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Nod Factor Thin-Layer Chromatography Profiling as a Tool to Characterize Symbiotic Specificity of Rhizobial Strains: Application to *Sinorhizobium saheli, S. teranga,* and *Rhizobium* sp. Strains Isolated from *Acacia* and *Sesbania*

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Rhizobia isolated from *Acacia* or *Sesbania* belong to several taxonomic groups, including the newly described species *Sinorhizobium saheli, Sinorhizobium teranga,* and the so-called cluster U. A collection of strains belonging to these different groups was analyzed in order to determine whether the host range of a strain could be correlated with various molecular nodulation determinants. Nodulation tests showed that, independently of their taxonomic position, all the strains isolated from the same plant genus exhibited a similar host range, which was different for *Sesbania* and *Acacia* isolates. The fact that *S. teranga* strains nodulate either *Acacia* or *Sesbania* led us to subdivide this species into biovars *acaciae* and *sesbaniae*. Thin-layer chromatography (TLC) analysis of the Nod factors synthesized by overproducing strains showed that (i) strains isolated from the same plant genus exhibited similar TLC profiles and (ii) profiles of *Acacia* and *Sesbania* symbionts were easily distinguishable, *Acacia* strains producing, in particular, sulfated molecules. In contrast, no correlation could be established between the host range of a strain and its plasmid content, the nature of the *nod* gene inducers or the presence of DNA sequences homologous to specific *nod* genes. We thus propose that Nod factor TLC profiling may be used as an easy and powerful tool for the classification of rhizobial strains on the basis of their symbiotic properties.

Additional keywords: nitrogen fixation.

Soil bacteria which symbiotically interact with leguminous plants to form nitrogen-fixing nodules are very diverse in regard to their taxonomic position. These bacteria, termed rhizobia, have been shown to belong to about 15 different named species and several unamed genomic species, distributed in the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium,* and *Sinorhizobium* (see Young 1994; de Lajudie et al. 1994). In phylogenetic trees, these genera are intermixed with nonsymbiotic bacteria like *Agrobacterium* or *Rhodopseudomonas* (see Young 1994; Martinez-Romero 1994). The current classification is mainly based on universally approved techniques in microbial taxonomy, like auxanographic tests, DNA:DNA and DNA:rRNA hybridizations, whole-cell protein SDS-PAGE, and 16S rRNA sequencing (Graham et al. 1991). Such a classification no longer considers symbiotic properties as a taxonomic criterion, since the ability to nodulate a given legume species can be shared by taxonomically dissimilar symbionts, especially in the case of tropical legumes. For example, soybean is nodulated by *B. japonicum* (Jordan 1984), *S. fredii* (Jarvis et al. 1992), and *B. elkanii* (Kuykendall et al. 1992). Conversely, strains belonging to the same species can exhibit different host spectra. For example, in *R. leguminosarum*, three different biovars have been defined on the basis of their host range (Jordan 1984). Therefore, the host range of a given strain cannot be predicted from its taxonomic position. When rhizobial strains need to be classified on the basis of their host specificity, as in agro-ecological studies, or for analysis of biodiversity and plant-bacteria coevolution, their host range is analyzed by nodulation tests on various potential host plants. However, such experiments, though representing heavy work, provide only a partial characterization of the host range, and can be poorly discriminating, depending on the number and on the choice of the hosts tested.

To develop more accurate tools for the characterization of the symbiotic potentialities of a strain, we examined possible correlations between the host range and diverse elements involved in the molecular dialogue which controls the recognition process between the bacterial symbiont and its host plant. The bacterial nodulation genes (*nod, nol,* and *noe*), which are generally located on large plasmids in *Rhizobium* spp., play a central role in this molecular dialogue (Fellay et al. 1995; Dénarié et al. 1996). These genes are expressed in response to plant inducers present in root exudates, generally flavonoids. The induction process is mediated by the products of the *nodD* regulatory genes, which bind to conserved DNA consensus sequences located upstream of the *nod* operons, called *nod*

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boxes (Spaink et al. 1987; Schultze et al. 1994; Fellay et al. 1995). In the presence of appropriate flavonoids, the NodD proteins activate the transcription of the structural *nod* genes. The recognition between a given NodD protein and particular flavonoids determines a first level of specificity in the symbiotic interaction. The expression of the structural *nod* genes leads to the production of extracellular bacterial signals, called Nod factors, which are essential for the infection process and nodule organogenesis (for reviews see Dénarié et al. 1992; Fellay et al. 1995). These Nod factors are lipooligosaccharides basically consisting of a chito-oligosaccharide backbone of three to five β-1,4-linked *N*-acetyl-D-glucosamine residues, substituted by an *N*-acyl chain at the nonreducing end. Other diverse substitutions are present at both ends of the oligochitin chain. The nature of all these substitutions, which is controlled by various combinations of specific *nod* genes, make the Nod factors plant specific (Dénarié and Cullimore 1993; Spaink 1994; Dénarié et al. 1996). For instance, in *S. meliloti*, the *nodH* gene is involved in the addition of the sulfate group, which is required for alfalfa nodulation (Roche et al. 1991; Schultze et al. 1995). In the same way, the *nodS* and *nodU* genes of *Sinorhizobium* sp. NGR234, respectively, determine Nod factor *N*-methylation and *O*-carbamoylation, which enable this strain to nodulate *Leucaena leucocephala* (Lewin et al. 1990; Jabbouri et al. 1995).

Taking advantage of this current understanding of the molecular basis of host specificity, we looked for possible relationships between host range and several bacterial nodulation-related features. For this study, a collection of rhizobial strains isolated in Senegal from *Acacia* and *Sesbania* appeared of special interest, since strains isolated from the same host belong to different species, whereas strains isolated from different hosts belong to a same species (de Lajudie et al. 1994). We characterized the host range of these strains by testing their ability to nodulate various legumes, and we attempted to establish correlations between this host range and various nodulation-related determinants such as plasmid content, nature of *nod* gene inducers, Nod factor production and presence of DNA sequences homologous to host-specific *nod* genes.

RESULTS

Grouping *Acacia* and *Sesbania* isolates on the basis of their host range.

Tropical legumes of the genus *Sesbania* are known to be nodulated by the highly specific species *Azorhizobium caulinodans* (Dreyfus et al. 1988). In a recent report, de Lajudie et al. (1994) performed a taxonomic analysis of a collection of rhizobial strains isolated in Senegal either from *Sesbania* or from *Acacia* hosts: All the strains classified into the new species *Sinorhizobium saheli* were isolated from *Sesbania*, whereas strains belonging to the genospecies Ua and Ub of the so-called cluster U were all isolated from *Acacia.* Strains of the new species *Sinorhizobium teranga* were isolated either from *Sesbania* or from *Acacia. S. teranga* and *S. saheli* are taxonomically closely related to *R. meliloti* and *R. fredii*, which have been reclassified in the emended genus *Sinorhizobium* (de Lajudie et al. 1994). Genospecies Ua and Ub belong to the [*R.*] *loti* branch, which is currently proposed to form the new genus *Mesorhizobium* (Lindström et al. 1995).

