Competitive Nodulation Blocking of cv. Afghanistan Pea Is Related to High Levels of Nodulation Factors Made by Some Strains of *Rhizobium leguminosarum* bv. *viciae*

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Cultivar Afghanistan peas are resistant to nodulation by many strains of *Rhizobium leguminosarum* bv. *viciae* but are nodulated by strain TOM, which carries the host specificity gene *nodX*. Some strains that lack *nodX* can inhibit nodulation of cv. Afghanistan by strain TOM. We present evidence that this “competitive nodulation-blocking” (Cnb) phenotype may result from high levels of Nod factors inhibiting nodulation of cv. Afghanistan peas. The TOM *nod* gene region (including *nodX*) is cloned on pIJ1095, and strains (including TOM itself) carrying pIJ1095 nodulate cv. Afghanistan peas very poorly but can nodulate other varieties normally. The presence of pIJ1095, which causes increased levels of Nod factor production, correlates with Cnb. Nodulation of cv. Afghanistan by TOM is also inhibited by a cloned *nodD* gene that increases *nod* gene expression and Nod factor production. Nodulation of cv. Afghanistan can be stimulated if *nodD* on pIJ1095 is mutated, thus severely reducing the level of Nod factor produced. Repression of *nod* gene expression by *nolR* eliminates the Cnb phenotype and can stimulate nodulation of cv. Afghanistan. Addition of Nod factors to cv. Afghanistan roots strongly inhibits nodulation. The Cnb⁺ strains and added Nod factors inhibit infection thread initiation by strain TOM. The *sym2^A* allele determines resistance of cv. Afghanistan to nodulation by strains of *R. leguminosarum* bv. *viciae* lacking *nodX*. We tested whether *sym2^A* is involved in Cnb by using a pea line carrying the *sym2^A* region introgressed from cv. Afghanistan; nodulation in the introgressed line was inhibited by Cnb⁺ strains. Therefore, the *sym2^A* region has an effect on Cnb, although another locus (or loci) may contribute to the stronger Cnb seen in cv. Afghanistan.

Additional keywords: lipo-chitin-oligosaccharide, symbiosis.

Specificity in the symbiosis between leguminous plants and bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium*, and *Mesorhizobium* (collectively known as rhizobia) is determined principally by various substituent groups on the lipo-chitin-oligosaccharide nodulation factors. These are synthesised by the products of the various nodulation (*nod*, *nol*, and *noe*) genes (Downie 1998). In addition to these genes, which specify which genera of plants can be nodulated, some *nod* genes can affect the nodulation characteris-

tics of different cultivars within a single genus. Such genotype-specific nodulation loci have been identified in several bacteria including *Rhizobium leguminosarum* bv. *viciae* (Davis et al. 1988), *Bradyrhizobium japonicum* (Sadowsky et al. 1991), *Sinorhizobium fredii* (Krishnan and Pueppke 1991, Meinhardt et al. 1993) and *R. leguminosarum* bv. *trifolii* (Lewis-Henderson and Djordjevic 1991a, 1991b). The *nodX* gene present in some native isolates of *R. leguminosarum* bv. *viciae* enables these isolates to nodulate cv. Afghanistan peas (Davis et al. 1988), and the *nodX* gene was originally isolated from strain TOM, which can nodulate cv. Afghanistan (Götz et al. 1985).

Spaink and associates (1991) demonstrated that one strain of *R. leguminosarum* bv. *viciae* makes four lipo-chitin-oligosaccharide Nod factors corresponding to oligomers of four or five β -1-4-linked *N*-acetyl glucosaminyl residues in which the terminal nonreducing residue carries an O-linked acetyl group and either a C18:1 or C18:4 N-linked fatty acyl group. Firmin and associates (1993) demonstrated that *nodX* from strain TOM determines acetylation of C6 of the terminal-reducing glucosamine residue of the pentameric Nod factors, resulting in the production of two additional Nod factors. Subsequently, Ovtsyna and associates (1998) demonstrated that fucosylation (by NodZ) of C6 of the terminal-reducing glucosamine could also overcome the nodulation resistance of cv. Afghanistan.

The nodulation resistance characteristic segregates as a single locus when cv. Afghanistan is crossed with cultivated lines that show no resistance (Holl 1975; Lie 1984). The resistance locus has come to be referred to as *sym2^A* (the ‘A’ referring to cv. Afghanistan), and the corresponding allele from cultivated peas has been called *sym2^C* (Geurts et al. 1997). A similar resistance was found in Iran peas and found to be allelic with *sym2^A*. When the *sym2* alleles were introgressed into a cultivated pea line, the introgressed lines were found to have the same nodulation specificity as cv. Afghanistan and cv. Iran (Kozik et al. 1995). These lines were used to precisely map *sym2* onto the pea genetic map on linkage group 1 (Kozik et al. 1996).

