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The *Rhizobium meliloti* regulatory *nodD3* and *syrM* genes control the synthesis of a particular class of nodulation factors *N*-acylated by (ω-1)-hydroxylated fatty acids

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Rhizobia elicit the formation of nitrogen-fixing nodules on specific legume hosts. *Rhizobium meliloti* nodulation (*nod*) genes control a signal exchange between the two symbiotic partners during infection and the early steps of nodulation. The regulatory *nodD1*, *nodD2* and *nodD3* genes are involved in the specific perception of different plant and environmental signals and activate the transcription of the *nod* operons. Once activated, the structural *nod* genes specify the synthesis of diffusible lipo-oligosaccharides, the Nod factors, which signal back to the plant. *R.meliloti* Nod factors are sulfated chito-oligosaccharides which are mono-*N*-acylated by unsaturated C16 fatty acids or by a series of C18 to C26 (ω-1)-hydroxylated fatty acids. In this paper we show that the regulatory *nodD3* gene and another symbiotic regulatory gene, *syrM*, which mediate bacterial responses to plant signals that differ from those involving *nodD1* and *nodD2*, determine the synthesis of Nod factors with different acyl moieties. *nodD3* and *syrM* are required for the synthesis of Nod factors *N*-acylated by the (ω-1)-hydroxylated fatty acids. This regulatory mechanism makes possible the qualitative adaptation of bacterial Nod signal production to plant signals in the course of the symbiotic process.

Key words: lipo-oligosaccharides/nitrogen fixation/Nod factors/*Rhizobium*/symbiosis

Introduction

Bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively referred to as rhizobia, elicit nitrogen-fixing nodules on the roots of leguminous host plants. Nodule induction is specific and a given rhizobial strain can infect a limited number of hosts. For example, *Rhizobium meliloti* nodulates *Medicago*, *Melilotus* and *Trigonella* species, and *R.leguminosarum* bv. *viciae* nodulates *Pisum* and *Vicia* species (Young and Johnston, 1989).

A number of *Rhizobium* genes which control infection, nodulation and host specificity, the nodulation (*nod*) genes, have been shown to be involved in a signal exchange between the two symbiotic partners (Dénarié *et al.*, 1992; Fisher and Long, 1992). In a first step proteins encoded by the *nodD* regulatory genes activate the transcription of *nod* operons in the presence of plant signals present in root exudate. Once activated, structural *nod* genes, common and species-specific, determine the production of extracellular lipo-oligosaccharide signals, the Nod factors, which signal back to the host and elicit a number of plant responses similar to those induced by the bacteria. The common *nodABC* genes are structurally conserved among all rhizobia and mutations in these genes completely abolish the ability to induce root hair curling and infection, cortical cell division and nodule formation. The species-specific *nod* genes are involved in defining the *Rhizobium* host range.

Nod factors produced by strains from different species of *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* have been characterized (for reviews see Dénarié *et al.*, 1992; Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993). They share a common basic structure: they are β-1,4-linked tetramers or pentamers of D-glucosamine, mono-*N*-acylated on the terminal non-reducing residue and *N*-acetylated on the other residues (Lerouge *et al.*, 1990; Roche *et al.*, 1991a,b; Spaink *et al.*, 1991; Price *et al.*, 1992; Sanjuan *et al.*, 1992; Schultze *et al.*, 1992; Mergaert *et al.*, 1993; Poupot *et al.*, 1993). They differ in the substituents linked to the chitin oligomer backbone. For example, the *R.meliloti* nodulation factors (NodRm) are *O*-sulfated on the terminal reducing amino sugar, are *O*-acetylated and are *N*-acylated by either unsaturated C16 fatty acids or a series of C18 to C26 (ω-1)-hydroxylated fatty acids on the terminal non-reducing amino sugar (Lerouge *et al.*, 1990; Roche *et al.*, 1991a,b; Schultze *et al.*, 1992; Demont *et al.*, 1993) (Figure 1). In *R.leguminosarum* bv. *viciae* the NodRlv factors are not sulfated and are *N*-acylated by either vaccenic acid (C18:1) or a highly unsaturated (C18:4) fatty acid (Spaink *et al.*, 1991).

The role of the various structural *nod* genes in the control of the Nod factor biosynthesis is presently under study. The common *nodABC* genes are involved in the synthesis of lipo-oligosaccharide core molecules (Spaink *et al.*, 1991; Atkinson *et al.*, 1992; Debellé *et al.*, 1992; John *et al.*, 1993). A function of species-specific *nod* gene products is to decorate these core molecules with various substituents, making the Nod factors plant-specific. For example in *R.meliloti* the species-specific NodPQ and NodH proteins, homologous to ATP sulfurylase, APS kinase and sulfotransferases, have been shown to control the sulfation of the NodRm factors (Schwedock and Long, 1990, 1992; Roche *et al.*, 1991a; Leyh *et al.*, 1992). The NodF and NodE proteins, homologous to acyl carrier proteins and β-ketoacyl synthases respectively, are involved in the synthesis of the particular polyunsaturated fatty acids which *N*-acylate the Nod factors of *R.leguminosarum* bv. *viciae* (C18:4) and

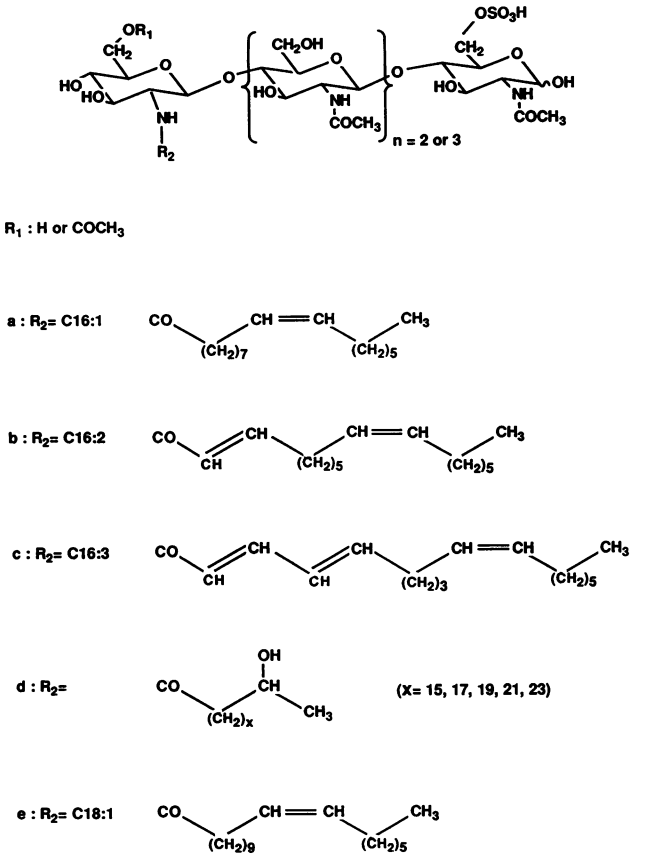


Fig. 1. Structures of *R.meliloti* Nod factors and of their various *N*-acyl substituents. R2 represents the acyl groups. (**a**–**c** and **e**) unsaturated acyls; (**d**) the (ω-1)-hydroxylated acyl series.

R.meliloti (C16:1, C16:2 and C16:3) (Spaink *et al.*, 1991; Demont *et al.*, 1993).

The regulatory NodD proteins are transcriptional activators of the LysR family (Henikoff *et al.*, 1988). They activate the expression of *nod* genes in the presence of specific plant signals and thus the *nodD* genes determine a first level of control of host specificity (Peters *et al.*, 1986; Horvath *et al.*, 1987; Spaink *et al.*, 1987). Most rhizobia possess multiple copies of *nodD* genes (Schlaman *et al.*, 1992; Göttfert, 1993). For example, *R.meliloti* possesses three copies of *nodD* genes and each NodD protein is involved in the response to different plant or environmental signals (Györgypal *et al.*, 1988, 1991; Honma *et al.*, 1990). NodD1 is activated by plant flavonoids, namely flavones such as luteolin (Peters *et al.*, 1986), whereas NodD2 is activated by betaines such as trigonelline and stachydrine (Phillips *et al.*, 1992). NodD3, in conjunction with SyrM, another transcriptional activator of the LysR family, is also involved in the control of *nod* gene expression by the level of ammonia, the primary product of symbiotic nitrogen fixation (Dusha and Kondorosi, 1993). In addition, *nodD3* and *syrM* when present in multiple copies elicit a constitutive expression of *nod* operons (Györgypal *et al.*, 1988; Mulligan and Long, 1989; Maillet *et al.*, 1990; Kondorosi *et al.*, 1991).