To classify these rhizobial strains on the basis of their symbiotic properties, 42 strains of *S. teranga*, *S. saheli*, genospecies Ua and genospecies Ub originating from either *Acacia* or *Sesbania* (Table 1) were inoculated onto several *Acacia* (*A. nilotica, A. senegal, A. raddiana,* and *A. seyal*) and *Sesbania* (*S. pubescens*, *S. rostrata,* and *S. grandiflora*) species. As cross inoculation between *Acacia*, *Leucaena,* and *Prosopis* has been observed (Zhang et al. 1991), *Prosopis juliflora* and *Leucaena leucocephala* were also tested.

Results (Table 1) showed that *Acacia* isolates (belonging to *S. teranga*, genospecies Ua or genospecies Ub) and *Sesbania* isolates (belonging to *S. teranga* or *S. saheli*) exhibit distinct host spectra: (i) all *Acacia* isolates induced nodules on *Acacia* species (data not shown for *A. seyal*) and most of them formed efficient nodules fixing nitrogen. They also nodulated *P. juliflora* and *L. leucocephala*, but failed to induce nodule formation on any of the *Sesbania* species tested, in the exception of *S. rostrata*, on which several strains were able to induce pseudonodules. (ii) *Sesbania* isolates induced nitrogen-fixing nodules on all *Sesbania* species tested, but formed non-fixing nodules on *Acacia seyal* (data not shown), and were unable to nodulate *A. nilotica, A. senegal, P. juliflora,* and *L. leucocephala. A. raddiana* was poorly nodulated by *S. teranga* strains. The strain ORS1013 is atypical, since, though isolated from *A. senegal*, it exhibited on other hosts the same specificity as *S. teranga* strains isolated from *Sesbania* (Table 1).

Therefore, in contrast to *S. saheli*, which only comprises *Sesbania* strains, the species *S. teranga* clusters both *Acacia* and *Sesbania* isolates with very distinct host ranges. In the following, we will designate *S. teranga* strains nodulating *Acacia* as *S. teranga* bv. *acaciae* and strains nodulating *Sesbania* as *S. teranga* bv. *sesbaniae* (Table 1).

S. teranga strains isolated from *Acacia* and *Sesbania* exhibit similar plasmid profiles.

In most fast-growing *Rhizobium*, the *nod* genes are located on a large plasmid called the pSym (Rosenberg et al. 1981; Appelbaum et al. 1985) and it has been suggested that transfer of symbiotic plasmids could be responsible for modifications in host specificity (Martinez-Romero 1994; Segovia et al. 1993). Therefore, strains belonging to different species but isolated from the same legume (*Acacia* or *Sesbania*) in the same area could be expected to exhibit similarities in their plasmid content. Conversely, *S. teranga* strains with differerent host ranges could be expected to have different plasmid contents. We thus visualized the plasmids present in the strains by agarose gel electrophoresis (see Materials and Methods). However, our results showed that *S. saheli, S. teranga* bv. *sesbaniae,* and *S. teranga* bv. *acaciae* strains could not be distinguished on the basis of their plasmid content (Fig. 1). One band migrating more slowly than pSyma and pSymb from *S. meliloti* was detectable in all the *Sinorhizobium* strains tested. As this band was routinely observed using a technique which does not usually allow chromosome visualization, and as no equivalent band could be detected with any of the control strains, we assigned it to a megaplasmid. All the *Sinorhizobium* strains also contain another large plasmid of more than 350 kb, and a few of them (ORS611, ORS1071, and ORS22) an additional smaller plasmid (data not shown). In contrast, no plasmids were detected in the cluster U strains studied, except for ORS1018 and ORS1020, which both contained one plasmid (data not shown).

Luteolin is a good *nod* gene inducer for both *S. teranga* and *S. saheli*.

The specific activation of a NodD protein by plant inducers determines a first level of specificity in the symbiotic interaction. We thus screened various flavonoid compounds for their ability to induce *nod* gene expression in strains chosen as representatives of the different taxonomic groups. Identification of *nod* gene inducers was performed using available transcriptional fusions of *E. coli lacZ* gene with the *nodA* promoter of *R. leguminosarum* bv. *viciae* (plasmid pMP240) or bv. *trifolii* (plasmid pRT311), and the *nodY* promoter of *B. japonicum* (plasmid pDT900). These fusions were introduced in streptomycin-resistant derivatives of *S. saheli* ORS611, *S. teranga* bv. *sesbaniae* ORS604, *S. teranga* bv. *acaciae* ORS1073, genospecies Ua ORS1001 and genospecies Ub ORS1040. Various commercial flavonoids, most of them known as *nod* gene inducers in other *Rhizobium* species (for a review see Fellay et al. 1995), were tested for their effect on β-galactosidase activity in these constructs.

Table 1. Original host plant and host specificity of members of *Sinorhizobium teranga*, *Sinorhizobium saheli*, and *Rhizobium* sp. from cluster U among *Sesbania*, *Acacia*, *Prosopis*, and *Leucaena* species[a]

Rhizobial strain	Isolated from	Plant tested							
		Sesbania			*Acacia*			*Prosopis*	*Leucaena*
		grandiflora	*rostrata*	*pubescens*	*senegal*	*raddiana*	*nilotica*	*juliflora*	*leucocephala*
Sinorhizobium branch									
S. saheli									
ORS 600	*S. pachycarpa*	+	+	+	–	–	–	–	–
ORS 609	*S. cannabina*	+	+	+	–	–	–	NT	–
ORS 611	*S. grandiflora*	+	+	+	–	–	–	–	–
S. teranga bv. *sesbaniae*									
ORS 15	*S. sp.*	+	+	+	–	± d	–	NT	–
ORS 19	*S. cannabina*	+	+	+	–	+	–	NT	–
ORS 604	*S. aculeata*	+	+	+	–	± d	–	–	–
ORS 613	*S. sesban*	+	+	+	–	+	–	–	–
ORS 8	*S. rostrata*	+	+	+	–	± d	–	NT	–
ORS 22	*S. rostrata*	+	+	+	–	± d	–	–	–
ORS 51	*S. rostrata*	+	+	+	–	± d	–	NT	–
ORS 52	*S. rostrata*	+	+	+	–	± d	–	–	–
ORS 53	*S. rostrata*	+	+	+	–	± d	–	NT	–
ORS 1013	*A. senegal*	+	+	+	+	± d	–	–	–
S. teranga bv. *acaciae*									
ORS 1071	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1072	*A. senegal*	–	–(pn)	–	+	+	+	±	+
ORS 1073	*A. senegal*	–	–	–	+	+	+	+	+
ORS 1007	*A. laeta*	–	–(pn)	–	+	+	+	NT	+
ORS 1009	*A. laeta*	–	–(pn)	–	+	+	+	+	+
ORS 1016	*A. laeta*	–	–	–	+	+	+	+	+
ORS 1045	*A. raddiana*	–	–	–	+	+	+	NT	+
ORS 1047	*A. horrida*	–	–	–	+	+	+	NT	+
ORS 1057	*A. mollissima*	–	–	–	+	+	+	NT	+
ORS 1058	*A. mollissima*	–	–	–	+	+	+	NT	+
ORS 929	*A. sp.*	–	–(pn)	–	–	+	+	NT	+
[*Rhizobium*] *loti* branch *									
Cluster U, genospecies Ua#									
ORS 1001	*A. senegal*	–	–	–	+	+	+ d	+	+
ORS 1004	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1010	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1014	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1015	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1018	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1020	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1024	*A. senegal*	–	–(pn)	–	+	+	+	±	±
ORS 1029	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1035	*A. senegal*	–	–	–	+	+	+ d	+	+
ORS 1036	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1037	*A. senegal*	–	–	–	+	+	+	NT	+
Cluster U, genospecies Ub§									
ORS 1030	*A. senegal*	–	–	–	+	+	+	+	+
ORS 1031	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1032	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1038	*A. senegal*	–	–	–	+	+	+	NT	+
ORS 1040	*A. senegal*	–	–	–	+	+	+ d	± d	+
ORS 1088	*A. seyal*	–	–	–	+	+	+	±	+

