

Molecular Basis of Symbiotic Promiscuity

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INTRODUCTION

Studies of nitrogen-fixing symbioses began in Europe, largely on Northern hemisphere plants. Thus, in 1542, the German physician and botanist Leonhard Fuchs (88) published drawings of nodulated legumes. During the 17th century, Malpighi (167), who worked mostly in Bologna, Italy, observed nodules on the roots of beans (*Phaseolus vulgaris* and *Vicia faba*, members of the Leguminosae). Almost 200 years later, the Russian botanist Woronin (280) noted that the nodules of *Alnus glutinosa* (Betulaceae) and *Lupinus mutabilis* (Leguminosae) were filled with minute bodies resembling bacteria. Although the observations that both legumes and non-legumes possess nodules were historically important, the origin of nodules was controversial (86). Frank (85) found nodules on the roots of all healthy legumes in a study in Germany and demonstrated that incinerating soil prevented the nodulation of *Pisum sativum*. Hellriegel (120), and Hellriegel and Wilfarth (121) showed, in a study in Germany, that nodule formation results from external infection of *Lupinus* spp., *P. vulgaris*, *P. sativum*, *Ornithopus sativa*, *Trifolium* spp., and *Vicia sativa*. However, it was Beyerinck (13–17), working in The Netherlands, who furnished the first proof that bacteria provoke nodules; he did this by preparing pure cultures of nodule organ-

isms from *V. faba* and using them to infect Fab beans growing in sterile soil (18). Prazmowski (204, 205), working in Poland, inoculated *P. sativum* with pure cultures and showed that the bacteria penetrate legumes via infection threads in root hairs. In fact, it is now clear that many diverse soil bacteria harbor symbiotic loci (28), including the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*. Collectively, they are called rhizobia.

Root hairs, which develop near the root apex, are unicellular extensions of root epidermal cells. Most root hairs emerge at the apical end of particular epidermal cells and elongate by polar growth of the tip (43). The tips of root hairs have high exocytotic and cell wall assembly activities (221). In the soil, they are extensively colonized by soil-borne microorganisms, including nitrogen-fixing bacteria. In the rhizosphere, young, growing root hairs play an important role in symbiotic recognition. Rhizobia and the Nod factors they secrete (for reviews, see references 42, 63, 115, 173, 240, and 256) stimulate the reorientation of root hair cell wall growth (47, 139), resulting in curled root hairs. Within these curled root hairs, Nod factors promote the formation of infection threads, and it is through these tubular structures that the bacteria enter most (but not all) plants (Fig. 1 and 2).

Work on the molecular basis of host specificity also began at the end of the last century. In a study in Germany, Hiltner (123) prepared aqueous, bacterium-free filtrates from mature *P. sativum* nodules and demonstrated that they contain a substance that induces root hair formation (Hai) and deformation

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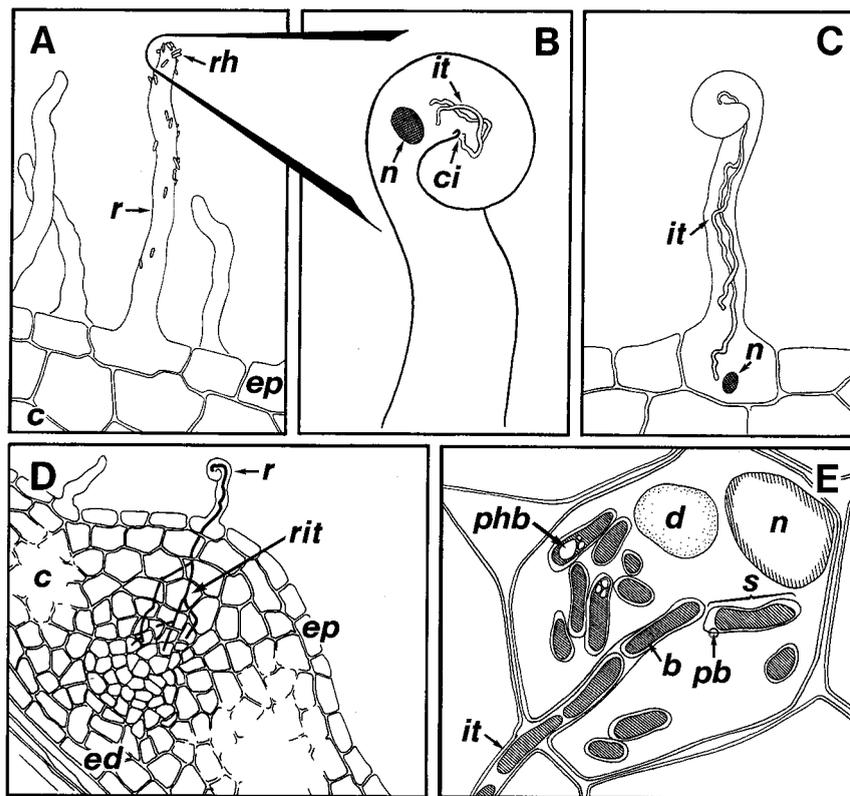


FIG. 1. Invasion of legume root hairs by *Rhizobium*. (A) Rhizobia (*rh*) colonize the rhizosphere and attach to the root hairs (*r*). (B) Opening the "outer door." Nod factors induce root hair curling and permit bacterial penetration at the center of infection (*ci*). The plant nucleus (*n*) precedes the growing infection thread(s) (*it*). (C) Crossing the inner doors. Still accompanied by the nucleus (*n*) an elongating infection thread (*it*) reaches the base of the root hair cell. (D) A developing infection thread ramifies (*rit*) near the nodule primordia formed by dividing cortical cells. (E) Bacteroids (*b*) are released from the infection thread (*it*) and form symbiosomes (*s*) in nodule cells. Granules of poly- β -hydroxybutyrate (*phb*) accumulate in bacteroids surrounded by the peribacteroid membrane (*pb*). Other abbreviations: *c*, cortex; *d*, digestive vacuole; *ep*, epidermis; *ed*, endodermis.

of the root hairs (Had) in this plant. Many attempts to define the structure of these deformation factors followed (213), but these investigations yielded fruit only in 1990, when Lerouge et al. (154), working in France, showed that the substances responsible are N-acylated oligomers of N-acetyl-D-glucosamine. Since then, the Nod factor structures (the products of *nod* genes) of a number of rhizobia have been elucidated (see "Baroque decorations to the Nod factor core" below).

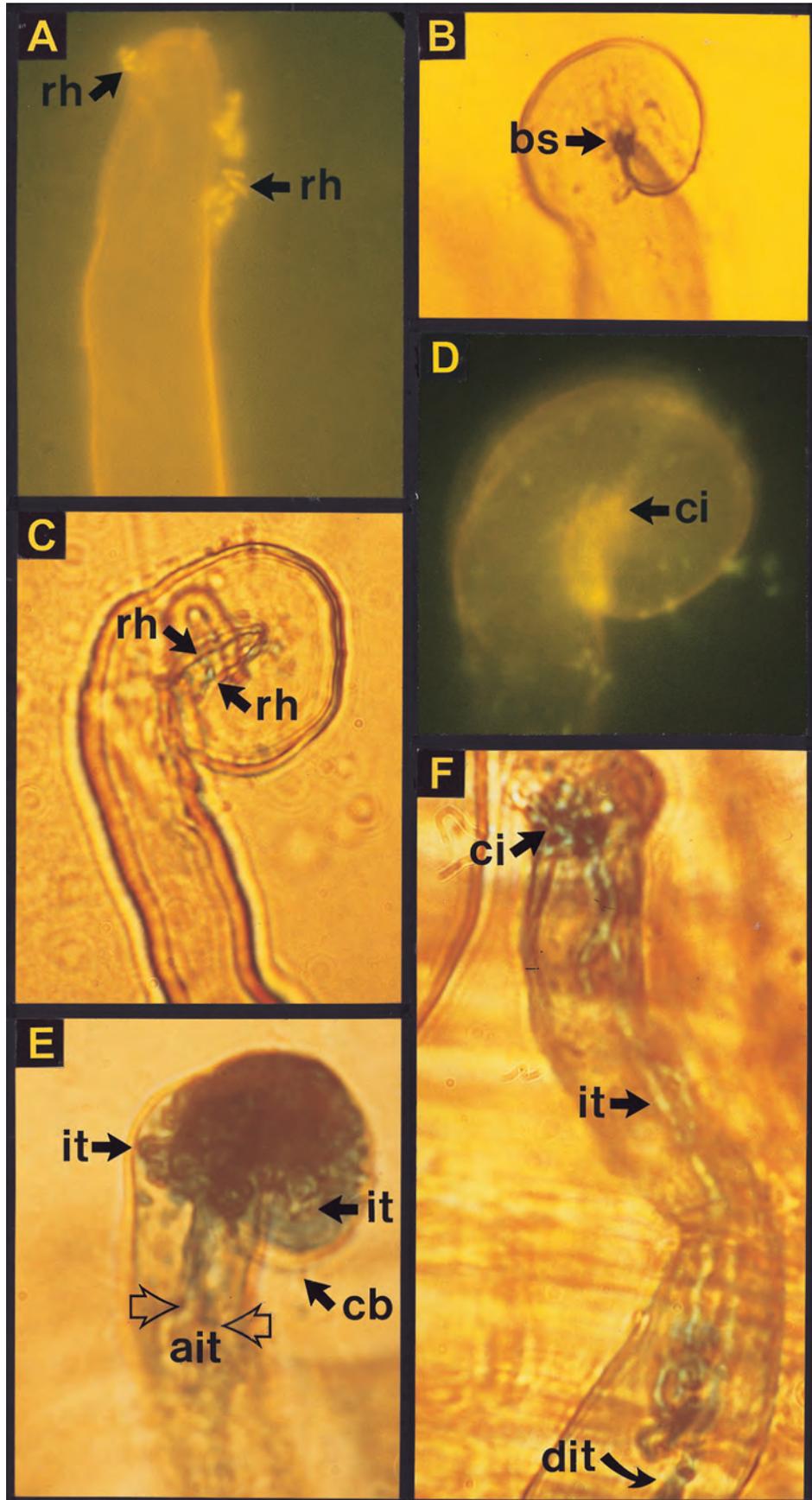
In other words, research on symbiotic nitrogen fixation was concentrated on European legumes, and although this has become less so with time, the focus on genera such as *Medicago*, *Pisum*, *Trifolium*, and *Vicia* has prevailed. Of course, model plants have advantages, but it is our contention that this bias toward European plants for historic reasons may have given a false impression of symbiotic specificity. What follows is an analysis of the molecular mechanisms governing broad host range (i.e., promiscuity) in rhizobia that may be regarded as a paradigm for other microbial symbioses. Readers are referred to the reviews by Irving et al. (128) and Downie and Walker (64) for more information on the role of the plant in nodulation.

BACTERIAL PROMISCUITY

Doubts that bacteria were faithful to the legume from which they were isolated also surfaced early. In experiments that are probably unrepeatable (Table 1), Bréal (25) induced nodule formation on *Lupinus*, *Medicago sativa*, and *P. sativum* by bac-

teria isolated from nodules of alfalfa (*M. sativa*). In similar, questionable experiments, Laurent (149) suggested that nodules could be produced on *P. sativum* by bacteria isolated from 36 species of plants. Perhaps the first reproducible data of this kind was obtained by Nobbe et al. (183, 184), who showed in experiments performed in Germany at the end of the 19th century that bacteria from *P. sativum* nodules were unable to nodulate plants of the tribes Genisteae and Hedysareae. Indeed, rhizobia which nodulate *Lupinus*, *Medicago*, *Pisum*, *Vicia*, etc., are now known to have restricted host ranges (Table 1).