Another unusual characteristic of cv. Afghanistan peas is that their nodulation by strains such as TOM can be significantly inhibited by certain strains of *R. leguminosarum* bv. *viciae* that cannot induce nodule formation (Winarno and Lie 1979). This effect was called “competitive nodulation blocking” (Cnb) (Dowling et al. 1989). Since mutations affecting genes required for the biosynthesis and decoration of Nod factors abolished the Cnb effect (Dowling et al. 1989; Firmin et al. 1993), it is evident that Nod factors are involved in the nodulation blocking. Mutations resulting in the production of only C18:1-substituted Nod factors (*nodE* mutant) or Nod factors lacking the acetyl group on the terminal-nonreducing glyco-

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saminyl residue (*nodL* mutant) abolished the Cnb effect (Dowling et al. 1989; Firmin et al. 1993), even though these mutations did not block nodulation on pea varieties other than cv. Afghanistan (Downie et al. 1985; Surin and Downie 1988).

A simple model for Cnb might have been that Nod factors lacking the NodX-dependent *O*-acetylation could competitively block access of the cognate (NodX acetylated) Nod factors to some kind of Nod factor receptor. However, the observation that strain TOM makes a mixture of Nod factors containing the *nodX*-dependent acetyl group (Firmin et al. 1993) indicates that such a model is unlikely since, in effect, strain TOM might tend to inhibit its own nodulation.

In addition to the *nodX*-dependent qualitative change to Nod factors, we noticed that there was a quantitative difference in the amounts of Nod factors made by those strains that could nodulate cv. Afghanistan and those that blocked nodulation. A Cnb⁺ strain produced much more Nod factor than strain TOM. This difference in the amount of Nod factors made was ascribed to a relatively low level of *nod* gene induction in strain TOM (Firmin et al. 1993; Kiss et al. 1998). Strain TOM expresses the *nodR* gene, which encodes a repressor of *nod* gene expression (Kiss et al. 1998). This may account, at least in part, for the lower levels of Nod factor formation in strain TOM.

In this study, we have analyzed Cnb and have found indications that the relatively high levels of Nod factors made by some strains of *R. leguminosarum* bv. *viciae* account for the nodulation blocking.

RESULTS

Cloned *nod* genes from strain TOM suppress nodulation and induce Cnb on cv. Afghanistan.

Plasmid pIJ1095 carries the *nod* gene region from *R. leguminosarum* bv. *viciae* strain TOM cloned in the cosmid vector pLAFR1 (Hombrecher et al. 1984). As reported previously (Götz et al. 1985), *nodX* subcloned from pIJ1095 into pIJ1357 enables strain A34 to form nodules on cv. Afghanistan, albeit at a somewhat lower level than strain TOM (Table 1). Furthermore, pIJ1095 can enable a derivative of *R. leguminosarum* cured of its symbiotic plasmid (8401) to form many nodules on the commercial pea variety Wisconsin Perfection (Table 1). The level of nodulation is comparable to that seen with the wild-type strains A34 and TOM, and similar high levels of nodulation were seen with other pea varieties such as Frisson and Rondo (data not shown).

However, when 8401 carrying pIJ1095 was tested on cv. Afghanistan peas, very poor nodulation was seen (Table 1). This low level of nodulation is a property of pIJ1095, as its transfer to strain TOM severely inhibited nodulation of cv. Afghanistan peas (Table 1). Strain TOM nodulates Wisconsin Perfection very well, and the presence of pIJ1095 resulted in only a small reduction in nodule number (Table 1).

We considered the possibility that the suppression of cv. Afghanistan nodulation by pIJ1095 might be related to the ability of some Cnb⁺ strains of *R. leguminosarum* bv. *viciae* to inhibit nodulation of cv. Afghanistan peas by strain TOM (Dowling et al. 1989; Firmin et al. 1993). This was tested with a Cnb assay in which strain 8401/pIJ1095 was inoculated onto cv. Afghanistan peas, which were subsequently inoculated with strain TOM. As shown (Fig. 1), 8401/pIJ1095 almost completely blocked nodulation by strain TOM. A similar result was seen with TOM carrying pIJ1095 (Fig. 1). This strong nodulation-blocking effect is not specific to pIJ1095, as the cloned *nod* gene region (on pIJ1089) from *R. leguminosarum* bv. *viciae* strain 248 also conferred a strong Cnb⁺ phenotype on 8401 and strain TOM (data not shown).

Mutation of *nodD* in pIJ1095 enhances nodulation on cv. Afghanistan.

