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The *nodH* and *nodQ* Host Range Genes of *Rhizobium meliloti* Behave as Avirulence Genes in *R. leguminosarum* bv. *viciae* and Determine Changes in the Production of Plant-Specific Extracellular Signals

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Transfer of a plasmid carrying the *Rhizobium meliloti* host range *nod* genes to *R. leguminosarum* bv. *viciae* 1) enabled *R. l.* bv. *viciae* to infect and nodulate alfalfa, the normal host of *R. meliloti*, and 2) strongly inhibited the ability of *R. l.* bv. *viciae* to infect and nodulate its normal host, common vetch. Using transposon Tn5-linked mutations, we showed that *R. meliloti nodH* and *nodQ* genes were responsible for the changes in the symbiotic specificity of *R. l.* bv. *viciae*. Using root hair deformation (Had) and root deformation (Tsr) bioassays on alfalfa and vetch, respectively, we demonstrated that the sterile supernatant solutions of cultures of the various *R. l.* bv. *viciae* derivatives, in which the *nod* genes had been induced, contained specific extracellular factors active on alfalfa or vetch. A correlation was observed between the specificity of the symbiotic behavior of bacterial cells in the nodulation tests and the specificity of their sterile filtrates in the bioassays, which indicates that in the *R. l.* bv. *viciae* hybrids the *R. meliloti nodH* and *nodQ* genes determine the changes in host range by helping to convert the vetch-specific signal(s) into an alfalfa-specific one(s). Both types of signals active on alfalfa or vetch are heat-stable, have a molecular mass less than 5,000 Da, and have some hydrophobic properties.

Additional keywords: host specificity, symbiosis.

The symbiotic interaction between fast-growing *Rhizobium* species and their leguminous host plants can be highly specific (Long 1984; Djordjevic *et al.* 1987). For example, *R. meliloti* Dangeard strains nodulate *Medicago*, *Melilotus*, and *Trigonella* species, while *R. leguminosarum* bv. *viciae* can form nodules on *Pisum* and *Vicia* species. In these symbiotic associations, the formation of nodules is a complex process involving, schematically, the following steps: root hair curling (Hac), infection thread formation (Inf) within root hairs, and nodule initiation and organogenesis (Nod) (Vincent 1980; Vasse and Truchet 1984; Debellé *et al.* 1986).

In *R. meliloti*, genetic, cytologic, and molecular studies have shown that several common and specific nodulation (*nod*) genes are clustered on approximately 16 kilobases (kb) of the pSym megaplasmid (Kondorosi *et al.* 1984; Debellé *et al.* 1986; Swanson *et al.* 1987). The *nodABC* genes are called common *nod* genes, because mutations in one of these genes can be complemented by cloned nodulation genes from another *Rhizobium* species without changing the host range (Kondorosi *et al.* 1984; Fisher *et al.* 1985). Three allelic regulatory *nodD* genes are responsible for activating the expression of *nod* genes in the presence of plant root exudates (Mulligan and Long 1985; Gottfert *et al.* 1986; Honma and Ausubel 1987). These *nodD* alleles could be involved in defining the host range by controlling *nod* gene expression as a function of the flavonoid composition of the legume host root exudates (Horvath *et al.* 1987; Györgypal *et al.* 1988).

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The following observations indicate that the *R. meliloti nodH*, *nodFEG*, and *nodQ* genes are host range determinants: 1) mutations in *nodH* and *nodFEG* genes cannot be fully complemented by the cloned nodulation genes from other *Rhizobium* species (Kondorosi *et al.* 1984; Debellé *et al.* 1986; Debellé and Sharma 1986; Horvath *et al.* 1986; Swanson *et al.* 1987); 2) in contrast to the wild type, strains having mutations in *nodFE*, *nodH*, and *nodQ* show altered infection and nodulation of the homologous host alfalfa and elicit root hair curling on heterologous hosts such as clover and vetch (Debellé *et* al. 1986; Horvath *et al.* 1986; Cervantes *et al.* 1989); and 3) the transfer of *R. meliloti* host range into *R. l.* bv. *trifolii* Jordan requires the *nodFE*, *nodH*, and *nodQ* genes (Debellé *et al.* 1988; Cervantes *et al.* 1989).

What could be the mechanisms by which these host range genes determine the plant-specific recognition? Van Brussel *et al.* (1986) and Zaat *et al.* (1987) have devised bioassays, based on vetch root deformation (Tsr phenotype) or root hair deformation (Had), that allow the detection of an extracellular factor(s) produced by *R. l.* bv. *viciae*. The *nodABC* genes are required for the production of such a factor(s). Using a Had bioassay with alfalfa, we have recently shown that the *nodH* host range gene of *R. meliloti* determines the production of an alfalfa-specific extracellular signal (Faucher *et al.* 1988).

In this paper, we report the effect of the transfer of the *R. meliloti* host range genes into *R. l.* bv. *viciae*. The *R. l.* bv. *viciae* transconjugants acquired the ability to infect and nodulate alfalfa and showed a decreased infectivity for their normal host, common vetch. Parallel changes in the Had and Tsr specificity of the sterile filtrates of their cultures were observed. The *nodH* and *nodQ* genes were

shown to play an essential role in these changes of specificity. We also discuss how these results support, and extend to *R. l.* bv. *viciae*, the model that we previously proposed for the functions of *R. meliloti nod* genes. These results show that the *nodABC* genes might determine the production of a "common" low molecular mass heat-stable factor, which would be modified by the host range gene products into a specific signal(s).

MATERIALS AND METHODS

Microbiological techniques. Bacterial strains and plasmids are described in Table 1 and Figure 1. Conditions used for bacterial growth and conjugation experiments have been described elsewhere (Truchet *et al.* 1985; Debellé *et al.* 1988). The introduction of *R. meliloti nod*::Tn5 and *nod*::Tn5-233 insertions into pGMI515 was performed by marker exchange experiments as follows. The self-transmissible plasmid pGMI515 was introduced into the various *R. meliloti* strains carrying *nod*::Tn5 insertions (see Table 1). The recombinant pGMI515 derivatives carrying the *nod*::Tn5 insertions were selected by mating with *Escherichia coli* (Migula) Castellani & Chalmers GMI3540 and selecting the plasmid transfer in the presence of tetracycline (10 μg/ml), kanamycin (40 μg/ml) for Tn5, gentamycin (25 μg/ml), and spectinomycin (100 μg/ml)

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Origin
Escherichia coli		
ED8767	*supE supF met hsdS recA56*	Faucher *et al.* 1988
GMI3540	Spontaneous Nal^r derivative of ED8767	Faucher *et al.* 1988
Rhizobium meliloti		
RCR2011	= SU47. Wild type; Nod$^+$ Fix$^+$ on *Medicago sativa*	Rosenberg *et al.* 1981
GMI5390	Region IIa *nod2412*::Tn5	Debellé *et al.* 1986
GMI5514	Region IIa *nod2110*::Tn5	*idem*
GMI5388	*nodQ115*::Tn5	*idem*
GMI5392	*nodQ2402*::Tn5	*idem*
GMI5381	*nodE2309*::Tn5	*idem*
GMI5378	*nodF2407*::Tn5	*idem*
GMI5598	*nodG314*::Tn5	Faucher *et al.* 1988
GMI5429	*nodH2219*::Tn5	Debellé *et al.* 1986
GMI5375	*nodH2121*::Tn5	*idem*
GMI5624	Δ(*nodEFGH*)DEK10	Debellé *et al.* 1988
Rm1021	Spontaneous Str^r derivative of SU47	Long *et al.* 1982
RmD3-1	Derivative of 1021; *nodD$_3$*::Tn5-233 Sp^r Gm^r	Honma and Ausubel 1987
JT314	Derivative of 1021; *nodG314*::Tn5	Swanson *et al.* 1987
R. leguminosarum bv. *viciae*		
248	Wild type; Nod$^+$ Fix$^+$ on *Vicia hirsuta* and *V. sativa* subsp. *nigra*; pRL1JI	Van Brussel *et al.* 1986
RBL1387	248 cured of its pRL1JI	*idem*
RBL1409	RBL1387 pRL1JI*nodA*::Tn5	Zaat *et al.* 1987
RBL1412	RBL1387 pRL1JI*nodC*::Tn5	*idem*
R. l. bv. *trifolii*		
ANU843	Wild type; Nod$^+$ Fix$^+$ on *Trifolium repens*	Schofield *et al.* 1983
ANU845	ANU843 cured of its symbiotic plasmid	*idem*
IncPl plasmids		
RP4	Tc^r Ap^r Km^r Tra$^+$	Datta *et al.* 1971
pGMI515	RP4′ (*in vitro*), Tc^r Ap^r (Fig. 1)	Truchet *et al.* 1985

[a]Nal = nalidixic acid, Str = streptomycin, Sp = spectinomycin, Gm = gentamycin, Tc = tetracycline, Ap = ampicillin, Km = kanamycin, and ^{r}= resistant.

