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Extension of Host Range of *Rhizobium leguminosarum* bv. *trifolii* Caused by Point Mutations in *nodD* That Result in Alterations in Regulatory Function and Recognition of Inducer Molecules

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The positive activation of several nodulation genes in strain ANU843 of *Rhizobium leguminosarum* biovar *trifolii* is mediated by the product of the *nodD* gene and by the interaction of NodD with plant-secreted inducer and anti-inducer compounds. We have mutagenized the *nodD* gene of strain ANU843 with nitrosoguanidine and have found that the ability of the mutated *nodD* products to interact with inducer and anti-inducer compounds is affected by the amino acid sequence in at least two key regions, including a novel area between amino acids 77 and 123. Several novel classes of mutants were recognized by phenotypic and molecular analysis of the mutant *nodD* genes. Classes 1 and 4 mutants were able to induce *nodA* expression independently of the addition of inducer and anti-inducer compounds and were unable to mediate autoregulation of the *nodD* gene. Classes 2 and 3 mutants retained several properties of the wild-type *nodD*, including the ability to interact with inducer and anti-inducer compounds and the capacity to autoregulate *nodD* expression. In addition, class 2 mutants showed an inducer-independent ability to mediate *nodA* expression to 10-fold higher levels over control strains. The class 3 mutant showed reactivity to compounds that had little or no inducing ability with the wild-type *nodD*. An alteration in NodD function was demonstrated with classes 2 and 3 mutants, which showed greatly enhanced ability to complement a Tn*5*-induced mutation in the *nodD*1 gene of strain NGR234 and to restore nodulation ability on the tropical legume siratro. Mutants of *nodD* possessing inducer-independent ability to activate *nod* gene expression (classes 1, 2, and 4) were capable of extending the host range of *R. l.* bv. *trifolii* to the nonlegume *Parasponia*. DNA sequence analysis showed that single base changes were responsible for the altered phenotypic properties of five of six mutants examined. Four of the six mutations affected amino acid residues in a putative receiver domain in the N-terminal end of the *nodD* protein.

Additional keywords: in vivo point mutations, NodD receiver domain, *Rhizobium*-legume symbiosis.

Nodulation (*nod*) genes required for infection of specific legumes are clustered on the native "symbiosis" (Sym) plasmid in several *Rhizobium* species (Schofield *et al.* 1984; Kondorosi *et al.* 1984; Downie *et al.* 1983). In strain ANU843 of *Rhizobium leguminosarum* biovar *trifolii*, which nodulates clover species, expression of the *nodD* gene is constitutive (Innes *et al.* 1985; Djordjevic *et al.* 1987). In several *Rhizobium* species NodD is a regulatory protein that controls *nod* gene expression (Rossen *et al.* 1985; Mulligan and Long 1985; Innes *et al.* 1985; Horvath *et al.* 1987; Spaink *et al.* 1987a) but requires flavonoid inducer molecules for positive regulatory ability (Redmond *et al.* 1986; Firmin *et al.* 1986; Peters *et al.* 1986; Zaat *et al.* 1987).

In some cases, functional homology can be demonstrated between *nodD* genes from different *Rhizobium* species (Djordjevic *et al.* 1985) despite the predicted protein product only showing ~48% amino acid sequence homology with other NodD proteins (Horvath *et al.* 1987). This demonstration of functional homology does not extend to all combinations of *nodD* genes. The NGR234 *nodD* mutant strain ANU1255, which is Nod⁻ on all tested host plants of the parental strain, is complemented for root hair curling and nodule induction (albeit very poorly) by the introduction of the *R. l.* bv. *trifolii nodD* (Djordjevic *et al.* 1985). In contrast, the *nodD*1 genes of *R. l.* bv. *viciae* and *R. meliloti* fail to complement the NGR234 *nodD* mutant (Djordjevic *et al.* 1985; Horvath *et al.* 1987). Complementation ability is, in some cases, thought to be dictated by the ability of the introduced *nodD* product to recognize specific inducer molecules in the exudate of the host plant (Spaink *et al.* 1987b).

The most significant homology between different NodDs occurs in the amino-terminal end, although several areas of homology also occur in parts of the C-terminal end (Horvath *et al.* 1987). NodD also shares significant homology at the N-terminal end with other positive regulatory proteins (e.g., the proteins LysR and AraC of *E. coli*), which also require small inducer molecules for positive regulatory activity (Shearman *et al.* 1986; Appelbaum *et al.* 1988). Recent analysis has shown that NodD has homology to a 100 amino acid "receiver" domain common to regulatory proteins that, together with other proteins, form dyadic relays that are involved in detecting and responding to environmental signals (Kofoid and Parkinson 1988). These dyadic relays are exemplified by two component regulatory systems involving a sensor protein (which contains a transmitter domain) and a regulator protein (which contains a receiver domain) (Ronson *et al.* 1987).

As in other closely related *Rhizobium* species, several *nod* genes of *R. l.* bv. *trifolii* are expressed in high levels only when rhizobia are incubated in the presence of root exudates from legumes (Innes *et al.* 1985; Rossen *et al.* 1985; Mulligan and Long 1985). Active compounds isolated from white clover roots that induce *nod* gene expression are flavones (e.g., 7,4′ dihydroxyflavone [DHF]), which are active at concentrations as low as 50 nM (Redmond *et al.* 1986;

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Djordjevic *et al.* 1987). Isoflavones and coumarins (such as formononetin and umbelliferone) are anti-inducers that antagonize the induction of *nod* genes by inducers (Djordjevic *et al.* 1987). Anti-inducers are present in the root washings of white clover plants in higher concentrations than inducers (Redmond *et al.* 1986). Both inducers and anti-inducers are important intermediates in the phenylpropanoid pathways in legumes (Vickery and Vickery 1981). With the exception of strains NGR234 (Bassam *et al.* 1988) and USDA123 (Kosslak *et al.* 1987), similar compounds have been identified as inducers and anti-inducers in other legume-*Rhizobium* interactions (Peters *et al.* 1986; Firmin *et al.* 1986).

The *nodD* gene itself has been shown to be a key determinant of host specificity in some *Rhizobium*-legume interactions (Spaink *et al.* 1987b; Horvath *et al.* 1987). The strain NGR234 *nodD* gene product is capable of interacting with a broad range of low molecular weight phenolic compounds including flavonoid, isoflavonoid, and coumestan-like molecules (Bassam *et al.* 1988). Transfer of this gene to either *R. l.* bv. *trifolii* or to *R. meliloti* results in the extension of the host range of the recipient strain to include a range of tropical legumes (Horvath *et al.* 1987; Bassam *et al.* 1988), as well as the nonlegume *Parasponia* (Bender *et al.* 1988). By using a chimeric *nodD* fused at a conserved *Bam*HI site, Horvath *et al.* (1987) showed that specificity for inducer molecules secreted by tropical legumes is determined in NGR234 by DNA sequences between the *Bam*HI site and the 3′ end of the *nod*D gene.