Tn5-mutant derivatives of pIJ1095 were screened to identify mutations that allowed increased nodulation of cv. Afghanistan pea. No mutated derivatives that resulted in high levels of nodules were identified, but one (pIJ7244) was found that had higher levels of nodulation (Table 1). In contrast, the mutation in pIJ7244 strongly reduced nodulation of Wisconsin Perfection (Table 1). When the mutated derivative of pIJ1095 was transferred to TOM, normal nodulation of cv. Afghanistan was seen (Table 1). Strain 8401/pIJ7244 was Cnb⁻, demonstrating that the mutation abolished the Cnb⁺ phenotype conferred by pIJ1095 (Fig. 1).

To identify the gene affected, the site of Tn5 insertion was mapped with restriction endonucleases. Part of the Tn5, plus flanking DNA, was then subcloned from pIJ7244, and the DNA sequence adjacent to the site of Tn5 insertion was determined. The data were all consistent with a single Tn5 insertion within the *nodD* gene 130 nt downstream of the predicted translation start. This observation might seem somewhat surprising, since mutation of *nodD* in *R. leguminosarum* bv. *viciae* blocks *nod* gene induction and nodulation (Downie et al. 1985). However, we observed previously that a cosmid carrying the cloned *nod* genes with Tn5 in *nodD* could enable a pSym⁻ strain to form a few nodules on Wisconsin Perfection peas (Downie et al. 1985), as is observed with 8401/pIJ7244 (Table 1).

The *nodD*::Tn5 allele from pIJ7244 was recombined into strain TOM to form A195, which was unable to form any nodules on cv. Afghanistan or Wisconsin Perfection (Table 1). The *nodD* mutation in A195 completely abolished hesperetin-induced *nod* gene induction when assayed with the *nodC-lacZ* fusion on pIJ1477 (data not shown), confirming that this mutation has the expected effect of a *nodD* mutation.

These observations suggest that the poor level of nodulation of cv. Afghanistan by strains carrying pIJ1095 might be due to this cultivar being hypersensitive to Nod factors. Mutation of *nodD* on pIJ1095 would be expected to block Nod factor production, although the low level of nodulation suggests that some Nod factors were being produced. We extracted Nod factors from strains TOM, TOM/pIJ1095, 8401/pIJ1095, and 8401/pIJ7244 after growth in medium containing the *nod* gene inducer hesperetin. As was observed previously (Firmin et al. 1993), strains carrying pIJ1095 produce about 20-fold more Nod factors than strain TOM, and TOM/pIJ1095 also produces high levels of Nod factors (Fig. 2). The *nodD* mutation in pIJ7244 severely reduced the level of Nod factor production.

Table 1. Effect of cloned *nod* genes on nodulation of cv. Afghanistan peas^a

Strain	Nodule number after 24 days	
	cv. Afghanistan	Wisconsin Perfection
8401	0	0
8401/pIJ1095	1 ± 1	74 ± 15
8401/pIJ7244	11 ± 3	10 ± 2
TOM	62 ± 9	89 ± 11
TOM/pIJ1095	4 ± 2	61 ± 7
TOM/pIJ7244	43 ± 9	87 ± 12
TOM/pIJ1597	8 ± 2	59 ± 8
A34	0	78 ± 13
A34/pIJ1357	34 ± 4	79 ± 12
A195	0	0

^a Data are average numbers of nodules ± standard error.

However, strain 8401/pIJ7244 did produce a small amount of Nod factors (about 3% of the level produced by TOM).

Repression of *nod* gene expression by *nolR* abolishes Cnb and enhances nodulation.

The data described above are consistent with a model suggesting that strains producing relatively high levels of Nod factors cause inhibition of nodulation of cv. Afghanistan peas. Such a model would predict that decreasing *nod* gene expression by other means should reduce Cnb. In strain TOM, *nod* gene expression is induced by *nodD* and repressed by *nolR*, which encodes a repressor similar to the NolR repressor found in *S. meliloti* but is functionally absent from some strains of *R. leguminosarum* bv. *viciae*, including A34 (Kiss et al. 1998). We transferred the cloned *nolR* gene (on pKE66) into *R. leguminosarum* bv. *viciae* strain A34, and this strongly reduced Cnb by A34 (Fig. 1). Previously, Kiss and associates (1998) demonstrated that *nolR* on pKE66 decreased both flavonoid-inducible *nod* gene expression and Nod factor production in this strain. Therefore, the decrease in Cnb caused by the cloned *nolR* gene is correlated with a decrease in Nod factor production. Cloned *nolR* (on pKE66) could also reduce the Cnb effect of strain 8401/pIJ1095 (Fig. 1).

Stimulation of *nod* gene expression in TOM decreases nodulation.