The U.S. researcher J. K. Wilson was one of the first non-Europeans to publish extensive information on different symbiotic associations (279). He tested the host range properties of rhizobia isolated from 31 genera of legumes on 160 different legume species. All isolates, including those from *Vicia* spp., nodulated legumes of different tribes. The average number of plant species nodulated by a particular strain was 33%, but it ranged from 6% (for an isolate from *Spartium scoparium*) to 66% (for a slow-growing, and therefore probably *Bradyrhizobium*, strain isolated from *Mucuna* nodules). Indeed, four of Wilson's isolates nodulated more than half the legumes tested. These data are included in Table 2 to show the patterns of nodulation provoked by broad-host-range rhizobia. Both *Bradyrhizobium* and *Rhizobium* species can be promiscuous, as shown in Table 2, and broad-host-range rhizobia were even isolated from *Albizia julibrissin* (Mimosoideae). Isolates from *P. vulgaris*, which include *R. etli* and *R. tropici*, were shown to



nodulate 17 and 18 different legume genera, respectively (122). It thus seems as if rhizobia associated with most tribes of the Leguminosae have various degrees of promiscuity. The principal exceptions to this rule are the more specialized rhizobia associated with the tribes Cicereae, Trifolieae, and Viceae, which have restricted host ranges (28).

LEGUME FIDELITY

As expected, hosts also vary greatly in their ability to enter into symbiosis with different rhizobia. In Wilson's study (279), six plants formed nodules with at least 90% of the isolates (Table 2). At the other extreme, *Hippocrepis* sp. and *Ornithopus sativa* formed nodules with only one other isolate. In further studies of this kind, numerous legumes have been inoculated with large collections of rhizobia and the capacity of the legumes to nodulate have been evaluated. Again, promiscuity exists among diverse legumes (Table 3). Mimosoid legumes not only harbor broad-host-range rhizobia but also are capable of interacting with diverse bacteria; *Leucaena leucocephala*, for example, is nodulated by a moderately broad spectrum of symbionts. The fact that *Azorhizobium caulinodans*, a microsymbiont of *Sesbania rostrata*, has a very restricted host range (Table 1) gives the impression that *Sesbania* spp. have very defined rhizobial requirements. However, Wilson (279) showed that *S. drummondii*, along with another tree (*Robinia pseudoacacia*), had the broadest capacity to nodulate among the legumes he tested.

However, most broad-spectrum hosts known are annual and climbing legumes of the tribe Phaseoleae. These include *Macroptilium atropurpureum*, which has been widely used as a test plant for nodulation by uncharacterized rhizobia (27, 248); *Lablab purpureus*, the legume from which *Rhizobium* sp. strain NGR234 was isolated (269); and *P. vulgaris* (175). Equally, the other plants listed in Table 3 are considered to have broad host ranges (185). The capacity of *Vigna unguiculata* to nodulate with many different rhizobia has been exploited in the search for *nod* genes (156, 157). Taken together, these data suggest that symbiotic promiscuity is widely dispersed in nature. It is not associated with a particular bacterial or plant taxonomic group and is not correlated with the growth habit of the legume. Broad-host-range legumes are also almost equally distributed between those that form indeterminate as opposed to determinate nodules (Table 3). Perhaps the only underlying similarity is that promiscuous bacteria and plants are found mostly in warm or tropical parts of the world.

MOLECULAR BASES

Nodulation leads to the colonization of plant cells by invading bacteria. Although many host plants and effective rhizobia have the ability to enter into symbiosis with more than one partner, only certain combinations of symbionts result in the formation of nitrogen-fixing nodules. Ineffective associations lead to empty or nonfixing bacteroid-containing nodules. Spec-

ificity among compatible partners minimizes the chances of infection by pathogens and the formation of ineffective associations that are detrimental to both symbionts. Experimental evidence suggests that the progression of invasive rhizobia towards nodule primordia is challenged at various "doors" (Fig. 1). Codes contained in molecular signals open these checkpoints. During the initial phases of nodulation (root hair curling and bacterial entry), these codes are given by flavonoids and Nod factors. In both cases, NodD proteins are the chief interlocutors of molecular traffic in the rhizosphere.

Flavonoids and Regulation of Nodulation Genes

Plants jettison surprisingly large amounts of organic matter into the soil, most of which supports the growth of rhizospheric microorganisms. By labelling whole plants with $^{14}\text{CO}_2$, Helal and Sauerbeck (119) showed that 19% of the photosynthate was released as organic material into the rhizosphere. These compounds include carbohydrates, organic acids, vitamins, amino acids, and phenolic derivatives. Of these, flavonoids (2-phenyl-1,4-benzopyrone derivatives) are the most important from the symbiotic perspective. Although found throughout the plant kingdom, flavonoids specifically trigger the expression of the rhizobial genes required for nodulation (*nod*, *nol*, and *noe*). The inducing capacity varies with flavonoids and rhizobia; and in some cases flavonoids may inhibit induction (61, 79, 196, 197, 199). Other molecules, such as the betaines and erythronic and tetriconic acids, may also act as inducers (90, 198).

Regulation of *nod* gene expression in rhizobia varies from strain to strain but is almost always mediated by NodD (for a review, see reference 237). NodD proteins belong to a family of LysR-like transcriptional regulators that bind to highly conserved 47-bp DNA motifs (*nod* boxes) found in the promoter regions of many nodulation loci (81, 227). Although NodD proteins bind to *nod* boxes even in the absence of an inducer, flavonoids are generally required for the expression of *nod* genes (81, 82, 101, 110). Thus, NodD acts both as a sensor of the plant signal and as an activator of transcription of *nod* loci (236). In *R. leguminosarum* bv. *viciae*, NodD proteins are localized in the cytoplasmic membrane (234), where the inducing flavonoid, naringenin, also accumulates (211). Direct binding of inducers to NodD has not been demonstrated, but point mutations in *nodD* affect the recognition of inducing molecules and cause an extension of the host range (30, 174). Furthermore, comparison of the NodD structure with various nuclear receptors has shown that the two types of proteins share conserved ligand-interacting domains located at the C-terminal end of their respective DNA-binding motifs (111).

Although *nodD* genes are ubiquitous in rhizobia, their symbiotic characteristics vary from one species to another. Some strains, such as *R. leguminosarum* bv. *trifolii*, have only one *nodD* gene, and in these cases, its mutation renders the strain incapable of nodulation (Nod⁻). In contrast, *B. japonicum*, *Rhizobium* sp. strain NGR234, *R. meliloti*, and *R. tropici* pos-

FIG. 2. Early steps in nodulation of legumes showing that continued development of infection threads is under the control of the *nodD1* gene in *Rhizobium* sp. NGR234. (A) Fluorescent image of a *Vigna unguiculata* root hair inoculated with a mutant incapable of producing NodNGR factors (NGR Δ nodABC) and concomitantly treated with 10^{-7} M NodNGR(S) factors. Arrows point to rhizobia (rh). (B) Commencement of curling of a root hair stained with methylene blue. Extreme curling leads to the formation of a bright spot (bs), where rhizobia are often entrapped (same treatment as in panel A). (C) A bright-field image of a root hair inoculated with NGR Δ nodABC::GUS3, treated with 10^{-7} M NodNGR(S) factors, and stained for β -glucuronidase activity. Arrows point to the entrapped rhizobia within the curl. (D) Fluorescent image of a root hair curled in the shape of a shepherd's crook, showing the center of infection (ci). (E) Experiment in which the *nodABC* mutant was replaced with NGR Δ nodD1::GUS3, but incubated with 10^{-7} M NodNGR(S) factors, and stained for β -glucuronidase activity. Apparently, the *nodD1* mutant lacks a factor(s) that is necessary for the continued development of infection threads (it). In its absence, infection threads abort (ait), forming a structure that resembles a cerebellum (cb). (F) Photomicrograph of root hairs inoculated with wild-type NGR234 marked with GUS3. Infection threads develop along the length of the root hair (dit). B. Relić and W. J. Broughton (unpublished results; see reference 215 for further details).

TABLE 1. Nodulation capacities of symbiotic members of the bacterial family *Rhizobiaceae*^a

Species	Typical host(s)	Host range	Examples of nonhosts	Reference(s)
<i>Azorhizobium caulinodans</i>	Robinieae (P), <i>Sesbania</i> spp.	Compatible only with <i>Sesbania punctata</i> and <i>S. rostrata</i>	MIMOSOIDEAE, PAPILIONOIDEAE (except <i>Sesbania</i>)	M. Holsters, personal communication
<i>Bradyrhizobium</i> sp. <i>Lupinus</i> spp.	Genisteae (P), <i>Lupinus</i> spp.	Acaciae (M), Mimoseae (M), Desmodieae (P), Loteae (P), Psoraleae (P), Phaseoleae (P), Acasynomeneae (P)	Vicieae (P), Cicereae (P), Trifolieae (P)	C. E. Pankhurst and C. W. Ronson, personal communication; H. Meyer z. A. and W. J. Broughton, unpublished data
<i>Bradyrhizobium elkanii</i>	Phaseoleae (P), <i>Glycine</i> spp.	Phaseoleae (P), Macroptilium, <i>Vigna</i> spp.	Vicieae (P), Cicereae (P), Trifolieae (P)	G. Stacey, personal communication
<i>Bradyrhizobium japonicum</i>	Phaseoleae (P), <i>Glycine</i> spp.	Aeschynomeneae (P), <i>Arachis</i> spp., Phaseoleae (P), <i>Macroptilium</i> , <i>Vigna</i> spp.	Vicieae (P), Cicereae (P), Trifolieae (P)	G. Stacey, personal communication
<i>Mesorhizobium huakuii</i>	<i>Astragalus sinicus</i>	Galegae (P), <i>Astragalus</i> spp.	MIMOSOIDEAE, PAPILIONOIDEAE	282
<i>Mesorhizobium loti</i>	Loteae (P), <i>Lotus</i> spp., Genisteae (P), <i>Lupinus</i> spp.	Mimoseae (M), <i>Mimosa pudica</i> , <i>Leucaena leucocephala</i> ; Phaseoleae (P), <i>Macroptilium atropurpureum</i>	Vicieae (P), Cicereae (P), Trifolieae (P)	G. Stacey, personal communication
<i>Rhizobium</i> sp. strain NGR234	Phaseoleae (P), Desmodieae (P)	Mimoseae (M), Acaciae (M), Ingeae (M), Sophoreae (P), Dalbergieae (P), Amorpheae (P), Millettieae (P), Robinieae (P), Indigoferae (P), Loteae (P), Galegae (P), Bossiaceae (P), Mirbelieae (P), Podalyriaceae (P), Crotalarieae (P), Thermopsidae (P), Genisteae (P), Psoraleae (P)	Vicieae (P), Cicereae (P), Trifolieae (P)	207
<i>Rhizobium etli</i>	Phaseoleae (P), <i>Phaseolus</i> spp.	Ingeae (M), Crotalarieae (P), Galegae (P), Mimoseae (M), Desmodieae (P), Robinieae (P)	Robinieae (P)	122; E. Martínez-Romero, personal communication
<i>Rhizobium fredii</i>	Phaseoleae (P), Desmodieae (P)	Mimoseae (M), Ingeae (M), Sophoreae (P), Amorpheae (P), Millettieae (P), Robinieae (P), Indigoferae (P), Loteae (P)	Vicieae (P), Cicereae (P), Trifolieae (P)	207
<i>Rhizobium galegae</i>	Galegae (P), <i>Galega</i> spp.	Compatible only with <i>Galega</i> spp.	MIMOSOIDEAE, PAPILIONOIDEAE (except <i>Galega</i>)	K. Lindström, personal communication
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>	Phaseoleae (P), <i>Phaseolus</i> spp.	Phaseoleae (P)	MIMOSOIDEAE, Trifolieae (P)	E. Martínez-Romero, personal communication
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>	Trifolieae (P), <i>Trifolium</i> spp.	Phaseoleae (P), Desmodieae (P), Trifolieae (P), <i>Medicago</i> spp.	MIMOSOIDEAE, Phaseoleae (P)	H. P. Späink, personal communication
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	Vicieae (P), <i>Pisum sativum</i> , <i>Vicia</i> spp.	<i>Lathyrus</i> spp. (e.g., <i>L. sativa</i>)	MIMOSOIDEAE, Phaseoleae (P), Desmodieae (P)	J. A. Downie, personal communication
<i>Rhizobium meliloti</i>	Trifolieae (P), <i>Medicago</i> spp., <i>Melilotus</i> spp., <i>Trigonella</i> spp.	Trifolieae (P), <i>Medicago</i> spp., <i>Melilotus</i> spp., <i>Trigonella</i> spp.	Acaciae (M), Desmodieae (P), Mimoseae (M), Phaseoleae (P), Viciae (P)	J. Dénarié, personal communication
<i>Rhizobium saheli</i>	Robinieae (P), <i>Sesbania</i> spp.	Robinieae (P), <i>Sesbania</i> spp.	Acaciae (M), Mimoseae (M)	165; C. Boivin, personal communication
<i>Rhizobium teranga</i> bv. <i>acaciae</i>	Acaciae (M), <i>Acacia</i> spp.	Mimoseae (M), <i>Leucaena leucocephala</i> , <i>Prosopis juliflora</i>	Robinieae (P), <i>Sesbania</i> spp.	165; C. Boivin, personal communication
<i>Rhizobium teranga</i> bv. <i>sesbaniae</i>	Robinieae (P), <i>Sesbania</i> spp.	Robinieae (P), <i>Sesbania</i> spp.	Acaciae (M), Mimoseae (M)	165; C. Boivin, personal communication
<i>Rhizobium tropici</i>	Phaseoleae (P), <i>Phaseolus</i> spp.	Galegae (P), Crotalarieae (P), Desmodieae (P), Mimoseae (M), Robinieae (P), Loteae (P)	Vicieae (P), Cicereae (P), Trifolieae (P)	E. Martínez-Romero, personal communication

^a Plants are listed by their subfamilies, tribes, genera, and species. Associations are listed only where fully effective nodulation (i.e., Fix⁺) has been reported. Subfamily names are listed in capital letters (Caesalpinioideae [C], Mimosoideae [M], and Papilionoideae [P]) (201).