In *R. l.* bv. *viciae*, Burn *et al.* (1987) have shown that a point mutation in the 3′ end of *nodD* results in the capacity of inducer-independent expression of *nodA*, which is enhanced by the addition of either inducer of anti-inducer compounds. Other mutant types isolated by Burn *et al.* (1987) were either defective in the ability to autoregulate *nodD* expression and/or to induce *nodA* expression. Recently, Hong *et al.* (1987) and Fisher *et al.* (1988) demonstrated that the NodDs of *R. l.* bv. *viciae* and *R. meliloti* are capable of binding to DNA sequences in the intergenic region of *nodD* and *nodA* in the absence of inducer molecules.

In this paper, we report the mutagenesis of the *nodD* gene of *R. l.* bv. *trifolii* (strain ANU843) with the chemical mutagen nitrosoguanidine (NTG) to determine: if the implied interaction of flavones with NodD could be confirmed by direct mutation of the *nodD* gene; whether the spectrum of inducer compounds normally utilized could be changed by mutation; whether functional changes in the *nodD* product could be induced by mutation; and whether changes in *nodD* can result in mutants that are capable of conferring altered host range ability. We show that single base changes in *nodD* can result in mutant products that are capable of mediating inducer-independent expression of the *nodA* promoter. Two classes of *nodD* mutants possess markedly enhanced capacity to complement mutations in the *nodD*1 gene from strain NGR234 where, normally, inefficient complementation occurs with the introduction of the wild-type *nodD* into this strain. One of these mutants, class 3, shows an enhanced inducing ability with compounds that normally have little or no detectable inducing activity with the wild-type *nodD*. Moreover, all mutants showing an ability to mediate *nod* gene induction in the absence of inducers (classes 1, 2, and 4) are capable of extending the host range of *R. l.* bv. *trifolii* to the nonlegume *Parasponia*.

Part of this work was presented at the Fourth International Symposium on the Molecular Genetics of Plant-Microbe Interactions.

MATERIALS AND METHODS

Strains and media. The *E. coli* strain HB101 carrying plasmid pRt032::*nodA*114 was used in mutagenesis experiments. Plasmid pRt032*nodA*114 carries the nodulation and host range genes of *R. l.* bv. *trifolii* cloned on a 14-kb *Hin*dIII DNA fragment (Schofield *et al.* 1984) and possesses a translation fusion of Mu*d*II 1734 (Castilho *et al.* 1984) inserted into the *nodA* gene (Djordjevic *et al.* 1987). Mu*d*II 1734 carries a kanamycin-resistance (Km^r) marker. The insertion site of the Mu*d*II 1734 in this derivative lies 64 bases from the predicted start codon of *nodA*. Strains ANU843 (wild type), ANU845 (Sym plasmid-cured ANU843), and ANU851 (ANU843 Tn*5*::*nodD*) of *R. l.* bv. *trifolii* were as previously described (Schofield *et al.* 1983). A Nod^- mutant of *Rhizobium* strain NGR234 (ANU1255), which contains a Tn*5* insertion in the *nodD*1 gene, was also used (Djordjevic *et al.* 1985). The *Parasponia Rhizobium* strain CP279 was used as a positive nodulation control on *Parasponia* plants (Bender *et al.* 1987). All media used (BMM, LB, TY, and TMR) have been described elsewhere (Rolfe *et al.* 1980; Innes *et al.* 1985).

Mutagenesis procedure. To minimize possible effects on gene regulation due to increased plasmid copy number, plasmid pRt032::*nodA*114 was chosen for mutagenesis because it maintains a copy number in *Rhizobium* of two to three per cell (as estimated by comparative hydbridization analysis). The mutagenesis procedure followed was essentially the same as that of Miller (1972). Strain HB 101(pRt032::*nodA*114) was grown in 12 ml of LB to stationary phase (10^9 cells per milliliter). NTG was added to 50 μg/ml of final concentration, and cultures were shaken slowly at 30° C. Samples (4 ml) were taken at 15, 30, and 45 min after NTG addition, washed in sterile water, and resuspended in 2 ml of LB. The viable count at this stage was between 2×10^5 and 5×10^6 cells per milliliter. The NTG-treated cells were shaken for 1 hr and then used as donor cells in a conjugal mating with *Rhizobium* strain ANU845. Cells were mated for a period of 3 hr only to minimize the generation of siblings before plating onto selective media.

Selection of *nodD* mutants. Km^r transconjugants were plated en masse onto TMR media containing kanamycin (200 μg/ml) and X-gal (at approximately 30 μg/ml) supplemented either with the inducer 7,4′ DHF (at 10^{-7} M) plus the competitive anti-inducer umbelliferone (at 10^{-5} M) or the poor inducer chrysin (at 1.25×10^{-7} M), or quercetin (at 5×10^{-7} M), a compound normally with no detectable inducing activity. Colonies that were excessively blue (indicative of an alteration in *nodA* gene expression) on plates containing DHF + umbelliferone, quercetin, or chrysin were picked and purified. All flavonoid inducer and anti-inducers were used as previously described (Djordjevic *et al.* 1987).

Measurements of *nodA* expression. Putative mutants were tested in assays that measured *nodA* expression in the presence and absence of a variety, or combination of, inducer and anti-inducer compounds. These were used at a concentration of 1×10^{-6} M, unless otherwise stated. *In vitro*

assays with ONPG as substrate to measure β-galactosidase activity from the *nodA:lacZ* fusion were done as described previously (Djordjevic *et al.* 1987), except that the 42° C incubation was deleted. Inductions of *nodA* were measured after a 1.5-hr exposure of putative *Rhizobium* mutants to inducer, anti-inducer, or a mixture of both compounds.

In vivo indications of *nodA* expression in the presence of clover plants were obtained by incorporating ANU845 cells containing the mutagenized plasmids into soft agar lawns containing X-gal (0.16 mg/ ml^{-1}). Freshly germinated clover plants were placed onto lawns before incubation (Djordjevic *et al.* 1987).

