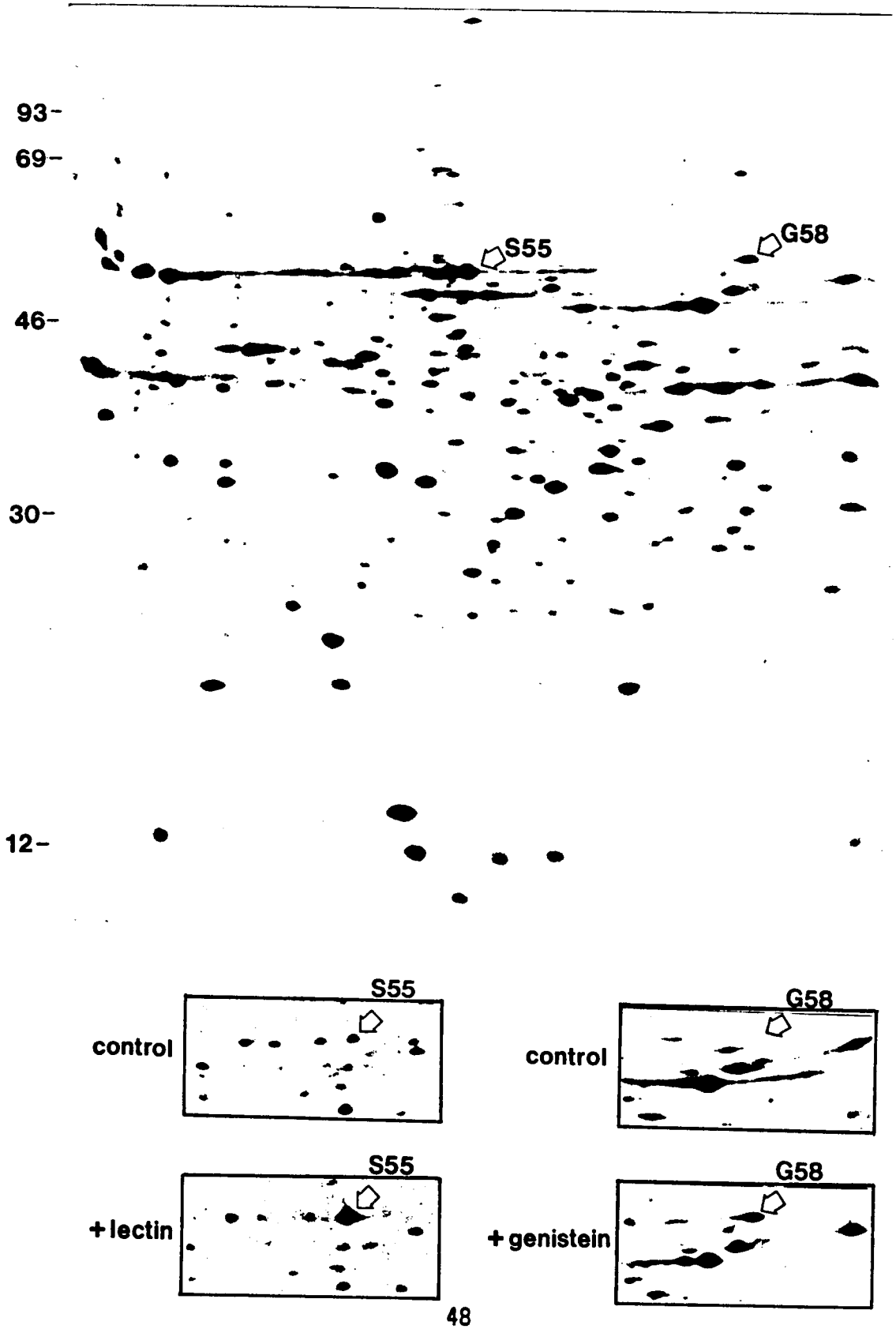
Control



Lectin

Figure 3 (continued)

Figure 5. Two-dimensional separations of [^{35}S]-proteins from B. japonicum USDA110 treated with soybean lectin and genistein in yeast extract-mannitol medium. Arrows indicate proteins induced by soybean lectin (S55) and Genistein (G58). Boxed gel portions are shown from cells treated as indicated.


93-
69-
46-
30-
12-
S55
G58
control
S55
control
G58
+ lectin
S55
+ genistein
G58

expression of at least 8 genes in USDA110. Nevertheless, these genes are transcribed at relatively low levels as judged by the relatively low β-galactosidase activities of nod-lac fusions (Kosslak et al. 1988, Banfalvi et al. 1988, Deshmane and Stacey, submitted, see HIL3 and HIL4 below). Curiously, only a single protein was clearly induced by genistein on 2-D protein gels (Figure 5). This protein, designated G58, has a molecular weight of ~ 58kd and a relatively basic pI of ~ 7.5-8. The intensity of the induced/uninduced spot G58 indicates it is induced > 50 fold. The size and pI of G58 do not correlate with any known nodulation genes.

NodD complementation. Since the induction of nod genes in other rhizobia is mediated by nodD genes (Mulligan and Long 1985, Horvath et al. 1987), a homologous gene from B. japonicum USDA110 was investigated to test its role in B. japonicum. The $nodD_1$ gene of B. japonicum was identified initially by its homology to the $nodD_1$ gene of R. meliloti (Nieuwkoop et al. 1987). In order to establish its functionality and investigate its specificity, the $nodD_1$ gene of B. japonicum USDA110 was used to complement R. meliloti $nodD_1$::Tn5 mutant MG107. Inactivation of $nodD_1$ from R. meliloti causes a marked delay in nodulation of Medicago sativa, since $nodD_2$ and $nodD_3$ play less important roles in alfalfa nodulation (Gottfert et al. 1986). A 3.9 kb HindIII fragment containing the $nodD_1$ from USDA110 was subcloned into wide host range vector pVK102 to form pDG39. This clone was moved into R. meliloti MG107 by conjugation, and the resulting transconjugant was tested for ability to nodulate alfalfa.

Figure 6 shows that the ~ 12 day delay in nodulation in MG107 was reduced to a ~ 3 day delay by complementing R. meliloti $nodD_1$ mutant MG107 with pDG39 carrying the B. japonicum USDA110 nodD gene.

Host-Inducible Genes in B. japonicum

Since the previously-studied Rhizobium species carry their nodulation genes tightly clustered, the DNA surrounding the common nod genes in B. japonicum USDA110 was examined for host-inducible genes concurrent with the search for inducible proteins. This search for host-inducible genes was performed by mutagenizing a DNA region containing the common nod genes (called pR32), with a reporter gene, and screening the resultant pR32::Tn3-lac constructs for host-inducible reporter-gene enzyme activity.

Tn3-lac mutagenesis of pR32. Specifically, Tn3-lac was allowed to transpose "randomly" into pR32 (Figure 7), and individual pR32::Tn3-lac constructs were selected in E. coli SF800. Plasmid DNA was isolated from these strains and restriction-mapped using EcoRI, HindIII, and BamHI to determine the Tn3-lac insertion sites. Approximately 120 pR32::Tn3-lac constructs were restriction mapped, of which 35 occurred in vector DNA, and 8 could not be unequivocally mapped. Three insertions generated large deletions (not shown in Figure) within pR32. Figure 7 shows the positions of 42 Tn3-lac insertions. Multiple insertions mapping to a single site (within 50 base pairs), with similar B-galactosidase activities, were presumed to be identical siblings and are omitted from Figure 7.

Some 92 pR32::Tn3-lac constructs (including siblings) were

Figure 6. Nodulation of alfalfa (*Medicago sativa* 'Buffalo') by wild-type *R. meliloti* AK631 "■", $nodD_1$ mutant MG107 "○", and MG107(pDG39) containing *B. japonicum* $nodD_1$ "●".

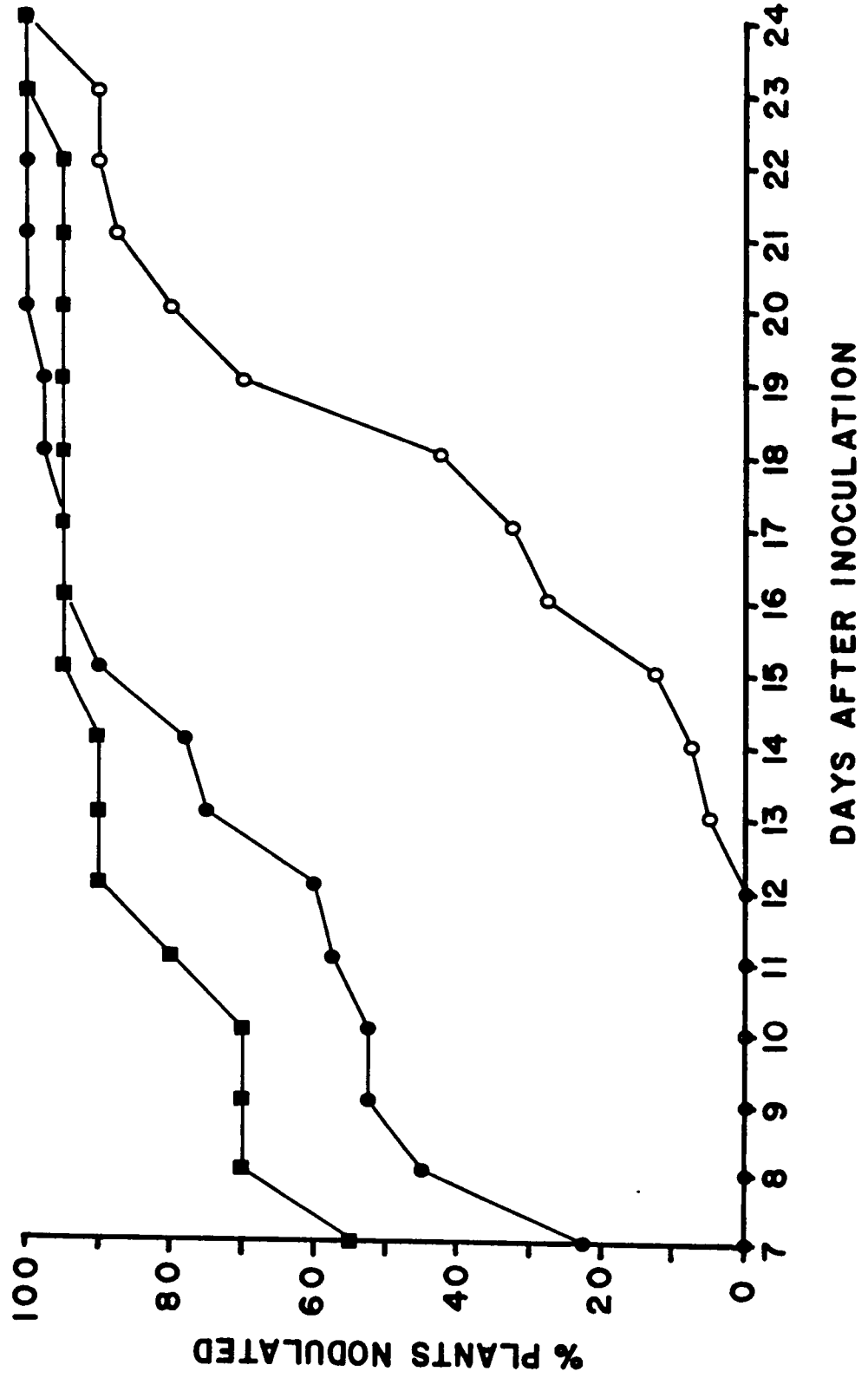
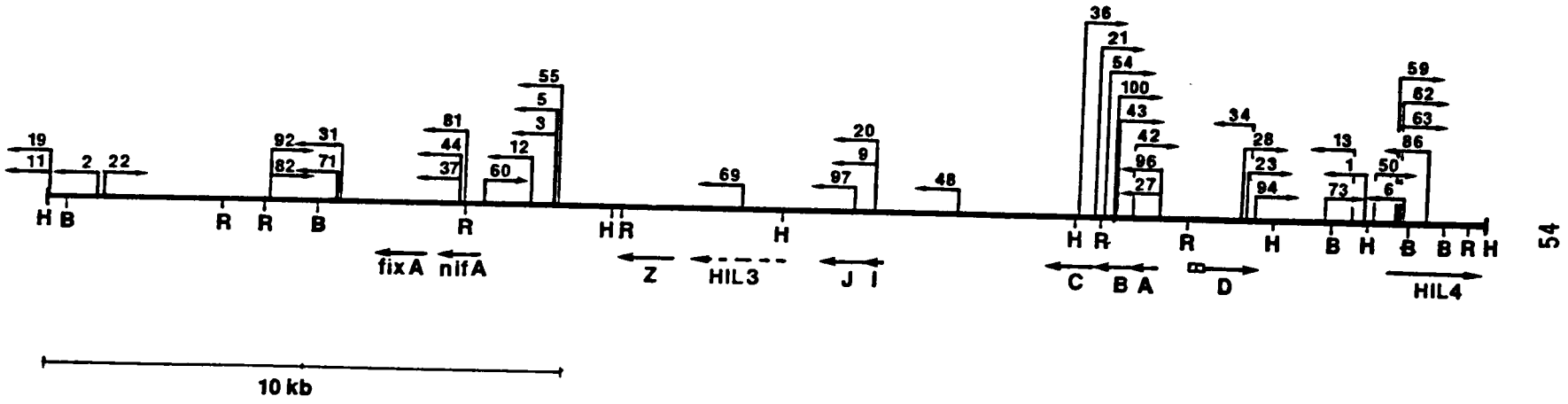

% PLANTS NODULATED
0
20
40
60
80
100
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
DAYS AFTER INOCULATION

Figure 7. Cosmid clone pR32 from B. japonicum USDA110 showing known nodulation genes and Tn3-lac insertions. EcoRI sites (R) and HindIII sites (H) are indicated.

