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I am submitting herewith a dissertation written by Joyce Pui-yee Yuen entitled "Factors Affecting Nodulation Gene Regulation in *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:

Associate Vice Chancellor and
Dean of The Graduate School

FACTORS AFFECTING NODULATION GENE REGULATION IN *Bradyrhizobium japonicum*

A Dissertation

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Degree

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This dissertation is dedicated to my God,
my parents, Kwok-leung Yuen and Sai-tim Yu,
my husband-to-be, Frank Tsai, brothers and sisters in my family and
in the church, and my friends. They have given me
invaluable support, encouragement,
and inspiration for the study.

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ABSTRACT

This study explored the role of various chemical and physical factors in regulating nodulation gene expression in *B. japonicum*. In addition, experiments examined the expression of NodD1 and its physiological importance to *Bradyrhizobium japonicum*. Two xanthones - 1,3,7-trihydroxyxanthone and 1,6-dihydroxy-2,8-dimethoxyxanthone - were found capable of inducing *nodD1* and *nodYABC* gene expression. This is the first report that xanthone compounds can be *nod* gene inducers. When certain organic acids such as L-malate, succinate, fumarate, acetate, α-ketoglutarate, and oxaloacetate, were added to the cultures, *nodD1* and *nodYABC* gene expression was significantly repressed. This inhibition was specific to *nod* gene expression and was not due to chelation of Ca^{2+} ions or pH change. This repression affected *nod* gene induction mediated by both NodD1 and NodV/NodW. L-malate and acetate added together to cultures exerted an additive inhibition exceeding 90%. A remarkable level of L-malate inhibition of *nod* gene expression occurred when cells were grown or resuspended in medium containing D-gluconate, L-glutamate, and yeast extract as the carbon and nitrogen sources. In contrast, lower or no inhibition occurred in cells grown or resuspended in medium containing glycerol as carbon source. It is believed that intracellular accumulation of L-malate, which occurred when cells did not readily utilize this organic acid, accounts for the different response of cells to L-malate. Other results showed that induction of *nodYABC* expression was suppressed when cells were grown under low oxygen tension. Furthermore, *nod* gene expression was tested at different pH values in RDY and MM. Nearly complete inhibition of *nod* gene expression in RDY occurred at pH 8.5 and pH 5.5

or lower in the presence or absence of L-malate. β-galactosidase activity in bacteroids containing a chromosomal *nodC-lacZ* fusion showed that *nod* gene expression was suppressed in bacteroids even though *nod* gene inducers were present. Since bacteroids *in planta* are exposed to an abundance of organic acids under microaerobic and acidic (pH 5.5 to 6.0) conditions, these results suggest that these nodule-specific conditions account for the suppression of *nod* gene expression in bacteroids.

The inducibility of *nod* gene expression was found higher in cells harvested from earlier growth phase or when induced at lower initial cell densities. Surprisingly, it was found that expression of NodD1 occurred in uninduced cultures at absorbance (A_{600nm}) values greater than 0.4. These results suggest *nodD1* expression may be regulated by a quorum sensing mechanism at higher cell densities. Elevated NodD1 expression may account for lower inducibility of cells at later growth phase and at higher cell densities.

Finally, it was found that NodD1 is important for the normal growth of *B. japonicum*. Mutation of *nodD1* resulted in a slower growth rate and lower cell viability. This effect was not due to defective production of Nod factors.

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CHAPTER 1

INTRODUCTION

Intensive research attention has been focused on the *Rhizobium*-legume symbiosis for about half a century. This interest is attributed not only to the ecological and economical importance of the biological nitrogen fixation process (90×10^6 ton N per year) (Siqueira *et al.*, 1991) to agriculture and forestry, but also to the fact that the symbiotic relationship is itself scientifically attractive. Through a complex series of developmental steps, the symbiont bacteria and the host plants influence each other in activities such as signal transduction, gene regulation and expression, cell division, metabolic function, cell morphogenesis, etc. This cell-cell interaction, which brings about a broad range of biochemical, genetic, and cellular activities, provides a valuable ground for studies of bacterial genetics, plant molecular biology, and cell biology. Although considerable knowledge is already available regarding the mechanism of nodule formation, much remains to be discovered of the molecular and biochemical aspects of this process.

Legume Diversity, and the Taxanomy and Host Specificity of *Rhizobium* Sp.

Nitrogen-fixing symbiosis with rhizobia is restricted to plants of the family Leguminosae (Fabaceae), which is the third largest family in the Angiosperms. The only

exception is the genus *Parasponia*, a non-legume plant of the family Ulmaceae (Trinick and Galbraith, 1976) that can be nodulated by rhizobia. The Leguminosae family comprises three subfamilies, Caesalpinioideae, Mimosideae, and Papilionoideae, each of which contains genera able to form root nodules (Allen and Allen, 1981; Polhill and Raven, 1981). Leguminous plants are very diverse in morphology, habitat, and ecology, ranging from Arctic annuals to tropical trees (de Faria *et al.*, 1989)

Bacteria that are able to establish N_2-fixing symbioses with leguminous plants are Gram-negative soil bacteria belonging to five genera - *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium, Mesorhizobium, and Sinorhizobium* - in the family Rhizobiaceae. The use of modern methods of bacterial systemics, such as numerical taxonomy, nucleic acid hybridization, and 16S rRNA analysis, however, has shown that there is genetic diversity among these genera (Young *et al.*, 1991). *Rhizobium* species are fast growing with a doubling time of 2 to 4 hours which produce acidic products from mannitol catabolism. They have many of their symbiotic genes localized on large plasmids, and induce indeterminate nodules on their host plants (Elkan, 1992). In contrast, *Bradyrhizobium* species are slow growing with a doubling time of 7 to 20 hours, produce alkaline products from mannitol catabolism. They have their symbiotic genes localized in the chromosome, and induce determinate nodules on their host plants (Elkan, 1992). *Rhizobium* and *Bradyrhizobium* are only distantly related (Martinez-Romero, 1994). The genus *Azorhizobium* is unique in that it can elicit stem and root nodules on its host plant and is able to fix nitrogen under symbiotic and free-living conditions (de Bruijn et al., 1988).

The current taxonomic classification of rhizobia is shown in Table 1. The species names of the bacteria are mostly related to their corresponding host plant. This reflects the fact that symbiotic interaction is host specific. The degree of host specificity varies from having a very narrow host range, like *Rhizobium leguminosarum* bv. trifolii., to having a very broad range, like *Rhizobium* sp. strain NGR234 (Young and Johnston, 1989).

Determination of Host Specificity

A specific *Rhizobium* strain only establishes a symbiosis with certain hosts (Table 1). The establishment of a host-specific symbiosis involves several factors - which include signal exchange and cellular recognition - that are contributed by both the rhizobia and the host plant.

Host-Specific Determination by Host Plant Flavonoids

Early studies on the effect of phenolic compounds present in legume seeds and root exudates found that these compounds can be stimulatory or toxic to nodulation (Rao *et al.*, 1973; Bhagwat and Thomas, 1982). At low concentrations, some compounds, such as the flavone, luteolin, and isoflavonoids, were found to serve as specific chemoattractants to certain rhizobia (Bergman *et al.*, 1988; Kape *et al.*, 1991; Dharmatilake and Bauer, 1992). Using nodulation gene fusions to the *E. coli lacZ* gene, Rossen et al. (1985) and Mulligan and Long (1985) showed that *nod* gene expression was

Table 1. Taxonomic classification of rhizobial species and their host plants (modified from van Rhijn and Vanderleyden, 1995).

Rhizobial species	Host plants
Rhizobium meliloti	*Medicago, Melilotus,* and *Trigonella spp.*
Rhizobium leguminosarum	
bv. viciae	*Pisum, Vicia, Lathyrus,* and *Lens* spp.
bv. trifolii	*Trifolium* spp.
bv. phaseoli	*Phaseolus vulgaris*
Rhizobium huakuii	*Astragalus sinicus*
Rhizobium ciceri	*Cicer arietinum*
Rhizobium sp. strain NGR234	Tropical legumes, *Parasponia spp.* (non-legume)
Rhizobium tropici	*Phaseolus vulgaris, Leucaena* spp., and *Macroptilium* spp.
Rhizobium etli	*Phaseolus vulgaris*
Rhizobium galegae	*Galega officinalis, G. orientalis*
Rhizobium fredii	*Glycine max, G. soja,* and other legumes
Mesorhizobium loti[a]	*Lotus* spp.
Bradyrhizobium japonicum	*Glycine max, G. soja,* and other legumes
Bradyrhizobium elkanii	*Glycine max, G. soja,* and other legumes
Bradyrhizobium sp. strain *Parasponia*	*Parasponia* spp.
Bradyrhizobium sp. strain *Lupinus*[b]	*Lupinus* spp.
Bradyrhizobium sp. strain *Vigna*[c]	*Macroptilium, Vigna* spp.
Azorhizobium caulinodans	*Sesbania* spp. (stem nodulating)
Sinorhizobium saheli[d]	*Sesbania* spp.
Sinorhizobium teranga bv. *acaciae*[e]	*Acacia* spp.
Sinorhizobium teranga bv. *sesbaniae*[f]	*Sesbania* spp.

[a] Stacey, G., personal communication

[b,c] Ronson, C. W., personal communication

[d, e, f] Boivin, C., personal communication

induced by plant phenolic compounds. Consequently, the use of transcriptional and translational *nod* gene fusions in different rhizobial species established a broad list of inducing compounds able to specifically activate *nod* gene expression (Table 2). These *nod* gene inducers are mostly flavonoid compounds and vary among rhizobial hosts nodulated by different microsymbionts.

Host-Specificity Determination by Rhizobia

The rhizobial endosymbionts, also take part in controlling nodulation and host specificity. The nodulation genes (*nod*, *nol*, and *noe* genes) that determine host specificity are classified into three groups: (1) the regulatory *nodD* genes which activate the expression of the other nodulation genes in response to compounds secreted by the host plant, (2) the common *nodABC* genes which are found in all rhizobial species and are functionally conserved between these species, and (3) the host-specific *nod* (*hsn*) genes, such as *nodH, nodL,* or *nodFE* in *R. meliloti*, which determine host range. Mutations in the *hsn* genes generally result in an alteration of the host spectrum. In addition, transfer of these genes has been shown to be able to extend the host range of the recipient rhizobial species (Debellé et al., 1995).

NodD is an important determinant of host specificity. It was found that some mutations in *nodD* cannot be complemented by a NodD from other *Rhizobium* species An example is the *R. meliloti nodD1* gene. It is required for nodulation of alfalfa, and it cannot complement the *nodD* mutation of NGR234 to allow nodulation of siratro

Table 2. Inducers for *nod* genes in Rhizobial species (modified from Siqueira et al., 1991).

	nodABC inducers *	*nodD* inducers
Rhizobium		
meliloti	a - Luteolin, 7,4'-dihydroxyflavone, galagin b - Narigenin, eriodictyol, 7,4'-dihydroxyflavanone d - Chrysoeriol, 4,4'-dihydroxy-2'-methoxychalcone, luteolin-glycoside, apigenin-glycoside, formononetin-7-O-glycoside	d- Trigonelline, stachydrine, 4,4'-dihydroxy-2'-methoxychalcone
leguminosarum		
bv. trifolii	a - Luteolin, chrysin, apigenin, 7,4'-dihydroxyflavone, 7-hydroxyflavone, 7,8-dihydroxyflavone b - Narigenin, eriodictyol, hesperetin, kaempferol, 7,4-dihydroxyflavonone, 4',7-dihydroxyflavonone, taxifolin, 3-methoxy-taxifolin, homoeriodicytol, pinocembrin c - 5,7-dihydroxyisoflavone d - Luteolin-glycoside, eriodictyol-glycoside, narigenin-glycoside, apigenin-glycoside	
bv. viciae	a- Luteolin, chrysin, apigenin, , 7-hydroxyflavone b- Narigenin, eriodictyol, hesperetin, 7,3'-dihydroxy-4'-methoxyflavanone, 3,5,7,3'-hydroxy-4'-methoxyflavanone d- Luteolin-glycoside, apigenin-7-O-glucoside,	
bv. phaseoli	b- Narigenin, eriodictyol, myricetin, quercetin, kaempferol c- Genistein d- Coumestrol, delphinidin, petunidin, malvidin	
fredii	c- Daidzein, genistein	
Bradyrhizobium		
japonicum	a- Apigenin b- Kaempferol, 4',7-dihydroxyflavonone c- Daidzein, genistein, formononetin, biochanin A, 5,7-dihydroxyisoflavone, prunetin, genistin, 7-hydroxyisoflavone d- Coumestrol	a- Apigenin b- Genistein, diadzein, biochanin A, genistin, formononetin d- 6"-O- malonyldaidzin, 6"-O-malonylgenistin, ?"-O-acetyldaidzin

* a: flavones; b: flavonones/flavonols; c: isoflavones; d: miscellaneous (other phenolic compounds or glycosidic conjugates of class a, b, and c.

(Horvath et al., 1987). This lack of complementation is due to the inability of the *R. meliloti* NodD1 to respond to the signals exuded by siratro. In fact, each rhizobial species contains a NodD which interacts optimally with the flavonoids produced by its host plant. Extension of nodulation ability to non-host plants can be achieved by the transfer of foreign or mutated *nodD* genes. For example, this was shown in the transfer of the *nodD1* gene from *Rhizobium* sp. strain NGR234 to *R. meliloti*, resulting in the ability of *R. meliloti* to nodulate siratro (Horvath et al., 1987). In addition, some point mutations in the *nodD* gene of *R. trifolii* can also extend the host range of this strain to the nonlegume *Parasponia* (Mclver et al., 1989). A *nodD* hybrid gene was constructed by Spaink et al. (1989a) from *R. meliloti* and *R. trifolii nodD* genes. This hybrid NodD allowed extension of host range to tropical legumes. This result showed that the various NodD proteins, though divergent in plant signal specificity, have a common ability to activate *nod* genes. Flavonoid signal recognition by the NodD homologs, therefore, provides a primary level of host specificity (Spaink et al., 1987b). Conceivably, the broadness of the host range of a rhizobial species usually correlates with the spectrum of flavonoid specificity of its NodD protein. NodD proteins from narrow-host-range rhizobia, like *R. meliloti*, *R. leguminosarum* bv. viciae, and *R. leguminosarum* bv. trifolii, respond to a few flavonoids, whereas the NodD from the broad-host-range *Rhizobium* NGR234 has a larger spectrum of inducing compounds, including the monocyclic aromatic compounds vanillin and isovanillin (Györgypal et al., 1991b).

The common and host specificity *nod* genes are involved in the synthesis of chito-lipooligosaccharides (i.e. the Nod factors) which act as host-specific symbiotic

signals during infection and nodule formation (Dénarié and Cullimore, 1993). The Nod factor backbone consists of a β-1,4-linked *N*-acetyl-*D*-glucosamine oligosaccharide varying in length from three to five sugars and substituted by an *N*-acyl chain at the non-reducing end (Carlson et al., 1994). Its synthesis is directed by the NodA, NodB, and NodC proteins, which are common in all rhizobial strains. NodA, NodB, and NodC are thought to function as an *N*-acyl transferase, a chito-oligosaccharide deacetylase, and an *N*-acetylglucosaminyltransferase, respectively (Rohrig, et al., 1994; John et al., 1993; Geremia et al., 1994).

Although the *nodABC* genes are present in all rhizobial strains, they are not readily interchangeable among all rhizobial strains. For example, a *nodA* mutant of *R. meliloti* and *R. tropici* cannot be complemented by the *nodA* gene from one another because their NodA proteins respectively *N*-acylate their Nod factors with different fatty acids; i.e., C16:1 or C16:2 for *R. meliloti* and C18:1 for *R. tropici* (Debellé et al., 1995). Likewise, a *nodC* mutant of *R. meliloti* and *R. tropici* cannot be complemented by *nodC* from the other strain because the NodC protein in *R. meliloti* synthesizes a major Nod factor of four glucosamine residues whereas, in *R. tropici*, the NodC protein synthesizes a major Nod factor of five glucosamine residues (Debellé et al., 1995). These results show that the common *nodABC* genes are not functionally conserved between all rhizobial species. In certain rhizobial strains, *nodA* and *nodC* could be important determinants of host range.

In addition to the length of the Nod factor backbone, the presence of different Nod factor substitutions, which are controlled by the *hsn* genes present in different species and

strains, are also important in host range determination. Host-specific *nod* genes can be non-allelic or allelic and, in most cases, mutations cannot be fully complemented by the introduction of the corresponding genes from other rhizobia. Most mutations result in an alteration or extension of the host range.

Non-allelic variation of host-specific *nod* genes is frequently observed among rhizobia. For example, the *nodH* gene is found in *R. meliloti*, but not in *R. leguminosarum* bv. *viciae*. Mutations in *nodH* prevent *R. meliloti* from nodulating alfalfa, its normal host, but enable it to nodulate vetch, a host *of R. leguminosarum* bv. viciae (Horvath et al., 1986). When *nodH* is transferred to both *R. leguminosarum* bv. viciae and bv. trifolii, they gain the ability to nodulate alfalfa (Faucher et al., 1989). This shows that the *nodH* gene is an important host range determinant. Genetic and biochemical studies showed that NodH is a sulfotransferase responsible for the sulfation of *R. meliloti* Nod factors (Roche et al., 1991). Non-sulfated Nod factors produced by *nodH* mutants are active on vetch but not on alfalfa. Therefore, sulfate substitution is important for Nod factor activity on alfalfa. However, *R. tropici* produces sulfated Nod factors (Poupot et al., 1993), but does not nodulate alfalfa. Major differences between the Nod factors from these two species are the following: The *R. tropici* Nod factors (1) are not *O*-acetylated, (2) have vaccenic acid (C18:1) as *N*-acyl chain instead of a C16 unsaturated fatty acids as in *R. meliloti*, and (3) have a backbone of chitin pentamers. Among these features, *O*-acetylation was found important for nodulation of alfalfa by *R. meliloti*. When *nodL*, which encodes an acetyltransferase (Bloemberg et al., 1994) is

transferred to *R. tropici*, the transconjugant is able to produce *O*-acetylated Nod factors and nodulate alfalfa.

Some of the host range genes are present as allelic variants in different rhizobial strains. For examples, the *nodFE* genes found in *R. meliloti* and *R. leguminsarum* bv. viciae play an important role in the control of host specificity in both species. In spite of their highly conserved nucleotide sequences, these genes are not functionally conserved between the two species because mutations in *R. meliloti nodFE* cannot be complemented by the *R. leguminosarum* bv. viciae *nodFE* genes. NodFE proteins specify the synthesis of acyl chains, which contribute to the specificity of the Nod factors (Demont et al., 1993).

The activity of Nod factors can also be determined by the presence of plant enzymes involved in the metabolism of the Nod factor. Staehelin et al. (1994) have shown that the Nod factors of *R. meliloti* are rapidly inactivated in the rhizosphere of alfalfa by the action of chitinases and that the rate of degradation depends on the structural modification of the Nod factor. The presence of a sulfate group on the *O*-6 position of the reducing end of the *R. meliloti* Nod factor strongly protects it against degradation by purified chitinases and intact plant roots (Staehelin et al., 1994). The finding that the Nod factors are substrates for plant chitinases suggests the difference in rates at which Nod factors are degraded might be an important determinant in host specificity (Schultze et al., 1993).

Infection and Nodulation Mechanism

The establishment of a nitrogen-fixing symbiosis between rhizobia and legumes is a complex, multi-step process. The initial nodulation steps are characterized as a two-way molecular conversation. The host legume releases signal compounds that stimulate the coordinate expression of bacterial genes (*nod*, *nol*, and *noe*) that are required for nodulation. These genes, in turn, encode enzymes involved in the synthesis of Nod factors that cause morphological changes in the plant roots.

Rhizobia are rhizosphere bacteria. They are found in much higher numbers in the rhizosphere of plants than in soil. Data indicate that *B. japonicum* cells grow very slowly in soil with a doubling time of 241 to 361 hours (Schmidt, 1974). However, those found in the rhizosphere have a doubling time of 9 to 12 hours (Bowen and Rovira, 1976). The rhizosphere stimulates rhizobial growth. Additionally, the higher cell numbers in the rhizosphere could also be due to chemoattraction of rhizobia to roots. Rhizobial cells can be chemoattracted to compounds released from legume roots. These compounds had been identified as amino acids, sugars, organic acids, and plant secondary metabolites, such as the *nod* gene inducers, flavonoids (Caetano-Anollé et al., 1988a; Aguilar et al., 1988; Barbour et al., 1991), and *nod* gene inhibitors, such as umbelliferone and acetosyringone (Aguilar et al., 1988). Crude root exudates are usually found to be the best chemoattractants (Gittie et al., 1978; Hunter and Fahring, 1980; Barbour et al., 1991).

Data from Caetano-Anollé et al. (1988b) and Mellor et al. (1987) support the notion that chemotaxis plays an important role in early colonization. Motility and chemotaxis were found to be determinants of competition between bacterial strains and to affect the efficiency of nodulation. However, chemotaxis is not essential for nodulation because flagellum-deficient mutants are still able to nodulate normally (Liu et al., 1989; Malek, 1992).

Rhizobial attachment is considered to be the first step of infection. Attachment to the root hair surface appears to involve multiple mechanisms. Reports have shown that host plant lectin (Halverson and Stacey, 1985), rhizobial fimbriae (Vesper and Bhuvanewari, 1988), and cellulose microfibrils (Smit et al., 1987) are important for effective attachment. The effect of these mechanisms vary with respect to the physiological conditions (growth phase and nutrient limitation) of the rhizobial cells (Roth and Stacey, 1991). Effective attachment may be a host-specific step. However, this has not yet been demonstrated conclusively.

At the surface of the root or very probably from a distance, rhizobia cause root hair branching, deformation, and curling into a "shepherd's crook" (van Brussel et al., 1986). These phenomena are also observed with the supernatants of induced rhizobia cultures. In all *Rhizobium*-plant interactions studied, the active substances in the supernatant have been identified as Nod factors, which are synthesized by means of rhizobial nodulation genes (Carlson et al., 1994).

In legumes, the region most suspectible to *Rhizobium* infection is just behind the apical meristem at the site of emerging root hairs (Bhuvaneswari et al., 1981). In the

"shepherd's crook", bacteria are entrapped in a pocket of the host cell wall. After entrapment, a local lesion of the root hair cell wall is formed by hydrolysis. The mechanism of hydrolysis of the cell wall is not known. It could be that the bacteria induce hydrolytic enzymes that are responsible for localized cell wall dissolution, or the bacteria exploit plant mechanisms such as those used when epidermal cells grow out into root hairs (Kijne, 1992). Rhizobia enter the roots at the sites where root hair cell walls are hydrolyzed. The penetration occurs by invagination of the plasma membrane. The host plant reacts by depositing new cell wall material around the lesion in the form of an inwardly growing tube. The tube is filled with proliferating bacteria surrounded by a matrix and becomes an infection thread. The infection thread grows toward the inner tangential wall of the root hair cell tip by a process of tip growth. Concomitantly with formation of the infection thread, specific cortical cells divide to form a nodule primordium, and the infection thread grows towards these primordia and ramifies into the plant cortex until it reaches a host cell, into which the bacteria are released. The bacteria released by exocytosis into the plant cell are contained in symbiosomes, the functional unit for nitrogen fixation (Roth et al., 1988). The symbiosome membrane [(SBM) or peribacteroid membrane (PBM)] acts as the interface between the symbiotic partners across which signals and metabolites are exchanged. It may also prevent the "intracellular" bacteria from inducing a plant defense response (Nap and Bisseling, 1990a; Werner, 1992). The metabolites exchanged through the SBM include ammonium, the product of nitrogen fixation, and heme, the prosthetic group of the oxygen transport protein leghemoglobin, which are exported by the bacteroids to the host cytoplasm.

Similarly, carbon sources and probably also assimilated ammonia are supplied by the host to the bacteroids (Werner, 1992; Guan et al., 1995). In soybean and alfalfa, cell division in the cortex can occur prior to the invasion of the root hairs. The root cortical cells through which infection threads will pass on their way to the nodule primordia change markedly in preparation for infection thread penetration (van Brussel et al., 1992).

There are two types of nodule structures which can be distinguished by the location of the nodule primordia and the plant species. In general, in temperate legumes such as pea, vetch, and alfalfa, the primordium is formed from cells in the inner cortex (Dudley et al., 1987). These legumes form indeterminate cylindrical nodules, which have a persistent apical meristem (Guan et al., 1995). This persistent meristem allows nodule elongation, since new cells are constantly added to the distal end of nodule. While the meristem is active, rhizobia are released from the infection threads into the plant cell cytoplasm (Brewin, 1991). The differentiation of the bacteria and the host plant cells leads to an establishment of a central zone of the nodule, in which nitrogen is reduced (Nap and Bisseling, 1990a). Thus, in indeterminate nodules, nodule growth and functioning occur simultaneously, and all intermediates in differentiation can be observed in a single longitudinal section of a nodule.

On the other hand, in most tropical legumes, such as soybean and French bean, nodules have a determinate growth pattern. A nodule meristem is induced in the root outer cortex, and the bacteria are released into actively dividing meristematic cells, of which each daughter cell receives rhizobia. Meristematic activity is restricted to a relatively short period (Guan et al., 1995). Following a round of successive divisions, the

invaded meristematic cells differentiate simultaneously to form the nitrogen-fixing central tissue. As a result of this developmental pathway, nodule growth and function are dissociated. Determinate nodules do not elongate but enlarge, thus only a single stage of plant and bacterial differentiation can be observed at any particular moment.

Besides the formation of infection threads through root hairs, some rhizobia may enter through cracks in the epidermis. In legumes such as *Arachis hypogaea* (peanut) and *Stylosanthes* spp., rhizobia infect their host by "crack entry". In the presence of rhizobia, cell divisions are induced in the cortex and epidermal cells enabling entry and intercellular spread of rhizobia. No infection threads are formed. Rhizobia colonize the root apoplast, possibly by cell wall digestion, and the progressive collapse of outer root cells. Continuous host cell divisions result in development of a uniformly infected central tissue resembling the determinate nodule type (Sprent and de Faria, 1988).

Stem and root nodules of *Sesbania rostrata* are induced following crack entry by *A. caulinodans* at the base of dormant root primordia, which are present in rows along the length of stem, or at the base of secondary roots in the case of root nodules (Ndoye et al., 1994). Direct intercellular infection is followed by very active multiplication of the bacteria, forming wide intercellular spaces filled with infection threads which spread into the meristematic zone induced in the cortex. The subsequent release of bacteria into the cytoplasm of newly induced meristematic cells leads eventually to the development of determinate nodules (Ndoye et al., 1994).

A third mode of infection is observed in *Mimosa scabrella*, a tropical tree, where the rhizobial infection sites are at junctions of epidermal cells (de Faria et al., 1988). The

bacteria penetrate the radial walls and proliferate intercellularly. Occasionally, subepidermal root hairs are formed upon inoculation, but they are never infected. Penetration of outer root cells starts from irregular cell wall ingrowth and progresses in the cortical region through the primary wall layer, rather than by separating cells at the middle lamella. This usually results in elicitation of a plant host defense response. However, rhizobia can be released into the cells of the developing nodule meristem, and a normally indeterminate nodule is formed (de Faria et al., 1988).

The route of infection is characteristic for the host because the same bacteria can penetrate different host species by either crack entry, infection through intact epidermis cells, or root hair infection threads. A given legume is infected by the same type of mechanism regardless of the infecting strain (Rolfe and Gresshoff, 1988). Similarly, the structural and developmental characteristics of an efficient nodule are specified by the plant and not by the rhizobial strain, indicating that the host possesses the genetic information for symbiotic infection and nodulation and that the role of the bacteria is to switch on this plant development program (Dénarié et al., 1992).

Function and Regulation of Bacterial Genes involved in Nodulation and Symbiosis

The establishment of a *Rhizobium*-legume symbiosis is a process involving the reciprocal exchange of signals between micro- and macro-symbionts in a sequential fashion resulting in the development of a highly specialized organ, the nodule. Within the nodule, nitrogen fixation takes place. Generally, two approaches are employed to

identify bacterial genes involved in symbiosis. The first approach is random mutagenesis, usually by Tn*5* (Beringer et al., 1987). Complementation of the mutants defective in nodule formation led to the identification of rhizobial genes, which are named according to the functions they serve in different stages of symbiosis. These genes include *nod*, *nol*, *noe*, *ndv*, *exo*, *fix*, and *nif* genes (Long, 1992). Table 3 shows the phenotype of bacteria with mutations in each gene class. The linkage maps of *nod*, *nol*, *fix*, *nif*, and other related genes of *R. meliloti*, *R. leguminosarum* bv. viciae, and *B. japonicum* are shown in Figure 1 (Sanjuan et al., 1993).

Rhizobial *nod*, *nol*, and *noe* Genes

nod, *nol*, and *noe* genes are nodulation genes whose products are involved in the early steps of nodule formation. Of those genes characterized, most are involved in the production of plant growth regulating substances, Nod factors, that induce root hair curling and cortical cell division (Fisher and Long, 1992). Nod factors are considered to be the main *Rhizobium* nodulation signal molecules, since the purified molecules are able to induce many of the plant responses observed in early stages of symbiosis (Truchet et al., 1991). Nod factors from several species of rhizobia are able to elicit root hair deformation and induce nodule primordia in a host-specific way, indistinguishable from the nodule meristem in the first stage of normal nodule organogenesis (Heidstra et al.,

Table 3. Phenotype of bacterial mutants of *nod*, *nol*, *noe*, *ndv*, *exo*, *fix*, and *nif* genes (Modified from Long et al., 1992).

Gene Name	Phenotype in Mutant
nod, *nol*, *noe*	Failure to synthesize signal molecule(s) such as Nod factors; no nodule formation; nodule delay; host range change
ndv, *exo*, *lps*	Nodule development defective; surface polysaccharide or metabolic changes; empty nodules form
fix	Infected nodules form; no symbiotic N_2 fixation; may get some N_2 fixation in free-living cells
nif	Infected nodules form; no symbiotic N_2 fixation; no N_2 fixation in free-living cells

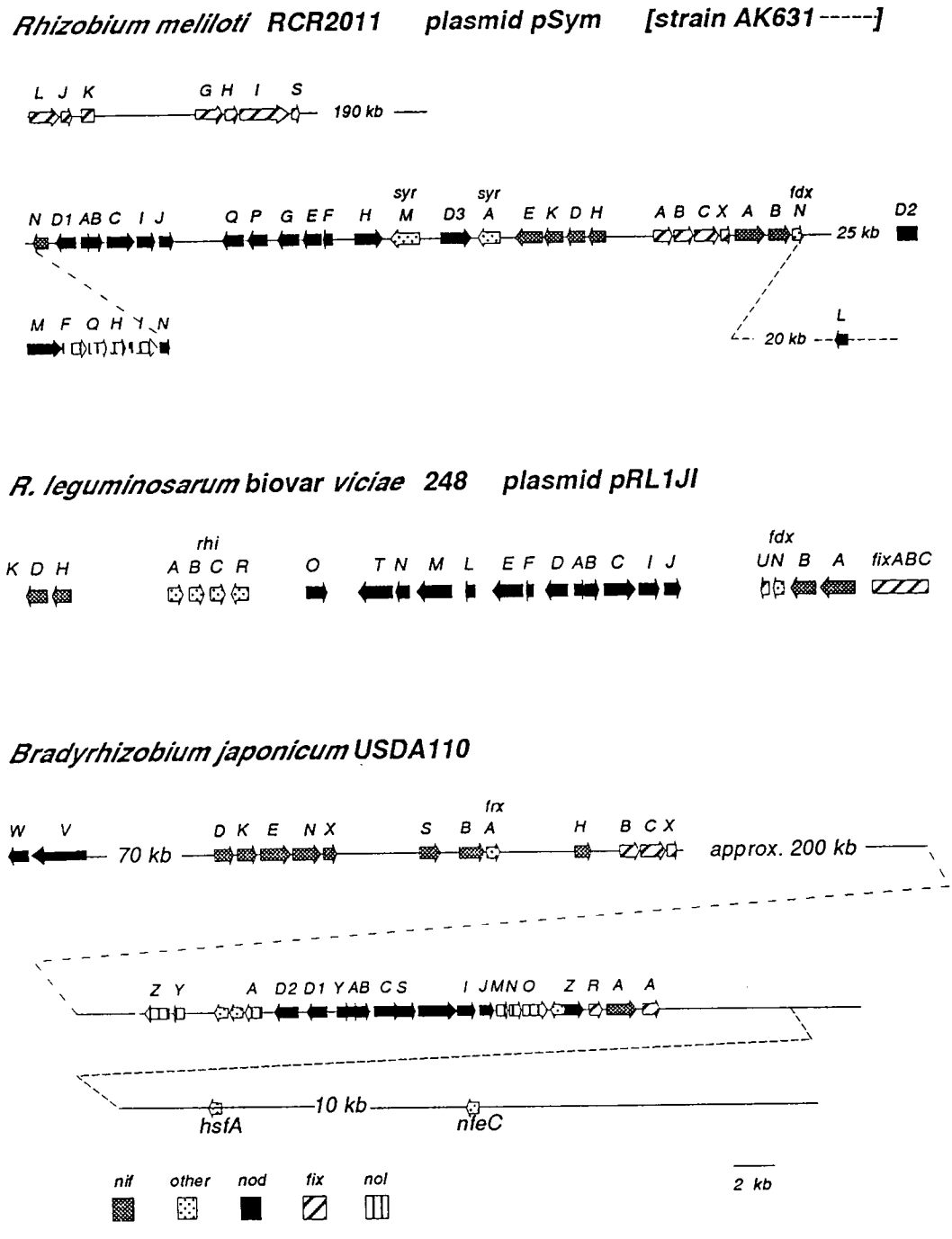


Figure 1. Linkage map of *nif, fix*, and *nod* genes of *R. meliloti, R. leguminosarum* bv. viciae, *B. japonicum* (modified from Sanjuan et al., 1993).

1994; Lerouge et al., 1990; Relic et al., 1993; Sanjuan et al., 1992; Spaink et al., 1991; Truchet et al., 1991). Nodule-like structures following treatment with Nod factors have been observed with soybean (Stokkermans and Peters, 1994), siratro (Relic et al., 1993), bean (Martinez et al., 1993), and *Sesbania* species (Mergaert et al., 1993). However, purified Nod factors are not able to promote the formation of genuine infection threads, but certain effects of the Nod factors have been observed which suggest that they are involved in the infection process. The formation of pre-infection thread structures is observed after treatment of *Vicia* roots with the Nod factors of *R. leguminosarum* bv. viciae (van Brussel et al., 1992). Another indication comes from the observation that the transcription of some early nodulins, which are specifically expressed in the infection thread, is also induced with isolated Nod factors (Horvaths et al., 1993; Journet et al., 1994; Nap and Bisseling, 1990b; Scheres et al., 1990a).

The first indication of the involvement of the *nod* genes in the synthesis of Nod factors came from the work of van Brussel and colleagues (van Brussel et al., 1986; van Brussel et al., 1990; Zaat et al., 1987; Zaat et al., 1988). They reported that sterile culture supernatants from *R. leguminosarum* bv. viciae cultures elicited a thick and short root phenotype on seedlings of common vetch. Banfalvi and Kondorosi (1989) reported that the expression of the *R. meliloti nodABC* genes in *E. coli* resulted in the release of substances into the culture supernatants, which could induce root hair curling on alfalfa and a few other plants.

Since the first report on the importance of the nodulation genes in nodule formation (Long et al., 1982), more than 50 nodulation genes have been identified in

various rhizobia and most of their gene products have been characterized (Table 4). In most cases, the expression of these *nod* genes only occurs in the presence of exudates or extracts of the host plant (reviewed by Long, 1989) and the transcriptional activator, NodD. In *B. japonicum*, a two-component system NodV and NodW is also involved (Sanjuan et al., 1994; Loh et al., 1997). The plant phenolic compounds that are responsible for this induction belong to the flavonoid family. The first such inducer identified was luteolin from alfalfa seed extracts, capable of inducing a *nodC-lacZ* fusion in *R. meliloti* (Peter et al., 1986). Subsequently, other flavonoid compounds that would specifically induce the *nod* genes in other *Rhizobium* and *Bradyrhizobium* species were identified (Zaat et al., 1987; Zaat et al., 1988; Djordjevic et al., 1987; Rossen et al., 1985; Kosslak et al., 1987; Maxwell et al., 1989; Göttfert et al., 1988; Hartwig et al., 1990; Banfalvi et al., 1988). In most *Rhizobium* species studied so far, the *nod* genes reside on large symbiotic plasmids (pSym) that also carry the *nif* and *fix* nitrogen-fixing genes (Martinez et al., 1990). In *R. loti*, *Bradyrhizobium* and *Azorhizobium* spp., the symbiosis-related genes are localized on the chromosome (Martinez et al., 1990).