[a] +, more than 50% of the plant tested were nodulated. –, none of the plants tested were nodulated. ±, between 10% and 50% of the plants tested were nodulated. d, delayed nodulation. NT, not tested. (pn), occasional formation of pseudonodules. * A new genus, *Mesorhizobium*, has been proposed for the *R. loti* branch (Lindström et al. 1995). # Ua is constituted of subclusters U1 and U2 described in de Lajudie et al. (1994). § Ub comprises strains from subcluster U3 described in de Lajudie et al. (1994). Ua and Ub represent two different genospecies (P. de Lajudie, unpublished).

No induction of the *nodY-lacZ* fusion could be detected in any of the five strains (data not shown). In contrast, significant induction levels were observed in *Sinorhizobium* strains containing the plasmid pMP240 carrying the *R. leguminosarum* bv. *viciae nodA::lacZ* fusion (Fig. 2), and, to a lesser extent, the plasmid pRT311 (data not shown). For these strains, several flavonoids, i.e. apigenin, naringenin, butein, and kaempferol had an inducing effect, but the best inducer was the flavone luteolin (Fig. 2). No induction or a very weak induction was observed with daidzein, genistein (Fig. 2), eriodictyol, umbelliferone, myricetin, and formononetin (data not shown). No induction could be detected for genospecies Ua ORS1001 and genospecies Ub ORS1040, bearing either pMP240, pRT311, or pMP194, a *S. meliloti nodABC::lacZ* fusion (data not shown, except for ORS1001(pMP240), Fig. 2).

Construction of derivatives overproducing Nod factors.

For the *Sinorhizobium* representatives, for which *nod* gene inducers have been identified, we attempted to characterize Nod factor production. *S. saheli* ORS611, *S. teranga* bv. *sesbaniae* ORS604, and *S. teranga* bv. *acaciae* ORS1073 were grown in a medium containing 10 μM luteolin, and ^{14}C acetate as a Nod factor precursor. Culture supernatants were analyzed by the TLC system previously described (Spaink et al. 1992). No luteolin-inducible spots were detected on TLC plates after a 2-week exposure, indicating a very low level of Nod factor production. This led us to attempt to increase Nod factor production by introducing plasmids bearing *nodD* genes from other rhizobial species into wild-type strains representative of the five taxonomic groups. We tested different *nodD* genes: *nodD1* of the broad host range strain *Sinorhizobium* sp. NGR234 (= plasmid pA28), *nodD2* of the tree-nodulating strain *S.* sp. BR816 (= plasmid pVK31), *nodD1* of the taxonomically closely related species *S. meliloti* (= plasmid pMH901), all of them known to activate *nod* gene expression through flavonoid induction (Bassam et al. 1988; van Rhijn et al. 1994; Maillet et al. 1990) and the *nodD3-syrM* genes of *S. meliloti* (plasmid pMH682), which direct flavonoid-independent *nod* gene transcription (Maillet et al. 1990).

Results (Table 2) showed that *nodD1* of *Sinorhizobium* sp. NGR234 allowed Nod factor overproduction in the five taxonomic groups. In contrast, the inducing effect of the other *nodD* genes was dependent on the species: *nodD3-syrM* genes of *S. meliloti* were efficient in the *Sesbania*-nodulating strains *S. saheli* ORS611 and *S. teranga* ORS604, whereas *S. meliloti nodD1* gene increased Nod factor production in both cluster U representatives ORS1001 and ORS1040, which nodulate *Acacia*. The *nodD2* gene of *Sinorhizobium* sp. BR816 was only active in the *S. teranga* bv. *acaciae* strain ORS1073.

Moreover, introducing plasmids pMH682, pVK31, or pMH901 into other *Sesbania* symbionts, *S. teranga* bv. *acaciae* and cluster U strains, respectively, resulted in the production of detectable Nod factors (see Fig. 3 for examples). Thus, for taxonomically closely related rhizobia, the ability of a strain to respond to a given exogenous *nodD* gene seems to be linked to its host range. However, such a link cannot be observed for taxonomically distant strains.

It has been reported that the nature of the regulatory *nodD* gene can influence Nod factor structure (Demont et al. 1994). Therefore, when possible, we compared the effect of different exogenous *nodD* genes on Nod factor production. However, for a given strain, similar TLC patterns were obtained, whatever the exogenous *nodD* gene responsible for Nod factor overproduction (data not shown).

***Acacia* symbionts produce sulfated Nod factors, whereas *Sesbania* symbionts do not.**

Several overproducing derivatives were constructed for each taxonomic group, and their Nod factor production was analyzed by TLC, using ^{14}C acetate or ^{35}S sulfate as precursors (see Materials and Methods). In ^{14}C-labeled *S. teranga* bv. *acaciae* strains harboring pVK31, several major flavonoid-inducible spots were detected in the culture supernatants (Fig. 3A, lanes 1ai, 2ai and 3ai). With ^{35}S as precursor, one of these spots was strongly labeled, and one or two others appeared as weaker signals (Fig. 3A, lanes 1si, 2si, and 3si). Flavonoid-induced cultures of the *Acacia*-nodulating genospecies Ua and Ub containing pMH901, exhibited an analogous TLC profile (Fig. 3B, lanes 4ai, 5ai, and 6ai), with also one major sulfate-labeled spot (Fig. 3B, lanes 4si, 5si, and 6si). However, the faster migrating spots were lacking or less intense than in *S. teranga* bv. *acaciae*. (Fig. 3D, lanes 6i and 14i). The TLC profile of these cluster U strains was very similar to that obtained with a ^{14}C-labeled culture of *R. tropici* CFN299 (Fig. 3D, lanes 6i and 13i), a tropical strain nodulating *L. leucocephala* (Martinez-Romero et al. 1991), but also *A. senegal* and *A. nilotica* (data not shown).