If relatively high levels of Nod factors are responsible for the poor nodulation of cv. Afghanistan by TOM/pIJ1095 (or by 8401/pIJ1095), then alternative means of stimulating *nod* gene expression might be anticipated to inhibit nodulation by TOM. Previously, Burn and associates (1987) identified a mutant form of *nodD* that enhanced *nod* gene expression in *R. leguminosarum* bv. *viciae*. The plasmid carrying this mutant form of *nodD* (pIJ1597) was transferred to strain TOM, and nodulation on cv. Afghanistan was analysed. As shown (Table 1), TOM/pIJ1597 nodulated considerably less well than TOM, al-

though nodulation on Wisconsin Perfection was not significantly affected. To analyze the effects of pIJ1597 on *nod* gene expression in strain TOM, we introduced the *nodC-lacZ* reporter on pIJ1477 into TOM/pIJ1597 and measured induction of β -galactosidase activity following the addition of hesperitin. Whereas the level of *nodC-lacZ* expression in strain TOM was around 650 ± 30 units (Kiss et al. 1998), the strain carrying pIJ1597 was found to have $5,500 \pm 450$ units of activity. This increased *nod* gene induction is correlated with increased Nod factor production by strain TOM/pIJ1597 compared with TOM (Fig. 2).

Nod factor extracts induce nodulation blocking on cv. Afghanistan.

Nod factors were extracted from strains 8401/pIJ1089 and 8401/pIJ1095 as described previously (Firmin et al. 1993). A fraction containing the Nod factors from each was collected from a reverse-phase column and added to cv. Afghanistan plants 24 h before inoculation with strain TOM. In each case, the addition of the crude Nod factor preparation resulted in almost complete nodulation blocking (Fig. 3A), demonstrating that the Nod factors from both strains could block nodulation. This inhibition of nodulation required the Nod factors, as no inhibition of nodulation was observed if an identical isolation was done using an extract from a *nodC* mutant that makes no Nod factors (Fig. 3A).

Similar experiments with Nod factors were done using Wisconsin Perfection instead of cv. Afghanistan. The complete blocking of nodulation observed with cv. Afghanistan was not seen, although the Nod factor preparation did delay the onset of nodulation (Fig. 3B). This delay in nodulation is not specific to nodulation by strain TOM, as a similar Nod factor-induced delay in nodulation was observed when strain A34 was used as inoculant (data not shown).

To determine which of the various Nod factors causes nodulation blocking, two individual Nod factor peaks, correspond-

Fig. 1. Competitive nodulation blocking tests. Seedlings of cv. Afghanistan were inoculated with the strains shown and 24 h later were inoculated with strain TOM. In the control with TOM alone, the seedlings were inoculated with sterile water before TOM. The data show averages $\pm$ standard error from at least eight matched plants per test.

ing to *Rlv*.IV (Ac C18:4) and *Rlv*.V (Ac C18:4), were purified from the supernatant of 8401/pIJ1089. When either of these Nod factors was added to the roots of strain TOM (at the same estimated concentration as that for the total Nod factor preparation), no significant blocking was observed (data not shown). To identify the active component, we then assayed different fractions of high-pressure liquid chromatography (HPLC)-separated Nod factor preparation, but none showed strong nodulation inhibition. When the fractions were recombined, however, blocking similar to that seen with the unfractionated extract (Fig. 3A) was seen (data not shown). These results suggest that the Nod factor-induced blocking may involve cooperative activity of more than one component in the Nod factor extract.

Cnb is not a systemic effect.

The Cnb effect could be somewhat similar to the systemic control of nodulation in legumes. This can be observed in split-root experiments in which one half of a root system is inoculated with a nodulating strain of the genus *Rhizobium*, which then inhibits subsequent nodulation on the other half of the root system (Caetano-Anolles and Gresshoff 1991). To determine if Cnb exerts such a systemic effect, cv. Afghanistan peas were grown in a split-root system; one side was inoculated with the Cnb⁺ strain A34, and after 24 h, the other side was inoculated with strain TOM. As shown in Figure 4, a slight reduction of nodulation was seen with A34, although this difference was not significant at the 95% confidence level. Very strong inhibition of nodulation was seen in a control experiment in which TOM was inoculated into the same side as A34 (Fig. 4), confirming that blocking could occur in this assay system. On the basis of these results, we conclude that the Cnb effect is not systemic.

It is known that ethylene can inhibit nodulation and that aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis, can stimulate nodulation in plants where ethylene accumulation causes nodulation inhibition (Fearn and LaRue 1991; Zaat et al. 1989). When AVG (1 μ M) was added to a Cnb assay with strain A34 as a blocking strain, no effect was observed on the ability of A34 to inhibit nodulation of cv. Afghanistan peas by strain TOM (data not shown). Higher levels of AVG (10 μ M) resulted in strong inhibition of root growth.

Infection events.