Rhizobial *exo*, *lps*, and *ndv* Genes

The other class of genes that are essential for infection and nodule formation include the *exo*, *lps*, and *ndv* genes. *exo* genes determine the synthesis of exopolysaccharides (Gray and Rolfe, 1990). *lps* genes control lipopolysaccharide synthesis (Noel, 1992). The *ndv* gene products are involved in the synthesis of capsular

Table 4. Functions and properties of nodulation genes (summarized from Stacey, 1990; Dénarié et al., 1992; Fisher and Long, 1992; Meinhardt et al., 1993; Carlson et al., 1994; van Rhijn and Vanderleyden et al., 1995; Bellato et al., 1997; Hanin et al., 1997; Quesada-Vincens et al., 1997; Lohrke et al., 1998).

nod genes	Species biovar[a]	Possible functions and properties
nodA	All	*N*-Acyltransferase, required for nodulation
nodB	All	Chitooligosaccharide de-*N*-acetylase, required for nodulation
nodC	All	UDP-*N*-Acetylglucosaminyltransferase, required for nodulation
nodD	All	*nod* gene transcription activator, LysR family
nodE	Rl, Rt, Rm	β-Ketoacyl synthase, host-range determinant
nodF	Rl, Rt, Rm	Acyl carrier protein, host-range determinant
nodG	Rm	Alcohol dehydrogenase, β-ketoacyl reductase, host-range determinant
nodH	Rm	Sulfotransferase, host-range determinant
nodI	Rl, Rt, Rm, Re, Ac, Bj, Bp	ABC cassette transport protein for Nod factor secretion
nodJ	Rl, Rt, Rm, Re, Ac, Bj, Bp	ABC cassette transport protein for Nod factor secretion
nodK	Bp	Unknown
nodL	Rl, Rt, Rm, Bp	*O*-Acetyltransferase, host-range determinant for Rl
nodM	Rl, Rt, Rm, Bp, Bj	*D*-Glucosamine synthase, Nod factor subunit synthesis
nodN	Rl, Rt, Rm, Bp	Host-range determinant
nodO	Rl	Hemolysin, Ca^{2+}-binding excreted proteins, mediates early stage of nodulation
nodP	Rm	ATP-sulfurylase, host-range determinant
nodQ	Rm	Adenosine-5'-phosphosulfate (APS) kinase, host-range determinant
nodR	Rtr	Host-range determinant
nodS	Re, Rtr, Rf, Bj, NGR, Ac	*S*-Adenosyl methionine methyltransferase, possible host-range determinant
nodT	Rl, Rt	Transit sequences, host-range determinant
nodU	Re, Rtr, Rf, Bj, NGR, Ac	Carbamoyl transferase
nodV	Bj, Ba	Sensor, two-component regulatory family, host-range determinant
nodW	Bj, Ba	Regulator, two-component regulatory family
nodX	Rl*	Acetyl transferase, host-range determinant
nodY	Bj	Unknown
nodZ	Bj, NGR	Fucosyl transferase, host-range determinant

Table 4. (continued)

nod genes	Species biovars	Possible functions and properties
nolA	Bj	DNA-binding protein, host-range determinant
nolB	Rf	Host-range determinant
nolC	Rf	Sequence similarity to the heat-shock protein DnaJ
nolD	Rm	Host-range determinant
nolE	Rm, Rp	Host-range determinant
nolF	Rm	Host-range determinant
nolG	Rm	Host-range determinant
nolH	Rm	unknown
nolI	Rm	unknown
nolJ	Rf	unknown
nolK	Ac	Sugar epimerase
nolM	Bj	unknown
nolN	Bj	Nod factor production
nolO	Bj	Nod factor production
nolP	Rp	unknown
nolR	Rm	*nod* gene transcription repressor
nolT	Rf	Host-range determinant, sequence similarity to *hrp* genes
nolU	Rf	Host-range determinant
nolV	Rf	Host-range determinant
nolW	Rf	Host-range determinant, sequence similarity to *hrp* genes
nolX	Rf	Host-range determinant, sequence similarity to *hrp* genes
nolY	Bj	unknown
nolZ	Bj	unknown
noeD	Bj	Host-range determinant, genotype specific
noeE	NGR	Host-range determinant

[a] *nod/nol* genes are present in *R. leguminosarum* bv. viciae (Rl), *R. leguminosarum* bv. trifolii (Rt), *R. leguminosarum* bv. phaseoli (Rp), *R. meliloti* (Rm), *R. tropici* (Rtr), *R. etli* (Re), *R. fredii* (Rf), *Rhizobium* sp. strain (NGR), *B. japonicum* (Bj), *Bradyrhizobium* sp. strain *Parasponia* (Bp), *Bradyrhizobium* (*Arachis*) sp. strain N92 (Ba) (Gillette and Elkan, 1996), *A. caulinodans* (Ac), and *R. leguminosarum* bv. viciae TOM (Rl*).

polysaccharide or K antigens, and β-1,2-glucans (Reuhs et al., 1993). Mutations in these genes result in defective nodule development (Long, 1992). A possible role of *exo* and *lps* genes in the determination of host specificity has been suggested, but there is no unequivocal evidence showing that *Rhizobium* surface components are major determinants of host-range specificity (Reuber et al., 1991).

Rhizobial *nif* and *fix* Genes

The rhizobial *nif* and *fix* genes were identified due to their importance for forming Fix^+ nodules or fixing nitrogen in free-living cells (Long, 1992). Rhizobial *nif* genes are structurally homologous to some of the *K. pneumoniae nif* genes. Therefore it is inferred that a conserved *nif* gene plays a similar role in rhizobia as in *K. pneumoniae*. At least ten different rhizobial *nif* genes have been identified in *R. meliloti, B. japonicum,* and *A. caulinodans*. Rhizobial genes which are not homologous to *nif* genes of *K. pneumoniae* but are essential for symbiotic nitrogen fixation are by convention named *fix* genes. The functions of *nif* and *fix* genes that have been identified in *R. meliloti*, *B. japonicum*, and *A. caulinodans* are shown in Table 5.

All of the *nif* and *fix* genes, and other related genes whose expression is also dependent on oxygen status are under the control of either the NifA or FixK transcriptional activator (Table 6). It is currently uncertain whether the *fixNOQP* genes are essential for nitrogen fixation in all rhizobia. For example, mutations in these genes in *R. meliloti* and *B. japonicum* result in a Fix^- phenotype. However, the inactivation of

Table 5. *nif* and *fix* genes identified in *R. meliloti* (Rm), *B. japonicum* (Bj), or *A. caulinodans* (Ac) and their known or proposed functions (modified from Fischer, 1994).

Gene	Product and/or (proposed) function
nif genes	
nifH	Fe protein of nitrogenase
nifD	α-Subunit of MoFe protein of nitrogenase
nifK	β-Subunit of MoFe protein of nitrogenase
nifE	Involved in FeMo cofactor biosynthesis
nifN	Involved in FeMo cofactor biosynthesis
nifB	Involved in FeMo cofactor biosynthesis
nifS (in Bj)	Cysteine desulfurase; activation of sulfur for metallocluster synthesis
nifW (in Ac)	Unknown function; required for full activity of FeMo protein
nifX	Unknown function
nifA	Positive regulator of *nif*, *fix*, and additional genes
fix genes	
fixABCX	Unknown function; required for nitrogenase activity; FixX show similarity to ferredoxins
fixNOQP	Microaerobically induced, membrane-bound cytochrome oxidase
fixGHIS	Redox process-coupled cation pump
fixIJ	Oxygen-responsive two-component regulatory systems involved in positive control of *fixK* (Rm, Bj, Ac) and *nifA* (Rm)
fixK/fixK2	Positive regulator of *fixNOQP* (Rm, Bj, Ac), *nifA* (Ac), *rpoN1*, and nitrate respiration (Bj); negative regulator of *nifA* and *fixK* (Rm)
fixK'(in Rm)	Reiterated, functional copy of *fixK*
fixK1(in Bj)	*fixK* homolog of unknown function; not essential for nitrogen fixation
fixR (in Bj)	Unknown function; not essential for nitrogen fixation
nfrA (in Ac)	Regulation of *nifA*

Table 6. *nif*, *fix*, and other genes under control of NifA or FixK.

	nif genes	*fix* gene	Other genes
		Under control of NifA	
R. meliloti	*nifHDKE, nifN, nifB*	*fixABCX*	*fdxN, mos, nfe*
B. japonicum	*nifDKENX, nifS, nifH*	*fixBCX, fixA*	*glnII, ndp, groES, groEL*
A. caulinodans	$nifH_1DKE$, $nifH_2$, *nifW*	*fixABCX*	/
		Under control of FixK	
R. meliloti	/	*fixNOQP, fixGHIS*	/
B. japonicum	/	*fixNOQP, fixGHIS*	Genes for nitrate respiration
A. caulinodans	/	*fixNOQP*	/

fixNOQP in *A. caulinodans* decreases symbiotic nitrogen fixation by only two-fold. The different phenotypes suggest that there is no one unique, conserved, oxidase system supporting nitrogen fixation in all rhizobia but rather that there are different respiratory chains and terminal oxidases supporting nitrogen fixation in different organisms (Batut and Boistard, 1994).

Function and Regulation of Plant Genes Involved in Nodulation and Symbiosis

More is known about the rhizobial symbiotic genes than those of the host plants due to the relative ease of such studies in prokaryotes. However, considerable progress has been made recently in explaining nodulation genetics in legumes. Host plant proteins that are expressed during nodule formation are called nodulins. Early nodulin (*ENOD*) genes are detected during the "infection and nodule formation" stage (i.e. within 72 hours after inoculation). The expression of late nodulin (*NOD*) genes starts at about the onset of nitrogen fixation (Nap and Bisseling, 1990b). Therefore, the study of nodulin expression defines the various stages of the nodulation process (Scheres, 1990b).

Expression of Early Nodulins

Root Hair (RH)-proteins have been detected in the root hairs of some legumes during invasion by rhizobia (preinfection stage). Five to ten specific RH-proteins in soybean (Bauer, 1981) and two RH-proteins (RH-42 and RH-44) in pea (Gloudemans et

al., 1989) were detected when whole-root and root hair RNA preparations were compared. However, the functions of these root hair proteins have not been established.

Many early nodulins genes encode cell wall structural proteins. The first early nodulin gene involved in nodule organogenesis was isolated from soybean. This gene, *ENOD2*, encodes a protein with a putative N-terminal signal peptide and repeating proline-rich pentapeptide motifs. *ENOD2* is expressed in the inner cortex of determinate and indeterminate nodules (van de Wiel et al., 1990). *ENOD2* homologs are also found in other legumes such as pea (van de Wiel et al., 1990), alfalfa (Dickstein et al., 1988), vetch (Moerman et al., 1987), and *Sesbania* (Srittmatter et al., 1989), suggesting a conserved role for ENOD2 in legume nodules. Moreover, the expression of the *ENOD2* gene in empty nodules indicates its role in nodule morphogenesis rather than a function in the infection process. The location of *ENOD2* expression suggests that it may contribute to the physical barrier in nodules to oxygen (van de Wiel et al., 1990). *ENOD2* usually acts as a developmental gene marker, which permits distinction of nodule-like structure from tumors or secondary roots.

Other nodulins associated with nodule organogenesis are soybean GmENOD13 (Franssen et al., 1988), pea Nps-40' (Govers et al., 1986), vetch Nvs-40 (Moerman et al., 1987), and alfalfa Nms-30 (Dunn et al., 1988).

Using the in situ hybridization technique, Scheres et al. (1990b) was able to show a sequential expression of early pea nodulins, namely *ENOD3*, *ENOD5*, *ENOD12*, and *ENOD14*, in a temporal and spatial manner. Expression of *ENOD12* was detected in the invasion zone (Scheres et al., 1990a). Maximal expression of *ENOD5* was then found in

the early symbiotic zone followed by expression of *ENOD3* and *ENOD14* in the youngest layers of the late symbiotic zone. When the cells of this zone got old, expression of leghemoglobin was highest with appearance of bacteroid nitrogenase mRNA. *ENOD3* and *ENOD14* mRNA concentrations in pea nodule can be used to mark the stage at which the infected cell has been fully differentiated into a functional nodule cell. Expression of *ENOD12* and *ENOD5* can be induced by addition of purified Nod factors from *R. leguminosarum* bv. viciae (Bisseling et al., 1990).

Expression of Late Nodulins

Most of the late nodulins have been well-characterized. They include enzymes involved in nitrogen assimilation, carbon metabolism, amide and ureide biogenesis, proteins present in the symbiosome membrane (SBM), and the leguminoglobins (Lbs). These proteins are either nodule-specific or are expressed at a higher level in nodules than elsewhere in the plant.

Nitrogen-assimilation enzymes identified are glutamine synthetase (GS) and glutamate synthase (GOGAT). They are responsible for assimilating ammonia, which is produced from nitrogen fixation in the nodules. Their activity varies with the amount of ammonia produced (Hirel et al., 1987).

Sucrose synthase (SS) (Morell and Copeland, 1985) and phosphoenolpyruvate carboxylase (PEPC) (Vance and Stade, 1984) were found to be highly and specifically expressed in nodules. SS converts sucrose into fructose and UDP-glucose, which yields

phosphoenolpyruvate (PEP) after glycolysis. PEPC carboxylates PEP to oxaloacetate, which is converted into the C_4-dicarboxylic acid, malate, by malate dehydrogenase. Therefore, SS and PEPC are important in production of C_4-dicarboxylic acids, major carbon sources for bacteroids, which are readily transported and consumed by bacteroids to produce ATP and reducing equivalents for nitrogen fixing (Streeter, 1991).

Nodulated legumes transport assimilated nitrogen from nodules to shoots in the form of amides, principally asparagine (indeterminate nodules) or ureides, principally allantoin and allantoic acid (determinate nodules) (Schubert, 1986). Plant enzymes involved in production of amides and ureides are specifically expressed in the nodule. Aspartate aminotransferase (AAT) catalyzes synthesis of asparagine from aspartate and glutamine (Reynolds et al., 1982). High levels of this enzyme were found in asparagine exporting nodules (Sawhney, 1987). Uricase (uricase II) is the key enzyme required for the production of allantoin via oxidation of uric acid (Reynolds et al., 1982) and was found localized in the peroxisomes of uninfected cells in the central tissue of soybean nodules (Nguyen et al., 1985).

As an interface between the symbiosome space and plant cytosol, the symbiosome membrane (SBM) plays a major role in controlling the exchange of metabolites between the bacteroids and host plants (Franssen et al., 1992). Currently, SBM nodulins have only been characterized in soybean. They are choline-kinase I, H^+ ATPase, Ngm-24, Ngm-26, and Ngm-23. Ngm-24 is localized in the inner surface of the SBM, so it is unlikely that it functions as a transmembrane transport protein (Katinakis and Verma, 1985). Ngm-26 is a transmembrane protein and probably functions in

exchange of metabolites across the SBM (Fortin et al., 1987). The amino sequence of Ngm-23 suggests that it may be a metal-binding protein (Franssen et al., 1992).

Leghemglobin is the most abundant nodulin. It functions as an oxygen carrier to facilitate the oxygen supply to bacteroids under microaerobic conditions (Appleby, 1984). Its expression is strongly activated before nitrogen fixation takes place.

Purified Nod factors can induce a spatial pattern of early nodulin expression which precisely corresponds to the pattern after *Rhizobium* infection. Nod factors, in the absence of bacteria, are able to induce cell divisions in the inner cortex of the root and the expression of early nodulin genes *ENOD12* and *ENOD40* in such primordia (Horvath et al., 1993; Minami et al., 1996). Of those nodulins known to be induced rapidly upon inoculation, *ENOD40* (Yang et al., 1993; also called GmN36, Kouchi and Hata, 1993) was shown to be expressed very early during soybean nodule initiation. Therefore, *ENOD40* expression can be used as an early molecular marker for the initiation of nodule primordia. How the Nod factor signaling system affects the well-defined pattern of gene expression is still to be analyzed. The Nod factor molecules that induce the initial steps of nodulation have a very specific structure and are active at nanomolar to picomolar concentrations. This strongly suggests that they act through receptors. The nature of this possible receptor-Nod factor interaction remains to be determined (van Kammen, 1995).

Function and Regulation of *nodD*

The *nodD* gene was first characterized in *R. meliloti* (Jacobs, et al., 1985) and *R. leguminosarum* (Downie et al., 1985) where *nodD* mutants were found to be defective in nodulation. The *nodD* gene in both bacteria are transcribed divergently from *nodABC* (Egelhoff et al., 1985 Rossen et al., 1985). Subsequently, the *nodD* gene product was identified as the transcriptional activator of flavonoid-inducible *nod* gene expression in rhizobia and it was later found to exist in all *Rhizobium*, *Bradyrhizobium*, and *Azorhizobim* strains examined (van Rhijn et al., 1993). Inactivation of the *nodD* gene consequently confers a Nod^- phenotype in rhizobia. However, *B. japonicum* is exceptional because of the presence of NodV/NodW regulators for *nod* gene expression (Sanjuan et al., 1994).

By amino acid sequence analyses, NodD proteins have been shown to belong to a large family of prokaryotic regulatory proteins, called LysR-type regulators. A feature of these regulators is that both environmental receptor and gene activator functions are harbored on the same protein. Examples include *Escherichia coli* LysR, IlvY, and CysB; *Salmonella typhimurium* MetR; and *Enterobacter cloacae* AmpR (Schell, 1993). *nodD* genes in different rhizobia are conserved at the nucleotide sequence level. These proteins all require an inducing compound for activation and all possess a putative helix-turn-helix motif in the amino region, which is characteristic for the DNA-binding ability (Schlaman et al., 1992b). Mutations in the helix-turn-helix motif affect the autoregulatory ability of NodD (Burn et al., 1989).

Most of *Rhizobium* and *Bradyrhizobium* species appear to possess multiple copies of *nodD*. *R. meliloti* and R. *leguminosarum* bv. phaseoli have three copies of *nodD* (Davis and Johnston, 1990; Göttfert et al., 1986; Honma and Ausubel, 1987). A mutation in one of the three *nodD* homologs of *R. meliloti* reduces the nodulation host range (Honma et al., 1990). Two copies of *nodD* have been identified in *R. fredii* (Appelbaum et al., 1988) and *B. japonicum* (Göttfert et al., 1989). *R. leguminosarum* bv. viciae and bv. trifolii (Wijffelman et al., 1989), and *Azorhizobium caulinodans* (Goethals et al., 1990a) possess only one *nodD* gene. Up to five copies of the *nodD* gene have been identified in *R. tropici* CIAT899 (van Rhijn et al., 1993). They are all called *nodD* genes because of their DNA and protein sequence conservation. The biological significance of having multiple copies of *nodD* is not clear. For example, there is no correlation between the number of *nodD* genes and the broadness of the host range. For instance, the narrow-host-range *R. leguminosarum* bv. phaseoli has three copies (Davis and Johnston, 1990), whereas the broad-host-range *Rhizobium* sp. strain NGR234 possesses two *nodD* copies, of which only one seems to be involved in *nod* gene induction (Broughton et al., 1991).

The generalized model of the regulatory scheme for *nod* gene induction in rhizobia is that NodD activates gene expression in the presence of flavonoid inducers (Mulligan and Long, 1985). The mechanism by which these compounds induce *nod* gene expression is not completely understood. NodD is thought to interact directly with the inducing flavonoid and stimulate transcription. However, there is only circumstantial evidence to substantiate the direct interaction of flavonoids with NodD. For example, a number of research groups have shown that the modification of the primary sequence of

NodD can change the specificity of the inducer (Burn et al., 1989; Hong et al., 1987; Spaink et al., 1987a ; Spaink et al., 1989b). Other indirect evidence involves the transfer of a *nodD* gene to a foreign *Rhizobium* strain resulting in the transfer of sensitivity to a given set of flavonoids (Spaink et al., 1987b; Horvath et al., 1987). Since flavonoids are required for activation of the NodD protein, they presumably induce a conformational change in the protein. This notion is supported by the fact that there are mutant and hybrid *nodD* alleles which can activate transcription in the absence of flavonoids (Spaink et al. 1989b).

It is apparent that the spectrum of flavonoid specificity of the endogenous NodD protein correlates with the broadness of the host range. NodD proteins from narrow-host-range rhizobia, like *R. meliloti*, *R. leguminosarum* bv. viciae, and *R. leguminosarum* bv. trifolii, respond to few flavonoids, whereas NodD from the broad-host-range *Rhizobium* NGR234 has a larger spectrum of inducing compounds, including even the monocyclic aromatic compounds vanillin and isovanillin (Györgypal et al., 1991b).

The inducer specificity of each different NodD occurs at the level of amino acid sequence. Several hybrid Nod proteins have been constructed and exhibit the flavonoid specificity of the NodD product constituting their C-terminal end (Spaink et al., 1989b; Horvath et a., 1987). This indicates that the signal specificity is located primarily at the C-terminal part of the protein, which is less highly conserved than the N-terminal part. In addition, the carboxyl part of the protein exhibits some resemblance to animal steroid receptors, which are also known to interact with flavonoid ligands (Györgypal and Kondorosi, 1991). However, mutations that modify flavonoid specificity can occur in the

N-terminal region (Mclver et al., 1989; Spaink et al., 1989b). Thus, flavonoid specificity depends on the overall tertiary structure of NodD protein.

A region in the N-terminal half of NodD is similar in structure to the so-called receiver module found in many bacterial regulatory proteins (Mclver et al., 1989). This motif is thought to be involved in protein-protein communication. The hypothesis that NodD contains a receiver module raises the possibility that NodD interacts with an unidentified protein factor(s) or forms multimeric associations with other NodD molecules.

NodD is apparently associated with the cytoplasmic membrane of the cell where it is probably in a good position to interact with the hydrophobic flavonoid molecules (Schlaman et al., 1989; Recourt et al., 1989). In *R. leguminosarum* bv. viciae, the NodD protein is localized in the bacterial cytoplasmic membrane, presumably inserted only in the cytoplasmic monolayer (Schlaman et al., 1989). Flavonoids have been reported to accumulate in the cytoplasmic membrane (Recourt et al., 1989; Hubac et al., 1993). Hubac et al. (1993) have shown that the absorption of luteolin by *R. meliloti* in the inner and outer membranes involves the NodD proteins. These observations support the suggestion that the site of interaction between NodD and the flavonoid is likely to occur in the inner membrane (Schlaman et a., 1992b). A membrane-associated hydrophobic alpha helix segment is predicted between amino acid residues 101 and 117 of NodD from *Rhizobium leguminosarum* bv viciae. This is in accordance with the finding that this NodD protein was localized in the inner membrane fraction the cell (Schlaman et al., 1989).

NodD in *R. leguminosarum* bv. viciae and *R. meliloti* has the ability to bind to the *nod* gene promoter in the absence of an inducer (Kondorosi et al., 1989; Fisher et al., 1988; Hong et al., 1987; Fisher and Long, 1989). Therefore, it is likely that binding of the inducer may increase the affinity of NodD for the promoter and/or induce a conformational change in the protein which is essential for transcriptional initiation. Consistent with this, in *R. meliloti* AK41 and *Azorhizobium* species, the binding of NodD to the *nod* promoter is enhanced in the presence of its inducer (Kondorosi et al., 1989; Goethals et a., 1992).

The binding site of NodD 5' to the *nod* genes has been identified by DNA footprint analysis (Fisher and Long, 1989; Kondorosi et al., 1989) and is, in general, well conserved upstream of the *nod* genes in all species of *Rhizobium* and *Bradyrhizobium* analyzed (Rostas et al., 1986; Spaink et al., 1987b). This sequence has been termed the "*nod* box". Deletion and mutation of the *nod* box sequence abolishes the ability of NodD to stimulate transcription (Hong et al., 1987; Rostas et al., 1986; Spaink et al., 1987b; Wang and Stacey, 1991). Likewise, mutations in NodD affect the ability of the protein to bind to the *nod* box sequence (Hong et al., 1987; Burn et al., 1989; Mclver et al., 1989).

Fisher and Long (1993) have shown that flavonoid-independent NodD3 induces or stabilizes a bend in the *nod* box of *nodA*, *nodF*, and *nodH* upon binding and that the bend center lies between the two NodD-binding sites. It is possible that the specific bend induced by NodD3 is already in an "active" mode and that this protein may bend the *nod* box more or less than that produced by the flavonoid-dependent NodD alone.

Therefore, it has been suggested that upon the putative binding of an inducer molecule to NodD, a conformational shift in the tertiary structure of the protein might take place (Kondorosi et al., 1989). This activation event might exclude the binding of the *nod* repressor and promote RNA polymerase (Kondorosi et al., 1989) to start up the coordinate transcription of *nod* regulon.

Another suggestion about the mechanism of binding of NodD to the promoter is that the expression of *nodD* gene(s) may result in a homo- or heterogeneous pool of NodD proteins depending on the number of *nodD* copies in the strain (Maillet et al., 1990; Hartwig et al., 1990; Györgypal et al., 1988). Flavonoids accumulate in the inner membrane (Recourt et al., 1989), which can be the site of their interaction with the inner membrane associated NodD (Schlaman et al., 1989). Depending on the available set of signal molecules, the putative ligand binding site of NodD could be occupied by either inducers or anti-inducers, or remain unoccupied if there are no available signal molecules able to bind (Djordjevic et al., 1987; Kondorosi et al., 1989; Hong et al., 1987; Fisher and Long, 1989). NodD molecules unoccupied or occupied with anti-inducers retain their original conformation (Djordjevic et al., 1987; Kondorosi et al., 1989). This conformation would not activate transcription of *nod* genes but could presumably compete with the flavonoid-activated form of NodD for binding to the inducible *nod* promoters (Kondorosi et al., 1989). In most *R. meliloti* strains, a negative regulatory factor, the NolR protein, binds to the overlapping sites of *nodD1* and *nodA* genes, as well as to the *nodD2* promoter (Kondorosi et al., 1991). The *nod* repressor weakens the

binding of NodD to the *nod*-box presumably by competition with RNA polymerase, hence decreasing the expression of *nodD* and inhibiting *nod* gene induction.

The *nodD* gene, originally studied in *Rhizobium* species, was found to be constitutively expressed. In *R. leguminosarum* bv viciae, the *nodD* gene is constitutively expressed, but the introduction of multiple copies of the *nodD* gene resulted in reduced expression (Rossen et al., 1985). In this organism, NodD appears to negatively regulate its own transcription. Similarly, NodD negatively regulates its own transcription in *R. leguminosarum* bv. trifolii (Spaink et al., 1987a).

In *R. meliloti*, the expression of *nodD3* and *syrM*, another member of the LysR family, is strongly intertwined in a complex way. For example, the *syrM* gene product activates the expression of *nodD3*, which in turn activates the expression of *syrM* (Swanson et al., 1993). The two genes constitute a self-amplifying positive regulatory circuit. Whereas NodD1 and NodD2 are specifically activated by plant compounds, plant inducers have little effect on the *syrM-nodD3* interaction. In the free-living state, *R. meliloti* strains with *syrM* and *nodD3* present in single copy show no detectable expression of either gene (Swanson et al., 1993). However, when both are carried on a multicopy plasmid, SyrM and NodD3 are present at sufficiently high concentrations to induce high levels of *nodABC* expression in the absence of an inducer. This suggests that one or both genes might be repressed in the free-living state. To a lesser extent, the *nodD2* gene influences *syrM* expression and *nodD1* influences *nodD3* expression (Maillet et al., 1990). Interestingly, SyrM also regulates the expression of the *exo* genes, indicating that SyrM could coordinately regulate the metabolism of exopolysaccharide

and Nod factors. SyrM has also been identified in *R. leguminosarum* bv. phaseoli (Michiels et al., 1993).

In *B. japonicum* (Wang and Stacey, 1991), *R. leguminosarum* bv. phaseoli (Davis and Johnston, 1990), and *R. fredii* (Appelbaum et al., 1988) the *nodD* gene is preceded by a *nod* box sequence. In the first two species, the *nodD* transcription level is enhanced in the presence of NodD protein and certain flavonoids, independently of other *nod* genes. The *nod* box was originally defined in *R. meliloti* as a 47-bp consensus sequence required for *nod* gene induction (Rostas et al., 1986). In the genetically distant *B. japonicum* and *A. caulinodans*, less highly conserved *nod* boxes have been identified and various consensus sequences have been proposed (Wang and Stacey, 1991; Goethals et al., 1992).

The classical consensus sequence of the *nod* box derived from Rostas et al. (1986) that is contained in most of the flavonoid-inducible *nod* genes characterized in *Rhizobium* and *Bradyrhizobium* species consists of three highly conserved regions of 7, 5, and 25 bp (Fisher and Long, 1989; Nieuwkoop et al., 1987; Rostas et al., 1986). Wang and Stacey (1991) characterized the *nod* box sequence 5' to *nodD1* gene and found that it does not fit this consensus. The *nodD1 nod* box is conserved at its 3' end and divergent at its 5' end with respect to the classic *nod* box sequences. Including other divergent *nod* boxes found in *B. japonicum* (Göttfert et al., 1989) and *A. caulinodans* (Goethals et al., 1990b), they re-examined all the published *nod* box sequences and suggested that *nod* box can best be viewed as a composition of a repeat of a 9-bp sequence. This interpretation confers four repeats on the classical *nod* box sequence and two on the 3' region of *B. japonicum nodD1 nod* box. This proposed structure of *nod* box is consistent with the structure of the

binding sites of members of LysR-type regulators such as MomR/OxyR (Bolker and Kahmann, 1989) and IlvR (Wek and Hatfield, 1988). In this model, an interaction of the *nod* box by a tetramer of NodD1, of which each binds to a 9-bp repeat, or a dimer of NodD1, of which each bind to a paired repeat, was suggested (Wang and Stacey, 1991). The difference in the *nodYABC nod* box and *nodD1 nod* box in *B. japonicum* may account for the much lower levels of *nodD1* expression than those of *nodYABC* expression (Banfalvi et al., 1988), and that the *nodD1* gene can specifically induced by certain inducers, which do not induce *nodYABC* genes (Smit et al., 1992).

In contrast to the proposal of Wang and Stacey (1991), Goethals et al. (1992) proposed the structure of *nod* box as two inverted repeats A-T-C-N_9-G-A-T, which have a repeat structural feature typical for DNA targets that are symmetrically bound by protein dimers or tetramers (Pado and Sauer 1984; Honma et al., 1990). A similar structure can be found in the binding sites of LysR-type regulators, such as MetR, OxyR, NahR, IlvY, and TrpI proteins, which show a more of less elaborate inverted repeat structure built around an invariable T-N_{11}-A motif (LysR motif) (Goethals et al., 1992). This structure is present in all *nod* boxes and favors the hypothesis that NodD binds to the *nod* box as multimers (Goethals et al., 1992).

The level of *nodD* expression is under the control of a complex set of environmental and intracellular factors. On the whole, *nodD* is constitutively expressed (Horvath et al., 1987; Mulligan and Long, 1985; Rossen et al., 1985). Beyond this, various *nodD* alleles differ with respect to their regulation by plant signal compounds (Milligan and Long, 1985; Rossen et al., 1985; Djordjevic et al., 1987; Banfalvi et al.,

1988), combined nitrogen (Wang and Stacey, 1990; Dusha et al., 1989), and regulatory proteins, including activator protein SyrM (Maillet et al., 1990), the NolR repressor protein (Kondorosi et al., 1989), and NodD itself (autoregulation) (Mulligan and Long, 1985; Rossen et al., 1985; Banfalvi et al., 1988; Davis and Johnston, 1990).

nod Gene Inducers - Flavonoids

The natural plant phenols arise biogenetically from two main pathways; the shikimate pathway, which directly provides phenylpropanoids such as the hydroxycinnamic acids and coumarins; and the polyketide (acetate) pathway, which can produce simple phenols and also many quinones. By far the largest group of phenolics, the flavonoids, are derived from a combination of these two pathways. The most important single group of phenolics are the flavonoids. They can be subdivided into 13 classes as shown in Table 7 (Harborne, 1989).

Flavones, flavonols and their glycosides constitute a biosynthetically related group of natural products which vary in color from white to yellow. The essential difference between flavones and flavonols is the presence of oxygenation at C-3 in the flavonols. Glycoside derivatives are those linked with a sugar. No universal function for flavones and/or flavonols in plants has yet been established, in spite of their being the most common and widely distributed of flavonoids. However, many functions in individual plants or plant groups have either been demonstrated or proposed. These

Table 7. Different classes of flavonoids (Harborne, 1989).

Class	Number of known substances
Anthocyanins	256
Chalcones	197
Aurones	29
Flavones, flavonols, and their *O*-glycosides	1660
C-Glycosylflavonoids	303
Flavanones	319
Dihydrochalcones	71
Dihydroflavonols	110
Flavans, leucoanthocyanins, and proanthocyanidins	309
Biflavonoids	134
Isoflavonoids	630
Neoflavonoids	70
Miscellaneous structures	100

include: (1) protection of the plant from UV light, insect, fungi, viruses and bacteria; (2) pollinator attraction; (3) antioxidants; (4) plant hormone controllers; (5) stimulants of nodule production by *Rhizobium* bacteria; (6) enzyme inhibitors; and (7) allelopathic agents (Markham et al., 1989).

The isoflavonoids are distinct from other flavonoid classes in that they contain a rearranged C_{15} skeleton based on 3-phenylchroman. The isoflavones form the largest group of this class. The four most common isoflavones are daidzein, formononetin, genistein, and biochanin. The isoflavonoids, unlike most other flavonoids, show a very limited distribution in the plant kingdom, being largely confined to the subfamily Papilionoideae of the Leguminosae with only occasional occurrence in the two other subfamilies, the Caesalpiniodeae and Mimosoideae (Williams and Harborne, 1989).

Phenolic compounds are found throughout the plant kingdom but the type of compounds present varies considerably according to the phylum. Phenolics are uncommon in bacteria, fungi, and algae. Flavonoids are almost completely absent from these groups of organisms. It is in the vascular plants that the full range of polyphenols are found. These include isoflavonoids and xanthones.

The structures of xanthones are related to those of flavonoids and their chromatographic behavior is also similar. Whereas flavonoids are frequently encountered in nature, xanthones have been found in a limited number of families. As aglycones, they occur in eight families, namely in the Gentianaceae, Guttiferae, Polygalaceae, Leguminosae, Lythraceae, Moraceae, Loganiaceae, and Rhamnaceae, among which

xanthones widely occur in only the first two families (Hostettmann and Hostettmann, 1989).

The natural inducers of *nod* genes isolated from host plants are various flavonoids belonging to the subclasses of flavones, flavanones, isoflavones, chalcones, and aurones (Hartwig et al., 1990; Redmond et al., 1986; Zaat et al., 1989; Bassam et al., 1988; Kosslak et al., 1987; Györgypal et al., 1991b). A repertoire of chemical compounds other than flavonoids, including coumarins, benzoic acids analogs, betaines, benzophenones and anthocyanins (Bender et a., 1990; Cunningham et al., 1991; Hungria et al., 1991a; Maxwell et al., 1989; Peters and Long, 1988; Phillips et al., 1992) have also been found able to induce *nod* gene expression.

Different flavonoid molecules can act synergistically to enhance *nod* gene induction (Hartwig et a., 1989). Flavonoids exert their *nod* gene regulatory effects at nanomolar concentrations (Zaat et al., 1989). In several cases, flavonoids without inducing properties have been shown to inhibit *nod* gene activation by effective inducers (Djordjevic et al., 1987; Firmin et al., 1986; Kosslak et al., 1990; Peters and Long, 1988; Cunningham et al., 1991). The anti-inducers usually have similar structures to those of the inducers, and inhibition can be overcome by increasing the concentration of the inducers (Peters and Long, 1988).

Flavonoids that are released from the legumes are mostly aglycones or glycosidic conjugates. The latter are less active but have a higher solubility in water, and they can be converted to active forms by bacterial glycosidases (Maxwell and Phillips, 1990).

The nature and amounts of the flavonoid compounds exuded can be dependent on the plant and its stage of development. For alfalfa, soybean, and bean, the spectrum of flavonoids present in seed exudates is different from that present in root exudates (Hartwig et al., 1989; Graham 1991; Hungria et al., 1991a; Hungria et al., 1991b).

Inoculation with an infective rhizobial symbiont can cause a change in the internal flavonoid pool of the root. The second wave of flavonoid can lead to increased *nod* gene-inducing activity, by as much as 10-fold, as detected in white clover (Rolfe et al., 1988), vetch (van Brussel et al., 1990), soybean (Schmidt et al., 1994), bean (Dakora et al., 1993b), *Lotus* species (Cooper and Rao, 1992), and alfalfa (Dakora et al., 1993a).

Nodulation Gene Regulation in *Bradyrhizobium japonicum*

B. japonicum is unique among other rhizobia in that *nod* gene regulation is not only controlled by NodD, but is regulated through a complex interaction of three types of regulators. They are NodD1, a LysR-type regulator (Wang and Stacey, 1991), NodV and NodW, a two-component regulatory system (Göttfert et al., 1990), and NolA, a MerR-type regulator (Garcia et al., 1996).

nod Gene Regulation by NodD1

Induction of the *nodYABC* operon, as measured by expression of a translational *nodY-lacZ* or *nodC-lacZ* fusion, requires NodD1 in the presence of isoflavonoids such as

genistein or daidzein (Banfalvi et al., 1988). This operon, similar to *nod* genes in other rhizobia which are flavonoid-inducible, contains a *nod* box in the promoter region, which was originally defined in *R. meliloti* as a 47-bp consensus sequence (Wang and Stacey, 1991). Interestingly, NodD1 was found to be inducible by the same isoflavonoid inducers and the induction required its own gene product (Banfalvi et al., 1988). Smit et al. (1992) found that in soybean root exudates, derivatives of genistein, glycitein, and diadzein containing glucose, malonyl, and acetyl groups can specifically induce *nodD1* expression but not *nodYABC* expression.