TABLE 2. Nodulation capacities of some broad-host-range rhizobia isolated from various legumes

Plant nodulated ^a	Nodulation ^{a,b} by rhizobia isolated from:					
	<i>Mucuna</i> (P10) ^c	<i>Albizia</i> (M5)	<i>Desmodium</i> (P11)	<i>Glycine</i> (P10)	<i>Lablab</i> (P10) NGR234	<i>Sesbania</i> (P8)
Amorpheae (P6) <i>Amorpha</i> spp.	+	+	+	+	+	+
Mirbelieae (P24) <i>Chorizema</i> spp.	+	+	+	-	+	+
Phaseoleae (P10) <i>Centrosema</i> spp.	+	+	+	-	+	+
<i>Phaseolus</i> spp.	+	+	+	+	+	+
Robinieae (P8) <i>Robinia</i> spp.	+	+	+	+	+	+
<i>Sesbania</i> spp.	+	+	+	+	+	+

^a Letters in brackets represent the subfamily to which the legume belongs (M, Mimosoideae; P, Papilionoideae), and the numbers represent the tribe (201).

^b +, effective (Fix⁺) nodulation; -, failure to nodulate (Nod⁻). Data for *Mucuna*, *Albizia*, *Desmodium*, and *Sesbania* rhizobia from reference 279; data for *Glycine* (rhizobial strain USDA257) and *Lablab* (rhizobial strain NGR234) rhizobia from reference 207.

^c We assigned this rhizobial isolate to *Bradyrhizobium* because of its slow growth.

sess two to five copies of *nodD* (71, 105, 194, 269c). In *R. meliloti*, mutation of all three copies of *nodD* is required to abolish nodulation (124), whereas inactivation of *nodD1* is sufficient to render strain NGR234 Nod⁻ (213). *nodD* products of various *Rhizobium* species vary in that they respond to different sets of flavonoids (72, 249). Moreover, NodD homologues from the same strain may have different flavonoid preferences (112, 117, 198). In *R. meliloti*, NodD1 is activated when cells are supplied with a complex plant seed extract or the flavonoid luteolin; NodD2 is activated only with the complex extract, not with purified luteolin; and NodD3 apparently mod-

ulates the expression of *nod* genes even in the absence of any plant factor (180). Together with *syrM*, *nodD3* of *R. meliloti* constitutes a self-amplifying positive regulatory circuit that is involved in the regulation of *nod* genes within the developing nodule (266). In contrast, NodD1 of strain NGR234 responds to a wide range of inducing molecules that include flavonoids known to be inhibitors in other rhizobia (e.g., vanillin and isovanillin) (4, 72, 155) and several estrogenic compounds (112). Transfer of the *nodD1* gene of strain NGR234 to restricted-host-range rhizobia extends the nodulation capacity of the recipients to new hosts, including the nonlegume *Parasponia andersonii* (4, 11, 126). Although a number of correlations exist between the spectrum of flavonoids able to interact with NodD proteins and the breadth of the host range (11, 54, 126, 249, 250, 263, 269d), variations in the ability of these proteins to differentially sense inducing molecules (and regulate the expression of *nod* genes) are insufficient to explain the phenomenon of host specificity.

Nonetheless, NodD genes represent a molecular interface between the bacterium and the plant. However, other species-specific sensor-activator systems also contribute to the control of bacterial host range. For instance, *nodV* and *nodW* of *B. japonicum* are essential for the nodulation of *M. atropurpureum*, *Vigna radiata*, and *V. unguiculata* but contribute only marginally to the symbiosis with *G. max* (104). Modulation of two-component regulatory systems such as *nodV* and *nodW* is mediated by a series of phosphorylation steps. NodV is thought to be a membrane-bound protein that senses signals from the plant and transduces the signal to NodW, which in turn activates the expression of *nod* genes (230). Both in vitro and in vivo studies have confirmed that phosphorylation of NodW is induced by genistein and depends on both acetyl phosphate and its cognate kinase, NodV (159). Also, comparison of the biological activities of the wild-type and mutant proteins indicates that phosphorylation of NodW is essential for *nod* gene activation (159). A search for genes whose transcription is dependent on NodW led to the identification of two suppressor genes, *nwsA* and *nwsB*. Together, these genes also form a

TABLE 3. Responses of known promiscuous legumes to diverse rhizobia

Legume ^a	Geographical origin ^b	% Nod ⁺		Reference
Mimosoideae (M3) <i>Leucaena leucocephala</i>	Tropical America	I	44	157
Amorpheae (P6) <i>Amorpha fruticosa</i>	North America	I	91	279
Mirbelieae (P24) <i>Chorizema ilicifolium</i>	Southeast Australia	I	91	279
Phaseoleae (P10) <i>Centrosema virginianum</i>	Warm America	D	91	279
<i>Lablab purpureus</i>	Tropical Africa		50	157
<i>Macropitium atropurpureum</i>	Tropical America		41	H. Meyer z. A. and W. J. Broughton, unpublished
<i>Phaseolus coccineus</i>	Tropical and warm America		91	279
<i>Vigna unguiculata</i>	Old World Tropical		56	157
Robinieae (P8) <i>Robinia pseudoacacia</i>	Tropical and warm America	I	94	279
<i>Sesbania drummondii</i>	Old World Tropical		94	279

^a Letters in parentheses represent the subfamily to which the legume belongs (M, Mimosoideae; P, Papilionoideae), and the numbers represent the tribe (201). D = determinate nodules; I, indeterminate nodules.

^b Data from reference 166. The data were taken from the reports of Wilson (279), who tested the nodulation capacity of 32 rhizobial isolates (from 31 legume genera) on the nodulation capacities of 160 species (78 genera), and Meyer z. A. and Broughton (see reference 157), who assayed the ability of 50 rhizobial isolates to nodulate 16 species (13 genera) of legumes.

dual-component regulatory system (109). When overexpressed, *nwsB* can complement a *nodW* mutant as well as a *nodD1 nodD2* double mutant, suggesting that in *B. japonicum* at least, alternatives to NodD- and flavonoid-sensing regulatory systems coexist (105).

nod gene expression is also subject to negative control. After initial induction by flavonoids, repression of several nodulation genes is required for optimal nodulation of *M. sativa* by *R. meliloti* (142) and of *P. sativum* by *R. leguminosarum* bv. viciae strain Tom (141). In *R. meliloti*, repression of several *nod* genes is controlled by NolR, a 13-kDa product that contains a helix-turn-helix (HTH) motif which is homologous to other regulators of the LysR family such as NodD and SyrM (143). In the dimeric form, NolR binds to conserved (A/T)TTAGN₆A(T/A) target sequences found in the promoter regions it controls (46) and thus represses the expression of the *nodD* genes as well as those necessary for the synthesis of the core Nod factor structures (*nodABC*). However, it does not affect host-specific *nod* loci such as *nodH*. Interestingly, the absence of NolR repressor activity in *R. meliloti* strain 1021 is due to a single insertional mutation in the C-terminal coding sequence, which abolishes the DNA binding ability of the protein without affecting its HTH motif (45).

With a few exceptions, transcription of most inducible *nod* loci is repressed before rhizobia differentiate into functional bacteroids (235, 244), although transcription of *nodD* and *nodE* of *R. leguminosarum* bv. viciae in *P. sativum* nodules and *nodA* of *A. caulinodans* in stem nodules of *S. rostrata* still occurs (56, 235). *R. meliloti* is no exception, although NolR does not play a direct role in the down regulation of flavonoid-inducible nodulation genes in bacteroids (46). In *B. japonicum*, repression of *nod* gene expression by Nola is probably an indirect effect, mediated perhaps by *nodD2* (91). *B. japonicum nola* and *R. meliloti nolR* mutants retain the ability to nodulate their respective hosts, albeit at lower efficiency, suggesting that fine-tuning of *nod* gene expression is required for optimal nodulation. Similarly, a *nodD2* mutant of strain NGR234 fails to repress the expression of the *nodABCInolOnoel* operon after initial flavonoid induction and, in contrast to the wild-type strain, is unable to form nitrogen-fixing nodules on *V. unguiculata* and *Cajanus cajan* (73).

***nod* Boxes: Variations on a Theme**

Sequence analysis showed that pNGR234a carries 19 sequences homologous to conserved NodD-dependent promoter elements (*nod* boxes), 5 of which control the expression of known *nod* operons (87, 195). No significant difference was found between the consensus sequence derived for *nod* boxes of pNGR234a and those published for other rhizobia (269d, 278). Interestingly, analysis of the promoter regions of the *nodABCInolOnoel*, *nodSU*, *nodZ*, *noeE*, and *nolL* loci showed that *nod* boxes with up to 11 base mismatches (compared to the consensus sequence) are still functional and regulate gene expression in a NodD1-dependent manner (195). It is possible, however, that some of these *nod* boxes have higher affinities for certain NodD1-inducer complexes than do others.

Thus, a microsymbiont such as strain NGR234 has many possibilities for fine-tuning *nod* gene expression. First, NodD1 may interact with a broad spectrum of inducing molecules to produce complexes that may preferentially trigger transcription from certain *nod* boxes over others. Second, supplementary activators of transcription (e.g., SyrM1 [114] and y4xI [274]) could, together with NodD1, form a regulatory network which exerts interlaced control over dispersed nodulation loci. Third, repressors such as NodD2 and NolR (73) could further

modulate *nod* gene expression. Together, these systems control the expression of NGR234 nodulation genes in a host-specific way, possibly resulting in the secretion of different sets of molecular signals in response to the macrosymbiont. Nod factors are obviously the most important of these signals, and their synthesis depends upon the timely and coordinated expression of many *nod* genes.