USDA 110 (pR32::Tn3-lac) map



moved from E. coli into B. japonicum USDA110 via triparental matings, and assayed for β-galactosidase activities with and without soybean root exudate. Four fusions, designated DG59, DG62, DG63, and DG69 (Figure 7), were identified as being inducible several-fold by soybean root exudate or seed extract (Table 2).

Host-inducible locus 4 (HIL4). DG59, DG62, and DG63 apparently represent a single transcriptional unit characterized by moderate levels of β-galactosidase activity which is inducible by soybean seed extract or genistein (Table 2). Restriction mapping and induction data suggest DG62 and DG63 represent siblings, whereas DG59 occurs 50-100 bp further upstream (Figure 7). These three mutants of B. japonicum are merodiploid for the pR32 insert DNA, however, all three share an apparently dominant phenotype. Colonies from DG59, DG62, and DG63 are > 10-times larger than the wild-type when grown on RDM plates (Figure 8). Further testing showed that minimal medium sponsored growth of normal colonies unless supplemented with 1 g/l glutamate, which allowed formation of large colonies. Surprisingly, attempts to cause the large colony phenotype by replacing glutamate with 1% glutamine, glycine, citrate, or succinate, failed as these dicarboxylates and amino acids inhibited growth of USDA110.

The role of HIL4 in nodulation of soybeans has been investigated by Deshmane and Stacey (submitted) in our laboratory, and M. Sadowski, a collaborator at USDA, Beltsville, MD. Dr. Deshmane finds that HIL4::Tn5 insertion mutant NAD27 is drastically impaired in nodulation of Glycine max 'Essex.' This mutant

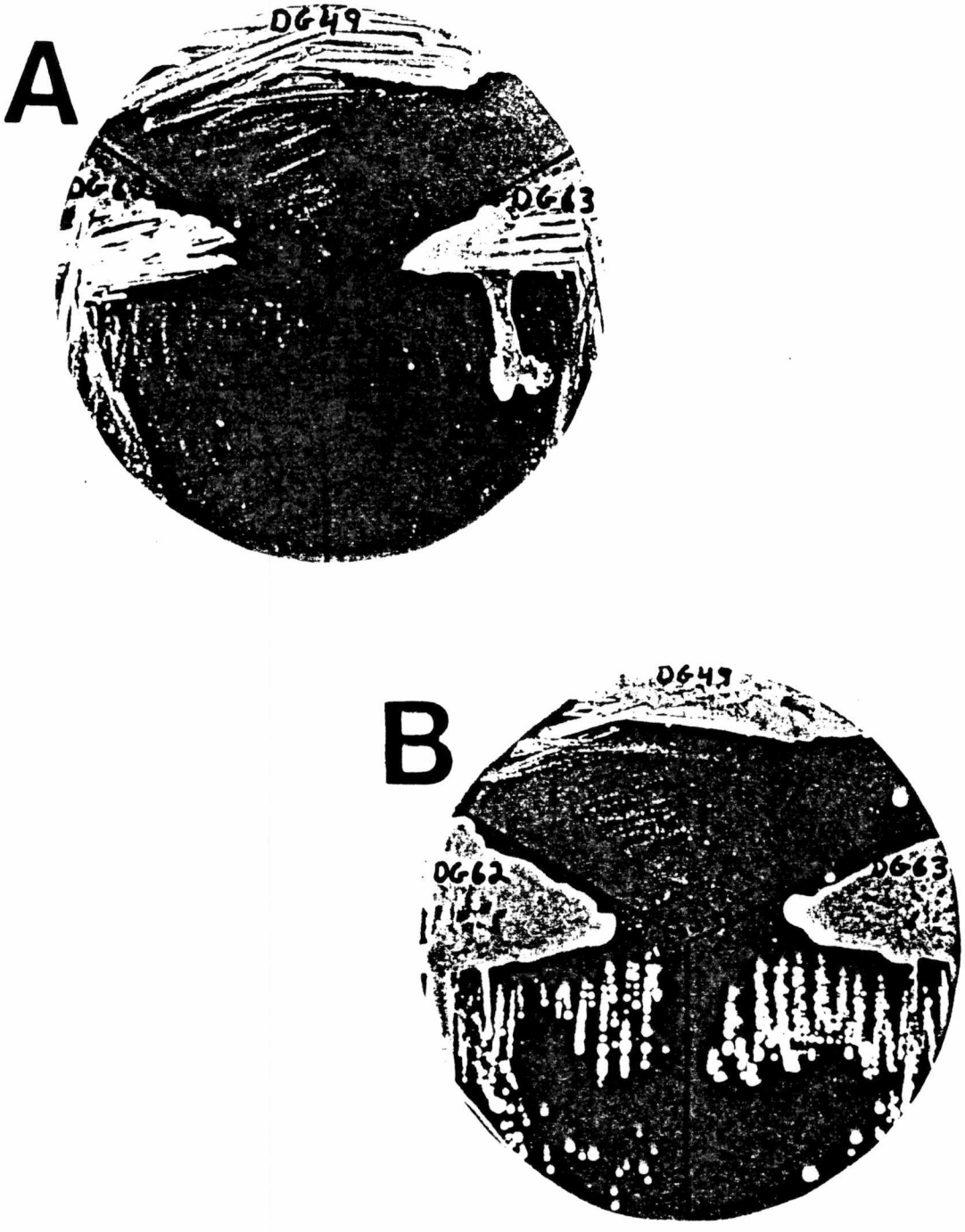
Table 2. β-Galactosidase Activities of B. japonicum nod-lac Fusions

Strain	Genotype	β-Galactosidase Activity[a]		
		Control	+2 μM Genistein	+ Soybean Seed Extract
USDA110	wild-type	< 2	< 2	< 2
DG62	HIL4::lacZ	43.5	112	119
DG69	HIL3::lacZ	5.4	41.1	38.8
USDA110(pZB32)[b]	nodY::lacZ	4.1	160	205

[a] β-galactosidase activities were determined essentially according to Miller (1972) using CPRG (Boehringer-Mannheim Bochemicals), and divided by eight to correct for CPRG's increased sensitivity compared to ONPG.

[b] Data from Banfalvi et al. (1988).

Figure 8. Colony Morphology of HIL4 mutants DG62, DG63, and control strain DG49. YEM medium (A) lacks glutamate whereas RDM medium (B) contains glutamate.


A
DG49
DG63
B
DG49
DG62
DG63

apparently has a normal colony morphology unlike the HIL4::Tn3-lac fusion strains. Independently, Dr. Sadowski found that HIL4 is important during competition between B. japonicum strains competing to nodulate particular soybeans.

Nearly all arable land in the U.S. contains a native population of bradyrhizobia. These native Bradyrhizobium japonicum strains can be classified predominantly into two "serotypes" by their reactions with antibodies made to strains USDA110 and USDA123. USDA123 strains usually outcompete USDA110 strains for nodulation of commercial soybean cultivars, however USDA123 strains usually fix nitrogen inefficiently. Even when commercial soybean seeds are coated with B. japonicum inocula, the inoculum strains rarely occupy more than 10-20% of resulting nodules (Cregan and Keyser 1986). A survey of 1,286 soybean isolates identified 13 which could exclude nodulation by USDA123 but not by USDA110 (Cregan and Keyser 1986). These 13 isolates reportedly share a dominant gene which acts to exclude USDA123 (Sadowski M., Cregan P., Keyser H., personal communication). USDA123 can be complemented for the ability to nodulate the 13 "excluding" soybeans by either pR32, or the 2.3Kb HindIII subcloned fragment carrying HIL4 (Sadowski M., Gerhold D., Stacey G., Cregan P., Keyser H. manuscript in preparation). This result suggests a possible pragmatic use for HIL4 in inocula.

Host-inducible locus 3 (HIL3). A second inducible locus was also identified by Tn3-lac mutagenesis, and designated HIL3. Lac-fusion mutant DG69 (Figure 7) is induced by genistein or soybean seed

extract like HIL4, and is expressed at a lower basal level than the HIL4 fusions (Table 2). This region occurs upstream of the siratro host specificity gene, nodZ, and is designated HIL3 (Figure 7). Tn5 mutants - 1kb upstream and - 0.3Kb downstream of the DG69 site do not greatly affect Glycine max 'Essex' nodulation (N. Deshmane, Ph.D. thesis-U.T., 1988). The HIL3 locus is currently being sequenced in our laboratory (S. Luka, unpublished) and transposon/lac-fusion constructs are being made to further delineate the locus and study its phenotype. Neither HIL3 nor HIL4 regions hybridize to a nod box-specific probe (M. Schell, unpublished), thus raising the question of how these genes are induced by genistein.

Host-Inducible Genes in R. meliloti

The apparent absence of nod boxes preceding HIL3 and HIL4 led me to critical testing of the role of nod boxes in the organism in which they were discovered, R. meliloti (Rostas et al. 1986). R. meliloti represents the best understood nodulation genetics system, providing a foundation for the study of nod gene regulation and function in other Rhizobium and Bradyrhizobium species. Hybridizations using a 25bp oligonucleotide representing the highly conserved nod box core sequence from R. meliloti allowed identification of homologous sequences in a variety of Rhizobium and Bradyrhizobium species, and identification of a total of six nod box sequences in R. meliloti strain AK631 (Rostas et al. 1986) (Figure 1). Previous work indicated that the genes associated with nod boxes n1 (nodABCIJ), n2 (hsnABC), n3 (hsnD), and n6 (the efficiency of

nodulation--efn locus including $nodD_2$), are sufficient for efficient nodulation of alfalfa (Putnoky and Kondorosi 1986). Thus it was of interest to determine whether n4 and n5 are associated with functional symbiotic genes and whether they regulate transcription in response to flavonoids.

Subcloning the n4 and n5 loci. The n4 and n5 regions were subcloned into wide host range vectors to facilitate their study. Restriction mapping of the 3.5Kb EcoRI fragment bearing n4 indicated that it contained only about 0.4 kb of DNA "downstream" (by analogy to n1-nodA, see Figure 9) of n4. In order to identify restriction fragments carrying more downstream sequences, cosmids bearing n4 and n5 (pEK5121 and pEK3148, respectively; Kondorosi et al. 1984) were digested with several restriction enzymes, electrophoresed through 1% agarose, blotted onto nitrocellulose, and probed with the nod box oligonucleotide (Figure 10). Thus identified, the 6.0 kb KpnI fragment bearing n4 was subcloned into pPR33 (a pRSF1010 derivative, P. Ratet unpublished) to yield pDG60 (see Figure 9). Similarly, the 5.2 PstI fragment carrying n5 was subcloned into pSF6 (Selvaraj et al. 1984), yielding pDG52 (Figure 9). The 10kb HindIII fragment containing n5 was later subcloned into pVK100 (Knauf and Nester 1982) yielding pDG100 in order to use for site-directed Tn5 mutagenesis.

Lac-fusions to n4 and n5 are luteolin inducible. To determine whether n4 and n5 are linked to symbiotically regulated genes, the promoterless lacZYA genes were inserted into pDG52 and pDG60 using miniMu-lac fusion vehicles. The transcriptional fusion vehicle

Figure 9. EcoRI restriction map of the Rhizobium meliloti nodulation region (@ 100kb). n1-n6 denote nod box sequences, and numbered arrows mark positions of plant-inducible lac-fusions. "Lollipops" indicate positions of Tn5 insertions causing delayed nodulation (open circles) or normal nodulation (darkened circles) upon recombination into the AK631 genome. This map is amended from (25), however the order of the five EcoRI fragments left of pDG100 is uncertain. R = EcoRI, K = KpnI, B = BamHI, b = BglII, and P = PstI.

