Entry of *Rhizobium leguminosarum* bv. *viciae* into Root Hairs Requires Minimal Nod Factor Specificity, but Subsequent Infection Thread Growth Requires *nodO* or *nodE*

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Using various mutant strains of *Rhizobium leguminosarum* bv. *viciae*, we have investigated the role of *nodO* in stimulating infection thread development in vetch and pea. Analysis of *R. leguminosarum* bv. *viciae* *nodE* and *nodO* mutants revealed no significant difference from the wild-type infection phenotype. Conversely, an *R. leguminosarum* bv. *viciae* *nodE nodO* double mutant was severely impaired in its ability to form normal infection threads. This strain displayed a number of novel infection-related events, including intracellular accumulations of bacteria at the base of root hairs, distended and enlarged infection threads, and reversed threads growing up root hairs. Since normal infection was seen in a *nodE* mutant, *nodO* must suppress these abnormal infection phenomena. A deletion mutant, retaining only the *nodD* and *nodABC* genes, also formed intracellular accumulations at the base of root hairs. Addition of *R. leguminosarum* bv. *viciae* *nodO* could alleviate this phenotype and restore some infection thread formation, although these threads appeared to be abnormal. Exogenous application of *R. leguminosarum* bv. *viciae* Nod factors could not alleviate the aberrant infection phenotype. Our results show that the most basic Nod factor structure can allow bacterial entry into the root hair, and that *nodO* can promote subsequent infection thread development.

The specificity of symbiotic interactions between rhizobia and their legume hosts is governed by a number of factors acting at various stages. Of greatest significance are bacterially secreted lipo-chitin oligosaccharide “Nod” factors, which are the major determinants of host range for a given *Rhizobium* species (Dénarié et al. 1996). Application of purified Nod factors to legume roots can elicit several responses including root hair deformation, cortical cell divisions, and, in some cases, the formation of nodule-like outgrowths (Heidstra and Bisseling 1996; Spaink 1996). Nod factors are usually made up of three to five β -1,4-linked *N*-acetyl glucosamine

residues with an acyl group replacing the N-linked acetyl group at the nonreducing end (Heidstra and Bisseling 1996). Specificity of Nod factors for a particular host is influenced by various modifications and substitutions to this basic structure. Nod factor synthesis absolutely requires the *nodABC* genes to form the acylated backbone, while other *nod* genes can determine host-specific modifications (Downie 1998).

Some functionally distinct *nod* gene products appear to operate synergistically. The *nodE* gene of *Rhizobium leguminosarum* bv. *viciae* encodes a protein with a condensing-enzyme-like domain similar to that found in fatty acid synthase (Spaink et al. 1989; Bibb et al. 1989). *R. leguminosarum* bv. *viciae* normally makes a mixture of Nod factors substituted with either a C_{18:4} acyl chain or a C_{18:1} (*cis*-vaccenic acid) acyl chain. A *nodE* mutant makes only Nod factors containing a C_{18:1} group, and this results in reduced (around 60% of wild type) nodulation of pea and vetch (Spaink et al. 1991; Economou et al. 1994). In an *R. leguminosarum* bv. *viciae* *nodE* mutant background, mutation of *nodO* reduces nodulation of pea and vetch to about 10 and 30% of wild-type levels, respectively (Economou et al. 1994). In the absence of host-specific *nod* genes, *nodO* is essential for nodulation (Downie and Surin 1990). Therefore, the *nodO* gene can compensate for the loss of appropriate Nod factor structure.

The benefits of *nodO* are not only restricted to compensating for lack of polyunsaturated acyl chain. The *nodO* gene can also extend the host range of various *Rhizobium* strains. Thus, *nodO* from *R. leguminosarum* bv. *viciae* allows an *R. leguminosarum* bv. *trifolii* *nodE* mutant to nodulate vetch (Economou et al. 1994), and *nodO* from *Rhizobium* sp. strain BR816 significantly increases the ability of *R. etli*, *S. meliloti*, *R. leguminosarum* bv. *trifolii*, and *Azorhizobium caulinodans* to nodulate the nonhost legume *L. leucocephala* (van Rhijn et al. 1996; Vlassak et al. 1998). Furthermore, *nodO* is reported to have a marked effect on the ability of *R. leguminosarum* bv. *viciae* to infect and nodulate a variety of pea carrying an allele that confers resistance to nodulation by some strains (Geurts et al. 1997).