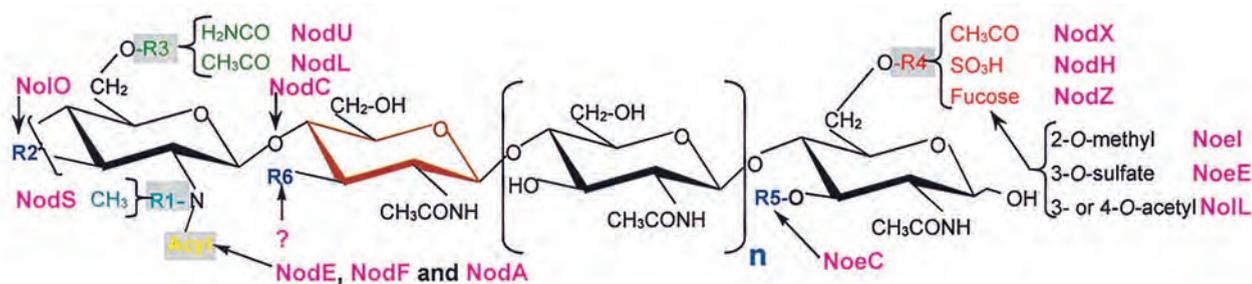
***nod* Enzymes and Nod Factor Synthesis**

Nod factor core. In response to the release by host plants of appropriate inducers, rhizobia synthesize and secrete a family of lipochitooligosaccharides (LCOs) called Nod factors. The first step in Nod factor assembly is performed by an *N*-acetylglucosaminyltransferase encoded by *nodC* (95). Chain elongation by NodC takes place at the nonreducing terminus (134, 135, 170). Then the deacetylase NodB removes the *N*-acetyl moiety from the nonreducing terminus of the *N*-acetylglucosamine oligosaccharides (132, 253). Finally, an acyltransferase encoded by *nodA* links the acyl chain to the acetyl-free C-2 carbon of the nonreducing end of the oligosaccharide (51, 226). Although not essential, NodI and NodJ seem to be involved in the export of Nod factors (34, 74, 254).

However, recent work suggests that *nodA* and *nodC* are also components of host-specific nodulation (*hsn*) (225). NodA varies in its specificity for different fatty acid substrates, thus contributing to the host range. For instance, replacement of *R. meliloti nodA* by *R. tropici nodA* leads to the production of Nod factors acylated with vaccenic acid instead of C_{16:2} (51), whereas *Bradyrhizobium* sp. strain ANU289 NodA is unable to direct the transfer of the *R. leguminosarum* bv. viciae *nodFE*-dependent multiunsaturated fatty acids to the chitin oligosaccharide acceptor (222). NodC is also a determinant of the length of the Nod factor backbone and thus of host specificity (133, 135).

Baroque decorations to the Nod factor core. Properly expressed *nodABC* genes are all that is needed for the synthesis of acylated dimers to pentamers of *N*-acetyl-D-glucosamine that possess symbiotic activity on certain plants (3, 200, 252). Thus all other Nod factor substituents must play more subtle roles in nodulation, perhaps by permitting interaction with certain plants or by protecting Nod factors from degradation. In this sense, these substituents can be likened to baroque decorations—they enhance rather than support the basic structure (Table 4). By definition, the loci that control these additions are unique to one or a few rhizobia and are thus *hsn* genes.

(i) **Fatty acids (*nodEF*).** Two basic types of fatty acids are N-linked to the terminal nonreducing sugar of the chitomeric core by NodA (Table 4): (i) the relatively common saturated or monounsaturated fatty acids (e.g., stearic and vaccenic acids) and (ii) the rarer, highly unsaturated compounds containing two to four double bonds as found in *R. leguminosarum* and *R. meliloti* strains containing *nodEF* (54, 252). NodF has homology to acyl carrier proteins, while NodE is a β -acetoacetyl synthase (21, 22, 49, 245). Transfer of *R. meliloti nodEFGHPQ* into *R. leguminosarum* bv. trifolii or bv. viciae confers on these strains the ability to nodulate *M. sativa* but prevents nodulation of the normal hosts, *Trifolium repens* and *V. sativa*, respectively (50, 70). Inactivation of *R. leguminosarum* bv. viciae *nodE* leads to the synthesis of vaccenic acid rather than the polyunsaturated fatty acids (C_{18:4}) produced by the parent strain (252). Insertional inactivation of *R. leguminosarum* bv. trifolii *nodE* severely inhibits the nodulation of several *Trifolium* species and simultaneously enhances the nodulation of *P. sativum* and *V. sativa* (59, 251). Mutation of *R. leguminosarum* bv. viciae

TABLE 4. Nod factors and their baroque decorations^a

Strains	Acyl	R1	R2	R3	R4	R5	R6	n	Ref.
<i>A. caulinodans</i> ORS571	C _{18:1} , C _{16:0}	Me	OH	Cb, H	Fuc, H	Ara, H	OH	1, 2	172
<i>B. elkanii</i> USDA61	C _{18:1}	Me, H	Cb, H	Ac, H	MeFuc	H	OH	1, 2	35
<i>B. japonicum</i> USDA110	C _{16:0} , C _{16:1} , C _{18:1}	H	OH	H	MeFuc	H	OH	2	229
<i>Rhizobium</i> sp. strain GRH2	C _{16:0} , C _{18:0} , C _{18:1} , C _{20:1}	Me, H	OH	H	S, H	H	OH	1, 2, 3	160
<i>Rhizobium</i> sp. strain ORS1645	C _{18:0} , C _{18:1}	Me	Cb ^c	Cb ^c	S, H	H	OH	2	164
<i>Rhizobium</i> sp. strain NGR234	C _{16:1} , C _{18:0} , C _{18:1}	Me	Cb, OH	Cb, H	MeFuc, AcMeFuc, SMeFuc	H	OH	2	206
<i>R. etli</i> CFN42	C _{18:1}	Me	Cb, OH	H	AcFuc	H	OH	2	33
<i>R. etli</i> CE3	C _{18:0} , C _{18:1}	Me	Cb, OH	H	AcFuc	H	OH	2	203
<i>R. fredii</i> USDA191 ^b	C _{18:1} , C _{18:0} , C _{16:1}	H	H	H	Fuc, MeFuc	H	H	0, 1, 2	7
<i>R. fredii</i> USDA257	C _{18:1}	H	OH	H	Fuc, MeFuc	H	OH	0, 1, 2	6
<i>R. galegae</i>	C _{18:1} , C _{18:2} , C _{18:3} , C _{20:2} , C _{20:3}	H	OH	Cb	H	H	Ac	1	282
<i>R. huakuii</i> ^d	C _{18:4}	H	OH	H	S	H	OH	2	282
<i>R. leguminosarum</i> bv. trifolii ANU843	C _{16:0} , C _{16:1} , C _{18:0} , C _{18:1} , C _{18:2} , C _{20:3}	H	OH	H, Ac	H	H	OH	0, 1, 2	188
bv. trifolii LPR5045	C _{18:0} , C _{18:1} , C _{20:3} , C _{20:4}	H	OH	Ac	H	H	OH	2	269b
bv. viciae RBL5560	C _{18:1} , C _{18:4}	H	OH	Ac	H	H	OH	1, 2	252
bv. viciae TOM	C _{18:1} , C _{18:4}	H	OH	Ac	Ac	H	OH	1, 2	80
<i>R. loti</i> NZP2213	C _{16:0} , C _{16:1} , C _{18:0} , C _{18:1} , C _{20:0} , C _{20:1} , C _{22:1}	H	Cb	H	Fuc, AcFuc	H	Fuc	-1, 0, 1, 2	187
<i>R. loti</i> NZP2037	C _{18:0} , C _{18:1}	Me	Cb	Cb	AcFuc	H	OH	2	161
<i>R. meliloti</i> RCR2011	C _{16:1} , C _{16:2} , C _{16:3}	H	OH	H, Ac	S	H	OH	1, 2	2, 154
<i>R. tropici</i> CFN299	C _{18:1}	Me	OH	H	S, H	H	OH	2	202
<i>S. saheli</i> ORS611	C _{16:0} , C _{18:1}	Me	Cb ^c	Cb ^c	Fuc, H	Ara, H	OH	2	163
<i>S. teranga</i> bv. acaciae ORS1602	C _{16:0} , C _{18:0} , C _{18:1}	Me	Cb ^c	Cb ^c	S, H	H	OH	2	164

^a Abbreviations: Ac, acetyl; Ara, arabinosyl; Cb, carbamoyl; Fuc, fucosyl; H, hydrogen; Me, methyl; S, sulfate; MeFuc, methylfucose; AcMeFuc, acetylated methylfucose; SMeFuc, sulfated methylfucose.

^b In USDA191, a minor fraction of the *N*-acetylglucosamine marked in brown is replaced by a glucose.

^c Carbamoyl group is either on R2 or R3.

^d *R. huakuii* Nod factors are partially glycosylated at the C-2 position of the reducing terminus.

nodE renders the strain Nod⁻ on *V. sativa* (32). Replacement of *R. meliloti nodEF* with *R. leguminosarum* bv. *viciae nodEF* leads to the synthesis of Nod factors containing a polyunsaturated C₁₈ fatty acid side chain which resembles those produced by *R. leguminosarum* bv. *viciae* (52). Thus, *nodEF* and the unsaturated Nod factors they produce appear to be specializations necessary for nodulation of the legume tribes Trifolieae and Viciae.

(ii) **6-O glycosylation.** An additional sugar, which can be either arabinose or fucose, represents another type of decoration.

(a) **Arabinosylation (*noeC*).** *noeC* and/or downstream genes are essential for arabinosylation of *A. caulinodans* Nod factors (171). In these Nod factors, the reducing terminus can be fucosylated, arabinosylated, or both (172). Perhaps this suggests that both arabinosylated and fucosylated Nod factors are

necessary for nodule formation on the stems of *Sesbania rostrata* (163, 164). D-Arabinosylated Nod factors result in larger numbers of nodules on the roots of *S. rostrata*, whereas the presence or absence of L-fucose has no effect. In contrast, other hosts of *A. caulinodans* prefer fucosylated Nod factors (75).

(b) Fucosylation (*nodZ*, *nolK*). Fucose is a frequent substitution, and its addition to Nod factors is encoded by *nodZ* in many rhizobia (162, 171, 208, 210, 261). Since all the usual *Glycine max* symbionts (*B. elkanii*, *B. japonicum*, *R. fredii*, and strain NGR234) secrete fucosylated Nod factors, the expectation that mutation of *nodZ* would prevent the nodulation of soybeans was strong. In fact, *B. japonicum nodZ* is required for nodulation of *M. atropurpureum* but not of *G. max* (261). Similarly, *nodZ* mutants of strain NGR234 produce nonfucosylated Nod factors and are unable to nodulate *Pachyrhizus tuberosus* but retain the capacity to nodulate soybeans (208). Since transconjugants of *R. leguminosarum* containing *B. japonicum nodZ* acquire the capacity to nodulate *Macroptilium* (but not to fix nitrogen) (162), it seems as if fucosylated Nod factors play a role in nodulation of only a few legumes. In *A. caulinodans*, *NolK* is involved in the synthesis of GDP-fucose, which is used by *NodZ* as the fucosyl donor in fucosylation of *A. caulinodans* Nod factors (171).

(iii) Sulfation (*nodH*, *noeE*). *NodH* and *NoeE*, the only two characterized sulfotransferases, are specific to the reducing terminus of Nod factors (68, 154, 209, 224, 239). Both enzymes use 3'-phosphoadenosine-5'-phosphosulfate as the sulfate donor. 3'-Phosphoadenosine-5'-phosphosulfate is synthesized by the enzymes encoded by *nodPQ* (242, 243). *NodH*-dependent sulfation of the reducing terminus at C-6 of *R. meliloti* Nod factors is a major determinant of host range in *R. meliloti* (154, 224). *nodH* mutants which fail to produce sulfated Nod factors were thought to completely lose the capacity to nodulate *M. sativa* but gain the ability to nodulate the nonhost *V. sativa* (49, 224). Although other data indicate that nodulation of alfalfa by some *nodH* mutants is strongly impaired rather than abolished (125, 186), these results confirm that optimal nodulation of *M. sativa* requires sulfated Nod factors whereas *V. sativa* does not tolerate them (70).

In contrast, *NoeE* is a fucose-specific sulfotransferase (113, 209). Mutation of strain NGR234 *noeE* blocks the nodulation of *P. tuberosus*, while its introduction into the closely related strain USDA257 extends the host range of *R. fredii* to include *Calopogonium caeruleum* (113). Surprisingly, these transconjugants do not acquire the ability to nodulate *P. tuberosus* (M. Hanin, unpublished results).