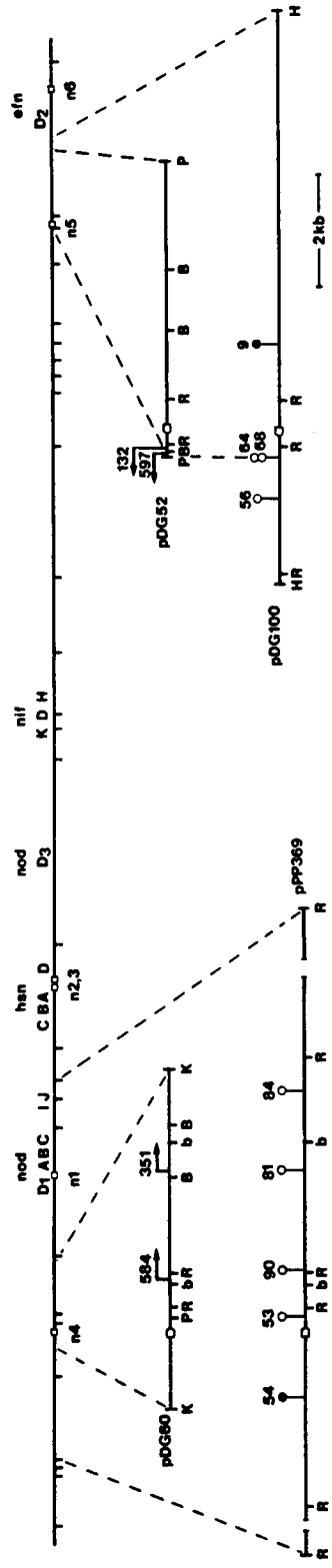
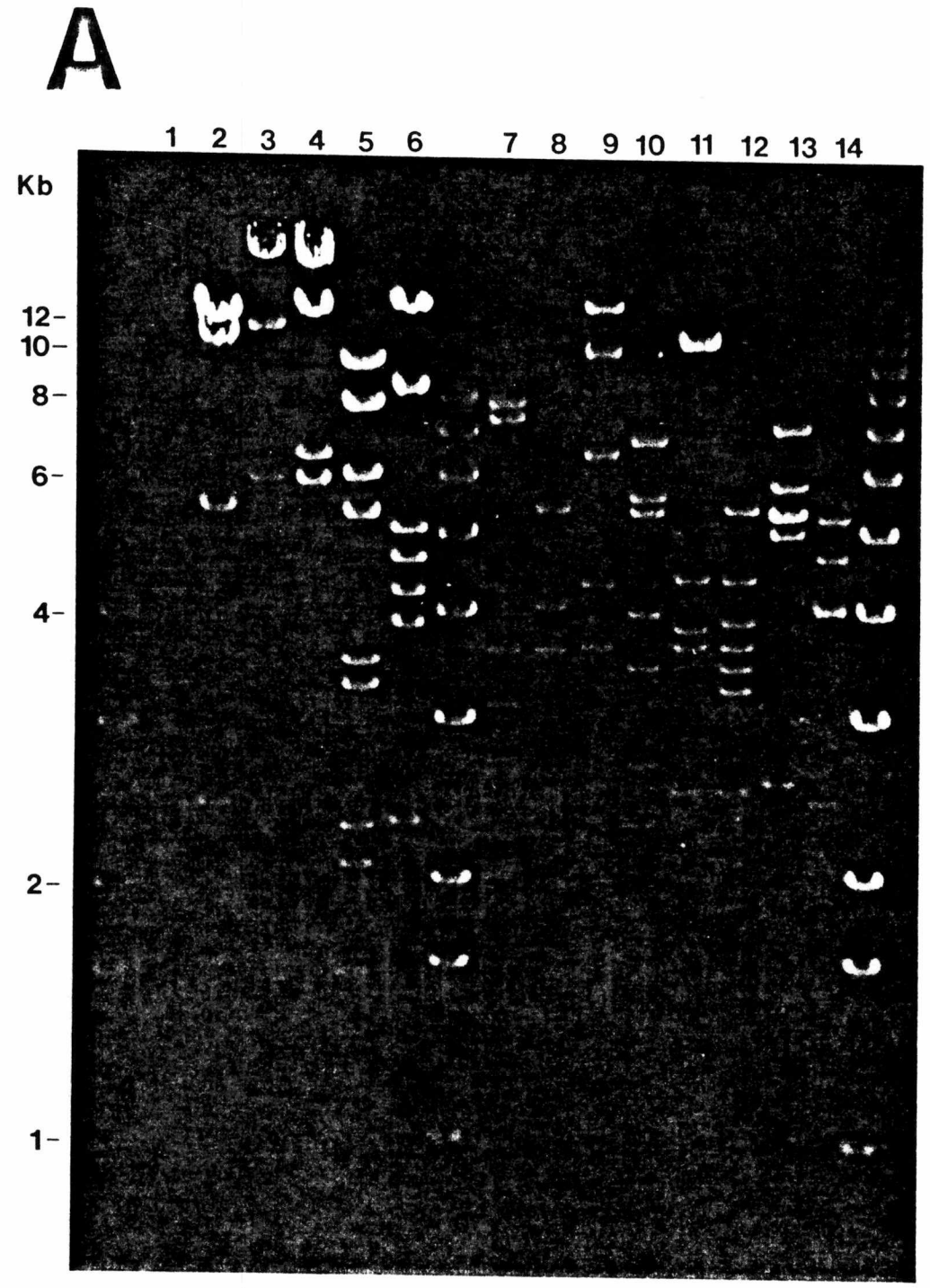

nod D1 A B C I J
hsn C B A D
nod D3
nif K D H
eln D2
n1
n2,3
n4
n5
n6
pDG80
pDG52
pDG100
pPP369
2kb

Figure 10. Restriction analysis (A) and nod box hybridization (B) of R. meliloti cosmids (Kondorosi et al. 1984) from the pSym nodulation region. Cosmids are: pBR3.5 BglII/EcoRI bearing n4 (lane 1), pEK2039 BglII bearing n4 (lane 2), pEK5121 KpnI bearing n1-n4 (lane 3), pEK2039 bearing n4 cut with: KpnI (lane 4), BamHI (lane 5), and HindIII (lane 6). Cosmid pEK3148 bearing n5 and n6 was cut with: BamHI (lane 7), BamHI/EcoRI (lane 8), HindIII (lane 9), HindIII/EcoRI (lane 10), BglII (lane 11), BglII/EcoRI (lane 12), PstI (lane 13), and PstI/EcoRI (lane 14). The three unmasked lanes represent DNA size standards. A synthetic nod box oligonucleotide was used as the probe (Rostas et al. 1986). The 6.0kb KpnI fragment bearing n4 was subcloned, as were the 5.2 PstI and 10kb HindIII fragments bearing n5 (see text).


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Kb
12-
10-
8-
6-
4-
2-
1-

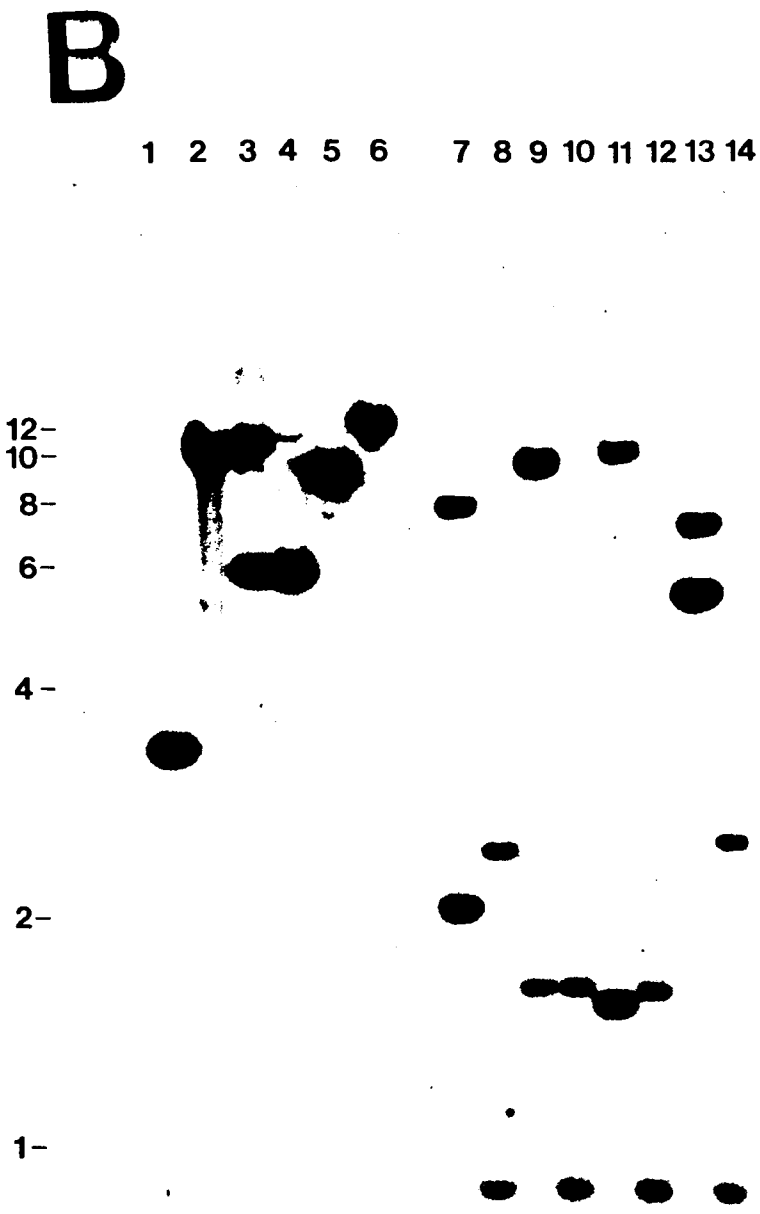


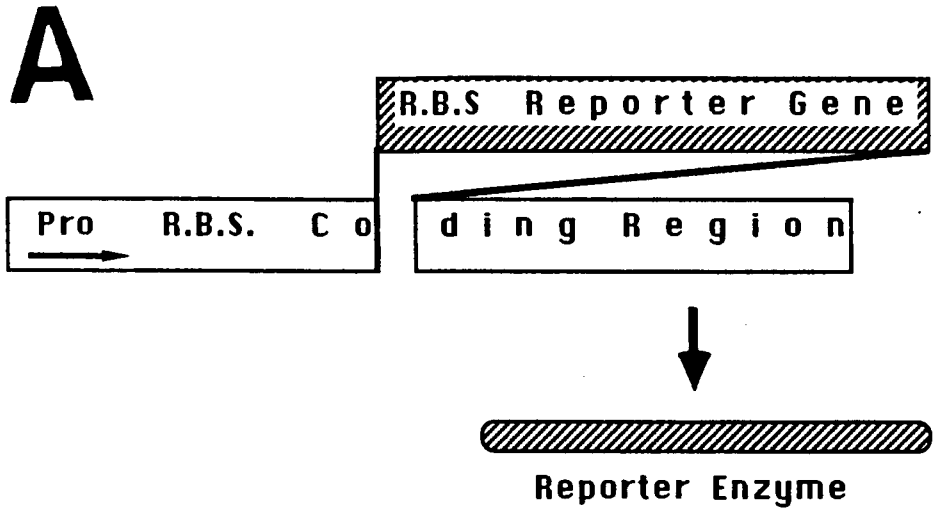
Figure 10 (continued)

MudI1734 (Castilho et al. 1984) yielded fusions with very high backgrounds in Rhizobium (>250 Miller B-galactosidase units) regardless of location. This background probably arose from a Rhizobium-recognized promoter within the vehicle's trp fragment (Groisman et al. 1986), therefore MudlacIIPR13 (Ratet et al. 1988) was used instead to construct translational fusions (Figure 11).

In order to select relatively rare lac-fusion constructs which might be flavonoid-inducible, populations of pDG52::Mudlac or pDG60::Mudlac were mated en masse into Rhizobium species MPIK3030. MPIK3030, like the closely related strain NGR234, has an exceptionally broad host range, and a correspondingly "promiscuous" nodD which is able to recognize luteolin, cumestrin, and other flavonoids as inducers of the nod-regulon (Horvath et al. 1987). Resulting transconjugants were replica-plated onto selective plates containing 50 µg/ml 5-bromo,4-chloro,3-indolyl,β-D-galactoside (Xgal), with and without 5 µM luteolin. Strains which appeared darker blue in the presence of luteolin than in its absence were grown in broth and assayed using the highly sensitive β-galactosidase substrate chlorophenol-red-β-D-galactoside (CPRG). MPIK3030 was used as recipient instead of R. meliloti AK631 because nod-lac fusions are expressed poorly in an AK631 background (see Discussion).

The pDG52::Mudlac n5-fusion constructs designated #132 and #597 (Figure 9) were induced approximately 6-fold and 3-fold, respectively, by luteolin (Table 3). Similarly, pDG60::Mudlac n4-fusions #584 and #351 were induced about 6-fold and 5-fold in MPIK3030. The relative orientations of nod boxes n4 and n5 inferred

Figure 11. Transcriptional (A) and translational (B) reporter gene fusions. The promoter (Pro), ribosome binding site (R.B.S.), and coding region, of the gene under investigation are indicated.