B. japonicum possesses two *nodD* genes arranged tandemly (Göttfert et al., 1992). The predicted NodD1 and NodD2 proteins share 62% identical amino acid residues at corresponding positions and exhibit different degrees of homology with NodD proteins of other *Bradyrhizobium*, *Azorhizobium*, and *Rhizobium* strains. The highest homology (91.7% identical amino acid residues) was found between NodD1 of *B. japonicum* and NodD of *Bradyrhizobium* sp. *Parasponia* (Scott, 1986), the lowest homology (47.9% identity) was between NodD2 of *B. japonicum* and NodD of *A. caulinodans* (Goethals et al., 1990a). Induction of the *nodYABCSUIJ* operon, as measured by expression of a translational *nodC-lacZ* fusion, required the *nodD1* gene, but not *nodD2* (Göttfert et al., 1992). When *B. japonicum* was deleted for both *nodD* copies, the mutant (strain Δ1267) still showed residual nodulation activity (Göttfert et al., 1992).

nod Gene Regulation by NodV and NodW

Early results indicated that mutation in *nodD1* in strain AN314 drastically reduced *nod* gene expression (Banfalvi et al., 1988), but had no effect on soybean nodulation (Nieuwkoop et al., 1987; Göttfert et al., 1992). Moreover, a *nodD1-nodD2* deletion mutant is still Nod^+ (Göttfert et al., 1992). However, when *nodW* was mutated as well, the ability of this triple mutant to nodulate soybean was severely reduced (Sanjuan et al., 1994). In addition, bacteria with mutations in *nodVW* lost the ability to nodulate cowpea, mungbean, and siratro, and displayed delayed nodulation on soybean (Göttfert et al., 1990). These results indicated that NodW is essential for nodulation of cowpea, siratro, and mungbean. In the absence of NodD1, NodW appears to be sufficient to allow nodulation of soybean. Interestingly, *nodY*- and *nodD1-lacZ* expression in a *nodD1-nodD2-nolA* triple mutant strain (i.e. strain Δ1267) were equivalent to the wild-type level (Dockendorff et al., 1994a). A *nodW* mutant derivative of strain Δ1267 eliminated all *nod* gene expression. Therefore, NodW is alone sufficient for *nod* gene induction by isoflavones, but NodW, in conjunction with NodD1, is essential for maximal induction of the common *nod* genes and *nodD1* (Sanjuan et al., 1994).

Sequence analyses indicates that NodV and NodW belong to the family of two-component regulators. This was confirmed by the study of Loh et al. (1997). They demonstrated that NodV could be autophosphorylated in vitro by the addition of [γ-^{32}P]ATP. Phosphorylated NodV was capable of phosphorylating an aspartate residue at position 70 of NodW in vitro. The in vitro phosphorylation of NodW occurred only when

genistein was added. A *B. japonicum nodW* mutant was used to test *nod* gene expression and nodulation of mungbean. When the mutant was complemented with a wild-type NodW protein, *nodC-lacZ* expression was induced and the strain nodulated mungbean. In contrast, no *nod* gene expression or nodulation of mungbean occurred when the mutant was complemented with a NodW harboring a mutation at the Asp70 residue.

nod Gene Regulation by NolA

The *nolA* gene was first identified by its ability to extend the host range of *B. japonicum* serogroup 123 strains to specific soybean genotypes that normally exclude nodulation by these strains (Sadowsky et al. 1991). Subsequent studies in our laboratory showed that NolA may act as a repressor of *nod* gene transcription (Dockendorff et al., 1994a); that is over-expression of *nolA* from a multicopy plasmid resulted in a reduction of *nod* gene expression. However, when *nolA* was mutated, there was no corresponding elevation of *nod* gene expression. The contradiction of these observations with regard to the proposed function of *nolA* as repressor was reconciled by the results of Garcia et al. (1996). They found that expression of *nolA* led to the expression of *nodD2*. Over-expression of *nodD2* from a multicopy plasmid reduced *nodC-lacZ* expression (Garcia et al., 1996). This result suggests that NodD2, instead of NolA, may function as repressor of *nod* gene expression. Consistent with this result, NodD2 has been proposed to be a repressor of *nod* genes in *Bradyrhizobium* sp. (*Arachis*) NC 92 (Gilette and Elkan, 1996).

Sequence analysis of NolA indicates that it contains a N-terminal helix-turn-helix domain, which is similar to the DNA binding domains of the MerR family of bacterial regulatory proteins (Sadowsky et al., 1991). The C-terminal end of these regulators are thought to interact with the regulatory compounds to activate binding in a dimeric form to its target promoter. The target promoters are similar in that the -35 and -10 regions are separated by 19 bps instead of the normal 16 to 17 bps. Several members of this family are autoregulatory, either positively or negatively. NolA was found to be positively autoregulatory (Garcia et al., 1996). In addition, the -35 and -10 regions of its promoter are separated by 19 bps, where an inverted repeat sequence was identified and proposed to be a putative NolA binding site. A similar promoter sequence was also identified in the 5' region of *nodD2* gene, which was found to be activated by NolA.

Recent studies in our laboratory revealed that three alternative ATG sites are involved in *nolA* gene translational initiation (Loh et al., 1998). Thus, *nolA* has the unusual feature of encoding for three distinct proteins. Immunoblot analysis *of B. japonicum* cell extracts using polyclonal anti-NolA antibodies confirmed the presence of three distinct polypeptides of molecular weights similar to those predicted from the *nolA* sequence. These polypeptides have been designated as $NolA_1$ (translated from ATG1), $NolA_2$ (ATG2), and $NolA_3$ (ATG3). Recent results show that these three NolA gene products have different functions in *B. japonicum*. For instance, $NolA_1$ possesses a helix-turn-helix DNA binding motif and was found to be required for the expression of $NolA_2$ and $NolA_3$. At least two independent transcriptional start sites, P1 and P2, have been identified for the *nolA* gene. Transcription from P1 requires an unidentified plant signal

and is independent of NolA1, whereas the presence of NolA1 is needed for transcription from P2. This is consistent with the presence of a putative NolA binding site in a region 5' to P2. The presumptive model of NolA regulation may be summarized as follows: *nolA* is transcribed from P1 in response to an unidentified plant signal. From the P1 transcript, $NolA_1$ is translated which then activates transcription from P2. $NolA_2$ and $NolA_3$ are subsequently translated from the P2 transcript (Loh et al., 1998).

nod Gene Regulation by Other Systems

Groβ et al. (1993) reported the identification of the *B. japonicum nwsB* gene, which when overexpressed from a strong promoter was able to suppress the Nod^- phenotype of a *nodW* mutant. The *nwsB* gene is preceded by a long open reading frame, *nwsA*. The protein products of the *nwsAB* gene pair also appear to be two-component regulators and are most similar to the protein products of the *B. japonicum* gene pair *nodVW* (Groβ et al., 1994). Cross talk occurs between NwsAB and NodVW, since the activity of NwsB depends on either the NodV or the NwsA sensor kinase. The physiological importance of cross talk between NodVW and NwsAB in *B. japonicum* remains unclear (Groβ et al., 1994).

A number of additional factors have also been shown to affect *nod* gene expression. For example, *nod* gene expression in *R. meliloti* (Dusha et al., 1989) and *B. japonicum* (Wang and Stacey, 1990) was found to be repressed by adding ammonia to the cultures. This result suggests that a coupling of nodulation and nitrogen fixation

functions may be mediated by ammonia regulation of both events. The *R. meliloti* NodD3 and *B. japonicum* NodD1, respectively, are involved in this repression.

Carbon Metabolism and Uptake in Bacteroids

Nitrogen fixation is an energy demanding process. Carbon sources are supplied by the host plant to the bacteroids to cope with the production of ATP for this reaction. However, nitrogenase is readily subjected to irreversible inactivation by oxygen. Therefore, bacteroids respire carbon at very low oxygen concentration. Many studies on the quantities and transport of carbon metabolites, as well as the levels and distribution of metabolic enzymes in nodules, have provided a useful background for understanding this process.

Carbon Metabolism in Nodules and Bacteroids

The flow of carbon through the various pools in host plants has been studied by providing labeled CO_2 to shoots and following the time-dependent distribution of label in nodule metabolites. Sucrose is found to be the most heavily labeled compounds in the nodules after short time delays (Reibach and Streeter, 1983). The suggestion that sucrose is the primary source of reducing equivalents supplied by plants is supported by the findings of high levels of invertase and sucrose synthase in nodules. The latter is believed to be the major sucrose hydrolytic enzyme because its activity in nodules is well

correlated with nitrogenase activity (Anthon and Emerich, 1990). Sucrose synthase catalyzes the following reaction:

$$\text{Sucrose} + \text{UDP} \rightarrow \text{Fructose} + \text{UDP-Glucose}$$

A very high activity of phosphenolpyruvate carboxylase (PEPC) is also found in the nodule cytosol (Deroche and Carrayol, 1989). This enzyme converts phosphoenol-pyruvate (PEP) and HCO_3^- into oxaloacetate and inorganic phosphate. Provision of the substrate PEP for PEPC is consistent with the high activity of glycolytic enzymes found in the nodule cytosol (Reibach and Streeter, 1983). Another important enzyme present at high activity in the nodule is malate dehydrogenase. It catalyzes the conversion of oxaloacetate into malate. Its activity is also correlated with nitrogenase activity (Nautiyal and Modi, 1987). Labeling studies with intact nodules show a rapid and substantial labeling of four-carbon organic acids, especially malate, and this activity is closely associated with nitrogen fixation activity in nodules (Vance et al., 1983). Werner (1992) found that the levels of PEPC and malate dehydrogenase is increased 5- to 50- fold in nodules compared to roots. In addition, studies on $^{14}CO_2$ fixation in effective nodules showed that 80% of the ^{14}C label was found in malate and about 10% in citrate (Rosendahl et al., 1990). This indicates that the major role of these two enzymes is to supply the bacteroids with malate. Several studies have shown that sugars, oxoacids, and glutamate were not readily transport into symbiosomes, whereas C_4-dicarboxylic acids such as malate and succinate were transported via a dicarboxylic transport system (Price et al., 1987; Udvardi et al., 1988a; Udvardi et al., 1988b). These results indicate that malate is the major carbon source supplied to the bacteroids by the host plant.

The general picture of carbon metabolism in nodules concluded from many studies is as follows: Sucrose is released in large quantities from the phloem of vascular bundles imbedded in the inner cortex and is presumably transported symplastically through the inner cortex towards the infected cells. Along the way, sucrose is hydrolyzed by sucrose synthase. This process, as well as the subsequent conversion of hexose to triose, may be accomplished in the inner cortex or in the infected cells. An important branch point occurs at PEP, most of PEP being carboxylated to form oxaloacetate and malate and a smaller portion being converted to pyruvate for consumption by mitochondria. Mitochondria probably play a much more restricted role than they do in most plant cells because of the "non-oxidative" environment in which they must operate. This idea is consistent with the very low level of labeled α-ketoglutarate found in nodules due to microaerobic inhibition of isocitrate dehydrogenase (King et al., 1986). The carbon nutrition needs of the bacteroids can be met without mitochondrial metabolism by coupling glycolysis to massive generation of four-carbon acids via PEPC (Streeter, 1991).

Indeed, although labeling studies indicate that there is transfer of sugars into symbiosomes (Reibach and Streeter, 1983), the isolated bacteroids do not appear to absorb sugars efficiently (Reibach and Streeter, 1984; Salminen and Streeter, 1987). Similarly, though cultured *R. leguminosarum* bv. viciae will transport sugars efficiently, bacteroids will not (Hudman and Glenn, 1980). These data indicate that bacteroids do not use sugars as a major carbon source.

In contrast to sugars, all rhizobia studied absorb dicarboxylic acids rapidly with active transport systems. Typically, Km values for C_4-dicarboxylic uptake are 100 μM or

less with uptake rates 30-50 times higher than those for sugars (Glenn et al., 1980; Reibach and Streeter, 1984; McRae et al., 1989). For amino acids, uptakes rates are generally intermediate between that of sugars and dicarboxylic acids and vary among different rhizobia (Reibach and Streeter, 1984; McRae et al., 1989). The minor function sugars serve in bacteroids is in line with the finding that levels of glycolytic enzymes in bacteroids are low relative to the host cytoplasm (Reibach and Streeter, 1983; Salminen and Streeter, 1987). Furthermore, bacteroids convert ^{14}C labeled sugars to $^{14}CO_2$ slowly in comparison with dicarboxylic acids (Salminen and Streeter, 1987). In fact, all *Rhizobium* mutants studied that lacked various enzymes of carbohydrate metabolism formed nodules capable of nitrogen fixation (Cervenansky and Arias, 1984; Arias et al., 1979; Guezzar et al., 1988; Glenn et al., 1984; Dilworth et al., 1986; Arwas et al., 1985; LaFontaine et al., 1989; Ronson and Primrose, 1979). Taken together, available data show that carbohydrates are not utilized by bacteroids for nitrogen fixation.

While the metabolism of sugars is inefficient, the metabolism of dicarboxylic acids is rapid. Labeled acetate and pyruvate are rapidly incorporated into TCA cycle intermediates (Stovall and Cole, 1978), and there is a substantial enzymatic capacity for the synthesis of acetyl CoA in bacteroids (Preston et al., 1989). The levels of TCA cycle enzymes are high in bacteroids (McKay et al., 1988), especially malate dehydrogenase (Waters et al., 1985), a key enzyme for converting malate to oxaloacetate.

Malic enzyme is present in bacteroids of *B. japonicum* and *R. leguminosarum* at a relatively high level (McKay et al., 1988; Copeland et al., 1989a). This enzyme converts malate into pyruvate and CO_2 with production of one NAD(P)H. The abundance of this

enzyme suggests how malate is metabolized to generate energy and reducing sources for bacteroids.

Another experiment supporting the idea that dicarboxylic acids are the preferred carbon sources for bacteroids was done with *B. japonicum* bacteroids. The bacteroids were isolated anaerobically and supplied with ^{14}C-labeled succinate, malate, aspartate, or glutamate in the presence of myoglobin. Conversion of substrate to CO_2 was most rapid for malate, followed by succinate, glutamate, and aspartate. The % of total carbon transported was 66, 37, 50, and 38% for malate, succinate, glutamate, and aspartate, respectively. More than 95% of the absorbed glutamate remained as glutamate in the bacteroids, whereas 39-66% of the absorbed succinate, malate, or aspartate was converted to glutamate acids (Salminen and Streeter, 1987). The results show that organic acids transported into bacteroids are quickly converted to either CO_2 or amino acids.

The importance of dicarboxylic acids for bacteroid metabolism is also shown by studies of various mutants with defective TCA cycle enzymes, such as succinate dehydrogenase (Gardiol et al., 1982) and α-ketoglutarate dehydrogenase (Duncan and Fraenkel, 1979). These mutants form Fix^- nodules.

The catabolism of dicarboxylic acids is highly oxygen-consuming and may explain the preference of bacteroids for the consumption of dicarboxylic acids as carbon source. The resultant reduction of oxygen pressure in the nodule may protect the nitrogenase enzyme complex from oxygen damage (Jording et al., 1994).

Radiorespirometric experiments using ^{14}C-succinate, -pyruvate, and -acetate suggest that even though under a low oxygen condition, oxidation of these substrates was

through a complete TCA cycle in *B. japonicum* bacteroids (Tajima and Kouchi, 1990). The microaerophilic nature of the nodule environment may bring about conditions leading to the repression of some TCA cycle enzymes such as citrate synthase, NADP-isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. The latter two are also subject to NADP(H) inhibition. In order to bypass these rate-limiting reactions in the TCA cycle for the maintenance of a continuous process of carbon oxidation and energy generation, several unique pathways have been identified in *B. japonicum* bacteroids which are not employed by their free-living counterparts. These pathways include the PHB (poly-β-hydroxybutyric) pathway, malate-aspartate shunt, and α-ketoglutarate-glutamate pathways (Figure 2) (McDermott et al., 1989).

A special feature of the *B. japonicum* bacteroid is the accumulation of a massive amount of the carbon polymer poly-β-hydroxybutyric (PHB) acid during the onset of nitrogen fixation through the PHB shunt (Figure 2) (McDermott et al., 1989). In spite of microaerobic conditions, high NAD^+/NADH values were maintained during organic acid oxidation (Tajima and Kouchi, 1990). This is possibly related to the synthesis of PHB, which can account for 50% of bacteroid cell dry weight (Wong and Evans, 1971), since, in this organism, NADH is the preferred cofactor for acetoacetyl-CoA reductase which catalyzes synthesis of PHB. The conversion of acetyl-CoA into PHB lowers the level of

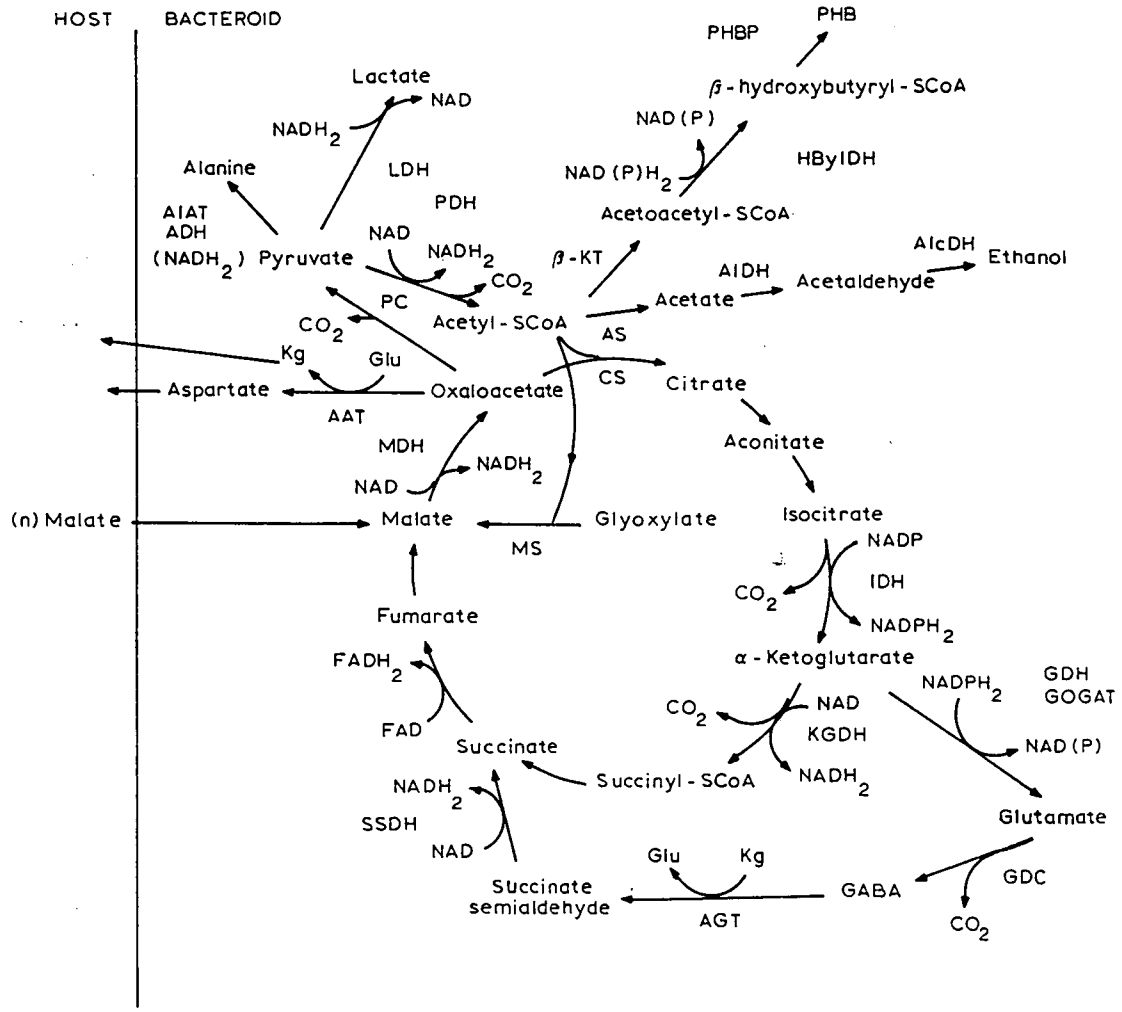


Figure 2. Carbon metabolic pathways in *B. japonicum* bacteroids (from McDermott et al., 1989).

CS: citrate synthase; IDH: NADP-isocitrate dehydrogenase; KGDH: α-ketoglutarate dehydrogenase; MDH: malate dehydrogenase; GDH: glutamate dehydrogenase; GOGAT: glutamine:oxoglutarate aminotransferase; GDC: glutamate decarboxylase; AGT: γ-aminobutyric-glutamic transaminase; SSDH: succinate semialdehyde dehydrogenase; MDH: malate dehydrogenase; AAT: aspartate aminotransferase; MS: malate synthase; PC: pyruvate carboxylase; PDH: pyruvate dehydrogenase; AS: acetyl-SCoA synthetase; AIDH: aldehyde dehydrogenase; AlcDH: alcohol dehydrogenase; LDH: lactate dehydrogenase; β-KT: β-ketothiolase; HBylDH: β-hydroxybutyryl-SCoA reductase; PHBP: poly-β-hydroxybutyrate polymerase; AIAT: alanine aminotransferase; ADH: alanine dehydrogenase; Glu: glutamate; Kg: α-ketoglutarate

NAD(P)H, thereby reducing the inhibition of citrate synthase and isocitrate dehydrogenase (McDermott et al., 1989). PHB can be used by the bacteroids when nodules are severely stressed for carbohydrate (Wong and Evans, 1971). However, not all bacteroids of different species accumulate PHB. Except for *R. leguminosarum* bv. phaseoli, bacteroids of the fast-growing genus *Rhizobium* do not accumulate PHB or accumulate far less than *Bradyrhizobium* spp. This may suggest fundamental differences between these two genera (McDermott et al., 1989).

The malate-aspartate shunt (Figure 2) in *B. japonicum* is believed to be essential in removing or utilizing excessive oxaloacetate during continuous malate uptake and in the reduction of malate in order to prevent inhibition of succinate dehydrogenase (McDermott et al., 1989). In addition, it provides an outlet of oxaloacetate under conditions where citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase may be repressed or inhibited relative to malate dehydrogenase. In this pathway, imported malate is oxidized to oxaloacetate and then transaminated to aspartate using glutamate. α-ketoglutarate produced from glutamate would be exported out of the bacteroids (Streeter and Salminen, 1988).

A significant portion of carbon entering the TCA cycle was found to be converted to glutamate (Salminen and Streeter, 1987). In the bacteroid, ammonium is abundant as a result of nitrogen fixation. When α-ketoglutarate dehydrogenase activity is repressed or inhibited due to the presence of NADH, α-ketoglutarate accumulates. In the presence of the α-ketoglutarate-glutamate shunt (Figure 2), α-ketoglutarate is converted into glutamate by glutamine:α-ketoglutarate aminotransferase. Glutamate will be either used

as the amino donor in the malate-aspartate shunt or decarboxylated to form γ-aminobutyrate (GABA), which was then deaminated and oxidized to succinate. The significance of the latter reaction is to allow decarboxylation of α-ketoglutarate into succinate even when α-ketoglutarate dehydrogenase is deactivated (Kouchi and Fukai, 1988).

Therefore, in spite of microaerobic conditions, bacteroids are able to employ various anaplerotic pathways to supply acetyl-CoA to the TCA cycle to generate enough ATP and NAD(P)H for nitrogen fixation.

Carbon Uptake by Bacteroids

C_4-dicarboxylate transport (Dct) mutants of *R. meliloti*, which were unable to grow on medium containing succinate as sole carbon source, were generated by random transposon mutagenesis (Jording et al., 1994). It was found that all Dct^- mutants obtained failed to grow with succinate, fumarate, and malate as a sole carbon source, whereas growth with other carboxylic acids, such as pyruvate and glutamate, and different sugars, was similar to that of wild type. These results suggest that the Dct system is specific for C_4-dicarboxylic acids. Common Dct systems have also been shown for *R. leguminosarum* (Finan et al., 1981; Ronson et al., 1981) and *B. japonicum* (McAllister and Lepo et al., 1983; San Francisco and Jacobson, 1985). The DNA region coding for C_4-dicarboxylate transport was first analyzed in detail in *R. leguminosarum* (Ronson et al., 1981; Ronson et al., 1984; Ronson et al., 1987). In this species, the *dct* cluster

contains the structural gene *dctA*, and the regulatory genes *dctB* and *dctD* organized in the two divergently transcribed operons *dctA* and *dctBD*. Based on sequence comparisons, the *dctB* and *dctD* genes were proposed to encode a two-component regulatory system (Nixon et al., 1986). In this regulatory system, DctB functions as a membrane-anchored sensor for C_4-dicarboxylates and is thought to activate the DctD protein by phosphorylation. The DctD protein contains a DNA binding site and, in its phosphorylated form, is able, together with the RpoN protein, to activate the expression of *dctA*, which encodes the C_4-dicarboxylate permease located in the cytoplasmic membrane (Jording et al., 1994).

Expression of *dctA* was studied in free-living *R. meliloti* (Jording et al., 1994). The expression rate of the *dctA* gene in wild type responds to succinate and is increased by about ten-fold (Jording et al., 1992). A ten-fold increase in succinate expression was also reported for the *dctA* gene of *R. leguminosarum* and *R. meliloti* grown in a microaerobic culture (Ronson and Astwood, 1987; Wang et al., 1989). However, NifA, FixL and FixJ have no effect on *dctA* expression (Jording et al., 1992; Pühler et al., 1990). This indicates that there may be another unidentified regulator(s) affecting *dctA* gene expression in response to a stimulus such as low oxygen tension.

The nodulation and nitrogen fixing ability of various rhizobial mutants with defects in dicarboxylate uptake have been compared (Streeter, 1991). *R. meliloti* (Bolton et al., 1986; Watson et al., 1988; Yarosh et al., 1988; Hornez et al., 1989), *B. japonicum* (El-Din, 1992), *Rhizobium* sp. strain NGR234 (van Slooten et al., 1992), *R. leguminosarum* bv. viciae (Glenn and Brewin, 1981; Arwas et al., 1985), *R.*

leguminosarum bv. phaseoli (LaFontaine et al., 1989), or *R. trifolii* (Ronson et al., 1981) mutants defective in dicarboxylic acid transport (i.e. *dct* mutants) can nodulate, but form nodules that do not fix nitrogen. Bacteroids that have reduced dicarboxylate uptake (Finan et al., 1983; Engelke et al., 1987; Humbeck and Werner, 1989) produced delayed nodules with reduced nitrogen fixing ability. Likewise, mutants with increased uptake ability exhibited better nitrogen fixation (Birkenhead et al., 1988; O' Gara et al., 1988). These results again indicate that dicarboxylic acids are the major source of reduced carbon supporting nitrogen fixation in bacteroids

Catabolite Repression and cAMP in Rhizobia

When *R. meliloti* was grown in the presence of succinate and glucose as carbon sources, it showed diauxic growth, with the utilization of succinate during the first and glucose during the second growth phase (Jording et al., 1994). The *dctA* gene is highly expressed only during the first growth phase, indicating that preference for the utilization of succinate is regulated at the transcriptional level. Preferred catabolism of C_4-dicarboxylates or TCA intermediates to other carbon sources, e.g. carbohyrates, had also been observed in other rhizobia (Ucker and Signer, 1978; Dilworth et al., 1983; McGetrick et al., 1985; de Vries et al., 1982; Stowers and Elkan, 1985; Röhm and Werner, 1985). In *E. coli*, diauxic growth is regulated by catabolite repression and involves the phosphoenolpyruvate-dependent phosphotransferase system (PTS). However, in rhizobia no PTS has been identified (Stowers, 1985). Although it is not

known what signal molecules regulate carbon metabolism in *Rhizobium* spp., cyclic mononucleotides may play a role in the regulation of rhizobial gene expression. In *B. japonicum*, exogenous cAMP was found to repress three enzymes involved in ammonia assimilation, including glutamine synthetase, glutamate synthase, and glutamate dehydrogenase. The levels of these enzymes were reduced two to five-fold by the addition of 1 mM cAMP (Upchurch and Elkan, 1978). In *B. japonicum*, it has been shown that the suppression of hydrogenase activity by malate is relieved by external addition of cAMP (Lim and Shanmugam, 1979). The same effect resulted when the internal concentration of cAMP was raised by gene-dosage (McGetrick et al., 1985). In *B. japonicum*, pool sizes of cAMP have been shown to vary with carbon source. Intracellular levels were low when the cells were grown in malate, but high on glutamate. It was proposed that the cAMP levels vary in response to the availability of a readily oxidizable carbon source (Lim and Shanmugam, 1979).

In *R. meliloti*, catabolite repression-like phenomena, such as the repression of formate metabolism / uptake or the repression of CO_2 fixation by a variety of carbon sources (McGetrick et al., 1985), do not correlate with fluctuations in the intracellular levels of cAMP, nor are they affected by the exogenous addition of cAMP. The *cya* gene, which encodes adenylate cyclase, has been identified *in R. meliloti* (Kiely and O'Gara, 1983) and *B. japonicum* (Guerinot and Chelm, 1984). These genes show no sequence similarity to either the *E. coli* and *S. cerevisiae cya* genes (O'Gara et al., 1989). *Rhizobium cya* mutants were found to have a reduced level of cAMP, but the production

of cAMP was never completely abolished (O'Gara et al., 1989). This indicates that these organisms must have alternative ways of producing cAMP.

Oxygen Regulation of Rhizobial Genes

In the *Rhizobium*-legume symbiosis, the rhizobial endosymbiont synthesizes enzymes that specifically function in nodules. A key enzyme is nitrogenase. This enzyme catalyzes an energy-consuming reaction, which reduces atmospheric nitrogen into ammonia. This is shown as follows,

$$N_2 + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi$$

This reaction has a high demand for ATP, which can be provided by oxidative phosphorylation. In addition, nitrogenase is readily inactivated, irreversibly, by oxygen. In order to provide the bacterial symbiont with an environment which meets the requirement for a high, respiration-derived, energy supply to nitrogenase but at a low free-oxygen tension, both the plant host and the bacteria have established special strategies.

In both indeterminate and determinate nodules, a physical oxygen diffusion barrier is found in the inner cortex, which is constituted of a few layers of thin-walled cells lacking intercellular spaces. This layer of cells has been called the boundary layer (Witty and Minchin, 1990) where nodulin ENOD2 is expressed and thus might contribute to the oxygen barrier (Van de Wiel et al., 1990). The diffusion barrier results in a low oxygen concentration in the infected zone of the nodule, below 30 nM, in comparison with 250

μM dissolved oxygen outside the nodule. Therefore bacteroids are in a microaerobic environment. In order to supply the bacteroids with enough oxygen for respiration, leghemoglobin, a plant protein with high affinity for oxygen, is synthesized and present inside the infected plant cells. This protein facilitates diffusion of oxygen to the bacteroids, which possess terminal oxidase with a high affinity for oxygen (reviewed in O' Brian and Maier, 1989).

The microaerobic condition created in the nodule not only fulfills the requirement of O_2-sensitive nitrogenase, but also exerts a tight regulatory control over nitrogen fixation (Batut and Boistard, 1994). When rhizobia are exposed to a microaerobic condition, they respond by an expression of new electron carriers that function optimally at low oxygen tension, synthesis of the nitrogenase complex, and the induction of a hydrogen uptake system which recycles the hydrogen evolved as a byproduct of nitrogenase activity.

Oxygen Control on *nif* and *fix* Genes of Rhizobia

The *nif* and *fix* genes identified and characterized in different rhizobia (*R. meliloti, B. japonicum, and A. caulinodans*) are listed in Table 5. Strains of *R. meliloti* and *R. leguminosarum* do not display nitrogenase activity *ex planta* under microaerobic conditions. They are strict symbiotic fixers. In contrast, *B. japonicum* and *A. caulinodans* do fix nitrogen *ex planta* under microaerobic conditions. The ability of the

latter two rhizobial strains to fix nitrogen after being exposed to microaerobiosis suggests that oxygen status and nitrogen fixation are closely linked.

In *R. meliloti*, the expression of *nif* genes including *nifHDK* and some *fix* genes can be induced in free-living cells under microaerobic conditions. Maximal *nifA* expression in *R. meliloti* could be obtained when the oxygen concentration in a pure culture was reduced to about 1% (Ditta et al., 1987). Likewise, *fixK* and *fixNOQP* expression can be induced under the same conditions (David et al., 1988). Thus, microaerobiosis mimics symbiotic conditions for induction of nitrogen fixation gene expression. Therefore, microaerobic conditions inside the nodule are likely the major trigger for inducing nitrogen fixation gene expression.

Expression of all *nif* and *fix* genes, and several other genes in different rhizobia have been shown to be activated by NifA or FixK (Table 6). The *fixL* and *fixJ* regulatory genes were first identified in *R. meliloti* and their products activate expression of *fixNOQP* and *fixGHIS*, as well as *nifA*, under microaerobic conditions (David et al., 1988). Hybridization techniques allowed the identification of homologous genes in *B. japonicum* (50% and 48% amino acid similarity in FixL and FixJ) (Anthamatten and Hennecke, 1991) and in *A. caulinodans* (51% and 54% amino acid similarity in FixL and FixJ) (Kaminski and Elmerich, 1991). FixL and FixJ proteins constitute the central element of the oxygen response regulatory pathway. They belong to the family of two-component regulatory proteins (Pakinson and Kofoid, 1992), in which FixL acts as the sensor and FixJ acts as the regulator. The central non-conserved region of FixL is cytoplasmic and carries a non-covalently attached heme group, which is believed to be

responsible for the modification of the catalytic properties of FixL in response to oxygen tension. Under anoxic conditions, the phosphatase activity of FixL is repressed and autophosphorylation activity is enhanced. FixL phosphorylates FixJ in a reaction which is independent of oxygen concentration (Lois et al., 1993). After phosphorylation, FixJ transcriptional activity is enhanced at least 100-fold (Reyrat et al., 1993). Phosphorylated FixJ allows transcriptional activation of *nifA* and *fixK* by an as yet unknown mechanism.

The NifA transcriptional activator was identified because it was required for the transcription of the *nifHDK* structural genes (Szeto et al., 1984). Sequence data indicated that *R. meliloti* NifA is homologous to *K. pneumonia* NifA and NtrC (Drummond et al., 1986). However, NifA does not contain a N-terminal receiver domain and thus is not classified as a member of the two-component family. *Rhizobium* NifA activity is regulated by oxygen (Beynon et al., 1988). Sensitivity of *B. japonicum* NifA to oxygen has been correlated with the presence of cysteine residues which could be part of a metal (Fe) binding motif located between the central and the terminal domains (Fischer et al., 1988). NifA identified in various rhizobia show extensive amino acid similarity (53-72%) to *K. pneumoniae* (Morett and Segovia, 1993).

FixK is the another regulatory protein involved in the oxygen control of nitrogen fixation gene expression which was first identified in *R. meliloti* (Batut et al., 1989). Homologs were later found in *B. japonicum* (Anthamatten et al., 1992), *A. caulinodans* (Kaminski et al., 1991), and *R. leguminosarum* (named as FnrN) (Colonna-Romano et al., 1990) with 38%, 37%, and 30% amino acid similarity, respectively. FixK acts as a positive regulator of the *fixNOQP* operon which likely encodes proteins involved in

electron transport. Oxygen control of *nif* gene expression is exerted at two levels, i.e., at the level of FixLJ and at the level of NifA. It is possible that oxygen control of *fixNOQP* expression is only mediated by FixLJ regulation of *fixK*. However, FixK could be detecting a yet unidentified regulatory signal different from oxygen (Batut and Boistard, 1994).

In different rhizobia, FixL/FixJ, FixK, and NifA cooperate in different schemes to control the expression of nitrogen fixation apparatus, respiratory chains, and other target genes (Fischer, 1994). A recent model for the genetic regulatory pathways controlling *nif* and *fix* gene expression in *B. japonicum* will be described here.

This model accommodates two largely independent regulatory cascades that respond to cellular oxygen or Redox status, the FixLJ/FixK cascade and the NifA cascade. Unlike *R. meliloti*, FixLJ are not involved in regulating NifA expression in *B. japonicum*. However, FixLJ and NifA cascades are connected through *rpoN1*, one of two σ^{54} genes, which is also regulated by oxygen in a FixLJ-dependent fashion (Kullik et al., 1991). *B. japonicum* has two *fixK*-like homologs, only one of which is FixLJ-regulated. However, in some cases the second *fixK* gene can functionally substitute for the mutated one (Hennecke et al., 1993). Expression of *fixNOQP* depends on FixJ which is probably regulated through FixK. There is a typical "anaerobox" located upstream of the promoter region of *rpoN1*, which could serve as a binding site for FixK. Therefore, it is possible that expression of *rpoN1* is regulated by FixLJ via FixK (Hennecke et al., 1993). An additional control of *nif* gene expression is provided by oxygen-dependent activation of *rpoN1* by the FixLJ/FixK cascade (Fischer et al., 1993). *rpoN1* is not essential for

nitrogen fixation, but the microaerobic induction of *rpoN1* ensures optimum σ^{54}-dependent transcription of *nif* gene.

