Rhizobium nodulation protein NodA is a host specific determinant of the transfer of fatty acids in Nod factor biosynthesis

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Rhizobium nodulation protein NodA is a host-specific determinant of the transfer of fatty acids in Nod factor biosynthesis

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Abstract In the biosynthesis of lipochitin oligosaccharides (LCOs) the *Rhizobium* nodulation protein NodA plays an essential role in the transfer of an acyl chain to the chitin oligosaccharide acceptor molecule. The presence of *nodA* in the *nodABCIJ* operon makes genetic studies difficult to interpret. In order to be able to investigate the biological and biochemical functions of NodA, we have constructed a test system in which the *nodA*, *nodB* and *nodC* genes are separately present on different plasmids. Efficient nodulation was only obtained if *nodC* was present on a low-copy-number vector. Our results confirm the notion that *nodA* of *Rhizobium leguminosarum* biovar *viciae* is essential for nodulation on *Vicia*. Surprisingly, replacement of *R. l.* bv. *viciae nodA* by that of *Bradyrhizobium* sp. ANU289 results in a nodulation-minus phenotype on *Vicia*. Further analysis revealed that the *Bradyrhizobium* sp. ANU289 NodA is active in the biosynthesis of LCOs, but is unable to direct the transfer of the *R. l.* bv. *viciae nodFE*-dependent multi-unsaturated fatty acid to the chitin oligosaccharide acceptor. These results lead to the conclusion that the original notion that *nodA* is a common *nod* gene should be revised.

Key words *Rhizobium* · Nodulation · Nod factors · Acyl transfer · *nodA*

Introduction

The interaction of bacteria of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*, collectively called rhizobia, with the roots of a leguminous plant or the non-legume tree *Parasponia* leads to the formation of a new organ, the nodule, in which atmospheric nitrogen is fixed. A certain rhizobial species can nodulate only a restricted number of host plants and this host specificity is determined by signal molecules that are exchanged between rhizobia and their hosts. Plants secrete flavonoid molecules that can be recognized by the regulatory NodD protein of the rhizobia. This recognition activates the transcription of *nod* genes, which are involved in the biosynthesis of nodulation signals, the Nod factors. These Nod factors consist of a backbone of three to six β-1,4-linked *N*-acetylglucosamine residues with a fatty acid attached to the amine of the non-reducing sugar, and hence are designated lipochitin oligosaccharides (LCOs) (Schultze et al. 1994; Dénarié and Cullimore 1993, López-Lara et al. 1995).

The *nodABC* genes, which are present in all rhizobia, are involved in biosynthesis of the basic structure of the nodulation signals. Studies on the *nodABC* genes have led to the following model of how LCOs are synthesized. Using UDP-*N*-acetylglucosamine as a precursor, it has been shown that NodC is involved in the biosynthesis of chitin oligosaccharides (Geremia et al. 1994, Kamst et al. 1995). Subsequently, the non-reducing terminal saccharide of the chitin oligosaccharide is de-*N*-acetylated by NodB, resulting in a free amine (John et al. 1993). NodA is essential for transfer of a fatty acid to the amine, resulting in a core LCO (Atkinson et al. 1994; Röhrig et al. 1994).

Other *nod* genes are responsible for modifications present in the LCOs produced by various rhizobia. For example, *Rhizobium leguminosarum* bv. *viciae* produces LCOs with a multi-unsaturated fatty acid that contains three *trans*-conjugated double bonds (Spaink et al. 1991). NodF, which is homologous to acyl carrier protein (Shearman et al. 1986), and NodE, which is homologous to β-ketoacyl synthases (Bibb et al. 1989), are responsible for the biosynthesis of this special fatty acid

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(Spaink et al. 1991; Geiger et al. 1994; Ritsema et al. 1994; Bloemberg et al. 1995).