Molecular cloning techniques and DNA sequencing. *Cla*I-generated DNA fragments of 2.6 kb that contained the intact *nodD* genes were cloned into either of the broad host range vectors pLAFR3 (a gift from B. Staskawicz) or pWB5a (a gift of W. Buikema). Both are plasmid derivatives of pRK290 (Tc^r) containing polylinker sequences (see Fig. 1). These derivatives were mobilized into *Rhizobium* by using pRK2013 as a helper plasmid (Ditta *et al.* 1980). Specific DNA fragments were subcloned from the 2.6-kb *Cla*I fragments either into the M13 bacteriophage vectors mp18 and mp19 or into the appropriate Bluescribe or Bluescript vector (Stratagene, San Diego, CA) and were sequenced by using the dideoxy chain-termination method with the Kelnow fragment of DNA polymerase I (Sanger *et al.* 1977) or Sequenase (United States Biochemical Corporation, Cleveland, OH) with supplied sequencing mixes (see Fig. 1).

A 0.7-kb *Bam*HI fragment containing both the *nod*A and the *nod*D promoter was excised from the plasmid pRt032::*nodA*114 and was cloned into the promoter cloning vectors pMP220 (*Inc*P1) and pMP190 (*Inc*Q) (Spaink *et al.* 1987a; see Fig 1). In one orientation the promoterless *lacZ* gene of the vector was fused to the *nodA* promoter and in the other to the *nodD* promoter. All DNA manipulations were done by using standard procedures (Maniatis *et al.* 1982).

Plant tests and ultrastructure studies. *Trifolium repens* (white clover), *T. subterraneum* (subterranean clover), *Pisum sativum* (pea), and *Macroptilium atropurpureum* (siratro) plants were germinated, grown, and inoculated as previously described (Rolfe *et al.* 1980; Chen *et al.* 1985). *Parasponia andersonii* plant assays were carried out as previously described (Bender and Rolfe 1985).

RESULTS

Analysis of mutated pRt032 derivatives with altered expression of *nodA*. Strain HB101 of *E. coli* containing plasmid pRt032::*nodA*114 (Cb^r, Km^r) was mutagenized with NTG, and surviving cells that retained kanamycin resistance were mated en masse to strain ANU845 ($pSym^-$). The resulting transconjugants were plated onto TMR minimal media containing X-gal and either DHF plus an excess of umbelliferone, chrysin, or quercetin. DHF and umbelliferone were used in combination (see Materials and Methods section) to detect mutants that could express the *nodA* gene in the presence of umbelliferone (a potent antagonist of induction by DHF), and this combination of compounds also reduced the background levels of β-galactosidase activity observed in colonies growing on TMR medium. Normally, chrysin has a weak stimulatory ability and quercetin has no detectable stimulatory activity (Djordjevic *et al.* 1987). Because all the mutants picked for further analysis retained β-galactosidase activity, the selection criteria used thus screened for NodD mutants that retained the ability to mediate *nodA* expression.

After the initial selection for mutants with altered levels of β-galactosidase activity on the selection media, 50 potential mutants were detected. Seven mutants (mostly obtained from survivors of the 30- and 45-min NTG treatments) were analyzed in detail, and four classes of potential *nodD* mutants were recognized after examining the ability of the strain ANU845 transconjugants to mediate *nodA*

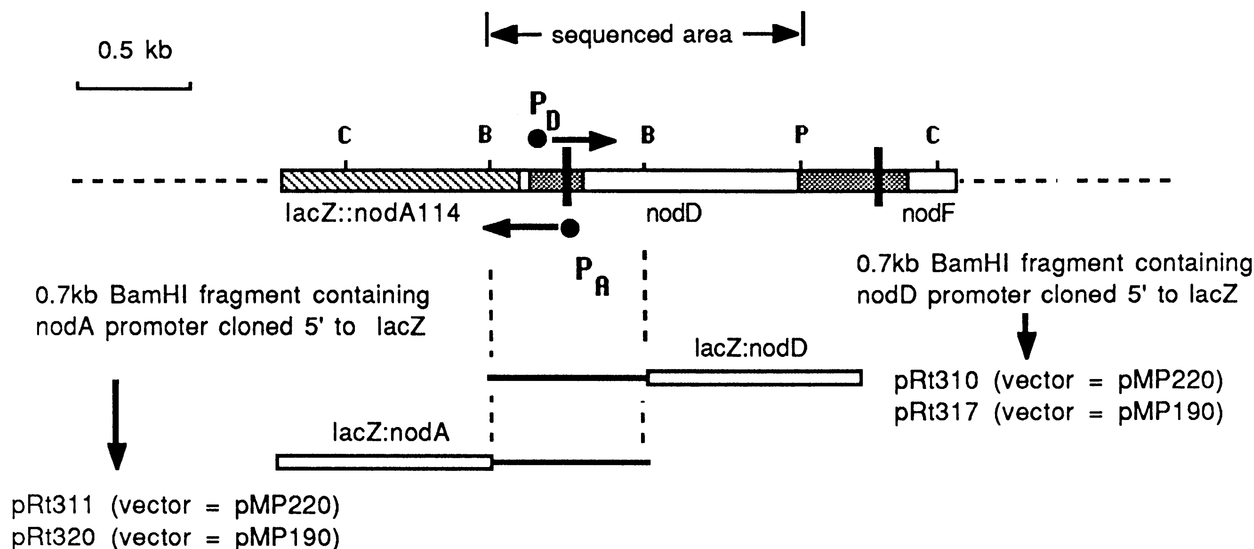

Fig. 1. Cloning and sequencing strategy used. The cross-hatched area represents part of the right-hand side of translational fusion element Mu*d*I11734 that is inserted into the *nodA* gene (*nodA*114). The 0.7-kb *Bam*HI fragment, containing the *nodD*/*nodA* intergenic region and the *nodA* and *nodD* promoters, was cloned in both orientations into pMP220 (*Inc*P1) or pMP190 (*Inc*Q). The 2.6-kb *Cla*I fragment containing either the intact mutagenized or wild-type *nodD* gene only was cloned into either pLAFR3 or pWB5a. The entire *nodD* gene as well as the intergenic regions between *nodA* and *nodD* was sequenced. The shaded areas represent the intergenic regions, and the unshaded regions represent coding sequence of *nodD* and parts of *nodA* and *nodF*. C = *Cla*I, B = *Bam*HI, P = *Pst*I. P_D and P_A represent the *nodD* and *nodA* promoters, respectively. Vertical bars represent the position of nod-box sequences (Schofield and Watson 1986; Rostas *et al.* 1986).

expression (β-galactosidase activity) in the presence and absence of a range of inducer and anti-inducer compounds (Table 1; see below).