Expression of *B. japonicum* NifA is controlled by a complex regulatory mechanism. The extraordinary feature is that NifA is expressed under both aerobic and anaerobic conditions (Morett et al., 1991). In other rhizobia, *nifA* expression only occurs under microaerobic conditions. A basal aerobic expression is detected which is independent on NifA and σ^{54} and is about one fourth of the microaerobic level, which is dependent on NifA (autoactivation) and σ^{54} (Morett et al., 1991). Expression of genes under the control of NifA requires RNA polymerase containing the alternative sigma factor σ^{54} which, in *B. japonicum*, is encoded by *rpoN1* or *rpoN2* (Hennecke et al., 1993).

The formation of the cellular chaperonin pool (GroES and GroEL) (Fischer et al., 1993b) is under control of NifA. Hennecke et al. (1998) reported that there are other regulator proteins (σ^{32}, HrcA) that are likely involved in controlling GroESL synthesis after a heat shock.

Cell-Density Regulated Gene Expression

Bacteria have been known to gather information from inanimate features of their environments, or/and their animal or plant hosts (Parkinson, 1993). Recently, it has been shown that bacteria can perceive information from other bacteria (Kaiser and Losick, 1993). The best characterized example is the autoinduction of luminescence in the symbiotic marine bacterium *Vibrio fischeri*, which occurs when cell density exceeds a

critical level. This gene signaling phenomenon is called quorum sensing. In the case of *V. fischeri*, autoinduction of luminescence involves the following mechanism: The bacteria produce a diffusible compound, the autoinducer, that accumulates in the surrounding environment during growth. At low cell densities, the autoinducer does not accumulate. However, the concentration of the autoinducer increases as the cell density increases. At high cell densities, the level of the autoinducers is sufficient to activate transcription of the *lux* genes. In the case of *V. fischeri*, the autoinducer was identified as *N*-3-(oxohexanoyl) homoserine lactone (Eberhard et al., 1981). The bacterial cell membrane is readily permeable to the autoinducer; therefore, an equal concentration of autoinducer exists within the cells and in their local environment (Kaplan and Greenberg, 1985). This sensing system allows the bacteria to become luminescent only when they reach a high density (10^{10}-10^{11} cells per ml) inside the light organs of their host fishes, not in sea water where fewer than 100 cells per ml are present (Fuqua et al., 1994). This species-specific intercellular signaling system allows *V. fischeri* to distinguish the free-living, low cell-density state from the host-associated, high cell density state; thus, rendering expression of certain genes only in symbiotic conditions. The regulatory region that enables induction of luminescence consists of two genes, *luxR*, which encodes an autoinducer-responsive transcriptional activator, and *luxI*, which encodes a protein required for autoinducer synthesis (Engebrecht et al., 1983; Engebrecht and Silverman, 1984). At low cell densities, *luxI* is transcribed at a basal level and the autoinducer accumulates slowly in the growth medium. At a sufficiently high concentration, the autoinducer, interacts with LuxR, activating transcription of the *luxICDABEG* operon.

Luciferase, encoded by this operon, renders the cells luminescent within their fish host light organ (Fuqua et al., 1994). More recently, it has become clear that similar regulatory systems are utilized by many non-luminescent bacteria to control a wide variety of functions in a cell density dependent manner (Table 8). LuxR and the homologous regulatory proteins identified in bacteria of other genera as shown in Table 1. This family of regulators has been designated the LuxR superfamily (Fuqua et al., 1994). The LuxR-type proteins appear to be composed of two modules. A C-terminal DNA binding domain containing a helix-turn-helix motif is predicted to mediate binding to target promoters. The general mechanism of activation of the LuxR-type proteins is that the N-terminal domain acts negatively to prevent an interaction between the C-terminal domain and target DNA binding sites. Upon autoinducer binding, there is a conformational change in the N-terminus and interference with the C-terminal domain is relieved. This event allows binding of the regulator as a dimer with σ^{70} RNA polymerase to the target promoter to activate transcription (Greenberg et al., 1997). All the signal compounds identified from different bacteria are homoserine lactones differing in the length and structure of their acyl groups.

Bacteriocin (*small*) was firstly isolated from *Rhizobium leguminosarum* (Hirsch, 1979; Wijffelman et al., 1983; van Brussel et al., 1985). It can inhibit growth of *R. leguminosarum* bv viciae and similar strains containing the Sym plasmid pRL1JI. However, it did not inhibit growth when the plasmid was cured (Hirsch, 1979; Wijffelman et al., 1983). *small* was later identified as a *N*-acyl-L-homoserine lactone

Table 8. Summary of *N*-acyl homoserine lactone-based regulatory systems (modified from Fuqua et al., 1996).

Bacterial species	Signal molecules[a]	Regulatory Proteins[b]	Target genes and function(s)	References
Vibrio fischeri	*N*-3-(oxohexanoyl)-homoserine lactone (VAI-1)	LuxI/LuxR	*luxICDABEG*, *luxR* Luminescence	Eberhard et al., 1981 Engebrecht et al., 1983 Engebrecht and Silverman, 1984
	N-(octanoyl)-L-homoserine lactone (VAI-2)	AinS/AinR[c]	*luxICDABEG*, ?	Kuo et al., 1994 Gilson et al., 1995
Vibrio harveyi	*N*-β-(hydroxybutyryl)-homoserine lactone (HAI-1)	LuxM/LuxN, LuxO, LuxR[d]	*luxCDABEG* Luminescence and polyhydroxy-butyrate synthesis	Cao and Meighen, 1989 Martin et al., 1989 Bassler et al., 1993 Bassler et a., 1994a Sun et al., 1994
	HAI-2	Lux?/LuxPQ, LuxO, LuxR[d]	*luxCDABEG*	Bassler et al., 1994b
Pseudomonas aeruginosa	*N*-3-(oxododecanyoyl)-L-homoserine lactone (PAI-1)	LasI/LasR	*lasB, lasA, aprA, toxA* Virulence factors	Gambello and Iglewski, 1991 Gambello et al., 1993 Passador et al., 1993 Pearson et al., 1994
	N-(butyryl)-L-homoserine lactone (PAI-2)	RhlI/RhlR	*rhlAB* Rhamnolipid synthesis; Virulence factors	Ochsner et al., 1994 Brint and Ohman, 1995 Latifi et al., 1995 Pearson et al., 1995 Ochsner and Reiser, 1995 Winson et al., 1995
Pseudomonas aureofaciens	PRAI[e]	PhzI/PhzR	*phz* Phenazine biosynthesis	Pierson et al., 1994 Wood and Pierson, 1996
Agrobacterium tumefaciens	*N*-3-(oxooctanoyl)-L-homoserine lactone (AAI)	TraI/TraR, TraM	*tra* genes, *traR* Ti plasmid conjugal transfer	Piper et al., 1993 Zhang et al., 1993 Fuqua and Winans, 1994 Fuqua et al., 1995 Hwang et al., 1995
Erwinia carotovora subsp. *carotovora* SCRI193	VAI-1[f]	ExpI/ExpR	*pel, pec, pep* Exoenzyme synthesis	Bainton et al., 1992 Jones et al., 1993 Pirhonen et al., 1993
Erwinia carotovora subsp. *carotovora* SCC3193	VAI-1[f]	CarI/CarR	*cap* Carbapenem antibiotic synthesis	Bainton et al., 1992

Table 8. (continued)

Bacterial species	Signal molecules[a]	Regulatory Proteins[b]	Target genes and function(s)	References
Erwinia carotovora subsp. *carotovora* SCC3193	VAI-1[f]	CarI/CarR	*cap* Carbapenem antibiotic synthesis	Bainton et al., 1992
Erwinia carotovora subsp. *carotovora* 71	VAI-1[f]	HsII/?	*pel, pec, pep* Exoenzyme synthesis	Chatterjee et al., 1995 Cui et al., 1995
Erwinia stewartii	VAI-1[f]	EsaI/EsaR	*wts* genes Exopolysaccharide synthesis; Virulence factors	Beck von Badman and Farrand, 1995
Rhizobium leguminosarum	*N*-(3R-hydroxy-7-*cis*-tetradecanoyl)-L-homoserine lactone, small bacteriocin (RLA1)	?/RhiR	*rhiABC* Rhizosphere genes and stationary phase	Wijffelman et al., 1983 Cubo et al., 1992 Gray et al., 1996 Schripsema et al., 1996
Enterobacter agglomerans	VAI-1[f]	EagI/EagR	Function unclear	Bainton et al., 1992 Swift et al., 1993
Yersenia enterocolitica	VAI-1[f]	YenI/YenR	Function unclear	Throup et al., 1995
Serratia liquifaciens	*N*-butanoyl-L-homoserine lactone (SAI-1)	SwrI/?	Swarming motility	Eberl et al., 1996
	N-hexanoyl-L-homoserine lactone (SAI-2)	SwrI/?	Swarming motility	Eberl et al., 1996
Aeromonas hydrophila	AHAI[e]	AhyI/AhyR	Function unclear	Swift et al., 1995
Escherichia coli/?[g]		?/SdiA	*ftsQAZ*, cell division	Wang et al., 1991

[a] Only the primary autoinducers synthesized by each organism and predicted to be involved in gene regulation are listed.

[b] Indicates the LuxI-like protein/LuxR-type regulator.

[c] AinS and AinR show no homology with LuxI and LuxR. However, these proteins show significant sequence similarity with the *V. harveyi* LuxM and LuxN proteins.

[d] The *V. harveyi* LuxR regulator is not dependent on HAI-1 and shares no significant similarity to *V. fischeri* LuxR.

[e] The structures of these compounds have not been elucidated.

[f] These compounds have structures identical to VAI-1.

[g] A compound capable of activating SdiA-dependent genes has been reported in *E. coli*, but the structure has not been determined. Non-acylated homoserine lactone has been implicated in the *E. coli* starvation response (Huisman and Kolter, 1994).

(RLAI) (Schripsema et al., 1996) with the same structure as an autoinducer firstly detected in *R. leguminosarum* culture extracts (Gray et al., 1996). In *R. leguminosarum* bv. viciae, four *rhi* (rhizosphere-expressed) genes, namely *rhiABC* and *rhiR*, borne on the Sym plasmid pRL1JI, were identified. The sequence of *rhiR* gene is homologous to *luxR*. The *rhiR* gene is expressed constitutively under all growth conditions (Cubo et al., 1992). Expression of the *rhiA* gene occurs only in late exponential and stationary phase (Gray et al., 1996). Expression of *rhiABC* requires *rhiR* (Economou et al., 1989) and RLAI (Schripsema et al., 1996). Expression of *rhiABC* is repressed by flavonoids in the presence of NodD (Cubo et al., 1992). The biochemical roles of the *rhi* gene products have not been established. The genes encoding autoinducer synthesis are not on the Sym plasmid and remain to be identified. One interesting feature is that the presence of Sym plasmid, pRL1JI, blocks the production of the RLAI, although genes on this plasmid are required for the response to RLAI (Gray et al., 1996). The mechanism by which autoinducer synthesis is inhibited by the presence of pRL1JI remains to be determined. As mentioned previously, RLAI (i.e., bacteriocin - *small*) can inhibit the growth of *R. leguminosarum* cells containing pRL1JI, but not cells lacking this plasmid (Hirsch, 1979; Wijffelman et al., 1983). The presence of *rhiR* and *rhiABC* alone was insufficient for growth inhibition (Gray et al., 1996). Gray et al. (1996) showed that RLAI and RhiR induce functions on pRL1JI, other than *rhiABC*, and these other functions interfere with the growth of *R. leguminosarum* cells. They speculated that this response to the autoinducer is required to limit growth of *R. leguminosarum* bv. viciae during host root

infection; thereby, avoiding excessive bacterial growth that might induce a plant defense response.

Overview of the Study

Induction of *nod* gene expression in *B. japonicum* is regulated by a complex interaction of transcriptional activators and repressors. The activators identified so far are NodD1 and NodV/NodW. The nature of NodD1 in *B. japonicum* is different from other rhizobia since it is positively autoregulatory in the presence of flavonoid inducers. A unique feature of *B. japonicum* is the involvement of a two-component regulator, NodV and NodW on *nod* gene regulation. In addition, the previously reported inhibitory effect of NolA (Dockendorff et al., 1994a) on *nod* gene expression is now thought to occur via NodD2, whose expression is activated by NolA. Therefore, NolA may play an indirect role in *nod* gene expression (Garcia et al., 1996). The involvement of these various positive and negative transcriptional factors finely tunes the level of *nod* gene expression in *B. japonicum*. Past studies have focused on identifying the genetic components involved in *nod* gene regulation. However, other than the flavonoid inducers, few studies have focused on the physical and chemical factors that may regulate *nod* gene expression. For example, previous work from our laboratory showed that combined nitrogen represses *nod* gene expression in free-living *B. japonicum* (Wang and Stacey, 1990).

It is well known that *nod* gene expression is repressed in bacteroids. However, the mechanism for this repression is unknown (Schlaman et al., 1991; Schlaman et al.,

1992a; Sharma and Signer, 1990). The lack of information on physical and chemical factors affecting *nod* gene regulation and their possible roles in repressing gene expression in bacteroids stimulated my research. In addition, the effect of cell growth phase and cell density on *nod* gene expression was investigated. Furthermore, *nodD1* expression relating to growth of the cells was also examined.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used are listed in Table 9. Generation of bacterial strains and plasmids are described in text.

Culture Media and Growth Conditions

All strains of *Bradyrhizobium japonicum* were grown and maintained on RDY medium (So et al., 1987) at 30 °C. RDY consists of 1 g/L yeast extract, 0.12 g/L KH_2PO_4, 0.12 g/L K_2HPO_4, 0.1 g/L $MgSO_4$, 1 ml/L of 1000-fold concentrated trace element solution, 1 g/L sodium L-glutamate, and 5 g/L sodium D-gluconate, pH 7.0. 1000-fold concentrated trace element solution is composed of 3 g/L H_3BO_3, 2.23 g/L $MnSO_4 \cdot 4H_2O$, 0.29 g/L $ZnSO_4 \cdot 7H_2O$, 0.125 g/L $CuSO_4 \cdot 7H_2O$, 0.065 g/L $CoCl_2$, 0.12 g/L $Na_2MoO_4 \cdot 2H_2O$, and 1 mM $FeCl_3$. Minimal medium (Bergersen, 1961) was used for growth of *B. japonicum* for induction of the *nodD1* and other *nod* genes. MM consists of 0.3 g/L KH_2PO_4, 0.3 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 1 ml/L of 1000-fold concentrated trace element solution, 0.5 g/L NH_4NO_3, 0.4% glycerol, 5.8 g/L MOPS, 0.0002 g/L biotin, 0.0001 g/L thiamine, and 0.0001 g/L calcium pantothenate, pH 7.0. *E.*

coli strains were cultured in LB (Sambrook *et al.*, 1989) at 37 °C for routine growth. For growth of *B. japonicum* in soft agar slants (0.8%), nitrate medium (Stacey et al., 1994) was used, which consists of 0.25 g/L yeast extract, 0.12 g/L KH_2PO_4, 0.12 g/L K_2HPO_4, 0.1 g/L $MgSO_4$, 1 ml/L of 1000-fold concentrated trace element solution, 10 mM KNO_3, and 5 g/L sodium D-gluconate, pH 7.0. The antibiotics used for plasmid maintenance and selection are: 200 μg/ml ampicillin, 100 μg/ml kanamycin, 20 μg/ml chloramphenicol, and 20 μg/ml tetracycline for *E. coli.*; 100 μg/ml kanamycin, streptomycin, spectinomycin, and tetracycline, and 30 μg/ml chloramphenicol for *B. japonicum.*

β-Galactosidase Activity Assays

Induction of *nod* gene expression in *B. japonicum* was determined by the activity level of β-galactosidase activity expressed from *nod-lacZ* fusions. Prior to transfer into induction medium, bacterial cells were grown to an A_{600nm} of 0.2 to 0.8 in RDY. Inducers, such as genistein, and compounds to be tested for inducing ability were dissolved in ethanol and stored at 4 °C. Unless specified, cells were diluted in 2 ml of MM to an initial cell density of A_{600nm} of 0.025 and induced. As an uninduced control, an identical volume of ethanol was added to one culture. Compounds, such as organic acids and amino acids, tested for their effect on *nod* gene expression, were dissolved in water, and adjusted to pH 7 before addition to the induction medium. An identical volume of water was added to the "No addition" control. Induction cultures were incubated at 28-30 °C with shaking at 200 rpm for 10 to 14 hours. The β-galactosidase assay was done as

described by Banfalvi et al. (1988). The substrate used was chlorophenol red-β-galactopyranoside (CPRG) (Boehringer Mannheim, Indianapolis, IN).

Enzymes and DNA Primers

Most enzymes used in this study were purchased from Promega (Madison, WI) and Stratagene (La Jolla, CA), and used following the protocols suggested by the manufacturers. Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

DNA Isolation and Manipulation

Plasmids from *E. coli* were isolated using the alkaline lysis methods described in Sambrook et al. (1989) or by using the Wizard® Plus Minipreps DNA Purification System (Promega Corporation, Madison, WI).

Isolation of DNA Fragments

DNA restriction endonuclease fragments used in cloning were separated by electrophoresis in agarose gels as described by Sambrook et al. (1989). Gel purified DNA fragments were isolated using a Geneclean II kit (Bio 10 Inc., La Jolla, CA).

Table 9. Bacterial strains and plasmids.

Strain or Plasmid	Relevant Characteristics	Source or Reference
Bacterial Strains		
B. japonicum biovar USDA110		
Bj110	wild type, Cm^r	USDA, Beltsville, MD
573	wild type, chromosomal *nodC-lacZ* fusion, Tc^r	Dockendorff et al., 1994a
ZB977	wild type, pZB32 with *nodY-lacZ* fusion, Tc^r	Banfalvi et al., 1988
SW100	wild type, pZB22 with *nodD1-lacZ* fusion, Tc^r	Banfalvi et al., 1988
Δ1267	also named as 1267-32, Δ*nodD1D2nolA*, pZB32 with *nodY-lacZ* fusion, Sp^r, Sm^r, Tc^r	Göttfert et al., 1992
SR15-32	also named as SR15-1, Δ1.5 kb deletion in putative repressor sequence, pZB32 with *nodY-lacZ* fusion, Tc^r	Sanjuan et al., 1992
GS300	wild type, pIJ1025 with *nifD-lacZ* fusion, Tc^r	Stacey et al., 1994
SL101	wild type, pGD499 with *nptII-lacZ* fusion, Tc^r	Ditta et al., 1985
AN314	*nodD1*::Ω, Km^r, Sm^r	Nieuwkoop et al., 1987
168	*nodC*::Ω, Km^r	Göttfert et al., 1989
B. japonicum biovar USDA135		
LB100	wild type, pZB32 with *nodY-lacZ* fusion, Tc^r	Banfalvi et al., 1988
LB101	wild type, pZB22 with *nodD1-lacZ* fusion, Tc^r	Banfalvi et al., 1988

Table 9. (continued)

Strain or Plasmid	Relevant Characteristics	Source or Reference
E. coli		
HMS174(DE3)pLysS	F^-, *recA*, r_{K12}^-, m^+, DE3 lysogen, Rif^r; pLysS plasmid, Cm^r	Studier et al., 1990
JM109	*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*, (r_K^-, m_K^+), *relA1*, *supE44*, Δ(*lac-proAB*), [F', *traD*36, *proAB*, $lacI^q$ZΔM15]	Messing, 1983
Plasmids		
pRSETB	Ap^r, T7 promoter	Invitrogen, San Diego, CA
pCR™2.1	Ap^r, Km^r, T7 promoter	Invitrogen, San Diego, CA
pZB22	*nodD1-lacZ*, Tc^r, Ap^r	Banfalvi et al., 1988
pIJ1025	*nifD-lacZ*, Tc^r	H. Hennecke
pGD499	*nptII-lacZ*, Tc^r	G. Ditta
pZB32	*nodY-lacZ*, Tc^r, Ap^r	Banfalvi et al., 1988
pCR-D1-1	*nodD1* cloned in pCR™2.1, Ap^r, Km^r	This study
pRSETBD1-1	*nodD1* cloned in pRSETB, Ap^r	This study

Construction of a Plasmid Expressing a His-tag NodD1 Fusion Protein from a T7 Promoter

A NodD1 fusion protein was expressed in *E. coli* cells from the T7 promoter of expression vector pRSETB (Invitrogen, San Diego, CA). This allowed production of a high level of protein for antibody generation. The plasmid used in expressing NodD1 was constructed as follows: Based on the *nodD1* sequence, a 5' primer (5'-GGA TCC ATG CGG TTG AAG GGA CTT G-3') and a 3' primer (5'-GGT AAG CCT AAC AGC GCC TGC G-3') were designed to generate a PCR product containing the full length *nodD1* gene (945 bp), as well as a BamHI restriction site introduced upstream to the translational start site (ATG) of *nodD1*. The PCR product (951 bp) was cloned into pCR^{TM}2.1 (Invitrogen). The orientation of the insert was determined by PstI digestion. A BamHI-BamHI fragment containing *nodD1* (987 bp) was released from pCR^{TM}2.1 (pCR-D1-5) and cloned into a BglII-digested pRSETB (Invitrogen) to generate pRSETBD1-1. The resulting plasmid (pRSETBD1-1) was transformed into *E. coli* HMS174 (DE3) (pLysS). The in-frame translational fusion of *nodD1* in this plasmid was confirmed by DNA sequence analysis by the dideoxy chain termination method (Sanger et al., 1977) using an ABI Prism 377 automated DNA sequencer (Perkin-Elmer, Norwalk, CT).

Purification of NodD1 Fusion Protein

E. coli strain PRSETBD1-1 was grown overnight in 100 ml LB containing 200 μg/ml Ap and 20 μg/ml Cm at 37 °C and 200 rpm. This culture was diluted into two

liters of LB containing the same concentration of antibiotics and incubated under the same conditions. When an A_{600nm} of 0.6 was reached, 1.5 ml of the culture was taken as the uninduced control. IPTG (Sigma, St. Louis, MO) was added into the remaining culture to a final concentration of 0.2 mM and the cells were induced for 4 hours at 37 °C and 200 rpm. After this incubation, 1.5 ml of the culture was taken as the induced control. In order to determine if the fusion protein expressed was soluble, cell lysates were prepared from 50 ml culture under both native (non-denaturing) conditions (for soluble protein) and denaturing conditions (for both soluble and insoluble protein) according to QIAGEN protocols (QIAGEN, Valencia, CA). (Appendix 1). 1 ml of a 50% nickel-charged resin slurry (Ni-NTA agarose, QIAGEN) was added to the lysate and the lysate-resin mixture was shaken gently on a rotary shaker for 60 min (at 4 °C for native lysate and at room temperature for denaturing lysate). The resin was washed in a 10-ml column (Bio-Rad, Hercules, CA) with wash buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole for native lysate, or 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris.HCl, pH 6.3 and then pH 5.9 for denaturing lysate) until the wash fractions reached an A_{280nm} less than 0.01. The fusion protein was eluted from the resin with elution buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole for native lysate, or 8M urea; 0.1 M NaH_2PO_4; 0.01 M Tris.HCl, pH 4.5 for denaturing lysate). Eluate was analyzed by SDS-PAGE and visualized by silver staining (Sambrook et al., 1989). As these initial results indicated that the fusion protein was found in the insoluble fraction, all cell lysates were subsequently prepared under denaturing conditions. Cells harvested from 2-L cultures were lysed in 40 ml lysis buffer. The eluate, containing the eluted

fusion proteins, was dialyzed two times against 10 mM Tris.HCl (pH 7.9) containing 0.1% Triton X-100, and once against 10 mM Tris.HCl (pH 7.9). The dialysate was dried using a Speed vac concentrator (Savant, Holbrook, NY). Precipitated proteins were re-dissolved in a small amount of distilled water and mixed with an equal volume of 2X SDS-PAGE sample-loading buffer (100 mM Tris.HCl, pH 6.8; 200 mM DTT; 4% SDS; 0.2% Bromophenol blue; 20% glycerol). The samples were boiled for 15-20 min to enhance the solubilization of proteins. Undissolved proteins were removed by centrifugation at 14,000 rpm in a microcentrifuge for 1 min, and the soluble proteins in the supernatant were separated by a 12% preparative SDS-PAGE. After electrophoresis, the fusion protein was purified by gel electroelution (Harlow and Lane, 1989). The procedure is described as follows: The gel was stained with fresh 0.05% Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA) in distilled H_2O for 30 min and was destained in distilled H_2O for several hours with several changes until the bands were distinguishable. The band of the fusion protein (about 40 kD) was cut out and sliced. The gel slices were combined with dialysis buffer (0.2 M Tris/acetate, pH 7.4; 1.0% SDS; 100 mM DTT) in a ratio of 1.0 ml for 0.1 g of wet gel. The mixture was placed in a dialysis tubing (Spectrum Laboratories, Inc., Laguna Hills, CA) and the protein was electroeluted in a horizontal electrophoresis chamber filled with electrophoresis buffer (50 mM Tris/acetate, pH 7.4; 0.1% SDS; 0.5 mM sodium thioglycolate) for 3 hours at 100 V. Electroeluted proteins were then dialyzed three times against 0.2 M $NaHCO_3$ containing 0.02 % SDS at room temperature, and three times against 10 mM Tris (pH 7.9) to remove SDS. The proteins were concentrated in a Speed vac concentrator

(Savant, Holbrook, NY) and precipitated by 50% acetone (vol/vol) at -20 °C overnight. Proteins were redissolved in PBS and the concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). The purity of protein was analyzed by SDS-PAGE and immunoblotting using anti-His peptide antibodies (Penta·His Antibody, QIAGEN).

Immunization of Rabbits

For the first immunization, a solution containing 0.6 mg purified His-tag NodD1 fusion protein was emulsified with an equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO), and injected subcutaneously at 10 sites into a female New Zealand white rabbit. Booster injections were administered twice at a three-week interval with 0.8 mg of the fusion protein emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO). Blood was collected 7 to 10 days after each injection. The pre-immune and immune sera were processed (Harlow and Lane, 1989) and kept at -20 °C in aliquots. Titers of the antisera were determined by ELISA using purified His-tag fusion protein as the antigen (Harlow and Lane, 1989).

Purification of Antibodies by Immunoaffinity Chromatography

Electroeluted His-tag NodD1 fusion proteins were loaded and bound to 1 ml Ni^{2+}-charged resin (Ni-NTA resin). The resin was washed with wash buffer (50 mM

NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole) to remove unbound proteins. Antibodies in the antiserum were first partially purified by precipitation with 50% $(NH_4)_2SO_4$, re-dissolved in one-fourth volume of PBS, and dialyzed against PBS overnight at 4 °C with three changes. The $(NH_4)_2SO_4$-purified antibody solution, which also contained non-specific antibodies, was then passed over the NodD1-bound Ni^{2+}-column to allow complete binding of anti-NodD1 antibodies. The resin was extensively washed with 10 mM Tris (pH 7.9) containing 300 mM NaCl. The bound antibodies were eluted with 4 M urea in 50 mM NaH_2PO_4 and 5 mM Tris.HCl (pH 8.0). The eluate was dialyzed against PBS overnight at 4 °C with three changes. Antigen-purified antibodies were kept at -80 °C in aliquots.

Immunoblotting

E. coli samples analyzed for protein expression were obtained by boiling the cells in SDS-PAGE sample buffer for 15 min. Lysed cell debris was pelleted by brief centrifugation and the supernatant containing the proteins was subjected to SDS-PAGE analysis. For *B. japonicum*, cell extracts were prepared by washing the cells in 50 mM Tris.HCl (pH 7.5) and resuspending in the same buffer containing 1 mM EDTA , 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 8 μg/ml leupeptin, 2 μg/ml chymostatin, and 10 μg/ml pepstatin A (Sigma, St. Louis, MO). Cells were lysed in an eppendorf tube by sonication on ice for six to eight 30-s cycles at an output of 8-9 using a Sonifier model 450 (Branson Ultrasonics, Danbury, CT). Cell extracts were obtained

after removing the cell debris by centrifugation in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. Proteins contained in the samples were separated by 12 % SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA) using a Hoeffer Scientific (San Francisco, CA) electrophoretic transfer apparatus. Membranes were blocked in TBS (20 mM Tris.HCl, pH7.5; 0.5 M NaCl) containing 3% BSA (TBS-BSA) overnight at room temperature with gentle shaking on a rotary shaker. Membranes were then incubated with 0.1 μg/ml Penta·His Antibodies (for detection of His-tag NodD1 fusion protein) or with a 1:3000 diluted antigen purified anti-NodD1 antibodies in TBS-BSA for 5 hours to overnight at room temperature. Unbound antibodies were removed by washing three times (10 min each time) with TTBS, which is TBS containing 0.05% Tween 20 (Sigma, St. Louis, MO). The membranes were incubated with 1:3000 diluted phosphatase-conjugated goat anti-rabbit IgG antibodies in TBS-BSA at room temperature for 1 hour. After removing the unbound secondary antibodies by three washes with TTBS, protein bands bound by the antibodies were visualized by developing the membrane in NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate) (Bio-Rad, Hercules, CA) as chromogenic substrates. The reaction was stopped by rinsing the blot a few times in distilled water.

Isolation of Bacteroids from Root Nodules

Root nodules were collected from 28-day old *Glycine max* (soybean) infected with *B. japonicum* USDA110 strain 573 carrying a chromosomal *nodC-lacZ* fusion and intact

nod genes on the chromosome. Bacteroids were isolated as described by Sarath et al. (1986). Sterilization of seeds before germination was done as described by Nieuwkoop et al. (1987). Inoculation, transfer, and growth of seedlings in Leonard jars was described by Wacek and Brill (1976). Plants were grown in the greenhouse under 16-hour daylight.

Preparation of Nodule Extract

Root nodules were collected from 28-day old *Glycine max* infected with *B. japonicum* USDA110 wild-type cells. 1 g of nodules were crushed in 1 ml 50% ethanol. Plant and bacteroid debris were removed by centrifugation at 10,000 rpm in a Beckman JA-17 rotor at 4 °C for 20 min. Supernatant was collected and filter-sterilized before use.

CHAPTER 3

RESULTS

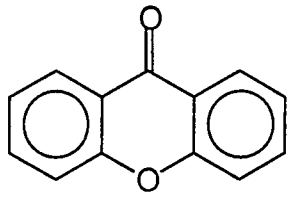
Screening of 40 Aromatic Compounds for Their Ability to Induce *nodD1*- or *nodY-lacZ* Expression

The 40 compounds shown in Table 10 were tested at a concentration of 1 μM for their ability to induce *nod* gene expression in strains ZB977, 573, Δ1267, and LB101. Compared to genistein, which is a known inducer of *B. japonicum nod* gene expression (Banfalvi et al., 1988), 1,6-dihydroxy-1,3-dimethoxyxanthone (compound 19), 1,3,7-trihydroxyxanthone (compound 26), biochanin A (compound 29), and formononetin (compound 39) were able to induce significant *nod* gene expression in all strains examined (Table 10). The relative levels of expression by these four compounds were approximately the same in these strains. It was previously reported that *nod* gene induction in strain Δ1267 was dependent on the presence of NodV and NodW, which are members of the two-component regulatory protein family (Göttfert et al., 1990). NodV and NodW were found to respond to isoflavones and activate *nod* gene expression (Loh et al., 1997). Expression of *nod* genes in strain Δ1267 in response to these inducers suggests that the NodV/W regulatory system may also recognize these compounds. The structures of the compounds are shown in Figure 3.

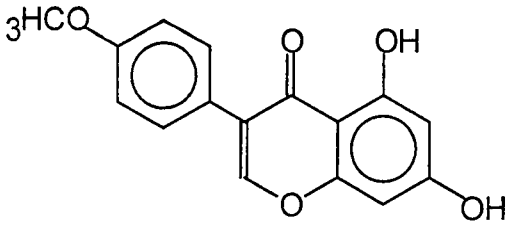
Table 10. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977, *nodC-lacZ* expression in strain 573, *nodY-lacZ* expression in strain Δ1267 (*ΔnodD1nodD2nolA*), and *nodD1-lacZ* expression in *B. j.* USDA135 strain LB101 in the presence of 1 μM of compounds isolated from plants collected in the Brazilian rain forest.

No.	Compounds	M.W.	β-Galactosidase Activity (U)[a]			
			ZB977	573	Δ1267	LB101
1	2-Hydroxyxanthone	212	3	1	1	2
2	3-Hydroxy-1,5,6-trimethoxyxanthone	302	3	1	2	1
3	1,3-Dimethoxyxanthone	256	3	1	1	1
4	4-Hydroxyxanthone	212	3	1	1	1
5	1,3-Dihydroxy-5,6-dimethoxyxanthone	288	3	1	1	1
6	4-Hydroxy-2,3-dimethoxyxanthone	272	2	1	1	1
7	2,8-Dihydroxy-1-methoxyxanthone	258	8	1	5	2
8	2,6-Dihydroxy-1,8-dimethoxyxanthone	288	3	1	2	1
9	2,8-Dihydroxy-1,6-dimethoxyxanthone	288	3	1	2	1
10	3-Hydroxy-2,4-dimethoxyxanthone	272	3	1	2	1
11	1-Hydroxy-7,8-dimethoxyxanthone	272	9	1	12	3
12	2,3-Dimethoxyxanthone	256	2	1	2	1
13	1,3,5-Trimethoxyxanthone	286	3	1	2	1
14	3-Hydroxyxanthone	212	2	1	2	1
15	1,5-Dihydroxy-3-methoxyxanthone	258	2	1	2	1
16	2,3,4-Trimethoxyxanthone	286	2	1	2	1
17	1,8-Dimethoxyxanthone	256	2	1	2	1
18	5,6-Dihydroxy-1,3-dimethoxyxanthone	288	2	1	4	1
19	1,6-Dihydroxy-2,8-dimethoxyxanthone	288	1840	398	1651	67
20	4-Methoxyxanthone	226	6	1	N.D.	3
21	2-Methoxyxanthone	226	7	1	1	2
22	1,8-Dihydroxyxanthone	228	2	1	1	1
23	1,7-Dihydroxyxanthone	228	3	1	2	1
24	1-Hydroxyxanthone	212	2	1	2	1
25	8-Hydroxy-1,2-Dimethoxyxanthone	272	3	1	2	1
26	1,3,7-Trihydroxyxanthone	244	524	140	523	9
27	2,3-Dihydroxy-4-methoxyxanthone	258	2	1	2	1
28	2,5-Dihydroxy-1-methoxyxanthone	258	3	1	2	2
29	Biochanin A	284	2121	604	1918	46
30	Quercetin	302	2	1	2	1
31	Morin	302	2	1	2	2
32	Acetilated Rutin	792	3	1	3	2
33	Acetilated Morin	512	2	1	1	1
34	Acetilated Naringenin	398	2	1	3	1
35	Naringenin	272	5	1	5	2
36	Quercetrin	448	2	1	3	1
37	Kaempherol	286	4	1	4	2
38	Isoquercetrin	464	2	1	3	2
39	Formononetin	268	1042	48	1479	18
40	Modified xanthone (nucleus)	196	2	1	3	1
	Genistein	270	2270	866	2885	85
	No addition		2	1	2	1

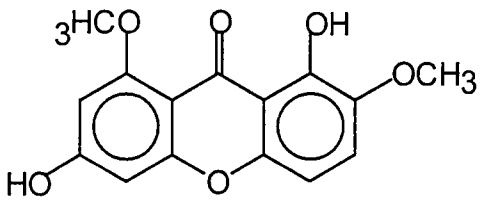
[a] Values of β-Galactosidase activity are the average of two or more assays with a deviation less than or equal to 20%.



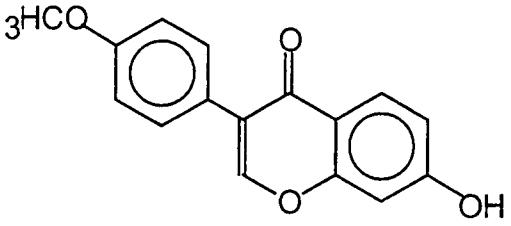
Xanthone



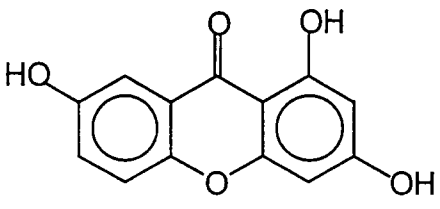
Biochanin A



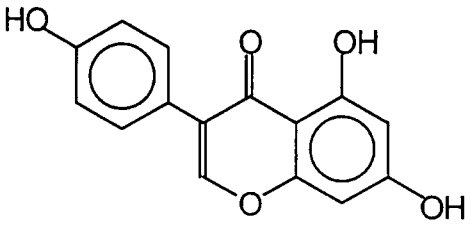
1,6-Dihydroxy-2,8-dimethoxyxanthone



Formononetin



1,3,7-Trihydroxyxanthone



Genistein

Figure 3. Structure of xanthone and tested compounds which were found to be able to induce *nod* gene expression.