An important question is whether the various NodD proteins have the same affinity for the promoters of the different operons involved in the biosynthesis of the Nod factors and thus control only quantitatively the production of these signal molecules. Alternatively, various NodD proteins could exert selective activation of different operons, thus modifying the composition and stoichiometry of the Nod enzymes and controlling not only the quantity but also the structure of the bacterial Nod factors. Such a mechanism would make possible the synthesis of particular bacterial signals in response to specific plant or environmental signals, thus refining the molecular dialogue between the two symbiotic partners. In this paper, by studying the structure of the Nod factors secreted by *R.meliloti* strains carrying various combinations of NodD and SyrM transcriptional activators, we show that the regulatory *nodD3* and *syrM* genes, and not *nodD1* and *nodD2*, are required for the synthesis of a particular class of Nod factors *N*-acylated by a series of (ω-1)-hydroxylated fatty acids. This class of Nod factors is characteristic of *R.meliloti* and was not found in other rhizobial species.

Results

Influence of cloned nodD regulatory genes on the acylation of Nod factors

Rhizobium meliloti 2011 has been reported to produce a number of different NodRm sulfated factors which differ in the following features (see Figure 1). First, the length of the glucosamine oligosaccharide backbone varies, with a majority of tetramers and a minority of pentamers (Roche *et al.*, 1991a,b; Schultze *et al.*, 1992). Secondly, the C6 of the non-reducing terminal glucosamine residue is frequently *O*-acetylated (Roche *et al.*, 1991a,b; Truchet *et al.*, 1991). Thirdly, the *N*-acyl moiety is made of either mono-, di- or tri-unsaturated C16 fatty acids with the unsaturations in positions Δ9, Δ2,9 or Δ2,4,9 respectively (Lerouge *et al.*, 1990; Schultze *et al.*, 1992) or a series of C18 to C26 (ω-1)-hydroxylated fatty acids (Demont *et al.*, 1993).

The very low levels of Nod factor production by the wild-type *Rhizobium meliloti* 2011 strain has made necessary the construction of genetically engineered overproducing strains. To study the influence of the regulatory *nodD* genes on Nod factor production we introduced, in a triple *nodD1/nodD2/nodD3* mutant, IncP multicopy plasmids carrying either *nodD1* (plasmid pMH901), *nodD2* (pMH93), *nodD3* (pMH903) or *nodD3/syrM* (pMH682) genes (see Figure 2). Trigonelline was added as a *nod* gene inducer to the cultures of transconjugants carrying the *nodD2* gene, whereas luteolin was added to the other cultures. Separation of Nod factors on a semi-preparative C18 reversed phase HPLC column showed a multiplicity of poorly resolved peaks which were collected together for structural analysis. Absence of peaks with a longer retention time suggested the absence of non-sulfated species (Roche *et al.*, 1991b).

Positive ion mass spectra (LSIMS mode) of the Nod factors from the four strains looked similar. They corresponded mostly to glucosamine tetramers, sulfated on the reducing end, and both *O*-acetylated and *N*-acylated by different C16-containing fatty acids on the non-reducing end. The following major compounds were found: NodRm-IV(Ac,S,C16:1) (M_r = 1146), NodRm-IV(Ac,S,C16:2) (M_r = 1144) and NodRm-IV(Ac,S,C16:3) (M_r = 1142). Pentamers, and non-*O*-acetylated compounds, were also detected, such as NodRm-V(Ac,S,C16:2) (M_r = 1347) and NodRm-IV(S,C16:2) (M_r = 1102). Thus the *nodD* genes

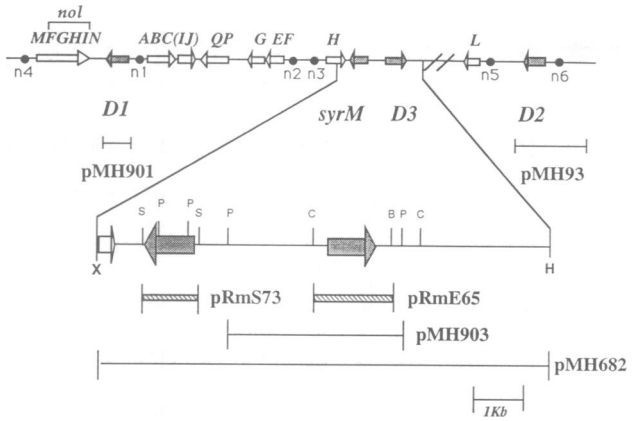


Fig. 2. Physical and genetic map of the *R.meliloti* nodulation region. The upper line represents the genetic map of the nodulation region. The open and shaded arrows represent the structural (*nod* and *nol*) and regulatory nodulation genes respectively. The black circles indicate the '*nod* box'. Below the genetic map are represented an enlarged physical map of the *syrM/nodD3* region and the various plasmids used in this study. Hatched lines represent regions cloned into the expression vector pTE3 under the control of the *trp* promoter. The short vertical lines indicate restriction sites. Abbreviations: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; S, *Sma*I (only cloning sites are represented); X, *Xho*I.

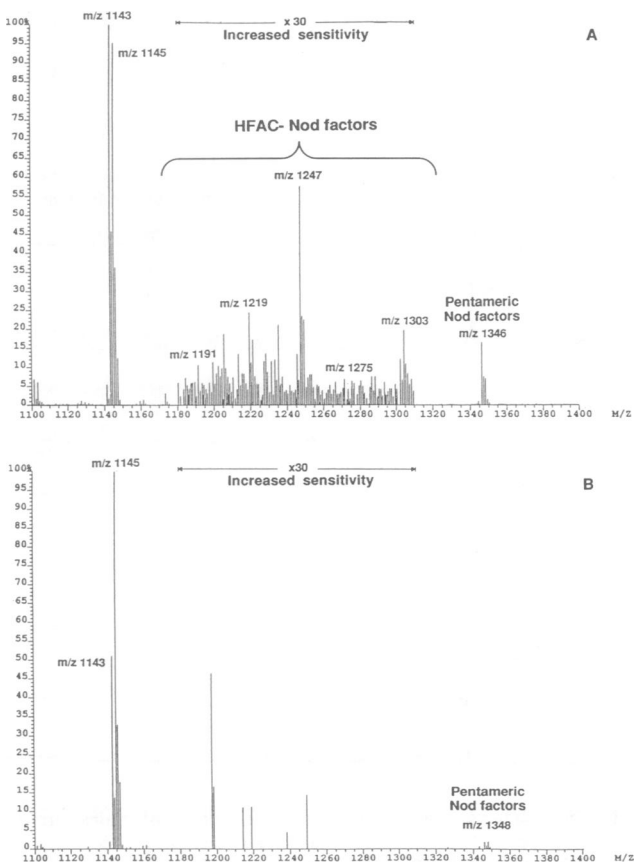
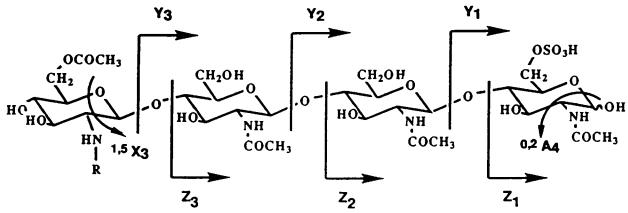


Fig. 3. Negative ion FAB-mass spectra of Nod factors from strains carrying either the *nodD3* or the *nodD1* regulatory genes. Nod factors were purified from (**A**) RmD1D2D3(pMH903) and (**B**) RmD1D2D3(pMH901) strains. NodRm-IV(Ac,S) gave peaks at *m/z* 1143 with the C16:2 acyl group and 1145 (C16:1), for factors acylated by unsaturated fatty acids, and at *m/z* 1191 (C18-OH), 1219 (C20-OH), 1247 (C22-OH), 1275 (C24-OH), 1303 (C26-OH) for the HFAC factors. Note the absence of HFAC factors on spectrum B.