A
R.B.S Reporter Gene
Pro R.B.S. C o
d i n g R e g i o n
Reporter Enzyme

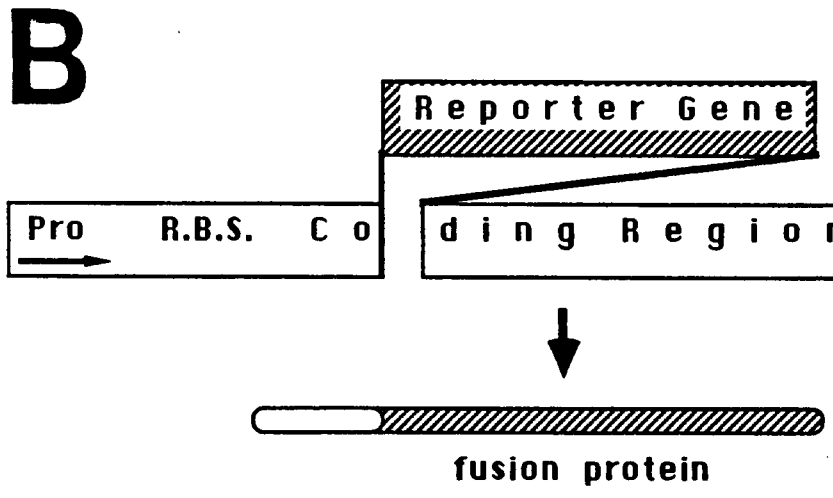

B
Reporter Gene
Pro R.B.S. C o
d i n g R e g i o n
fusion protein

Table 3. β-Galactosidase Activities of n4 and n5 Lac-Fusions.

Lac-Fusion Construct (in MPIK3030)	β-Galactosidase Activity[a]	
	Control	+5μM Luteolin
none	3.1	2.8
n4-351	5.3	26.4
n4-584	5.2	29.4
n5-132	10.2	64.0
n5-597	7.7	25.4

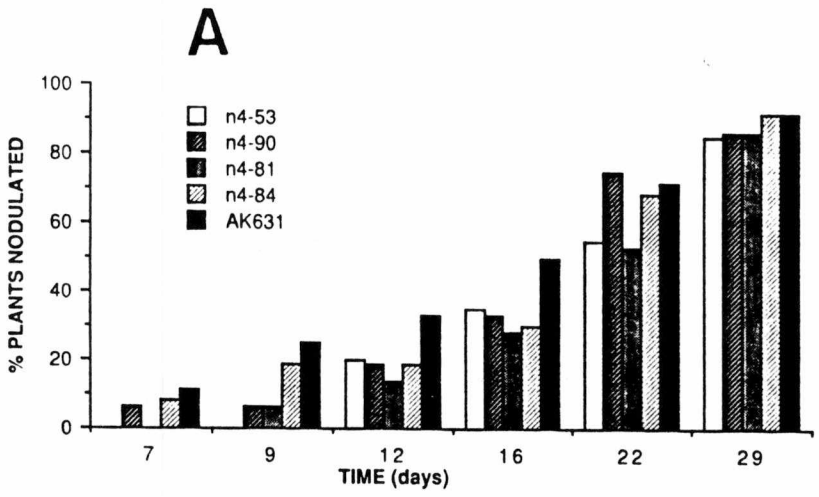
[a] β-galactosidase values were determined essentially according to Miller (1972) using CPRG (Boehringer-Mannheim Biochemicals), and divided by eight to correct for CPRG's increased sensitivity compared to ONPG.

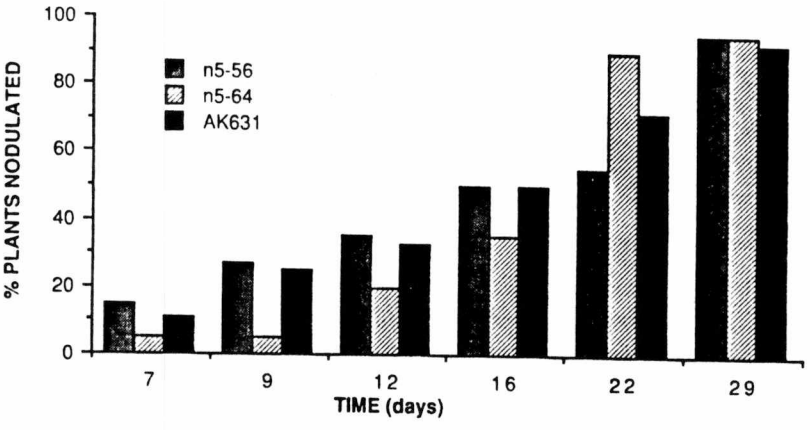
from the directionality of these lac-fusions are in agreement with their polarity determined earlier by sequencing (Rostas et al. 1986), and are indicated in Figure 9. The host-inducible nature of these fusions suggests that n4 and n5 may regulate functional symbiotic genes. This possibility was investigated using site-directed Tn5 mutagenesis.

Tn5 mutagenesis. The P-incompatability group cosmids pPP369 (Putnoky and Kondorosi 1986) and pDG100 were selected for Tn5 mutagenesis, which was performed using P1::Tn5 (Quinto and Bender 1984). Tn5 insertions which were located near n4 in pPP369, and n5 in pDG100 (Figure 9), were recombined into the R. meliloti AK631 symbiotic plasmid via marker-exchange (Ruvkun and Ausubel 1981) (Figure 2, page 34). Putative homogenotes were verified by probing Southern blots of EcoRI digests of genomic DNA with [32 P]-labelled pPP369 or pDG100.

Symbiotic phenotypes of mutants. These Tn5 mutants were then compared with the wild-type for the ability to nodulate alfalfa plants. Figure 12 and Table 4 are data from a representative experiment using 30-50 plants for each mutant. The data from several experiments consistently indicate that mutations downstream of either n4 or n5 impair the bacterium's ability to nodulate alfalfa. The n5-64 and n5-68 mutations, two independent Tn5 insertions which map very close together (see Figure 9), delayed alfalfa nodulation by about 4 days. Mutant n5-56 did not produce a detectable delay in nodulation (Figure 12b), however the number of nodules per plant formed by n5-56

Figure 12. Nodulation of alfalfa (A,B), and sweetclover (C) by Tn5 mutants of R. meliloti AK631. Data presented are from a representative experiment using 30-50 plants for each mutant. Nodule numbers formed by each mutant are given in Table 4.


A
100
80
60
40
20
0
% PLANTS NODULATED
n4-53
n4-90
n4-81
n4-84
AK631
7
9
12
16
22
29
TIME (days)


B
100
80
60
40
20
0
% PLANTS NODULATED
n5-56
n5-64
AK631
7
9
12
16
22
29
TIME (days)

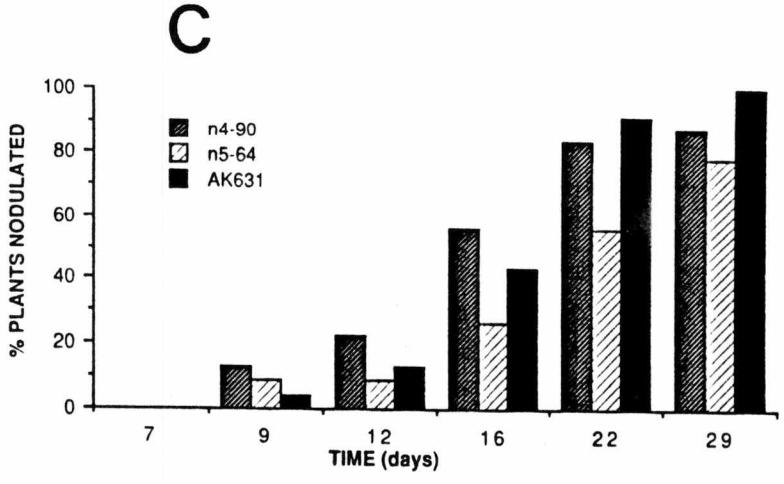

C
100
80
60
40
20
0
% PLANTS NODULATED
n4-90
n5-64
AK631
7
9
12
16
22
29
TIME (days)

Table 4. Nodule Numbers Formed on Alfalfa and Sweetclover by Tn5 Mutants of R. meliloti AK631.

Mutant	Day 7	Day 9	Day 12	Day 16	Day 22	Day 29
			Average # Nodules per Alfalfa Plant:			
AK631	.11 +/-.05	.33 +/-.06	.42 +/-.07	.78 +/-.15	3.1 +/-.55	6.5 +/-.89
n4-53	0	0 **	.30 +/-.15	.65 +/-.22	2.8 +/-.76	5.3 +/-.98*
n4-90	.06 +/-.04	.06 +/-.04*	.33 +/-.09	.72 +/-.20	2.3 +/-.38**	5.3 +/-.85*
n4-81	0	.06 +/-.04*	.28 +/-.12	.56 +/-.17*	1.5 +/-.32***	4.5 +/-.67**
n4-84	.08 +/-.05	.28 +/-.11*	.44 +/-.18	.72 +/-.27	2.5 +/-.43	6.1 +/-.67
n5-64	.05 +/-.04	.10 +/-.08**	.95 +/-.40	1.2 +/-.44	3.2 +/-.53	5.7 +/-.69
n5-56	.10 +/-.04	.40 +/-.11	.60 +/-.15	.95 +/-.24	1.9 +/-.37***	4.0 +/-.63***
			Average # Nodules per Sweetclover Plant			
AK631	0	.04 +/-.04	.13 +/-.07	.65 +/-.17	3.0 +/-.42	4.9 +/-.52
n4-90	0	.30 +/-.16	.52 +/-.23	1.4 +/-.40$$	4.3 +/-.69$	6.3 +/-1.2$
n5-64	0	.10 +/-.06	.17 +/-.08	.35 +/-.14**	1.2 +/-.27***	3.0 +/-.58***

Values are expressed as mean +/- standard error.

Astericks indicate significantly fewer nodules per plant at the 0.1 level "**", .05 level "***", or .025 level of significance "****". Mutant n4-90 formed more nodules than the wild type on sweetclover with significance indicated at the 0.1 level "$", and the .05 level "$$".

The Student's t-test was used for statistical analyses of data from days 16-29. Data from days 7-12 contained many unnodulated plants necessitating use of a different (median chi-squared) test.

was significantly less than the wild-type at the .0025 level of significance (Table 4).

Tn*5* insertions in the n4 region delayed alfalfa nodulation by 3 to 6 days, including mutants n4-53, n4-90, n4-81, and n4-84 (Figure 12a). All of the n4 mutants also tended to form fewer alfalfa nodules than the wild-type (Table 4). These differences showed some statistical significance for all mutants except n4-84. Mutants n4-90 and n5-64 were selected for testing on sweetclover (*Melilotus alba* 'white blossom'). Interestingly, mutation n5-64 delayed sweetclover nodulation by 7 days, whereas mutation n4-90 actually increased sweetclover nodulation (Figure 12c and Table 4). In general, the short delays in nodulation observed with these n4 and n5 mutants correlated with formation of fewer nodules per plant, and are reminiscent of the phenotypes of *nodFEG* mutations (Horvath et al. 1986). Mutations upstream of n4 (369-54) and n5 (100-9, Figure 9) were also tested on alfalfa plants (data not shown) and found to nodulate in a manner comparable to AK631.

R. meliloti AK631 and mutant strains n5-64, and n4-90, were used to inoculate alfalfa plants on Fahreus slides (Fahreus 1957) and observed after 6 and 11 days with a phase-contrast microscope to look for differences in the infection process caused by these mutations. The mutants appeared to curl root hairs, form infection threads, and initiate nodules in a manner qualitatively indistinguishable from that of the wild-type strain, (data not shown).

Hybridization of n4 and n5 regions to other rhizobia. The

evidence that the n4 and n5 loci carry host-induced nodulation genes prompted us to search for homologous genes in other rhizobia. The n4 probe used in Figure 13 was the 2.3kb PstI-BamHI fragment from pDG60 which was cut out of an M13mp18 clone called pDG2.3 and gel purified (see Figure 9; Norrander et al. 1983), whereas the n5 probe was the EcoRI fragment isolated from pDG100. Under nonstringent conditions the n4-locus probe showed no homology to DNA from a variety of other Rhizobium and Bradyrhizobium species (Figure 14). The n5 probe, however, hybridized strongly to total DNA from R. trifolii and R. leguminosarum, and very weakly to R. fredii, R. sp. MPIK3030, and several strains of Bradyrhizobium japonicum (Figure 14). No hybridization of n5 to USDA110 genomic DNA could be detected, however weak hybridization was observed to a 3.8Kb EcoRI fragment in pRjUT14 (Russell et al. 1985). The linkage of this hybridizing fragment to the common nod region, and adjoining a 7.0Kb EcoRI fragment thought to encode nod functions (Nieuwkoop, A. unpublished), suggests it may contain a related nod gene.