Relative Effectiveness of Xanthones as Inducers of *nod* Gene Expression

The effectiveness of 1,6-dihydroxy-1,3-dimethoxyxanthone and 1,3,7-trihydroxy-xanthone as *nod* gene inducers was tested at different concentrations (0.005 to 5 μM). Genistein and formononetin were included for comparison. Genistein is the primary, natural inducer of *nod* gene transcription, while formononetin had previously been shown to be a weak inducer (Banfalvi et al., 1988). As shown in Figure 4, 1,3,7-trihydroxyxanthone and formononetin showed a similar pattern of *nod* gene induction. At a level of 2 μM, the expression was maximal with an 18-fold and 25-fold increase, respectively. However, a high level of *nodY-lacZ* induction was observed with a relatively low concentration of 1,6-dihydroxy-2,8-dimethylxanthone (i.e., 6-, 18-, and 40-fold at 0.005, 0.01, and 0.025 μM, respectively). By comparison, genistein induced 12-, 62-, and 98-fold expression at equivalent concentrations. Induction of *nod* gene expression in response to either 1,6-dihydroxy-2,8-dimethoxyxanthone or genistein leveled off at approximately 0.025 μM and was maximal at 1 μM. Thus, these results show that xanthone compounds can be very effective inducers of *nod* gene expression in *B. japonicum*.

Enhancement or Inhibition of *nodY-lacZ* Expression by Different Carbon Compounds

Regulation of *nod* genes in *B. japonicum* and *vir* gene regulation in *Agrobacterium tumefaciens* are similar in that they both involve a two-component

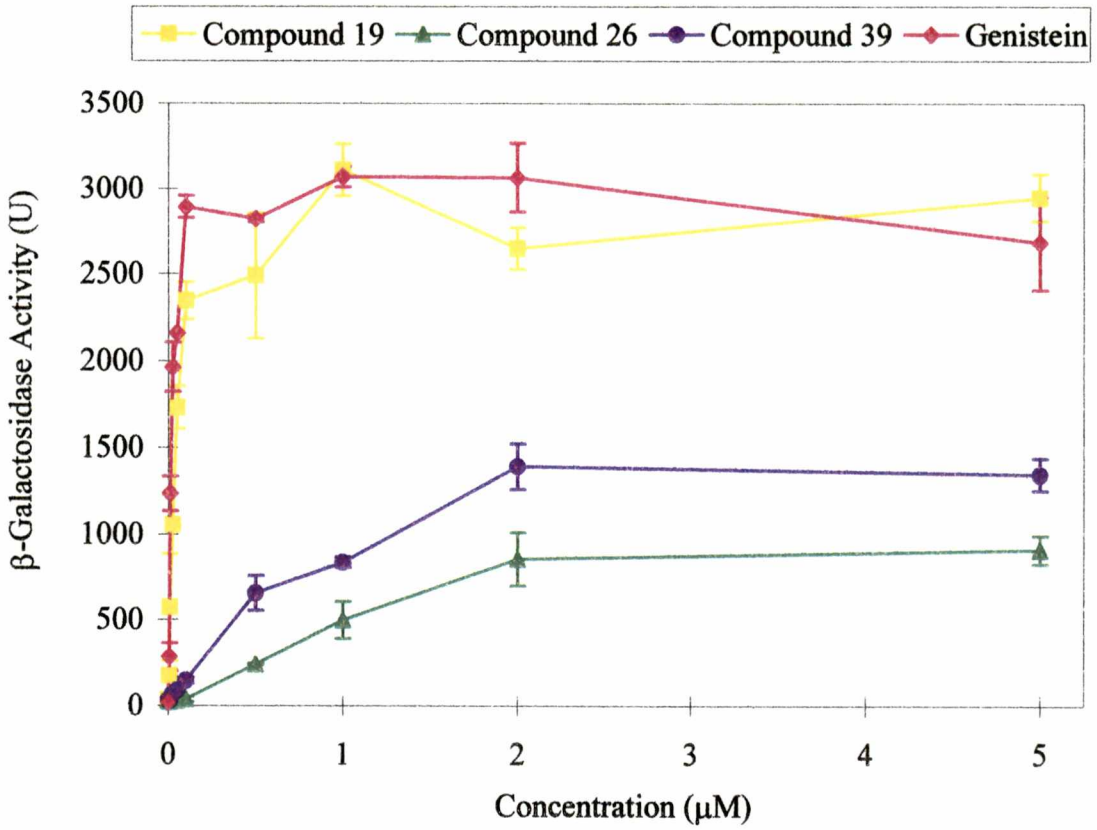


Figure 4. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 in the presence of compound 19 (1,6-dihydroxy-2,8-dimethoxyxanthone), compound 26 (1,3,7-trihydroxyxanthone), compound 39 (formononetin), and genistein at different concentrations.

regulatory system. Ankenbauer and Nester (1990) showed that certain sugars (e.g. L-(+)-arabinose) can act synergistically with the plant phenolic metabolite, acetosyringone, to induce *A. tumefaciens vir* gene expression at a high level. The following experiment was designed to determine if a similar mechanism is involved in *nod* gene regulation in *B. japonicum*. In order to test the effect of various organic acids, sugars, and amino acids on *nod* gene expression, strain ZB977 (USDA110; *nodY-lacZ*) was induced with 0.025 μM genistein in the presence of 1 or 10 mM of each compound listed in Table 11. As shown, the expression of *nodY-lacZ*, as compared to that of the negative control ("no addition"), was increased by 20 to 50% (less than two-fold) when malonate, L-(+)-tartarate, D-(+)-gluconate, D-(+)-xylose, D-(+)-fructose, D-(+)-galactose, D-(+)-glucose, D-mannitol, D-(+)-mannose, or sucrose was added. However, addition of acetate, fumarate, L-malate, succinate, α-ketoglutarate, or oxaloacetate resulted in a significant inhibition of *nod* gene expression. Gene expression was reduced approximately 50 and 70%, respectively, at 1 and 10 mM of fumarate, L-malate, or succinate. The inhibitory effect of 10 mM acetate was 3.8-fold more than that at 1 mM. Interestingly, the expression of *nodY-lacZ* was reduced 54% at 10 mM oxaloacetate, and 89% at 10 mM α-ketoglutarate, but were enhanced to 41% and 46%, respectively, when 1 mM of these organic acids was added. A 30% reduction of expression occurred with the addition of 1 mM pyruvate, citrate, or L-aspartate, or 10 mM L-glutamate.

Table 11. Induction of *nodY-lacZ* expression in *B. japonicum* strain ZB977 in the presence of different carbon sources. Induction was done in MM in the presence of 0.025 μM genistein as inducer. Inhibitory compounds are shown in bold.

Carbon Sources	Mean β-Galactosidase Activity (U) ± SD[a]					
	1 mM			10 mM		
	Ind.[b]	Unind.[b]	%[c]	Ind.	Unind.	%
	Experiment 1A[d]			Experiment 2A		
Acetate	**1817 ± 319**	**59**	**-18**	**559 ± 108**	**52**	**-68**
Pyruvate	1553 ± 133	58	-30	1402 ± 169	23	-19
Malonate	2632 ± 101	40	+18	1912 ± 115	17	+11
Fumarate	**1142 ± 125**	**79**	**-49**	**460 ± 24**	**22**	**-73**
L-Malate	**1144 ± 132**	**41**	**-49**	**592 ± 98**	**25**	**-66**
Oxaloacetate	**3147 ± 14**	**44**	**+41**	**790 ± 73**	**16**	**-54**
Succinate	**1084 ± 480**	**61**	**-51**	**543 ± 77**	**26**	**-69**
L-(+)-Tartarate	3068 ± 311	41	+39	2533 ± 59	26	+47
α-Ketoglutarate	**3258 ± 253**	**48**	**+46**	**197 ± 51**	**25**	**-89**
Citrate	2493 ± 605	46	0	1101 ± 63	15	-36
D-(+)-Gluconate	3516 ± 138	31	+58	2107 ± 129	15	+18
No addition[e]	2228 ± 46	28	/	1727 ± 157	36	/
	Experiment 1B			Experiment 2B		
L-Aspartate	1965 ± 172	46	0	1154 ± 8	44	-30
L-Glutamate	2079 ± 323	52	0	1189 ± 176	46	-28
L-(+)-Arabinose	2187 ± 73	49	0	1732 ± 129	36	0
D-(+)-Xylose	2690 ± 203	33	+41	2096 ± 78	39	+28
β-(+)-Fructose	2517 ± 93	62	+32	2252 ± 216	36	+37
D-(+)-Galactose	2505 ± 81	50	+31	1937 ± 100	42	+18
D-(+)-Glucose	2694 ± 183	42	+41	2277 ± 66	37	+39
D-Mannitol	2423 ± 219	47	+21	2224 ± 166	38	+36
D-(+)-Mannose	2280 ± 254	41	+20	2045 ± 126	40	+25
Lactose	2618 ± 103	57	+37	1335 ± 127	32	-19
Sucrose	2804 ± 18	35	+35	2278 ± 326	34	+32
No addition	1906 ± 183	50	/	1640 ± 85	35	/

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] Ind. stands for Induced cultures. Unind. stands for Uninduced cultures. No replicate was done for the uninduced cultures.

[c] % stands for % of enhancement (indicated by "+") or inhibition (indicated by "-") of the activity. It is equal to [(act. with no addition – act. with that carbon source) / act. with no addition] × 100%. Data used for calculating % of inhibition are within the same experiment.

[d] Testing of different carbon sources was done in four separate experiments.

[e] Equal volume of water was added.

The Effect of Inducer Concentration on the Inhibition of *nod* Gene Expression by L-Malate

The results shown in Figure 5 indicate that the *nod* gene induction level is a function of inducer concentration. The possibility that L-malate inhibition of *nod* gene expression can be relieved by using higher inducer concentrations was tested. *nodY-lacZ* expression in strain ZB977 and chromosomal *nodC-lacZ* expression in strain 573 were induced with different concentrations of genistein (0.025 to 5 μM) in the presence of 1 mM L-malate (Table 12 and Figure 5). The induction level of *nod-lacZ* expression in these strains was 45- and 72-fold, respectively, at 0.025 μM genistein (50% of the maximal level at 4 μM genistein for strain ZB977), and then leveled off with increasing amount of genistein added. The effect of 1 mM L-malate on reducing the expression level was different when induction was assayed at different concentrations of genistein. The highest inhibition, 77% and 64% in strain ZB977 and 573, respectively, occurred when the lowest concentrations (0.025 and 0.01 μM) of genistein were used. Induction with higher concentrations of genistein resulted in a reduction of the inhibitory effect, which was 17 % in strain ZB977 and 5% in strain 573 at 2 μM genistein. A similar dose-response curve was observed when *nod* gene expression was induced with soybean seed extract. As shown in Table 13, the greatest level of *nod* gene induction in both ZB977 and 573 strains occurred when 10 μl/ml of soybean seed extract was used. The decrease of expression in the presence of greater amounts of soybean seed extract was not due to a change in pH or growth upon its addition. Addition of 5 mM L-malate did not cause any inhibition of *nodY-lacZ* expression when cells of strain ZB977 were induced with an

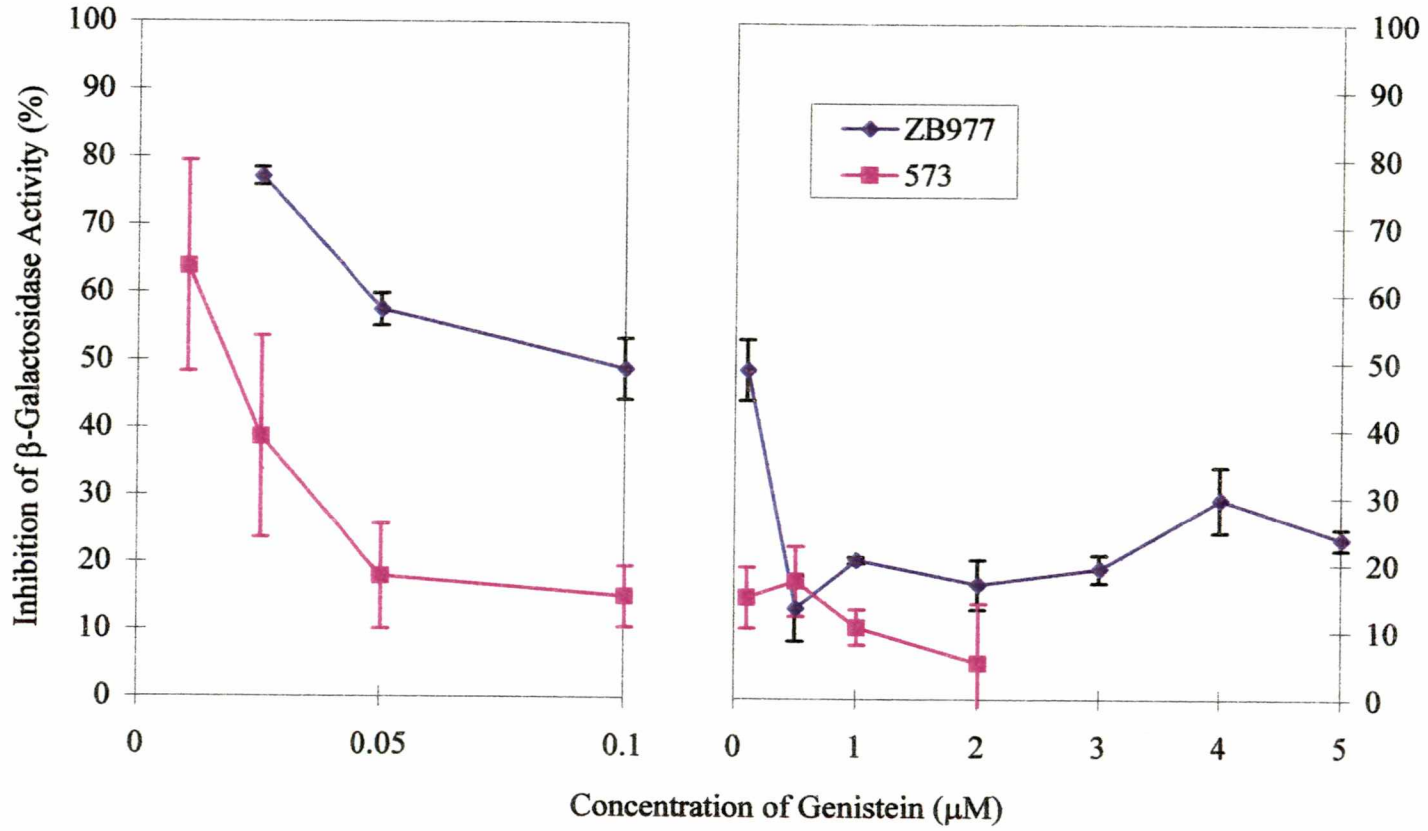


Figure 5. Inhibition (%) of *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 and chromosomal *nodC-lacZ* expression in strain 573 by 1 mM L-malate induced with genistein at different concentrations.

Table 12. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 and chromosomal *nodC-lacZ* expression in strain 573 induced by genistein at different concentrations in the presence or absence of 1 mM L-malate. Induction was done in MM.

	ZB977				573		
	β-Galactosidase Activity ± SD[a]				β-Galactosidase Activity ± SD[a]		
Conc. of genistein (μM)	L-Malate added		% Inh.[b]	Conc. of genistein (μM)	L-Malate added		% Inh.
	0 mM	1 mM			0 mM	1 mM	
0	52 ± 2	47 ± 9	10	0	16 ± 5	9 ± 8	43
0.025	2345 ± 183	537 ± 43	77	0.01	380 ± 87	137 ± 84	64
0.05	2900 ± 65	1233 ± 97	57	0.025	1154 ± 208	709 ± 239	39
0.1	3323 ± 14	1702 ± 213	49	0.05	1248 ± 159	1025 ±130	18
0.5	3209 ± 142	2778 ± 222	13	0.1	1368 ± 16	1163 ± 88	15
1	2918 ± 1	2317 ± 19	21	0.5	1689 ± 136	1395 ± 123	17
2	3337 ± 76	2769 ± 175	17	1	1674 ± 80	1496 ± 58	11
3	3725 ± 168	3005 ± 111	19	2	1711 ± 27	1620 ± 198	5
4	4229 ± 281	2975 ± 292	30				
5	3149 ± 81	2400 ± 71	24				

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] % Inhibition is equal to [(act. without L-malate – act. with 1 mM L-malate) / act. without L-malate] × 100% at each concentration of genistein tested.

Table 13. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 and chromosomal *nodC-lacZ* expression in strain 573 induced by soybean seed extract (SSE) at different concentrations. pH and the absorbance at 600nm of the induction cultures are shown. Induction was done in MM.

	ZB977			573		
SSE (µl/ml)	β-Galactosidase Activity ± SD (U)[a]	pH	A_{600nm}	β-Galactosidase Activity ± SD (U)	pH	A_{600nm}
0	38 ± 8	7.02	0.070	6 ± 1	7.02	0.083
0.25	3530 ± 5	7.02	0.069	1143 ± 66	7.03	0.084
0.5	2941 ± 86	7.03	0.072	1486 ± 165	7.03	0.085
1	4168 ± 116	7.03	0.071	1713 ± 252	7.05	0.086
2.5	3935 ± 182	7.04	0.070	2078 ± 1	7.05	0.084
5	4438 ± 407	7.04	0.066	2280 ± 149	7.05	0.082
10	5026 ± 50	7.04	0.062	2332 ± 19	7.04	0.073
30	3215 ± 178	7.02	0.062	1383 ± 48	7.03	0.069
50	2655 ± 102	7.02	0.065	1029 ± 8	7.01	0.071
70	1643 ± 47	7.00	0.067	937 ± 20	7.00	0.072

[a] Results are presented as the mean of three independent assays followed by the standard deviation (SD).

optimal amount of soybean seed extract (i.e. 10 μl/ml) (Table 14). Though experiments investigating the effect of L-malate on *nod* gene expression at lower levels of soybean seed extract were not performed, we believe that inhibition of *nod* gene expression will occur in the presence of soybean seed extract at sub-optimal levels.

Inhibition of *nodD1-lacZ* Expression by L-Malate

Since induction of the *nodYABC* operon requires NodD1 (Banfalvi et al., 1988), an experiment was performed to test whether the effect of L-malate on *nod* gene expression was a result of inhibiting *nodD1* expression. Induction of *nodD1-lacZ* expression in both strain SW100 (USDA110 wild type) and strain LB101 (USDA135 wild type) was assayed in the presence of different of L-malate concentrations (Table 15). The results show that 1 mM L-malate inhibited *nodD1-lacZ* expression by about 60%. The induction level of *nodD1* expression in SW100 was very low (only 12 U as compared to 450 U in LB101). This may account for the relatively lower L-malate inhibition of *nodD1* expression in this strain.

Specific Inhibitory Effect of L-Malate on *nod* Gene Expression

To examine if L-malate inhibition is specific to *nod* genes, induction of *nod* gene expression in strain ZB977, Δ1267, and 573 was compared to *nptII-lacZ* expression (constitutive) in strain SL101 in the presence of 0.5 to 70 mM L-malate (Table 16 and

Table 14. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 induced by 10 µl/ml soybean seed extract (SSE) in the presence of L-malate at different concentrations. Induction was done in MM.

	β-Galactosidase Activity ± SD[a]			
L-Malate (mM)	Induced	Uninduced[b]	pH	% Inhibition[c]
0	5598 ± 337	87	6.96	/
0.5	6079 ± 431	83	6.96	0
1	6486 ± 418	84	6.96	0
5	6632 ± 979	66	6.98	0

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] No replicate was done for the uninduced cultures.

[c] % Inhibition is equal to [(act. with no addition – act. with L-malate) / act. with no addition] × 100%.

Table 15. Induction of *nodD1-lacZ* expression in *Bradyrhizobium. japonicum* wild-type strains USDA110 and USDA135 in the presence of L-malate at 1 mM and 5 mM. Induction was done in MM.

	Mean β-Galactosidase Activity (U) ± SD[a]							
Concentration of L-malate	0 mM		1 mM			5 mM		
Strains	Ind.	Unind.[b]	Ind.	Unind.	Inh. %[c]	Ind.	Unind.	Inh. %
B. j. USDA110[d] strain SW100	12 ± 1	2	9 ± 1	3	25 %	11 ± 2	2	8 %
B. j. USDA135[e] strain LB101	450 ± 69	18	195 ± 18	15	57 %	207 ± 8	13	54 %

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] There was no replicate done for uninduced cultures.

[c] % Inhibition is equal to [(act. with no addition – act. with L-malate)/act. with no addition] × 100.

[d] *B. j.* USDA110 strain SW100 was induced with 0.1 μM genistein. This strain exhibits very low *nodD1-lacZ* fusion expression.

[e] *B. j.* USDA135 strain LB101 was induced with 0.025 μM genistein.

Table 16. Level of *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 (wt) and strain Δ1267 (Δ*nodD1D2nolA*), chromosomal *nodC-lacZ* expression in strain 573 (wt), and *nptII-lacZ* expression in strain SL101 as a function of L-malate concentration. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Conc. of L-Malate (mM)	ZB977 β-Galactosidase Activity			Δ1267 β-Galactosidase Activity		
	Ind.[a]	Unind.[b]	%[c]	Ind.	Unind.	%
0	1710 ± 205	69	100	1136 ± 88	37	100
0.5	809 ± 24	70	47	837 ± 56	52	74
1	1022 ± 129	98	60	754 ± 92	39	66
10	629 ± 204	62	37	789 ± 45	36	69
30	500 ± 14	65	29	544 ± 120	32	48
50	631 ± 15	80	37	650 ± 204	40	57
70	618 ± 141	68	36	591 ± 88	40	52

	573 β-Galactosidase Activity			SL101 β-Galactosidase Activity		
	Ind.	Unind.	%	Ind.	Unind.	%[d]
0	436 ± 32	2	100	2378 ± 279	2748	100
0.5	231 ± 22	2	53	2420 ± 513	3067	100
1	279 ± 14	9	64	2854 ± 476	2127	100
10	187 ± 36	2	43	3253 ± 355	4041	100
30	124 ± 35	2	28	3704 ± 1062	3738	100
50	98 ± 3	9	22	5300 ± 635	5200	100
70	55 ± 18	4	13	5057 ± 449	6115	100

[a] Ind. stands for Induced cultures. Results are presented as the mean of three independent assays followed by the standard deviation.

[b] Unind. stands for Uninduced cultures. No replicate was done for the uninduced cultures.

[c] % stands for % of β-galactosidase activity in the presence of L-malate at each tested concentration compared to the β-galactosidase activity in the presence of 0 mM L-malate.

[d] Since there was no inhibition on *nptII-lacZ* expression in strain SL101 in the presence of L-malate, % of β-galactosidase activity was taken as 100% at all the concentrations of L-malate tested.

Figure 6). The results show that L-malate inhibited *nod* gene (*nodY-lacZ* and chromosomal *nodC-lacZ*) expression, but not *nptII-lacZ* expression. When L-malate concentrations were varied from 30 to 70 mM, inhibition of *nod* gene expression in ZB977 (wild type) or Δ1267 (*nodD1D2nolA* mutant) did not increase. However, a linear increase of inhibition of chromosomal *nodC-lacZ* expression was observed in strain 573 with increasing L-malate concentrations. Inhibition of *nodY-lacZ* expression was generally less pronounced in strain Δ1267. Since *nod* gene expression in this mutant was shown to be mediated by NodV and NodW (Sanjuan et al., 1994), the results suggest that the inhibitory effect of organic acids on *nod* gene expression probably involves NodV and NodW (Figure 6 and Table 16), as well as NodD1 (Table 15).

Inhibitory Effect of a Mixture of Organic Acids on *nod* Gene Expression

The organic acids, especially L-malate and succinate, that inhibited *nod* gene expression (Table 11) are thought to be the major carbon sources for bacteroids (Stowers 1985). Since *nod* gene expression is repressed in planta (Schlaman et al. 1991), the presence of these organic acids in nodules may account for the lower level of *nod* gene expression in bacteroids. In order to study the possible combined effect of organic acids, *nodY-lacZ* expression in strain ZB977 in the presence of L-malate, succinate, or a mixture of both was examined (Figure 7). A 50% reduction in expression occurred with either 5 mM L-malate or succinate. A combination of both did not have an additive or synergistic effect on inhibition. In contrast, addition of acetate to L-malate did produce

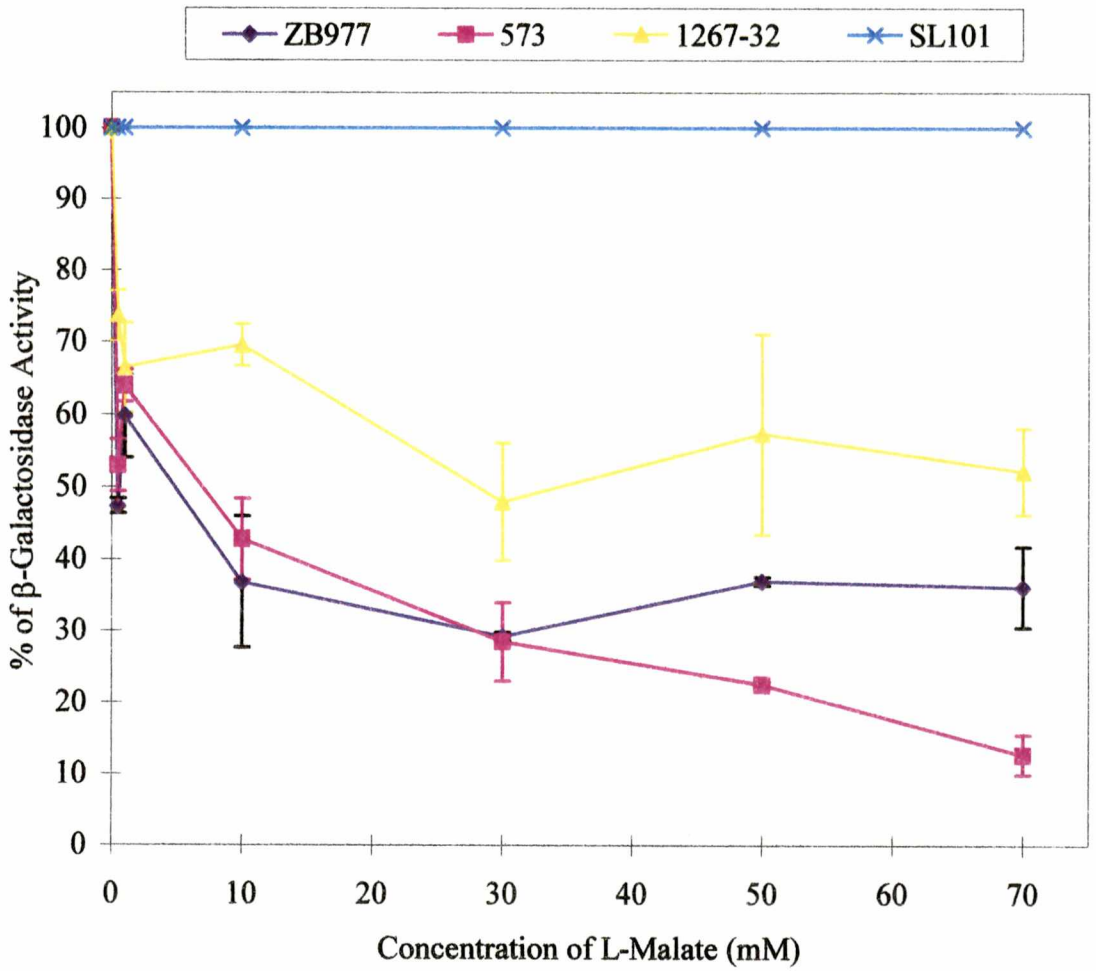


Figure 6. Level of *nodY-lacZ* expression in *Bradyrhizobium japonicum* strain ZB977 (wt) and strain 1267-32 (Δ*nodD1D2nolA*) and chromosomal *nodC-lacZ* expression in strain 573 (wt) as compared to *nptII-lacZ* expression in strain SL101 as a function of L-malate concentration. 0.025 μM genistein was present as inducer.

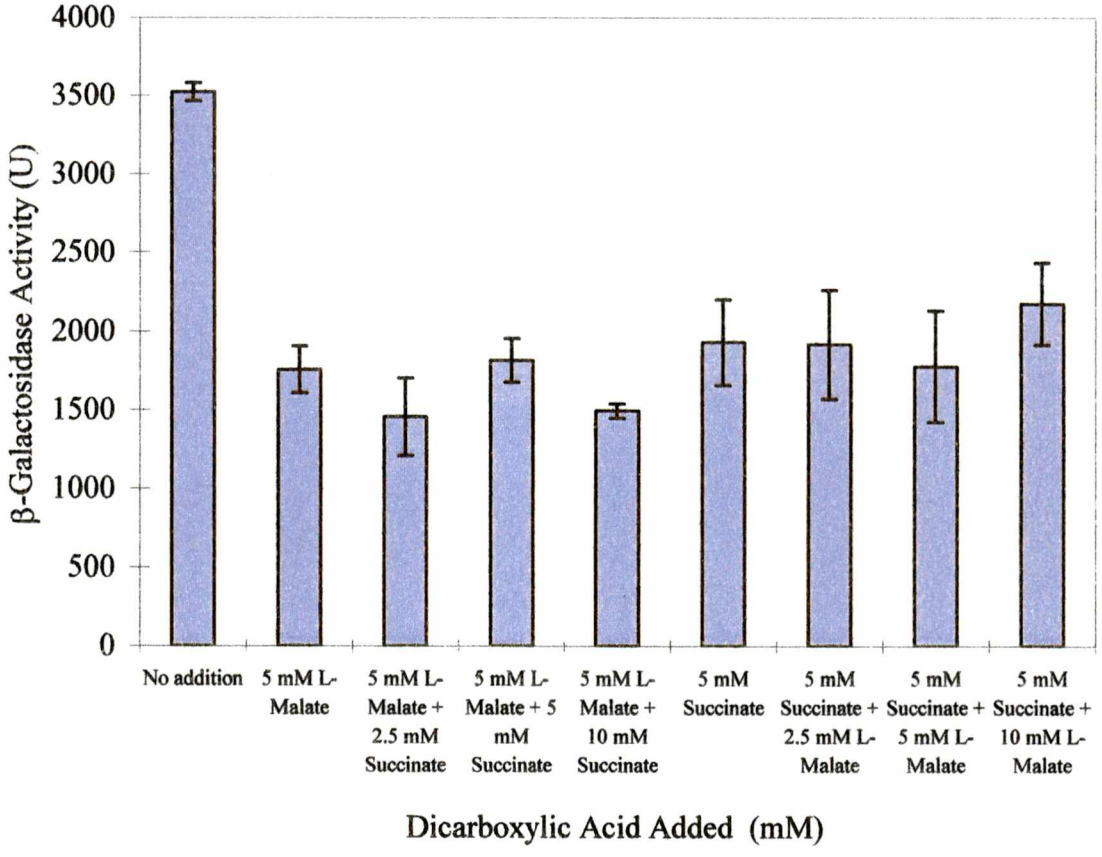


Figure 7. *nodY-lacZ* expression of *Bradyrhizobium japonicum* USDA110 strain ZB977 in the presence of a mixture of L-malate and succinate at different concentrations. Induction was done in MM in the presence of 0.05 μM genistein as inducer.

an additive inhibition on *nod* gene expression in both strain ZB977 and 573 (Table 17). The inhibition increased with increasing amounts of acetate added. More than 90% inhibition was achieved in the presence of 0.5 mM L-malate and 10 mM acetate in both strains. Under the same conditions, no inhibition of *nptII-lacZ* expression in strain SL101 was seen.

Effect of Calcium Ions on L-Malate Inhibition of Chromosomal *nodC-lacZ* Expression

Since it was possible that the addition of L-malate into medium would cause chelation of ions, such as calcium, the following experiment was done to determine whether L-malate inhibition of *nod* gene expression was due to chelation of calcium ions. Table 18 details the effect of calcium ions on chromosomal *nodC-lacZ* expression in strain 573. As indicated, L-malate inhibition in cultures with or without addition of calcium ions was about the same. These results show that L-malate inhibition on *nod* gene expression cannot be relieved by supplementing the cultures with more calcium.

Effect of Pre-growth with L-Malate and Resuspension of Cells in Fresh MM on the Induction of *nodY-lacZ* Expression

Up to this point, the data shown represent the determination of the inhibitory effect of L-malate by measuring the level of *nod* gene induction in cells incubated with L-malate for just 10 to 14 hours. However, bacteroids in nodules are exposed to organic acids for a longer period of time and are capable of metabolizing them (McDermott et al., 1989). This metabolism may account for the inability of L-malate to completely

Table 17. Induction of *nodY-lacZ* expression in *Bradyrhizobium japonicum* strain ZB977 and *nptII-lacZ* expression in *B. japonicum* strain SL101 at different concentrations of sodium acetate (NaAc) in the presence or absence of 0.5 mM L-malate. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

		Mean β-Galactosidase Activity (U) ± SD[a]					
	Conc. of NaAc	Without 0.5 mM L-malate			With 0.5 mM L-malate		
Strain	(mM)	Ind.[b]	Unind.[b]	%[c]	Ind.	Unind.	%
ZB977	0	2119 ± 102	34	/	1472 ± 182	7	31
	1	1488 ± 106	37	30	1032 ± 138	27	51
	5	968 ± 176	49	54	581 ± 245	12	73
	10	504 ± 39	43	76	188 ± 23	29	91
573	0	339 ± 34	5	/	245 ± 27	3	28
	1	264 ± 27	6	22	229 ± 61	2	32
	5	114 ± 55	10	66	113 ± 38	6	77
	10	47 ± 31	7	86	22 ± 27	9	94
SL101	0	2118 ± 12	2224	/	3141 ± 508	2896	0
	1	2347 ± 118	2156	0	2934 ± 7	2615	0
	5	2295 ± 66	2299	0	3252 ± 169	2751	0
	10	2790 ± 212	3101	0	3638 ± 135	3380	0

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] Ind. stands for Induced cultures. Unind. stands for Uninduced cultures. No replicate was done for the uninduced cultures.

[c] % of inhibition of activity in the presence of different concentrations of sodium acetate. It is equal to [(β-galactosidase activity with no addition – β-galactosidase activity at a concentration of NaAc +/- 0.5 mM L-malate) / β-galactosidase activity with no addition] × 100%.

Table 18. Chromosomal *nodC-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain 573 in the presence 0, 0.5, 10, or 70 mM L-malate with supplement of 0, 0.1, or 0.2 mM calcium. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Conc. of L-malate (mM)	β-Galactosidase Activity ± SD[a]								
	No Ca^{2+} added			0.1 mM Ca^{2+} added			0.2 mM Ca^{2+} added		
	Ind.	Unind.[b]	% Inh.[c]	Ind.	Unind.	% Inh.	Ind.	Unind.	% Inh.
0	366 ± 39	14	/	452 ± 7	12	/	342 ± 23	5	/
0.5	168 ± 33	11	54	185 ± 3	22	59	134 ± 14	9	61
10	108 ± 39	7	71	167 ± 13	21	63	101 ± 5	9	71
70	56 ± 30	8	85	89 ± 39	16	80	70 ± 15	11	80

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] No replicate was done for the uninduced cultures.