Experiments in which DNA fragments encoding *nod* genes were exchanged between rhizobia have been the basis for a division of these *nod* genes into common and host-specific *nod* genes. The *nodABC* genes were designated as common *nod* genes, because introduction of the *nodABC* genes from heterologous rhizobia into *nodA*, *nodB* and *nodC* mutants restored nodulation and did not affect the host range (Kondorosi et al. 1984; Fisher et al. 1985; Marvel et al. 1985).

In the work presented in this paper we have analyzed the role of NodA in more detail. We developed a genetic test system in order to test the *in vivo* function of *nodA*. Surprisingly, replacement of *R. l.* bv. *viciae nodA* by that of *Bradyrhizobium* sp. ANU289 led to a nodulation-minus phenotype on *Vicia*. We show here that *Bradyrhizobium* sp. ANU289 *nodA* is active in the biosynthesis of LCOs, but it is not able to direct the transfer of the *R. l.* bv. *viciae nodFE*-dependent multi-unsaturated fatty acid to the chitin oligosaccharide backbone. Our results show that the original notion that NodA is a common nodulation protein should be revised.

Materials and methods

Bacterial strains and growth conditions

The rifampicin-resistant strain of *Rhizobium leguminosarum* bv. *viciae* RBL5560, which confers wild-type nodulation on *Vicia*, harbours the symbiosis plasmid pRL1JI that contains a Tn*5* insertion in the gene for medium bacteriocin production (Zaat et al. 1987). The *nodA*::Tn*5* mutant RBL5562 is equivalent to strain RBL5560, differing only with respect to the position of the Tn*5* insertion (Wijffelman et al. 1985). Rhizobial strains were grown at 28° C on YMB medium (Hooykaas et al. 1977) solidified with 1.8% agar. For the production of LCOs bacteria were grown in B^- medium (Van Brussel et al. 1977). Plasmids were mobilized from *Escherichia coli* into *Rhizobium* as described previously, using plasmid pRK2013 as a helper plasmid (Ditta et al. 1980). Rifampicin (20 mg/l) was used to select against *E. coli*. Rhizobial strains harboring plasmids were grown on media containing the appropriate antibiotics. Concentrations used were: spectinomycin, 100 mg/l (IncW vectors); tetracycline, 2 mg/l (IncP vectors); chloramphenicol, 10 mg/l; streptomycin, 500 mg/l (IncQ vectors). When used in *E. coli* the same concentrations were employed except that tetracycline was applied at a concentration of 20 mg/l. Kanamycin (50 mg/l) was used to select for pET9-derived vectors in *E. coli* (Studier et al. 1990).

Construction of plasmids

Recombinant DNA techniques were performed as described by Sambrook et al. (1989) and Innes et al. (1990). Restriction, polymerase and ligation enzymes were obtained from Pharmacia LKB (Uppsala, Sweden). The construction of plasmids is outlined in Fig. 1. Plasmids pMP247 and pMP292 have been described by Spaink et al. (1995). The primers used for the polymerase chain reaction (PCR) were, oMP138 (5′-AAAACCATGGCTTCTGAAGTGCGATGGAAAATATG-3′) and oMP139 (5′-CCCGGATCCTCATAGTTCCGACCCGTTTCG-3′) (Isogen Bioscience, Maarssen, The Netherlands). The *nodA* gene of *Bradyrhizobium* sp. ANU289 was isolated by PCR amplification from total DNA of strain *Bradyrhizobium* sp. (*Parasponia*) strain ANU289 (Scott 1986) with primers oMP140 (5′-GGAATACATATGAATATTGCCGTGTCGCGG-3′) and oMP141 (5′-AAAGGATCCTCACAACTCGGGCCCGTTCCG-3′).

For expression in *E. coli*, the genes were cloned into pET vectors. The *nodA* gene of *R. l.* bv. *viciae* was cloned in pET9d, resulting in pMP4150 and the *nodA* gene of *Bradyrhizobium* sp. ANU289 was cloned into pET9a, resulting in pMP4112. For use in *Rhizobium*, the *nodA* genes were cloned from the pET vector into an IncP plasmid. This resulted in the plasmids pMP4156 (*nodA* of *R. l.* bv. *viciae*) and pMP4116 (*nodA* of *Bradyrhizobium* sp. ANU289). The genes are under the control of the promoter of the *nodABCIJ* operon and the Shine-Dalgarno sequence is derived from the pET vector (Fig. 1C).