The *nodO* gene from *R. leguminosarum* bv. *viciae* encodes a secreted protein that apparently exists in its native form as a dimer, of approximate *M_r* 67,000 (Sutton et al. 1994 1996). The predicted amino acid sequence contains repeated Ca²⁺-binding domains in the N-terminal region, and they are ho-

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mologous to those of a number of bacterial toxins collectively known as RTX proteins (Economou et al. 1990). In vitro studies have shown that the purified NodO protein forms cation-selective channels in lipid bilayers (Sutton et al. 1994). Although it has been proposed that NodO may stimulate an ion flux across the plant plasma membrane and thereby trigger subsequent downstream events, a true structure-function relationship remains to be elucidated. Indeed, although it is likely that *nodO* is expressed throughout many stages of nodulation (Vlassak et al. 1998), there is currently no hard evidence defining where NodO is required.

Many rhizobia enter their host plants through infection threads, which are plant-derived structures initiating at points where bacteria become entrapped in root hairs (Kijne 1992). In wild-type *Rhizobium*-legume interactions, the number of infections initiated in the epidermis can exceed the final number of nodules formed by up to 100-fold (Nutman 1962; Calvert et al. 1984; Vasse et al. 1993). Both the host legume and the bacterial strain can contribute to the efficiency of infection (i.e., the proportion of infections that are not aborted), and successful infections can vary from 1 to 99% of the infections initiated. It was suggested that the reduced numbers of nodules induced by an *R. leguminosarum* bv. *viciae* *nodE* mutant and a *nodE nodO* double mutant on peas and vetch might be the result of limiting numbers of infection threads (Economou et al. 1994). In a parallel study, an *S. meliloti nodF nodL* double mutant was unable to penetrate host plants to stimulate the formation of normal infection threads (Ardourel et al. 1994). This *nodF, nodL* mutant produces Nod factors lacking an O-linked acetyl group at the nonreducing end and has altered acyl chain structure (Ardourel et al. 1994). Although this mutant did not stimulate normal infection, it produced biologically active Nod factors because early nodulation responses like root hair deformation and cortical cell divisions were induced (Ardourel et al. 1994). This led to the hypothesis that there are (at least) two Nod factor receptors in the epidermal cells of legume plants: one with low stringency for substitutions at the nonreducing end transducing signals that lead to early morphological changes; the other, more stringent for all Nod factor substitutions responsible for bacterial entry and infection thread initiation.

Here we describe the effects on infection thread formation of mutating various *R. leguminosarum* bv. *viciae* genes either in the presence or absence of functional *nodO*. With strains marked with a constitutively expressed *lacZ* gene, it was possible to identify different infection phenotypes for the different strains, and it became clear that *nodO* plays a significant role in promoting infection thread development.

RESULTS

Preliminary experiments examined the infection threads formed on pea (*Pisum sativum*) and vetch (*Vicia hirsuta*) roots 2, 4, 8, 12, and 20 days after inoculation with various *R. leguminosarum* bv. *viciae* strains (data not shown). Vetch plants typically formed a primary root, which provided a good means for accurate quantitative measurements of infection numbers. Around 12 days after inoculation, the root was a suitable length for microscopic analysis and displayed a variety of symbiotic characteristics. These included many infection threads, infected nodule primordia, young nodules, and nod-

ules reaching maturity. Hence, all results described concerning vetch are from roots 12 days after inoculation. The extensively branched root system of pea made accurate quantitation of infection events more difficult. To limit error, experiments were done on lateral roots, generated after excision of the primary root tip. However, vetch remained the simplest plant with which to work, and the experiments done on pea were primarily used to substantiate some of the observations made with vetch.

Mutation of either *nodE* or *nodO* of *R. leguminosarum* bv. *viciae* does not affect the formation of infection threads on vetch.