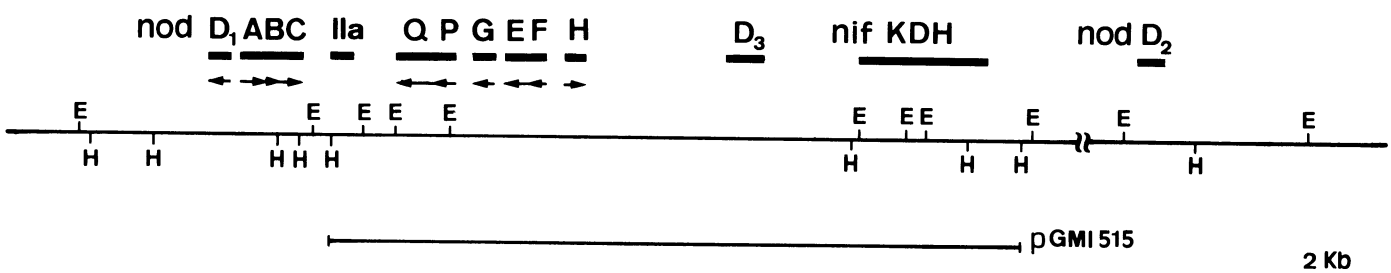

Fig. 1. Physical and genetic map of the nodulation (*nod*) region of *Rhizobium meliloti* 2011. The horizontal line represents the restriction map (E, *Eco*RI; H, *Hin*dIII). The plasmid pGMI515 is shown below the physical map. Above the physical map, the arrows indicate the direction of transcription of the *nod* genes.

for Tn5-233. The pGMI515 derivatives were then mated from *E. coli* GMI3540 into the appropriate *R. l.* bv. *viciae* strains. The *R. l.* bv. *trifolii* ANU843 derivatives containing the various *nod*::Tn5 insertions and the *nod* deletion in pGMI515 were described previously (Debellé *et al.* 1988).

DNA biochemistry. The pSym megaplasmid and the other plasmids were visualized after agarose gel electrophoresis (Rosenberg *et al.* 1981). The location of the *nod*::Tn5 insertions, after their introduction into the appropriate plasmids, was checked by restriction fragment analysis. Restriction endonucleases were purchased from Boehringer Mannheim, London, U.K., and New England Biolabs, Bishops' Stortford, Herts, U.K. Plasmid isolation, digestion by restriction enzymes, and agarose gel electrophoresis were conducted by standard procedures (Maniatis *et al.* 1982).

Plant cultivation. Seeds of *Medicago sativa* L. cv. Gemini were obtained from Tourneur Frères (F77120 Coulommiers, France), and seeds of *V. sativa* subsp. *nigra* and of *V. hirsuta* were provided by G. Genier (Station d'Amélioration des Plantes Fourragères, INRA, F86600, Lusignan, France). Alfalfa and common vetch nodulation tests were performed as previously described (Truchet *et al.* 1984) on Jensen agar slopes (Vincent 1970), with a *Rhizobium* inoculum of approximately 10^5 bacteria per tube; 40 plants were used for each strain. Light microscopy of presumptive nodules was performed as described elsewhere (Truchet *et al.* 1989). Reisolation of bacteria from vetch and alfalfa nodules was performed as already described (Faucher *et al.* 1988). Root hair infection phenotypes were analyzed by light microscopy as described previously (Vasse and Truchet 1984); 10 seedlings were examined for each strain.

Tsr and Had bioassays. The vetch Tsr bioassay was conducted as described previously (Van Brussel *et al.* 1986). Twenty plants were used for *R. l.* bv. *viciae* strains and 10 plants for *R. l.* bv. *trifolii*. The significance of length differences between two sets of plant roots was calculated by the test of comparison of means (Scheffe 1959). Sterile supernatant fluids from flavonoid-induced *R. l.* bv. *viciae* cultures were obtained using low density cultures (approximately 5×10^5 bacteria per milliliter) as already described (Faucher *et al.* 1988). The flavonoid inducers naringenin or luteolin were used at a final concentration of 1.5 μM.

The vetch and alfalfa Had bioassays were performed as follows. Five germinated seeds with rootlets about 1-cm long were aseptically transferred onto Jensen agar plates. The plates were sealed with Parafilm and placed vertically for 24 hr (with alfalfa) or 48 hr (with vetch) in a plant growth chamber (at 22° C with a 16-hr light period, a relative humidity of 75%, Osram vFluora L 77 as the type of light, and light intensity at the level of the top of the plates of 30 $\mu E \cdot m^{-2} \cdot s^{-1}$) to allow plant growth and root hair development. Then 2 ml of a *Rhizobium* sterile supernatant was poured to cover the alfalfa root system (5 ml with vetch), and after 30 min, the excess liquid was removed. A further incubation was performed for 16 to 24 hr in the plant growth chamber. The roots of the five plants were transferred between slide and cover slip and observed by bright field microscopy after staining by methylene blue (Vasse and Truchet 1984). For each strain, two series of 10 plants were prepared with two independent preparations of sterile supernatants.

Preliminary fractionation of bacterial filtrates. The sterile supernatant (50 ml) of a flavonoid-induced culture was passed successively through ultrafilters with decreasing pore diameter using an ultrafiltration cell (Type TCF10, Amicon Corp., Danvers, MA). YM5 and YM2 filters (Diaflo ultrafiltration membranes, Amicon) allowed permeation of molecules with an apparent molecular mass of less than 5,000 and 1,000 Da, respectively. Each desalted fraction was adjusted to the initial volume with Jensen medium before Had assays.

Another type of fractionation was done by passing 5 ml of sterile supernatant through a C18 Sep-Pak reversed-phase cartridge (Waters Associates, Milford, MA) and eluting with ethanol. The ethanol eluate was dried, and the dry extract was dissolved in Jensen medium and adjusted to the original volume before Had assays.

RESULTS

Symbiotic behavior of *R. l.* bv. *viciae* (pGMI515) on alfalfa. The pGMI515 plasmid is an RP4-derivative that carries the *R. meliloti* RCR2011 *nod* region II (including the *nodPQ* genes), the host range genes *nodFEG* and *nodH*, and the $nodD_3$ regulatory gene. It does not carry the common *nodABC* genes or the regulatory $nodD_1$ gene (see Fig. 1). This plasmid was previously shown to be appropriate for *in planta* studies because of its stability in *Rhizobium* (Debellé *et al.* 1988). *R. l.* bv. *viciae* 248, as well as its $RP4^+$ derivative, neither infected (Hac^- Inf^-) nor nodulated (Nod^-) alfalfa. In contrast, *R. l.* bv. *viciae* 248 carrying the pGMI515 plasmid elicited the formation of root-derived structures (Fig. 2A), which morphologically resembled ineffective (Fix^-) nodules.