Classes 1 and 4 mutants. In the absence of inducer molecules, mutants Au24 and C51 (class 1) and C58 and Q22 (class 4) showed elevated levels of constitutive *nodA* activity (10- to 15-fold above control levels for class 1 mutants and 50-fold over control levels for class 4 mutants). With the exception of the addition to class 1 mutants of umbelliferone, β-galactosidase activity was not significantly enhanced or inhibited by the addition of a wide range of inducer or anti-inducer compounds. In the presence of increasing concentrations of umbelliferone, (normally a potent antagonist of *nodA* induction), both class 1 mutants, Au24 and C51, consistently showed between 150–260% enhanced expression of the *nodA* promoter. Class 4 mutants consistently gave lower β-galactosidase activity (15–30% decrease) in the presence of inducer molecules and a 13–36% increase in the presence of the anti-inducer umbelliferone. The addition of other isoflavonoid anti-inducers did not have the same effect as the addition of umbelliferone.

Class 2 mutants. Mutants Q21 and Au35 were characterized by elevated constitutive expression (10 times background levels in the absence of inducers). In addition, these mutants retained the ability to induce expression of the *nodA* gene after the addition of inducers. No change was noted in the types of compounds shown to have inducing activity. As with the control strain, no *nodA* induction occurred after addition of anti-inducer compounds (Table 1), and sensitivity of *nodA* induction to the addition of increasing concentrations of anti-inducers was evident in competition experiments (data not shown).

Class 3 mutant. Mutant C70 was the only candidate for a mutant with an altered specificity for inducing compounds. The distinguishing feature of this mutant was that it showed significantly enhanced responses to both chrysin (normally a weak inducer) and quercetin (a compound that normally has no effect on activation). Slightly elevated levels of *nodA* expression (two to three times background levels) were also consistently recorded in the absence of inducer molecules.

Table 1. Ability of putative *nodD* mutants to affect *nodA* expression in the presence of inducer and anti-inducer compounds[a]

Compound added (1×10^{-6} M)	Control	Mutant Classes			
		1	2	3	4
None	22	326	218	60	904
DHF	1,432	321	2,181	1,794	651
Umbelliferone	17	566	224	45	1,022
Umbelliferone[b]	20	839	227	51	1,237
Chrysin	376	307	1,777	1,153	783
Daidzein	21	401	249	47	681
Formononetin	20	325	202	126	809
Quercetin	22	334	346	260	858

[a] Results given for mutants Au24 (class 1), Q21 (class 2), C70 (class 3), and Q22 (class 4). The results for mutants in the same class were essentially identical for the compounds used. Prefix Au means that the mutant was selected on media containing both umbelliferone and DHF; Q, mutant selected on plates containing quercetin; C, mutants selected on plates containing chrysin (see Materials and Methods). Colonies of all mutants appeared a more intense blue color than those surrounding them on the selection medium used. The results were averages of two duplicates, and the same trend of results was apparent in repeat experiments. Variation of β-galactosidase levels between duplicate samples was within 5% in all experiments.

[b] Umbelliferone was added at 1×10^{-5} M in this row.

***In vivo* assays.** When strain ANU845 (pRt032::*nodA*114) is incorporated into soft agar overlays containing X-gal and incubated in the presence of clover seedlings, rhizobia are observed to express *nod* genes in a distinct zone near the root tip (Djordjevic *et al.* 1987). This is due to the combined effect of the release of net inducer and anti-inducers from the plant root tissue at distinct locations. To determine if the *in vitro* assays (Table 1) were giving a true indication of the level of *nod* gene expression that would occur in the environment of a clover root, the mutants were incorporated into soft agar overlays and incubated in the presence of freshly germinated seedlings and the chromophoric substrate X-gal. When class 1 mutants were used, a small zone of blue appeared surrounding the upper root and hypercotyl (but not the root tip, as occurs with wild-type bacteria). When classes 2 and 3 mutants were used, the blue zone occurred in the same location as with the wild-type construction, except that the size of the zone was notably increased—this being consistent with the elevation in inducer-independent activity of these mutants. The class 4 mutants gave a blue coloration throughout the soft agar overlay that was less intense around the plant (data not shown).

Phenotypic and molecular analysis of *nodD* mutants. To confirm that a mutation in the *nodD* gene was responsible for the altered phenotypes seen in the mutants, each *nodD*, including the wild type, was excised from the plasmid pRt032::*nodA*114 derivatives by using the restriction enzyme *Cla*I. These 2.6-kb *Cla*I fragments (see Fig. 1), which contained the intact *nodD* gene only, were cloned into pRK290 derivatives. Each recombinant plasmid was introduced into strains ANU851 (*nodD*::Tn*5*) or ANU1255 ($nodD_{NGR234}$::Tn*5*) for complementation analysis and to determine if altered specificity to inducer molecules resulted in the ability of the transconjugants to nodulate nonhost plants. Transconjugants were inoculated onto clover, pea, siratro, and *Parasponia* plants.

The introduction into *R. l.* bv. *trifolii* strain ANU851 of the Tc^r plasmids carrying mutated *nodD* genes resulted in strains that were able to nodulate clovers, although at varying efficiencies. Nodules were not induced by the strains on other nonhost legumes such as pea or siratro. In contrast, some mutants were able to initiate the earliest stages of nodule induction in the nonlegume *Parasponia* (Fig. 2). All mutant *nodD* genes that were able to mediate *nodA* expression in the absence of inducer molecules (classes 1, 2, and 4) were capable of initiating small nodules on *Parasponia* that, in most cases, were devoid of bacteria (Fig. 2, a–c). In two separate experiments (10 plants inoculated per strain), 63–100% of *Parasponia* plants were nodulated. For example, compared with the *Parasponia Rhizobium* strain CP 279, which induced 2.5 nodules per plant, an average of 5.3, 3, and 7 nodules per *Parasponia* plant were induced by strain ANU851 carrying class 1 (Au24), class 2 (Q21), or class 4 (C58) mutant *nodD* genes, respectively. In contrast to other hybrid strains tested, strain ANU851 carrying the C58 (class 4) *nodD* continued nodule development, initiating small, branched structures that notably contained a defined meristem as well as intracellular bacteria (Fig. 2, d–f). Strain ANU851, carrying either the wild-type *nodD* gene or the class 3 *nodD* mutant, was unable to initiate nodules on *Parasponia* plants. Similar