Previously, it was observed that a *nodO* mutant of *R. leguminosarum* bv. *viciae* nodulates vetch to a similar level as wild type, whereas a *nodE* mutant is reduced to approximately 60% of wild-type levels (Economou et al. 1994). The results shown in Figure 1A show a similar though not identical picture. With *R. leguminosarum* bv. *viciae* strains containing a *hemA::lacZ* fusion (on pXLGD4), stained for β -galactosidase activity, it was possible to get a much more accurate count of nodule numbers, as even small nodules appeared distinctly blue. It was also possible to count the number of infected nodule primordia formed, as these appeared as swollen regions with blue threads penetrating into the root cortex. This meant the total numbers of nodules counted 12 days after inoculation were higher than previously reported and it became apparent that, on vetch at least, the *nodE* mutant (A42) was capable of nodulating at up to 80% of wild-type levels. As described previously (Knight et al. 1986), the *nodE* mutant induced more extensive root hair deformation and branching than the wild type (Table 1).

With the *lacZ* system, visualizing infection threads, root hair curling, and entrapped bacteria within curled root hairs was relatively easy. Infection threads formed on vetch by the wild type (A34) initiated at the base of root hairs (Fig. 2A). This differed slightly from what was seen in pea, where infections typically initiated from the tips of curled root hairs (Fig. 2B). In vetch, the presence of the bacteria apparently stimulated branched outgrowths at the base of root hairs, which curled (Hac⁺) and entrapped the bacteria as illustrated in Figure 2A and C. Most observed infection threads initiated at this point of entrapment. Such threads usually aborted in the epidermal cells and, for the wild type, infection threads were seen at a frequency of between 100 and 200 per root 12 days after inoculation (Fig. 1B). Threads that did penetrate into the lower cortical layers were narrow and usually followed a fairly direct course to a developing nodule primordium (Fig. 2C). Similar types and numbers of infections were seen with both the *nodE* (A42) and *nodO* (A67) mutants (Fig. 1B). In pea, infection threads induced by the wild type were seen at an average frequency of 27.9 ± 5.9 per lateral root section ($n = 8$). As with vetch, these appeared straight and narrow, following a direct route to developing nodule primordia.

Mutations in both *nodE* and *nodO* result in aberrant infections.

Vetch roots inoculated with the *nodE nodO* double mutant (A91) containing pXLGD4 looked completely different from those inoculated with wild-type *R. leguminosarum* bv. *viciae*. Extensive root hair deformation and branching (Hac⁺⁺) were

seen, as was observed with the *nodE* mutant. Root hairs formed branches at their base and entrapped bacteria where the branched root hair formed apparently normal root hair curling events. However, instead of infection threads, large aggregations of bacteria were seen (Fig. 2D and E). Bacteria had apparently initiated infection, but were unable to stimulate the formation of a normal infection thread. Twelve days after inoculation, these abnormal infection events were seen at a frequency of around 800 per root, indicating these aberrant infections occur at a significantly higher frequency than infection threads induced by the wild-type (Fig. 1B). The *nodE nodO* double mutant was also seen entrapped in pea root hairs,

forming aberrant infection foci (Fig. 2F). These were seen at an average frequency of 24.3 ± 3.0 per lateral root section ($n = 8$), with very few infection threads (average 0.75 ± 0.3 per root section, $n = 8$). However, unlike the distinctive heavy staining seen in vetch, the blocked infections in pea were seen as more discrete foci (Fig. 2F).

Although aberrant infections formed the majority of the infection structures seen in vetch root hairs inoculated with the *nodE nodO* double mutant, a few infection threads were formed and hence some nodules were generated (Fig. 1A). However, the few threads formed by the *R. leguminosarum* bv. *viciae* *nodE nodO* double mutant were abnormal, having a

Fig. 1. Influence of *nodO* on stimulating nodule and infection thread formation on vetch. Events were counted on root sections 12 days after inoculation with various strains of *Rhizobium leguminosarum* bv. *viciae*. **A**, Average number of nodules and infected primordia. **B**, Average number of infections and aberrant infections (aggregations of bacteria at base of root hairs). All values represent averages from two separate experiments totaling 16 plants for each strain. Bars represent $\pm$ SE. Asterisk: Total number of aberrant infections exceeded 1000 per root section with this strain. Inaccuracies when counting such high numbers increase significantly; therefore, values were scored as ">1000" and, as such, error bars are not applicable. True value is higher than that indicated.

distended, enlarged appearance that continued along the length of the thread into the developing nodule primordium (Fig. 2G and H). This phenotype was also seen in pea.