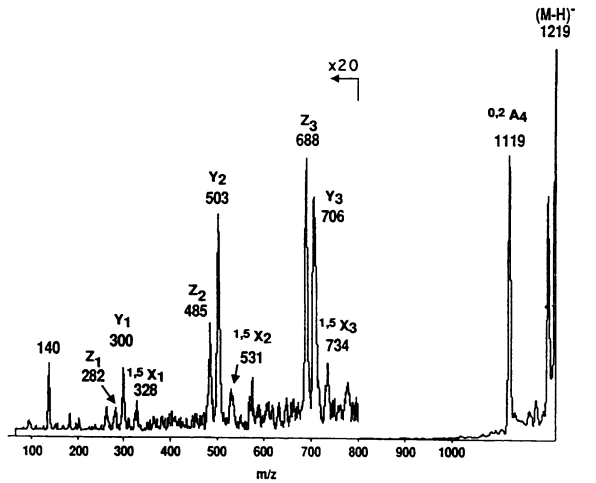


Fig. 4. Characterization by MIKE spectrometry of (ω-1)-hydroxylated fatty acid-containing compounds. Negative ion collision-activation-decomposition (CAD) spectrum of the deprotonated molecular ion from NodRm-IV(Ac,S) (19-OH C20:0) at *m/z* 1219 (MIKE spectrometry). All fragment ions retained the sulfate group. Cleavages at each glucosamine interlinkage induced three ions: two of them resulted from cleavages at both sides of the interglycosidic oxygen (Y and Z fragmentations), the third one at the sugar ring ($^{1,5}X$). The $^{0,2}A_4$ fragmentation is characteristic of a free reducing end. The nomenclature is that from Domon and Costello (1985). The mass difference between the $(M-H)^-$ ion and the Y_3 ion corresponds to the mass of the sugar residue at the non-reducing end. Thus, all MIKE spectra of each $(M-H)^-$ ion from Nod factors differing by modifications on the non-reducing terminal glucosamine should be identical, with the exception of the $^{0,2}A_4$ ion which retains the non-reducing end. For example, the $(M-H)^-$ ion of NodRm-IV(Ac,S)(21OH C22) (28 mass units above the former) gave the same CAD-MIKE spectrum, except for the $^{0,2}A_4$ ion which was shifted up by 28 mass units (spectrum not shown).

do not influence significantly the length of the sugar backbone or the degree of *O*-sulfation or *O*-acetylation of Nod factors.

However, positive spectra seemed to differ for minor ions that we have recently shown correspond to NodRm factors *N*-acylated by a series of (ω-1)-hydroxylated fatty acids and that are more clearly detected in the negative ion mode. These fatty acid components have previously been identified clearly by GC/MS techniques (Demont *et al.*, 1993). In the negative ion mode of ionization of Nod factors, differences between strains could be observed (Figure 3). In the Nod factors isolated from the strain carrying the *nodD3* gene, many additional species of higher mass (M_r of 1192, 1220, 1248, 1276 and 1304; Figure 3A) were observed, corresponding to NodRm-IV(Ac,S) molecules amidified by the series of C18 to C26 (ω-1)-hydroxylated fatty acids. The same was observed with the strain carrying both *nodD3* and

Table I. Proportion of fatty acids in Nod factors of *R.meliloti nod* regulatory mutants

Strains	Fatty acids (%)			
	Nod factor (mg/l)	ΣC16	Δ11 C18:1	Σ(ω-1)-hydroxylated
2011(p*nodD3syrM*)	1.0	58	2	40
2011(pGMI149)	2.5	60	5	35
RmD1D2D3(p*nodD1*)	0.3	90	10	–
RmD1D2D3(p*nodD2*)	0.1	64	36	–
RmD1D2D3(p*nodD3*)	0.2	75	3	22
RmD1D2D3(p*nodD3syrM*)	2.2	84	4	12
RmD1D2(p*nodD1*)	3.3	87	3	10
RmD1D2(p*nodD2*)	0.1	87	7	6
RmD1D2(p*nodD3*)	0.4	51	7	42
Rm*syrM*::Tn*5*(pP*trp-nodD3*)	1.3	40	5	55
Rm*nodD3::sp/g-1*(pP*trp-syrM*)(p*nodD1*)	3.5	66	4	30
Rm*nodD3::MudIIlac*(pP*trp-syrM*)(p*nodD1*)	4.0	71	3	26

Proportions were estimated by gas chromatography and mass spectrometry as described in Materials and methods. For each strain at least two independent cultures were analyzed. P*trp* signifies under the control of the *trp* promoter. Σ corresponds to the summed proportions of fatty acids within the same family, as indicated.

syrM (data not shown). Negative mode MS/MS analysis by mass-analyzed ion kinetic (MIKE) spectrometry of NodRm-IV(Ac,S) factors containing the C20 or the C22 (ω-1)-hydroxylated fatty acid (M_r = 1220 and 1248) confirmed the general chito-oligosaccharide structure of these factors and showed that the acyl substituent amidified the glucosamine residue at the non-reducing end (see Figure 4). With strains carrying the *nodD1* or *nodD2* genes only, no Nod factors containing (ω-1)-hydroxylated fatty acids could be detected (Figure 3B).

To determine the relative ratios of the different types of *N*-acylation of Nod factors from strains carrying different regulatory genes, we examined the GC profile of the fatty acids released after Nod factor methanolysis (Table I). Extracts from strains carrying the *nodD3* or *nodD3/syrM* plasmids contained, in addition to the unsaturated C16 fatty acids, the series of C18 to C26 (ω-1)-hydroxylated fatty acids (Figure 5A). Relative abundances of the C18, C20, C22, C24 and C26 hydroxylated species were estimated to be 2:5:17:7:1, respectively. Vaccenic acid (C18:1) was present only in trace amounts, not significantly different from the 2011(p*nodD3/syrM*) control strain (Table I). In contrast, none of the hydroxylated fatty acids could be detected in Nod factors from the strains containing the *nodD1* or *nodD2* plasmids (Figure 5B). The strain carrying the *nodD2* plasmid contained, in addition to the unsaturated C16 fatty acids, one C18:1 fatty acid representing a high proportion (~36%) of the lipid fraction (Table I). This C18:1 fatty acid exhibited the GC retention time characteristic of vaccenic acid (C18:1 Δ11). No correlation could be found between the total amount of Nod factors excreted by the different strains and the ratio of Nod factors acylated by (ω-1)-hydroxylated, showing that the *N*-acylation with these particular fatty acids is not simply due to an increase in the level of Nod factor synthesis (Table I).

We can thus conclude that the presence of multiple copies of different regulatory *nodD* genes of *R.meliloti* determines the production of Nod factors which differ in their acyl moiety. In addition to the C16 unsaturated fatty acids which

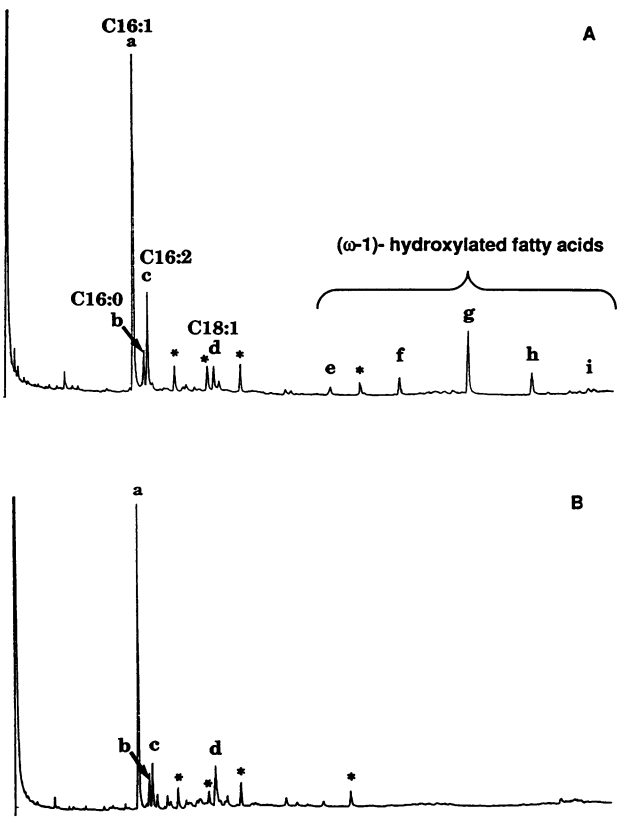


Fig. 5. Comparison of fatty acids from Nod factors of strains carrying either the *nodD3* or the *nodD1* regulatory genes. GC profiles of methyl ester trimethylsilyl ether derivatives of fatty acids from *R.meliloti* Nod factors. (**A**) RmD1D2D3 (pMH903) and (**B**) RmD1D2D3 (pMH901) strains. Non-hydroxylated fatty acids: a: Δ9 C16:1; b: C16:0; c: Δ2,9 C16:2; d: Δ11 C18:1; (ω-1)-hydroxylated fatty acids: e: 17-OH C18; f: 19-OH C20; g: 21-OH C22; h: 23-OH C24; i: 25-OH C26. Note that on non-polar stationary phases, fatty acids having a conjugated double bond, such as c, eluted later than saturated fatty acids. All the structure assignments were done by GC/MS coupling. Unidentified non-lipid components are indicated by an asterisk.