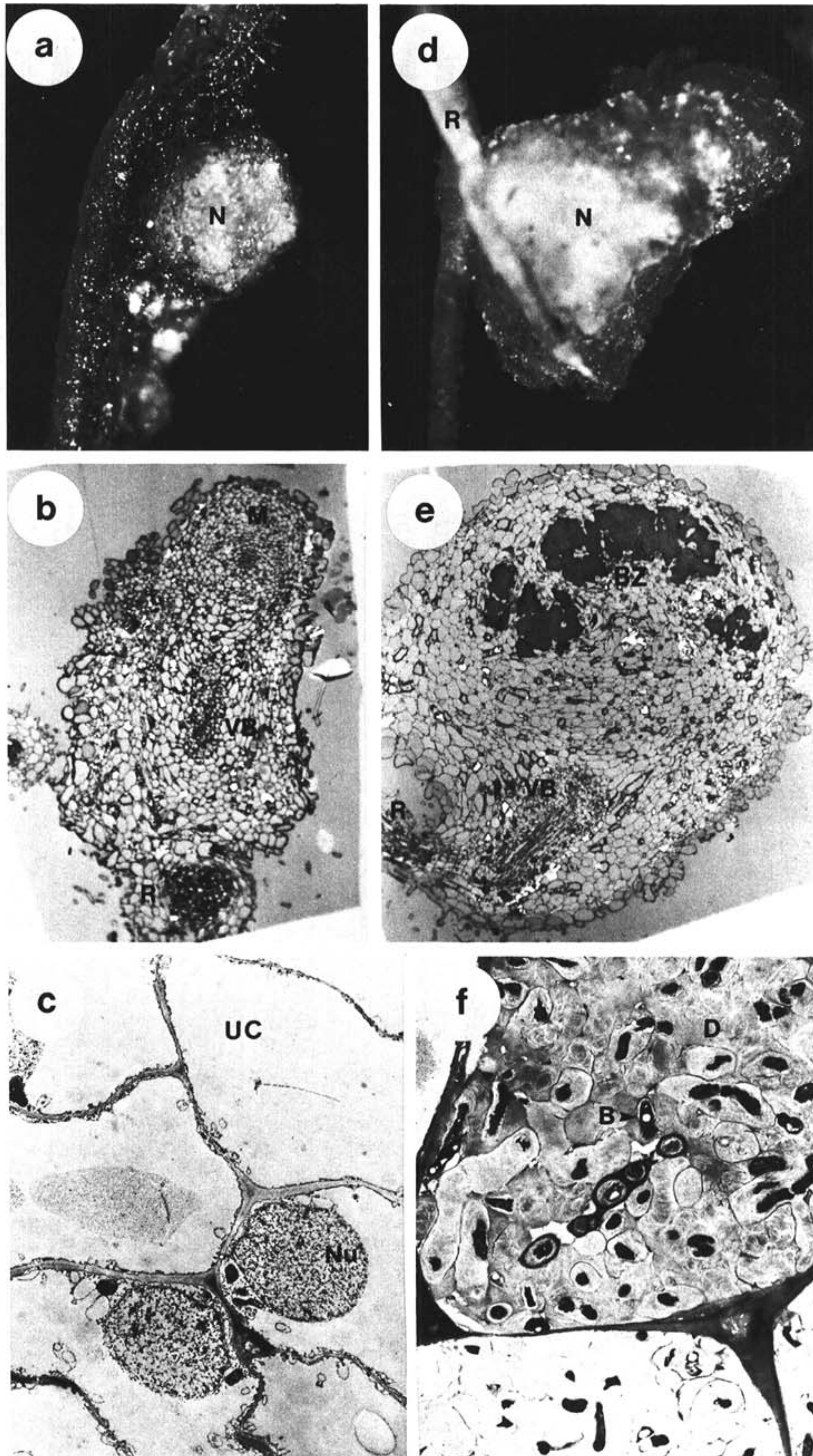

Fig. 2. Response of *Parasponia* to inoculation with *R. l.* bv. *trifolii* strain ANU851 containing mutated *nodD* genes. **A,** *Parasponia* root nodule (N) induced by strain ANU851 (*nodD*::Tn*5*) carrying the Au24 *nodD* (R = root × 18). **B,** Light micrograph section of a nodule formed by the same strain. Note the presence of a meristem (M) and vascular bundle (VB). No infection zone was observed (×40). **C,** Transmission electron micrograph of the uninfected cells (UC). Nu = nucleus (×11,000). **D–F,** *Parasponia* nodules induced by strain ANU851 carrying the C58 *nodD*. **D,** Branched nodule on a *Parasponia* root (R) (×16); **E,** Light micrograph of the same nodule. Note the darkly stained zone of bacterial infection (BZ) (×40). **F,** Transmission electron micrograph of a swollen host cell in the infection zone showing bacteria (B) and the exclusion of the host cytoplasm by an intracellular deposit (D) (×15,000).

results were obtained if strain ANU851 carrying the originally mutated pRt032*nodA*114 derivatives were used instead of the corresponding strains carrying the cloned *nodD* gene alone. Bacteria possessing the antibiotic resistance markers of the inoculum strains were recovered from the *Parasponia* nodules induced by ANU851 carrying the C58 *nodD* gene, but no viable bacteria could be recovered from plants inoculated with other hybrid strains.

When inoculated onto clover plants, strain ANU851 carrying class 1 and class 4 mutant *nodD* genes induced root nodules on some plants, but these were delayed 1–2 days. Between 40–75% of plants had no obvious signs of nodulation. With the introduction of classes 2 and 3 mutant *nodD* genes into ANU851, the nodulation response was more akin to that achieved when the wild-type *nodD* gene was used, except that 20% of the plants remained without nodules after exposure of plants to strains carrying the class 2 *nodD*.

To test for the occurrence of recombination events that might explain the variability in the complementation results on clover plants, analysis of bacteria obtained from nodules showed that: 100% of isolates retained the Km^r (Tn*5*) marker; only 50–70% of isolates retained the Tc^r marker of the introduced plasmid (indicating that some plasmid instability was apparent); and transformation of *E. coli* with plasmids from lysates of the Tc^r nodule bacteria resulted only in Tc^r (but not Km^r) transformants. Therefore, no evidence for double reciprocal recombination was found because double reciprocal crossover events would result in strains that contained both Km^r and Tc^r markers on the introduced plasmid. Nevertheless, some single crossover events would integrate the introduced plasmid into the genome to restore a functional *nodD*. Such events may account for the results seen.

Complementation of the NGR234 *nodD*. Because the $nodD_{NGR234}$ interacts with a different but overlapping spectrum of inducer compounds (Bassam *et al.* 1988) and is usually not complemented by *nodD* genes from several fast-growing *Rhizobium* strains (Djordjevic *et al.* 1985; Horvath *et al.* 1987), a potential functional change in NodD can be detected by enhanced complementation of the $nodD_{NGR234}$ mutant (ANU1255). The recombinant plasmids, containing only the mutant ANU843 *nodD* genes or the corresponding wild-type *nodD* gene, were introduced into strain ANU1255 and the transconjugants were inoculated onto siratro. The class 2 (Q21 and Au35) and class 3 (C70) *nodD* fragments were able to complement the $nodD_{NGR234}$ mutation efficiently, whereas the introduction of other mutant *nodD* fragments gave the same (poor) response as that of the wild-type *nodD* gene (only occasional nodules resulted after examination of more than 20 plants). Five to 10 nodules per plant resulted when the class 2 *nodD* genes were introduced, and three to five nodules per plant resulted when the class 3 *nodD* was introduced. The ability of classes 2 and 3 mutants to complement the $nodD_{NGR234}$ mutation was similar to the complementation result achieved when the homologous $nodD_{NGR234}$ gene was introduced into strain ANU1255. Further evidence of the efficiency of complementation was indicated by the formation of nitrogen-fixing nodules on the majority of siratro plants (as judged by acetylene reduction assays and the difference in plant growth over uninoculated and nonnodulated controls 5 wk postinoculation).