Another unusual aspect of vetch inoculated with the double mutant was the large numbers of infection threads growing up root hairs in the opposite direction to normal. This is illustrated in Figure 2I, in which the origin of the infection thread is arrowed. This “reversed thread” phenomenon was never seen with the wild type, seen rarely with the *nodO* mutant (0.88 ± 0.51 per root, $n = 18$), occasionally with the *nodE* mutant (2.94 ± 0.74 per root, $n = 18$) but was seen at a relatively high frequency of 9.00 ± 2.38 per root ($n = 18$) for the *nodE nodO* double mutant. Such reversed threads were not, however, observed in pea.

A *nodC* mutant, which cannot produce Nod factors, was not detected within root hairs (data not shown). This control confirms that Nod factors are required for the aberrant infection phenotype.

***nodO* can alleviate the blocked infection phenotype of an *R. leguminosarum* bv. *viciae* *nodFELMNTO* deletion mutant.**

To determine whether *nodO* is capable of stimulating normal infection threads by a strain unable to make host-specific substitutions to the Nod factors, an *R. leguminosarum* bv. *viciae* *nodFELMNTO* deletion mutant was tested on vetch. This strain retains the *nodD* and *nodABCIJ* genes necessary for Nod factor synthesis and secretion. However, since it lacks *nodFE* it can only produce Nod factors with a C_{18:1} acyl chain. Additionally, due to the absence of *nodL*, the Nod factors must

lack the O-linked acetyl group on the terminal nonreducing glucosamine residue (Bloemberg et al. 1994). Although this deletion mutant cannot nodulate vetch at all (Downie and Surin 1990), it causes substantial root hair branching and deformation (Table 1). Several of the branches near the base of the root hairs curled (Hac⁺) and entrapped bacteria (Table 1 and Fig. 2K).

The *R. leguminosarum* bv. *viciae* *nodFELMNTO* deletion strain (A69) formed aberrant infections at an even higher frequency than the *nodE nodO* double mutant (Fig. 1B). The aberrant infections formed by the double mutant (Fig. 2D and J) looked similar to those induced by the deletion mutant (Fig. 2K) and both involved entrapment of bacteria within the curled region of a branched root hair. However, unlike the double mutant, the deletion mutant did not form threads of any kind, including reversed threads (Fig. 1B). Addition of the *R. leguminosarum* bv. *viciae* *nodO* gene (on pIJ1997) to the deletion mutant almost completely abolished the formation of bacterial aggregations at the site of infection, stimulated infection thread formation, and allowed a low level of nodulation (Fig. 1A and B). The (near) absence of aberrant infections seen as aggregations at the base of root hairs is somewhat surprising, especially in view of the fact that the level of nodulation is lower than that of the *nodE nodO* double mutant. However, the number of infection threads was lower than that of the wild type and the threads themselves tended to terminate very quickly and looked rather disjointed (Fig. 2L). Some reversed threads were also seen. Threads that penetrated the cortex had a form similar to those seen with the *nodE nodO* double mutant.