Table II. Proportion of fatty acids in Nod factors of various *R.meliloti* strains

Strains	Fatty acids			
	ΣC16	Δ11 C18:1	C20:1	Σ(ω-1)-hydroxylated
102F28(p*nodD3syrM*)	22	7	2	69
102L4(p*nodD3syrM*)	54	3	12	31
CC2017(p*nodD3syrM*)	49	7	3	41
CC2093(p*nodD3syrM*)	65	4	4	27
L5-30(p*nodD3syrM*)	17	10	1	72
L5-30(p*nodD1*)	53	4	–	43
L5-30(p*nodD2*)	72	1	–	27
L5-30(p*nodD3*)	48	6	–	46

Proportions were estimated by gas chromatography and mass spectrometry as described in Materials and methods. For each strain at least two independent cultures were analyzed. Σ corresponds to the summed proportions of fatty acids within the same family, as indicated.

are always present, Nod factors can be acylated by a C18:1 fatty acid (vaccenic acid), especially when *nodD2* is the only active *nodD* gene, and they can be acylated by a series of C18 to C26 (ω-1)-hydroxylated fatty acids in the presence of *nodD3*.

Role of the nodD3 and syrM genes

To investigate whether a single copy of *nodD3* on the pSym megaplasmid is sufficient to determine the synthesis of (ω-1)-hydroxylated fatty acid-containing Nod factors (=HFAC-Nod factors), we constructed strains isogenic to the strains used in the previous paragraph with only one difference, the presence of a wild-type *nodD3* gene on the pSym. We introduced the plasmids pMH901 (*nodD1*), pMH93 (*nodD2*) and pMH903 (*nodD3*) in a double *nodD1/nodD2* mutant. Interestingly, the presence of a single *nodD3* copy was sufficient to allow the production of HFAC-Nod factors by strains having multiple copies of *nodD1* and *nodD2* (Table I). Thus *nodD3* is epistatic on *nodD1* and *nodD2* and determines the synthesis of HFAC-Nod factors even in the presence of an excess of these genes. The observation that the proportion of HFAC-Nod factors is higher in the presence of *nodD1* is consistent with the previous observation that *nodD1* activates the expression of *nodD3* (Maillet *et al.*, 1990). Increasing the copy number of *nodD3* resulted in an increase in the proportion of HFAC-Nod factors. HFAC-Nod factors were also abundant when nodD1, nodD3 and syrM were present in equal copy numbers (with the pGMI149 plasmid).

The *nodD3* and *syrM* genes of *R.meliloti* are known to interact. The NodD3 protein activates the expression of the *syrM* gene and, reciprocally, the SyrM protein activates *nodD3* (Kondorosi *et al.*, 1991; Swanson *et al.*, 1993). *nodD3* could thus control the synthesis of HFAC-Nod factors, either directly or by the activation of the *syrM* gene.

Recently Swanson *et al.* (1993) have devised tools to dissect the respective roles of these two genes. They have constructed two IncP plasmids, one in which *nodD3* is expressed from the *trp* promoter and is thus independent of SyrM (=plasmid pRmE65) and another one in which *syrM* is expressed from the *trp* promoter and is thus independent of NodD3 (=plasmid pRmS73). We have introduced the *nodD3* constitutive clone in a strain having a *syrM*::Tn*5* mutation in the pSym*a* megaplasmid. It can be seen in Table I that a very high proportion of the Nod factors of this strain is amidified by (ω-1)-hydroxylated fatty acids, showing that *nodD3* does not require the presence of *syrM* to induce the synthesis of HFAC-Nod factors.

The constitutive *syrM* clone was introduced in a *nodD3*::Tn*5* mutant. Since SyrM is reported not to activate directly the transcription of operons preceded by a *nod* box such as *nodABC* (Honma *et al.*, 1990; Kondorosi *et al.*, 1991; Swanson *et al.*, 1993), we introduced, to increase the production of Nod factors, a *nodD1* gene cloned into an IncQ plasmid (=plasmid pGMI1394) compatible with the IncP plasmid carrying the constitutive *syrM* gene. This strain was found to produce HFAC factors (Table I). To confirm this result the pRmS73 and pGMI1394 plasmids were introduced in a strain carrying another *nodD3* mutation, *nodD3*::*Mud*II*lacZ*# V40, for which the insertion has been localized within the *nodD3* ORF by DNA sequencing (F.Debellé, personal communication). HFAC factors were produced by this strain. It can thus be concluded that both *nodD3* and *syrM* can independently elicit the production of HFAC factors.

(ω-1)-hydroxylated fatty acids in Nod factors of various R.meliloti strains

To assess the generality of the induction by the *nodD3/syrM* genes of the synthesis of HFAC-Nod factors, we introduced the plasmid pMH682 into various *R.meliloti* strains. In an attempt to ensure a broad genetic diversity we selected strains from varied geographical origins and symbiotic specificity. For example, strain L5-30 originates from Central Europe, 102F28 from North America, 102L4 from North Africa and CC2017 and CC2093 from Australia.

Mass spectra analysis of the Nod factors from the pMH682$^+$ transconjugants from the five strains showed that all the factors share the basic structure already described for strain 2011(pMH682): a chito-oligosaccharide backbone, with a majority of tetramers, sulfated at the reducing end, and *O*-acetylated and *N*-acylated at the non-reducing end.

The Nod factors from the five strains were hydrolyzed and the released fatty acids were analyzed by GC. All the strains were found to produce a mixture of Nod factors comprising the unsaturated C16 fatty acids, small amounts of a C18:1 and a C20:1 fatty acid, and the series of C18 to C26 (ω-1)-hydroxylated fatty acids. The proportions of the various fatty acids are given in Table II. Interestingly, the proportion of (ω-1)-hydroxylated fatty acids was high, ranging from 27 to 72%. The introduction of plasmids containing either *nodD1*, *nodD2* or *nodD3* into strain L5-30

did not eliminate the production of HFAC-Nod factors (Table II), suggesting that as with strain 2011, only one copy of *nodD3* is sufficient to trigger the synthesis of these Nod factors. We can conclude that the ability to synthesize HFAC-Nod factors is general among *R.meliloti* strains. It is worth noting that HFAC-Nod factors could not be detected using the same analytical procedures in other rhizobial species, such as *R.fredii* (M.P.Bec, personal communication), *R.leguminosarum* bv. *viciae* (NodRlv factors were kindly provided by H.Spaink, Leiden University), *R.tropici* (R.Poupot, personal communication), and *Rhizobium* sp. NGR234 (F.Talmont, personal communication).

nodD3/syrM genes and (ω-1)-hydroxylated fatty acids in lipid A

(ω-1)-hydroxylated fatty acids are very uncommon in bacteria and their presence has not been reported in phospholipids. However, an (ω-1)-hydroxylated fatty acid with a C28 chain has recently been identified as a component of lipopolysaccharides (LPS) from *R.meliloti* strain 2011, but no mention was made of homologs with a shorter chain length (Bhat *et al.*, 1991). It thus appears that two glycolipids of the *R.meliloti* cell surface, LPS and Nod factors contain (ω-1)-hydroxylated fatty acids. Moreover, in both lipid A and Nod factors the (ω-1)-hydroxylated fatty acids amidify a glucosamine residue. The series of C18 to C26 (ω-1)-hydroxylated fatty acids could be precursors in the synthesis pathway of the 27-hydroxy-octacosanoic acid. It was thus important to explore the possibility that *nodD3* and *syrM* might simply increase the synthesis of (ω-1)-hydroxylated fatty acids resulting in their non-specific accumulation in both Nod factors and lipid A.