The wild-type *nodD* gene suppresses constitutive activity of several mutants. To test for the ability of the mutant NodD derivatives to compete with the wild-type NodD for potential target sites on the DNA, the original mutagenized pRt032::*nodA*114 plasmids were transferred to strains ANU843 ($nodD^+$), ANU845 ($pSym^-$), and ANU851 (*nodD*::Tn*5*). The levels of β-galactosidase activity obtained was determined in the presence and absence of DHF. The results (Table 2) show that in the absence of inducer molecules, the presence of a wild-type *nodD* gene suppresses the flavone-independent *nodA* expression of classes 1, 2, and 4 mutants to 20–24% of the activity in the absence of a wild-type *nodD* gene. Therefore, the uninduced wild-type *nodD* is dominant to the mutant *nodD*, despite being present at a lower copy number, and reduces *nodA* transcription. In contrast, in the presence of the inducer DHF, the activity of the *nodA* gene was less than that obtained when the wild-type *nodD* gene was absent for classes 3 and 4 but was enhanced for class 1 mutants. The presence of either the class 3 or 4 *nodD* together with the wild-type *nodD* leads to antagonistic effects on the expression of *nodA* in the presence of inducers. When several other inducer compounds were used instead of DHF (e.g., apigenin or luteolin), the same trend of results was obtained with all classes of *nodD* mutants (data not shown).

The ability to regulate *nodD* expression is impaired in some *nodD* mutants. The ability of NodD to "autoregulate" *nodD* expression occurs in *R. l.* bv. *viciae* (Rossen *et al.* 1985) but not in *R. meliloti* (Mulligan and Long 1985) or in NGR234 (Bassam *et al.* 1988). To test for the ability of the wild-type and mutant NodD derivatives to autoregulate *nodD*, plasmid pRt317 was used. Plasmid pRt317 contains a promoterless *lacZ* gene fused to the *nodD* promoter of strain ANU843 (Fig. 1). Expression from the promoter was shown to be regulated by the presence of a wild-type copy of the *nodD* gene by comparing levels of β-galactosidase activity obtained in strains ANU843 ($nodD^+$) to those obtained in strains ANU851 (*nodD*::Tn*5*) and ANU845 ($pSym^-$) (Fig. 3).

As an additional test, the plasmids carrying the cloned 2.6-kb *Cla*I fragments spanning either the wild-type *nodD* gene or the mutated *nodD* genes were introduced into strain ANU845(pRt317) to determine if the presence of these *nodD* genes enabled autoregulation of the expression of the *nodD* gene. Both the wild-type *nodD* gene as well as mutants C70 (class 3) and Q21 (class 2) were able to repress *nodD* expression, whereas mutants C51 and Au24 (class 1) and C58 and Q22 (class 4) were unable to do so. The addition of DHF to the bacteria at a concentration sufficient

Table 2. Induction of *nodA* (from mutagenized pRt032::*nodA*114 derivatives) by DHF in the presence and absence of *nodD*

	β-Galactosidase activity			
	Strain ANU845[a]		Strain ANU843	
	−DHF	+DHF	−DHF	+DHF
Control	30	1,235	30	967
Mutant				
Class 1	413	340	35	903
Class 2	218	1,561	36	949
Class 3	72	1,429	32	741
Class 4	1,085	837	260	383

[a] Similar results were obtained when plasmids containing putative *nodD* mutations were introduced into strain ANU851(*nodD*::Tn*5*). The results obtained were consistent in three separate experiments, and the variation in β-galactosidase levels between duplicates was within 5%.

to induce *nodA* expression had no effect of the levels of *nodD* expression obtained.

Determination of the mutation site by DNA sequence analysis. Both wild-type and mutant *nodD* genes from class 1 (Au24, C51), class 2 (Q21), class 3 (C70), and class 4 (Q22 and C58) mutants were subjected to DNA sequence analysis. Each *nodD* was completely sequenced (Fig. 1), and single mutations were found in five of six mutants examined (Fig. 4). Consistent with the phenotypic similarity of class 1 mutants, a single base substitution had occurred in each of these mutants that affected the same (predicted) *nodD* codon. Two alterations were found in the class 4 mutant C58 (Fig. 4). With all mutants, no change was noted in the *nodD*/*nodA* intergenic region that contains the *nodA* and *nodD* promoters. Computer-aided analysis of the effects of the changes in the predicted structure by using Chou-Fasman (1978) and hydrophobicity analysis (Kyte and Doolittle 1982) showed no significant effect of the mutation on the predicted protein secondary structure (data not presented). The two class 1 mutants (Au24 and C51), the class 3 mutant (C70), and one of the class 4 mutants (Q22) were located in *nodD* clustered around the *Bam*HI site. This area of NodD has recently been reported to be homologous to a postulated receiver protein consensus domain (Kofoid and Parkinson 1988). This homology is indicated in Figure 4. One of the two substitutions in the class 4 mutant (C58a) occurs immediately after this region of homology. The site of the other two amino acid substitutions (the class 2, Q21, and the second alteration to C58) occur, respectively, immediately before, and in, a short domain that computer analysis (Chou and Fasman 1978) predicts to contain two strong β-turn tetrapeptides. The β-turn tetrapeptides -NSDP- (P_T score $= 2.72 \times 10^{-4}$) and the overlapping -DPGN- (P_T score $= 7.6 \times 10^{-4}$) are functionally strongly conserved among the predicted sequences of NodD for *R. meliloti* (Egelhoff *et al.* 1985), different biovars of *R. leguminosarum* (Schofield and Watson 1986; Shearman *et al.* 1986), and in strain MPIK3030 (Horvath *et al.* 1987).