(iv) Acetylation (*nodL*, *nodX*, *noll*). Unlike sulfate groups, acetyl residues may be found at both extremities of Nod factors, i.e., on C-6 of the reducing or nonreducing termini, as well as on the fucose (Table 4). Furthermore, acetate groups on the fucose can move from one α -hydroxyl group to another (12). Mutation of *nodX* provokes a drastic reduction in Nod factor production by *R. leguminosarum* strain TOM and the ability to nodulate primitive cultivars of *P. sativum* (48). In *R. leguminosarum* bv. *viciae* however, a fucosyl group added by *NodZ* can functionally replace the missing acetyl group in a *nodX* mutant (189). Although *NodL* is responsible for acetylation of C-6 of the nonreducing terminus in *R. meliloti* Nod factors (2), *R. meliloti nodL* mutants are impaired in their ability to elicit infection thread formation on *M. sativa* but form nodules with only a moderate delay (96). A similar phenotype was observed when the corresponding mutant of *R. leguminosarum* bv. *viciae* was used to inoculate *V. hirsuta*, while *nodL nodF* double mutants of *R. meliloti* are unable to penetrate their hosts (2). In strain NGR234, disruption of the flavonoid-inducible *noll*

gene results in the synthesis of NodNGR factors that lack the 3-O- or 4-O-acetate group (12). Interestingly, the nodulation capacity of the mutant NGR Δ *noll* is not impaired whereas transconjugants of USDA257 containing *noll* of NGR234 produce acetylated Nod factors and nodulate the nonhosts *C. caeruleum*, *L. leucocephala*, and *Lotus halophilus* (12). Acetylation of the fucose on Nod factors of *R. etli* also conditions efficient nodulation of some *P. vulgaris* cultivars and of the alternate host *Vigna umbellata* (44).

(v) N methylation and carbamoylation (*nodS*, *nodU*, *nolO*). *NodS* is an N-methyltransferase (93, 94, 129), while *NodU* and *NolO* control carbamoylation at C-6 and C-3 (or C-4), respectively, on the nonreducing N-acetyl-D-glucosamine (57, 129, 131). Inactivation of *nodS* in *A. caulinodans*, strain NGR234, and *R. tropici* abolishes the nodulation of *L. leucocephala* and *P. vulgaris* (158, 276). Introduction of either *nodS* or *nodU* into *R. fredii* extends its host range to include *Leucaena* spp. (129, 144).

(vi) 2-O methylation (*noeI*). The fucose group of Nod factors of *B. japonicum* USDA110, NGR234, and USDA257 is mostly 2-O methylated (Table 4). In strains NGR234, *NoeI* controls this function, and mutation of *noeI* leads to the production of fucosylated Nod factors that are not 2-O methylated (131). Introduction of *noeI* into *R. loti* allows the production of 2-O-methylated NodNGR factors. However, nodulation tests using *L. leucocephala*, *Lotus japonicus*, *M. atropurpureum*, and *V. unguiculata* and the NGR Δ *noeI* mutant have failed to identify a host which requires 2-O-methylated NodNGR factors (S. Jabbouri and M. Hanin, unpublished results).

Nod enzymes, which are responsible for the elaboration of Nod factors, are among the principal determinants of host specificity. Indeed, a number of them were called *hsn* determinants before the unified *nod* gene nomenclature was established. Most of them catalyze the adjunction of baroque decorations to the core Nod factor molecule (which is synthesized by *NodC*, *NodB*, and *NodA*); the effect of these different families of molecules on legumes is discussed below.

Nod Factors and Host Specificity

Although much information on the influence of Nod factor substituents on host range exists, no strict correlation can be drawn between the types of LCOs produced by rhizobia and the plants they nodulate. For example, although the Nod factors produced by *R. etli* and *R. loti* are identical, the two species have distinct host ranges (*Phaseolus* spp. and *Lotus* spp., respectively) (33). Also, the major Nod factors secreted by *R. leguminosarum* bv. *trifolii* are the same as two of the major LCOs produced by *R. leguminosarum* bv. *viciae* (188, 255), yet the two biovars nodulate distinct plants. Although the inability of *NodD* proteins to recognize the host flavonoids (and thus to activate *nod* gene expression) might be responsible for some of these phenotypes, these data emphasize the point that Nod factor structures alone cannot be used to predict host range. Furthermore, two rhizobia that nodulate the same plant may secrete different Nod factors. *R. tropici* and *R. etli* produce sulfated and acetylfucosylated Nod factors, respectively (Table 4), but both effectively nodulate *P. vulgaris* (202, 203). *B. elkanii*, *B. japonicum*, strain NGR234, and strain USDA257 have a number of common hosts (207), but their Nod factors vary considerably (Table 4).

The amounts of Nod factors released by rhizobia are also important in determining the host range. For instance, introduction of strain NGR234 *nodDI* into *R. meliloti* increases Nod factor production by about twofold and permits the nodulation of *V. unguiculata*, a nonhost (214). Similarly, conjugation of the

TABLE 5. Effects of apigenin, *nodD1*, and *nodSU* on Nod factor production and nodulation of *L. leucocephala* by strains NGR234 and USDA257

Strain ^a	Nod factor production ^b		Nodulation of <i>L. leucocephala</i>
	- Apigenin	+ Apigenin	
NGR234	0.4 ± 0.2	61.8 ± 9.7	Nod ⁺ Fix ⁺
NGRΔ <i>nodD1</i>	0.4 ± 0.3	0.3 ± 0.2	Nod ⁻
NGRΔ <i>nodSU</i>	0.3 ± 0.3	5.7 ± 2.5	Nod ⁻
USDA257	0.2 ± 0.2	1.5 ± 1.1	Nod ⁻
USDA257(<i>nodSU</i>)	0.1 ± 0.1	38.3 ± 9.8	Nod ⁺ Fix ⁺

^a NGRΔ*nodD1*, *nodD1* mutant of *Rhizobium* sp. strain NGR234; NGRΔ*nodSU*, *nodSU* mutant of strain NGR234; USDA257 (*nodSU*), *R. fredii* USDA257 transconjugant containing the functional *nodSU* genes of NGR234.

^b Nod factors were quantified using the tomato cell suspension assay (259). The results are expressed in nanomoles per milliliter of culture corrected to an absorbance at 700 nm of 1 and were taken from references 144, 158, and 214.

nodSU genes of strain NGR234 into *R. fredii* USDA257 not only extends the host range of the transconjugant to include *L. leucocephala* (144) but also vastly increases Nod factor production (Table 5). In contrast, the NGRΔ*nodSU* mutant, which secretes only 1/10 the amount of Nod-factor produced by the wild type, is incapable of nodulating *L. leucocephala* (Table 5). One interpretation of these data is that higher levels of Nod factors (produced when a functional NodS or NodU is present) (129), not the presence of N-methylated or 6-O-carbamoylated LCOs, is necessary for nodulation of *Leucaena*.

Although the supporting evidence is lacking, it also seems likely that in addition to variations in the levels of Nod factors, different members and various proportions of the respective Nod factor families are secreted into the rhizospheres of specific legumes. There, only certain combinations of Nod factors, present at optimal levels, are capable of inducing deformation (Had) and curling (Hac) in homologous legumes (118, 148, 154, 169, 206, 213, 238, 252, 269a).

Cooperation among Nod factors and related signal molecules has also been demonstrated. Minami et al. (177, 178) examined the effects of LCOs on changes in the expression of early nodulin genes in *G. soja* roots and found that any combination of one active Nod factor with a nonspecific LCO was sufficient to induce mRNA expression of early nodulin genes such as *Enod2*. In contrast, both synthetic Nod factors and the chitin pentamer PACT, which is unable to provoke Had on *G. soja*, induced the transient accumulation of *Enod40* mRNA. It would thus seem that not only are absolute levels of Nod-factors important but also the composition and relative proportions of the mixtures excreted by rhizobia are necessary for the induction of different components of the nodulation pathway (190).

Nod factor binding proteins. The extent to which specific Nod factors bind to their respective plant receptors is still unknown. In *Medicago*, two putative Nod factor binding sites (NFSB) have been characterized (23, 181). Some properties of NFSB1 of *M. truncatula* suggest that it may also be involved in processes other than nodulation. First, its affinity and specificity for LCOs are low. Second, a similar site exists in particulate fractions of tomato roots (23). In contrast, NFSB2, which was isolated from the microsomal fraction of *Medicago varia* cell suspension cultures, is sensitive to proteases and shows high affinity for Nod factors (181). Covalent linkage of both the lipid and the chitoooligosaccharide moieties, as well as O acetylation of the nonreducing sugars of Nod factors, is required for high-affinity binding to NFSB2 (108). In competition experiments in which the ligand specificity of NFSB2 was assayed by titrating

³⁵S-labelled *R. meliloti* Nod factors against synthetic LCOs, those with short (C₈) acyl chains were poor competitors compared to those with C₁₆ and C₁₈ fatty acid side chains (108). However, NFSB2 does not select for the presence of sulfate on the reducing sugar or for the number and/or positions of double bonds in the acyl chain. Since sulfated *R. meliloti* Nod factors are required for optimal nodulation of *Medicago* (see above) and as synthetic LCOs containing at least two double bonds are more active biologically than are monounsaturated compounds (53), it seems doubtful that these putative receptors are host-specific determinants of the *Medicago-R. meliloti* interaction.

Similarly, a root surface lectin of *Dolichos biflorus* has apyrase (Ca²⁺-dependent ATP diphosphatase) activity and binds Nod factors from both heterologous and homologous rhizobia (69). Reverse PCR-based techniques using mRNAs of *L. japonicus* and *M. sativa* root confirmed the presence of orthologues in these two legumes. Similar experiments were also performed with *D. biflorus* and *Arabidopsis*, leading to a second lectin in the former and a nonlegume lectin in the latter (223). Since much work still has to be done on these Nod factors binding lectin/nucleotide phosphohydrolases (LNPs), it is too early to pronounce on their exact symbiotic role. It is intriguing, however, that *D. biflorus* has two LNPs. Close orthologues of LNP1 are found only in legumes, while *D. biflorus* LNP2 is more closely related to apyrase sequences of nonleguminous plants (223).

Although Nod factors are bacterial products, they share many characteristics with plant hormones (213). One of these is that perturbations in the auxin-cytokinin balance mimic Nod factors in inducing meristematic activity of nodule meristems (128). Another is that the range of concentrations over which both hormones and Nod factors are active can vary by a millionfold. This suggests a desensitisation or an adaptation by the plant to increasing hormone concentrations (137). In turn, this implies that covalent and reversible modification to the "receptors" occurs; these suggestions are supported by the fact that the ethylene receptor is homologous to two-component chemoreceptors of bacteria (38). Perhaps this explains why the search for Nod factor receptors in direct Nod factor binding studies has been only moderately successful.