To assess whether the presence of cloned *nodD3/syrM* genes might result in changes in the lipid composition of LPS, we purified lipid A from three related *R.meliloti* 2011 derivatives: (i) the wild-type strain 2011 as a control, (ii) GMI6390 = 2011(*pnodD3/syrM*) and (iii) 2011*nodC* (*pnodD3/syrM*) a mutant which does not synthesize the Nod factor glucosamine backbone, a substrate for the Nod factor acyl-transferase, and therefore a strain in which competition for the (ω-1)-hydroxylated fatty acid pool between Nod factor and lipid A synthesis should be suppressed. The three strains were grown in the presence of luteolin. Fatty acids released upon hydrolysis were subjected to GC analysis. Figure 6 allows the comparison of the GC profiles of fatty acids liberated from lipid A and from Nod factors extracted from cultures of the same strain 2011(*pnodD3/syrM*). This shows that the series of C18 to C26 (ω-1)-hydroxylated fatty acids present in Nod factors (as well as the C16:1 and 16:2 unsaturated fatty acids) cannot be detected in lipid A. In contrast, the C28 (ω-1)-hydroxylated fatty acid is abundant in lipid A and absent in Nod factors. The fatty acid profiles of lipid A from the three strains were similar with one difference, an enhancement of the C12 homolog from the 3-hydroxylated series, with strains carrying the p*nodD3/syrM* plasmid (data not shown). The series of C18 to C26 (ω-1)-hydroxylated fatty acids could not be detected in the lipid A of the *nodC* mutant indicating that these fatty acids were not transferred on lipid A even in the absence of their acceptor, the Nod factor chito-oligosaccharide backbone. These results indicate that the *N*-acyl-transferases involved in the amidification by (ω-1)-hydroxylated fatty acids of the glucosamine residues of Nod factors and lipid A have different specificities.

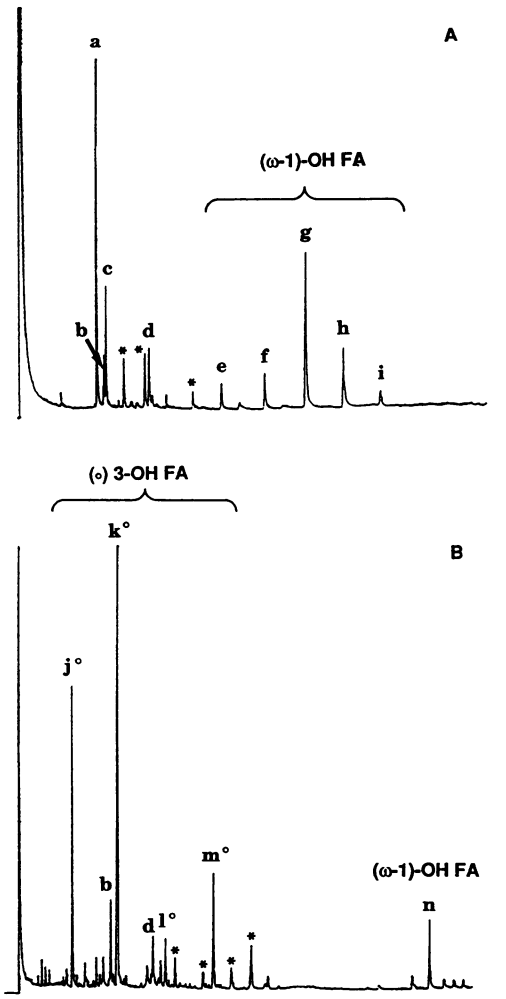


Fig. 6. Comparison of fatty acids of Nod factors and LPS from a *R.meliloti* strain overexpressing *nod* genes. GC profiles of methyl ester trimethylsilyl ether derivatives of fatty acids from *R.meliloti* 2011(*pnodD3/syrM*) Nod factors (**A**) and the corresponding LPS (**B**). (A) same peak assignments as in Figure 5A. (B) j: 3-OH C12:0; b: C16:0; k: 3-OH C14:0; d: Δ11 C18:1; l: 3-OH C16:0; m: 3-OH C18:0; n: 27-OH C28:0, the (ω-1)-OH C28 fatty acid. Unidentified non-lipid components are indicated by an asterisk. 3-hydroxy-acids, labelled with an open circle, were identified by GC/MS coupling.

Root hair-deforming and nodulation ability of Nod factors with various N-acyl moieties

Nod factors from *R.meliloti* are able to elicit, on axenic alfalfa (*Medicago sativa*) seedlings, a number of responses, such as root hair deformations (Lerouge *et al.*, 1990; Roche *et al.*, 1991a; Schultze *et al.*, 1992), induction of mitosis in the inner cortex and nodule formation (Truchet *et al.*, 1991; Schultze *et al.*, 1992). An important question is whether for NodRm factors the replacement of unsaturated C16 fatty acids by saturated acyl chains such as the (ω-1)-hydroxylated fatty acids results in changes in hair-deforming and nodule-inducing activities.

Seedlings were treated with the following Nod factors. (i) 'Wild-type' Nod factors were prepared from the RmD1D2D3(p*nodD3/syrM*) strain and contained a mixture

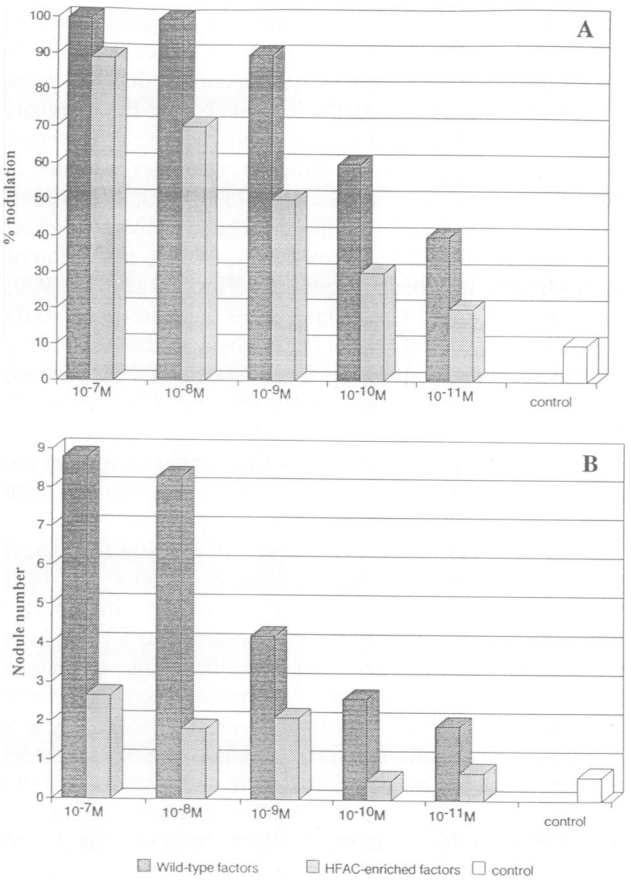


Fig. 7. Nodule-inducing properties of Nod factors with various *N*-acyl moieties. (**A**) Proportion of tubes containing nodulated seedlings. (**B**) Number of nodules per tube. Nodules were scored 35 days after addition of the Nod factors. Twenty plants were used for each dilution with two seedlings per tube. The proportions of Nod factor acyl substituents were as follows: (i) wild-type factors: unsaturated C16/C18:1/(ω-1)-hydroxylated fatty acids (21:1:3); (ii) HFAC-enriched factors: C18:0/(ω-1)-hydroxylated fatty acids (2:8). Control seedlings were treated with water.

of unsaturated (88%) and (ω-1)-hydroxylated (12%) fatty acids (Table I); (ii) HFAC-free Nod factors were prepared from the RmD1D2D3(p*nodD1*) strain and contained only unsaturated fatty acids, 90% of C16 and 10% of C18:1 (Table I); (iii) HFAC-enriched Nod factors were prepared from a *nodFE* (p*nodD3*/*syrM*) mutant and hydrogenated to saturate the fatty acids: they contained ~80% of (ω-1)-hydroxy fatty acids, 20% of C18:0, and were free of unsaturated C16 and C18. To try to identify possible antagonistic or synergistic effects of HFAC factors, mixtures (50:50) of HFAC-enriched factors with 'wild-type' and with HFAC-free preparations were also assayed. Three independent assays of alfalfa hair deformation were performed and showed that the various Nod factors, whatever the fatty acyl substitutions, were very active. They elicited root hair deformations at concentrations down to $10^{-12}-10^{-13}$ M (data not shown).