Cytological studies by using a clearing procedure (Truchet *et al.* 1989) revealed that some of these root deformations had the histological traits of genuine nodules. No bacteria could be reisolated from these surface-sterilized root-derived structures. Light microscopy of the roots 4–7 days after inoculation showed that *R. l.* bv. *viciae* (pGMI515) elicited strong reactions on root hairs of alfalfa seedlings. Numerous root hairs had marked curling, most of them without an infection focus in the center of the curl (Hac^s) and some of them being genuine shepherds' crooks with a refractile infection focus (Hac^+, Fig. 3A). The few infection threads seen within small root hairs did not stain with methylene blue at 7–11 days after inoculation (Fig. 3B). The introduction of pGMI515 into *R. l.* bv. *viciae* enables this bacterium to infect alfalfa root hairs and to elicit nodule formation on alfalfa roots.

Symbiotic behavior of *R. l.* bv. *viciae* (pGMI515) on vetch. The wild-type strain *R. l.* bv. *viciae* 248, as well as its $RP4^+$ derivative, infected the homologous host *V. s.* subsp. *nigra* effectively. Four days after inoculation numerous shepherds' crooks were formed, and infection threads within root hairs were stained by methylene blue (Fig. 3C). In contrast, with the *R. l.* bv. *viciae* $pGMI515^+$ transconjugant the vetch infection was abnormal. Root hairs with a marked curling were rare, and most of the

curled hairs did not show an infection focus (Hac^s) (Fig. 3D). In addition, infection threads were rare, short, and often distorted, and only a small proportion were stained (Fig. 3E).

In Figure 2B, it can be clearly seen that the presence of pGMI515 inhibited the nodulation of *V. s.* subsp. *nigra*. In three of the five nodules collected, the reisolated bacteria had lost the plasmid pGMI515, as indicated by the loss of tetracycline-resistance and by plasmid profile studies. This suggests that the plants selected the *R. l.* bv. *viciae* that had lost the infection-inhibitory plasmid pGMI515. To test the generality of this inhibitory effect, strains 248 and 248(pGMI515) were inoculated on another species of vetch, *V. hirsuta*; similar results were obtained (data not shown). Thus, the presence of pGMI515 in *R. l.* bv. *viciae* results in a strong inhibition of infection and nodulation of the homologous host, vetch. That this inhibition was not due to a curing of the *R. l.* bv. *viciae* pSym plasmid by pGMI515 was shown by plasmid profile experiments (data not shown).

***R. meliloti* genes determining the symbiotic changes.** To know which *R. meliloti* genes are responsible for the symbiotic changes of *R. l.* bv. *viciae*, we introduced Tn5 insertions in the *R. meliloti nod* genes carried by pGMI515. The various mutants were tested on vetch (*V. s.* subsp. *nigra* and *V. hirsuta*) and alfalfa. Figure 2B shows that mutations in either *nodH* or *nodQ* restored a normal common vetch nodulation. Tn5 insertions in *nodD$_3$*, *nodG*, and region *nod* IIa did not clearly suppress the inhibition of vetch nodulation. Mutations in *nodFE* genes had an intermediary effect. Similar results were obtained with *V. hirsuta* (data not shown).

Cytology of common vetch infection revealed that *nodH* or *nodQ* mutants were Hac$^+$ Inf$^+$ at four days after inoculation, as were the *R. l.* bv. *viciae* 248 wild-type strain and its RP4$^+$ derivative (compare Figs. 3C and 3F). Mutants in the *nod* IIa locus *nodD$_3$* and *nodG* genes had an infection phenotype similar to the *R. l.* bv. *viciae* (pGMI515) strain. Insertions in *nodFE* resulted in an intermediate phenotype; these mutants were Hac$^+$ Inf$^+$, but to a lesser extent than *nodH* and *nodQ*.

To check whether mutations in *nodH* or *nodQ* were restoring vetch nodulation by causing an instability of pGMI515, bacteria were reisolated from 15 nodules of vetch seedlings inoculated by mutants carrying these mutations: all 50 clones isolated from each nodule were tetracycline-resistant. Four representative clones had the plasmid profile of pGMI515$^+$ transconjugants.

It can be seen in Figure 2A that whereas Tn5 insertions in *nodD$_3$*, *nodFEG*, and *nod* locus IIa did not significantly

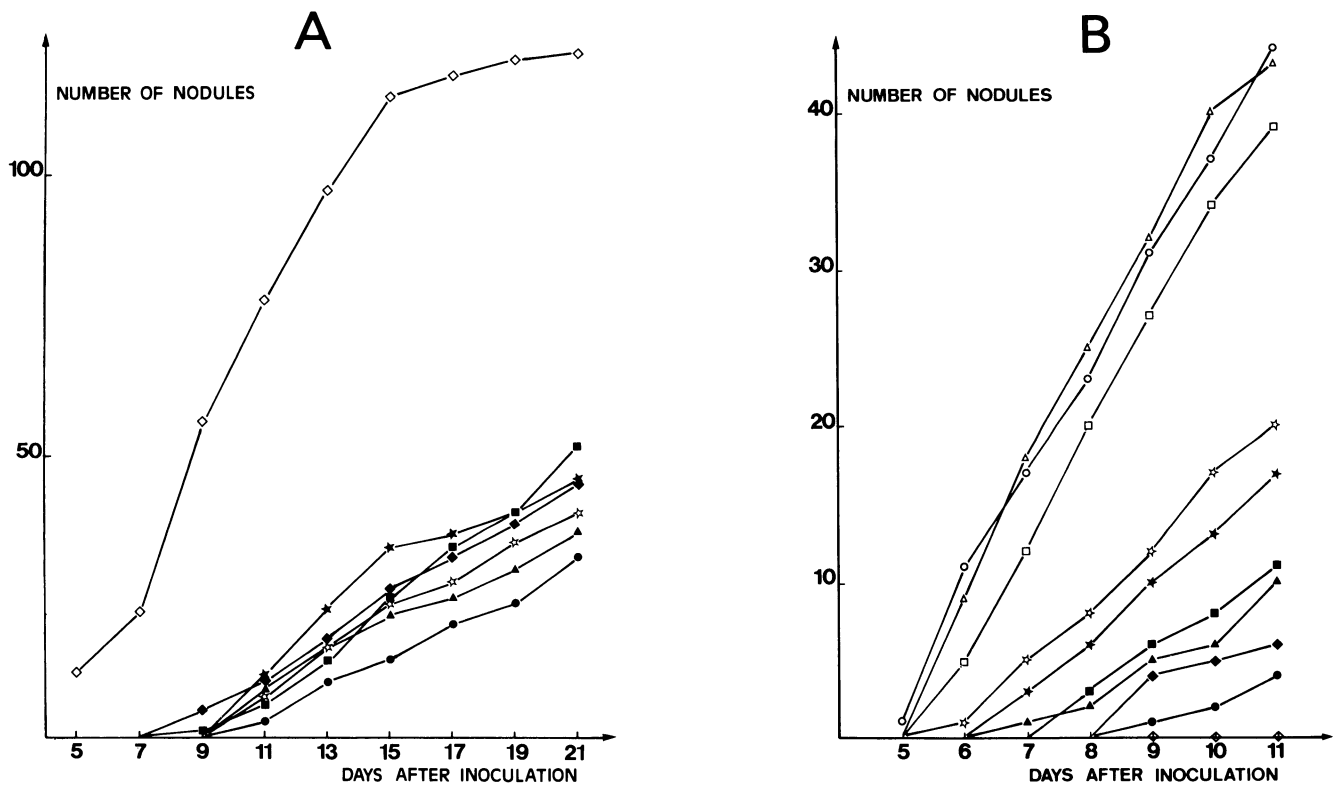

Fig. 2. Nodulation kinetics of *Rhizobium leguminosarum* bv. *viciae* transconjugant strains on *Medicago sativa* (**A**) and *Vicia sativa* subsp. *nigra* (**B**). Each point represents the mean value of 20 plants from two independent experiments. In **A**, the number of nodules represent the total number of observed root-derived structures, some of them being genuine nodules. On alfalfa, no nodule could be observed even 4 wk after inoculation with the following strains: *R. l.* bv. *viciae* 248 harboring the plasmid RP4 (= vector) or pGMI515 derivatives containing a Tn5 insertion in *nodH* or *nodQ*. Symbols are given as follows. *R. meliloti* 2011 (◇) and *R. l.* bv. *viciae* 248 harboring the following plasmids: RP4 (○), pGMI515 (●), pGMI515 *nodD$_3$*::Tn5-233 (■), pGMI515 *nodH2121*::Tn5 (△), pGMI515 *nodF2407*::Tn5 (☆), pGMI515 *nodE2309*::Tn5 (★), pGMI515 *nodG314*::Tn5 (▲), pGMI515 *nodQ115*::Tn5 (□), and pGMI515 region IIa *nod2412*::Tn5 (◆).