Protein production

For the production of NodA protein, derivatives of *E. coli* strain JM101 harboring the constructs pMP4150 (*nodA R. l.* bv. *viciae*) and pMP4112 (*nodA* ANU289) were grown in LC (medium 10 g/l Bactotrypton, 5 g/l Yeast extract, 8 g/l NaCl, 2.45 g/l $MgSO_4.7H_2O$; pH 6.6) to an OD_{620nm} of 0.2. Protein production was induced by the addition of phage mGP1-2 (Tabor and Richardson 1987) at a titer of 10^9 per ml and cells were allowed to produce protein for 5 h. Cells were lysed and proteins were denaturated by boiling for 10 min in loading mix for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Proteins were separated on 15% SDS-PAGE gel and protein bands were visualized using Coomassie Brilliant Blue.

Radioactive labelling of LCOs and TLC analysis

For LCO production, rhizobia were induced at an OD_{620} of 0.03 by incubation in the presence of 4 μM naringenin (Sigma, St. Louis, Mo., USA) for 16 h. To obtain labeled compounds, 0.2 μCi/ml D-[1-^{14}C]-glucosamine with a specific activity of 53 Ci/mol (Amersham International, Amersham, UK) was added to the culture at the moment of naringenin addition. After 16 h cultures were extracted with 0.5 volume of water-saturated *n*-butanol, dried under vacuum and redissolved in 0.01 volume of 50% acetonitrile in water. Thin-layer chromatography (TLC) analysis was performed as described by Spaink et al. (1991) using ODS plates (Sigma) with 50% acetonitrile as the mobile phase. Radioactivity was detected using a phosphorimaging system and ImageQuant software (Molecular Dynamics, Sunnyville, Calif., USA).

Chitinase treatment of the labeled compounds from 0.5 ml culture of rhizobia was performed with 10^{-4} units of chitinase from *Streptomyces griseus* (Sigma) for 16 h at room temperature.

HPLC analysis of LCOs

Volumes of 2 l for RBL5560 and RBL5562.pMP2777 (*nodB*, *nodC*).pMP4116 (*nodA* ANU289) and of 4 l for RBL5562.pMP2777 (*nodB*, *nodC*).pMP4156 (*nodA R. l.* bv. *viciae*) were used for high-performance liquid chromatography (HPLC) analysis of LCOs. Induced cultures were extracted with 0.3 volume of *n*-butanol, dried under vacuum and redissolved overnight in 25 ml of 60% acetonitrile in water. Samples were purified and concentrated using a 1 ml ODS column (J. T. Baker N.V., Deventer, The Netherlands), as follows. Samples were diluted to 30% acetonitrile, applied to the column and LCOs were eluted using 3 ml of 60% acetonitrile. Following dilution to 30% acetonitrile, 200 μl was applied to a reversed phase PEP-S (5 μm, 4 × 250 mm, Pharmacia LKB, Uppsala, Sweden) HPLC column. Elution took place with step