The *sym*2⁴ locus of cv. Afghanistan peas blocks the initiation of infection threads by strains of *R. leguminosarum* bv. *viciae* lacking *nodX* (Geurts et al. 1997). To analyze where nodulation blocking occurs, we constructed a derivative of strain TOM carrying a constitutively expressed *lacZ* gene and stained cv. Afghanistan roots to observe infection threads and infection foci in the presence and the absence of the nodulation-blocking strain A34. Observations on infection events were limited to the primary root. Early infection events were grouped into three classifications: infection foci, in which bacteria have accumulated in 'shepherd's crook' structures in root hairs; initiated infection threads, in which some infection thread growth is seen, but it does not extend beyond the root hair cell; and infection threads, which extend through two or more root cells. The data are summarized in Table 2. Strain TOM induces many infections, but Cnb⁺ strain A34 almost completely abolished infection thread formation by TOM. A similar inhibition of infection thread initiation was induced by a Nod factor preparation containing a mixture of Nod factors. These results indicate that nodulation blocking is a result of inhibition of infection events. As a control, strain A34 was marked with the constitutive *lacZ* gene, and early events were scored. As expected (Geurts et al. 1997), this strain produced very few infection events; the numbers of infection foci produced were somewhat

higher than that seen with strain TOM, perhaps because these do not develop into normal infection threads.

Nodulation inhibition is linked to the cv. Afghanistan *sym*2⁴ locus.

Although genetic segregation data and analysis of introgressed lines demonstrate that the resistance of cv. Afghanistan to nodulation is due to the *sym*2⁴ locus, it has not been described whether the Cnb phenotype is also located at, or close to, *sym*2⁴. This possibility was tested by analyzing Cnb in a derivative of the cultivated line Rondo, which had been introgressed with *sym*2⁴. As expected, strain TOM nodulates this line normally (Fig. 5). The Cnb⁺ strain A34 showed an intermediate Cnb phenotype. It clearly inhibited nodulation by strain TOM (Fig. 5), but the blocking effect was not as clear as

Fig. 2. Analysis of amounts of Nod factors in the Nod factor preparations from different strains. Nod factor preparations were analyzed by analytical high-pressure liquid chromatography monitoring absorbance at 303 nm. In each trace, the loading is equivalent to the amount isolated from 100 ml of culture supernatant. The different absorbance scales on each trace give a measure of the relative amounts of Nod factors present. The vertical brackets correspond to the absorbance units (ΔA) indicated. The traces correspond to samples from the following strains: A, TOM; B, 8401/pIJ1095; C, 8401/pIJ7244; D, TOM/pIJ1095; and E, TOM/pIJ1597.

that seen on cv. Afghanistan. This indicates that the blocking effect is, in part, linked to *sym2^A* but that other genes in cv. Afghanistan may be required for the full expression of the phenotype. When nodulation of the 'Rondo'-*sym2^A*-introgressed line was tested with 8401/pIJ1095 (which carries *nodX* but cannot nodulate cv. Afghanistan; Table 1), an intermediate level of nodulation was seen but, compared with strain TOM, the onset of nodulation was delayed and fewer nodules formed (Fig. 5). This intermediate phenotype suggests that the *sym2^A* region may cause a reduction of nodulation by strains producing high levels of Nod factors but that the very poor nodulation seen with cv. Afghanistan requires other genotype-specific determinants.

DISCUSSION

It was clear from previous work (Dowling et al. 1989; Firmin et al. 1993) that Cnb depends on the Cnb⁺ strain being able to produce Nod factors carrying the NodFE-determined C18:4 fatty acyl chain and the NodL-determined acetyl group on the terminal-nonreducing *N*-acetyl-glucosaminyl residue. However, not all strains of *R. leguminosarum* bv. *viciae* that are Nod⁻ on cv. Afghanistan have the ability to block nodulation by strain TOM (Winarno and Lie 1979). The results presented here provide an explanation as to why only some strains of *R. leguminosarum* bv. *viciae* are Cnb⁺. This is correlated with high levels of Nod factor production by blocking strains, rather than the presence or absence of the NodX-determined acetyl group. Thus, by increasing Nod factor production, a Cnb⁻ strain can be made Cnb⁺ and, by decreasing Nod factor production, a Cnb⁺ strain can be made Cnb⁻. These results also fit with observations with other strains. For example, *R. leguminosarum* bv. *viciae* strain PRE is Cnb⁻ (Winarno and Lie 1979) and produces low levels of Nod factors (Kozik et al. 1995), and strain 248, which is Cnb⁺ (Dowling et al. 1989), produces high levels of Nod factors (Kozik et al. 1995).