HFAC-enriched Nod factors were able to elicit nodule formation. At high concentrations, from 10^{-9} M to 10^{-7} M, >50% of the tubes contained nodulated seedlings, a proportion which was clearly higher than the background of spontaneous alfalfa nodulation (Figure 7A). HFAC-enriched Nod factors, however, exhibited a decreased nodulating activity compared with 'wild-type' factors. The comparison of the nodule numbers induced at different concentrations of factors shows that the activities of 10^{-10} M wild-type and 10^{-7} M HFAC-enriched factors were of the same order of magnitude, suggesting that the HFAC-enriched factors exhibit a nodule-inducing activity reduced by a factor >100-fold (Figure 7B). As compared with the wild-type factors, HFAC-free factors had a slightly decreased activity but the difference was not statistically significant. The addition of HFAC-enriched factors to wild-type or to HFAC-free factors did not result in significant effects (data not shown). We conclude that the replacement of unsaturated C16 by (ω-1)-hydroxylated fatty acids results in changes in the elicitation of plant responses: whereas the ability to deform root hairs remains high and unchanged, the nodule-inducing activity is clearly decreased.

Discussion

Rhizobium *nod* genes control a signal exchange during legume infection and nodulation (Dénarié *et al.*, 1992; Fisher and Long, 1992). In this paper we have described a novel function of *nod* regulatory genes in the control of this molecular dialogue: the regulatory NodD3 and SyrM proteins, which regulate *nod* gene transcription in the presence of signals different from those active with NodD1 and NodD2, control the synthesis of a particular class of Nod factors. Several lines of evidence show that the *nodD3* gene controls specifically the synthesis of Nod factors acylated by a series of C18 to C26 (ω-1)-hydroxylated fatty acids. In mutants having no active *nodD3* gene no HFAC-Nod factors can be detected. One *nodD3* copy in the pSym megaplasmid is sufficient to allow the production of HFAC factors, and increasing the *nodD3* copy number results in an increase in the proportion of HFAC factors. A single copy of *nodD3* present in the pSym megaplasmid triggers the synthesis of HFAC factors even in the presence of multiple copies of *nodD1* and *nodD2*. The regulatory functions and the regulation of *nodD3* are complex. *nodD3* activates expression of *syrM* and reciprocally *syrM* activates expression of *nodD3* (Kondorosi *et al.*, 1991; Swanson *et al.*, 1993). However, the fact that a *syrM*-deficient mutant produces HFAC factors in the presence of a constitutive *nodD3* gene indicates that *nodD3* is directly responsible for the activation of gene(s) involved in the synthesis of HFAC factors. Interestingly, the *syrM* gene, in the absence of an active *nodD3* gene, also provokes the production of HFAC factors, showing that both *nodD3* and *syrM* can independently induce the synthesis of these particular Nod factors.

Whereas NodD1, NodD2, NodD3 and SyrM are all transcriptional activators of the LysR family, NodD3 and SyrM genes have a number of particular characteristics. The NodD1 and NodD2 proteins exhibit higher sequence similarity between each other than with NodD3, and the SyrM protein has only ~30% similarity to *R.meliloti* NodD proteins (Barnett and Long, 1990; Kondorosi *et al.*, 1991; Rushing *et al.*, 1991). The activity of NodD1 and NodD2 is plant inducer-dependent whereas cloned *nodD3/syrM* genes can elicit a high constitutive expression of *nod* operons, *nodD3* forming a positively amplifying circuit with *syrM* (Györgypal *et al.*, 1988; Mulligan and Long, 1989; Maillet

et al., 1990; Kondorosi *et al.*, 1991; Swanson *et al.*, 1993). *syrM* is a global symbiotic regulator which controls not only the expression of *nod* operons via *nodD3* but also exopolysaccharide synthesis via *syrA*, thus controlling the major classes of signals required for the infection process (Mulligan and Long, 1989). Moreover, NodD3 is involved, together with SyrM, in the control of *nod* gene expression by ammonia, the primary product of nitrogen fixation (Dusha and Kondorosi, 1993). The NodD3 protein, as NodD1, has been shown to bind specifically to *nod* boxes located upstream of *nodABC*, *nodFE* and *nodH* operons and activate the transcription of the downstream operons (Fisher *et al.*, 1988). However, the DNA footprint of NodD3 on these *nod* gene promoters displays an additional hypersensitive cleavage site (Fisher and Long, 1989). In contrast to the other NodD regulatory proteins, NodD3 has been shown to activate not only the transcription of *nod* box-preceded operons, but also the transcription of a gene such as *syrM* that is not preceded by a *nod* box (Swanson *et al.*, 1993). The effect of SyrM on the activation of *nod* boxes has been reported to be indirect and to depend on NodD3, and SyrM activates the transcription of genes which are not preceded by a *nod* box, such as *nodD3* and *syrA* (Mulligan and Long, 1989; Swanson *et al.*, 1993). Thus the genes whose activation by *nodD3* and *syrM* results in the synthesis of HFAC-Nod factors are not necessarily classical *nod* genes (preceded by a *nod* box).

Different modifications of rhizobial metabolism could result in the synthesis of HFAC factors. First, the *nodD3* and *syrM* genes could activate genes that increase the (ω-1)-hydroxylated fatty acid pool. Second, the *nodD3* and *syrM* genes could activate gene(s) coding for acyl-transferases catalyzing the specific transfer of (ω-1)-hydroxylated fatty acids, present in the fatty acid pool, on to the Nod factor backbone. The series of C18 to C26 (ω-1)-hydroxylated fatty acids are likely to be precursors in the synthesis of 27-hydroxyoctacosanoic acid, a major component of lipid A from *Rhizobium* LPS (Bhat *et al.*, 1991). We have shown that the distribution of (ω-1)-hydroxylated fatty acids is complementary between these two types of surface glycolipids, with the series of C18 to C26 present in Nod factors and the C28 in LPS lipid A, and that more generally there seems to be a mutual exclusion, in *R.meliloti*, between the fatty acids present in lipid A and those present in Nod factors. The acyl-transferases which amidify the N-atom of glucosamine residues in the Nod factors on the one hand, and LPS on the other hand, seem therefore to have distinct and complementary specificities for acyl groups.

The *nodFE* genes specify the synthesis of polyunsaturated fatty acids, C18:4 in *R.leguminosarum* bv. *viciae* and C16:2/C16:3 in *R.meliloti*. In *nodFE* mutants, in the absence of the polyunsaturated symbiotic fatty acids, Nod factors are acylated by default with vaccenic acid, the most abundant fatty acid present in rhizobial cells (Spaink *et al.*, 1991; Demont *et al.*, 1993). We have shown that a *R.meliloti* triple *nodD1D2D3* mutant, carrying a *nodD2* clone and grown in the presence of the NodD2-specific plant inducer trigonelline, produces a high proportion (>30%) of factors acylated by vaccenic acid, as leaky *nodFE* mutants do (Demont *et al.*, 1993). This indicates that NodD2 activates the *nodFE* operon less efficiently than the *nodABC* operon involved in the synthesis of the chito-oligosaccharide backbone. Thus not only NodD3 and SyrM, but also NodD2 seem to have specific affinities for the promoters of different operons involved in the Nod factor biosynthesis. Since each of the three NodD proteins of *R.meliloti* responds to different plant and/or environmental signals, it is likely that these signals will influence the synthesis of Nod factors of different structures. NodD proteins bind to promoter regions in dimeric forms (Fisher and Long, 1989, 1993; Kondorosi, 1992). Different NodD proteins have been shown to interact in the control of *nod* gene expression, and the formation of heterodimers has been suggested (Honma *et al.*, 1990; Kondorosi *et al.*, 1991). Our results indicate that NodD homodimers have different affinities for the regulatory regions of genes controlling the synthesis of Nod factors, and suggest also that heterodimers (for example NodD2/NodD3) may have different activities: these differential affinities could allow fine tuning control of the ratio of the different Nod factors in response to various plant signals in the course of the symbiotic process.