affect the induction of nodule formation on alfalfa, insertions in *nodH* or *nodQ* completely suppressed nodulation. Tn5 insertions in *nodA* (RBL1409) or in *nodC* (RBL1412) genes of *R. l.* bv. *viciae* harboring the plasmid pGMI515 also suppressed nodulation on alfalfa. Cytology of alfalfa roots showed that *nodH* and *nodQ* mutants had

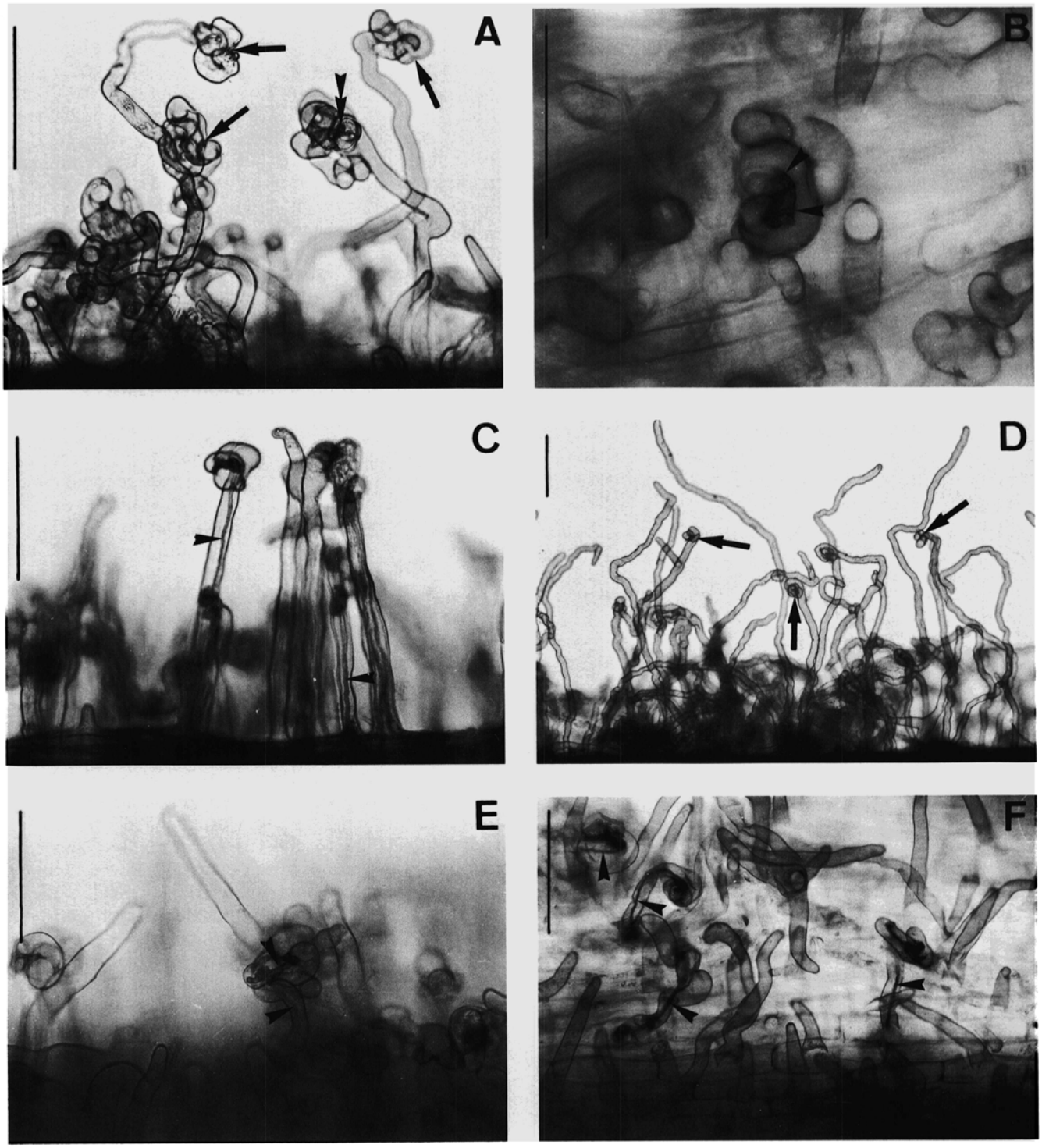

Fig. 3. Infection phenotypes. **A** and **B**, Alfalfa inoculated with *Rhizobium leguminosarum* bv. *viciae* (pGMI515). **A**, Hac^s (arrows) and Hac^+ (double arrowheads) phenotypes. **B**, Infection thread (arrowheads) in a curled root hair. **C**, Common vetch inoculated with the control strain *R. l.* bv. *viciae* (RP4) 4 days after inoculation. Hac^+ Inf^+ phenotype. Arrowheads point to infection threads. **D** and **E**, Common vetch inoculated with *R. l.* bv. *viciae* (pGMI515). **D**, Hac^s phenotype (arrows). **E**, Inf^+ phenotype, unstained infection thread (arrowheads). **F**, Common vetch inoculated by *R. l.* bv. *viciae* (pGMI515 *nodQ115*::Tn5) 4 days after inoculation. Hac^+ Inf^+ phenotype. Arrowheads point to infection threads. In all the micrographs, the bar equals 100 μm.

lost the ability to infect root hairs and were Hac$^-$ Inf$^-$, like the *R. l.* bv. *viciae* 248 (RP4) control strain. We can conclude that *nodH* and *nodQ* genes are responsible for the major symbiotic changes on both the homologous host vetch and the nonhomologous host alfalfa.

Tsr reaction on common vetch of *R. l.* bv. *viciae* hybrids. What could be the mechanisms by which *R. meliloti nodH* and *nodQ* genes modify the symbiotic behavior of *R. l.* bv. *viciae*? By comparison with nodulation assays, the common vetch Tsr (thick and short roots) bioassay has the advantage that it can be performed either with bacterial cultures or with sterile filtrates of cultures grown in conditions of *nod* induction (Van Brussel *et al.* 1986; Zaat *et al.* 1987; Faucher *et al.* 1988). This possibility obviously would facilitate the detection and isolation of symbiotic signals.

We first studied the influence of the *R. meliloti* host range genes on the Tsr-inducing ability of *R. l.* bv. *viciae* by inoculating vetch seedlings with bacterial cultures. The Tsr reaction was estimated by measuring the seedling root length, and the significance of the observed differences was estimated statistically. Results are given in Table 2. The introduction of pGMI515 into *R. l.* bv. *viciae* 248 significantly decreased the Tsr effect. Tn5-induced mutations in *nodH* or *nodQ* carried by pGMI515 restored fully the Tsr reaction, which was similar to the one induced by the *R. l.* bv. *viciae* wild-type strain. A Tn5-233 insertion in the *nod*D_3 gene significantly restored a slight Tsr reaction. Insertions into other pGMI515 *nod* genes did not restore the Tsr reaction: the Tsr phenotype was not significantly different from the one induced by the pGMI515$^+$ transconjugant.