Table 1. Summary of strains, Nod factors, and root hair responses

Strain	Source	Relevant characteristics	Expression of <i>nodO</i>	Nod factors ^a	Root hair phenotype (vetch) ^b	Infection event
A34	Downie et al. 1985	Wild type	+	R lv IV (Ac, C _{18:1}) R lv IV (Ac, C _{18:4}) R lv V (Ac, C _{18:1}) R lv V (Ac, C _{18:4})	Had ⁺ Hac ⁺	Normal ITs
A42	Downie et al. 1985	<i>nodE68::Tn5</i>	+	R lv IV (Ac, C _{18:1}) ^c R lv IV (C _{18:1}) R lv V (Ac, C _{18:1}) R lv V (C _{18:1})	Had ⁺⁺ Hac ⁺	Normal ITs
A56	Downie et al. 1985	<i>nodC128::Tn5</i>	+	None	Had ⁻ Hac ⁻	No infection
A67	Economou et al. 1994	<i>nodO93::Tn3</i> HoHo1	-	R lv IV (Ac, C _{18:1}) R lv IV (Ac, C _{18:4}) R lv V (Ac, C _{18:1}) R lv V (Ac, C _{18:4})	Had ⁺ Hac ⁺	Normal ITs
A69	Downie and Surin 1990	<i>nodΔ99::kan</i> (<i>ΔnodFELMNTO</i>)	-	R lv IV (C _{18:1}) R lv V (C _{18:1})	Had ⁺⁺ Hac ⁺	Abnormal infection foci; no ITs
A69/ pIJ1997	This work	<i>ΔnodFELMNTO</i> + cloned <i>nodO</i>	++	R lv IV (C _{18:1}) ^d R lv V (C _{18:1})	Had ⁺⁺ Hac ⁺	ITs formed; somewhat abnormal
A91	Economou et al. 1994	<i>nodE68::Tn5</i> <i>nodO93::Tn3</i> HoHo1	-	R lv IV (Ac, C _{18:1}) ^c R lv IV (C _{18:1}) R lv V (Ac, C _{18:1}) R lv V (C _{18:1})	Had ⁺⁺ Hac ⁺	Abnormal infection foci; few ITs

^a Nod factor nomenclature is as described by Spaink et al. (1991), where R. lv describes the strain (*Rhizobium leguminosarum* bv. *viciae*), IV or V defines the number of glucosamine residues, Ac the NodL-determined acetyl group, and C_{18:1} or C_{18:4} the acyl chain.

^b Had, root hair deformation, Hac, root hair curling.

^c The mutation in *nodE* has a polar effect on *nodL*, resulting in only a proportion of the Nod factors carrying an acetyl group on the C-6 of the non-reducing glucosamine.

^d Deletion of the *nodFELMNTO* genes results in reduced levels of Nod-factors, which are produced at about 1% of normal levels (K. E. Wilson and J. A. Downie, unpublished observations).

Exogenous application of Nod factors cannot prevent aberrant infections.

It has been reported that the *nodABC* deletion mutant of *Rhizobium* sp. strain NGR234 and the *nodA* mutant of *Bradyrhizobium japonicum* (which are both unable to synthesize Nod factors) can nodulate their respective host plants *Vigna unguiculata* and *Glycine max* if Nod factors are supplied exogenously (Relic et al. 1994). Here, we tried to suppress the formation of aberrant infection events by adding Nod factor exogenously. This could, in theory, enable proper infections to be initiated. However application of *R. leguminosarum* bv. *viciae* wild-type Nod factors to *Vicia hirsuta* roots inoculated with A69 (*nodFELMNTO* deletion mutant) made no difference to the presence of aberrant infections. Aggregations of bacteria at the base of root hairs were still present in numbers typically exceeding 1000 per root ($n = 18$), and no stimulation of threads or reversed threads was observed.

DISCUSSION

It has been proposed that Nod factor recognition is mediated by a two-receptor system (Ardourel et al. 1994). In this model, based on studies of *S. meliloti* interactions with alfalfa, a low-stringency signaling receptor perceives Nod factors and alters root hair growth and other cellular processes, to prepare for infection. A second receptor "having stringent structural requirements for substitutions at the non-reducing end of the [Nod factor], would be involved in the specific recognition of rhizobial cells to allow the formation of the [infection] sites and the initial bacterial ingestion, a kind of entry lock" (Ardourel et al. 1994). Our results, however, demonstrate that, in vetch and pea, the initial entry does not require host-specific substitutions on the Nod factors. However, proper, directed infection thread growth does require additional specificity, which can be determined by either a modified acyl group (*nodE*) or by *nodO* encoding a secreted protein. An *R. leguminosarum* bv. *viciae* deletion mutant, lacking all the host-specific nodulation genes, and hence secreting the most basic Nod factors, clearly penetrated root hairs (Fig. 2K). The inability of this strain to nodulate vetch stems from the fact that it cannot induce infection thread formation at these initial infection sites. Instead, large accumulations of bacteria are seen, forming aberrant infection foci. Similarly, a *nodE nodO* double mutant, producing Nod factors with a $C_{18:1}$ acyl group (the wild type produces a mixture of Nod factors with either a $C_{18:1}$ or $C_{18:4}$ group), penetrated vetch and pea root hairs, but was unable (on most occasions) to stimulate the formation of infection threads. When threads did initiate, they were usually seen to remain within root hairs, apparently lacking order and direction (Fig. 2D and I). These results show that in vetch and