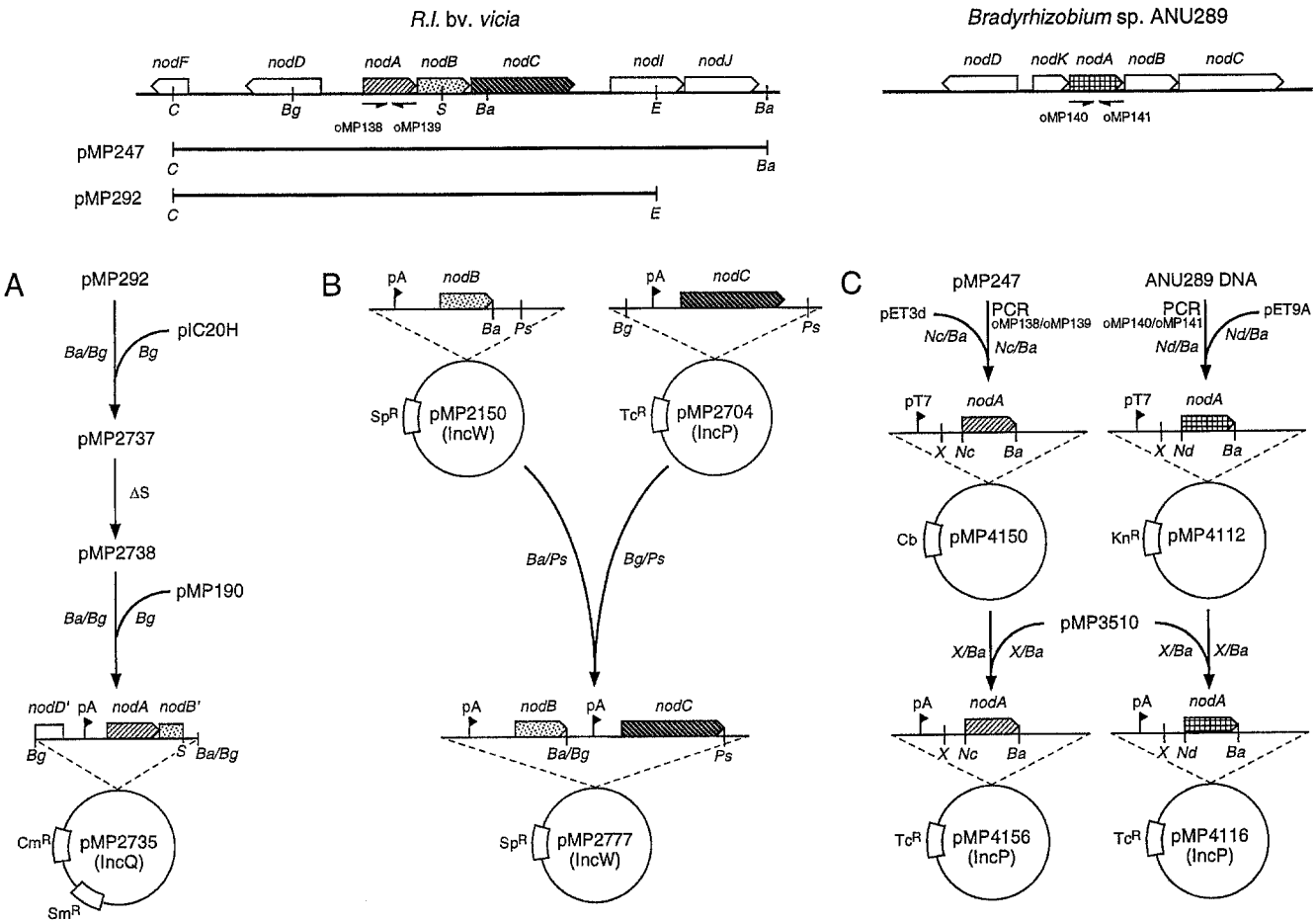

Fig. 1A–C Construction of plasmids. Parts of the *nod* regions of *R. l.* bv. *viciae* and *Bradyrhizobium* sp. ANU289 are shown. Indicated are the regions present on plasmids pMP247 and pMP292 (Spaink et al. 1995) and the positions of attachment of oligonucleotide primers oMP138, oMP139, oMP140 and oMP141. **A** Construction of pMP2735. The polylinker of pIC20H contains a *Sma*I site in such a position that deletion of a *Sma*I fragment of pMP2737 resulted in plasmid pMP2738 in which part of the *nodB* gene has been removed. To obtain a plasmid for use in *Rhizobium* the *Bam*HI–*Bgl*II fragment of pMP2738 containing *nodA* was cloned into the IncQ plasmid pMP190. **B** Construction of pMP2777. The plasmids pMP2150 and pMP2704 have been described by Spaink et al. (1994). **C** Construction of pMP4156 and pMP4116. These plasmids only differ in the nucleotide sequence of the *nodA* genes. Plasmid pMP3510 has been described by Spaink et al. (1995). Abbreviations: *Ba*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *Nc*, *Nco*I; *Nd*, *Nde*I; *Ps*, *Pst*I; *S*, *Sma*I; *X*, *Xba*I

gradients of 40% acetonitrile (30 min) and 45% acetonitrile (20 min), using a flow rate of 0.7 ml/min.