Although our data suggest that the Cnb⁺ phenotype is due to a quantitative effect of Nod factor production, we cannot easily distinguish a simple quantitative effect from the possibility that high levels of Nod factor production could be correlated with formation of (possibly low levels of) unusual Nod factors or changes in ratios of Nod factors that may induce blocking. In fact, one result described here suggests that a more complex model such as this might be valid. The observation that strain A34 is Cnb⁺ but that strain A34 carrying the cloned *nodX* gene can nodulate reasonably well implies that modification of Nod factors by NodX can decrease blocking. Otherwise, strain A34 carrying *nodX* should, in effect, block its own nodulation. Furthermore, a natural isolate of *R. leguminosarum* bv. *viciae* (strain A1), which contains *nodX* and nodulates cv. Afghanistan, was found to produce high levels of Nod factors in culture (Ovtsyna et al. 1999).

We tried to fractionate the Nod factor preparation to determine which fractions contained the blocking factor. We observed no strong inhibition of nodulation with either Nod *Rlv*.IV (Ac C18:4) or Nod *Rlv*.V (Ac C18:4). Our attempts to fractionate a blocking factor were unsuccessful, and no individual fraction blocked as effectively as the recombined fractions. This suggests that more than one component could be required for optimal blocking. Minami and associates (1996) observed that mixtures of Nod factors are more effective at inducing early nodulin gene expression than single Nod factors, so it is possible that some such synergistic effect may occur during nodulation blocking. It was also clear that the purified Nod factor preparation transiently inhibited nodulation in Wisconsin Perfection peas. This could be related to the inhibition of nodulation of Wisconsin Perfection by a strain of *R. leguminosarum* bv. *viciae* overexpressing the *nod* gene region (Knight et al. 1986). Taken together, these observations suggest that cv. Afghanistan peas may be hypersensitive to nodulation inhibition by some Nod factors. Ovtsyna and associates (1999) observed that strain A1, which nodulates cv. Afghanistan, produces many Nod factors (at least 23 were found). They also demon-

Fig. 3. Inhibition of nodulation by Nod factor preparations. A Nod factor preparation from 8401/pIJ1089 was added to **A**, cv. Afghanistan or **B**, Wisconsin Perfection seedlings at a concentration of 0.1 μ M Nod factor, and then 24 h later, strain TOM was inoculated (▲). Similar data (data not shown) were observed with a Nod factor preparation from 8401/pIJ1095. The nodulation by strain TOM without added Nod factors is shown as ◆. The inhibitory effect is Nod factor specific because no inhibition of nodulation of cv. Afghanistan was seen with an equivalent extraction from the supernatant of a *nodC* mutant strain (■); this extract had no significant effect on nodulation of Wisconsin Perfection, but for clarity the data are not shown. The data are averages $\pm$ standard error of at least 10 plants per test.

strated that there were significant quantitative differences in the Nod factors produced when comparing bacteria grown in pea root exudate with bacteria grown in the presence of a pure *nod* gene inducer. Therefore, specific plant rhizosphere effects, which could be difficult to mimic during in vitro bacterial culture, could contribute to the observed nodulation blocking by some strains of *R. leguminosarum* bv. *viciae*.

It is apparent that the suppression of nodulation by Cnb⁺ strains and by added Nod factors inhibits infection by strain TOM. This is consistent with the observation (Firmin et al. 1993) that an exopolysaccharide-deficient mutant that does not initiate infections retains a Cnb⁺ phenotype. Therefore, the blocking effect can be expressed independently of infection thread formation.

Suppression of nodulation in legumes is seen under various circumstances. For example, nitrate can suppress nodulation but is unlikely to be related to the Cnb effect. Ethylene arrests persistent infections, and the pea *sym5* mutant is Nod⁻ due to hypersensitivity to ethylene (Fearn and LaRue 1991), while an ethylene-insensitive mutant of *Medicago truncatula* has increased numbers of infections (Penmetsa and Cook 1997). However, our inability to prevent Cnb with an inhibitor of ethylene synthesis indicates that the Cnb effect is not due to an ethylene-signalling pathway. Plant defense responses have been associated with the arrest of infection thread growth (Vasse et al. 1993), but it seems unlikely to be the explanation in the absence of infections initiated by the Cnb⁺ strain, although we cannot eliminate such an effect in Cnb. There is a feedback mechanism called autoregulation (Caetano-Anolles and Gresshoff 1991) that inhibits nodule formation once nodules have been formed. Thus, nodulation in younger parts of the root can be suppressed by prior inoculation of a nodulating

strain. Furthermore, nodulation in a susceptible zone of the root can be suppressed by the application of a heavy second inoculation (Takats 1986, 1990). Perhaps the delayed nodulation of Wisconsin Perfection peas (Fig. 3B) induced by Nod factors is similar to suppression of nodulation by a second heavy inoculation. If this were the case, it is possible that the Cnb effect is related to autoregulation of nodulation, which may be strongly enhanced in this cultivar by a high sensitivity of cv. Afghanistan to some Nod factors.