[c] % Inhibition is equal to [(act. without L-malate – act. with L-malate) / act. without L-malate] × 100%.

inhibit *nod* gene expression. With these considerations, cells grown in RDY were compared to those grown in RDY with L-malate added to see if a greater inhibition of *nod* gene expression could be observed in cells pre-grown in the presence of L-malate. L-malate inhibition of *nodY-lacZ* expression in strain ZB977 grown in RDY or RDY in the presence of 5 mM L-malate was compared (Figure 8). To avoid carryover of L-malate into the induction medium, cells were washed and resuspended in MM before induction. As shown in Figure 6, when cells grown in RDY were diluted into induction medium (MM) (Direct Transfer), a 50 to 60% inhibition of *nodY-lacZ* expression by 0.5 to 15 mM L-malate was observed. The level of *nod* gene expression in cells pre-grown with 5 mM L-malate and then induced in the presence of different amounts of L-malate was about 2-fold lower than those in cells grown in RDY. Surprisingly, when cells grown in RDY were washed and resuspended in MM before induction, inhibition of *nodY-lacZ* expression was not seen in the presence of 0.5 to 5 mM L-malate (Figure 8). Only about 30% inhibition was seen at 15 mM L-malate. In order to define the possible effect of resuspending cells in MM on L-malate inhibition, L-malate inhibition of *nod* gene expression in cells grown in RDY and in RDY with 1 mM L-malate were compared in four conditions before induction: direct transfer of cells, centrifugation and resuspension of cells back into the supernatant, resuspension of cells in RDY, and resuspension of cells in MM. The results in Table 19 show that centrifugation had no effect. The level of *nod* gene expression in cells after centrifugation and resuspension into the supernatant was inhibited by L-malate in a similar fashion as cells subjected to direct transfer. Similar L-malate inhibition was also seen in L-malate-pre-grown cells after direct transfer or after

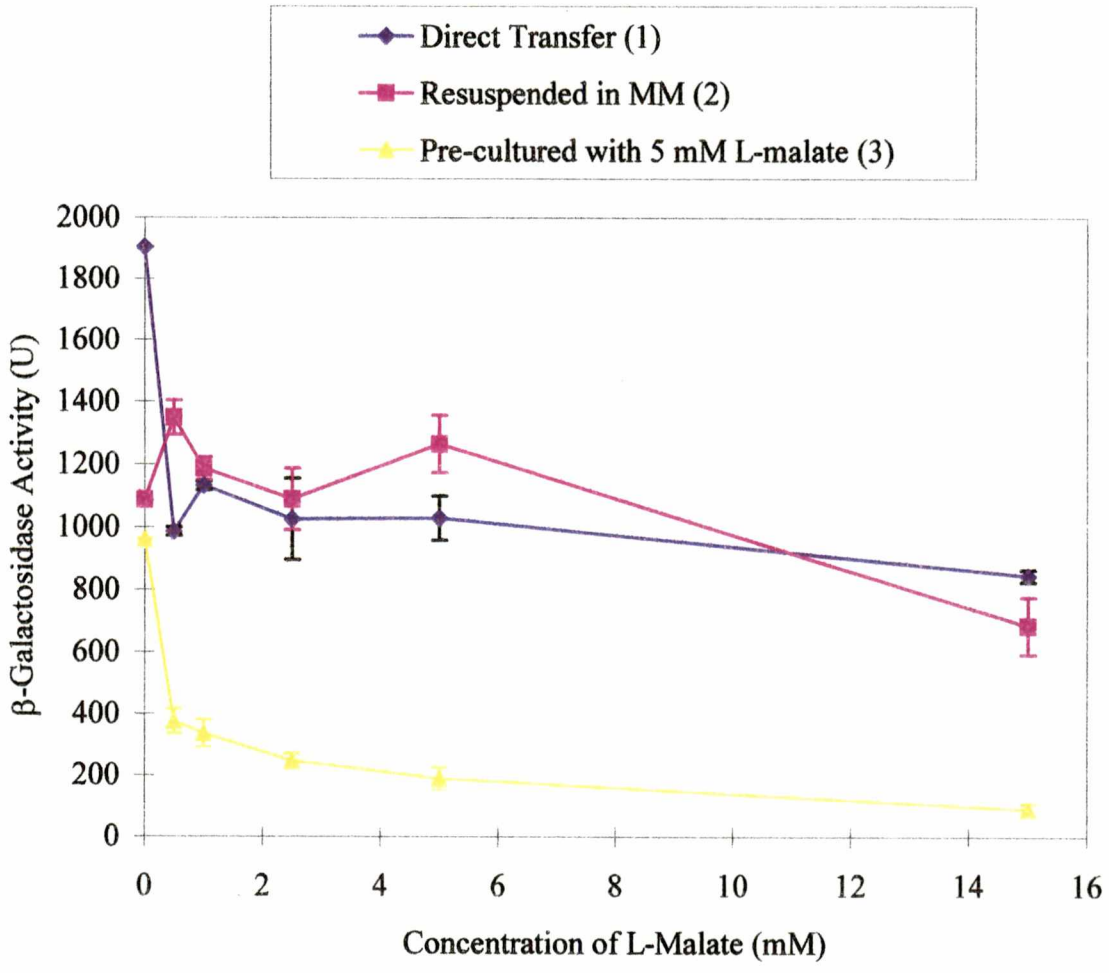


Figure 8. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 when cells were directly transferred (1), resuspended in minimal medium (MM) (2), and resuspended in MM after pre-cultured with 5 mM L-malate (3) before induction. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Table 19. Induction of *nodY-lacZ* expression in *B. japonicum* strain ZB977 when cells were directly transferred, centrifuged and resuspended, resuspended in RDY, or resuspended in MM before induction. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Treatments of the cells before induced	Conc. of L-malate (mM)	β-Galactosidase Activity (U) ± SD[a]					
		Cells grown in RDY			Cells grown in RDY with 1 mM L-malate		
		Ind.[b]	Unind.[b]	% inh.[c]	Ind.	Unind.	% inh.
Direct transfer	0	2205 ± 69	24	/	1539 ± 381	17	/
	1	946 ± 177	20	57	906 ± 258	27	41
	5	1126 ± 57	19	49	874 ± 114	19	43
	10	803 ± 213	17	64	1009 ± 48	22	34
Centrifuged & resuspended	0	2100 ± 43	16	/	1911 ± 371	20	/
	1	1253 ± 250	20	40	1062 ± 209	20	44
	5	1390 ± 243	16	34	943 ± 107	21	51
	10	988 ± 195	16	53	780 ± 45	17	59
Resuspended in RDY	0	3013 ± 129	18	/	2160 ± 435	20	/
	1	1510 ± 413	22	50	1233 ± 120	26	43
	5	1303 ± 108	18	57	951 ± 231	23	56
	10	918 ± 75	26	70	1026 ± 1	24	53
Resuspended in MM	0	2954 ± 333	16	/	3264 ± 55	25	/
	1	3370 ± 147	22	0	2073 ± 347	23	36
	5	3151 ± 685	20	0	2090 ± 180	33	36
	10	2728 ± 423	17	8	2418 ± 442	28	26

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] Ind. stands for induced cultures. Unind. stands for uninduced cultures. No replicate was done for the uninduced cultures.

[c] % inh. stands for inhibition of the activity. It is equal to [(act. with 0 mM L-malate – act. with L-malate at that conc.) / act. with 0 mM L-malate] × 100%.

centrifugation and resuspension. When both cells were resuspended in fresh RDY before induction, similar levels of L-malate inhibition were observed. However, when cells were resuspended in MM before induction, L-malate inhibition on *nod* gene expression in cells grown in RDY disappeared, whereas the inhibition still occurred in cells pre-grown with 1 mM L-malate. These results show that resuspension of cells in fresh MM before induction somehow removed L-malate inhibition of *nod* gene expression seen in earlier experiments. Growth of cells in the presence of L-malate can counteract the effect of resuspension in MM.

The effect of resuspending cells in MM on L-malate inhibition was further examined. Cells pre-grown in RDY or RDY with 5 mM L-malate were washed, resuspended in MM, and induced with genistein at different concentrations (0.01 to 1 μM). The induction level of *nod* gene expression was compared (Table 20). Recall that previous experiments showed that L-malate inhibition was most remarkable when cells were induced with a sub-optimal level of genistein (Table 5 and Figure 12). However, as shown in Table 20, the dependency of L-malate inhibition on inducer concentration was not seen in cells grown in RDY and resuspended in MM. Similar to previous studies, cells pre-grown with 5 mM L-malate and resuspended in MM showed less inhibition by L-malate with increasing inducer concentration (Table 20).

Table 20. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 grown in RDY or in RDY added with 5 mM L-malate, and resuspended in MM before induction with genistein at different concentrations in the presence of 0 mM or 1 mM L-malate. Induction was done in MM.

Conc. of Genistein (μM)	Grown in RDY			Grown in RDY with 5 mM L-malate		
	Addition of L-malate			Addition of L-malate		
	0 mM	1 mM		0 mM	1 mM	
	β-Galactosidase Activity ± SD[a]		Inh. %[b]	β-Galactosidase Activity ± SD		Inh. %
0	71 ± 1	48 ± 1	32	57 ± 9	40 ± 2	29
0.01	1073 ± 68	872 ± 35	19	509 ± 30	131 ± 58	74
0.025	2313 ± 234	1941 ± 54	16	2245 ± 160	657 ± 172	71
0.05	2807 ± 176	1837 ± 931	35	3667 ± 409	1401 ± 168	62
0.1	2621 ± 180	2908 ± 173	0	3466 ± 39	2222 ± 341	36
0.5	2610 ± 388	2465 ± 95	6	4009 ± 64	2931 ± 123	27
1	2967 ± 126	2330 ± 76	22	3916 ± 167	3083 ± 119	21

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] % Inhibition is equal to [(act. with 0 mM L-malate – act. with 1 mM L-malate) / act. with 0 mM L-malate] × 100%.

Inhibitory Effect of Yeast Extract on *nodY-lacZ* Expression

β-Galactosidase assays performed in the previous studies in our laboratory [e.g., Banfalvi et al. (1988), Wang and Stacey (1990, 1991), Dockendorff et al. (1994a, 1994b)] used RDY as the induction medium. RDY was replaced by MM as the induction medium in our studies where we examined the effect of various carbon compounds. It was found that *nod-lacZ* expression was much higher when induction was done in MM. In order to identify what components in RDY were responsible for the reduction in *nod* gene expression, *nodY-lacZ* expression in strain ZB977 was measured in MM, MM added with 0.1% yeast extract, and RDY. As shown in Table 21, the level of *nodY-lacZ* expression was lower when induction was done in RDY (65% reduced), as compared to MM. Moreover, the addition of 0.1% yeast extract, a component in RDY, to MM resulted in a 46% reduction of *nodY-lacZ* expression. Therefore, yeast extract has an inhibitory effect on *nod* gene expression contributing to the lower gene expression in RDY. Since RDY contains 22.9 mM D-gluconate, we tested whether the presence of D-gluconate in RDY also reduced *nod* gene induction. The level of *nod* gene expression in strain ZB977 and 573 in the presence of 5 to 30 mM D-gluconate, with or without 0.1% yeast extract, was examined (Table 22). The results show that D-gluconate alone did not inhibit *nodY-lacZ* expression in strain ZB977. However, the level of *nodC-lacZ* expression in strain 573 was reduced by 18 to 47% at a concentration of 10 to 30 mM. When 0.1% yeast extract was also present, a significant inhibition of *nod* gene expression was seen in both strains, which increased with D-gluconate concentration (70% to 90%

Table 21. *nodY-lacZ* expression of *Bradyrhizobium japonicum* USDA110 strain ZB977 induced in MM, MM added with 0.1 % of yeast extract, or RDY. 0.025 μM genistein was present as inducer. pH and absorbance at 600nm of the cultures after induction are shown.

	β-Galactosidase Activity ± SD[a]				
	Induced	Uninduced[b]	pH	% inhibition[c]	A_{600nm}
MM	2185 ± 64	32	6.95	/	0.107
MM + 0.1 % yeast extract	1178 ± 37	39	6.96	46	0.116
RDY	770 ± 79	51	7.08	65	0.148

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] No replicate was done for the uninduced cultures.

[c] % Inhibition is equal to [(act. in MM – act. in MM with 0.1 % yeast extract or in RDY) / act. in MM] × 100%.

Table 22. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 and chromosomal *nodC-lacZ* expression in strain 573 in the presence of D-gluconate at different concentrations with or without addition of 0.1% yeast extract. pH and absorbance at 600nm of the cultures after induction were shown. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Conc. of D-gluconate	With 0.1% yeast extract ?	β-Galactosidase Activity ± SD[a] Induced	Uninduced[b]	pH	% Inhibition[c]	A_{600nm}
		ZB977				
0 mM	No	2966 ± 138	34	6.99	/	0.077
5 mM	No	2890 ± 294	34	7.02	3	0.080
10 mM	No	2835 ± 310	35	7.05	4	0.078
20 mM	No	2999 ± 278	32	7.07	0	0.077
30 mM	No	2990 ± 115	30	7.10	0	0.075
0 mM	Yes	940 ± 236	36	7.02	68	0.079
10 mM	Yes	417 ± 103	36	7.07	86	0.076
20 mM	Yes	286 ± 62	34	7.09	90	0.077
30 mM	Yes	215 ± 29	33	7.10	93	0.069
RDY[d]	/	1001 ± 164	28	7.11	66	0.107
		573				
0 mM	No	374 ± 20	1	6.95	/	0.100
10 mM	No	305 ± 22	2	7.00	18	0.102
20 mM	No	250 ± 25	2	7.05	33	0.098
30 mM	No	197 ± 38	3	7.08	47	0.099
0 mM	Yes	120 ± 29	1	6.98	68	0.102
10 mM	Yes	75 ± 4	3	7.01	80	0.099
20 mM	Yes	44 ± 13	2	7.05	88	0.090
30 mM	Yes	35 ± 2	1	7.05	91	0.091
RDY[d]	/	126 ± 16	15	7.05	66	0.117

[a] Results are presented as the mean of three independent assays followed by the standard deviation.

[b] No replicate was done for the uninduced cultures.

[c] % Inhibition is equal to [(act. without addition – act. with addition or in RDY) / act. without addition] × 100%.

[d] RDY contains 0.1% yeast extract and approximately 23 mM D-gluconate.

inhibition at 0 to 30 mM D-gluconate). These results suggest an additive inhibitory effect of yeast extract and D-gluconate on *nod* gene expression. The presence of 0.1% yeast extract and 20 mM D-gluconate in MM resulted in 90% inhibition of *nod* gene expression, which was higher than the inhibition exerted by RDY (66%) though both media contained similar amounts of yeast extract and D-gluconate.

Effect of Carbon and Nitrogen Components on Inhibition of *nodY-lacZ* Expression by L-Malate

Results in Figure 8 and Table 19-22 showed that certain carbon and nitrogen components added to either the growth or induction medium inhibited induction of *nod* gene expression and also interfered with the inhibitory effect of L-malate. The following experiments were designed to further investigate these effects. Cells of strain ZB977 were grown in RDY, washed and resuspended in medium containing different carbon and nitrogen sources. Cells were then diluted to the same optical density and induced in MM in the presence of 0 to10 mM L-malate. The L-malate inhibition of *nodY-lacZ* expression in these cells were compared to the cells directly transferred. The results are shown in Table 23. The same comparison was also studied using cells of strain ZB977 grown in media containing different carbon and nitrogen sources. In this experiment, cells were directly transferred into the induction medium. The results were shown in Table 24. The results from Table 23 show that L-malate inhibition was present in cells directly transferred, or cells resuspended in "mineral RDY" containing the same components (1 g/L yeast extract, 1 g/L L-glutamate, 5 g/L gluconate) of RDY, only 5 g/L D-gluconate

Table 23. Induction of *nodY-lacZ* expression in *B. japonicum* strain ZB977 grown in RDY, and washed and resuspended in media containing different carbon and nitrogen sources before induction. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Media for washing & resuspending the cells	Conc. of L-malate added (mM)	Mean β-Galactosidase Activity (U) ± SD[a]		
		Ind.[b]	Unind.[b]	% inh.[c]
Direct transfer	0	1177 ± 28	35	/
	1	741 ± 102	33	37
	5	674 ± 125	32	43
	10	537 ± 49	42	54
"mineral" RDY[d] +	0	1207 ± 51	32	/
1 g/L yeast extract +	1	639 ± 88	61	47
5 g/L D-gluconate +	5	616 ± 75	33	49
1 g/L L-glutamate	10	531 ± 31	33	56
"mineral" RDY +	0	1421 ± 193	43	/
5 g/L D-gluconate +	1	830 ± 99	63	42
1 g/L L-glutamate	5	682 ± 49	34	52
	10	673 ± 99	37	53
"mineral" RDY +	0	553 ± 33	78	/
1 g/L yeast extract +	1	756 ± 127	26	0
4 ml/L glycerol +	5	787 ± 21	16	0
1 g/L L-glutamate	10	765 ± 89	19	0
"mineral" RDY +	0	863 ± 91	13	/
1 g/L yeast extract +	1	678 ± 128	16	21
5 g/L D-gluconate +	5	604 ± 111	15	30
0.5 g/L NH_4NO_3	10	366 ± 46	19	58
MM	0	1062 ± 211	13	/
	1	1136 ± 52	17	0
	5	1097 ± 52	18	0
	10	876 ± 105	18	18

[a] Results are presented as the mean of two independent assays three independent assays followed by the standard deviation.

[b] Ind. stands for Induced cultures. Unind. stands for Uninduced cultures. No replicate was done for the uninduced cultures.

[c] % inh. stands for inhibition of the activity. It is equal to [(act. with 0 mM L-malate – act. with L-malate at that conc.) / act. with 0 mM L-malate] × 100 %.

[d] "mineral" RDY is RDY containing no yeast extract, D-gluconate, and L-glutamate.

Table 24. Induction of *nodY-lacZ* expression in *B. japonicum* strain ZB977 grown in media containing different carbon and nitrogen sources before induction. Induction was done in MM in the presence of 0.025 μM genistein as inducer.

Media in which the cells grown	Conc. of L-malate added (mM)	Mean β-Galactosidase Activity (U) ± SD[a]		
		Ind.[b]	Unind.[b]	% inh.[c]
"mineral" RDY[d] +	0	1650 ± 63	25	/
1 g/L yeast extract +	1	316 ± 82	22	81
5 g/L D-gluconate +	5	341 ± 42	23	79
1 g/L L-glutamate	10	232 ± 95	22	86
"mineral" RDY +	0	1046 ± 164	21	/
5 g/L D-gluconate +	1	314 ± 76	23	70
1 g/L L-glutamate +	5	231 ± 22	25	78
	10	127 ± 32	19	88
"mineral" RDY +	0	2186 ± 167	26	/
1 g/L yeast extract +	1	1732 ± 180	31	21
4 ml/L glycerol +	5	1852 ± 239	30	15
1 g/L L-glutamate	10	1686 ± 141	29	23
"mineral" RDY +	0	1845 ± 135	32	/
1 g/L yeast extract +	1	513 ± 49	31	72
5 g/L D-gluconate +	5	575 ± 111	32	69
0.5 g/L NH_4NO_3	10	462 ± 40	36	75
"mineral" MM[e] +	0	2600 ± 186	43	/
4 ml/L glycerol +	1	2047 ± 142	37	21
0.5 g/L NH_4NO_3	5	1411 ± 70	36	46
	10	1430 ± 291	34	45
"mineral" MM +	0	908 ± 33	35	/
5 g/L D-gluconate +	1	760 ± 19	53	16
0.5 g/L NH_4NO_3	5	696 ± 75	27	23
	10	605 ± 45	31	33
"mineral" MM +	0	1982 ± 42	11	/
4 ml/L glycerol +	1	1753 ± 192	36	12
1 g/L L-glutamate	5	1603 ± 31	17	19
	10	1256 ± 143	17	37
"mineral" MM +	0	2615 ± 136	48	/
1 g/L yeast extract +	1	1927 ± 127	25	26
4 ml/L glycerol +	5	1701 ± 123	26	35
0.5 g/L NH_4NO_3	10	1589 ± 99	27	39

[a] Results are presented as the mean of two independent assays three independent assays followed by the standard deviation.

[b] Ind. stands for Induced cultures. Unind. stands for Uninduced cultures. No replicate was done for the uninduced cultures.

[c] % inh. stands for inhibition of the activity. It is equal to [(act. with 0 mM L-malate – act. with L-malate at that conc.) / act. with 0 mM L-malate] × 100 %.

[d] "mineral" RDY is RDY containing no yeast extract, D-gluconate, and L-glutamate.

[e] "mineral" MM is MM containing no glycerol and NH_4NO_3.

and 1 g/L L-glutamate, or 1 g/L yeast extract, 5 g/L D-gluconate, and 0.5 g/L NH_4NO_3. L-malate inhibition did not occur when cells were resuspended in MM, and in media containing 1 g/L yeast extract, 0.4% glycerol, and 1 g/L L-glutamate. The results suggest that D-gluconate present in the cell suspension, which was then added into the induction medium, was required for L-malate inhibition of *nod* gene expression. In the experiment comparing the effects of growth media containing different carbon and nitrogen sources (Table 24), a greater L-malate inhibition (75 to 88% inhibition at 10 mM L-malate) was seen in cells grown with D-gluconate as carbon source and yeast extract or/and L-glutamate as nitrogen source. A lower level of inhibition (23 to 45% inhibition at 10 mM L-malate) was seen when cells were grown with glycerol as carbon source and various nitrogen sources, or D-gluconate as carbon source and NH_4NO_3 as nitrogen source. These results suggest that L-malate inhibition on *nod* gene expression was more prominent in cells grown in rich media, i.e., those containing preferred carbon and nitrogen sources. D-gluconate has been reported to be the preferred carbon source for *B. japonicum* (Stowers 1985).

Effect of cAMP on L-Malate Inhibition of Chromosomal *nodC-lacZ* Expression

The above results show that certain organic acids and rich media could bring about an inhibition of *nod* gene expression in *B. japonicum*. One explanation for these results could be that inhibition of *nod* gene expression by carbon sources may be mediated by catabolite repression. Therefore, the effect of cAMP addition on L-malate

inhibition of chromosomal *nodC-lacZ* expression in strain 573 was examined. Table 25 shows that the addition of dibutyryl cAMP (20 to 200 μM) could enhance *nod* gene expression maximally by 33% in a culture without L-malate or 26% in a culture with 0.5 mM L-malate. However, the addition of dibutyryl cAMP did not appear to relieve the inhibition of *nod* gene expression caused by 0.5 mM L-malate. Indeed, 40 to 55% inhibition was observed in all the cultures treated with L-malate and the inhibition did not vary upon the addition of increasing concentrations of cAMP.

nodC-lacZ Expression in Bacteroids

Schlaman et al. (1991) showed that the *nod* genes were not expressed in *Rhizobium leguminosarum* bv. viciae bacteroids. To examine *nod* gene expression in *B. japonicum* bacteroids, a chromosomal *nodC-lacZ* fusion was constructed in strain 573, which has an intact set of *nod* genes and, therefore, can form wild-type nodules. Bacteroids of strain 573 were isolated. β-galactosidase assays were done using freshly isolated bacteroids. Bacteroids of wild type USDA110 were used as a negative control. Results in Table 26 show that β-galactosidase activity measured in bacteroids of strain 573 was about 4 U. The background β-galactosidase activity detected in wild-type bacteroids was 1 U. The low background activity in the wild-type bacteroid preparation showed that the bacteroids isolated were free of contamination by plant β-galactosidase activity. To test whether low *nodC-lacZ* expression in bacteroids was due to a lack of inducers in nodules, a nodule extract was prepared and tested for its ability to induce

Table 25. Chromosomal *nodC-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain 573 added with dibutyryl cAMP at different concentrations in the presence of 0 or 0.5 mM L-malate.

cAMP (μM)	β-Galactosidase Activity						
	0 mM L-Malate added			0.5 mM L-Malate added			
	Ind.[a]	Unind.[b]	% Incr.[c]	Ind.	Unind.	% Incr.	% Inh.[d]
0	479 ± 60	35	/	286 ± 49	22	/	40
20	578 ± 131	28	21	262 ± 71	19	0	55
50	637 ± 141	31	33	345 ± 21	24	21	46
100	600 ± 12	22	25	361 ± 45	19	26	40
200	635 ± 121	17	30	321 ± 70	22	12	49

[a] Ind. stands for induced cultures. Results are presented as the mean of three independent assays followed by the standard deviation.

[b] Unind. stands for uninduced cultures. No replicate was done for the uninduced cultures.

[c] % Incr. shows the increase of activity due to addition of cAMP. It is equal to [(act. with cAMP – act. without cAMP) / act. without cAMP] × 100%.

[d] % Inh. shows the inhibition of activity due to addition of 0.5 mM L-malate. It is equal to [(act. without L-malate – act. with L-malate) / act. without L-malate] × 100%.

Table 26. Chromosomal *nodC-lacZ* expression in bacteroids of *B. japonicum* strain 573

β-Galactosidase Activity (U) ± SD[a]	
Bacteroids of strain 573	Bacteroids of wild-type strain
3.9 ± 0.3	0.9 ± 0.1

[a] Results are presented as the mean of five independent assays followed by the standard deviation.

nodC-lacZ expression in strain 573. Figure 9 shows that the nodule extract did contain inducers capable of activating *nod* gene expression. These results show that *nod* gene expression is suppressed in bacteroids of *B. japonicum*, and the low level of expression was not due to a lack of inducers in nodules.

Inhibition of *nodY-lacZ* Expression under Microaerobic Conditions

The expression of a *nodY-lacZ* fusion in *B. japonicum* USDA110 strain ZB977, SR15-32 (Δ1.5kb putative *nolR* repressor sequence), 1267-32 (same as Δ1267) (Δ*nodD1D2nolA*), and USDA135 strain LB100, was compared to both *nifD-lacZ* expression in strain GS300, and *nptII-lacZ* expression in strain SL101. In order to generate a microaerobic growth condition, cells were grown in a soft agar slant (0.8%) of nitrate medium containing 40 μg/ml X-gal (Figure 10). 1 μM genistein was added as inducer. Strains exhibiting *lacZ* expression were identified by the appearance of blue color in the agar. In case of strain ZB977, SR15-32, 1267-32, or LB100, only cells growing near the surface of the agar exhibited a blue color. In contrast, *nifD-lacZ* and *nptII-lacZ* expression occurred in cells growing throughout the agar slant. These results demonstrated that *nod* gene expression was suppressed in cells grown under low oxygen conditions. Expression of the *nifD-lacZ* fusion was expected since the gene is induced by NifA under microaerobic conditions (Thöny et al., 1987; Thöny et al., 1989; Batut and Boistard, 1994). The fact that microaerobiosis did not suppress *nptII-lacZ* expression

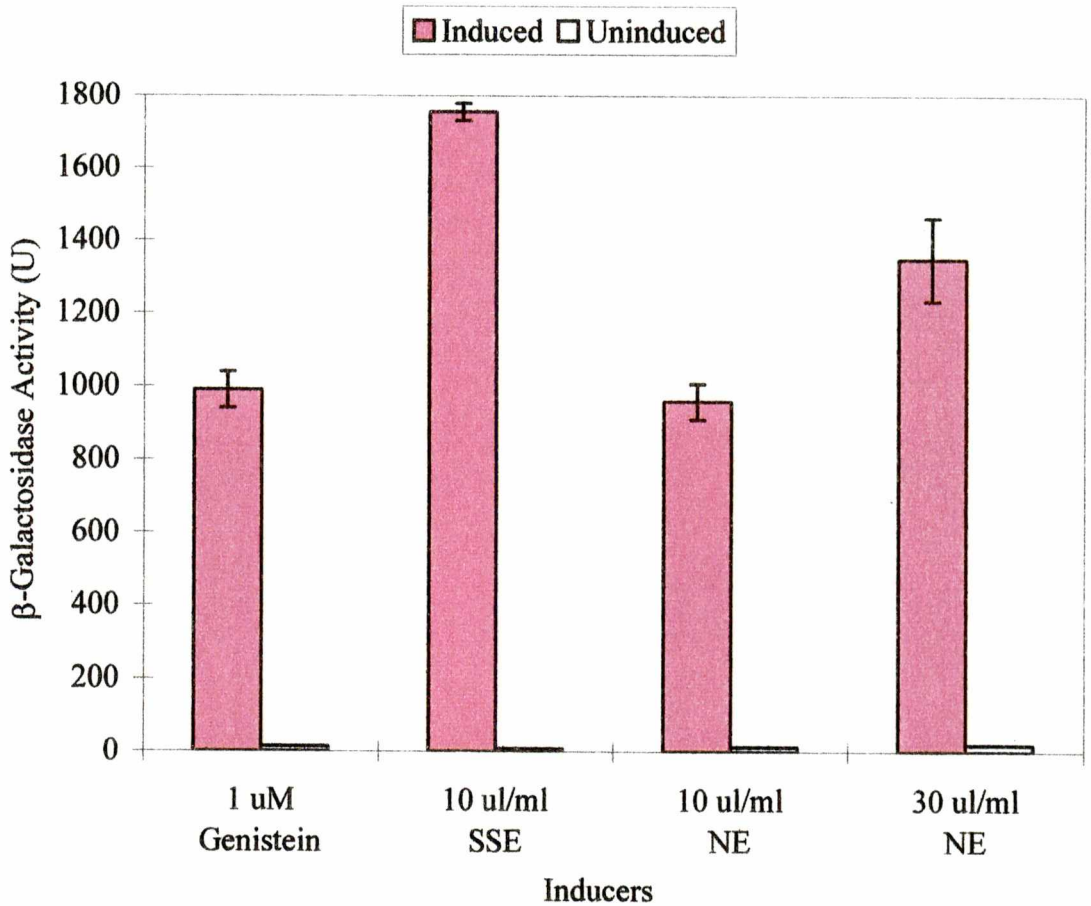


Figure 9. Chromosomal *nodC-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain 573 induced by genistein, soybean seed extract (SSE), and nodule extract (NE). Induction was done in MM.

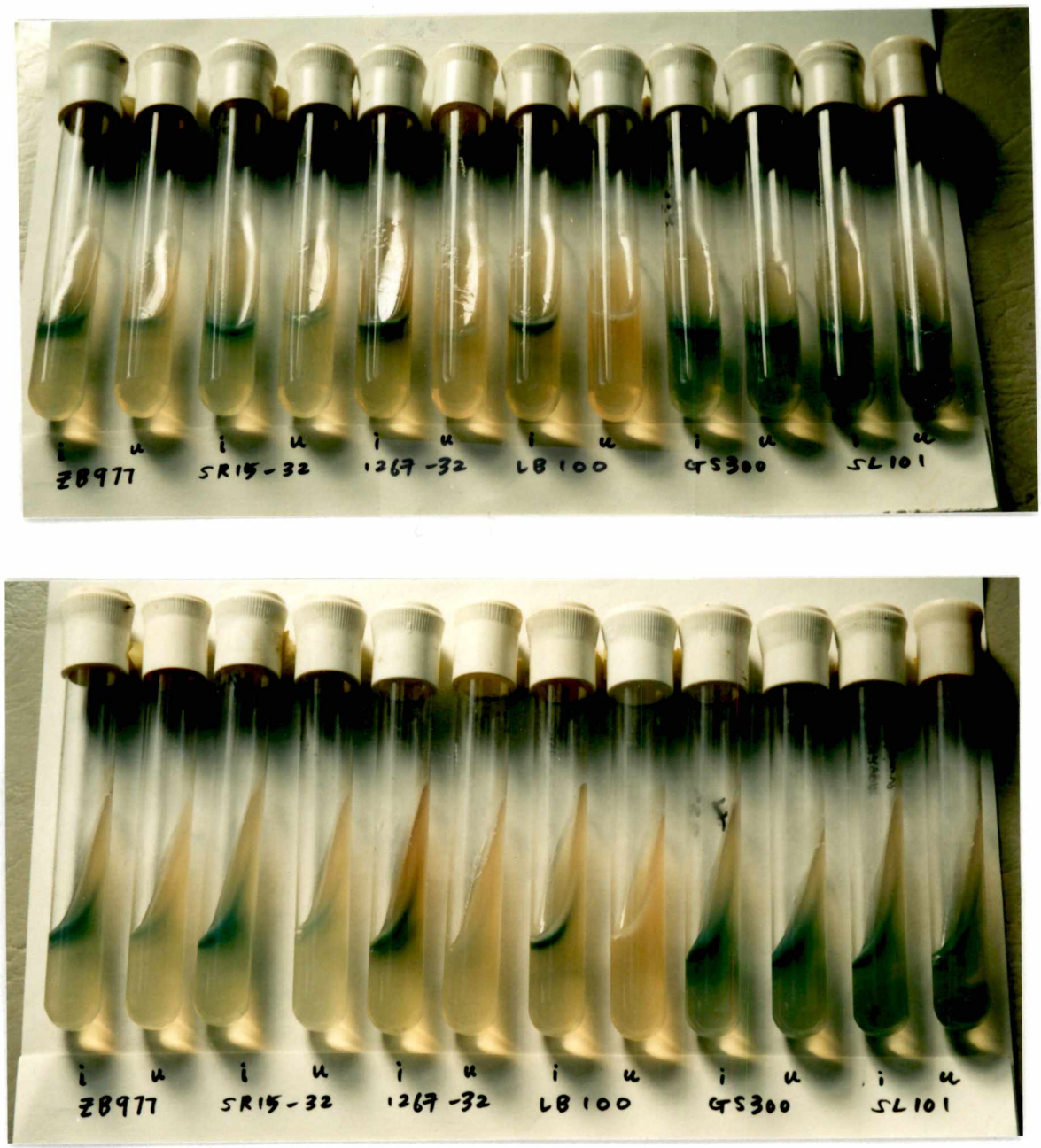


Figure 10. *nodY-lacZ* expression in *B. japonicum* USDA110 strain ZB977 (wild-type), SR15-32 (Δ1.5 kb deletion in putative repressor sequence), 1267-32 (same as Δ1267, Δ*nodD1D2nolA*), and USDA135 strain LB100 (wild-type), *nifA-lacZ* expression in USDA110 strain GS300 (wild-type), constitutive *nptII-lacZ* expression in USDA110 strain SL101 (wild-type) grown in agar slant (0.8%) of nitrate medium containing 40 μg/ml X-gal. (i) - "induced by 1 μM genistein"; (u) - "uninduced".

shows that suppression of *nod* gene expression under microaerobic conditions is not a general physiological effect.

Effect of pH on *nodY-lacZ* Expression

L-Malate inhibition on *nodY-lacZ* expression in strain ZB977 induced in RDY or MM at different pH (pH 4.0 to 8.5) was examined (Figure 11). Optimal *nodY-lacZ* expression in RDY occurred at pH 7 in the presence or absence of 5 mM L-malate. Expression of *nod* genes decreased linearly to a very low level from pH 7 to either pH 5.5 or pH 8. The presence of L-malate resulted in generally lower expression at all the pH values tested. Optimal *nodY-lacZ* expression in MM occurred at about pH 6.5. Lowest expression occurred at extreme pH (pH 4 and 8.5) but was still about 1000 U. The presence of L-malate reduced the expression by a similar extent at all tested pH values. The expression at pH 4 and 8.5 dropped to a very low level in RDY, regardless of the presence of L-malate.

Production and Purification of NodD1 Fusion Protein

In order to express NodD1 in *E. coli*, the *nodD1* gene was cloned into the expression vector pRSETB to produce pRSETB-D1-1. Cell extracts of *E. coli* harboring pRSETB-D1-1 were analyzed by SDS-PAGE and immunoblot using anti-His peptide antibodies (Figure 12). A dominant band of 40 kD was observed. This polypeptide had a

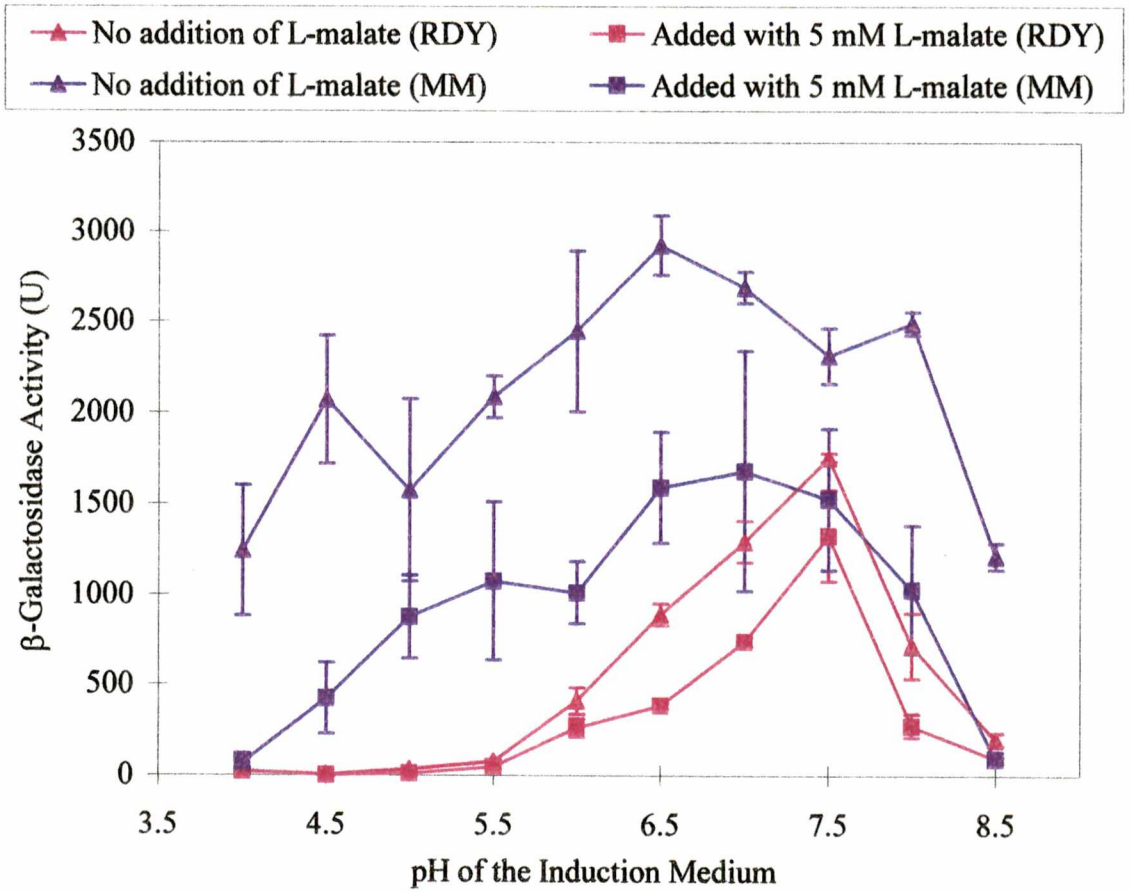


Figure 11. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 induced in RDY and MM at different pH with or without addition of 5 mM L-malate.