Hydrolysis of Nod factors. In principle, the chemical stability of Nod factors should influence the biological activity of the LCO mixtures secreted by rhizobia. In fact, chitinases and other enzymes rapidly cleave purified Nod factors in the host rhizosphere. The degradation products formed are only weakly active on their respective hosts (118, 258). Plant chitinases have been studied extensively in the context of plant-pathogen interactions. Among these are isoforms that are induced in symbiotic and/or pathogenic interactions (103, 192, 257, 273, 281). Distinct isoforms of chitinases and related hydrolases cleave specific β-1,4-linkages in the carbohydrate moiety of Nod factors (Tables 6 and 7), resulting in the formation of acylated degradation products that are either di-, tri- or tetrameric. In general, leguminous and nonleguminous chitinases that belong to the same chitinase class have similar substrate specificities. Interestingly, the baroque decorations on certain Nod factors have a strong influence on the stability of these molecules (241, 258, 259) and modify the cleavage specificity of a given chitinase. For example, pentameric LCOs are hydrolyzed in two positions by an *M. sativa* class I chitinase, but only one of these positions is susceptible in sulfated Nod factors (Table 7). Furthermore, the sulfate group at the reducing end and the O-acetyl group at the nonreducing terminus of *R. meliloti* Nod factors dramatically increase the stability of these LCOs, rendering tetrameric molecules re-

TABLE 6. Hydrolysis of Nod factors by chitinases and related enzymes^a

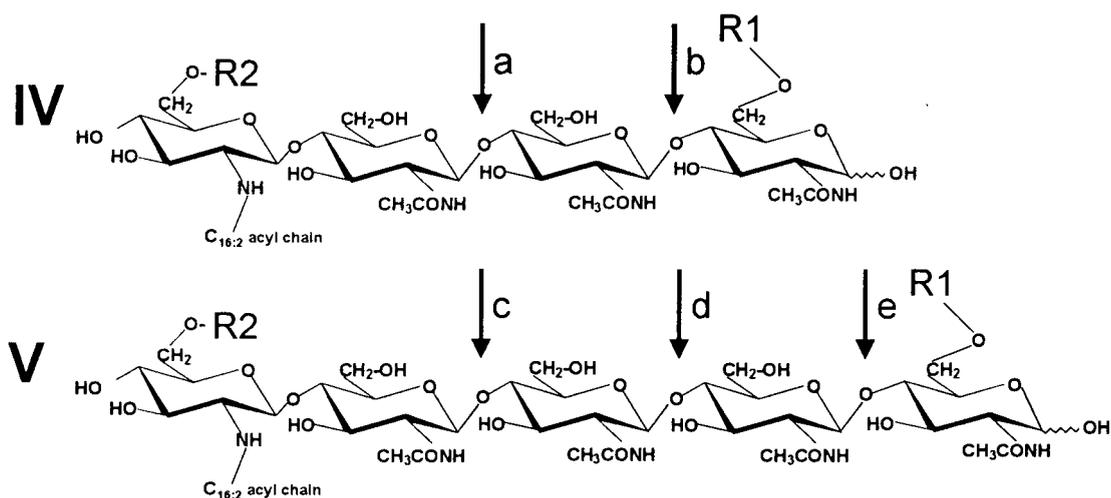
Enzyme	Plant isozyms ^b	Nod factors used as substrates ^{b,c}	Acylated products in hydrolysate ^{b,d}	Reference(s)
Chitinase class I	<i>Ms, Pv, Vs, Nt</i>	NodRm-V(C_{16:2}, S)	III	241, 258
	<i>Ms, Vs</i>	NodRm-V(C_{16:2})	IV, III	258
	<i>Ms, Pv, Vs, Nt</i>	NodRm-IV(C_{16:2}, S)	None	241, 258
	<i>Ms, Pv, Vs, Nt</i>	NodRm-IV(C_{16:2})	III	241, 258
	<i>Vu</i>	NodNGR-V(AcMeFuc)	Unknown structures	259
	<i>Vu</i>	NodNGR-V(SMeFuc)	Unknown structures	259
	<i>Ps, Pv, Vu</i>	NodNGR-V(MeFuc)	Unknown structures	259
	<i>Nt</i>	NodRm-IV(Ac, C _{16:2})	None	241
	<i>Nt</i>	NodRlv-V(Ac, C _{18:4})	AcIV, AcIII	Ovtsyna et al., submitted
	<i>Nt</i>	NodRlv-V(Ac, C _{18:4} , Fuc)	AcIII	Ovtsyna et al., submitted
	<i>Nt</i>	NodRlv-IV(Ac, C _{18:4})	None	Ovtsyna et al., submitted
	<i>Nt</i>	NodRlv-IV(Ac, C _{18:4} , Fuc)	None	Ovtsyna et al., submitted
	Chitinase class II	<i>Nt</i>	NodRm-V(C _{16:2} , S)	III
<i>Nt</i>		NodRm-IV(C _{16:2} , S)	None	241
<i>Nt</i>		NodRm-IV(C _{16:2})	III	241
Chitinase class III	<i>Ca, Bv, Nt</i>	NodRm-V(C_{16:2}, S)	III, II	241
	<i>Ca, Bv, Nt</i>	NodRm-IV(C_{16:2}, S)	II	241
	<i>Ca, Bv, Nt</i>	NodRm-IV(C_{16:2})	III, II	241
	<i>Sr</i>	NodAc factors	Unknown structures	103
	<i>Nt</i>	NodRm-IV(Ac, C _{16:2} , S)	None	241
	<i>Nt</i>	NodRm-V(Ac, C _{16:2} , S)	AcIII	241
	<i>Nt</i>	NodRlv-V(Ac, C _{18:4})	AcIV, AcIII	Ovtsyna et al., submitted
	<i>Nt</i>	NodRlv-V(Ac, C _{18:4} , Fuc)	AcIII	Ovtsyna et al., submitted
	<i>Nt</i>	NodRlv-IV(Ac, C _{18:4})	AcIII	Ovtsyna et al., submitted
<i>Nt</i>	NodRlv-IV(Ac, C _{18:4} , Fuc)	None	Ovtsyna et al., submitted	
Chitinase class IV	<i>Bv, Dc</i>	NodRm-V(C _{16:2} , S)	III	241
	<i>Bv, Dc</i>	NodRm-IV(C _{16:2} , S)	None	241
	<i>Bv, Dc</i>	NodRm-IV(C _{16:2})	III	241
Chitinase class V	<i>Nt</i>	NodRm-V(C _{16:2} , S)	III	241
	<i>Nt</i>	NodRm-IV(C _{16:2} , S)	None	241
	<i>Nt</i>	NodRm-IV(C _{16:2})	III	241
Chitinase class VI	<i>Nt</i>	NodRm-V(C _{16:2} , S)	III	241
	<i>Nt</i>	NodRm-IV(C _{16:2} , S)	None	241
	<i>Nt</i>	NodRm-IV(C _{16:2})	III	241
Novel chitinase/lysozyme	<i>Ms</i>	NodRm-V(C_{16:2}, S)	III	179
	<i>Ms</i>	NodRm-IV(C_{16:2}, S)	III	179
	<i>Ms</i>	NodRm-IV(C_{16:2})	III	179
	<i>Ms</i>	NodRm-IV(Ac, C_{16:2}, S)	AcIII	179
Nod factor hydrolase	<i>Ms</i>	NodRm-V(C_{16:2}, S)	II	258, 260
	<i>Ms</i>	NodRm-V(C_{16:2})	II	258
	<i>Ms</i>	NodRm-IV(C_{16:2}, S)	II	258, 260
	<i>Ms</i>	NodRm-IV(C_{16:2})	II	258, 260
	<i>Ms</i>	NodRm-IV(Ac, C_{16:2}, S)	AcII	260
	<i>Ms</i>	NodRm-V(Ac, C_{16:2}, S)	AcII	260
Roots	<i>Ms, Vs</i>	NodRm-V(C_{16:2}, S)	III, II	258, 260
	<i>Ms, Vs</i>	NodRm-V(C_{16:2})	IV, III, II	258
	<i>Ms, Vs</i>	NodRm-IV(C_{16:2}, S)	II	258, 260
	<i>Ms, Vs</i>	NodRm-IV(C_{16:2})	III, II	258
	<i>Ms</i>	NodRm-IV(C_{16:0}, S)	II	258
	<i>Ms, Ps, Vs</i>	NodRlv-V(Ac, C_{18:4})	AcIV, AcIII, AcII	97, 118
	<i>Vs</i>	NodRlv-IV(Ac, C_{18:4})	AcIII, AcII	118
	<i>Vs</i>	NodRlv-V(Ac, C_{18:0})	AcIV, AcIII, AcII	118
	<i>Sr</i>	NodAc factors	Unknown structures	103

^a Purified enzymes (as well as whole roots) of both legumes (bold) and nonlegumes were incubated with Nod factors, and the reaction products were examined for the presence of acylated chitin fragments.

^b Abbreviations are as follows. Hydrolases were isolated from the following legumes: *Cicer arietinum* (*Ca*), *Medicago sativa* (*Ms*), *Phaseolus vulgaris* (*Pv*), *Pisum sativum* (*Ps*), *Sesbania rostrata* (*Sr*), *Vicia sativa* (*Vs*), and *Vigna unguiculata* (*Vu*) and the nonlegumes *Beta vulgaris* (*Bv*), *Daucus carota* (*Dc*), and *Nicotiana tabacum* (*Nt*). Chemical groups are abbreviated as follows: acetyl (Ac), fucosyl (Fuc), methyl (Me), sulfate (S), methylfucose (MeFuc), acetylated methylfucose (AcMeFuc), sulfated methylfucose (SMeFuc).

^c Nod factors were purified from *A. caulinodans* (NodAc), *R. leguminosarum* bv. viciae (NodRlv), *Rhizobium* sp. strain NGR234 (NodNGR), and *R. meliloti* (NodRm). Details of their structure, such as the presence of certain baroque decorations (for abbreviations, see footnote b), the length of the acyl chain, and the number of double bonds, are shown in brackets. Tetramers and pentamers of *N*-acetylglucosamine are marked as IV and V, respectively. The nonsulfated NodRm factors and fucosylated NodRlv factors are derivatives of the wild type obtained by desulfation and fucosylation with NodZ, respectively. NodRm-IV(C_{16:0}, S) was prepared from NodRm-IV(C_{16:2}, S) by catalytic reduction.

^d Acylated products were separated from the substrate by reverse-phase high-pressure liquid chromatography (179, 241, 258, 260; A. O. Ovtsyna, M. Schultze, I. A. Tikhonovich, H. P. Spaink, É. Kondorosi, Á. Kondorosi, and C. Staehelin, submitted for publication) and thin-layer chromatography (97, 103, 118, 258). Abbreviations: lipotetrasaccharide (IV); lipotrisaccharide (III); lipodisaccharide (II); O-acetylated lipotetrasaccharide (AcIV); O-acetylated lipotrisaccharide (AcIII); O-acetylated lipodisaccharide (AcII); Nod factors resistant to hydrolysis by the given enzyme (None).

TABLE 7. Sites of hydrolytic cleavage of Nod factors of *Rhizobium meliloti* by enzymes isolated from the roots of *Medicago sativa*^a

Hydrolase (reference)	No. of N-acetylglucosamine residues	R1	R2	Cleavage site(s)
Intact roots (258, 260)	IV	Sulfatyl	H	a
	IV	H	H	a, b
	IV	Sulfatyl	O-Acetyl	a
	V	Sulfatyl	H	c, d
	V	H	H	c, d, e
Chitinase class I (241, 258)	IV	Sulfatyl	H	None
	IV	H	H	b
	IV	Sulfatyl	O-Acetyl	None
	V	Sulfatyl	H	d
	V	H	H	d, e
Novel chitinase/lysozyme (179)	IV	Sulfatyl	H	b
	IV	H	H	b
	IV	Sulfatyl	O-Acetyl	b
	V	Sulfatyl	H	d
Nod factor hydrolase (258, 260)	IV	Sulfatyl	H	a
	IV	H	H	a
	IV	Sulfatyl	O-Acetyl	a
	V	Sulfatyl	H	c
	V	H	H	c
	V	Sulfatyl	O-Acetyl	c

^a Distinct cleavage site preferences exist toward tetrameric (IV) and pentameric (V) Nod factors of *R. meliloti* as well as their desulfated and deacetylated derivatives, respectively.

sistant to degradation by a large number of different chitinases (Table 6). Novel enzymes have been identified in *Medicago*, and their cleavage preferences are different from those of known chitinases (Tables 6 and 7). One of them is an extracellular lipodisaccharide-releasing Nod factor hydrolase that cleaves all tetra- and pentameric *R. meliloti* Nod factors. Preincubation with *R. meliloti* Nod factors stimulated the activity of this enzyme in *Medicago* roots, suggesting that Nod factor inactivation is an early feedback response by the host plant.