It has been shown that the structure of the Nod factor acyl moiety is important for biological activity in both *R.leguminosarum* bv. *viciae* and *R.meliloti*. In NodRlv factors the replacement of the polyunsaturated C18:4 chain by vaccenic acid (C18:1) abolishes the ability to induce nodule primordia and pre-infection thread structures in vetch (Spaink *et al.*, 1991; Van Brussel *et al.*, 1992). Hydrogenation of the unsaturated C16 acyl chain of NodRm factors decreases the ability to elicit nodule formation in alfalfa (Truchet *et al.*, 1991). Moreover, *R.meliloti nodFE* mutants, which produce Nod factors in which the unsaturated C16 acyl chain is replaced by vaccenic acid, are strongly altered in their ability to elicit the formation of infection structures in *Medicago* hosts (M.Y.Ardourel, N.Demont, F.Debellé, F.Maillet, J.C.Promé, J.Dénarié and G.Truchet, submitted). In this paper we show that whereas HFAC-enriched Nod factors with saturated acyl moities elicit normal root hair deformations, their nodule-inducing ability is strongly reduced. Thus the changes in the acyl moiety composition of NodRm factors, controlled by the *nodD3* and *syrM* genes, might be important in fine tuning of the molecular dialogue between the symbiotic partners. It was not possible to isolate the five different species of the series of C18 to C26 (ω-1)-hydroxylated fatty acids, and to separate them from minor saturated fatty acids (such as C18:0) to check their individual biological activities. However, the complete chemical synthesis of major NodRm factors has been achieved recently (Nicolaou *et al.*, 1992), opening the possibility of synthesizing pure Nod factors differing only by subtle changes in the acyl chain. This will permit detailed studies of the importance of the carbon chain length and of the (ω-1)-hydroxylation.

Each rhizobial species studied so far has been found to produce a family of Nod factors varying in the number of glucosamine residues, the type of O-substitutions and the type of *N*-acyl groups (Roche *et al.*, 1991a; Price *et al.*, 1992; Schultze *et al.*, 1992; Mergaert *et al.*, 1993; Poupot *et al.*, 1993). The biological significance of the production of a set of different factors is not known. A model has been proposed suggesting that these families of molecules are important in modulating the symbiotic interactions that occur with different host plants or under different environmental conditions (Schultze *et al.*, 1992). Interestingly, the expression in pea root hairs of two early nodulin genes, *ENOD5* and *ENOD12*, is induced in a transient manner by

two different purified NodRlv factors while a mixture of these factors extends the period during which these genes are expressed (Horvath *et al.*, 1993). Future studies will address the question of the symbiotic role of HFAC factors, and may require the identification of new early nodulin genes.

It is interesting to note that the induction of the synthesis of HFAC-Nod factors by *nodD3* and *syrM* has been observed in a number of strains of *R.meliloti* of different geographic origin and thus seems to be a general characteristic of the control of *R.meliloti* Nod factor synthesis. In contrast, HFAC-Nod factors have not been found in other *Rhizobium* species so far. These results suggest that HFAC-Nod factors may have a particular role in the establishment of symbiosis with *R.meliloti* hosts.

The presence of multiple copies of *nodD* genes has been described in other rhizobia such as *R.tropici*, *R.etli*, *R.fredii*, *Rhizobium* sp. NGR234 and *B.japonicum* (Schlaman *et al.*, 1992; Göttfert, 1993) and a *syrM* gene has recently been identified in *R.leguminosarum* bv. *phaseoli* (Michiels *et al.*,

Table III. Bacterial strains and plasmids

Designation	Relevant characteristics	Reference/source
R.meliloti		
RCR2011	= SU47, wild-type, Australia isolate, Nod$^+$,Fix$^+$ on *M.sativa*	Rosenberg *et al.* (1981)
L5-30	Poland isolate	Rosenberg *et al.* (1981)
102F28	North America isolate	Rosenberg *et al.* (1981)
102L4	North Africa isolate	J.Burton
CC2093	Australia isolate, Nod$^+$,Fix$^-$ on *M.laciniata*	Rosenberg *et al.* (1981)
CC2017	Australia isolate, Nod$^+$,Fix$^+$ on *M.laciniata*	Rosenberg *et al.* (1981)
1021	= SU47, Sm^R derivative	Honma and Ausubel (1987)
RmD1D2-3	1021*nodD1*::Tn*5* *nodD2::tm*	Honma and Ausubel (1987)
RmD1D2D3-1	1021*nodD1*::Tn*5* *nodD2::tm* *nodD3::splg-1*	Honma and Ausubel (1987)
GMI6665	RmD1D2-3(pMH901)	this study
GMI6666	RmD1D2-3(pMH93)	this study
GMI6667	RmD1D2-3(pMH903)	this study
GMI6455	RmD1D2D3-1(pMH901)	this study
GMI6456	RmD1D2D3-1(pMH93)	this study
GMI6457	RmD1D2D3-1(pMH903)	this study
GMI6458	RmD1D2D3-1(pMH682)	this study
GMI6390	2011(pMH682)	Roche *et al.* (1991)
GMI6393	L5.30(pMH682)	this study
GMI6392	102F28(pMH682)	this study
GMI6511	102L4(pMH682)	this study
GMI6566	CC2093(pMH682)	this study
GMI6568	CC2017(pMH682)	this study
JT701	1021 *syrM*::Tn*5*	Swanson *et al.* (1987)
GMI6650	JT701(pRmE65)	this study
RmD3-1	1021*nodD3::sp/g-1*	Honma and Ausubel (1987)
GMI6669	RmD3-1(pRmS73)(pGMI1394)	this study
GMI5872	2011*nodD3::Mud*II*lacZ* # V40	Maillet *et al.* (1990)
GMI6683	GMI5872(pRmS73)(pGMI1394)	this study
GMI6690	2011*nodC*::Tn*5* # 2303(pMH682)	F.Debellé
GMI6365	2011Δ(*nodFE*)4(pMH682)	Demont *et al.* (1993)
E.coli		
GMI3550	S17.1	Simon *et al.* (1983)
GMI10847	S17.1(pMH903)	this study
GMI10726	HB101(pMH682)	Honma *et al.* (1990)
GMI10827	C600(pGMI1394)	this study
GMI3686	K12(pRK2013)	Ditta *et al.* (1980)
Plasmids		
pMH901	pWB5a-prime(IncP), insert from *R.meliloti pSyma* carrying *nodD1*, Tc^R	Honma *et al.* (1990)
pMH93	pRK290-prime(IncP), insert from *R.meliloti pSyma* carrying *nodD2*, Tc^R	Honma *et al.* (1990)
pMH903	pWB5a-prime(IncP), insert from *R.meliloti pSyma* carrying *nodD3*, Tc^R	Honma *et al.* (1990)
pMH682	pWB5a-prime(IncP), insert from *R.meliloti pSyma* carrying *nodD3* and *syrM*, Tc^R	Honma *et al.* (1990)
pGMI1394	pML132-prime(IncQ), insert from *R.meliloti pSyma* carrying *nodD1*, Gm^R	F.Debellé
pRK2013	helper plasmid for mobilization of IncP and IncQ plasmids, Km^R	Ditta *et al.* (1980)
pTE3	broad host range expression vector, IncP	Egelhoff and Long (1985)
pRmE65	*nodD3* expressed from the *trp* promoter in pTE3	Fisher *et al.* (1988)
pRmS73	*syrM* expressed from the *trp* promoter in pTE3	Swanson *et al.* (1993)
pGMI149	pRK290-prime (IncP), 30 kb insert from *R.meliloti pSyma nod* region, Tc^R	Debellé *et al.* (1986)

1993). Our finding opens the way to the analysis of the role of multiple *nodD* and *syrM* regulatory genes in the control of Nod factor structure in different rhizobial systems, as a mechanism for the fine control of bacterial signaling to plants in response to specific plant and environmental signals.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids are described in Table III and Figure 2. Conditions used for bacterial growth and conjugation experiments have been described previously (Truchet *et al.*, 1985). The transfer of the pGMI1394 IncQ plasmid and of the pMH682 IncP plasmid to *R.meliloti* was carried out by triparental mating using the helper plasmid pRK2013 (Ditta *et al.*, 1980). The other IncP plasmids (pMH901, pMH93 and pMH903) were first introduced by triparental matings into *Escherichia coli* strain S17.1 which has an RP4 sex factor integrated in the chromosome. The purified S17.1 transconjugants were then mated with the appropriate *R.meliloti* recipients. The transfer of IncP plasmids was selected with 10 μg/ml tetracycline, and of the IncQ plasmid (pGMI1394) with 150 μg/ml gentamicin. With *R.meliloti* recipients carrying the D3-1 mutation, spectinomycin (500 μg/ml) was used to counterselect *E.coli* donors on TY plates (Truchet *et al.*, 1985). In contrast, in matings with wild-type *R.meliloti* strains and with other mutants, auxotrophic *E.coli* donors were counterselected on sucrose-containing minimal Vincent agar plates (Vincent, 1970). For Nod factor production, *R.meliloti* cultures were grown in 5 l Erlenmeyer flasks filled with 2 l of culture medium containing either luteolin (10 μM) or trigonelline (1 mM) as *nod* gene inducers (Lerouge *et al.*, 1990; Phillips *et al.*, 1992). The flasks were agitated on a rotary shaker at 50 r.p.m. for ~24 h at 30°C. When the optical density (650 nm) reached 0.8–1.0, cultures were filtered through a 0.45 μm Millipore filter membrane and the cell-free culture medium was immediately extracted as described below.