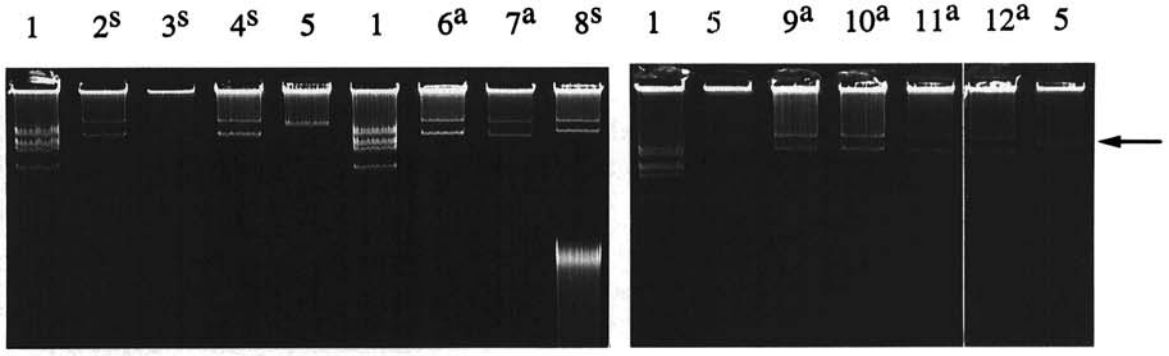

Fig. 1. Plasmid profiles of representative *Sinorhizobium teranga* bv. *acaciae* ([a]) and bv. *sesbaniae* ([s]) strains. ORS51 (lane 2); ORS52 (lane 3); ORS53 (lane 4); ORS1072 (lane 6); ORS1073 (lane 7); ORS19 (lane 8); ORS1007 (lane 9); ORS1009 (lane 10); ORS1016 (lane 11); ORS1045 (lane 12). *Rhizobium leguminosarum* bv. *viciae* VF39SM (lane 1, standard plasmid sizes: 135, 160, 205, 220, 350 and 600 kb) and *Sinorhizobium meliloti* RCR2011 (lane 5) exhibiting two megaplasmids (arrow) were used as standards.

In contrast, *S. saheli* and *S. teranga* bv. *sesbaniae* bearing pMH682 both produced only nonsulfated Nod metabolites, which migrated closely together (Fig. 3C, lanes 7ao, 8ao, and 9ao). Their TLC profiles appeared very similar to that of *A. caulinodans* ORS571 isolated from *S. rostrata* (Fig. 3D, lane 10i, 11o, and 12o). It is worth noting that the strain ORS1013, isolated from *A. senegal,* but able to nodulate *Sesbania* species, exhibited a typical *Sesbania* Nod factor pattern (Fig. 3C, lane 7ao), and thus we classified it as *S. teranga* bv. *sesbaniae* (Table 1).

Thus *Acacia* and *Sesbania* symbionts synthesize different Nod metabolites, and can be easily distinguished on the basis of their supernatant TLC profiles (Fig. 3D, lanes 10i, 11o, and 12o for *Sesbania* strains and 13i, 6i, and 14i for *Acacia* nodulating strains).

Genomic hybridizations using various *nod* gene probes.

From the recent determination of the structure of the Nod factors produced by representatives of the different taxonomic groups (J. Lorquin, G. Lortet, M. Ferro, N. Méar, B. Dreyfus, J.-C. Promé, and C. Boivin, unpublished), it appeared that for both *Acacia* and *Sesbania* isolates, the nonreducing end was substituted by a *N*-methyl and a *O*-carbamoyl group, whereas the reducing end was glycosylated for *Sesbania*-nodulating strains and partially sulfated for *Acacia*-nodulating strains. We thus looked for the presence in these strains of DNA sequences hybridizing to probes consisting of the *nodS* and *nodU, nodH,* or *nodZ* genes, which are involved, respectively, in *N*-methylation, *O*-carbamoylation (Jabbouri et al. 1995; Geelen et al. 1995; Mergaert et al. 1995b), sulfation (Lerouge et al. 1990; Roche et al. 1991) and glycosylation (Stacey et al. 1994; Mergaert et al., in press) of Nod factors. Results are summarized in Table 3. Surprisingly, hybridization of genomic DNA with an internal *nodH* probe from *S. meliloti* revealed that an homologous DNA sequence was present in all tested strains, even those for which no ^{35}S-labeled spots could be detected. In fact, except for ORS1047 and ORS1058, which form a subgroup in the *S. teranga* species (M. Neyra, unpublished results), all Senegalese sinorhizobia shared a common unique 5.5-kb *nodH*-hybridizing band. All of the strains tested, nodulating either *Acacia* or *Sesbania*, lacked detectable homology to an internal *nodZ* probe from *A. caulinodans* ORS571. Using the *nodSU* genes from *S. fredii* as a probe, *Acacia* and *Sesbania* strains hybridized differently: the former gave a clear (cluster U strains) or an intense (*S. teranga* strains) signal, while no hybridization or only a weak trace could be observed with the latter strains.

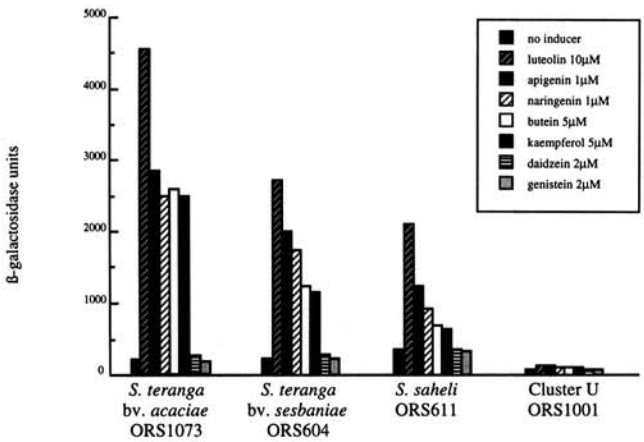

Fig. 2. Expression of a *Rhizobium leguminosarum nodA*::*lacZ* fusion in *Sinorhizobium saheli*, *S. teranga* and genospecies Ua in response to several flavonoids. Values represent average of at least three independent experiments.

DISCUSSION

Current *Rhizobium* taxonomy is based on a polyphasic approach, consisting of a set of biochemical and molecular techniques (Graham et al. 1991). In contrast, the classification of rhizobia as a function of their symbiotic properties still relies on nodulation tests, which are time- and space-consuming, and only provide a partial characterization of the host spectrum. To develop alternative methods for classifying rhizobia strains on the basis of their host range, we examined possible correlations between host spectrum and various molecular nodulation determinants.

Nodulation experiments were performed with a collection of strains isolated either from *Sesbania* or from *Acacia*, and belonging to the species *S. saheli*, *S. teranga* or to the geno-

Table 2. Nod factor overproduction by *Sinorhizobium saheli*, *S. teranga* and *Rhizobium* sp. from cluster U harbouring *nodD* genes from various sources[a]

Plasmid (genes present)	Inducer	TLC response after introduction of plasmid-borne *nodD* genes in *S. saheli* ORS611	*S. teranga* bv. *sesbaniae* ORS604	*S. teranga* bv. *acaciae* ORS1073	Genospecies Ua ORS1001	Genospecies Ub ORS1040
pMH901 (*nodD1* of *S. me*)	Luteolin	–	–	–	+	+
pMH682 (*nodD3* and *syrM* of *S. me*)	None	+	+	–	–	–
pVK31 (*nodD2* of *S.* sp. BR816)	Naringenin	–	–	+	–	–
pA28 (*nodD1* of *S.* sp. NGR234)	Apigenin	+	+	+	+	+

[a] *Rhizobium* and *Sinorhizobium* derivatives, harboring an exogenous *nodD* gene, were grown in the presence of ^{14}C acetate and with or without the appropriate inducing flavonoid. Culture supernatants were analyzed by TLC, using octadecyl silica plates. Presence and absence of flavonoid-inducible compounds are, respectively, designated + and –. However, rhizobia derivatives harbouring pMH982 containing the constitutively expressed *nodD3-syrM* genes are compared to the corresponding wild-type strains for the detection of new radiolabeled compounds. In this case, + and – designate, respectively, presence or absence of *nodD*-inducible compounds. Data are the results of at least three independent experiments.

species Ua and Ub from the so-called cluster U (Table 1). All strains isolated from the same plant genus exhibited similar host ranges, independently of their taxonomic position. *Sesbania* isolates nodulate all *Sesbania* species tested as well as the widely compatible *Acacia seyal* (Dreyfus and Dommergues 1981), but only poorly *A. raddiana*, and not other *Acacia* species. In contrast, the host spectrum of the *Acacia* isolates includes all the species of *Acacia* tested, *Prosopis juliflora* and *Leucaena leucocephala*, but not *Sesbania* species. Since strains with different host ranges belong to the species *S. teranga*, we propose to subdivide this species into biovars *acaciae* and *sesbaniae*.