Table 2. Tsr phenotype on *Vicia sativa* subsp. *nigra* inoculated with strains derived from *Rhizobium leguminosarum* bv. *viciae* and *R. l.* bv. *trifolii*

Recipient strain	Plasmid	Mean root length[a] (cm)
Medium		9.7 ± 1.2[b]
R. l. bv. *viciae* 248		
(Wild type)	None	3.9 ± 0.7[c]
	Vector = RP4	3.9 ± 0.5[c]
	pGMI515	7.3 ± 1.2[d]
	pGMI515 *nodH2121*::Tn5	4.0 ± 0.5[c]
	pGMI515 *nodH2219*::Tn5	3.9 ± 0.4[c]
	pGMI515 *nodQ115*::Tn5	4.2 ± 0.5[c]
	pGMI515 *nodF2407*::Tn5	6.6 ± 1.8[d]
	pGMI515 *nodE2309*::Tn5	6.0 ± 1.2[d]
	pGMI515 *nodG314*::Tn5	6.5 ± 1.2[d]
	pGMI515 *nod*IIa*2110*::Tn5	7.8 ± 1.1[d]
	pGMI515 *nod*IIa*2412*::Tn5	8.2 ± 1.3[d]
	pGMI515 *nod*D_3::Tn5-233	4.9 ± 0.9[c]
(*nodA*::Tn5)	None	9.9 ± 0.6[b]
	pGMI515	9.0 ± 0.8[b]
(*nodC*::Tn5)	None	10.1 ± 0.9[b]
	pGMI515	9.3 ± 0.7[b]
R. l. bv. *trifolii* ANU843		
(Wild type)	None	4.9 ± 1.0[f]
	Vector = RP4	4.4 ± 0.8[f]
	pGMI515	7.4 ± 0.6[g]
	pGMI515 *nodH2121*::Tn5	4.8 ± 0.6[f]
	pGMI515 *nodQ115*::Tn5	4.1 ± 0.2[f]
	pGMI515 Δ*nodEFGH*	4.4 ± 0.5[f]
ANU845 (pSym$^-$)	None	8.3 ± 1.1[g]

[a] Results were analyzed by comparison of means.
[b,c,d,e,f,g] Values followed by the same letter did not differ significantly ($P < 0.01$).

We had shown earlier that the introduction of *R. meliloti* host range genes into *R. l.* bv. *trifolii* drastically inhibited its ability to infect and nodulate its homologous host white clover (Debellé *et al.* 1988). As in the case of *R. l.* bv. *viciae*, these changes were determined by *nodH*, *nodFEG*, and *nodQ* (region IIb). Van Brussel *et al.* (1986) reported that Nod$^+$ strains of *R. l.* bv. *trifolii* are also able to elicit a Tsr reaction on common vetch. We checked the Tsr phenotype of *R. l.* bv. *trifolii* ANU843 and of its transconjugants carrying pGMI515 or pGMI515 derivatives having a Tn5 insertion in the various *nod* genes. The results are given in Table 2. In *R. l.* bv. *trifolii*, as in *R. l.* bv. *viciae*, the *R. meliloti nodH* and *nodQ* genes determine an inhibition of the Tsr effect on vetch.

Modifications in the production of extracellular symbiotic signals. Instead of bacterial suspensions, the vetch Tsr assay can be performed by using the sterile supernatant of a culture that has been grown with an appropriate *nod*-inducer flavonoid (Zaat *et al.* 1987; Faucher *et al.* 1988). We thus checked the Tsr reaction of the supernatants of the various hybrid strains, after induction with naringenin for 24 hours.

To study the specificity of the supernatants, we also checked them on alfalfa using a Had bioassay. The previously described alfalfa Had bioassay (Faucher *et al.* 1988), based on growing the seedling in the glass slide assembly of Fåhraeus (1957), was replaced by a method in which seedlings were grown on agar plates; a similar method has been devised independently by Dazzo *et al.* (1988) with clover. This new assay was quicker (18 hr instead of 6 days) and required smaller volumes of supernatants (2 ml instead of 20 ml). Moreover, screening for the Had phenotype was easier because hair branching was the most frequent deformation, as in the assay for white clover proposed by Bhuvaneswari and Solheim (1985). According to assays, branching affected only a part of the root, including the growing root hair zone and the beginning of the mature hair zone, or was extended over the whole root system (Figs. 4A and 4B). Occasionally branched root hairs could also be loosely undulated. While the Tsr reaction could be measured and analyzed statistically, the Had reaction was not easy to quantitate; however, the differences between straight and branched root hairs were very striking as can be seen in Figure 4. As a control, branching was never observed on plants treated with the Jensen medium alone, the Jensen medium containing the flavonoid inducer, or with the supernatant of uninduced rhizobial cultures (Figs. 4C and 4D).

Results of bioassays are given in Table 3. Both vetch Tsr and alfalfa Had activities were lost when either *nodA* or *nodC* was inactivated, showing that the supernatant activities on both hosts were dependent on the *R. l.* bv. *viciae* common *nodABC* operon. Supernatant activities required the addition of a flavonoid inducer to the rhizobial culture. It is worth noting that sterile filtrates containing the same *nod* inducer, naringenin, elicited vetch or alfalfa reactions according to the bacterial strain used. This shows

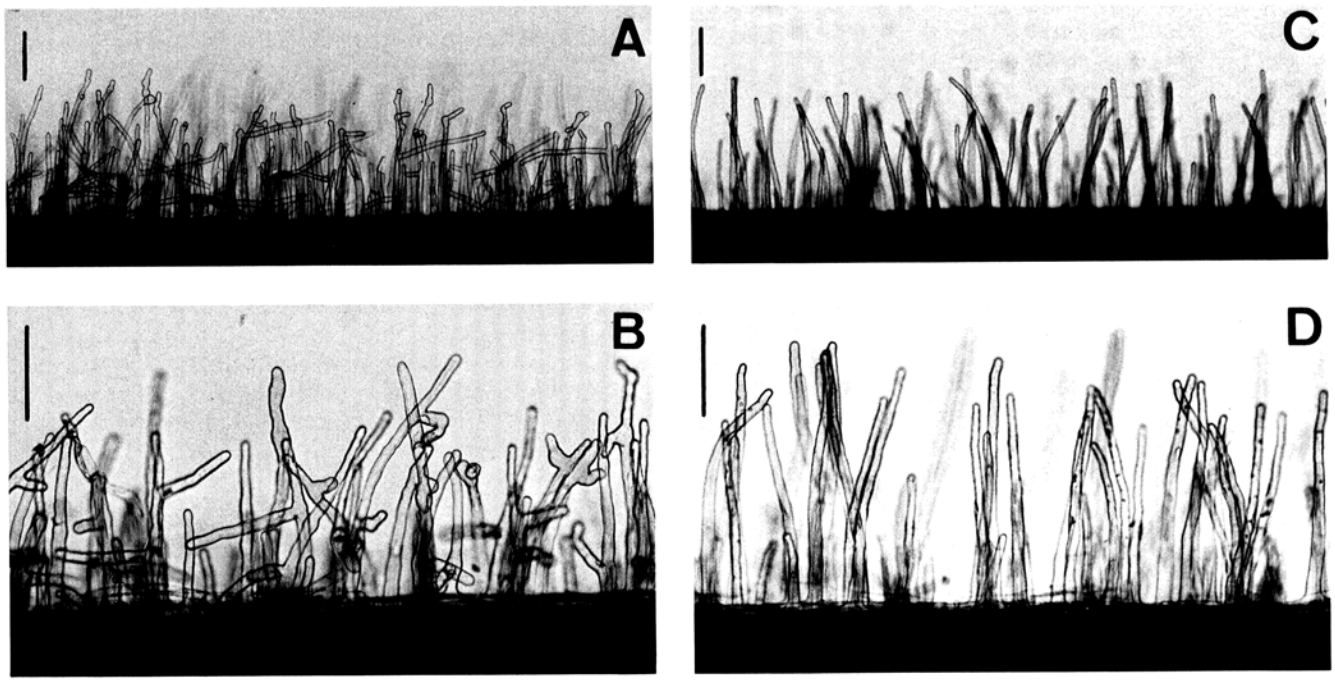

Fig. 4. Alfalfa Had bioassays. **A** and **B**, Hair branching induced by the sterile supernatants from luteolin-induced *Rhizobium meliloti* 2011 (**A**) and naringenin-induced *R. leguminosarum* bv. *viciae* (pGMI515) (**B**). **C** and **D**, Straight root hairs from control plants inoculated with Jensen medium containing the flavonoid inducer naringenin (**C**) or with the supernatant of noninduced *R. l.* bv. *viciae* (pGMI515) (**D**). In all the micrographs, the bar equals 100 μm.