Figure 13. Hybridization of the n4 locus (the 2.3kb PstI-BamHI fragment from pDG60) to EcoRI digested genomic DNA from: R. meliloti AK631 (lane 1), R. meliloti L5-30 (lane 2), R. 'trifolii' ANU843 (lane 3), R. leguminosarum 'Viciae' 897 (lane 4), R. leguminosarum species MPIK3030 (lane 5), R. fredii USDA193 (lane 6), Bradyrhizobium japonicum USDA110 (lane 7), and Agrobacterium tumefaciens 136 (lane 8). The relative lightness of the 3.5kb band in lane 1 is due to its short region of overlap with the probe (see Figure 1).

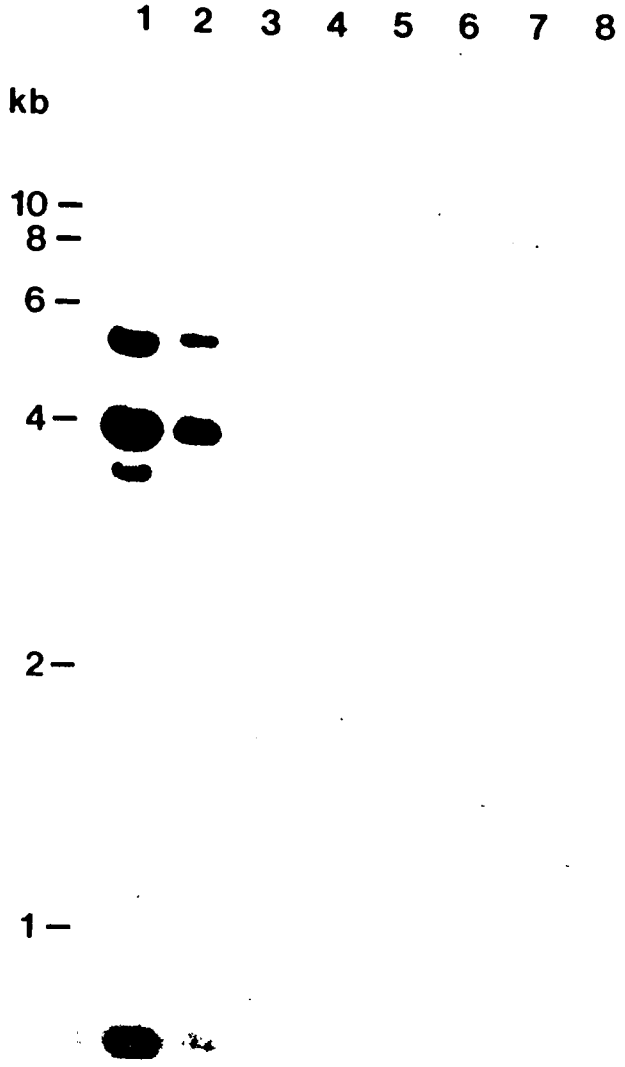
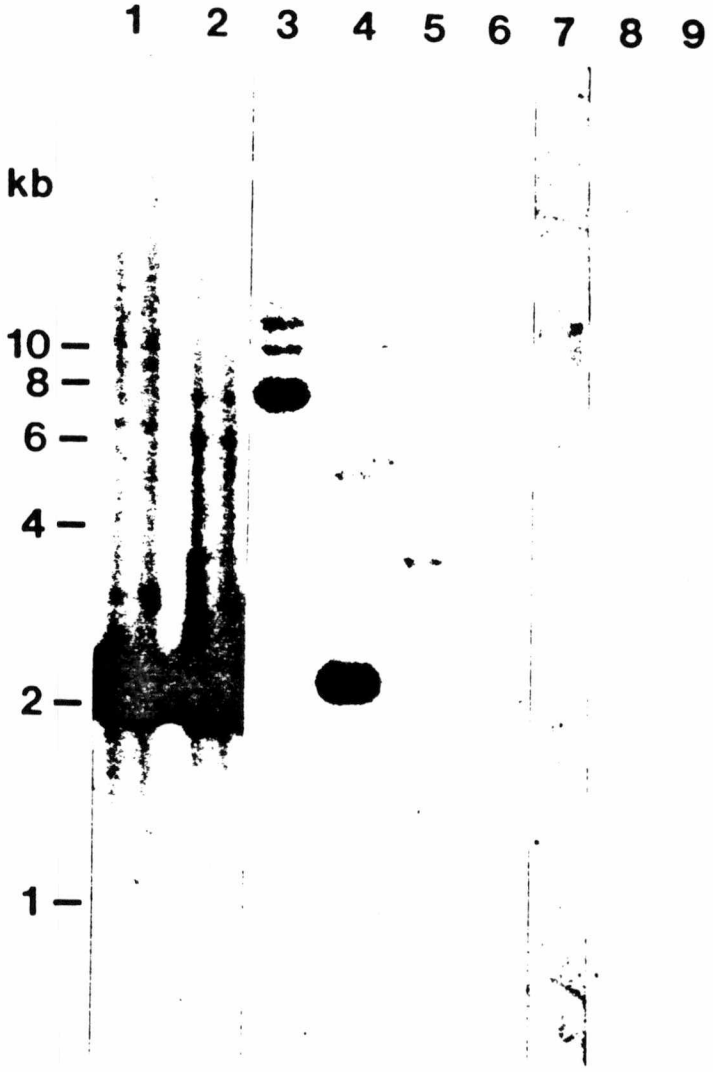

1 2 3 4 5 6 7 8
kb
10
8
6
4
2
1

Figure 14. Hybridization of the n5 locus (2.1kb EcoRI fragment from pDG100) to EcoRI digested genomic DNA from R. meliloti AK631 (lane 1), R. meliloti L5-30 (lane 2), R. leguminosarum 'trifolii' ANU843 (lane 3), R. leguminosarum 'Viciae' 897 (lane 4), R. fredii USDA 193 (lane 5), Agrobacterium tumefaciens 136 (lane 6), R. species MPIK3030 (lane 7), Bradyrhizobium parasponiae ANU 289 (lane 8), and B. japonicum 61A101C (lane 9).


1 2 3 4 5 6 7 8 9
kb
10
8
6
4
2
1

CHAPTER 5

DISCUSSION

The work described herein was performed in order to shed light on how rhizobia recognize and respond to their plant hosts in preparation for nodulation. Work in our laboratory focuses on Bradyrhizobium japonicum because of its unique features and the economic importance of its soybean host. Related investigations were also performed using Rhizobium meliloti since this organism is easier to study and is better characterized in terms of nodulation genetics. Thus, work on the R. meliloti-alfalfa/sweetclover model system has laid the foundation for our work, and provides an opportunity to form testable hypotheses concerning B. japonicum nodulation. The role of lectin in nodulation was studied using B. japonicum and soybean seed lectin, since this lectin is well-characterized and has a demonstrable physiological effect on B. japonicum nodulation (Halverson and Stacey 1984, 1986a, 1986b).

Identification of B. japonicum Proteins Induced by Host Signals

Root exudate-induced proteins. In early experiments, USDA110 cells were treated with soybean root exudate to determine its effect on B. japonicum gene expression. It is apparent from Figure 3 that root exudate profoundly affects expression of many USDA110 proteins. Since plant roots exude a diverse array of organic compounds, this "chicken noodle soup" can be expected to have many nutritional

effects on bacterial gene expression. The large number of induced and repressed proteins apparent in Figure 3B precludes one from identifying those which are germane to symbiotic differentiation. It does suggest, however, that many compounds exuded by soybean roots may contribute to root colonization and nodulation, even those that are not host-specific signals which induce nodulation-specific genes. One can easily envision the bacterial symbiont using root exudate components as carbon, nitrogen, and energy sources, for example, and as guides for chemotaxis to the root.

It has been previously demonstrated that B. japonicum is metabolically very diverse, being capable of using a large variety of organic compounds as carbon and energy sources. Most Bradyrhizobium strains have been shown to metabolize numerous hexoses and pentoses, as well as selected organic acids and amino acids. Curiously, strain USDA110 lacks mannitol dehydrogenase activity (M. Barbour, personal communication) and thus must use yeast extract components during growth on yeast extract mannitol medium. Unfortunately no comprehensive survey of the composition of soybean root exudate is available, nor are the carbon substrates used by Bradyrhizobium in the rhizosphere known. Because of these uncertainties, we decided to use two pure components of root exudate to define specific protein responses.

Soybean lectin- and genistein-induced proteins. Since soybean seed lectin (SBL) is known to signal B. japonicum to become "nodulation competent" (Halverson and Stacey 1986), USDA110 cells

were treated with SBL in order to identify protein/s which respond to this purified component of root exudate. As Figure 4, page 43, indicates, B. japonicum responds to SBL added to plant nutrient solution by increasing levels of some proteins (Figure 4, numbers 1-4) and decreasing levels of others (Figure 4, A-C). This result suggests a moderately complex physiological response. Although this experiment does not prove that the indicated proteins represent a response specific for SBL, treatment of the control cells bovine serum albumin was expected to rule out nonspecific responses to the added glycoprotein.

This response also corroborates the finding that chloramphenicol can, by inhibiting translation, block the induction of nodulation competence (Halverson and Stacey 1986c). Following the relative success in identifying SBL-induced proteins by 2-D electrophoresis, this method was also applied to search for genistein-induced proteins.

Following the identification of genistein as the optimal inducer of B. japonicum nod genes, this flavonoid was compared with SBL for ability to stimulate synthesis of particular proteins. In contrast to the SBL-treatment experiments presented in Figure 4, subsequent cultures were treated during growth in rich medium rather than while stationary in a nutrient-poor salts solution (Yeast Extract Mannitol instead of Plant Nutrient Solution). These growing cells were also treated in early logarithmic phase ($O.D._{600}$ = 0.1-0.2 rather than $O.D._{600}$ 0.3-0.4), a condition necessary for nod gene induction by genistein. Thus, only a single protein was observed to

respond to SBL under these conditions (Figure 5, S55, page 47). The different general patterns of proteins complicate comparison of Figure 4, page 43, to Figure 5, however it is likely that S55 corresponds to spot #1 on Figure 4. It is important to note that although two-dimensional electrophoresis is a powerful method for observing a vast number of gene products, it is relatively insensitive to subtle quantitative changes in protein levels. Thus, proteins which are induced or repressed 2-5-fold are not likely to be detected by this method.

Protein S55 is of particular interest because of its prominence and putative isoforms. The relatively high intensity of the S55 spot suggests it may be isolated and used for N-terminal protein microsequencing (Vanderckhove et al. 1985). Such a sequence could then be used to predict the sequence of a DNA oligonucleotide usable to identify the S55 gene by hybridization. Purification of > 20 pMoles (required for microsequencing) by preparative isoelectric focusing/gel electrophoresis, however, was judged to be prohibitively difficult. The presence of 3 spots which comigrate with S55 in the vertical dimension at more acidic pI's suggests that S55 can exist in 4 progressively modified isoforms. This putative covalent modification could be e.g. phosphorylation, methylation, adenosylation, or glycosylation. S55 has a larger apparent molecular weight than predicted by any known nodulation gene, nor are any of these genes (nodYABC, HIL3-HIL6) induced by SBL. These results suggest that the gene for S55 has yet to be discovered.

Electrophoretic separation of proteins from genistein-induced cells was performed in order to correlate protein data with genetic data concerning nod gene regulation. Lac-fusion data from our laboratory show that nodYABC, nodD, HIL3, HIL4, HIL5, and HIL6 are all induced by genistein (Banfalvi et al. 1988, Deshmane, and Stacey, submitted, and this work). These data differ from the in vivo situation in that the plasmid-borne gene-fusions probably occur with increased copy numbers, increased supercoiling, and sterically disturbed (chimeric) fusion proteins. Nevertheless, these data suggest that excepting nodYABC, the known B. japonicum nod genes are only induced 2-5 fold by genistein, a level which is difficult to discern in comparing protein gels. It is likely that the relatively low -galactosidase activities of the nod genes further hampers their detection on protein gels. Thus, the known nod-gene products could not be identified in 2-D protein gels, although a single new induced protein was identified.

Figure 5, page 47, shows that a relatively basic protein (pI ~ 8) was induced > 50-fold by genistein. The 58kd apparent molecular weight of this protein, G58, is larger than predicted by the sequence of any known nodulation gene, including nodYABC, nodD, and nodZ. The high rate of G58 induction suggests it is not a HIL3-HIL6 gene product, and therefore probably represents an unknown symbiotic gene. Thus, it seems that B. japonicum has a relatively large number of nodulation genes, and that more probably remain undiscovered. The difficulty in locating genes which correspond to identified proteins, and the relative ease with which proteins can be studied once their

genes are cloned, suggested a genetic approach for studying host-induction of nodulation responses.