Examining different possible host specificity markers, we showed that Nod factor TLC profile is the only character tightly correlated with the host range of the strains. Whatever their taxonomic position, strains isolated from the same plant genus exhibited similar TLC profiles, which are clearly different for *Acacia* and *Sesbania* isolates (Fig. 3D). In particular, TLC analysis of ^{35}S-labeled overproducing strain supernatants revealed the partial sulfation of Nod factors from *Acacia* iso-

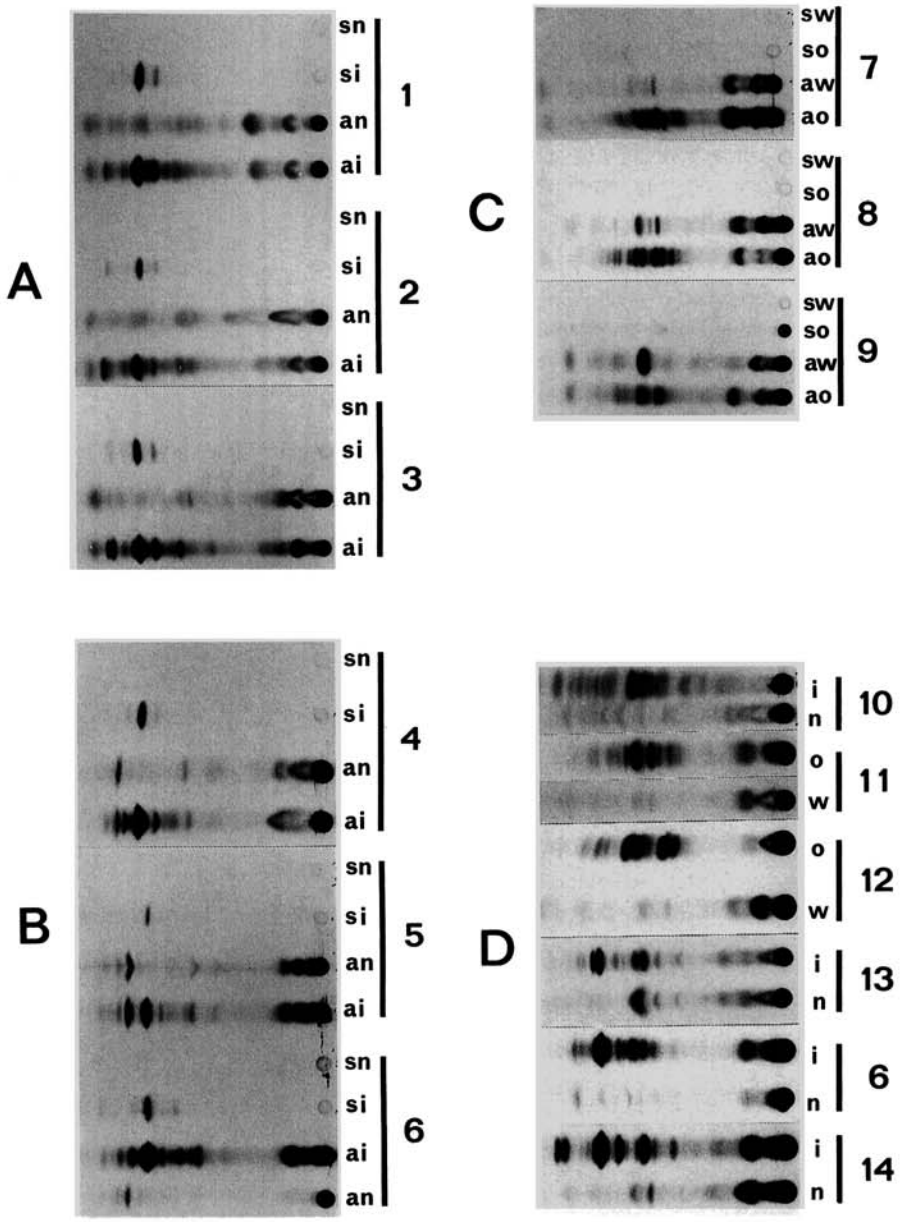

Fig. 3. Thin-layer chromatography (TLC) separation of Nod factors produced by various *Acacia* and *Sesbania* nodulating strains. Radioactivity was visualized after 3 to 8 days exposition to a Kodak X-OMAT K film. a, ^{14}C-labeled compounds; s, ^{35}S-labeled compounds; i, cells grown in presence of the appropriate flavonoid inducer; n, non induced cultures; w, wild-type strain; o, overproducing strain harboring pMH682. **A,** *Sinorhizobium teranga* bv. *acaciae* overproducing strains: ORS1071(pVK31) (lanes 1), ORS1007(pVK31) (lanes 2) and ORS929(pVK31) (lanes 3). **B,** *Rhizobium* sp. overproducing strains from cluster U: ORS1040(pMH901) (lanes 4), ORS1020(pMH901) (lanes 5) and ORS1001(pMH901) (lanes 6). **C,** *S. saheli* and *S. teranga* bv. *sesbaniae* wild type and overproducing strains: ORS1013 (lanes 7), ORS19 (lanes 8), and ORS609 (lanes 9). **D,** Comparison of TLC migration of ^{14}C-labeled compounds produced by *Azorhizobium caulinodans* wild-type strain ORS571 (lanes 10), *S. saheli* ORS611 (lanes 11), *S. teranga* bv. *sesbaniae* ORS604 (lanes 12), *R. tropici* wild-type strain CFN299 (lanes 13), *Rhizobium* sp. ORS1001 from cluster U (lanes 6) and *S. teranga* bv. *acaciae* ORS1073 (lanes 14).

lates, whereas no sulfation could be observed for those of *Sesbania* isolates (Fig. 3C). Furthermore, *A. caulinodans* strain ORS571 and *Sinorhizobium* strains nodulating *Sesbania* showed very similar Nod factors TLC profiles. The recent determination of the chemical structure of the Nod factors produced by *Sesbania*-nodulating *Sinorhizobium* (J. Lorquin, G. Lortet, M. Ferro, N. Méar, B. Dreyfus, J.-C. Promé, and C. Boivin, unpublished) confirmed that this similarity of the TLC profiles reflects the resemblance of the molecules. In the same way, *R. tropici* strain CFN299, which nodulates *A. senegal* and *A. nilotica* (unpublished results), exhibited a Nod factor profile similar to that of *Acacia* strains from cluster U. TLC analysis of Nod factors thus appears to be a powerful screening method to group or distinguish strains on the basis of their host spectra.