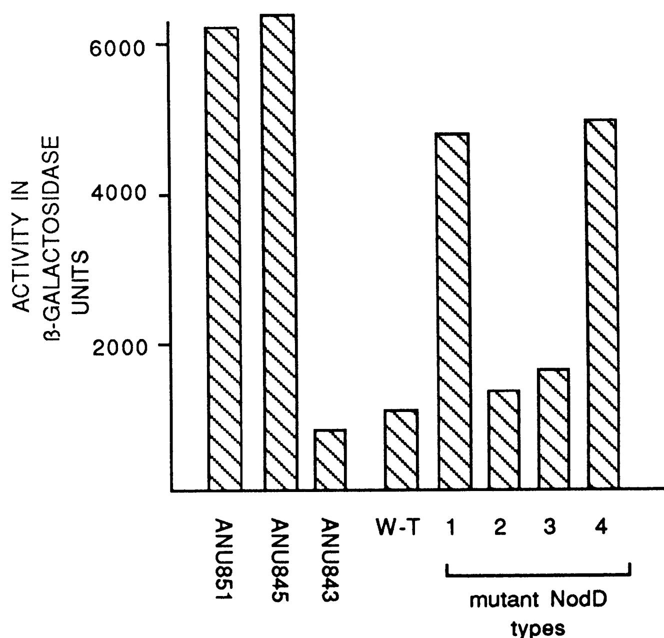

Fig. 3. Autoregulation ability of wild-type and mutant *nodD* genes. The level of β-galactosidase activity from plasmid pRt317 (*nodD* expression) was assessed in different backgrounds of *R. l.* bv. *trifolii* and was shown to be repressed by the presence of a wild-type *nodD* copy on the pSym. The introduction of the various *nodD* genes (on *Inc*P1 plasmids) into strain ANU845 (pRt317) showed that the presence of the wild-type or class 2 or 3 *nodD* genes also repressed expression from the *nodD* promoter. Classes 1 and 4 *nodD* mutants showed a loss of autoregulation ability. Values obtained for β-galactosidase activity in duplicates were within 10%, and the experiment was repeated three times.

DISCUSSION

This paper discusses our successful use of the chemical mutagen NTG to mutagenize the *nodD* gene of strain ANU843 of *R. l.* bv. *trifolii* and identify several regions of the predicted protein structure that affect normal function. Single base substitutions were found in five of six mutants sequenced, and the location of these mutations identify regions in NodD that affect inducer and anti-inducer recognition, autoregulation capacity, and the ability to mediate *nodA* expression. The selection procedure used was successful in isolating mutants (classes 2 and 3) with either altered affinity toward inducer molecules or altered regulatory properties that, in addition, retained several properties of the original NodD. Two classes of mutants (classes 1 and 4) were also isolated that possessed inducer-independent capacity to mediate *nodA* expression. The phenotypes of classes 1, 3, and 4 are novel and differ from the mutants isolated in *R. l.* bv. *viciae* (Burn *et al.* 1987). Class 2 mutants have similar properties to the class IV mutants isolated by Burn *et al.* (1987), except that the class 2 mutants isolated here retain their sensitivity to anti-inducer molecules such as formononetin and umbelliferone (Table 1; Djordjevic *et al.* 1987). Consistent with the similarity in phenotype of the classes 2 and IV mutant *nodD* genes in

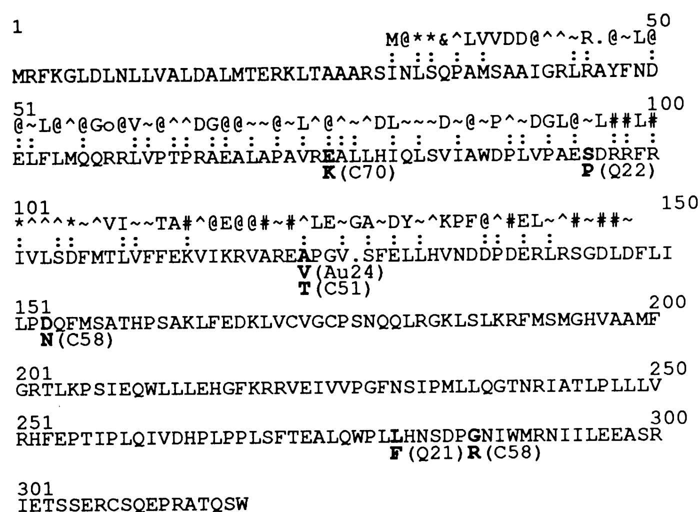

Fig. 4. Translated protein sequence of the *R. l.* bv. *trifolii* strain ANU843 *nodD* gene. The positions of the substitutions arising from the mutagenesis are indicated by bold type with the substituted amino acid given below. The mutations arise from the following triplet alterations: C70 GAG (Glu) to AAG (Lys); Q22 TCT (Ser) to CCT (Pro); Au24 GCA (Ala) to GTA (Val); C51 GCA (Ala) to ACA (Thr); C58a GAT (Asp) to AAT (Asn); Q21 CTT (Leu) to TTT (Phe); C58b GGG (Gly) to AGG (Arg). The protein receiver domain consensus (Kofoid and Parkinson 1988) is aligned against the ANU843 NodD sequence. Functional groups are indicated: @, acidic and related (DENQ); #, basic (KRH); ^, polar (STPAG), ~, nonpolar (VLIM); o, aromatic (FYW); *, anything. Alignment between functionally conserved residues of the ANU843 NodD and the receiver consensus is indicated by (:).

these two biovars, the mutation sites are both located in the 3′ end of *nodD* at (predicted) codons 280 and 283, respectively (Burn *et al.* 1987).

All mutants that were capable of mediating significant levels of inducer-independent *nodA* expression were capable of extending the host range of *R. l.* bv. *trifolii* to the nonlegume plant *Parasponia* but not to other legume species tested (pea and siratro). This result indicates that the continual expression of the *nod* genes is sufficient to establish at least the early steps of nodule formation on *Parasponia*, regardless of the presence of inappropriate host-specificity genes in the genetic background of the strain. There was a correlation between the level of inducer-independent expression mediated by the mutant NodD and the extent of nodulation achieved on *Parasponia* plants, with the nodules induced by strains containing one of the class 4 NodDs being more extensively developed. This suggests that the failure of any *Rhizobium* strain to nodulate *Parasponia* may simply reflect the inability to initiate sufficient sustained *nod* gene expression in the root environment and indicate that high concentrations of anti-inducer compounds are normally secreted by this plant. The failure of strains containing the mutated *nodD* genes to nodulate other nonhost legume species probably reflects a requirement both for precise *nod* gene regulation as well as the absence of the appropriate host-specificity genes.