NodD complementation. In order to understand host flavonoid induction of the nod genes of B. japonicum, it was necessary first to establish the presumed functionality of the regulatory gene, nodD. As shown in Figure 6, page 51, the $nodD_1$ gene from B. japonicum complemented R. meliloti $nodD_1$::Tn5 to allow nodulation at an efficiency close to that of wild-type R. meliloti. This result demonstrates that the B. japonicum $nodD_1$ is functional in R. meliloti, and that it has a broad enough flavonoid specificity to recognize alfalfa flavonoids. This finding parallels the results of a similar study using the nodD gene from broad host-range Rhizobium MPIK3030 (Horvath et al. 1987). In this study the MPIK3030 nodD gene was transferred into R. meliloti, and was able to extend R. meliloti's host range to include siratro. The flavonoids in alfalfa root exudate which are presumably recognized by the B. japonicum $nodD_1$ are unknown, although luteolin can induce a B. japonicum nod-lacZ fusion ~ 2-fold (Banfalvi et al. 1988). This report also established the dependence of nodYABC induction on $nodD_1$. A slight induction by soybean seed extract (1.5-3 fold) is also suggested by these data in the absence of $nodD_1$, hinting that a second nodD gene may also be present in USDA110.

Host-Inducible Genes in B. japonicum

Because nodulation and nitrogen fixation genes tend to be

tightly clustered in the highly studied Rhizobium species, a ~ 22kb region surrounding the common nod genes was searched for symbiotic genes using translational lac-fusions. Since translational fusions must occur in-frame as well as in the correct orientation, less than one-sixth of all fusions will fuse a (i.e., nod) promoter to 'lacZ. Thus a large number (120) of fusion constructs were analyzed, and two new symbiotic loci were identified.

Tn3-lac Mutagenesis of pR32

The Tn3-lac fusion vehicle proved to be an effective, although not ideal, way of constructing symbiotic-reporter gene fusions. Thus, the majority of Tn3-lac insertions occurred in the pR32 insert DNA, and only ~ 2.5% of these caused adjacent deletions. Curiously, eight unique Tn3-lac insertions occurred in the nodYABC operon, however only two of these insertions were in the proper orientation (Figure 7, page 53) and were inactive and thus apparently out of frame. The reason for this failure is unclear, however, in vitro nodY-lacZ fusions were recently constructed in our laboratory (Banfalvi et al. 1988). Significantly, the nodY-lacZ fusion, the HIL3 fusion (DG69), and the HIL4 fusions (DG59, DG62, DG63) and the HIL5 and HIL6 fusions (Deshmane and Stacey, submitted) (see Figure 1, page 14) were all induced by soybean root exudate, and by the same spectrum of flavonoid compounds (Table 2, page 56), implying a common regulatory scheme for the three operons. Indeed, a functional $nodD_1$ gene product has been found to be essential for nodYABC, $nodD_1$, HIL5, and HIL6 induction. Numerous attempts to form homogenotes were made

using pR32::Tn3-lac constructs, however the innate resistance of USDA110 to ampicillin prevented selection of Tn3-lac in the B. japonicum chromosome.

Host-inducible locus 4 (HIL4). Mutants DG59 and DG62/DG63 represent a single host inducible locus (HIL4), or operon, and share a curious phenotype. These Tn3-lac fusions display a considerable basal level of activity which is induced 2-3 fold by genistein or soybean root exudate/seed extract (Table 2, page 56). Although these data must be considered imprecise due to the vagaries of gene-fusions mentioned earlier, the high basal level of activity suggests that HIL4 is expressed at a significant level in the absence of inducer. This moderate basal activity seems to be unique among nod genes. The large colony phenotype of these mutants is also independent of inducer, supporting this conclusion. Glutamate, which is required for large colony formation, has no effect on the B-galactosidase activity of DG62/DG63, and thus may be a substrate or substrate precursor involved in phenotypic expression.

It is unusual to see a "negative dominant" mutation, as occurs in DG59, DG62 and DG63. Since these strains carry a normal HIL4 operon as well as the HIL4::Tn3-lac plasmid-borne copy, one would expect the mutant HIL4 allele to be complemented by (recessive to) the normal HIL4 allele. A possible explanation for this discrepancy is suggested by negative dominant mutations in the E. coli uxuR and exuR genes (Ritzenthaler et al. 1985). The repressor proteins encoded by these genes reportedly function as multimers. Interaction

between normal and mutant repressor monomers apparently results in formation of inactive multimeric repressors, and the mutant uxuR and exuR alleles are thus negative dominant. If the HIL4 locus similarly encodes a repressor gene, it may function to repress extracellular polysaccharide synthesis like the psi gene of R. phaseoli which is necessary for nitrogen fixation (Borthakur et al. 1985).

Investigations of the symbiotic phenotype of HIL4-mutants indicate this locus is important during competition for soybean nodulation. B. japonicum HIL4::Tn5 mutant NAD27 nodulates axenically grown Glycine max 'Essex' seedlings much more slowly than USDA110 does (N. Deshmane, U.T. PH.D. thesis, 1988). Tests of soybean nodulation performed in natural soil (which contains competing B. japonicum strains) indicate that the HIL4 locus is required for nodulation of a particular restrictive soybean genotype, but not required to nodulate nonrestrictive genotypes such as 'Essex.' These apparently conflicting data serve to highlight the difference between nodulation assays performed under artificial, axenic conditions, and those performed in natural soil.

The importance of HIL4 in nodulating the restrictive soybeans suggests a practical application for this locus. Classical plant breeding shows that the soybeans which restrict nodulation by USDA123 do so by virtue of a single dominant gene (M. Sadowski and P. Cregan personal communication). USDA123 serotype strains tend to be inefficient nitrogen fixers, but are the predominant bradyrhizobia in Mid-western soils (Cregan and Keyser 1986). These USDA123 strains consistently outcompete commercial inoculum strains for nodulation of

common, nonrestrictive, soybean cultivars; however these strains appear to lack a functional HIL4 allele (M. Sadowski personal communication). Thus, by breeding this restrictive plant gene into commercial soybean cultivars and including the HIL4 locus in B. japonicum inoculum strains, we could restrict nodulation of soybean crops predominantly to the effective inoculum strain.

Although the precise function of HIL4 remains elusive, evidence suggests a role in regulating extracellular polysaccharide synthesis. This hypothesis is supported by the large colony phenotype of the negative dominant mutants. The ability of antibodies to distinguish between B. japonicum strains USDA110 and USDA123, the latter strain being incapable of nodulating the restrictive soybean genotype, further suggests that HIL4 influences the cell surface of USDA110. The dependence of DG59, DG62, and DG63 on glutamate for expression of the large colony phenotype is puzzling in light of the inability of related amino acids or dicarboxylic organic acids to substitute for glutamate. It may thus be relevent that glutamate is the best nitrogen source for inducing nitrogen fixation in free-living bradyrhizobia (Kaneshiro and Kurtzman 1982). Thus, glutamate may serve as a signal to B. japonicum to begin differentiating in preparation for nitrogen fixation. It is possible that glutamate is a precursor to extracellular polysaccharide synthesis, or a post-translational effector of the HIL4 gene product activity.

If we address the hypothesis that the HIL4 gene/s regulate

extracellular polysaccharide synthesis, it may be instructive to compare HIL4 to other rhizobial genes which influence polysaccharide synthesis. HIL4 bears some resemblence to the psi gene of R. leguminosarum 'Viciae', a gene which inhibits EPS synthesis and nodulation if present on a multicopy plasmid (Latchford et al. 1988). This phenotype can be suppressed by increasing the copy number of the pss gene, although these two genes do not appear to affect one another's regulation. Although HIL4, like psi, appears to suppress polysaccharide production, addition of multiple HIL4 copies (i.e. on pR32) do not have an obvious phenotypic effect on B. japonicum USDA110.

The syrM gene of R. meliloti is reminiscient of HIL4 in that both appear to be regulatory genes which affect EPS synthesis. Curiously, syrM$^-$ mutants have no apparent EPS phenotype or nodulation phenotype (S. Long, personal communication). If syrM and $nodD_3$ are multicopy in R. meliloti, however, EPS is overproduced, and the nod genes (i.e. nodABC) are expressed constitutively at a high level. Perhaps the presence of multicopy HIL4 genes would elicit a similar effect on expression of the B. japonicum nodYABC operon.

Host-inducible locus 3 (HIL3). Locus HIL3 is inducible by genistein (Table 2, page 56), implying a role in symbiosis, however little more is known about this operon. The lac-fusion data obtained using DG69 show an ~ 7-fold induction by genistein and a very low basal activity, suggesting it is not expressed at a significant level except in the plant's presence. Additional lac-fusion mutagenesis

(using Mud-lacIIPR13) and sequencing (S. Luka unpublished) are in progress in order to delimit the operon and identify the promoter (a degenerate nod box?) which sponsors its induction. Tn5 mutants flanking the DG69 insertion site have no apparent effect on soybean or siratro nodulation (N. Deshmane, U.T. Ph.D. thesis, 1988). In view of the nodulation phenotypes of the n4 and n5 regions, it seems possible that the HIL3 locus may also have a subtle, as yet unidentified function. Sequencing of the HIL3 locus and further mutagenesis should clarify whether the Tn5 insertions interrupt or narrowly miss HIL3 and whether HIL3 indeed has a cryptic function.

The flavonoid-inducible nature of the HIL3 and HIL4 loci begs the question: where are the nod boxes responsible for nodD protein binding leading to induction? Virtually all flavonoid-inducible genes identified to date are associated with nod boxes and conversely, deletion of either the 5' or 3' end of the nod box abolishes promoter activity/induction of downstream genes (Spaink et al. 1987). The exception to this rule is a pair of flavonoid-inducible genes which occur in R. fredii, a fast growing Rhizobium species found in China which nodulates soybeans (Sadowski et al. 1988). A 17 bp conserved sequence unrelated to the nod box does occur 5' to these two genes. This 17 bp motif is presumably the binding site for a regulatory protein analagous to nodD protein. Since the synthetic nod box oligonucleotide (Rostas et al. 1986) does not hybridize to the HIL3-HIL6 loci (M. Schell, unpublished), these loci must contain either degenerate nod boxes, or nonhomologous cis-acting sequences which have analogous functions (e.g. the 17 bp R.

fredii motif). Since two or three base pair changes in the nod box sequence can abolish hybridization, sequencing will be necessary to answer this question.

Host-Inducible Genes in R. meliloti

The unusually large size of the nod box promoter, and its sequence conservation among rhizobia have allowed identification of two additional regions from the Rhizobium meliloti nod-regulon using simple DNA hybridization techniques. This approach may be applicable to other systems as well, in order to elucidate coordinate gene expression and identify groups of genes with related biological functions. This hybridization technique has also been important for the identification of two loci which may have been overlooked by standard techniques (e.g. random Tn5 mutagenesis) due to the relatively subtle nodulation phenotypes of the n4 and n5 mutants.

The induction of the n4 and n5 regions confirms the postulated role of the nod box sequence as a symbiotic promoter. LacZ-fusions to both n4 and n5 regions were reproducibly induced by luteolin, daidzein, and alfalfa- or siratro seed exudate in an MPIK3030 background, consistent with the promiscuous nature of the MPIK3030 nodD gene (Horvath et al. 1987). The two inducible lac-fusions downstream of both n4 and n5 probably define single transcriptional units (operons) associated with each nod box. The 6 to 7-fold induction of the n4 and n5 regions (Table 3, page 70) was quantitatively similar, despite the higher basal activity of the n5 fusions. This difference may be the result of the occurrence of the

n4- and n5-*lac* fusion constructs in two different replicons.

R. species MPIK3030 was used as host for the fusions because it sponsors considerable induction of *nod*-*lac* fusions without the addition of extra *nodD* copies as required in *R. meliloti* (Mulligan and Long 1985). Deletion of the n2/n3 *nod* boxes preceding the AK631 *nodFEG* and *nodH* genes does disrupt alfalfa nodulation (Rostas et al. 1986), suggesting that *nod* boxes are functionally important for *nod* gene expression in *R. meliloti* AK631. The possibility that AK631 exercises an additional regulatory control over induction is under investigation.

The directions of transcription and approximate sizes of the n4 and n5 nodulation loci can be inferred from the positions of host-inducible Mud-*lac* insertions and nodulation-impaired Tn*5* insertions. The n4 operon is transcribed rightward, including the host-inducible n4-351 and n4-584 Mu-*lac* insertion sites. Consistent with this transcriptional direction, the Tn*5* mutations n4-53, n4-90, and n4-81, share a similar delayed nodulation phenotype. Mutation n4-84, which is 4.2kb downstream of the *nod* box, has shown a small delay in nodulation (see Figure 12a, page 72, and Table 4, page 74) in each of three experiments. It is still unclear, however, whether this mutation occurs in the same operon as upstream mutations.

The n5 locus is transcribed leftwards from n5 as shown by Mu*d*-*lac* insertions n5-132 and n5-597. Tn*5* insertions n5-64 and n5-68 occur close to the inducible n5-Mu*d*-*lac* insertions and cause delayed nodulation of alfalfa and sweetclover. It is possible that mutation

n5-56 also occurs within this nodulation operon. Repeated nodulation tests using n5-56 and a total of 76 alfalfa seedlings, produced variable results (see Figure 12, page 72) although the number of nodules per plant was consistently lower than formed by the wild-type. Although relatively short nodulation delays are seen for n4 and n5 mutants, one cannot rule out the possibility that such mutations may have more profound effects on nodulation (e.g. competitive ability, host specificity, nodulation in soil) under other conditions.