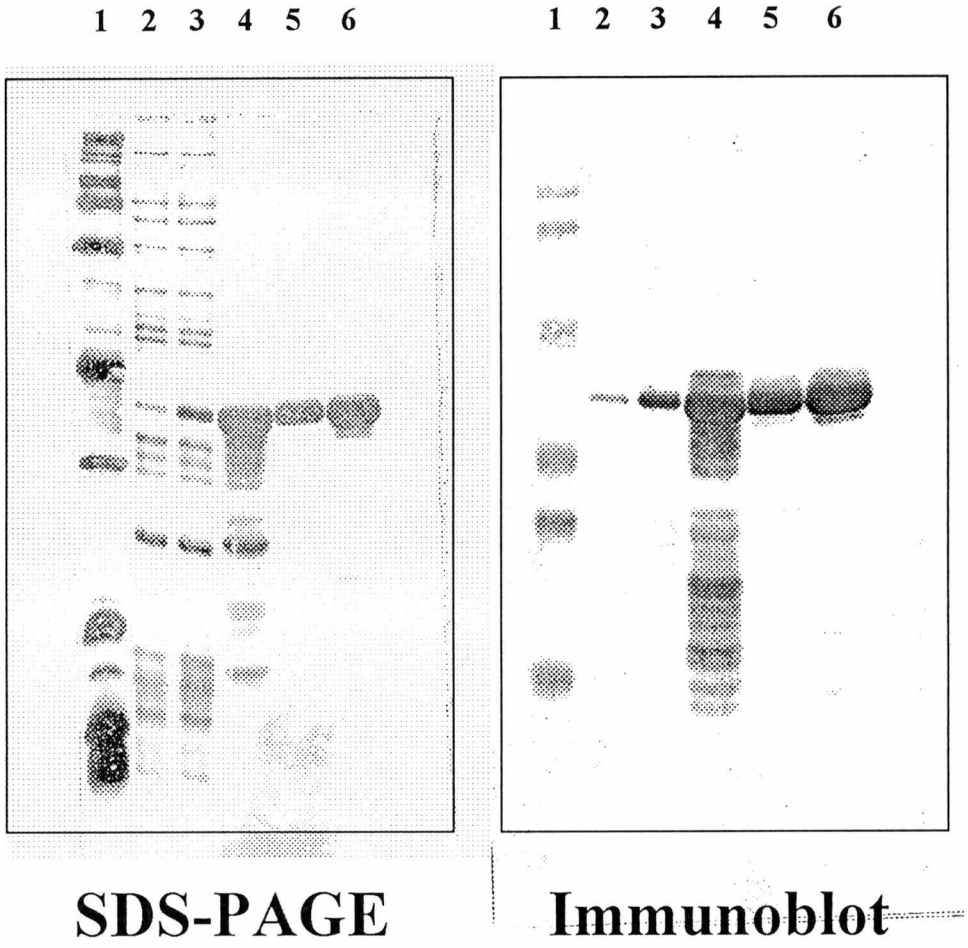


Figure 12. Silver-stained SDS-PAGE and immunoblot analysis of His-tag NodD1 fusion protein. Lane 1: Standards; Lane 2: Uninduced cells; Lane 3: Induced cells; Lane 4: Eluate from Ni^{2+}-column; Lane 5 and 6: Purified His-tag NodD1 fusion protein for 1st, and 2nd and 3rd immunization. Anti-His peptide antibodies were used.

molecular weight consistent with that predicted for the His-tag NodD1 fusion protein. The majority of the fusion protein was present in insoluble inclusion bodies. Therefore, the protein was solubilized and affinity-purified on a Ni^{2+}-charged column under denaturing conditions. Figure 12 shows the presence of the 40-kD fusion protein in a culture induced with IPTG. A lower amount was also present in the uninduced culture and was probably due to leaky expression from the T7 promoter of pRSETB. The eluate from the column exhibited several lower molecular weight bands that reacted with the antibodies and were likely the result of proteolysis. Using preparative SDS-PAGE and electroelution, the undegraded fusion protein was purified (Fig 12, lane 5 and 6), and used to immunize rabbits for the generation of anti-NodD1 antibodies.

Induction of NodD1 Expression

Previous studies of Banfalvi et al. (1988) had shown that *nodD1* was inducible by soybean seed extract. However, *nodD1* expression in strain USDA110 was very low (Table 15). Therefore, most of these earlier studies on *nodD1* expression were done using strain USDA135 which exhibits high activity. The availability of anti-NodD1 antibodies allowed us to re-examine *nodD1* expression in strain USDA110. For this purpose, cultures of *B. japonicum* USDA110 were induced for 2 to 10 hours by soybean seed extract and NodD1 expression was examined by immunoblot (Figure 13). As shown in Figure 13, a polypeptide of approximately 36 kD was labeled by the anti-NodD1 antibodies in all the induced samples. The molecular weight of this protein was the same

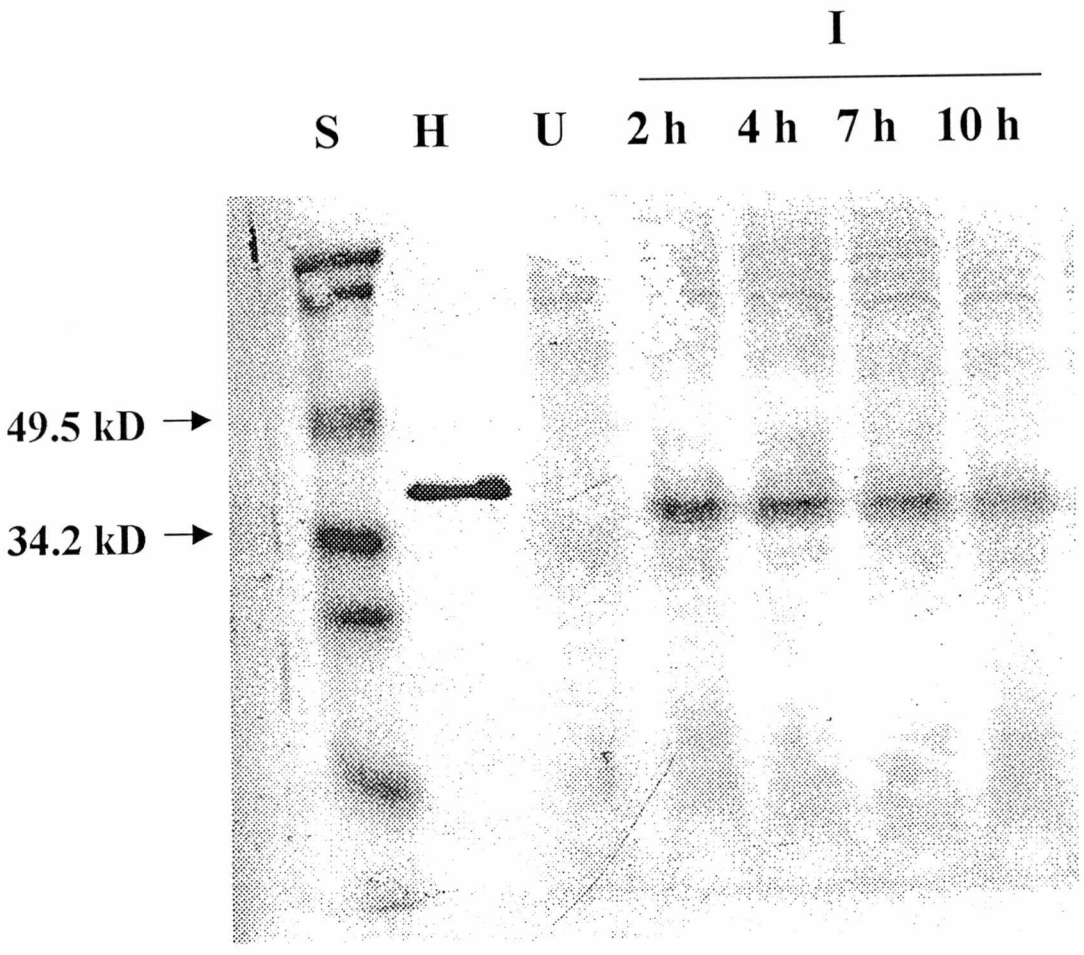


Figure 13. Induction of NodD1 expression in *B. japonicum* USDA110 wild-type strain. **S**: Standards; **H**: His-tag NodD1 fusion protein; **U**: Uninduced culture; **I**: Cultures induced for 2, 4, 7, and 10 hours with soybean seed extract. Rabbit anti-NodD1 antibodies were used.

as that predicted from the *nodD1* sequence (i.e. 35.9 kD). As a control, when pre-immune serum was used, secondary antibodies did not react with this protein and no labeling was seen. The expression of the NodD1 polypeptide was detected within two hours of induction. NodD1 expression continued but gradually faded after 10 hours of induction. In this experiment, no labeling of NodD1 was observed in the uninduced culture. These results are entirely consistent with those published previously by Banfalvi et al. (1988) using a *nodD1-lacZ* fusion.

Expression of NodD1 at Different Cell Densities

The induction of *nodD1* in *B. japonicum* USDA110 was studied at more defined time points (from 30 min to 8 hour) following induction. In one case, a *B. japonicum* culture at A_{600nm} = 2.6 was used as the inoculum for the experiment. Surprisingly, as shown in Figure 14, NodD1 was detected in the uninduced sample in this experiment. The level of NodD1 decreased with time of induction (from 30 min to 4 hours). The expression dropped to a minimal level after 4 hours of induction. After 5 hours of induction, NodD1 levels increased again. Expression continued 8 hours after induction during which the level of NodD1 was gradually reduced. The expression of NodD1 in uninduced cultures was puzzling. Therefore, we undertook a study to test for conditions where NodD1 could be expressed without induction.

As noted, the previous experiment used cells from cultures grown to A_{600nm} = 2.6. Earlier experiments had used younger cultures. Therefore, in order to determine the

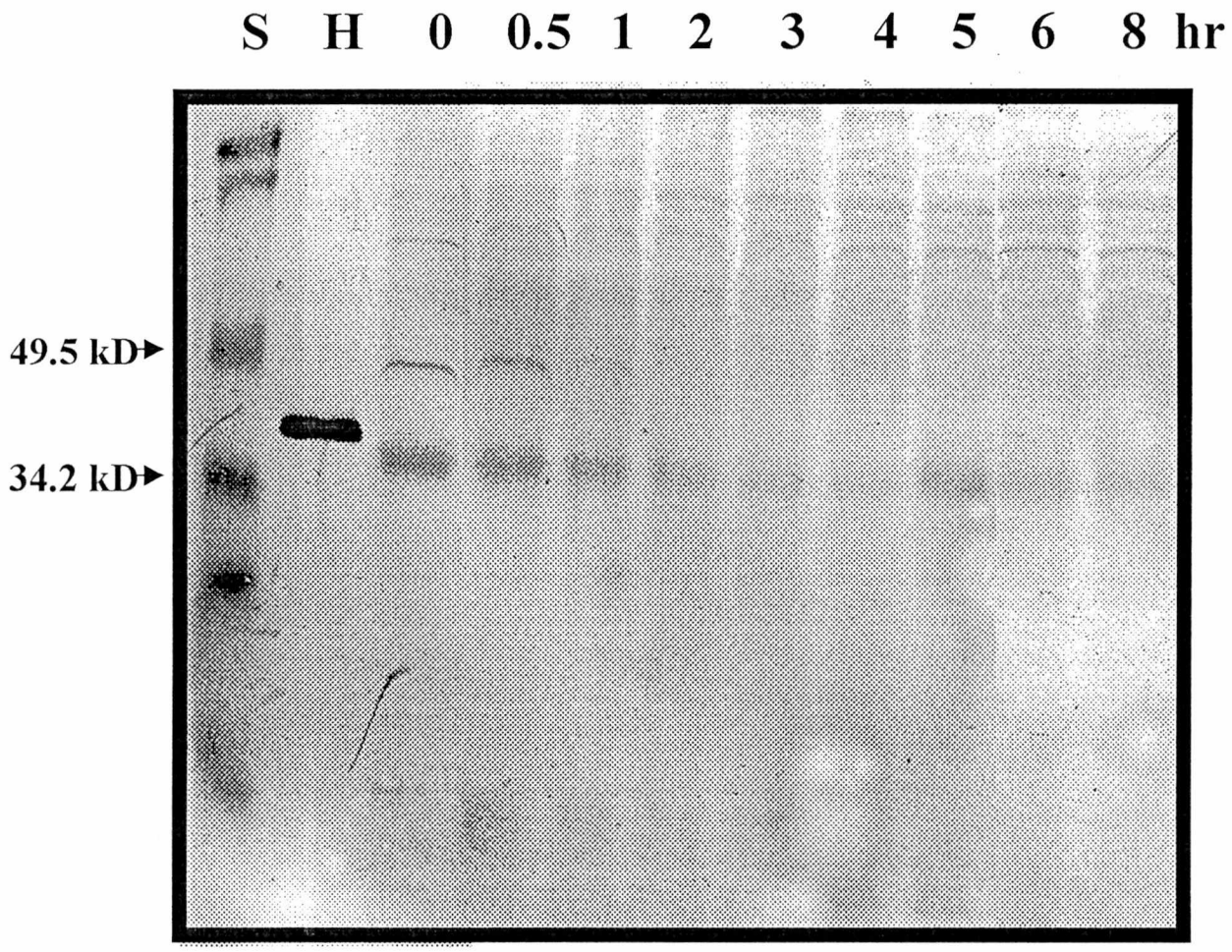


Figure 14. NodD1 expression in stationary-phase cells (A_{600nm}=2.6) of *B. japonicum* USDA110 induced with soybean seed extract for 0 to 8 hours. **S**: Standards; **H**: His-tag NodD1 fusion protein. Rabbit anti-NodD1 antibodies were used.

growth-phase dependency of NodD1 expression, immunoblots were made using extracts of *B. japonicum* USDA110 cells grown to different optical densities (i.e. starting culture with A_{600nm} = 0.074 grown to final A_{600nm} = 2.824, Figure 15). As found in our earlier experiments (Figure 13), no NodD1 expression was found in cultures A_{600nm} = 0.074 to 0.312. However, NodD1 was detected in uninduced cultures when A_{600nm} reached 0.39. The level of NodD1 expression subsequently increased and reached a constant level when the A_{600nm} of the culture reached 0.612. After this point, the level of expression remained constant (i.e. A_{600nm} = 0.708 to 2.824).

A similar result was also obtained from individual cultures of *B. japonicum* USDA110 grown to different final A_{600nm} (0.116 to 0.892) (Figure 16). No NodD1 was detected in cultures of A_{600nm} = 0.116, 0.120, 0.295, or 0.289. However, NodD1 was expressed in cultures of A_{600nm} = 0.668, 0.672, 0.884, or 0.892.

Specificity of Anti-NodD1 Antibodies

To ensure that the antibodies produced were specific to NodD1, cell extracts generated from USDA110 wild type and *nodD1* mutant (AN314) were compared (Figure 17). The NodD1 protein band was only present in induced and stationary-phase cultures of wild-type strain, whereas it was neither present in the uninduced culture of wild type nor in all three cultures derived from strain AN314. Since strain AN314 retains the *nodD2* gene, this result indicates that the antibodies generated are specifically directed to NodD1.

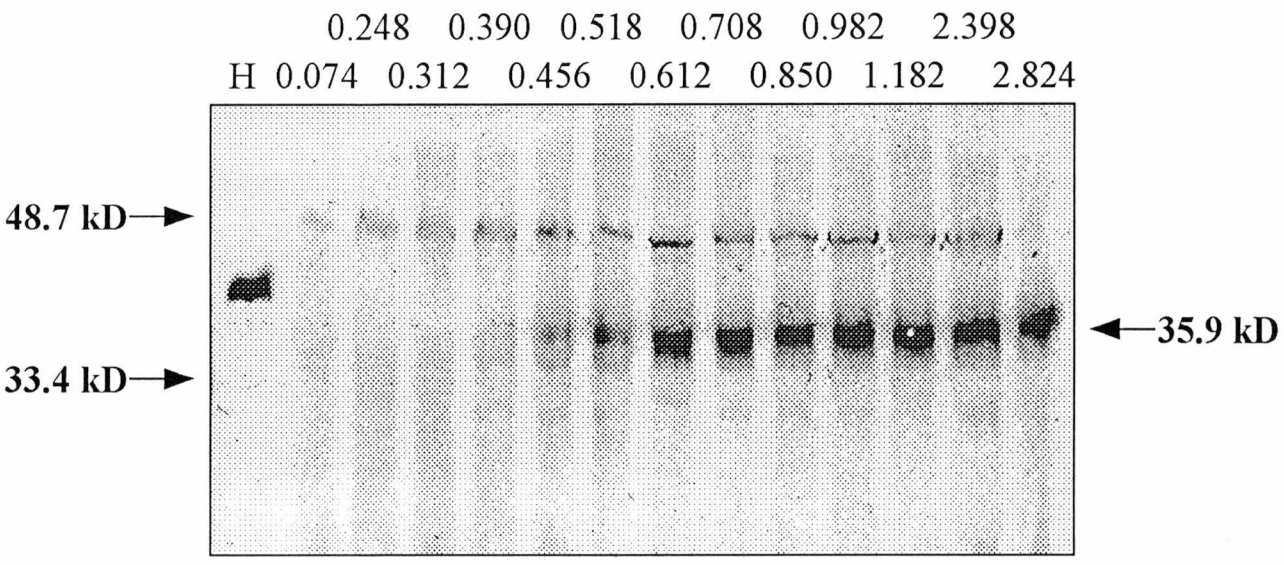


Figure 15. Expression of NodD1 in *B. japonicum* USDA110 grown in RDY at different cell densities (A_{600nm}). **H**: His-tag NodD1 fusion protein. Rabbit anti-NodD1 antibodies were used.

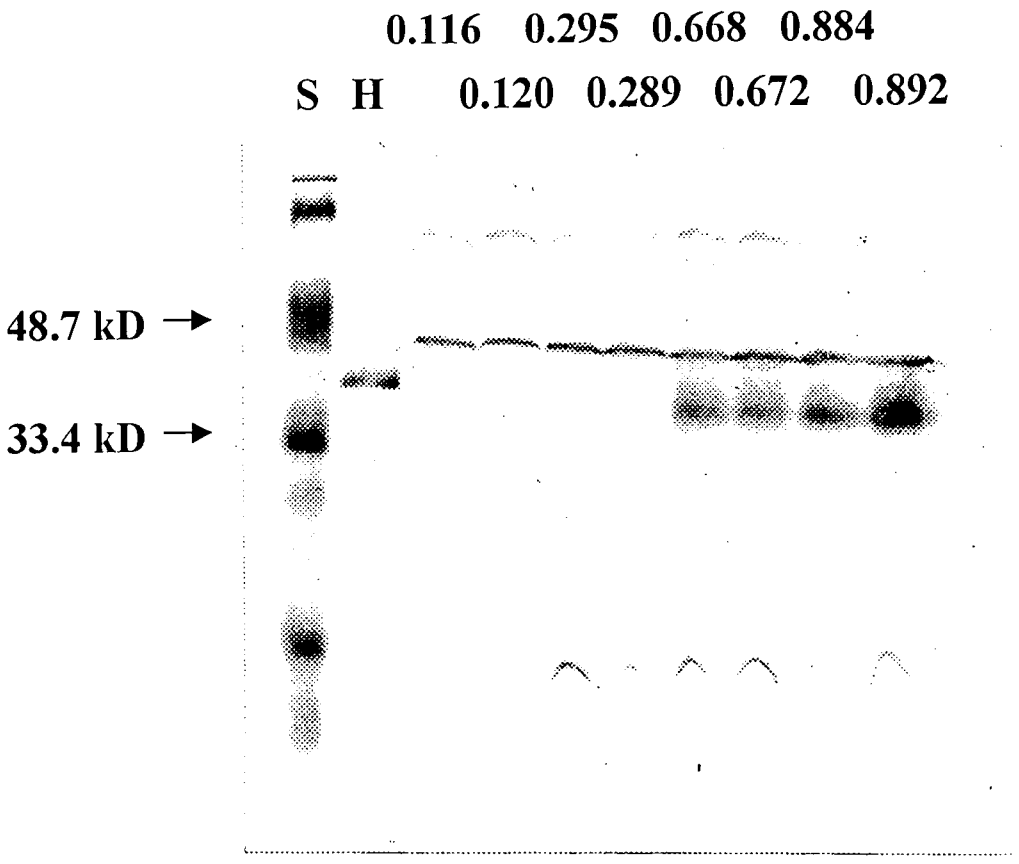


Figure 16. NodD1 expression in *B. japonicum* USDA110 grown to different cell densities (A_{600nm}) in RDY medium. **S**: Standards; **H**: His-tag NodD1 fusion protein. Rabbit anti-NodD1 antibodies were used.

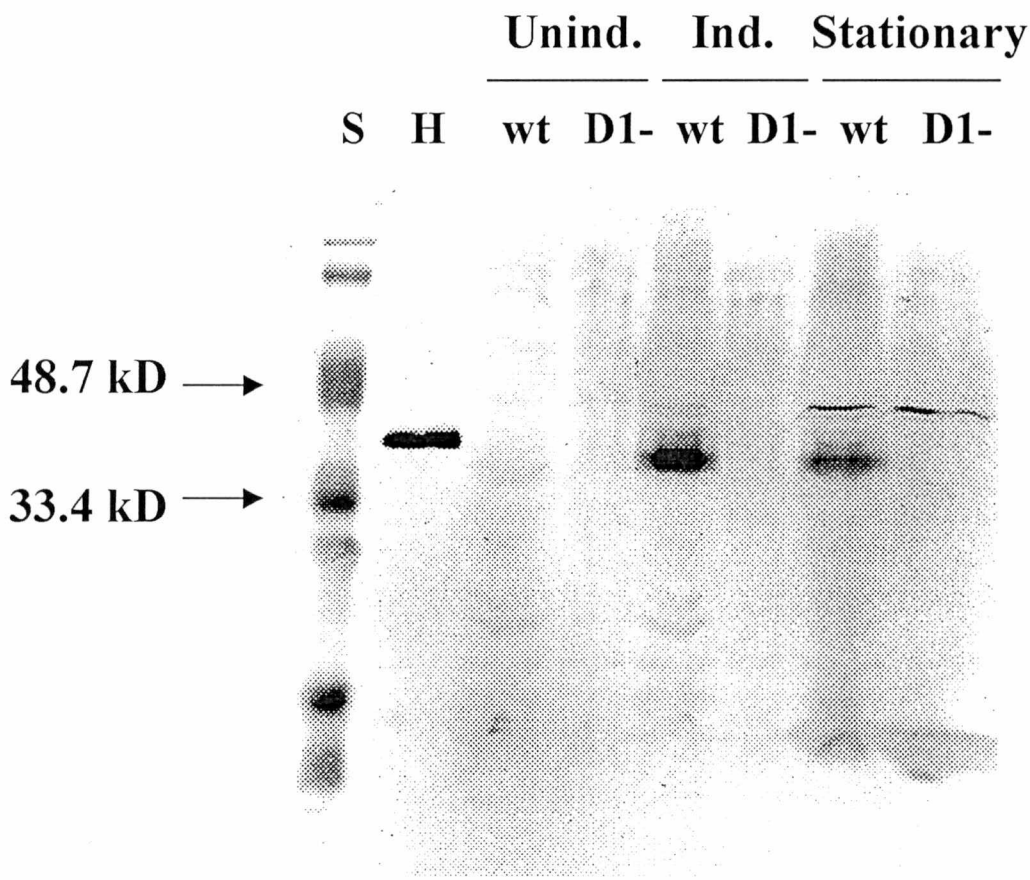


Figure 17. NodD1 expression in uninduced (**Unind.**) (A_{600nm} < 0.2), induced (**Ind.**), and stationary-phase cultures (A_{600nm} > 1.5) of *B. japonicum* USDA110 wild type (**wt**) and *nodD1* mutant strain AN314 (**D1-**). **S**: Standards; **H**: His-tag NodD1 fusion protein. Rabbit anti-NodD1 antibodies were used.

Inducibility of *nod* Gene Expression at Different Cell Densities and Growth Phases

Since NodD1 is required for *nod* gene expression, an experiment was done to examine *nodY-lacZ* expression in cells of strain ZB977 induced at different cell densities (A_{600nm} from 0.1 to 1.0) (Figure 18). It was found that higher induction levels of *nodY-lacZ* expression occurred in cultures induced at lower cell densities (Trial 1 in Figure 18). Lower *nod* gene expression in cultures induced at higher cell density was not due to depletion of inducers in the culture as 40 µl/ ml of soybean seed extract was used. It had been shown previously that as little as 0.25 µl/ml SSE induced significant *nod* gene expression (refer to Table 13). Figure 18 shows the data of two trials of the same experiment. The difference is that in trial 1 the starting A_{600nm} of the cultures was 0.55, whereas for trial 2 it was 2.24. As can be seen, the level of expression in trial 2 is significantly lower than trial 1.

The above experiment was modified and repeated. The level of expression of a chromosomal *nodC-lacZ* fusion in strain 573 was assayed in cultures harvested at different A_{600nm}, adjusted to different initial A_{600nm}, and then induced (Figure 19). The cultures were induced for 5 hours (cf. 10-14 hours for previous assays) in order to minimize any change in the cell growth phase during induction. It was found that the inducibility of *nod* gene expression was highest in cells harvested at the earliest growth phase (A_{600nm} = 0.068). For cells harvested at A_{600nm} = 0.192, *nod* gene induction was significantly reduced when cells were induced at a higher initial A_{600nm} (i.e., 0.3, 0.5, or 0.8). The inducibility of cells harvested at A_{600nm} of 0.068 and 0.192 was about the same

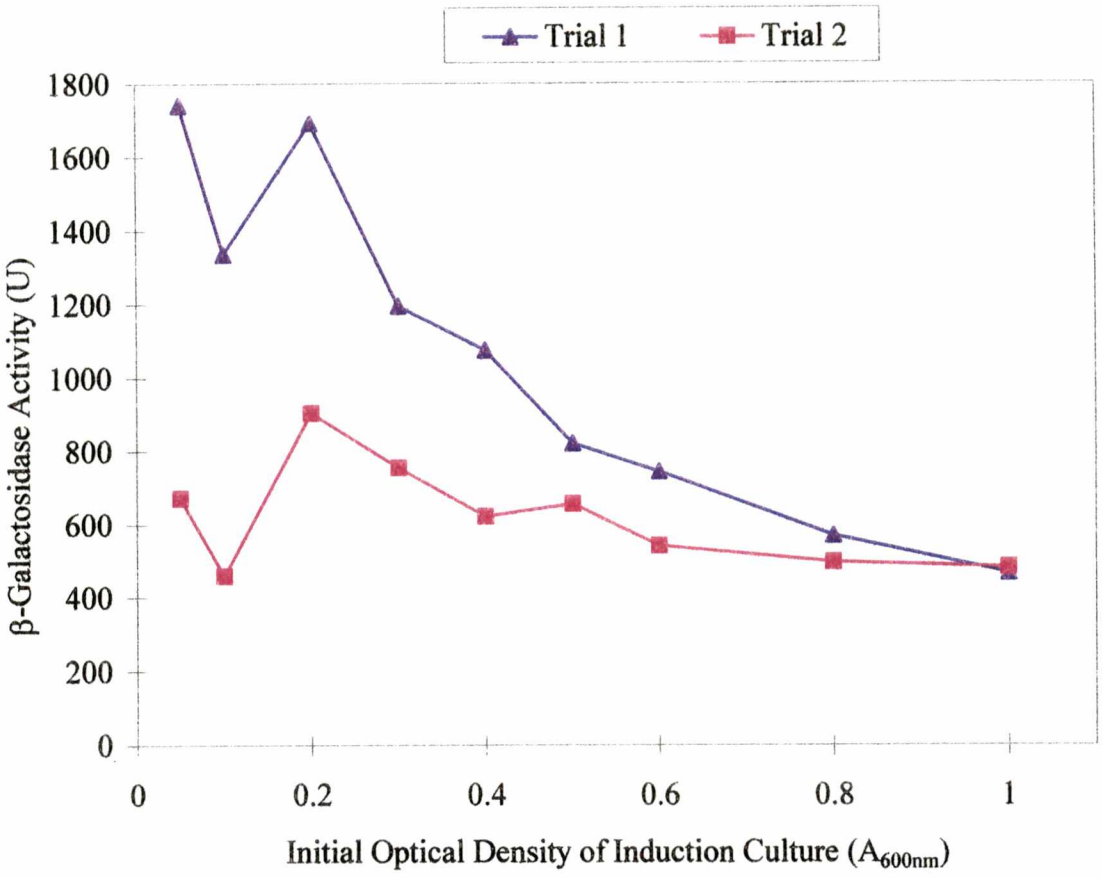


Figure 18. *nodY-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain ZB977 induced at different cell densities (A_{600nm}). A_{600nm} of inoculum for trial 1 was **0.55** and for trial 2 was **2.24**. 40 µl/ml of soybean seed extract was present as inducer. Induction was done in RDY medium.

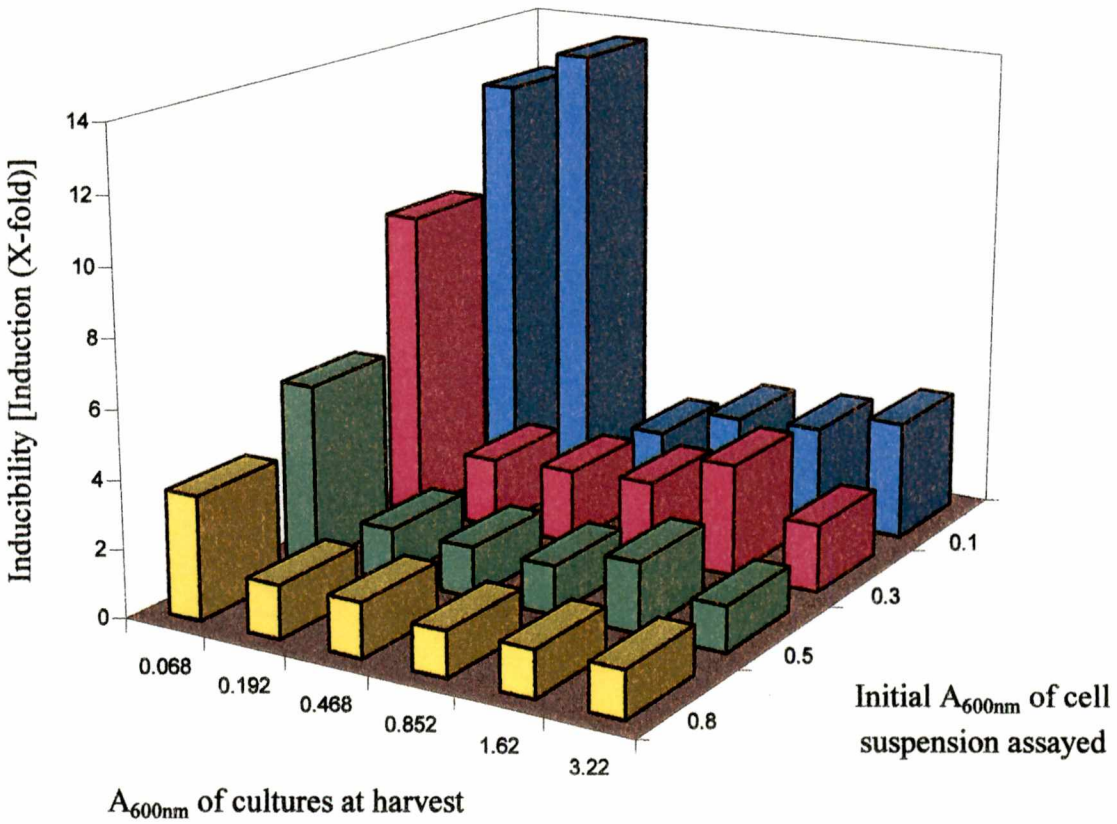


Figure 19. Inducibility of chromosomal *nodC-lacZ* expression in *Bradyrhizobium japonicum* USDA110 strain 573 harvested at different growth phases and induced at different initial cell densities (A_{600nm}). Induction was done in MM for 5 hours in the presence of 30 µl/ml soybean seed extract. Fold of induction is equal to β-galactosidase activity (act.) of induced culture / act. of uninduced culture at each cell density assayed. Standard deviation was less than 10% in each case.

as those induced at an initial A_{600nm} of 0.1. The inducibility of *nod* gene expression in cells harvested at later growth phases (A_{600nm} = 0.468, 0.852, 1.62, or 3.22) was similar. These data clearly show that cells are more inducible when harvested at lower A_{600nm} values and induced at a low cell density.

Growth of *B. japonicum nodC* and *nodD1* Mutants

The expression of NodD1 in uninduced cultures suggests a possible role of this protein in the normal growth and metabolism of *B. japonicum*. Therefore, the importance of NodD1 for normal growth of *B. japonicum* was examined in the following experiment.

Log-phase cells of *B. japonicum* USDA110 wild-type strain, 168 (*nodC* mutant), and AN314 (*nodD1* mutant) were inoculated into 50 ml RDY (in 250-ml conical flask) to give an initial A_{600nm} of 0.0004. Duplicate cultures of each strain were incubated with shaking (200 rpm) at 30 °C. Growth of the cultures were monitored by optical density (A_{600nm}) and viable cell counts (i.e. colony forming units CFU). For each strain, the generation time was calculated using an exponential equation best fit for the portion of curve where cells grew exponentially. The generation times are 9.3 ± 0.4, 9.7 ± 0.3, and 11.9 ± 1.1 hours respectively for wild type, 168, and AN314 (Figure 20). The difference in their growth rates was reflected in the pattern of their growth curves (CFU) (Figure 20). Stationary phase for the wild type, 168, and AN314 cultures was reached after 60, 75, and 110 hours of growth. The number of viable cells in all cultures declined after 150 hours of growth. After 250 hours of incubation, very few viable cells remained.

The growth curves reflected by A_{600nm} showed some differences from those generated using the viable counts. Figure 21 shows that all three strains had a similar pattern of growth and reached a maximum optical density (A_{600nm}) between 4.5 to 5.0. The generation time calculated from this plot for wild type, 168, and AN314 was about 13.4 ± 0.1, 12.0 ± 0.1, and 16.7 ± 0.6 hours, respectively (Figure 21). The initial growth phase and the relatively slower growth of AN314 are apparent in Figure 21. Surprisingly, unlike the CFU plots, there was a drastic drop in A_{600nm} values immediately after cultures reached the maximum density. The A_{600nm} of all three cultures decreased to the same value after about 250 hours, matching what was seen in Figure 20.

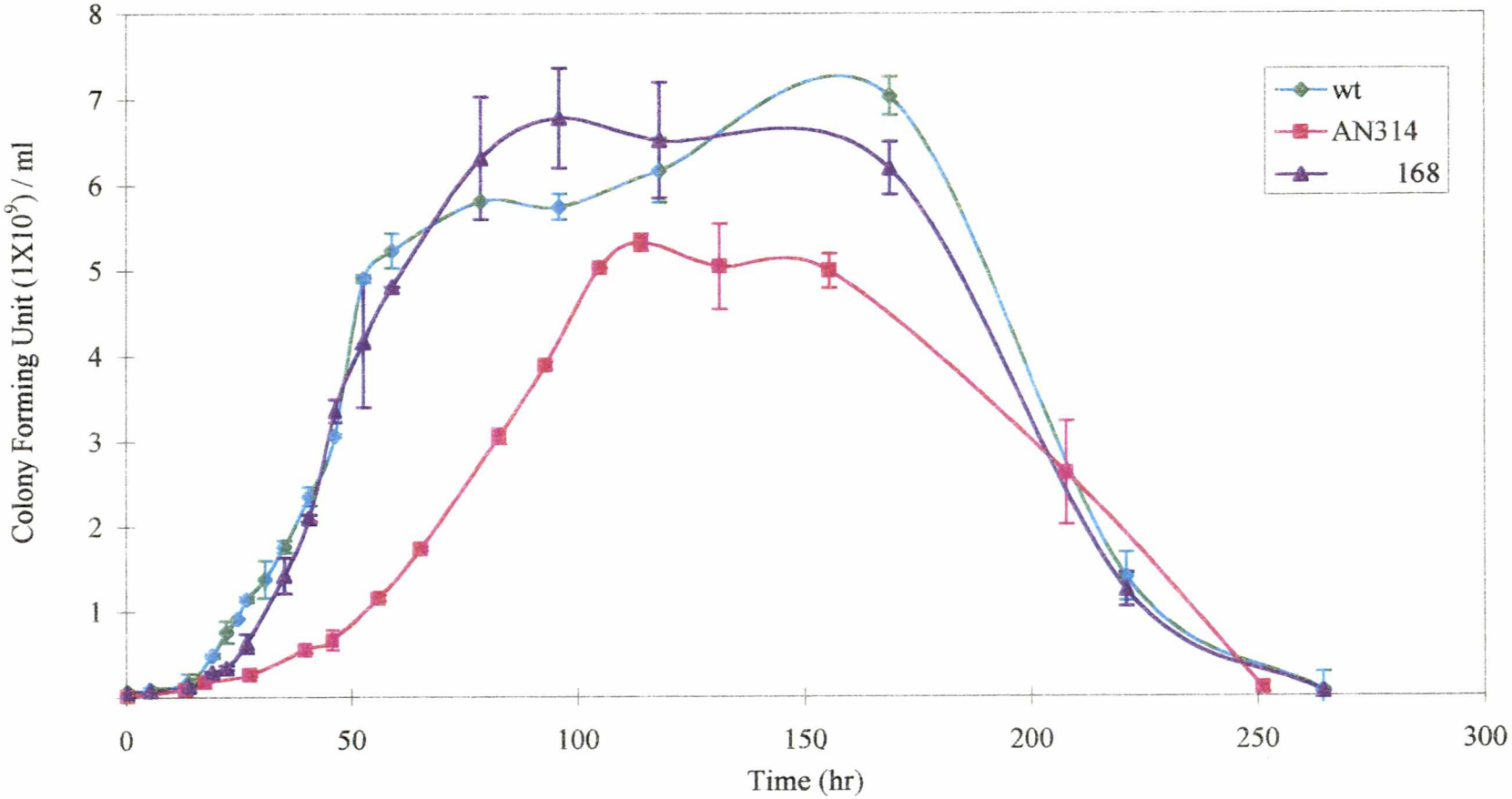


Figure 20. Growth curve (colony forming units) of *Bradyrhizobium japonicum* USDA110 wild type (wt), *nodD1* mutant strain AN314, and *nodC* mutant strain 168.