Of the four distinct classes of *nodD* mutants isolated, classes 1 and 4 mutants had the most severe mutations in terms of the effect on normal NodD function. These data suggest that the classes 1 and 4 mutant NodDs have undergone changes in their tertiary structure that render the active sites on the proteins insensitive to the direct or indirect effects of inducer and anti-inducer molecules and that affect the ability to associate with the autoregulatory site on the DNA molecule. The ability of classes 1 and 4 mutants to mediate expression of *nodA* in the absence of inducer molecules despite their inability to autoregulate *nodD* expression indicates separate sites for these functions and may suggest that at least two DNA binding sites for NodD occur in the *nodD*/*nodA* intergenic region: one that allows regulation of the expression of *nodD* and the other that permits NodD to facilitate expression of *nodA*. If these two sites are physically separable, this raises the possibility of the autoregulatory site being used elsewhere on the genome as a NodD-dependent regulatory site.

There is a similarity in phenotype of the class 1 mutants and class 4 mutants despite the mutation sites occurring at different locations. Similarly, the mutation sites in the class 4 mutants Q22 and C58 (which has two mutations) occur in different locations yet result in similar phenotypic effects. The site defined by the class 1 mutation sites must be essential for normal NodD function particularly because: the alteration in mutant Au24 results in the replacement of alanine by threonine (an amino acid in the same functional group [Dayhoff, 1978]); the DNA and amino acid sequence surrounding the mutation site is highly conserved in several NodD molecules (Horvath *et al.* 1987); and the Chou and Fasman analysis (1978) predicts no significant changes in the secondary structure of the mutant proteins compared with that of the wild type. It is possible that the classes 1 and 4 mutants are "locked" into weak and strong positive regulatory forms of NodD, respectively, and that the locations of these mutations defines two distant parts of the NodD protein that interact to form a single functional domain, or that interact to enable normal functioning to occur. This concept is supported by the strong β-turn forming potential of the tetrapeptide -DPGN- in which the C58b mutation occurs. The substitution resulting from the C58b mutation (resulting in -DPRN-) maintains the β-turn potential of this region. This structural feature would make this region sterically available for ligand formation or interactions with another domain of the protein.

The other classes of mutants recognized (classes 2 and 3) retain features that are similar to that of the wild-type NodD, including responsiveness to inducers and anti-inducers and the ability to regulate the expression of the *nodD* gene. The mutation site in the class 2 mutant Q21 occurs in the C-terminal end of NodD near the strong β-turn motif and results in the ability to mediate constitutive *nodA* expression in the absence of inducer molecules. In contrast, the class 3 mutant (C70) was capable of positively regulating *nodA* expression in the presence of compounds (chrysin and quercetin) that have poor inducing ability with the wild-type NodD. Despite the differences in phenotype of classes 2 and 3 *nodD* mutants and the location of the mutation sites, they were able to efficiently complement a Tn*5*-induced mutation in the *nodD* gene of strain NGR234. The NGR234 *nodD* gene is able to complement mutations in *nodD* genes from several fast-growing *Rhizobium* strains, confer extended host range ability, and in addition, impart the ability to recognize the *nodA* promoter of strain ANU843 in the presence of a wide range of inducer molecules (Bassam *et al.* 1988; Horvath *et al.* 1987). If the ability to recognize a broad range of phenolic compounds is requisite for broad host range ability, it is not surprising that mutations in $nodD_{NGR234}$ are either not complemented or inefficiently complemented by *nodD* genes from several *Rhizobium* species with narrow host ranges (Djordjevic *et al.* 1985), which have been shown to have a more stringent, narrow requirement for inducer molecules (Djordjevic *et al.* 1987; Spaink *et al.* 1987b) than strain NRG234 (Bassam *et al.* 1988). The ability of class 3 (C70) mutant to recognize different inducer molecules is evidence for an alteration in the shape of the putative active site on NodD for direct inducer recognition; however, no evidence for a direct interaction of NodD with inducer has been demonstrated. The alteration in the recognition of inducer molecules by the class 3 mutant may be important in the ability to complement the $nodD_{NGR234}$ mutation.

In experiments where both a mutant and a wild-type *nodD* were present in the same cell (Table 2), it was shown that the wild-type NodD protein could suppress the inducer-independent capacity to activate *nodA* expression by all of the mutant NodDs despite the mutant *nodDs* being present in a higher copy number. This is evidence for the capacity of the wild-type NodD, in the absence of inducers, to bind to the DNA (presumably at a site in the *nodA*/*nodD* intergenic region) and act as a repressor of *nodA* transcription. This may reflect a greater efficiency of the wild-type NodD to compete for this target site. In the presence of inducer molecules, class 1 mutants were shown to be recessive to the wild-type *nodD* as these mutant proteins were unable to interfere with the ability of the wild-type NodD to mediate the expression of the *nodA* gene. In contrast, when the class 3 or 4 and wild-type *nodD*s were present in the same cell, neither *nodD* type dominated and less *nodA* expression

resulted than would have occurred in the absence of the "competing" *nodD*. If NodD is a multimeric protein, the ability of the mutant and wild-type subunits to aggregate and form a heterologous combination of NodD subunits could explain this result. Monomeric NodD could autoregulate *nodD* expression and shut down *nodA* transcription. In the presence of inducer, NodD may become multimeric and aid in the initiation of transcription of *nodA*. Heterologous NodDs (consisting of mutant and wild-type monomers) may have antagonistic effects on *nodA* transcription initiation, whereas NodDs capable of inducer-independent transcription initiation of *nodA* may be locked into the positive regulatory (multimeric) form. This hypothesis is supported by the proposals of Kofoid and Parkinson (1988).

Four of six mutations examined affected amino acid residues in a putative receiver domain of NodD identified by Kofoid and Parkinson (1988) (see Fig. 4). Although this identification of a putative receiver domain in NodD is only based on sequence homology, it may serve as a useful model in the elucidation of the mechanism of inducer-NodD interactions. Further, the absence of a transmitter protein (to complete the dyadic relay common to two-component regulatory systems) suggests that the mode of interaction of inducer and NodD could be analogous to that of transmitter:receiver relays interactions (Kofoid and Parkinson 1988).

The results presented here indicate that NodD is the direct target for the binding of inducer and anti-inducer molecules. This is clearly supported by the phenotypic properties of the class 3 (C70) mutation that results in the alteration of the spectrum of phenolic compounds that induce *nodA* transcription. It is clear that mutation of *nodD* can result in altered inducer recognition, affects the regulation of both *nodD* and *nodA* expression, and influences host range. It is also apparent that mutations located closer to the 5′ end of *nodD* as well as the 3′ end affect NodD function. One of the first steps in the interaction of rhizobia with a potential host is the recognition of the particular set of inducer molecules secreted by the roots. Therefore, we support the conclusion of Spaink *et al.* (1987) that *nodD* is a key regulatory gene in the formation of nodules on legume plants. The isolated *nodD* mutants discussed in this paper will be useful tools for *in vitro* assays to determine the precise target for NodD binding to *nod* regulatory sequences and to determine the putative binding domain for inducers to NodD.

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