Interestingly, a mutation (n4-90) in the n4 locus which impairs alfalfa nodulation, appears to increase nodulation of sweetclover (Table 4, page 74). This result suggests that the n4 locus confers upon AK631 host-specificity for alfalfa.

Renalier et al. (1987) recently reported on several deletions in the R. meliloti 2011 symbiotic plasmid which define a delayed nodulation locus linked to a reiterated nitrogen fixation (fix) locus. The restriction map of this region is nearly identical to that of the AK631 n5 region and the delayed nodulation phenotypes of mutations in these loci corroborate this identification. Thus, our data generally supports that of Renalier et al. (1987), who delimited this nod locus to roughly the same region. We were, however, unable to detect an increase in root hair curling by the n5-mutant as might be expected from the report by Renalier et al. (1987), possibly due to the different bacterial strains and plant cultivars employed.

The n5 locus appears to be conserved among "fast growing" Rhizobium species whereas the n4 locus is probably unique to

Rhizobium meliloti strains. The strong hybridization of the n5-region probe to R. leguminosarum 'trifolii' and R. leguminosarum 'Viciae' suggests that this operon is conserved among Rhizobium species. Thus it is hoped that the homologous genes will be cloned from other rhizobia for further study. The very weak hybridization to Bradyrhizobium species may reflect evolutionary divergence between these genera, or may be merely coincidental. In USDA110 this hybridization appears to occur to a 3.8kb band in pUT14, a position distantly linked to the common nod genes.

The n4-downstream nodulation locus shows no significant homology to other rhizobia, suggesting it may have a function specific to nodulation of one or more R. meliloti hosts. In R. meliloti, the n4-containing fragment hybridizes to a @ 5kb fragment in addition to the expected 3.8kb, 3.5kb, and 0.6kb EcoRI bands (Figure 13, page 77, lanes 1,2). The significance of this @ 5kb fragment is unknown, however it is not present within the @ 100kb nod-nif region represented in Figure 9, page 62. The effect of n4-region Tn5 mutations in delaying nodulation of alfalfa, but not sweetclover, indicates that this region may have host-specific character, similar to the nodFEG and nodH operons described previously (Horvath et al. 1986). Thus host specificity appears to be a relatively complex phenomenon, determined to varying degrees by several nodulation operons. Alfalfa-specific nodulation, for example, appears to rely on nodFEG and nodH, the n4 locus, and one or more nodD genes which can recongize the particular flavonoids

secreted by alfalfa roots (Horvath et al. 1986, this report, Gyorgypal et al. 1988, Horvath et al. 1987).

Summary

In summary, this work is intended to clarify how rhizobia respond to molecular signals from their plant hosts. The 2-D protein gels reveal the complexity of B. japonicum's response to soybean root exudate, and the relative simplicity of its responses to soybean seed lectin and genistein. Proteins responding to lectin and genistein were thus identified. If technical improvements occur allowing isolation of sufficient amounts of host-inducible proteins for eliciting antibodies or N-terminal microsequencing, the corresponding genes may be identified.

Two new host flavonoid-inducible operons were also identified using molecular genetics techniques. No function is known for locus HIL3, however HIL4 seems to play a vital role in genotype-specific soybean nodulation, perhaps by regulating production of extracellular polysaccharides. Mutagenesis of the HIL3 locus is in progress. By evaluating the nodulation of these HIL3-mutants on various hosts of B. japonicum the presumed symbiotic function of this locus may be learned. Sequencing of the HIL3 and HIL4 loci is also in progress (S. Luka and M. Sqdowski, personal communication). Analysis of these sequences will reveal whether inducibility is sponsored by degenerate nod boxes or by unrelated promoter sequences, and may yield clues as to the biochemical functions of these genes.

Two new flavonoid-inducible operons were also identified in R.

meliloti. The n5 locus is functional during nodulation of alfalfa and sweetclover, whereas the n4 operon favors alfalfa nodulation. Future work on the n4 and n5 operons by DNA sequencing and interspecies complementation may yet elucidate the biochemical and symbiotic functions of these genes. Finally, a complementation experiment using *B. japonicum* and *R. meliloti* helped to define the role of *B. japonicum* $nodD_1$ in nodulation. It is hoped that this work will spur further basic research towards understanding plant-microbe interactions and nodulation; and will ultimately facilitate the exploitation of nitrogen fixing symbioses for Man's benefit.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ames, G.F. and Nikaido, K. 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry 15:616-623.

Bachem, C.W., Banfalvi, Z., Kondorosi, E., Schell, J. and Kondorosi, A. 1986. Identification of host-range determinants in the Rhizobium species MPIK3030. Mol. Gen. Genet.203:42-48.

Banfalvi, Z., Randhawa, G.S., Kondorosi, E., Kiss, A. and Kondorosi, A. 1983. Construction and characterization of R-prime plasmids carrying symbiotic genes of R. meliloti. Mol. Gen. Genet. 189:129-135.

Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L. and Stacey, G. 1988. Regulation of nod gene expression in Bradyrhizobium japonicum. Mol. Gen. Genet.

Bassam, B.J., Rolfe, B.G. and Djordjevic, M.A. 1986. Macroptilium atropureum (siratro) host specificity genes are linked to a nodD-like gene in the broad host range Rhizobium strain NGR234. Mol. Gen. Genet. 203:49-58.

Bauer, W.D. 1981. Infection of legumes by rhizobia. Ann. Rev. Plant Physiol. 32:407-449.

Beringer, J.E. 1974. Transfer of the drug-resistance transposon Tn5 to Rhizobium. J. Gen. Microbiol. 84:188-189.

Bhuvaneswari, T.V. and Bauer, W.D. 1978. Role of lectins in plant-microorganisms. III. Influence of rhizosphere/rhizoplane culture condition on the soybean lectin-binding properties of rhizobia. Plant Physiol. 62:71-74.

Bhuvaneswari, T.V., Pueppke, S.G. and Bauer, W.D. 1977. Role of lectins in plant-microorganism interactions. I. Binding of Soybean lectin to rhizobia. Plant Physiol. 60:486-491.

Bishop, P.E., Guevara, J.G., Engelke, J.A. and Evans, H.J. 1976. Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between Rhizobium japonicum and Glycine max. Plant Physiol. 57:542-546.

Bohlool, B.B. and Schmidt, E.L. 1974. Lectins: a possible basis for specificity in the Rhizobium-Legume root nodule symbiosis. Science 185:269-271.

Borthakur, D., Downie, J.A., Johnston, A.W.B. and Lamb, J.W. 1985. psi, a plasmid-linked Rhizobium phaseoli gene that inhibits exopolysaccharide production and which is required for symbiotic nitrogen fixation. Mol Gen. Genet. 200:278-282.

Carlson, R.W., Kalembasa, S. and Garcia, F. 1988. The expression of R. phaseoli CE3 lipopolysaccharide (LPS) O-antigen in R. leguminosarum 128C53. In proceedings of the 7th International Congress on N_2 Fixation (Bothe, H., deBruijn, F.J., and Newton, W.E., eds.) p. 471.

Casadaban, M. 1975. Fusion of the Eschericia coli lac genes to the ara promoter: A general technique using Mu-1 insertions. Proc. Nat. Acad. Sci. USA 72:809-813.

Castilho B.A., Olfson P. and Casadaban M.J. 1984. Plasmid insertion mutagenesis and lac gene fusion with mini-Mu Bacteriophage Transposons. J. Bacteriol. 158(2):488-495.

Cregan, P.B. and Keyser, H.H. 1986. Host restriction of nodulation by Bradyrhizobium japonicum strain USDA123 in soybean. Crop Science 26:911-916.

Dart J. 1974. The infection process. in:The Biology of Nitrogen Fixation. Quispel A, ed. pp381-429. Amsterdam Holland.

Dazzo, F., Hollingsworth, R., Hollingsworth, S.P., Robeles, M., Olen, T. Salzwedel, J., Djordjevic, M. and Rolfe, B. 1988. Recognition process in the Rhizobium trifolii--white clover symbiosis. In proceedings of the 7th International Congress on N_2 Fixation (Bothe, H., DeBruijn, F. J. and Newton, W. E., eds.). p. 431.

Dazzo, F.B. and Gardiol, A.E. 1984. Host specificity in Rhizobium-legume interaction, In D. P. S. Verma and T. H. Hohn (eds.), Genes involved in microbe-plant interactions. Springer Verlag, Berlin. pp. 3-31.

Dazzo, F.B. and Hubbell, D.H. 1975a. Antigenic differences between infective and noninfective strains of Rhizobium trifolii. Applied Microbiol. 30:172-177.

Dazzo, F.B. and Hubbell, D.H. 1975b. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Applied Microbiol. 30:1017-1033.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. 1980. Broad host range cloning system for gram negative bacteria: construction of a gene bank of Rhizobium meliloti. PNAS-USA 77(12):7347-7351.

Downie, J.A., Hombrecher, G., Ma, Q., Knight, C.D., Wells, B. and Johnston, A. 1983. Cloned nodulation genes of Rhizobium leguminosarum determine host-range specificity. Mol. Gen. Genet. 190:359-365.

Downie, J.A. and Johnston, A. 1986. Nodulation of legumes by Rhizobium: the recognized root? Cell 47:153-154.

Duncan, R. and Hershey, J.W.B. 1984. Evaluation of isoelectric focusing conditions during two-dimensional isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis: variation of gel patterns with changing conditions and optimized bioelectric focusing conditions. Anal. Bioch. 138:144-155.

Ellingboe, A.H. 1984. Genetics of host-parasite relations: an essay. Adv. in Plant Pathol. 2:131-151.

Etzler, M.E. 1983. Introduction. in: Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins. Etzler, M.E., and Goldstein, I.J. eds. Liss Publ. New York, N.Y.

Fahreus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. 16:374-381.

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

Fisher, R.F., Egelhoff, T.T., Mulligan, J.T. and Long, S.R. 1988. Rhizobium meliloti nodD binds to DNA sequences upstream of inducible nodulation genes. In abstracts of 7th International Congress on N Nitrogen Fixation, Cologne, FRG.

Geremia, R.A., Caraignac, S., Zorreguita, A., Toro, N., Olivares, J. and Ugalde, R.A. 1987. A Rhizobium meliloti mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form B-1,2,glucan. J. Bacteriol. 169:880-884.

Gottfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F. and Kondorosi, A. 1986. At least two nodD genes are necessary for efficient nodulation of alfalfa by Rhizobium meliloti. J. Mol. Biol. 191:411-420.

Gyorgypal, Z., Iyer, N. and Kondorosi, A. 1988. Three regulatory nodD alleles of diverged flavonoid-specificity are involved in host-dependent nodulation by Rhizobium meliloti. Mol. Gen. Genet. 212:85-92.

Hahn, M. and Hennecke, H. 1988. Cloning and Mapping of a novel nodulation region from Bradyrhizobium japonicum by genetic complementation of a deletion mutant. Appl. Environ. Microbiol. 54:55-61.

Halverson, L.J. and Stacey, G. 1984. Host recognition in the Rhizobium-soybean symbiosis. Plant Physiol. 74:84-89.

Halverson, L.J. and Stacey, G. 1986a. Plant-Microbial Communication. Microbiol. Rev. 50:193-225.

Halverson, L.J. and Stacey, G. 1986b. Effect of lectin on nodulation by wild-type Bradyrhizobium japonicum and a nodulation-defective mutant. Appl. Env. Microbiol. 51(4):753-760.

Halverson, L.J. and Stacey, G. 1986c. Effect of lectin on nodulation by wild-type Bradyrhizobium japonicum and a nodulation-defective mutant. Appl. Environ. Microbiol. 51:753-760.

Hombrecher, G., Gotz, R., Dibb, N.J., Downie, A.J., Johnston, A. and Brewin, N.J. 1984. Cloning and mutagenesis of nodulation genes from Rhizobium leguminosarum TOM, a strain with extended host range. Mol. Gen. Genet. 194:293-298.

Horvath, B., Bachem, C.W.B., Schell, J. and Kondorosi, A. 1987. Host-specific regulation of nodulation genes in Rhizobium is mediated by a plant signal, interacting with the nodD gene product. EMBO J. 6:841-848.

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Torok, I., Gyorgypal, Z., Barabas, I., Wieneke, U., Schell, J. and Kondorosi, A. 1986. Organization, structure, and symbiotic function of Rhizobium meliloti nodulation genes determining host-specificity for alfalfa. Cell 46:335-343.

Innes, R.W., Kuempel, P.L., Plazinski, J., Canter-Cremers, H., Rolfe, B. and Djordjevic, M.A. 1985. Plant factors induce expression of nodulation and host-range genes in Rhizobium trifolii. Mol. Gen. Genet. 201:426-432.