Table 3. Had phenotype on *Medicago sativa* and Tsr phenotype on *Vicia sativa* subsp. *nigra* of sterile supernatants obtained after flavonoid induction of cultures of *Rhizobium leguminosarum* bv. *viciae* 248 and its derivatives

Recipient strain	Plasmid	Inducer[a]	Alfalfa Had[b]	Vetch Mean root length[c] (cm)
Medium			−	10.1 ± 1.2[d]
		N	−	10.1 ± 1.1[d]
Wild type	None		−	9.5 ± 0.9[d]
	None	N	−	5.0 ± 1.3[e]
	None	L	−	ND
	RP4	N	−	4.9 ± 0.6[e]
	pGMI515		−	8.4 ± 1.2[d]
	pGMI515	N	+	8.5 ± 1.3[d]
	pGMI515	L	+	ND
	pGMI515 *nodH2121*::Tn5		−	9.1 ± 1.5[d]
	pGMI515 *nodH2121*::Tn5	N	−	6.3 ± 1.5[f]
	pGMI515 *nodH2121*::Tn5	L	−	ND
	pGMI515 *nodQ115*::Tn5		−	10.7 ± 0.5[d]
	pGMI515 *nodQ115*::Tn5	N	−	6.3 ± 0.7[f]
	pGMI515 *nodQ2402*::Tn5	N	−	6.1 ± 0.7[f]
	pGMI515 *nodE2309*::Tn5	N	+	7.2 ± 1.0[g]
	pGMI515 *nodG314*::Tn5	N	+	7.1 ± 0.7[g]
	pGMI515 *nod*IIa*2412*::Tn5	N	+	8.5 ± 0.9[d]
	PGMI515 *nodD*$_3$::Tn5-233	N	+	6.5 ± 0.8[f]
nodA::Tn5	None	N	−	ND
	pGMI515		−	9.1 ± 0.7[d]
	pGMI515	N	−	8.6 ± 1.1[d]
nodC::Tn5	None	N	−	ND
	pGMI515		−	8.9 ± 0.9[d]
	pGMI515	N	−	9.9 ± 0.7[d]

[a] Supernatants were obtained from cultures induced by naringenin (N) or by luteolin (L). Control supernatants were prepared without any flavonoid.
[b] Had$^+$ and Had$^-$ phenotypes correspond to those described in Figure 4.
[c] Results were analyzed by comparison of means. ND = not determined.
[d, e, f, g] Values followed by the same letter did not differ significantly ($P < 0.01$).

that the specificity of the symbiotic signals depends on the *Rhizobium nod* genes and not on the flavonoid inducer. Whereas the supernatant of the *R. l.* bv. *viciae* wild-type strain was Tsr^+ on vetch and Had^- on alfalfa, the supernatant of the pGMI515$^+$ transconjugant was Tsr^- on vetch and Had^+ on alfalfa. When *R. l.* bv. *viciae* derivatives carrying various *R. meliloti nod*::Tn5 insertions in the pGMI515 were tested, only the *nodH* and *nodQ* mutations significantly increased the Tsr effect and suppressed the alfalfa Had reaction. Thus, the correlation between the specificity of the symbiotic behavior of the various bacterial strains and the specificity shown by their sterile supernatants with the vetch Tsr and alfalfa Had bioassays is very clear. Filtrates of *Rhizobium* cultures that were Tsr^+ on vetch were shown to be also Had^+ on the same host.

The vetch Tsr and the vetch or alfalfa Had activities of the various supernatants were not detectably altered after autoclaving for 30 min at 110° C, showing that the extracellular Had factors are heat-stable. When supernatants active on vetch or on alfalfa were filtered with ultrafiltration membranes, the largest amount of Had activity was found to pass through a 5,000-Da cutoff membrane (YM5), but was retained over a 1,000-Da cutoff one (YM2). Reversed-phase chromatography with a C18 Sep-Pak cartridge showed that both Had factors, active on alfalfa or vetch, have some hydrophobic properties, because the supernatant Had activity was retained on the C18 phase and could be eluted with ethanol.

DISCUSSION

The transfer of plasmid pGMI515 (containing the *R. meliloti* host range genes) into *R. l.* bv. *viciae* modifies its symbiotic behavior in two ways: the hybrid strain has acquired the ability to infect and nodulate alfalfa, and it has a decreased ability to infect and nodulate its original host, common vetch. A similar type of interaction between symbiotic genes of different species was first reported by Beynon *et al.* (1980). The *R. meliloti* genes that are responsible for both types of changes are *nodH* and *nodQ*. *R. meliloti nodH* and *nodQ* mutants have an extended host range and are Hac^+ Inf^+ on common vetch. Thus, the *R. meliloti nodH* and *nodQ* genes, when present together, prevent *R. l.* bv. *viciae* and *R. meliloti* from infecting vetch. However, these two genes have different symbiotic roles, because for nodulating alfalfa, *nodH* is required in both *R. l.* bv. *viciae* and *R. meliloti*, whereas *nodQ* is essential in *R. l.* bv. *viciae* but not in *R. meliloti*.

The transfer of pGMI515 into *R. l.* bv. *trifolii* had also been shown to modify the symbiotic behavior of the recipient strain in two ways: by enabling it to nodulate and infect alfalfa and by suppressing its ability to infect and nodulate its original host, white clover (Debellé *et al.* 1988). The *R. meliloti* genes involved in this type of "dominance" were *nodH*, *nodFE*, and *nodQ*.

R. meliloti host range genes such as *nodFE*, *nodH*, and *nodQ* behave in *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* as the avirulence (*avr*) genes of phytopathogenic bacteria, that is they suppress or inhibit the virulence of recipient strains on some of their hosts (Staskawicz *et al.* 1984; Gabriel *et al.* 1986). In the case of *nodFE*, for which alleles are present in *R. l.* bv. *trifolii* as in *R. meliloti*, the *R. meliloti nodFE* genes are dominant. The *R. meliloti nodH* and *nodQ* genes seem not to have alleles in *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* (Rodriguez-Quinones *et al.* 1987; Surin and Downie 1988; Schwedock and Long 1989), and they are thus epistatic on *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* host-specific *nod* genes. Thus the host range genes of *R. meliloti* can be dominant or epistatic, as are the *avr* genes. This formal analogy might reflect similarities between the molecular mechanisms of host specificity in *Rhizobium* and phytopathogenic *Pseudomonas* and *Xanthomonas*.

The recent development of various biological assays has revealed that common *nod* genes of *R. l.* bv. *viciae* and *R. meliloti* determine the production of extracellular compounds. The use of Tsr and Had assays on common vetch has shown that in *R. l.* bv. *viciae*, the *nodD* and *nodABC* genes are required for the production of Tsr and Had factors (Van Brussel *et al.* 1986; Zaat *et al.* 1987). Indeed, *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* strains cured of their pSym and containing the cloned *nodDABC* genes allow the production of Tsr and Had factors when cocultivated with their host plants (Canter Cremers *et al.* 1986; A. A. N. Van Brussel, personal communication). Both Tsr and Had factors are heat-stable and of low molecular weight.