pea there is a low requirement for Nod factor specificity to gain bacterial entry into the root hair. Instead, specific Nod factors are needed to coordinate the formation and direction of an infection thread from the site of entrapment.

The results obtained with vetch indicate that entry and normal infection thread growth can be uncoupled in mutants lacking *nodE* and *nodO*. There is a qualitative difference in the extent of infection by these mutants of vetch compared with pea root hairs, with vetch accumulating larger numbers of bacteria in aberrant infection foci. Significant differences were observed by Ardourel et al. (1994) between two different hosts of *S. meliloti* inoculated with a *nodF nodL* double mutant. On *M. sativa*, no hair curling or bacterial entrapment were observed, whereas, on *M. truncatula*, the *nodFL* mutant bacteria accumulated inside curled root hairs (Ardourel et al. 1994). Thus, while the abnormal infections induced on vetch by the *R. leguminosarum* bv. *viciae nodE nodO* mutant differs from what was seen with the *S. meliloti nodFL* mutant on *M. sativa*, it is somewhat similar to what was seen with *M. truncatula*. Thus, entry (but not infection thread growth) can occur with minimal Nod factor specificity.

In the rare instances where infection threads initiated by the *nodE nodO* double mutant managed to cross into the cortical cell layers of vetch, infection thread growth was abnormal. Threads were distended and further aggregations were often seen, particularly at the interface between two adjacent cells, where the thread became grossly enlarged, sometimes filling most of the infected cell (Fig. 2G and H). This represents the point where the thread is trying to access the adjacent cell from the inner periclinal wall of the already infected cell. There is often some accumulation of thread material at this juncture during wild-type infections, where the infection thread will grow along the inner wall to meet the axis of the cytoplasmic bridge formed in the adjacent cell (see Figure 2C) (Kijne 1992). Once this point is located, the wall of the thread will fuse with the cell wall of the infected cell, and localized degradation of both adjacent cell walls will occur to facilitate passage of the thread (van Spronsen et al. 1994; Kijne 1992). Before the infection thread and plant cell wall fuse, there is a partial cell wall degradation associated with the formation of mature cytoplasmic bridges (van Spronsen et al. 1994). Purified mitogenic Nod factors (those having a $C_{18:4}$ acyl chain and an *O*-acetyl group; Spaink et al. 1991) can also induce cytoplasmic bridge formation and associated partial cell wall degradation in vetch and pea (van Brussel et al. 1992; van Spronsen et al. 1994). However, non-mitogenic Nod factors (those lacking a $C_{18:4}$ acyl chain or an *O*-acetyl group) cannot induce the formation of cytoplasmic bridges (van Brussel et al. 1992). One interpretation of our results is that impaired cytoplasmic bridge formation and lack of associated plant cell