A technical difficulty of this method could be the detection of the very small amounts of Nod factors produced by many wild-type strains. We solved this problem by the introduction of exogenous *nodD* genes cloned in multicopy plasmids into the studied strains. Whereas most of the *nodD* genes tested were only able to activate Nod factor production in a defined group of strains, the introduction of the *nodD1* gene from *Sinorhizobium* sp. strain NGR234 enabled all the strains to overproduce Nod factor in the presence of apigenin (Table 2). This *nodD1* gene had a similar effect on Nod factor production when introduced into various other temperate and tropical *Rhizobium* species (C. Boivin, unpublished data). Thus, in addition to its ability to respond to a wide range of inducing compounds (Bassam et al. 1988; van Rhijn et al. 1994), the NGR234 NodD1 protein seems to be able to recognize the *nod* boxes of various rhizobia. The *R. leguminosarum nodD* derivative FITA (flavonoid independent transcription activation) was also shown to allow Nod factor overproduction in several taxonomically different strains (Lopez-Lara et al. 1995a). The construction of overproducing strains carrying such exogenous *nodD* genes should greatly facilitate Nod factor characterization, whatever the production level of the wild-type strains. Furthermore, the use of 1-^{14}C glucosamine as Nod factor precursor (Price and Carlson 1995) could allow more specific labeling of these molecules, and should improve the sensitivity of the technique. When Nod factor profiles are closely related, highly resolutive techniques are needed. Resolution of TLC plates could be improved by standardizing the culture conditions, the extraction and elution processes, and by performing two-dimensional TLC (Price and Carlson 1995).

We therefore propose to use the TLC profiling technique, which is currently the fastest way to globally characterize Nod factors, for classifying rhizobial strains on the basis of their host range. This method should constitute a more comprehensive approach than nodulation tests, the confidence of which depends on the choice of appropriated plant species, and on their ability to grow and develop symbiotic interactions in laboratory conditions. An immediate application could be its use for the characterization of biovars in rhizobium taxonomy. To our knowledge, this is the first application of the recent advances in the understanding of the molecular basis of symbiotic specificity for ecological or classification purposes.

A potential limitation to this method could be due to the fact that Nod factor structure is not the only determinant of the specificity in the *Rhizobium*-legume interaction. For instance, *R. etli* and *R. loti* produce identical Nod factors (Lopez-Lara et al. 1995a; Cardenas et al. 1995; Poupot et al. 1995), but differ in host specificity presumably because of different perception of plant flavonoids (Cardenas et al. 1995). Thus, to classify isolates as a function of their host range, the identification of *nod* gene inducers could be a useful complement to Nod factor profiling. In the present work, all *Sinorhizobium* strains tested exhibited very similar induction patterns, the highest level of induction being obtained with 10 μM of luteolin (Fig. 2). Luteolin was already known as one of the best *nod* gene inducers for the closely related species *S. meliloti* (Peters et al. 1986), but was reported as a poor *nod* gene inducer for *A. caulinodans* (Goethals et al. 1989). No inducer could be identified for cluster U strains among a collection of commercial flavonoids. This could be due either to the fact that we did not find the right compound, or to the fact that the NodD proteins of cluster U strains were not able to interact with the foreign *nod* promoters fused to the *lacZ* gene. However, it is worth noting that in a *R. loti* strain, which is taxonomically related to the cluster U, no induction of a *R. loti nodA-lacZ* fusion could be obtained with commercial flavonoids (Zhang et al. 1995). Our results suggest that the nature of the *nod* gene inducers seems to be more related to the taxonomic position of a given strain than to its symbiotic properties, and therefore plant signal recognition may not interfere with Nod factor production for the determination of the host range of taxonomically close rhizobia.

From an evolutionary point of view, the fact that all *S. saheli* and *S. teranga* strains present similar responses to plant flavonoids suggests that, at least, the C-terminal part of the NodD proteins, which is mostly responsible for flavonoid recognition (Kondorosi 1992), is conserved among these strains. However, the *nodD* gene sequence was expected to vary as a function of the host plant, since it has been reported that the phylogenetic trees established from *nodD* 3′-sequences of various rhizobia strains follow plant taxonomy, and not rhizo-

Table 3. Presence of sequences homologous to *S. meliloti nodH, A. caulinodans nodZ* and *S. fredii nodSU* in *S. saheli, S. teranga* and *Rhizobium* sp. from cluster U

	Number of *Eco*RI fragments homologous to		
Strain	***S. me nodH***	***A. ca nodZ***	***S. fr. nodSU***
S. saheli			
ORS600	1[a]	NT	0
ORS609	1[a]	0	0
ORS611	1[a]	0	0
S. teranga bv. *sesbaniae*			
ORS604	1[a]	0	0
ORS613	1[a]	0	1
ORS51	1[a]	NT	0
ORS19	1[a]	0	0
ORS1013	1[a]	0	0
S. teranga bv. *acaciae*			
ORS1009	1[a]	0	1
ORS1016	1[a]	NT	2
ORS1073	1[a]	0	1
ORS1047	1	0	1
ORS1058	1	0	2
cluster U			
ORS1001	1	0	1
ORS1010	NT	NT	1
ORS1030	NT	NT	1
ORS1035	NT	0	1

[a] The *Eco*RI hybridizing fragment was 5.5 kb. NT = not tested.

bia taxonomy (Dobert et al. 1994). Thus, the fact that the NodD proteins of *S. teranga* bv. *acaciae* and *sesbaniae* and *S. saheli* exhibit a similar pattern of flavonoid recognition could just reflect the fact that *Acacia* and *Sesbania* root exudates contain common inducers.

The synthesis and transfer of the various substitutions present on the Nod factor oligochitin backbone is under the control of the specific *nod* genes (see Fellay et al. 1995). We therefore expected that it could be possible to discriminate between *Acacia* and *Sesbania* strains by detecting in these strains particular host specificity *nod* genes. TLC analysis of ^{35}S-labeled cultures indicated that Nod factors from *Acacia* strains were partially sulfated, whereas those of *Sesbania* strains were not (Fig. 3). However, both *Acacia* and *Sesbania* strains hybridized with a *nodH* probe. The *nodH*-like sequence present in *Sesbania* strains could therefore be a pseudo gene without biological activity. Such an example of nonfunctional nodulation genes has been described in *S. fredii* USDA257, where a deletion removing part of the upstream *nod* box is responsible for the loss of expression of the *nodSU* genes (Krishnan et al. 1992). However, the fact that the internal *nodH* probe hybridized to a single common band in most of the *S. teranga* and *S. saheli* strains tested indicates a high conservation of that sequence. A possible explanation is that the *nodH*-like gene encodes a protein also involved in the sulfation of other compounds, such as lipopolysaccharides, as it has been suggested for *S. meliloti nodQ* genes. (Cedergren et al. 1995). In the case of *Sesbania* strains, this protein would be unable to transfer the sulfate group to the Nod factors, possibly as a result of structural modifications of these factors.