John, M., Schmidt, J., Wieneke, U., Krussmann, H.-D. and J. Schell, J. 1988. Transmembrane orientation and receptor-like structure of the Rhizobium meliloti common nodulation protein nodC. EMBO J. 7:583-588.

John, M., Schmidt, J., Wieneke, U., Kondoresi, E., Kondorosi, A. and Schell, J. 1986. Expression of the nodulation gene nodC of Rhizobium meliloti in Eschericia coli: Role of the nodC gene product on nodulation. EMBO J. 4(10) 2425-2430.

Kamberger, W. 1979. An Ouchterlony double diffusion study on the interaction between legume lectins and rhizobial cell surface antigens. Arch. Microbiol. 121:83-90.

Kaneshiro, T. and Kurtzman, M.A. 1982. Glutamate as a differential nitrogen source for the characterization of acetylene-reducing Rhizobium strains. J. Appl. Bacter. 52:201-207.

Keen, N.T. 1982. Specific recognition in gene-for-gene host-parasite systems. Adv. Plant Pathol. 1:35-82.

Knauf, V.C. and Nester, E.W. 1982. Wide host range cloning vectors: A cosmid clone bank of an agrobacterium Ti plasmid. Plasmid 8:45-54.

Kondorosi, P., Banfalvi, Z. and Kondorosi, A. 1984. Physical and genetic analysis of a symbiotic region of Rhizobium meliloti: identification of nodulation genes. Mol. Gen. Genet. 193:445-452.

Latchford, J.W., Hawkins, F., Barkes, R.D. and Johnston, A. 1988. The role of exopolysaccharide in nodulation of peas by Rhizobium. in Proceedings of the 7th International Congress on N_2 Fixation (Bothe, H., deBruijn, F.J. and Newton, W.E., eds.) p. 493.

Leigh, J.A., Signer, E.R. and Walker, G.C. 1985. Exopolysaccharide-deficient mutants of Rhizobium meliloti that form ineffective nodules. Proc. Nat. Acad. Sci.-USA 82:6231-6235.

Lewin, A., Rosenberg, C., Meyer, H., Wong, C.H., Nelson, I., Manen, J.F., Stanley, J., Dowling, D.N., Denarie, J. and Broughton, W.J. 1987. Multiple host-specificity loci of the broad host-rage Rhizobium sp. NGR234 selected using the widely compatible legume Vigna unguiculata. Plant Mol. Biol. 8:447-459.

Lugtenburg, B. 1988. Regulation of nod gene expression and nodulation. Presented at the 7th International Congress on N_2 Fixation. Koln, W. Germany.

Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Press, New York.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Press, New York.

Mulligan, J.T. and Long, S.R. 1985. Induction of Rhizobium meliloti nodC expression by plant exudate requires nodD. PNAS-USA 82:6609-6613.

Nieuwkoop, A.J., Banfalvi, Z., Deshmane, N., Gerhold, D., Schell, M., Sirotkin, K.M. and Stacey, G. 1987. A locus encoding host range is linked to the common nodulation genes of Bradyrhizobium japonicum. J. Bacteriol. 169(6):2631-2638.

Norrander, J., Kempe, T. and Messing, J. 1983. Improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26:101-106.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 256:4007-4021.

Pueppke, S.F. 1984. Adsorption of slow- and fast-growing rhizobia to soybean and cowpea roots. Plant Physiol. 75:924-931.

Pueppke, S.G., Freund, T.G., Schultz, B.C. and Friedman, H.P. 1980. Can. J. Microbiol 26:1489-1497.

Putnoky P. and Kondorosi A. 1986. Two gene clusters of Rhizobium meliloti code for early essential nodulation functions and a third influences nodulation efficiency. J. Bacteriol. 167(3):881-887.

Puvanesarajah, V., Schell, F.M., Gerhold, D. and Stacey, G. 1987. Cell surface polysaccharides from Bradyrhizobium japonicum and a nonnodulating mutant. J. Bacteriol. 169:137-141.

Quinones, F.R., Banfalvi, Z., Murphy, P. and Kondorosi, A. 1987. Interspecies homology of nodulation genes in Rhizobium. Plant Molecular Biology 8:61-75.

Quinto, M. and Bender, R.A. 1984. Use of bacteriophage P1 as a vector for Tn5 insertion mutagenesis. Applied Environ. Microbiiol. 47:436-438.

Ratet, P., Schell, J. and deBruijn, F.J. 1988. Mini-Mulac transposons with broad-host-range origins of conjugal transfer and replication designed for gene regulation studies in Rhizobiaceae. Gene 63:41-52.

Recourt, K., VanBrussel, A., Driegsen, A. and Lugtenberg, B. 1988. The uptake of the nod gene inducer naringenin by Rhizobium leguminosarum. in: Proceedings of the 7th Intl. Congress of Nitrogen Fixation. Bothe, H., deBruijn, F. and Newton, W. (eds.)

Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. 1986. Flavones induce expression of nodulation genes in Rhizobium. Nature 323:632-635.

Renalier, M., Batut, J., Ghai, J., Terzaghi, B., Gherardi, M., David, M., Garnerone, A., Vasse, J., Truchet, G., Guguet, G. and Boistard, P. 1987. A new symbiotic cluster on the pSYM megaplasmid of Rhizobium meliloti 2011 carries a functional fix gene repeat and a nod locus. J. Bacteriol. 169:2231-2238.

Ritzenthaler, P., Blanco, C. and Mata-Gilsinger, M. 1985. Genetic analysis of uxuR and exuR monomer repressors interactions. Mol. Gen. Genet 199:507-511.

Rossen, L., Shearman, C.A., Johnston, A. and Downie, J.A. 1985. The nodD gene of Rhizobium leguminosarum is autoregulatory and in the presence of plant exudate induces the nodA,B,C genes. EMBO J. 4:3369-3373.

Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A. and Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in Rhizobium. PNAS-USA 83:1757-1761.

Russell, P. Schell, M.G., Nelson, K.K., Halverson, L.J., Sirotkin, K.M. and Stacey, G. 1985. Isolation and characterization oft he DNA region encoding nodulation functions in Bradyrhizobium japonicum. J. Bacteriol. 164:1301-1308.

Ruvkun, G. and Ausubel, F. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature 289: 85-88.

Sadowski, M.J., Olsen, E.R., Foster, V.E., Kosslak, R. and Verma, D.P. 1988. Two host-inducible genes of Rhizobium fredii and characterization of the inducing compound. J. Bacteriol 170:171-178.

Selvaraj, G., Fong, Y.C. and Iyer, V.N. 1984. A portable DNA sequence carrying the cohesive site (cos) of bacteriophage lambda and the mob (mobilization) region of the broad-host-range plasmid RK2: A module for the construction of new cosmids. Gene 32:235-241.

Shearman, C.A., Rossen, L., Johnston, A.W.B. and Downie, J.A. 1986. The Rhizobium leguminosarum nodulation gene nodF encodes a polypeptide similar to acyl-carrier protein and is regulated by nodD plus a factor in pea root exudate. EMBO J. 5:647-652.

Smit, G., Tubbing, D., Kijne, J. and Lugtenberg, B. Role of Ca in the activity of rhicadhesin, isolated from Rhizobium leguminosarum biovar viciae, which mediates the first step in attachment of Rhizobiaceae cells to plant root hair tips. Submitted.

Smit, G., Logman, T., Boerrigter, M.B., Kijne, J.W. and Lugtenberg, B. Purification and partial characterization of the Ca-dependent adhesin from Rhizobium leguminosarum, which mediates the first step in attachment of Rhizobiaceae cells to plant root hairs. Submitted.

Smit, G., Kijne, J.W. and Lugtenberg, B. Roles of flagella, lipopolysaccharide, and a Ca-dependent cell surface protein in attachment of Rhizobium leguminosarum to pea root hair tips. Submitted.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spaink, H.P., Okker, R., Wijffelman, C.A., Pees, E. and Lugtenberg, B. 1987. Promoters in the nodulation region of the Rhizobium leguminosarum SYM plasmid pRL1JI. Plant Mol. Biol. in press.

Spaink, H.P., Wijffelman, C.A., Dees, E., Okker, R. and Lugtenberg, B. 1987. Rhizobium nodulation gene nodD as a determinant of host specificity. Nature 328:337-340.

Stacey, G., Pocratsky, L.A. and Puvanesarajah, V. 1984. Bacteriophage that can distinguish between wild-type Rhizobium japonicum and a non-nodulating mutant. Appl. Environ. Microbiol. 48:68-72.

Stachel, S.E., An, G., Flores, C. and Nester, E.W. 1985. A Tn3 lacZ transposon for the random generation of B-galactosidase gene fusions: application to the analysis of gene expression in Agrobacterium. EMBO J. 4(4):891-898.

Strosberg, A.D., Buffard, D., Kominski, P.A., Chapot, M.P., Rossow, P.W. and Foriers, A. 1986. Lectin multigene families in leguminous and nonleguminous plants. In: Molecular Biology of Seed Storage Proteins and Lectins, Shannon, L. M. and Chrispeels, M. J., eds.

Tsien H.C. and Schmidt E.L. 1980. Accumulation of soybean lectin-binding polysaccharide during growth of Rhizobium japonicum as determined by hemagglutination inhibition assay. Appl. Environ. Microbiol. 39:1100-1104.

Tsien, H.C. and Schmidt, E.L. 1981. Localization and partial characterization of soybean lectin binding polysaccharide of Rhizobium japonicum. J. Bacteriol. 145:1063-1074.

Vanderckhove, J.F., Bauw, G., Puype, M., Van Damme, J. and Van Montagu, M. 1985. Protein blotting on polybrene-coated glass-fiber sheets. Eur. J. Bioch. 152:9-19.

Vesper, S.J. and Bauer, W.D. 1985. Characterization of Rhizobium attachment to soybean roots. Symbiosis 1:139-162.

Vincent, J.M. 1980. Factors controlling the legume-Rhizobium symbiosis. in: Nitrogen Fixation II Newton WE and Orme-Johnston eds. pp103-129. University Park Press, Baltimore.

Vodkin, L.O., Rhodes, P.R. and Goldberg, R.B. 1983. cA lectin gene insertion has the structural features of a transposable element. Cell 34:1023-1031.

Wacek, T.J. and Brill, W.J. 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci. 15:519-523.

Wolpert, J.S. and Albersheim, P. 1976. The lectins of legumes interact with the O-antigen-containing lipopolysaccharides of their symbiont rhizobia. Bioch. Biophys. Res. Comm. 70(3)729-737.

Wright, K. and Northcote, D.H. 1974. The relationship of root-cap slimes to pectins. Biochem. J. 139:525-534.

VITA

David Gerhold was born Dec 15, 1960 in Bellefonte, Pennsylvania. He was educated in State College, PA and gained an interest in plants through growing houseplants and Bonsai. After earning a B.S. at Penn State University, he started graduate school at Michigan State University. After gaining an interest in nodulation from Dr. Frank Dazzo at MSU, he transferred to work in Dr. Gary Stacey's Lab to learn molecular biology. A year spent in Dr. Kondorosi's lab broadened his cultural experiences, and taught him a lesson in practicality in scientific research.

His interests have included plant insect resistance, and the nodulation process, as indicated below in published work. David will now do postdoctoral work on plant pathogens with Dr. Noel Keen in Riverside California. A career is planned discovering how plants "recognize" harmful microbes and respond to protect themselves.

PUBLICATIONS

Gerhold D.L., Craig R. and Mumma R.D. 1984. Analysis of Trichome Exudate from Mite-Resistant Geraniums. J. Chem. Ecology 10:713-722.

Gerhold D.L., Dazzo F.B., and Gresshoff P.M. 1985. Selective Removal of Seedling Root Hairs for Studies of the Rhizobium-Legume Symbiosis. J. Microbiol. Methods 4:95-102.

Craig R., Mumma R.O., Gerhold D.L., Winner B.L., and Snetsinger R. 1985. Genetic Control of a Biochemical Mechanism for Mite Resistance in Geraniums. in Natural Resistance of Plants to Pests. pp168-177.

Puvanesarajah V., Schell F.M., Gerhold D., and Stacey G. 1987. Cell Surface Polysaccharides from Bradyrhizobium japonicum and a Nonnodulating Mutant. J. Bacteriol. 169:137-141.

Nieuwkoop A.J., Banfalvi Z., Deshmane N., Gerhold D., Schell M.G., Sirotkin K.M., and Stacey G. 1987. A Locus Encoding Host Range is Linked to the Common Nodulation Genes of Bradyrhizobium japonicum. J. Bacteriol. 169:2631-2638.

Gerhold D., Stacey G., and Kondorosi A. 1988. Use of a Promoter-Specific Probe to Identify Two New Loci from the Rhizobium meliloti Nodulation Regulon Plant Mol. Biol. in press.

Gerhold D., and Stacey G. Recent Advances in Molecular Biology Techniques for Studying Symbiotic Microbes. in Biotechnology of Plant-Microbe Interactions. Hagedorn C. and Nakas J. eds., in press.