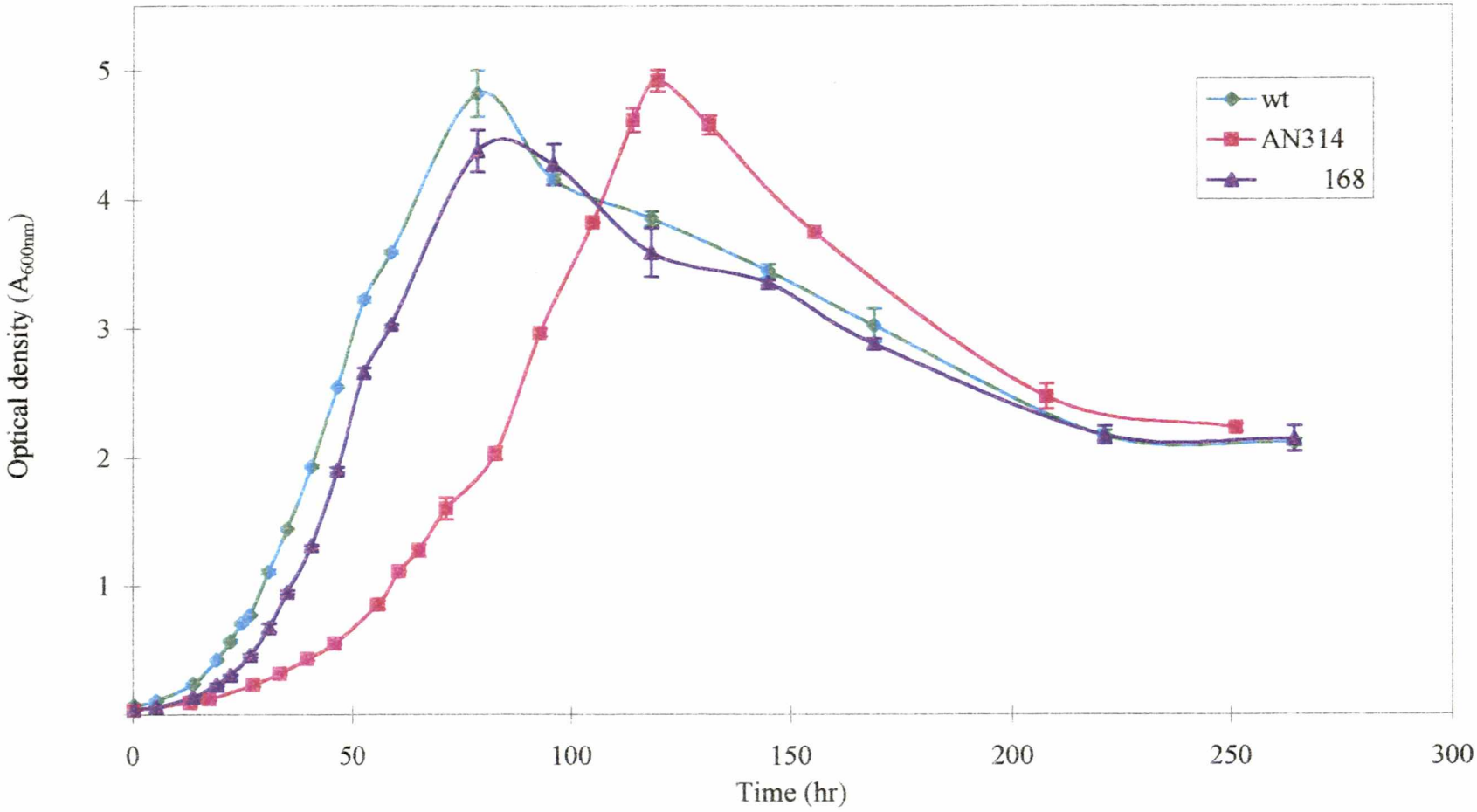


Figure 21. Growth curve (A_{600nm}) of *Bradyrhizobium japonicum* USDA110 wild type (wt), *nodD1* mutant strain AN314, and *nodC* mutant strain 168.

CHAPTER 4

DISCUSSION

Induction of *nod* Gene Expression by Xanthone Compounds

Besides flavones, flavanones, isoflavones, chalcones, and aurones, which belong to the flavonoid chemical class, compounds belonging to other classes of plant phenolic compounds are capable of inducing *nod* gene expression. Xanthones are C_{13} polyphenolic compounds (see structure in Figure 3) that occur in a limited number of plant families. They are less prevalent than flavonoids. However, their structures are very related to those of C_{15} flavonoids in terms of their hydroxylation patterns (Harborne and Simmonds, 1962) and chromatographic behavior (Hostettmann and Hostettmann, 1989). Our study is the first to show that a xanthone compound can induce *nod* gene expression in *B. japonicum*.

Like isoflavonoid inducers, the two xanthone compounds identified in our study were able to induce the expression of the *nodYABC* operon via NodD1 and NodV/NodW. These same compounds induced expression of *nodD1*. The NodD proteins in all rhizobia act as a primary determinant of host specificity by recognizing specific chemical inducers. Therefore, NodD1 and NodV/NodW of *B. japonicum* are able to recognize the two xanthone compounds perhaps due to the fact that xanthone hydroxyl or/and methoxyl groups are positioned similarly to the isoflavonoid inducers of *B. japonicum*. Several

studies (Györgypal et al., 1991a; Györgypal et al., 1991b; Cunningham et al., 1991) have examined the structure/function relationships of *nod* gene inducers. In the case of *B. japonicum*, such studies are complicated by the fact that a diverse range of compounds can serve as inducers (Cunningham et al., 1991). Thus, it has proved impossible to identify specific chemical traits required for *nod* gene induction. The finding that specific xanthones are efficient inducers of *B. japonicum nod* gene expression further complicates such structure/function analysis.

Inhibition of *nod* Gene Expression by Organic Acids and Rich Medium

TCA cycle intermediates such as the C_4-dicarboxylic acids, namely succinate, malate, and fumarate, are the major carbon sources for bacteroids in all rhizobial strains (Streeter, 1991). In this study, we found that these compounds (namely acetate, fumarate, L-malate, oxaloacetate, succinate, and α-ketoglutarate) reduced the level of *nod* gene induction in *B. japonicum* (Table 11). Although a diversity of organic acids and carbohydrates can be found in nodules (Reibach and Streeter, 1983), only C_4-dicarboxylic acids, are actively transported into bacteroids by the inducible Dct (**D**icarboxylic acid **t**ransporter system) system (Finan et al. 1981; McAllister and Lepo, 1983; San Francisco and Jacobson, 1985). Indeed, the presence of the Dct system in free-living *B. japonicum* may explain the different inhibitory effect of various organic acids. As shown in Table 11, *nod* gene expression was reduced approximately 50 and 70%, respectively, at 1 and 10 mM fumarate, L-malate, or succinate. The inhibitory effect at 10 mM acetate was 3.8-

fold more than that at 1 mM. Expression of *nodY-lacZ* was reduced 89% at 10 mM α-ketoglutarate, but was enhanced 46% at 1 mM. Differences in the transport kinetics of these organic acids may account for the differences in their inhibitory effect at higher concentrations. Succinate, L-malate, and fumarate are transported actively in both cultured cells and bacteroids of *B. japonicum* (McAllister and Lepo, 1983; Parke and Ornston, 1984). Although not determined for cultured cells of *B. japonicum*, the Km for L-malate transport in bacteroids is 40 μM (Reibach and Streeter, 1984). Therefore, assuming a similar affinity in cultured cells, active transport of fumarate, L-malate, or succinate would be saturated at 1 mM, apparently producing an intracellular level sufficient to give a 50% reduction of *nod* gene expression. Active transport of α-ketoglutarate into *B. japonicum* bacteroids has been reported (Reibach and Streeter, 1984). This uptake system has a relatively lower substrate affinity, but a higher substrate saturation point than that for succinate or L-malate (Reibach and Streeter, 1984). It is not known whether this transport system is also present in cultured cells. If present, these transport properties might explain the higher inhibition (89%) found at 10 mM α-ketoglutarate when compared with that of the other three dicarboxylic acids. Acetate can cross the bacterial cell membrane by passive diffusion as an undissociated acid (Visser and Postma, 1973; Baronofsky et al., 1984). Consistent with this fact, increased inhibition of *nod* gene expression was found with increasing acetate concentration. Oxaloacetate has been reported to be an inhibitor of succinate transport. However, it is unlikely that oxaloacetate is transported by the Dct system since *dct* mutants grew normally on oxaloacetate (Finan et al., 1981). As the case for α-ketoglutarate, the

oxaloacetate transport system may have a lower substrate affinity and, therefore inhibition effect was only seen at higher concentration (Table 11).

Since L-malate is the major dicarboxylic acid supplied to the bacteroids by the host plant (Vance et al., 1983; Rosendahl et al., 1990; Udvardi et al., 1988a), this compound was used as the representative C_4-dicarboxylic acid for further studies of *nod* gene expression. Both NodD1, a LysR type regulator, and NodW and NodV, a two-component regulatory system, are required for *nodYABC* expression in *B. japonicum*. Our results show that inhibition of *nodYABC* induction by L-malate is mediated through NodD1 (Table 15), as well as NodV and NodW (Table 16 and Figure 6). As shown in Table 15, 1 mM L-malate inhibited *nodD1* expression by 50%. This suggests that L-malate inhibition of *nodY-lacZ* expression in strain ZB977 (Table 11 and 16) was likely due to reduction of *nodD1* expression. In addition, results shown in Table 16 and Figure 6 show that *nodY-lacZ* expression in strain Δ1267 was also reduced in the presence of L-malate. Since *nodD1D2nolA* were deleted in this mutant strain, induction of *nod* gene expression is solely regulated by NodV and NodW. Therefore, this indicates that L-malate inhibition on *nod* gene expression is also mediated through NodV and NodW. Indeed, the fact that both NodD1 and NodV/ NodW are involved in L-malate inhibition explains the observation that L-malate was less inhibitory in strain Δ1267 compared to that found with the wild-type strain ZB977. Furthermore, the result that L-malate did not inhibit *nptII-lacZ* expression in strain SL101 indicates that the effect of L-malate is specific for *nod* gene expression (Table 16 and Figure 6).

The cumulative effect of various organic acids (Table 11) was tested to see if a higher inhibition of *nod* gene expression could be obtained. Clearly, bacteroids are exposed to a mixture of organic acids in nodules (Stumpf and Burris, 1981). The presence of both L-malate and succinate did not result in a greater inhibition of *nod* gene expression (Figure 7). This result is consistent with the fact that L-malate and succinate are transported by the same uptake system (Finan et al., 1981). However, addition of both acetate and L-malate did produce an additive inhibitory effect on *nod* gene expression (Table 17). The inhibition increased with increasing amounts of added acetate. More than 90% inhibition was achieved in the presence of 0.5 mM L-malate and 10 mM acetate. This result is again consistent with fact that acetate and L-malate are transported into cells by different routes.

The specific inhibition of L-malate and various organic acids on *nod* gene induction suggests that they may be involved in suppressing *nod* gene expression in nodules (Sharma and Signer , 1990; Schlaman et al., 1991). Bacteroids are continuously exposed to organic acids. Therefore, further experiments were done to examine if higher levels of inhibition could be obtained using cells pre-grown with L-malate. The results in Figure 8 show that pre-growth with 5 mM L-malate did result in a much higher inhibition of *nod* gene expression. However, a surprising result was also obtained. When cells grown in RDY were resuspended in MM before induction, inhibition of *nodY-lacZ* expression did not occur in the presence of 0.5 to 5 mM L-malate (Figure 8). In contrast, cells grown in RDY and directly transferred to MM showed a 50-60% inhibition by L-malate. The effect of resuspension of cells in MM was not due to the physical effects of

centrifugation or resuspension (Table 19). This effect was due to the chemical composition of the medium. The effects of the various carbon and nitrogen components in the growth and induction medium on *nod* gene induction were analyzed. The results in Figure 8, Table 19, Table 21, Table 22, Table 23, and Table 24 showed that L-malate inhibition on *nod* gene expression was more prominent in cells grown in rich medium, i.e. those containing preferred carbon and nitrogen sources.

We speculate that the type of medium used for *B. japonicum* growth affects the physiological response of cells to L-malate. Specifically, when *B. japonicum* cells are grown in rich medium such as RDY, the L-malate added is not readily metabolized since cells prefer to utilize D-gluconate. The latter compound is known to be a preferred carbon source for *B. japonicum* (Stowers 1985). Under these conditions, L-malate accumulates inside the cells at a higher concentration resulting in inhibition of *nod* gene expression. In contrast, when cells are grown in minimal medium, L-malate is immediately metabolized as the preferred carbon source. Therefore, L-malate is present at a very low intracellular level of L-malate unless an excess amount of L-malate is added so that it can accumulate inside the cells. This explains the interesting observation that resuspension of cells in MM reduced L-malate inhibition (Figure 8, Table 19, and Table 23). In this case, inhibition of *nod* gene expression did not occur at 1 mM and 5 mM L-malate likely due to rapid catabolism of this added carbon source. However, inhibition was seen at 10 mM L-malate, a level that might saturate metabolism, resulting in intracellular accumulation of L-malate to a level sufficient to inhibit *nod* gene expression. In the case of cells directly transferred or resuspended in medium containing D-gluconate,

and yeast extract or/and L-glutamate, these carbon and nitrogen sources were carried over into the induction medium, allowing the cells to utilize them before the switching to L-malate metabolism. Without active catabolism of L-malate, even though in the presence of very low L-malate concentration (i.e. 1 mM), L-malate still accumulates quickly in the cells to a level sufficient to inhibit *nod* gene expression.

Cells grown in different media respond differently to L-malate inhibition (Table 24). A greater L-malate inhibition (75 to 88% inhibition at 10 mM L-malate) was seen in cells grown on D-gluconate, yeast extract or L-glutamate because the added L-malate was not metabolized and accumulated in the cells. A lower level of inhibition (23 to 45% inhibition at 10 mM L-malate) was seen when cells were grown with glycerol or D-gluconate with NH_4NO_3 because the cells preferred to metabolize L-malate. Thus our model predicts that a lower level of L-malate in cells results in a lower inhibition of *nod* gene expression. In the case of growth on D-gluconate and NH_4NO_3, L-malate inhibition was still low. This indicates that when cells are grown with a poor nitrogen source, cells likely prefer to metabolize L-malate.

Organic acids, as well as amino acids, sugars, and phenolic compounds, are released from legume roots and are chemoattactive to rhizobia (Grittie et al., 1978; Barbour et al., 1991). These substances together with other factors, probably account for a more active growth of rhizobia in the rhizosphere (Bowen and Rovira, 1976; Schmidt, 1974). Our results do not suggest that the presence of organic acids in the rhizosphere is inhibitory to *nod* gene expression. Due to the oligotrophic nature of the soil habitat, rhizobia are generally nutrient limited and grow poorly. Upon encountering the organic

acids secreted from host roots, rhizobial cells metabolize them for growth. It is unlikely that these organic acids accumulate to a high level inside the cells. In fact, our results suggest that the presence of a low level of organic acids may facilitate induction of *nod* gene expression in suboptimally growing cells. Indeed, a slight increase in *nod* gene expression was observed in cells resuspended in MM or medium containing glycerol as the carbon source upon the addition of low levels of L-malate (1 to 5 mM) (Figure 8, Table 19, and Table 23). Therefore, our findings suggest that organic acids in the rhizosphere may enhance *nod* gene expression in rhizobia.

Bacteroids in nodules grow in conditions that favors the production of ATP for nitrogen fixation. A high level of L-malate is expected to be present inside the bacteroids because L-malate is the major carbon source supplied to the bacteroids (Rosendahl et al., 1990). Therefore, we suggest that the presence of high levels of L-malate, together with other organic acids (e.g., acetate, fumarate, succinate, α-ketoglutarate and oxaloacetate), in bacteroids may account at least in part for the suppression of *nod* gene expression in bacteroids (Schlaman et al., 1991; Sharma and Signer, 1990) (Table 26).

Further evidence in favor of the inhibition of *nod* gene expression by C_4-dicarboxylic acids was obtained by Mavridou et al. (1995). They reported that a *dctB* mutant of *R. leguminosarum* bv. viciae showed a significant reduction in *nodD* and *nodABC* gene expression. The effect of this mutation is to enhance dicarboxylic acid transport. Therefore, inhibition of *nod* gene expression by dicarboxylic acids may be a general feature of *Bradyrhizobium* and *Rhizobium* species.

The mechanism by which organic acids inhibit *nod* gene expression in *B. japonicum* is not known. The addition of dibutyryl cAMP did not affect L-malate inhibition of *nod* gene expression. However, this result was not conclusive because it is not certain that dibutyryl cAMP added was transported into the cells It has been proposed that cAMP levels vary in *B. japonicum* cells in response to the availability of readily oxidizable carbon sources (Lim and Shanmugan, 1979). Therefore, experiments monitoring the levels of intracellular cAMP in *B. japonicum* cells treated with L-malate and/or various organic acids are needed to further examine the involvement of cAMP-mediated catabolite repression in *nod* gene regulation.

Inhibition of *nod* Gene Expression by Microaerobic Conditions

Microaerobic conditions can induce *nif* and *fix* gene expression in free-living rhizobial cells (Ditta et al., 1987; David et al., 1988) FixL/FixJ, FixK, and NifA regulators perceive the microaerobic environment and induce the expression of various *nif* and *fix* genes (Table 6) (Batut and Boistard, 1994; Hennecke et al., 1993; Stigter et al., 1993). The results in Figure 10 showed that growth of *B. japonicum* cells in microaerobic conditions significantly reduced *nodYABC* expression. This repression was not mediated by the putative NolR-type repressor, NolA, NodD1, or NodD2 since mutants defective in these functions (i.e., strain SR15-32 and strain 1267-32) were subjected to the same inhibition as the wild type (strain ZB977). We did not test the possible involvement of FixK and NifA in the inhibition of *nod* gene expression. Direct interaction of FixK or

NifA on the σ^{70}-dependent *nod* gene promoter is unlikely since these proteins interact exclusively with σ^{54}-dependent promoters (Fischer 1994). Therefore, if FixK or NifA are involved in the *nod* gene inhibition, the effect is likely indirect. Alternatively, it is possible that there is another unidentified regulator which responds to microaerobiosis and mediates inhibition of *nod* gene expression. An example is the microaerobic induction of *dctA* expression in *R. leguminosarum* (Ronson and Astwood, 1985) and *R. meliloti* (Wang et al., 1989) which is not regulated by NifA, FixL, or FixJ.

The results of these experiments provide another possible mechanism by which *nod* gene expression is suppressed in bacteroids (Table 26). The isolation of mutants that do not respond to microaerobic inhibition of *nod* gene expression would allow the identification of genes involved in this process.

pH Effect on *nod* Gene Expression

The previous experiments showed that various organic acids such as L-malate, succinate, fumarate, and acetate can inhibit *nod* gene expression. The effect of pH on L-malate inhibition of *nod* gene expression was also examined.

The pH (pH 4.0 to 8.5) effect on *nod* gene expression induced in RDY was different from that found for cells grown in MM (Figure 11). When induction was done in RDY, nearly complete inhibition of *nod* gene expression occurred at pH 5.5 and 8.5. Addition of 5 mM L-malate lowered the level of *nod* gene expression at pH 6.0 to 8.0. The lowest level of *nod* gene expression occurred at extreme pH (pH 4 and 8.5) in MM,

but was still approximately a 1000 U. Addition of 5 mM L-malate lowered the expression at all the tested pH, resulting in a nearly complete inhibition at the extreme pH.

It has been proposed that the symbiosome is similar to a vacuole with internal pH values between 5.5 to 6.0 (Kahn et al., 1995). Since bacteroids are exposed to an abundant level of carbon sources, mainly dicarboxylic acids such as L-malate, we believe that their physiology more closely resembles that of free-living cells grown in rich medium. Therefore, the acidic pH in the symbiosome is another factor that could contribute to the suppression of *nod* gene expression in bacteroids.

Other published studies have shown that acidic pH inhibits *nod* gene expression in rhizobia. For example, *nodA*- and *nodF-lacZ* expression, in the presence of 50 μM 7,4'-dihydroxyflavonone as inducer, was significantly reduced at pH values less than 5.3 in *R. leguminosarum* bv. trifolii (Richardson et al., 1988). Similarly, *nodY-lacZ* expression, in the presence of either 7.3 μM genistein or daidzein as inducer, was significantly reduced at pH 4.5 in *B. japonicum* USDA110 strain ZB977 (Sutherland et al., 1990).

Inducibility of *nod* Gene Expression Depends on Growth Phase and Cell Density

The results in Figure 18 showed that higher induction levels of *nodYABC* expression occurred in cultures induced at lower cell densities (Figure 18). Another experiment was performed to assay *nod* gene expression in cultures harvested at different A_{600nm}, as well as induced at different initial A_{600nm}. The results showed that both cellular

growth phase and culture density affect *nod* gene expression (Figure 19). Cells are more inducible in early growth phase and when they are induced at lower cell densities.

The natural soil environment, especially outside the rhizosphere, is often oligotrophic with limiting organic matter. Many soil bacteria, such as rhizobia, do not produce resting cells and likely exist in soil as vegetative cells in a reduced state of metabolism (called starved state, Morita, 1997). As described earlier, *B. japonicum* cells found in soil grow very slowly with a doubling time of 241 to 361 hours (Schmidt, 1974). Cells in soil exhibit reduced metabolism, are nutrient limited and resemble cells in stationary phase, which is operationally defined as a condition of nongrowth elicited by nutrient deprivation (Morita 1997). In contrast, *B. japonicum* cells found in the rhizosphere have a doubling time of 9 to 12 hours (Bowen and Rovira, 1976). The active growth of cells in the rhizosphere indicates once cells enter the rhizosphere, they are able to grow actively on nutrients released from the plant. Therefore, upon entering the rhizosphere, rhizobial cells switch from a starved state to a metabolic active state. For example, this might occur when the root of a newly-germinated seed penetrates the soil. In this connection, our findings that *B. japonicum* cells are most inducible at early growth phase suggest that initial growth in rhizosphere is in fact a favorable condition for *nod* gene induction. Inducibility of *nod* gene expression decreases during later growth stages (i.e. higher A_{600nm}) when nutrients may become limiting. Therefore, we believe that starved cells in fallow soil are less inducible. When such cells enter the rhizosphere and are exposed to the appropriate flavonoids inducers, a good level of *nod* gene expression is induced resulting in efficient nodulation.

Our results also showed that cells are more inducible at lower cell densities. The decrease of inducibility at high cell densities was not due to inducer limitation because inducers were present in excess. These data suggest *nod* gene expression is cell-density dependent.

Indigenous NodD1 Expression is Cell-Density Dependent

The availability of anti-NodD1 antibodies allowed us to examine the expression of NodD1 in *B. japonicum* strain USDA110. Immunoblot results shown in Figure 13 confirmed previous results that measured *nodD1-lacZ* fusion expression (Banfalvi et al., 1988). These experiments showed that the *nodD1* gene can be induced by isoflavones found in soybean seed extract. The NodD1 protein was detected within two hours of induction. A surprising finding was the detection of NodD1 in uninduced cultures grown at high A_{600nm} (i.e. 2.6) (Figure 14). The addition of soybean seed extract to such cultures transiently reduced the level of NodD1 expression (Figure 14). In uninduced *B. japonicum* cells grown in RDY, NodD1 was detected when the cultures reach a A_{600nm} equal to about 0.4. The level of NodD1 reached a constant level starting from A_{600nm} equal to about 0.6 (Figure 15). Similar results were obtained in Figure 16. The growth curve in A_{600nm} shown in Figure 21 indicated that cells at $A_{600nm} = 0.4$ are in the early log phase. Therefore, expression of indigenous NodD1 is unlikely due to a general starvation response as controlled by the RpoS gene in *E. coli*. RpoS is a stationary-phase- or starvation-specific sigma factor in *E. coli* (Hengge-Aronis, 1993). In fact, expression of

indigenous NodD1 appears to be cell-density dependent. Cell-density regulated gene expression, which is also called quorum-sensing, has been found in many bacteria (Table 8). The *rhiABC* genes in *R. leguminosarum* encoded on the Sym plasmid, were the first genes in rhizobia known to be regulated by quorum-sensing (Gray et al., 1996). Further experiments are required to ascertain the mechanism of cell-density dependent regulation of NodD1 in *B. japonicum*. It is possible that an autoinducer (e.g. homoserine lactone) is involved in this regulation. In addition, it would be useful to examine whether *nodD* or other *nod* genes are controlled in a density-dependent manner in other rhizobia. Although NodD is constitutively expressed in various rhizobia (e.g., *R. meliloti*, *R. leguminosarum* bv. trifolii and bv. viciae, and *R. fredii*), it is possible that NodD expression is regulated by quorum sensing in these bacteria, but was not noticed if experiments were done only with log phase cultures.

The significance of quorum-sensing regulation of NodD1 expression in *B. japonicum* is unclear. However, indigenous expression of NodD1 in uninduced cells may relate to the observation that the inducibility of *nod* gene expression is affected by cellular growth phase and cell density (Figure 19). It has been suggested that the NodD protein, a LysR-type regulator, may bind to the *nod* promoters in the absence of inducer (Kondorosi et al., 1989; Fisher et al., 1988; Hong et al., 1987; Fisher and Long, 1989). NodD1 in this condition would not activate *nod* gene transcription and, indeed, could repress expression. In this connection, one could speculate that when *B. japonicum* cultures reach a critical cell density, autoinducers accumulate activating NodD1 expression. In the absence of *nod* gene inducers, an inactive NodD1 binds to the *nod* promoters repressing *nod* gene

expression. The negative effect of indigenous NodD1 proposed above may explain the observation in Figure 19 that cells are less inducible when they are harvested at higher A_{600nm} and induced at higher densities.

In fact, the suggested regulatory role of NodD1 in uninduced cells may also explain the observation that *nodD1-lacZ* expression is very low in USDA110 (Banfalvi et al., 1988; Wang and Stacey, 1991). Unless the cells used for induction are from a culture of an early growth phase culture or they are induced at a very low cell density, indigenous NodD1 present in cells will occupy the promoters controlling *nodD1-lacZ* expression, leading to very low expression of this fusion.

It has been reported that introduction of multiple copies of the *nodD* gene into *R. meliloti* reduced expression of *nodD* (Rossen et al., 1985). Similar negative autoregulation of *nodD* expression has also been shown in *R. leguminosarum* bv. trifolii (Spaink et al., 1987a). Therefore, NodD1 may be important in *B. japonicum* for negative autoregulation of *nodD1* gene expression.

Effect of *nodD1* Mutation on Cell Growth

In *B. japonicum*, NodD1 is important for *nod* gene induction (Banfalvi et al., 1988). However, *nodD1* mutants nodulate host plants (Göttfert et al., 1992) due to the presence of another *nod* gene regulatory system - NodV and NodW (Sanjuan et al., 1994). Cell-density-dependent regulation of *nodD1* expression suggests that NodD1 is important in normal cell growth. To study the possible physiological role of NodD1 in growth, we

compared the growth curves of wild type, a *nodD1* mutant, and a *nodC* mutant. The purpose of including the *nodC* mutant for comparison was to see if the effect of the *nodD1* mutation on growth was due to its effect on *nodYABC* regulation. Figure 20 and Figure 21 show the growth of the three strains monitored by colony forming units and A_{600nm}, respectively. As shown in both figures, the *nodC* mutation did not affect normal growth. This result suggests that the ability to produce Nod factors is not important for growth of *B. japonicum* under these conditions. In contrast, the *nodD1* mutation did affect normal growth. The *nodD1* mutant cultures had a slower growth rate (generation time of 11.9 hours compared to 9.3 hours of wild-type strain), and reached a lower maximum viable cell density (about 5.2×10^9 CFU/ml) than that of the wild type (about 7×10^9 CFU/ml). However, the *nodD1* mutant reached the same optical density as the wild type and *nodC* mutant. This latter result may be due to the production of more exopolysaccharide by the *nodD1* mutant. The viability of *nodD1* mutant cells appeared to be reduced in stationary phase. This can be seen in Figure 20 where, after reaching the peak value, the viable counts of *nodD1* mutant cells dropped faster than that of the wild type and *nodC* mutant (during time = 150 to 250 hours).

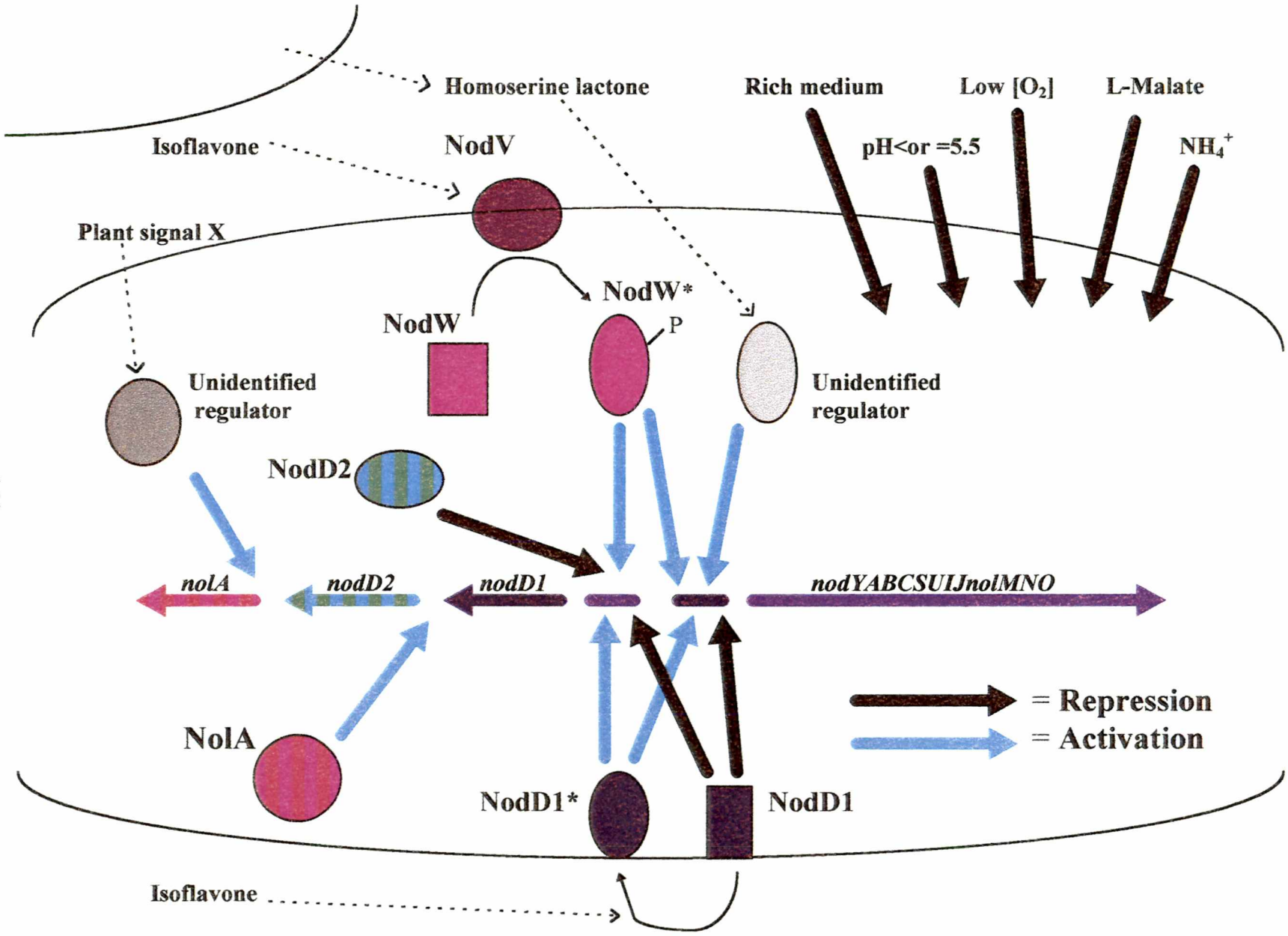
Taken together, these results suggest that the *nodD1* gene is important for normal growth of *B. japonicum*. Since a similar result was not obtained for the *nodC* mutant, we postulate that NodD1 controls the expression of other unknown genes required for optimal *B. japonicum* growth.

Summary of the Factors Affecting *nod* Gene Regulation in *B. japonicum*

Various chemical and physical factors have been found that inhibit *nod* gene expression in *B. japonicum*. This list include organic acids, such as L-malate, rich medium, microaerobiosis, and acidic pH (equal or less than 5.5). It is likely that all of these factors are encountered by bacteroids. As reported in other rhizobia (Schlaman et al., 1991; Sharma and Signer, 1990), *nod* gene expression is suppressed in *B. japonicum* bacteroids, even though *nod* gene inducers are present in nodules. Our results suggest that the physical and chemical conditions to which bacteroids are exposed in nodules may explain the lack of *nod* gene expression in bacteroids.

Constitutive expression of NodD1 occurs in uninduced cultures of *B. japonicum* at high cell densities (A_{600nm} more than 0.4). This cell-density dependent regulation may be due to the production of autoinducers, (i.e. homoserine lactone compounds), secreted from cells as the cell density increases. NodD1 binds to the *nod* promoter in an inactive form in an uninduced culture, and may repress *nod* gene expression under these conditions. This activity of NodD1 may account for the lower inducibility of *nod* gene expression found in cells at later growth phase and at higher cell densities. An updated model of *nod* gene regulation in *B. japonicum* adding the findings of this study is shown in Figure 22.

Figure 22. Proposed Model for *nod* gene regulation in *B. japonicum*. Expression of the *nodYABCSUIJnolMNO* genes and *nodD1* gene can be activated by NodD1 and NodV/NodW in the presence of isoflavones. Expression of NodD2 inhibits expression of the *nodYABC* operons. *nodD2* expression can be activated by $NodA_1$, whose expression is activated by an unidentified regulator in the presence of unknown plant signal(s). Expression of the *nodYABC* genes can be inhibited by rich medium, acidic pH (pH equal or less than 5.5), low oxygen tension, L-malate and various organic acids such as fumurate, succinate, acetate, α-ketoglutarate, and oxaloacetate, and ammonia. *nodD1* expression can be induced by NodD1 in the presence of isoflavones. Our results propose that at high cell densities or late growth phase, indigenous NodD1 expression is activated by an unidentified regulator in the presence of a sufficiently high level of homoserine lactone, which is secreted by other cells. In the absence of isoflavones, NodD1 binds to *nod* promoters hindering binding of the active NodD1 (labeled as "NodD1*" in the figure), which is able to induce *nod* gene expression upon activation by isoflavones.


Homoserine lactone
Rich medium
Low [O2]
L-Malate
Isoflavone
NodV
pH<or =5.5
NH4+
Plant signal X
NodW*
NodW
P
Unidentified regulator
Unidentified regulator
NodD2
nolA
nodD2
nodD1
nodYABCSUIJnolMNO
= Repression
= Activation
NolA
NodD1*
NodD1
Isoflavone

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APPENDIX

Preparation of Cell Lysate under Native Conditions

1. Cells were harvested from a 50-ml culture by centrifugation at 10,000 rpm for 5 min in a Beckman JA-14 rotor at 4 °C.
2. Cell pellet was resuspended in 10 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole).
3. 1 mg/ml of lysozyme was added and the suspension was incubated on ice for 30 min.
4. The cells were then sonicated for 30 sec for six times at an output of 15 using a microtip (Sonifier model 450, Branson Ultrasonics, Danbury, Conn.)
5. Lysate was centrifuged at 17,000 rpm in JA-17 rotor or 18,000 rpm in JA-20 rotor for 30 min at 4 °C to pellet cellular debris.
6. The clear lysate (the supernatant) was kept at -20 °C if not used immediately.

Preparation of Cell Lysate under Denaturing Conditions

1. Cells were harvested from a 50-ml culture by centrifugation at 10,000 rpm for 5 min in a Beckman JA-14 rotor at 4 °C.
2. Cells were resuspended in 10 ml of lysis buffer (6M guanidine.HCl; 0.1 M NaH_2PO_4; 0.01 M Tris.HCl, pH 8.0).
3. Cells were lysed by gentle shaking for 60 min at room temperature .

4. Cell suspension was sonicated on ice with six 15-second cycles at an output of 20 to shear DNA and RNA using a microtip (Sonifier model 450, Branson Ultrasonics, Danbury, Conn.).
5. Lysate was centrifuged at 17,000 rpm in JA-17 rotor or 18,000 rpm in JA-20 rotor for 30 min at 4 °C to pellet cellular debris.
6. The clear lysate (the supernatant) was kept at -20 °C if not used immediately.

VITA

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