S. saheli and *S. teranga* bv. *sesbaniae* Nod factors are fucosylated, whereas those of *S. teranga* bv. *acaciae* and cluster U strains are not (J. Lorquin, G. Lortet, M. Ferro, N. Mear, B. Dreyfus, J.-C. Prome, and C. Boivin, unpublished). As in *B. japonicum* (Stacey et al. 1994) and *A. caulinodans* (Mergaert et al., in press), the fucosylation of the Nod factors is under the control of the *nodZ* gene, we investigated the possibility that DNA homology with this gene could be a criterion to distinguish between *Sesbania* and *Acacia* strains. However, in *S. saheli* and *S. teranga* bv. *sesbaniae*, as well as in *Acacia* strains, no hybridization signal could be detected with a *nodZ* probe (Table 3). Similarly, *nodS* and *nodU* genes are, respectively, responsible for the *N*-methylation and the 6-*O* carbamoylation of the nonreducing terminal glucosamine residue in *A. caulinodans* (Mergaert et al. 1995a, 1995b) and *Sinorhizobium* NGR234 (Jabbouri et al. 1995). We used the *S. fredii nodSU* genes, which show a high degree of homology with their NGR234 counterparts (Krishnan et al. 1992), as a probe to detect the presence of homologous sequences in *Sinorhizobium* and cluster U strains. Strains isolated from *Acacia* exhibited homology with this probe, while most of the *Sesbania* strains from either *S. saheli* or *S. teranga* yielded no hybridization signal (Table 3). Such a result was unexpected, since *N*-methylation and/or *O*-carbamoylation of Nod factors are very common in tropical strains, and are encountered in both *Acacia* (*Rhizobium* sp. GRH2, *R. tropici*) and *Sesbania* (*A. caulinodans*) nodulating strains (Lopez-Lara et al. 1995b; Poupot et al. 1993; Mergaert et al. 1993). Furthermore, *O*-carbamoylation and *N*-methylation of Nod factors in both *Acacia* and *Sesbania* isolates was recently established (J. Lorquin, G. Lortet, M. Ferro, N. Méar, B. Dreyfus, J.-C. Promé, and C. Boivin, unpublished). Taken together, these results suggest that absence of hybridization with *Sesbania* strains was caused by the fact that *nodSU* and *nodZ* alleles diverged significantly in terms of DNA sequences from those of *S. fredii* and *A. caulinodans,* respectively, but yet retained full biochemical activity.

More generally, hybridization results show that in a given strain, the presence or the absence of particular *nod* genes, as assessed by Southern hybridization, is neither sufficient to predict the nature of the substituents present on the Nod factors, nor correlated with the host range. Such discrepancies are presumably due to sequence divergences in genes encoding the same function and presence of pseudo genes having lost their functional activity, as a consequence of genetic rearrangements.

The fact that strains belonging to different taxonomic groups show convergent host ranges strongly suggests that lateral gene transfer has occurred, possibly via plasmid transfer, since in most Rhizobia and Sinorhizobia, *nod* genes are located on transmissible plasmids. All *S. teranga* bv. *acaciae* and *sesbaniae* and *S. saheli* harbor a megaplasmid and one or two additional large plasmids, a profile similar to that of the closely related species *S. meliloti* (Bromfield et al. 1987). In contrast, *Acacia* strains from cluster U usually do not contain any plasmids. Thus, plasmid content of a strain appeared not to be related to its host specificity. This result indicates that a simple transfer of plasmids cannot account for the convergence of host ranges in different species nor for the divergence of host specificities within the same rhizobial species. Emergence of *Sesbania* and *Acacia* symbionts within the *Sinorhizobium* branch thus probably results from complex genetic events, possibly including plasmid transfer followed by genetic recombination, for the acquisition of new genes such as *nodZ*-like genes, and specific inactivation of other genes such as *nodH*. A given host plant would select bacteria for which these genetic events lead to the production of Nod factors adapted to specific plant receptors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

All the wild-type rhizobia listed in Table 1, were described by de Lajudie et al. (1994). Other bacterial strains and plasmids used in this study are described in Table 4. The *R. meliloti-R fredii* branch including *Rhizobium* sp. NGR234 and *Rhizobium* sp. BR816 (Martinez-Romero 1994) has been recently reclassified in the emended genus *Sinorhizobium* (de Lajudie et al. 1994). In the text *R. meliloti*, *R. fredii, R.* sp. NGR234, and *R.* sp. BR816 were thus referred to as *Sinorhizobium meliloti*, *S. fredii, Sinorhizobium* sp. NGR234 and BR816. The complete media for the growth of *E. coli* and *Rhizobium* strains were, respectively, LB (Sambrook et al. 1989) and yeast extract-mannitol medium YM (de Lajudie et al. 1994). The minimal medium for *nod* gene induction (V medium) was as described by Roche et al. (1991). TpBroth medium (in g/liter: 4, peptone; 0.5, yeast extract; 0.5, tryptone; 0.2, $MgSO_4$, 7H2O; 0.2, $CaCl_2$, $2H_2O$) was used for triparental matings and plasmid content. *E. coli*, *S. teranga, S. saheli,* and cluster U strains were grown at 37°C on solid medium and at 30°C in liquid medium.

Microbiological techniques.

All conjugation experiments were realized using as receptor spontaneous streptomycin-resistant derivatives of wild-type strains described in Table 1. Broad host range plasmids were mobilized from *E. coli* to Sm^r rhizobia derivatives with the helper plasmid pRK2013. *(Sino)rhizobium* and *E. coli* strains were grown overnight at 30°C in Tpbroth. Samples of donor, helper, and receptor cells (1:1:5, ratio) were pooled, centrifugated, and resuspended in TpBroth 1/50 initial volume. Mating mixtures were spread on TpBroth agar and incubated overnight at 37°C. Selection of transconjugants was performed on YM agar medium supplemented with 10 μg of tetracycline per ml (incP vectors) or 100 μg of chloramphenicol per ml (incQ vectors) for plasmid selection, and 100 μg of streptomycin per ml to select against *E. coli* donors. Presence of plasmids in transconjugants was confirmed by rapid plasmid DNA extraction and digestion with the appropriate enzymes according to standard procedures (Sambrook et al. 1989).

Nodulation tests.

Seeds were superficially sterilized with concentrated sulfuric acid for a duration of 30 (*Acacia senegal*), 150 (*A. raddiana*), 120 (*A. nilotica*), 30 (*Leucaena leucocephala, A. seyal*), 15 (*Prosopis juliflora*), or 60 min (*Sesbania grandiflora* pink flowers; *S. pubescens*; *S. rostrata*). Seeds were then abundantly rinsed with sterile water and allowed to soak for 6 to 24 h. Surface-sterilized seeds were germinated at 30°C for 24 to 48 h, and then transferred to Gibson tubes containing Jensen slant agar and liquid medium (Vincent 1970). Plants were grown under continuous light (20 W/m^2) at 28°C. Five to eight plants were tested for each strain. Plants were observed for nodule formation during 6 to 8 weeks, and effectiveness was estimated by visual observation of plant vigor and foliage color. *Acacia* and *Prosopis* seeds were obtained from DRPF, ISRA, Senegal.

β-Galactosidase assays.

Bacteria were grown overnight to mid-log phase in V medium supplemented with tetracycline (5 μg/ml) or chloramphenicol (50 μg/ml). Bacterial suspension was diluted in fresh medium to A_{600} = 0.02 and flavonoids added at a final concentration of 1 μM (apigenin, naringenin), 2 μM (daidzein, genistein), 10 μM (luteolin), or 5 μM (other flavonoid compounds). Induction was performed at 30°C for 24 h and enzyme activity was assayed as previously described (Boivin et al. 1991). The basal level of β-galactosidase activity of *Rhizobium* and *Sinorhizobium* strains containing pMP220 or pMP190 derivatives was high (150 to 300 units). Luteolin, apigenin, naringenin, butein, daidzein, kaempferol, and genistein were purchased from Extrasynthese (Genay, France). Eriodictyol, umbelliferone, myricetin, and formononetin were a gift from J. Dénarié.