Nod factor purifications

Nod factors were extracted from the culture medium by butanol, and purified by ethyl acetate washing as previously described (Roche *et al.*, 1991b). They were separated by HPLC (Kontron model 420 pumps fitted with a Kontron model 430 multiwavelength detector). Separations were first carried out on a semipreparative C18 reversed phase column (7.5 × 250 mm, Spherisorb ODS2, 5 μm, ColoChrom), using a linear gradient running from water/acetonitrile 80:20 (v/v) (solvent A) to pure acetonitrile (solvent B), at a 2 ml/min flow rate. The UV absorbance was monitored at both 220 and 260 nm. A broad peak eluting at ~55% acetonitrile corresponded to the mixture of Nod factors. This freeze-dried fraction was weighed to determine Nod factor yield. When necessary, this fraction was further purified on an analytical C18 reversed phase column (4.6 × 250 mm, Spherisorb ODS1, 5 μm) using 3 min isocratic solvent A, followed by a linear gradient from solvent A to solvent B for 30 min at a flow rate of 1 ml/min. The HFAC-enriched Nod factors were prepared as follows. Extracts from GMI6365, a 2011Δ*nodFE*(pMH682) strain producing Nod factors acylated with vaccenic acid (C18:1) and (ω-1)-hydroxylated fatty acids (but not with unsaturated C16 fatty acids), were purified by HPLC on both semi-preparative and analytical columns. All the different peaks, even those that were only partly resolved, were collected separately. Aliquots of each fractions were hydrolyzed and their hydroxylated fatty acid content was estimated by gas chromatography (GC). Fractions containing at least 80% hydroxylated fatty acids were pooled and hydrogenated twice on a Pd/C catalyst as described previously (Roche *et al.*, 1991b). A final purification was performed by analytical HPLC. HFAC-enriched Nod factors contained ~80% of (ω-1)-hydroxylated and 20% of C18:0 fatty acids. No C16 unsaturated fatty acids could be detected.

Isolation and purification of lipopolysaccharides

LPSs were extracted from *R.meliloti* strains by the hot phenol/water procedure (Westphal and Jann, 1965). The pooled water layers were dialyzed against distilled water for 48 h, and freeze-dried. About 200 mg of material were obtained from 10 g of dry bacteria. This residue was resuspended in 10 ml of distilled water and diluted with 100 ml of ethanol. The precipitate was collected by centrifugation at 5000 *g* for 15 min, washed with ethanol and redissolved into water. This solution was then mixed with an equal volume of ethanol and the precipitate was centrifuged and discarded. The supernatant was evaporated, dissolved in water and precipitated again with an equal volume of ethanol. The clear supernatant containing LPS was freeze-dried. Approximately 30 mg of LPS were purified from 200 mg of the crude extract obtained by water/phenol extraction.

Instrumentation

Mass spectra of Nod factors were recorded on an Autospec instrument (Fisons, VG-analytical, Manchester, UK) equipped with a fast cesium ion bombardment source (liquid secondary ion mass spectrometry, or LSIMS). The cesium gun operated at 20 kV; the secondary ion accelerating voltage was 8 kV. One microliter of Nod factor solution in methanol (concentration 1 μg/μl) was deposited on the stainless steel target loaded with 1 ml of matrix. In the positive ion mode, the matrix was a 1:1 mixture of *meta*-nitrobenzyl alcohol (MNBA) and glycerol, mixed with an equal volume of either 1% trifluoroacetic acid in water or a 1 μg/μl solution sodium iodide in water. In the negative mode, the matrix was a 1:1 mixture of MNBA and glycerol.

Collision-activation-decomposition (CAD) spectra were measured on a VG-ZAB HS instrument, using mass-analyzed ion kinetic (MIKE) spectroscopy. This analysis method allowed a good mass selection for the parent ion and a poor resolution for daughters, in contrast to the previously used B/E constant scans (Roche *et al.*, 1991b) which gave a good daughter ion resolution but a wide mass selection of parent ions. The MIKE analysis was thus preferred for the study of complex ionic mixtures. Ionization was done by fast atom bombardment source, using a 8 keV xenon beam. The matrix was identical to the experiment described above. Helium was the collision gas. The parent $(M-H)^-$ ions were selected at one unit resolution. Spectra from ~20 scans of 10 s of the deflecting voltage were averaged and mass calculated using a home-made computer program.

Gas chromatograms were performed on a Girdel 30 apparatus (Girdel, France) equipped with a flame ionization detector and a Ross type injector. Helium was the carrier gas. Separations were achieved on a OV1-coated capillary column (i.d. 0.32 mm, length 12 m, film thickness 0.1 μm; Altech, France) using a linear program from 100 to 280°C at 3°C/min. GC/MS experiments were performed on a Hewlett-Packard 5985B instrument, fitted with non-polar AT1 column (0.32 mm × 60 m, 0.25 μm; Altech). The carrier gas was helium.

Fatty acid analysis

Fatty acid release from either Nod factors or LPS was achieved by acid methanolysis. Approximately 100 μg Nod factors or 1 mg LPS were dissolved in 0.5 ml of 1 M HCl solution in methanol. After 18 h at 80°C, the mixture was evaporated, partitioned between diethyl ether and water. The ether phase was washed with water, evaporated and then dissolved in 20 μl *bis*-trimethylsilyl-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS). One microliter of this solution, containing both methyl esters of non-hydroxylated fatty acids and *O*-trimethylsilyl ether methyl esters of hydroxylated fatty acids, was analyzed immediately by GC or GC/MS. On GC/MS, the GC column fitted on the instrument did not allow the elution of very long chain fatty acids. In particular, the (ω-1)-hydroxylated C28 fatty acid could not be eluted. It was identified by extrapolating the retention time of the shorter fatty acids from the same family, that had previously been identified by GC/MS (Demont *et al.*, 1993) and the already described chromatographic behavior of the (ω-1)-hydroxylated C28 fatty acid isolated from *R.meliloti* LPS (Baht *et al.*, 1991).

To estimate the relative ratio between fatty acids in Nod factors we assumed that the recovery yield after hydrolysis was similar for fatty acids containing no conjugated double bond (C16:1 Δ9, C18:1 Δ11 and (ω-1)-hydroxylated fatty acids, for example). Conjugated fatty acids (such as the 2E,9Z hexadecadienoic acid) were released with a lower yield from their amide-bound form. Their ratio was adjusted by comparison with the negative ion mass spectra of intact Nod factors which give more accurate values for the ratio between the C16:2 and C16:1 fatty acid-containing molecules, as previously discussed (Demont *et al.*, 1993).

Plant assays

The alfalfa root hair deformation assay was performed as already described with *Medicago sativa* cv. Gemini (Roche *et al.*, 1991a). Ten plants were used for each treatment and 50 plants were used for the control. Results were analyzed statistically as previously described (Roche *et al.*, 1991a). The alfalfa nodulation assays were performed as previously described (Truchet *et al.*, 1991) with the following modifications. Seeds of *M.sativa* cv. Gemini were replaced by the cultivar AS-13 (Ferry-Morse Co., Modesto, CA) which exhibits a lower proportion of spontaneous nodulation. Seeds of AS-13 were kindly provided by Sharon Long (University of Stanford). Twenty plants were used for each treatment and the plant roots were protected from light by aluminium foil. The Nod factors were added in two steps, first, in the melted Fahraeus agar medium immediately before it was poured

into test tubes, and secondly 1 week after sowing the sterile germinated seeds on the agar slope, by adding 1 ml of Nod factor liquid solution on the root system with appropriate concentrations.

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