It seems likely that the same factor or factors are responsible for eliciting the Tsr and Had vetch reactions (Zaat *et al.* 1987). In an *R. meliloti* strain deleted of all the *nod* genes, the cloned *R. meliloti* $nodD_1ABC$ genes are sufficient to generate the production of a HadV factor(s) active on vetch (Faucher *et al.* 1988). Thus, the common *nodABC* genes of both *R. l.* bv. *viciae* and *R. meliloti* determine the production of HadV factors; we propose to call these factors HadVc (c for common). The *R. meliloti nodAB* genes are involved in generating compounds that stimulate mitosis of plant protoplasts. These compounds are also heat-stable, of low molecular weight, and partially hydrophobic (Schmidt *et al.* 1988). All these common *nod* gene-mediated factors that have been detected using various bioassays might belong to the same class of "common HadVc" molecules.

Using an alfalfa Had bioassay, we previously showed that in *R. meliloti* the host range *nodH* gene determines the production of an alfalfa-specific HadA extracellular signal (Faucher *et al.* 1988). We have now shown that the *R. meliloti nodQ* gene, when introduced into *R. l.* bv. *viciae*, is also involved in generating an alfalfa-specific HadA signal. Thus, at least two *R. meliloti* host range genes are involved in generating host-specific signals. The HadA signals, like the HadVc signals, are heat-stable, of low molecular weight, and partially hydrophobic. The changes of symbiotic specificity of *R. l.* bv. *viciae* hybrids carrying various combinations of *R. meliloti nodH* and *nodQ* genes strictly parallel the changes in the Tsr and Had specificity of sterile supernatants of flavonoid-induced cultures, which indicates that these two genes might contribute to host specificity by mediating the production of specific extracellular signals.

Mutations in *R. l.* bv. *viciae nodA* or *nodC* genes resulted, for the various hybrid strains, in the loss of the ability to infect alfalfa and vetch on one hand and to produce

extracellular factors active on alfalfa or vetch on the other. How can the epistatic effect of *nodABC* on *nodH* and *nodQ* be interpreted? What are the respective roles of common and specific *nod* genes in the production of the Had factors?

Two simple hypotheses can be proposed for the origin of the common and specific Had factors: HadVc and HadA are not chemically related and are produced by independent metabolic pathways, or HadVc and HadA are chemically related and part of the same biochemical pathway. Whereas the results of some bioassays are difficult to interpret in the frame of the "two-independent-pathways" hypothesis, all the bioassay results obtained so far either with *R. meliloti* mutants (Faucher *et al.* 1988) or with *R. l.* bv. *viciae* hybrids (this work) are compatible with the hypothesis that HadVc and HadA are part of the same pathway. For example: 1) mutations in *nodH* or in *nodQ* result in the simultaneous disappearance of HadA and appearance of HadV activity; 2) there is an epistatic effect of mutations in *nodABC* upon *nodH* and *nodQ*; and 3) both HadA and HadVc factors are heat-stable and behave similarly both in ultrafiltration and reversed-phase chromatography experiments.

If we hypothesize that the Had factors are synthesized by the same pathway, then the epistasy of *nodABC* over *nodH* and *nodQ* suggests that the common *nod* genes act upstream of the host range genes in the pathway. Both the *R. l.* bv. *viciae* and *R. meliloti nodABC* genes determine the production of a HadVc factor that might serve as a precursor for the synthesis of the species-specific Had signals; this might be the physiologic explanation of the mutual complementation of the *nodABC* genes from different species.

To produce an alfalfa-specific HadA signal(s), the *nodQ* gene is essential in *R. l.* bv. *viciae* but not in *R. meliloti*. This could be because to produce HadA signals in the *R. l.* bv. *viciae* hybrids, the *R. meliloti* host range *nodH* and *nodQ* genes might have to mediate the modification not only of the "common" precursor HadVc, but also of vetch-specific HadVs (s for specific) signals modified by the products of the species-specific *R. l.* bv. *viciae nod* genes. The fact that a mutation in either *nodH* or *nodQ* results in a loss of HadA activity suggests that these two genes act on a linear pathway. Our model to interpret these results is described in Figure 5. Schmidt *et al.* (1988) assumed that *R. meliloti nodC* is not involved in generating the mitosis-stimulating factor(s) and might be a potential signal transducer. We do not know whether *nodC* is involved in the export of an alfalfa-specific factor(s).

Several recent reports have shown that the *nodD* genes can control the host range of *Rhizobium* species by regulating the expression of other *nod* operons as a function of the flavonoid composition of root exudates of legume hosts (Horvath *et al.* 1987; Spaink *et al.* 1987; Györgypal *et al.* 1988). The presence of *R. meliloti* $nodD_3$ in *R. l.* bv. *viciae* hybrids had an influence on eliciting the Tsr reaction on vetch, which could be explained by the previously reported very high level of *nod* activity required for inducing the Tsr phenotype (Zaat *et al.* 1987). This result suggests that the $nodD_3$ gene is involved in the control of the level of expression of *R. meliloti nod* genes in *R. l.* bv. *viciae* and that it activates *nod* genes not only in the rhizosphere or root surface of a homologous host (Györgypal *et al.* 1988), but also of a nonhomologous host such as vetch.

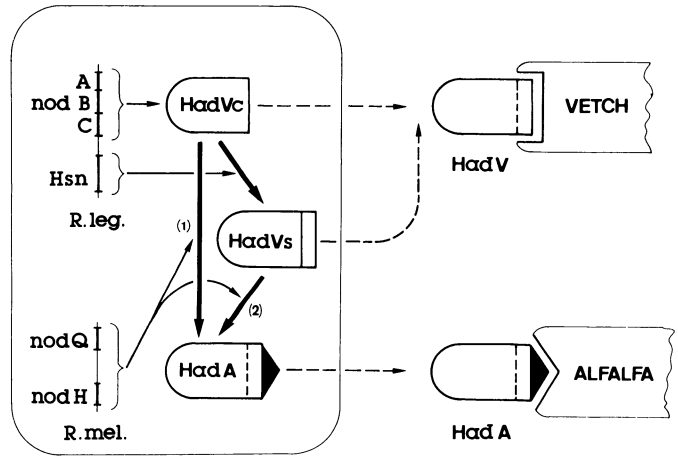

Fig. 5. A model for the role of *nod* genes in the production of symbiotic extracellular signals. Hypotheses to explain the "dominance" of *Rhizobium meliloti* host range *nod* genes over those of *R. leguminosarum* bv. *viciae* are: 1) the enzymes encoded by the *R. meliloti nodH* and *nodQ* genes have a higher affinity for the HadVc precursor than the enzymes encoded by the *R. l.* bv. *viciae* host range genes; and 2) the products of *nodH* and *nodQ* genes can convert the HadVs signal(s) into HadA signal(s). The model postulates the existence of Had signal receptors on the root hairs of common vetch and alfalfa hosts. Hsn = host-specific *nod* region of *R. l.* bv. *viciae*. The dotted line within the Had symbols means that they might represent either HadVc or HadVs, or both.

In contrast, the changes of the *R. l.* bv. *viciae* specificity of infection and nodulation, associated with the transfer of pGMI515, were not due to a *nodD* gene because a plasmid derivative containing a Tn5-233 insertion in $nodD_3$ caused the same symbiotic changes as did the pGMI515. In addition, the same flavonoid induced in different strains the production of Had signals of different specificity.

We can thus conclude that the specificity of infection and nodulation is determined in *R. meliloti* on at least two levels: 1) *nodD* genes activate the expression of other *nod* operons as a function of specific plant signals, and 2) host range genes such as *nodH* and *nodQ*, once activated, determine the production of bacterial extracellular signals that allows the recognition of specific legumes.