Fig. 2. Infection events in pea and vetch, induced by various *Rhizobium leguminosarum* bv. *viciae* strains containing pXLGD4 (*hemA::lacZ*). All images were taken from plants 12 days after inoculation. **A**, A34 (wild-type [wt]) infection thread in vetch, initiating in curled outgrowth at base of a root hair. **B**, A34 infection thread in pea, initiating in curled root tip. **C**, A34 infection thread in vetch, initiating in curled outgrowth at base of a root hair and traveling through cortical cell layers. Arrow, accumulation of thread material at interface between two adjacent cortical cells. **D**, A91 (*nodE nodO* double mutant) aberrant infection in vetch, formed at base of a root hair. **E**, A91 inducing multiple aberrant infections (typical) in vetch. **F**, A91 aberrant infection in a pea root hair. **G**, Longitudinal section through a vetch nodule, formed after inoculation with A91. **H**, Detail of A91 infection thread in vetch, penetrating epidermis into outer cortical cell layers. **I**, A91 reversed thread in a vetch root hair. Arrow, site where infection initiated. **J**, A91 trapped in a curled outgrowth at base of a vetch root hair, forming an aberrant infection. **K**, A69 (*nodFELMNTO* deletion mutant) aberrant infection in vetch, at base of a root hair. **L**, A69 + pIJ1997 (cloned *nodO*) infection thread in vetch, formed at base of a root hair. Bars: (**A–D**, **F**, **H–K**) 20 μ m, (**E**, **G**) 200 μ m, (**L**) 10 μ m.

wall degradation could be factors contributing toward the abnormal infection thread phenotype found with the *R. leguminosarum* bv. *viciae* *nodE nodO* mutant.

Infection thread initiation by entrapped bacteria and infection thread propagation into an adjacent cell are situations that can be considered analogous (Kijne 1992; Ardourel et al. 1994). In both circumstances, plant cell wall degradation and cytoplasmic rearrangements are required before the cell can initiate and direct the growth of the thread (van Spronsen et al. 1994). Invasion of alfalfa root hairs by infective bacteria stimulates the accumulation of microtubules, which form a dense parallel array extending from the site of infection (Timmers et al. 1999). The microtubule cytoskeleton then reorganizes to link the infection thread tip to the nucleus, which apparently directs infection thread growth (Timmers et al. 1999; Nutman 1959). Further longitudinal microtubules run parallel to the infection thread. Defective regulation of these processes could account for the appearance of aberrant infection foci in root hair cells.

Comparison of the *nodE* mutant to the *nodE nodO* double mutant shows that an apparently normal infection phenotype can be restored with restoration of *nodO* function. When *nodO* is expressed, infection threads initiate normally and are no longer distended and enlarged. The Nod factors synthesized and secreted by the *nodE* and the *nodE nodO* mutants are identical; therefore, it is demonstrated that, in a *nodE* mutant background, *nodO* stimulates both infection thread initiation and subsequent development of the thread. Adding *nodO* to the *R. leguminosarum* bv. *viciae* deletion mutant could largely abolish the appearance of abnormal infection foci. However, the infection threads that were formed still looked abnormal. This shows that infection thread initiation and infection thread development are differentially influenced by *nodO*. Starting an infection thread is apparently easier than propagating it through many cell layers.

Our data would be consistent with a modified two-receptor model, where the second higher specificity receptor is primarily responsible for reorganization of the root cell cytoskeleton—both in root hair cells to form and direct infection threads, and in cortical cells to stimulate cytoplasmic bridge formation and associated partial cell wall degradation. In this instance, NodO could be functioning either to bypass the receptor, amplify the signal from the receptor, or enhance Nod-factor binding to the receptor. Based on the known biochemical properties of NodO, amplifying the signal is the most attractive option. Movement of specific ions across the plasma membrane and oscillations in intracellular calcium are thought to be significant in early Nod factor signaling (Ehrhardt et al. 1996; Felle et al. 1998, 1999; Cárdenas et al. 1999). Although the influence of NodO over these specific processes has yet to be determined, its capacity to form ion channels in membranes suggests a potential role. Weak Nod factor interaction with a stringent receptor could elicit partial ion fluxes that are not sufficient to activate downstream signaling events, whereas in the presence of NodO they would be.