However, two different types of observations suggest that chitinases may not play major roles in the determination of host specificity. First, the class I, II, III, V, and VI chitinases isolated from *Nicotiana tabacum*, as well as the class III and IV chitinases of *Beta vulgaris* and *Daucus carota* (all nonlegumes), degrade Nod factors in similar ways to that used by legume chitinases (Table 6). One interpretation of these data is that chitinases form part of a generalized plant response system rather than tailoring Nod factor degradation to specific legume-*Rhizobium* associations. A second important consider-

ation is that root hydrolases take hours to degrade Nod factors (260), while the first responses of root hairs to inoculation by rhizobia (or to the presence of purified Nod factors) can be measured in seconds (64). Nevertheless, expression of a chitinase from *Serratia marcescens* by *R. fredii* USDA191 transconjugants resulted in delayed nodulation and a marked decrease in total nodule number on soybean cultivar McCall in comparison with the wild-type strain (146). What is undisputed, however, is that chitinases modulate the levels of Nod factors in the rhizosphere and, in so doing, help suppress defense responses stimulated by high concentrations of LCOs (231, 260). Since defense reactions also occur in aborted infection threads (273), it is possible that plant hydrolases help regulate Nod factor levels even after the bacterium has entered the plant. In other words, chitinases and related hydrolases cleave and so inactivate Nod factors. Local induction or down-regulation of these enzymes in leguminous roots would thus modulate local LCO concentrations. Since Nod factor levels seem to be host-range determinants, plant hydrolases might help tailor host specificity.

NOD FACTORS OPEN THE LEGUME OUTER DOOR

Although the mechanism of root hair deformation is poorly understood, it probably resembles that of root hair development (118). The tips of Nod factor-treated *V. sativa* root hairs swell before polar growth is induced. Induction of tip growth is preceded by local hydrolysis of the cell wall and followed by polar growth (118). Schiefelbein and Somerville (233) showed that the phenotype of root hair-specific mutants of *Arabidopsis thaliana* resembles root hair deformations caused by rhizobia. Thus, initiation and deformation of root hairs appear to be ontogenically related.

Nod factors are the signals required for entry of rhizobia into some legumes. Concomitant treatment of *G. max* and *V. unguiculata* roots with NodNGR factors and *nodABC* mutants of strain NGR234 or *B. japonicum* USDA110 permits these strains to nodulate and fix nitrogen on their respective hosts (215). NodNGR factors also allow the entry of *R. fredii* USDA257 into the roots of the nonhost *Calopogonium caeruleum* (215) and of the *nodABC* mutant of NGR234 into *Macroptilium atropurpureum* (213). Similarly, coapplication of purified *A. caulinodans* Nod factors and a *nodA* mutant of the same strain restores the formation of outer cortical infection pockets in *Sesbania rostrata* (56). These data leave little doubt that at least in some symbioses, Nod factors act like keys to legume doors. Interestingly, a recent report suggests that one of the "latches" that is opened by a Nod factor "key" has been found. A transposon-tagged mutant of *Lotus japonicus*, which is arrested at the stage of bacterial recognition, was identified (232). The mutation, which has been named *nin* (for nodule inception), is required for the formation of infection threads and is a transcription factor.

Once Nod factors have opened the root hair door to the invading rhizobia, additional bacterial signals appear necessary for continued development of the infection threads. This is clearly seen in variations of the Nod factor complementation experiments described above. These experiments work well if the strain deficient in Nod factor production is mutated in genes essential for Nod factor synthesis (*nodABC*) but not if the strain is mutated in *nodD1*. In this latter case, rhizobia bunch up in the curled part of the root hair and the infection thread fails to develop toward the root cortex, resulting in contortions that resemble a cerebellum (Fig. 2E). It thus seems probable that products of genes other than those needed for the synthesis of Nod factors, but also under the control of *NodD1*, are required for the continued development of infection threads.

DO INFECTION THREADS HAVE AN INNER DOOR?

Other Host Range Keys

NodD proteins, with their ability to recognize different inducers, the complex mixtures of Nod factors as well as the various levels at which they are maintained, are not the only determinants of host specificity. Although Nod factors permit rhizobia to enter the outer door of the legume host and may play a role during nodule development (56, 267), additional "keys" are necessary for the formation of symbiotically proficient nodules. In fact, later steps of the infection process such as infection thread formation and propagation, as well as bacterial release into the cytoplasm of infected cells, require constituents of the cell wall and in some cases secretion of specific proteins (for a review, see reference 275).

Polysaccharides and surface components. Symbiotically relevant components of the rhizobial cell wall include extracellu-

lar polysaccharides (also known as exopolysaccharides) (EPS), lipopolysaccharides (LPS), capsular polysaccharides (CPS and KPS), and cyclic β -glucans (Fig. 3). Surface polysaccharides (SPS) form a complex macromolecular structure at the bacterium-plant interface. They accumulate on the surface of the prokaryote as a capsular layer but are also released into the extracellular space as bacterial slime.

EPS I and EPS II represent the two major classes of EPS synthesized by *R. meliloti*. EPS I members are polymers of an octasaccharide repeating unit of succinoglycan ranging in size from 10^6 to 10^7 Da for the high-molecular-weight (HMW) fraction (152). In contrast, the low-molecular-weight (LMW) fraction is formed of monomers, dimers, and trimers (277). Although mutants of *R. meliloti* Rm1021 incapable of synthesizing succinoglycan (EPS I) produce normal root hair curling on *M. sativa*, the nodules are devoid of bacteria and bacteroids and thus are ineffective (76, 151). Addition of the LMW fraction, specifically trimers of succinoglycan, to roots of alfalfa restored the nodule invasion capability of the *exo* mutant (5, 277). Interestingly, EPS I mutant strains of *R. meliloti* derepressed for the synthesis of another class of EPS are capable of forming normal nodules on alfalfa (99). EPS II members are polymers of modified glucose-(β -1,3)-galactose (99), and only small amounts (as little as 7 pmol per plant) of the purified LMW fraction are sufficient to restore nodule invasion by noninfective strains (102). Thus, although EPS II members can replace succinoglycan in nodule invasion, they are not required when wild-type EPS I is produced.

Fluorescence microscopy analyses show that nodule invasion is aborted at various stages in different *exo* mutants (40). Cells that lack *ExoR*, a negative regulator of *exo* gene expression (212), vastly overproduce succinoglycan and are unable to colonize curled root hairs and form infection threads. In contrast, a mutant with a mutation in *exoY* that is incapable of synthesizing succinoglycan (since it lacks the first enzyme in the biosynthetic pathway) (217) colonizes curled root hairs but forms few, very short infection threads. Although the *exoH* mutant that produces symbiotically dysfunctional succinoglycan forms infection threads longer than those produced by the *exoY* mutant, they never extend as far as the base of the root hair cell. Thus, in *R. meliloti*, succinoglycan could be regarded as a symbiotic signal (or key) required for opening the inner root hair door(s). Mutants that fail to produce the symbiotically active forms of these EPSs in adequate quantities are incapable of penetrating the adjacent plant cell and thus remain blocked or trapped in the infected root hair (40, 153). Also, since nodules induced by EPS I mutants of *R. meliloti* show pronounced symptoms of plant defense, it is possible that a molecule derived from the EPS I biosynthetic pathway functions as a suppressor of plant defense reactions (182). It has been postulated that in other plants, EPS of *R. leguminosarum* bv. *viciae* accelerates root hair curling and infection in such a way that invasion by rhizobia precedes the plant defense response (272). Similarly, it is thought that the wild-type cyclic β -glucans of *B. japonicum* (Fig. 3) may function as suppressors of host defense responses (20).

Formation of symbiosomes (which involves the release of rhizobia from infection threads into the cytoplasm of infected nodule cells) in plants other than *M. sativa* requires different components including EPS, LPS, CPS, KPS, and cyclic β -glucans (Fig. 3, Table 8). Although EPS⁻ mutants of *M. lotii* are fully effective on *Lotus pedunculatus*, they provoke small, ineffective, tumor-like growths on *L. leucocephala* (127). Similarly, *Exo*⁻ mutants of *R. leguminosarum* bv. *trifolii* (271), *R. leguminosarum* bv. *viciae* (24), and strain NGR234 (39) are ineffective on their respective hosts, *T. repens*, *P. sativum*, and *L.*

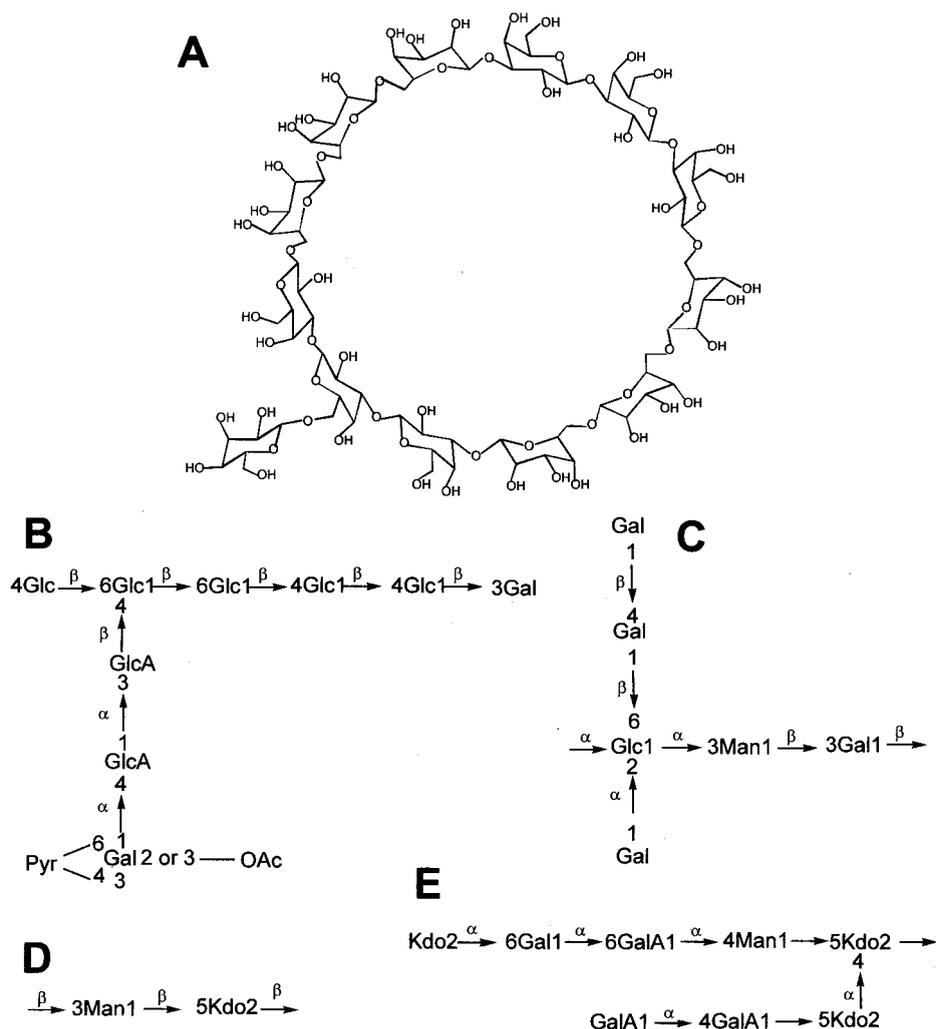


FIG. 3. Examples of the five classes of rhizobial polysaccharides. (A) Structure of cyclic β -(1,6)- β -(1,3)-glucans common to *B. japonicum*. Redrawn from reference 26. (B) Acidic EPS of *Rhizobium* sp. strain NGR234 (60). EPS I (succinoglycan) of *R. meliloti* resembles the EPS of strain NGR234. (C) KPS of *R. leguminosarum* bv. *trifolii* (98). (D) The somatic K antigen of *R. fredii* USDA257 (83). (E) Core structure of the LPS of *R. etli* (84). Abbreviations: Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; Man, mannose; Kdo, 3-deoxy-D-manno-2-octulosonic acid; OAc, acetate group; Pyr, pyridine.

leucocephala. In contrast, *Exo*⁻ mutants of *R. leguminosarum* bv. *phaseoli* (58) and *R. fredii* (140), are fully effective on the determinate legumes *P. vulgaris* and *G. max*. One possible explanation of these data is that EPS is required for the formation of fully effective symbioses on plants that produce indeterminate nodules but not on legumes which form determinate nodules. However, Parniske et al. (192) showed that an EPS-defective mutant of *B. japonicum* which forms nitrogen-fixing nodules on *G. max* was ineffective on *G. soja*. Furthermore, a mutant of *Rhizobium* strain TAL1145 that is deficient in EPS synthesis still nodulates various hosts, independently of their nodule type (193).