The mechanism by which the Cnb effect is induced remains unclear, although it is evident that a genetic determinant at or near the *sym2^A* locus is responsible, at least in part. One possible explanation for nodulation blocking could be related to the induction of Nod factor hydrolases, and indeed, strain TOM induces much lower levels of such hydrolases in pea roots than other strains of *R. leguminosarum* bv. *viciae* (Ovtsyna et al. 2000). It would be possible to test if the *sym2^A* locus contributes to enhanced levels of Nod factor hydrolases in cv. Afghanistan by using a derivative of cv. Afghanistan in which the *sym2^A* locus is replaced with *Sym2^C* from a cultivar that does not show a Cnb phenotype.

The lower level of blocking seen in the 'Rondo'-*sym2^A* introgressed line indicates that determinants other than *sym2^A* seem to contribute to the phenotype. This fits with the observation of Winarno and Lie (1979) that cv. Iran, which carries an allele of *sym2^A* (Kozik et al. 1995), exhibits a Cnb phenotype but at a lower level than cv. Afghanistan. The *sym2^A* locus seems to play a role in recognition of Nod factors, although it is unlikely to be an early step in Nod factor recognition, since cv. Afghanistan root hairs respond to Nod factors lacking the NodX-determined acetyl group by inducing calcium spiking and root hair deformation (Walker et al. 2000).

Fig. 4. Effects of a Cnb⁺ strain in a split root assay. Strain A34 was added to one side of the root system 24 h before inoculation of strain TOM on the other side (◆). The Cnb⁺ effect of adding strain A34 to the same side as TOM is shown as ▲, and nodulation by strain TOM with no other inoculation is shown as ■. The average numbers of nodules ± standard error on the side inoculated with TOM are shown based on data obtained with 12 plants.

Fig. 5. Nodulation and Cnb tests on 'Rondo' *sym2^A*. A derivative of line 'Rondo' (A.5.4.3) carrying homozygous *sym2^A* introgressed from cv. Afghanistan (Kozik et al. 1995) was tested for nodulation by strains TOM (◆), 8401/pIJ1095 (Δ), and A34 (□). Competitive nodulation blocking was assayed using strain A34 plus TOM (▲) by inoculating TOM 24 h after the test strain. The data are averages ± standard error of at least 10 plants per test.

The characterization of *sym2^A* and its product may give an insight into the mechanism of Cnb.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The characteristics and sources of the strains and plasmids are described in Table 3. Plasmids pXLGD4, pIJ1477, pIJ1597, and pKE66 were transferred to *Rhizobium* strains by triparental matings as described previously (Kiss et al. 1998; Walker and Downie 2000). Assays of β -galactosidase activity were done as described by Rossen and associates (1985). Plasmid pIJ7244 was made by mutagenising pIJ1095 with Tn5 and conjugating the mutated derivatives into strain 8401 as described previously (Downie et al. 1985). The transconjugants were screened for those that could nodulate cv. Afghanistan peas. Two independent plasmids that conferred nodulation were identified. Both had mutations in *nodD*, and only one (pIJ7244) was fully characterized. The location of the Tn5 was mapped with *Bam*HI, *Eco*RI, and *Hind*III onto the map of the TOM *nod* gene region described previously (Hombrecher et al. 1984). An *Eco*RI-*Bam*HI fragment carrying part of the Tn5 and the flanking DNA was subcloned from pIJ7244 into pUC18. The precise location of the Tn5 insertion was determined by DNA sequencing using a primer from within Tn5 as described previously (Crank and Downie 1994). The Tn5 mutation in pIJ1744 was transferred into the genome of strain TOM by homologous recombination to create strain A195, essentially as described by Downie and associates (1985). The recombinants were checked by DNA hybridization and by polymerase chain reaction using the Tn5 primer used for DNA sequencing and a primer from the sequence of DNA determined upstream of the *nodD* gene.

Plant growth.

Nodulation tests described in Table 1 were carried out as described previously (Beynon et al. 1980). Competitive nodulation tests were carried out essentially as described by Dowling and associates (1987), except that the low-nitrogen plant-growth medium used was as described by Beynon and associates (1980). Inoculants were grown on tryptone yeast extract agar plates (Beringer 1974) containing the appropriate antibiotics, and the bacteria were picked off with a sterile loop and resuspended in sterile water. Blocking strains were inoculated at a final density of about 10^5 per ml, and strain TOM was inoculated 24 h later at a similar density. Experiments with split roots were carried out similarly, except that, instead of using Erlenmeyer flasks containing approximately 300 ml of medium, two tubes, each containing about 100 ml of medium, were used. Split roots were generated, essentially as described by Kossak and Bohlool (1984), by aseptically excising the primary root tip from freshly germinated seedlings, which were then grown on agar plates until the lateral roots were long enough to be placed into the plant-growth tubes. All nodulation data are presented with respect to the number of days after TOM inoculation. The line of 'Rondo' carrying the *sym2^A* re-

gion introgressed from cv. Afghanistan was A.5.4.3 (Kozik et al. 1995).