Of course, NodO may have a more direct function. The images shown here suggest plant cell wall degradation may be impaired when infecting bacteria are secreting the wrong Nod factors. NodO may have a role in loosening the plant cell wall if endogenous plant enzymes have not been activated. However, even if the method by which NodO acts remains un-

known, the results here clearly show its function is to promote infection. This may account for the ability of *nodO* to extend the host range and nodule-forming capacity of rhizobia-secreting Nod factors that lack specific substitutions.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The strains used are described in Table 1. All strains are derivatives of the *R. leguminosarum* bv. *viciae* strain A34 (previously called 8401/pRL1JI), carrying *nod* mutant alleles on the pSYM pRL1JI. In some cases the strain numbers (but not the allele designations) have been altered from what was originally described. The plasmid pXLGD4 containing the constitutive *hemA* promoter fused to *lacZ* (Leong et al. 1985) was conjugated from *Escherichia coli* DH5 α into the *Rhizobium* strains by triparental matings with the helper pRK2013 (Figurski and Helinski 1979) selecting on TY (complete) medium (Beringer 1974) containing 400 $\mu\text{g ml}^{-1}$ streptomycin and 5 $\mu\text{g ml}^{-1}$ tetracycline. Plasmid pIJ1997 carries *nodO* cloned in the gentamicin-resistant vector pML123 (Crank and Downie 1994). Selection for the transfer of pML123 into A69/XLGD4 was made with streptomycin, tetracycline, and gentamicin (10 $\mu\text{g ml}^{-1}$) following a triparental mating as described above.

Nod factors were prepared by reverse phase chromatography as described previously (Firmin et al. 1993) with strain 8401 carrying the cloned *nod* gene region on pIJ1089 (Downie et al. 1985). The sample used contained a mixture of four components composed of tetrameric and pentameric *N*-acetylglucosamine oligomers carrying an acetyl group on C₆ of the terminal nonreducing glucosamine and either a C_{18:1} or C_{18:4} acyl group, essentially as described by Spaink et al. (1991).

Plant growth and inoculation.

For vetch experiments, freshly germinated seeds of *V. hirsuta* were grown on agar slopes (1% agar) in 2.5 $\times$ 15 cm tubes as described in Knight et al. (1986). Roots were inoculated with 200 μl of bacteria resuspended in sterile water corresponding to approximately 5 $\times$ 10³ bacteria. Where required, 0.5-ml aliquots of Nod factor, diluted in water to a final concentration of 10⁻⁷ M, were added to roots at 0, 2, 4, 6, and 8 days after inoculation. For pea experiments, freshly germinated seeds of *Pisum sativum* cv. Frisson were transferred to 10-cm-square petri dishes containing 1% agar medium as described by Knight et al. (1986). A hole had been cut in the side of the dish next to the seedling to allow outgrowth of the pea shoot. The primary root tip was excised to stimulate the growth of lateral roots. Once lateral roots had reached a length of 3 cm (after 3 to 4 days), they were inoculated as described above, except 100 μl of inoculum was used. Square dishes containing the seedlings were covered in black plastic to prevent exposure to light and held at a 45° angle with the hole in the dish uppermost. Seedlings were kept at room temperature in ambient light conditions.

Microscopy.

Microscopy was performed on root sections 12 days after inoculation. By this time, vetch roots were typically 10 cm long and pea roots 15 to 20 cm long. For vetch, all of the nodules and infected primordia and most of the infection threads

were usually present on the upper third of the root; therefore, this 3 to 4 cm of root was removed for microscopic analysis. For pea, 3- to 4-cm root sections were removed around the inoculation site.

Tissue was prepared essentially as described in Boivin et al. (1990); root segments were fixed in 0.2 M sodium cacodylate buffer (pH 7.2) containing 1.25% glutaraldehyde without vacuum at room temperature for 2 h. Segments were then washed twice for 15 min in 0.2 M sodium cacodylate buffer before staining overnight at room temperature in 0.2 M sodium cacodylate (pH 7.2), 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 0.1% 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). Root segments were then rinsed a further 3 times in the 0.2 M sodium cacodylate buffer and twice in sterile water before clearing for 1 min in a 10% solution of commercial bleach followed by a further five rinses in sterile water. Root segments were mounted in Fåhrus slides (Fåhrus 1957) containing sterile water and examined with a Zeiss Axiophot microscope. Images were recorded either onto 35-mm color-reversal photographic film or digitally recorded with computer-based imaging equipment. Where appropriate, stained and cleared root segments were embedded in 3% agarose and transverse sections approximately 800- μ m thick were made with a vibratome. Sections were mounted in water under a coverslip on multiwell microscope slides and examined under the microscope.

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