In this paper, we have described agar plate Had bioassays on vetch and alfalfa that are much more convenient than the assays previously described (Zaat *et al.* 1987; Faucher *et al.* 1988). They require small volumes of culture filtrates, are simpler to perform, and the results can be read rapidly. We are currently using these assays to purify the HadA and HadV signals. Determination of their chemical structure should allow us to decipher the role of the various *nod* genes in the synthesis and export of these symbiotic signals.

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LITERATURE CITED

Beynon, J. L., Beringer, J. E., and Johnston, A. W. B. 1980. Plasmids and host range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J. Gen. Microbiol. 120:421-429.

Bhuvaneswari, T. V., and Solheim, B. 1985. Root hair deformation in the white clover-*Rhizobium trifolii* symbiosis. Physiol. Plant. 63:25-34.

Canter Cremers, H. C. J., van Brussel, A. A. N., Plazinski, J., and Rolfe, B. G. 1986. Sym plasmid and chromosomal gene products of *Rhizobium trifolii* elicit developmental responses on various legume roots. J. Plant Physiol. 122:25-40.

Cervantes, E., Sharma, S. B., Maillet, F., Vasse, J., Truchet, G., and Rosenberg, C. 1989. The product of the host specific *nodQ* gene of *Rhizobium meliloti* shares homology with translation elongation and initiation factors. Mol. Microbiol. 3:745-755.

Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B., and Richmond, M. H. 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. 108:1244-1249.

Dazzo, F. B., Hollingsworth, R. I., Salzwedel, J. L., Philip-Hollingsworth, S., Robeles, M., Olen, T. A., Apenzeller, L., Wang, S., Toro, I., Squartini, A., Anderson, S. F., Chen, J., Chapman, K. A., Maya-Flores, J., Cargill, L. C., Baker, D., Djordjevic, M. A., and Rolfe, B. G. 1988. Signal recognition responses in the *Rhizobium trifolii*-white clover symbiosis. Pages 35-40 in: Molecular Genetics of Plant-Microbe Interactions. R. Palacios and D. P. S. Verma, eds. The American Phytopathological Society, St. Paul, MN.

Debellé, F., Maillet, F., Vasse, J., Rosenberg, C., de Billy, F., Truchet, G., Dénarié, J., and Ausubel, F. M. 1988. Interference between *Rhizobium meliloti* and *Rhizobium trifolii* nodulation genes: The genetic basis of *R. meliloti* dominance. J. Bacteriol. 170:5717-5727.

Debellé, F., Rosenberg, C., Vasse, J., Maillet, F., Martinez, E., Dénarié, J., and Truchet, G. 1986. Assignment of symbiotic developmental phenotypes to common and specific nodulation (*nod*) genetic loci of *Rhizobium meliloti*. J. Bacteriol. 168:1075-1086.

Debellé, F., and Sharma, S. B. 1986. Nucleotide sequence of *Rhizobium meliloti* RCR2011 genes involved in host specificity of nodulation. Nucleic Acids Res. 14:7453-7472.

Djordjevic, M. A., Gabriel, D. W., and Rolfe, B. G. 1987. *Rhizobium* - The refined parasite of legumes. Annu. Rev. Phytopathol. 25:145-168.

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

Faucher, C., Maillet, F., Vasse, J., Rosenberg, C., van Brussel, A. A. N., Truchet, G., and Dénarié, J. 1988. *Rhizobium meliloti* host range *nodH* determines production of an alfalfa-specific extracellular signal. J. Bacteriol. 170:5489-5499.

Fisher, R. F., Tu, J. K., and Long, S. R. 1985. Conserved nodulation genes in *Rhizobium meliloti* and *Rhizobium trifolii*. Appl. Environ. Microbiol. 49:1432-1435.

Gabriel, D. W., Burges, A., and Lazo, G. R. 1986. Gene-for-gene interactions of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum* with specific resistance genes in cotton. Proc. Natl. Acad. Sci. USA 83:6415-6419.

Gottfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F., and Kondorosi, A. 1986. At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. 167:881-887.

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

Honma, M. A., and Ausubel, F. M. 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proc. Natl. Acad. Sci. USA 84:8558-8562.

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

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

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

Long, S. R., Buikema, W. J., and Ausubel, F. M. 1982. Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod$^-$ mutants. Nature 298:485-488.

Long, S. R. 1984. Genetics of *Rhizobium* nodulation. Pages 265-306 in: Plant-Microbe Interactions, Vol. 1. T. Kosuge and E. W. Nester, eds. Macmillan, New York.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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

Rodriguez-Quinones, F., Banfalvi, Z., Murphy, P., and Kondorosi, A. 1987. Interspecies homology of nodulation genes in *Rhizobium*. Plant Mol. Biol. 8:61-75.

Rosenberg, C., Boistard, P., Dénarié, J., and Casse-Delbart, F. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. Mol. Gen. Genet. 184:326-333.

Scheffe, H. 1959. The Analysis of Variance. John Wiley Press, New York.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. 1988. *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells. Proc. Natl. Acad. Sci. USA 85:8578-8582.

Schofield, P. R., Djordjevic, M. A., Rolfe, B. G., Shine, J., and Watson, J. M. 1983. A molecular linkage map of nitrogenase and nodulation genes in *Rhizobium trifolii*. Mol. Gen. Genet. 192:459-465.

Schwedock, J., and Long, S. R. 1989. Nucleotide sequence and protein products of two new nodulation genes of *Rhizobium meliloti*, *nodP* and *nodQ*. Mol. Plant-Microbe Interact. 2:181-194.

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

Staskawicz, B. J., Dahlbeck, D., and Keen, N. T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.

Surin, B. P., and Downie, J. A. 1988. Characterization of the *Rhizobium leguminosarum* genes *nodLMN* involved in efficient host-specific nodulation. Mol. Microbiol. 2:173-183.

Swanson, J. A., Tu, J. K., Ogawa, J., Fisher, R. F., and Long, S. R. 1987. Extended region of nodulation genes in *Rhizobium meliloti* 1021. 1. Phenotypes of Tn5 insertion mutants. Genetics 117:181-189.

Truchet, G., Rosenberg, C., Vasse, J., Julliot, J. S., Camut, S., and Dénarié, J. 1984. Transfer of *Rhizobium meliloti* Sym genes into *Agrobacterium tumefaciens*: Host specific nodulation by atypical infection. J. Bacteriol. 157:134-142.

Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A. M., Rosenberg, C., Batut, J., Maillet, F., and Dénarié, J. 1985. Identification of a *Rhizobium meliloti* pSym 2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol. 164:1200-1210.

Truchet, G., Camut, S., de Billy, F., Odorico, R., and Vasse, J. 1989. The *Rhizobium*-legume symbiosis: Two methods to discriminate between nodules and other root-derived structures. Protoplasma 149:82-88.

van Brussel, A. A. N., Zaat, S. A. J., Canter Cremers, H. C. J., Wijfellman, C. A., Pees, E., Tak, T., and Lugtenberg, B. J. J. 1986. Role of plant root exudate and Sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of Tsr factor, which causes thick and short roots on common vetch. J. Bacteriol. 165:517-522.

Vasse, J. M., and Truchet, G. L. 1984. The *Rhizobium*-legume symbiosis: Observation of root infection by bright-field microscopy after staining with methylene blue. Planta 161:487-489.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. Pages 9-10 in: IBP Handbook 15. Blackwell Scientific Publications, Oxford.

Vincent, J. M. 1980. Factors controlling the legume-*Rhizobium* symbiosis. Pages 103-129 in: Nitrogen Fixation, Vol. 2. W. E. Newton and W. H. Orme-Johnson, eds. University Park Press, Baltimore.

Zaat, S. A. J., van Brussel, A. A. N., Tak, T., Pees, E., and Lugtenberg, B. J. J. 1987. Flavonoids induce *Rhizobium leguminosarum* to produce *nodDABC* gene-related factors that cause thick, short roots and root hair responses on common vetch. J. Bacteriol. 169:3388-3391.