Nod factor tests.

Nod factors were isolated and separated on reverse-phase columns as previously described (Firmin et al. 1993). Quantitative analysis of the levels of Nod factors produced involved isolation of Nod factors from 1 liter of culture. The data presented in Figure 2 are based on the amount of Nod factors produced by the equivalent of 100 ml of culture supernatant and were done as described by Firmin and associates (1993). To produce Nod factors for analysis of their effects on nodulation blocking, Nod factors were isolated from either strain A34, 8401/pIJ1089, or 8401/pIJ1095 using 10 liters of culture supernatant. The Nod factor region was eluted from a preparative C18 reverse-phase column with methanol, and the concentration of Nod factors present was estimated based on the ϵ_{mM} of 18 at 303 nm, using analytical HPLC and integrating the area under the peaks. This method assumes that the extinction coefficients of different C18:4 Nod factors are similar and ignores contributions from Nod factors that have a C18:1 acyl chain. These assumptions avoid problems with non-Nod-factor material that may be detected at lower wavelengths (200 nm) and seemed reasonable on the basis that strains which do not produce C18:4-containing Nod factors do not induce Cnb. The total amount of C18:4-containing Nod factor present was usually 10^{-7} M. In those experiments in which the Nod factors were separated into separate fractions, the fractions were separated by HPLC as described by Firmin and associates (1993).

Infection assays.

Germinated seedlings with a root length of about 2 cm were grown in liquid medium as was used in Cnb tests. Plants were grown for 24 h and were then inoculated at a density of 10^5 per ml, using strain A34 or strain 8401/pIJ1095 24 h before addition of strain TOM/pXLGD4. Five days after inoculation of TOM/pXLGD4, the roots were removed from the growth medium and the lateral roots were excised. The procedures used for staining of β -galactosidase in root tissue to visualize infection foci and infection threads were essentially as described by Walker and Downie (2000).

Table 2. Effects of Nod factors and a Cnb⁺ strain on infections^a

Inoculum	Infection foci	Infected root hairs	Infection threads
TOM/pXLGD4	2.0 $\pm$ 0.3	5.7 $\pm$ 0.5	31.3 $\pm$ 2.5
A34 + TOM/pXLGD4	2.0 $\pm$ 0.5	0.3 $\pm$ 0.1	0.9 $\pm$ 0.2
Nod factor + TOM/pXLGD4	1.3 $\pm$ 0.4	0.3 $\pm$ 0.1	0.7 $\pm$ 0.3
A34/pXLGD4	11.4 $\pm$ 2.6	2.0 $\pm$ 0.2	0.1 $\pm$ 0.05

^a Data are averages $\pm$ standard error of events measured on cv. Afghanistan primary roots stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) to identify the strains carrying pXLGD4.

Table 3. Bacterial strains and plasmids

Strain or Plasmid	Relevant characteristics	Source
8401	pSym ⁻ strain of <i>Rhizobium leguminosarum</i>	Lamb et al. 1982
A34	Derivative of 8401 carrying the symbiotic plasmid pRL1J1	Downie et al. 1985
TOM	<i>R. leguminosarum</i> bv. <i>viciae</i> native isolate; nodulates cv. Afghanistan	Winarno and Lie 1979
A195	Derivative of TOM carrying <i>nodD::Tn5</i> ; Str ^R	This work
pIJ1089	Cosmid carrying cloned <i>nod</i> gene region from A34; Tet ^R	Downie et al. 1983
pIJ1095	Cosmid carrying cloned <i>nod</i> gene region from TOM; Tet ^R	Hombrecher et al. 1984
pIJ1357	<i>nodX</i> cloned from pIJ1095	Götz et al. 1985
pIJ1477	<i>nodC-lacZ</i> reporter plasmid; Tet ^R	Rossen et al. 1985
pIJ1597	Cloned mutant allele of <i>nodD</i> that enhances <i>nod</i> gene expression; Kan ^R	Burn et al. 1987
pIJ7244	Derivative of pIJ1095 carrying Tn5 in <i>nodD</i>	This work
pKE66	<i>nolR</i> gene cloned from TOM; Gent ^R	Kiss et al. 1998
pXLGD4	<i>hemA-lacZ</i> fusion that expresses <i>lacZ</i> constitutively; Tet ^R	Leong et al. 1982

^R = Resistance

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