Detection of Nod factors by thin-layer chromatography.

An overnight culture was diluted in fresh V medium, supplemented with tetracycline (5 μg/ml) for overproducing strains, to A_{600} = 0.02, in 2.5 ml of final volume. Ten microcuries of (^{14}C) acetate (56 mCi/mmole, Amersham) or 10 μCi of (^{35}S) sulphate (1,000 Ci/mmol, Amersham) was added. Cells were simultaneously induced with 10 μM luteolin (strains bearing pMH901 and wild-type *S. saheli* and *S. teranga* strains), 2.5 μM naringenin (strains bearing pVK31), 20 μM naringenin (*A. caulinodans* ORS571, *R. tropici* CFN299) or 1 μM apigenin (strains bearing pA28), and incubated at 30°C for 24 h. No inducer was added for strains bearing pMH682. Supernatants of labeled cultures were extracted with n-butanol, applied to octadecyl silica TLC plates (Sigma), and visualized as described by Spaink et al. (1992), except that each extract loaded on a plate corresponded to 1.25 ml of the initial culture supernatant.

Table 4. Other bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Source or reference
Rhizobia		
RCR2011	= SU47, *Sinohizobium meliloti*[a] wild type, Nod$^+$, Fix$^+$ on *Medicago sativa*	Rosenberg et al. 1981
ORS571	*Azorhizobium caulinodans* wild type, Nod$^+$, Fix$^+$ on *Sesbania rostrata*	Dreyfus et al. 1988
CFN299	*Rhizobium tropici* type IIA wild type, Nod$^+$, Fix$^+$ on *Phaseolus vulgaris*	Martinez-Romero et al. 1991
VF39SM	*Rhizobium leguminosarum* bv. *viciae* VF39 derivative, Mel$^+$, Sm^r	Hynes et al. 1988
E. coli		
KMBL1164	del(*lac-proAB*) *thi* F$^-$	P. van der Putte
GMI3355	Nal^r, Rif^r derivative of K12	Sambrook et al. 1989
Plasmids		
pRK2013	ColE1, helper plasmid for mobilization of IncP and IncQ plasmids, Km^r	Figurski and Helsinski 1979
pMP240	pMP220 containing the *nodABC* promoter of *R. leguminosarum* bv. *viciae* towards *lacZ*, Tc^r, IncP	R. J. H. Okker
pRT311	pMP220 containing the *nodABC* promoter of *R. leguminosarum* bv. *trifolii* strain ANU843 towards *lacZ*, Tc^r, IncP	Mc Iver et al. 1989
pMP194	pMP190 containing the *nodABC* promoter of *S. meliloti*[a] towards *lacZ*, Cm^r, IncP	Spaink et al. 1987
pTD900	pPP375 containing the *nodY* promoter of *B. japonicum* towards *lacZ*, Tc^r, IncP	G. Stacey
pA28	2.2-kb, *nodD1* containing *Eco*RI-*Pst*I fragment from *S.* sp.[a] NGR234 pNGR234a; in pRK7813, Tc^r, IncP	Price et al. 1992
pMH682	9-kb, *nodD3* and *SyrM* containing *Hin*dIII fragment from *S. meliloti*[a] SU47; in pWB5a, Tc^r, IncP	Honma et al. 1990
pMH901	2.7-kb, *nodD1* containing *Pvu*II-*Hin*dIII fragment from *S. meliloti*[a] SU47; in pWB5a, Tc^r, IncP	Honma et al. 1990
pVK31	3.5-kb, *nodD2* containing *Bgl*II-*Xho*I fragment from *S.* sp.[a] BR816; in pVK100,Tc^r, IncP	Van Rhijn et al. 1993
pGMI174	6.1-kb, *nodGEFH* containing *Eco*RI-*Pst*I fragment from *S. meliloti*[a] RCR2011; in pBR322, Tc^r	Debellé and Sharma 1986
pHBK401	3.8-kb, *nodSU* containing *Eco*RI fragment from *S. fredii*[a] USDA257; in pGEM7ZF(+), Amp^r	Krishnan et al. 1992
pBBRNIJZ9	4.8-kb, *nodIJZnoeC* containing *Apa*I-*Hin*dIII fragment from *A. caulinodans* ORS571 in pBBR1MCS, Cm^r	Fernandez-Lopez et al. 1996

[a] The *R. meliloti-R fredii* branch including *Rhizobium* sp. NGR234 and BR816 (Martinez-Romero 1994) has been recently reclassified in the emended genus *Sinorhizobium* (de Lajudie et al. 1994).

Plasmid profiles.

Strains from *S. saheli* (ORS609, ORS611), *S. teranga* (ORS1007, ORS1009, ORS1016, ORS1045, ORS1047, ORS1071, ORS1072, ORS1073, ORS19, ORS22, ORS51, ORS52, ORS53, ORS604, ORS613, and ORS1013) and genospecies Ua (ORS1001, ORS1004, ORS1010, ORS1014, ORS1015, ORS1018, ORS1020, ORS1024, and ORS1029) were analyzed for plasmid content using a modified Eckhardt (1978) technique as described by Bromfield et al. (1987). *S. meliloti* RCR2011 and *R. leguminosarum* bv. *viciae* VF39SM were used as standards for estimation of plasmid size from their electrophoretic mobility.

Hybridization of total DNA with *nod* gene probes.

The pellet of a 20-ml culture grown overnight at 30°C was rinsed with TE buffer (Tris 10 mM, EDTA 1 mM, pH 8), and resuspended in 10 ml of TE, SDS 0.5%, proteinase K 0.3 mg/ml, RNAse 0.3 mg/ml and incubated overnight at 37°C with slight agitation. Total DNA was then cut through a syringe needle and extracted twice with phenol/chloroform/isoamyl (25:24:1), and once with chloroform. After ethanol precipitation, pellet was resuspended in 5 ml of TE. One microgram of total DNA was digested overnight with *Eco*RI (Pharmacia), separated by agarose gel, and blotted to Biodyne membrane using Southern blot standard procedures (Sambrook et al. 1989). DNA was fixed on Biodyne membrane with 0.4 N NaOH. The hybridization probes were 0.65-kb *Eco*RV fragment of pBBRNIJZ9 (*nodZ* of *A. caulinodans*), 2.1-kb *Pst*I-*Eco*RI fragment of pHBK401 (*nodSU* of *S. fredii*), and 0.8-kb *Ava*II fragment of pGMI174 (*nodH* of *S. meliloti*). Twenty to fifty nanograms of desired fragment was probed with 25 μCi (^{32}P) dCTP using the ready-to-go DNA labeling kit (Pharmacia), and purified on a microspin S200 HR column (Pharmacia). Prehybridization was performed at 60°C in 5× SSC, 0.5% SDS, 5× Denhardt (bovine serum albumin 0.02%, Ficoll 0.02%, polyvinylpyrrolidone 0.02%), 0.2 mg/ml denaturated salmon sperm DNA for 1 h. Hybridization was performed overnight in the same buffer without salmon sperm DNA, at the same temperature, using the whole labeled probe. The membrane was then rinsed using standard protocols (Sambrook et al. 1989) and exposed to Fuji RX film for 1 to 3 days.

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