A number of mutations that encode similar phenotypes but that occur in a variety of genes have been found (Table 8). For example, where the production of cyclic- β -(1 \rightarrow 2)glucans, rhamnose-rich SPS, LPS, SG, β -(1 \rightarrow 2)glucans, and KPS is impaired, *Nod*⁺ *Fix*⁻ phenotypes are also observed. Different regulators appear to control the expression of these diverse genes, and the resultant phenotype depends on the plant. Quite often an *Inf*⁺ *Fix*⁻ phenotype is observed, suggesting that these genes play similar roles. One possibility is that such

surface components are necessary for or form part of the developing infection thread. Hence, a plant that could not itself supply the missing carbohydrate would give a *Fix*⁻ phenotype while the rhizobial mutant in a plant that normally synthesizes the compound would have no effect.

Secreted proteins. Several strains of *Rhizobium* secrete symbiotically active proteins. Among these, the *nodO* product is required for nodulation of *V. hirsuta* by mutants of *R. leguminosarum* bv. *viciae* with *nodFELMNT* deleted (62), as well as for extension of the host range of an *R. trifolii nodE* mutant to include *V. sativa* (67). *nodO* encodes a Ca^{2+} binding protein that is thought to form cation-specific channels in membranes of leguminous plants (66, 264). Although *NodO* and *NodS* have distinct biochemical functions, a *nodO* homologue of *Rhizobium* sp. strain BR816 was shown to complement a *nodS* mutant of NGR234 for nodulation of *L. leucocephala* (269e), suggesting that secreted proteins may supplement some *Nod* factor deficiencies. Secretion of *NodO* is dependent on a C-terminal signal of about 24 residues (265) and on the *prsDE* genes, which encode two type I-like inner membrane proteins (77). In addition to *NodO*, at least three other proteins are

TABLE 8. Effects of mutations in nonnodulation genes on Fix phenotypes in various legumes

<i>Rhizobium</i> strain	Locus or gene(s)	Product ^a	Regulator	Phenotype ^a	Plant	Reference(s)
<i>A. caulinodans</i> ORS571	dTDP-D-gluc. synth.	Deoxy sugars		Fix ⁻	<i>Sesbania rostrata</i>	56
<i>B. japonicum</i> USDA110	<i>exoP</i>	EPS export		Fix ⁻	<i>Glycine max</i>	10
	<i>ndvC</i>	Cyclic β -glucans		Fix ⁻	<i>Glycine max</i>	19
	<i>ndvB</i>	Cyclic β -glucans		Fix ⁻	<i>Glycine max</i>	65
<i>M. loti</i> NZP2037		EPS		Fix ⁻	<i>Leucaena leucocephala</i>	127
				Fix ⁺	<i>Lotus pedunculatus</i>	
NGR234	<i>exoY</i>	EPS	<i>nodD1</i>	Fix ⁻	<i>Leucaena leucocephala</i>	106
	<i>fixF</i>	Rhamnose-rich SPS		Fix ⁻	<i>Vigna unguiculata</i>	130
<i>R. etli</i> CFN42	<i>lpsβ</i>	LPS		Fix ⁻	<i>Phaseolus vulgaris</i>	92
<i>R. leguminosarum</i> strain 300	<i>lps</i>	LPS		Fix ⁻	<i>Pisum sativum</i>	136
		LPS		Fix ⁻	<i>Trifolium pratense</i>	100
		EPS		Fix ⁻	<i>Trifolium pratense</i>	247
	<i>pssD</i>	EPS		Fix ⁻	<i>Trifolium repens</i>	270, 271
		EPS		Inf ⁺ Fix ⁻	<i>Trifolium pratense</i>	147
<i>R. meliloti</i> 2011	<i>exoAMONP</i>	EPS 1		Fix ⁻	<i>Medicago sativa</i>	8
	<i>exoUVWTI</i>	EPS 1		Fix ⁻	<i>Medicago sativa</i>	9
<i>R. meliloti</i> 1021	<i>exoY</i>	SG	<i>exoS, chv1</i>	Inf ⁺ Fix ⁻	<i>Medicago sativa</i>	40, 41
	<i>ndvA</i>	β -(1 \rightarrow 2)Glucan		Fix ⁻	<i>Medicago sativa</i>	
<i>R. meliloti</i> AK631	<i>rkpK</i>	KPS	<i>exoB (galE)</i>	Fix ⁺	<i>Medicago sativa</i>	138
	<i>rkpK1</i>	KPS		Fix ⁻	<i>Medicago sativa</i>	31
<i>R. tropici</i> CIAT899::Tn5		EPS		Exo ⁻ Fix ⁺	<i>Macroptilium atropurpureum</i>	176

^a Abbreviations: SPS, surface polysaccharide; SG, succinoglycans; Inf, formation of infection threads; Fix, nitrogen fixation; Exo, EPS synthesis.

secreted via this system, two of which (PlyA and PlyB) are glycanases involved in the processing of bacterial EPSs (78).

Sequencing the symbiotic plasmid of NGR234 revealed flavonoid-inducible genes encoding components of a type III secretion system (TTSS) (87). In a number of bacterial pathogens, TTSSs are induced upon contact with host cells (of plants or animals) and deliver virulence proteins directly into the eukaryotic cytosol (150). Strain NGR234, as well as several strains of *R. fredii*, was shown to excrete at least three to five proteins in a NodD1- and TTSS-dependent manner (145, 274). In USDA257, the *nolXWBTVU* cluster (corresponding to the *nolX*, *rhcC1*, *nolB*, *rhcJ*, *nolU* and *nolV* genes of NGR234) (274) regulates the nodulation of *G. max* in a cultivar-specific manner (168), whereas the TTSS of NGR234 profoundly affects the nodulation of various legumes such as *P. tuberosus* and *Tephrosia vogelii* (274). The absence of conserved *nod* box regulatory elements in the promoter regions of the *nol* and *rhc* operons indicates that NodD1-dependent transcriptional regulation of the TTSS genes is mediated by another factor. *y4xI*, an HrpG homologue which is under the control of a functional *nod* box, is thought to be the key intermediary in the regulatory cascade between flavonoids and activation of the TTSS machinery in NGR234 (274). The delayed induction of *rhc* genes in comparison with loci involved in the elaboration of the Nod factors suggests that the TTSS machinery is assembled after Nod factors have been elaborated and that protein export begins when intimate contact between bacteria and root hairs has been established. Thus, the most important role of TTSS-secreted proteins such as NodX and *y4xL* of NGR234 would occur after the bacteria have entered the plant, possibly during the development of infection threads.

It has been suggested that bacterial invasion of plant cells triggers nonspecific defense reactions and that successful pathogens overcome these defenses (89). Similarly, invading symbionts have probably evolved different strategies to lower host plant defenses. TTSS proteins and polysaccharides may contribute to this phenomenon. Some plants would perceive these compounds as part of the infection pathway and react to their presence by increased nodulation, as occurs in alfalfa inoculated with Exo⁺ *R. meliloti* and in *T. vogelii*, which favors strains of NGR234 with a functional TTSS. An alternate explanation is that these secreted substances function as suppressors of plant defense reactions, as proposed for various polysaccharides (20, 182). In contrast, *P. tuberosus* which is poorly nodulated by wild-type NGR234, produces many effective nodules when inoculated with TTSS mutants, suggesting that secreted proteins have detrimental effects (elicitors of defense reactions?) on certain hosts (274). Other rhizobia may have different keys to the inner door. Since apparently functional TTSSs are absent from *R. leguminosarum* and *R. meliloti*, other factors probably play similar roles. Some of these are undoubtedly polysaccharides, but it seems likely that a variety of keys exist. When they are used sequentially and in the correct combinations, infection thread development continues beyond the epidermal cells and a home for the invading rhizobia is constructed in the cortex of the root. Improper use of the keys results in abortion of the infection threads, as shown in Fig. 2E.

Clearly, various components of nodulating rhizobia, which include the NodD proteins, the spectrum of inducers, the palette of Nod factor substituents, levels of Nod factors, polysaccharides, and secreted proteins, contribute to the control of

host specificity. Although some of these may act synergistically (e.g., complementation of *nodS* mutants by NodO), most are part of a developmental process that leads to nodule invasion. Host plants are thus capable of controlling the successive steps of the rhizobial infection through checkpoints demanding specific keys. The absence of a suitable key does not necessarily result in a Nod⁻ or Fix⁻ phenotype, however. Most determinants of symbiotic specificity optimize the chances of forming effective nodules. Consecutive host specificity barriers are thus like hurdles of different heights that allow the host to select among many strains for the one best suited to its requirements.

EVOLUTION OF HOST SPECIFICITY

Broad-host-range rhizobia most probably have keys which fit many legume doors. Experiments with two different but closely related bacteria, *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257, and 452 different species of legumes showed that NGR234 and USDA257 form fully functional nodules on 136 and 66 species of legumes, respectively (207). Exactly which symbiotic determinants are shared by these two rhizobia is not known, yet both produce similar Nod factors. Those of USDA257 lack the baroque decorations encoded by *nodS*, *nodU*, *nolO*, *nolL*, *noeE*, and *noeI* of NGR234 (Table 4). Similarly, *nodZ* mutants of NGR234 still nodulate most plants tested, although the Nod factors lack all substituents carried on the fucose group (Table 4). In other words, it seems as if these additional Nod factor substituents extend an already broad host range but that they are not necessary for basic promiscuity.

If this logic reflects evolution, then ancestral Nod factors were unmodified N-acetylated oligomers of N-acetyl-D-glucosamine, possessing simple, unsaturated fatty acid side chains. It should be remembered that polymers of N-acetyl-D-glucosamine, i.e., chitin, are abundant in nature where they form an important part of the cell wall of many fungi, including mycorrhizae. Vesicular arbuscular mycorrhizae establish symbiotic associations with a broad range of vascular plants (116, 246). The existence of mycorrhizal-like structures in fossils strongly suggests that fungal-plant associations developed before rhizobial symbioses (216). Since highly sensitive recognition systems for chitin oligomers are found in diverse plants, it is not unreasonable to suggest that evolution of rhizobial perception in legumes resulted in a perception apparatus that is specific to N-acetylated chitin oligomers (i.e., Nod factors) (259).

Presumably, a bacterium producing ancestral Nod factors had the ability to nodulate many plants. Later, modifications followed which, in the case of NGR234, allowed it to nodulate even more plants. Acquisition of these broad-host-range genes was probably the result of lateral transfer. Concomitantly, Nod factor-cleaving chitinases coevolved in the legume host, thus directing selective pressure toward more stable Nod factors. In NGR234, this was provided through the acquisition of protective baroque decorations.

Morphological and molecular data suggest that both NGR234 and USDA257 have acquired the ability to nodulate three distinct subfamilies of plants, i.e., the Caesalpinioideae, Mimosoideae, and Papilionoideae (207). In the Papilionoideae, this ability arose early and was maintained through to the Amorphaeae, Robinieae, Indigofereae, Phaseoleae, and Desmodieae. Once acquired, the capacity to form symbioses with promiscuous rhizobia was only apparently lost in five tribes including the Cicereae, Viciae, and Trifolieae. Since the microsymbionts of these plants generally secrete fewer, less highly modified Nod factors (albeit possessing polyunsaturated

fatty acids), this implies that narrow host range is a specialization which developed for certain plants in restricted niches.

In other words, symbiotic promiscuity is probably ancestral to restricted host range. Support for this hypothesis comes from the observation that NGR234 and USDA257 also nodulate *Parasponia andersonii* (Ulmaceae) (207, 269). *Parasponia* spp. are small trees (up to 15 m high) which grow as pioneer plants in mountainous areas of Indonesia, Malaysia, and Papua New Guinea (1). Since Trinick isolated NGR234 in Papua New Guinea (268), this means that *Parasponia-Rhizobium* symbioses evolved in the same habitat. Most probably, the direct correlation that exists between solar energy input and species diversity (228) is driving evolution in tropical regions. Thus, ancestors of bacteria such as NGR234 could have been the first to intimately associate with legumes.

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