For the detection of sugars, a pulsed amperometric detection (PAD) system was used with PeakNet (Dionex Corporation, Sunnywale, Calif. USA) software. In this system sugars are detected with a gold electrode which requires charged sugar residues. A pH above the pK_a of *N*-acetyl glucosamine is needed to bring the sugars into a charged state. This pH was established by post-column addition of NaOH to a final concentration of 100 mM using an RP-1 pump (Dionex).

Nodulation experiments

Germinated seeds of *Vicia sativa* ssp. *nigra*, *Vicia hirsuta* and *Trifolium subterraneum* were inoculated with rhizobia and grown on Jensen medium, solidified with 0.8% agar if required (Van Brussel et al. 1982). In aqueous culture, roots could be shielded from the light, as described by Van Brussel et al. (1992).

Results

Development of a genetic system to test *nodA* function in vivo

Strain RBL5562 harbours the symbiotic plasmid pRL1JI of *R. l.* bv. *viciae*, which contains a Tn*5* insertion in *nodA* (Wijffelman et al. 1985). This Tn*5* insertion has a polar effect on the transcription of the other genes of the operon, the *nodBCIJ* genes. The *nodB* and *nodC* genes were provided on the plasmids pMP2150, of the IncW compatibility group, and pMP2704, of the IncP compatibility group, respectively. Both genes were cloned downstream of the promoter of the *nodABCIJ* operon (Spaink et al. 1994). The IncQ plasmid pMP2735 contains part of *nodD*, *nodA* including its promoter region, and part of *nodB* (Fig. 1A). The RBL5562-derived strain harboring the combination of plasmids pMP2735 (*nodA*), pMP2150 (*nodB*) and pMP2704 (*nodC*) was tested for nodulation on *V. sativa*, on agar with roots in the light or in liquid

medium with roots exposed to the light or shielded from the light. The results showed that under all these conditions the RBL5562 derivative can only induce a largely reduced and delayed nodulation reaction (data not shown).

Previous experiments had shown that a high level of *nodC* expression has a strong negative effect on nodulation (our unpublished data). Therefore, the low-copy-number IncW plasmid pMP2777 was constructed. This plasmid contains both *nodB* and *nodC* genes, each cloned downstream of the promoter region of the *nodABCIJ* operon (Fig. 1B). In order to standardize the expression of *nodA*, this gene was recloned in such a way that it was not surrounded by sequences of other *nod* genes. To this end, the *nodA* gene was obtained using the PCR technique and cloned into pET9d, resulting in plasmid pMP4150 (Fig. 1C). With this plasmid the expression of NodA protein in *E. coli* was tested. SDS-PAGE analysis of total proteins showed a high level of production of *R. l.* bv. *viciae* NodA protein (Fig. 2). Recloning of *nodA* into an IncP vector under the control of the promoter region of the *nodABCIJ* operon led to plasmid pMP4156, which can be used in *Rhizobium*.

Strain RBL5562 harbouring pMP2777 (*nodB*, *nodC*) and pMP4156 (*nodA*) was tested for nodulation. The results showed that nodulation was faster and more efficient than with strain RBL5562.pMP2735 (*nodA*).pMP2150 (*nodB*).pMP2704 (*nodC*) (data not shown). However, the nodulation was still not as efficient as observed with the wild-type nodulating strain RBL5560. A possible explanation for the low efficiency could be the absence of transcription of the *nodIJ* genes. It was shown by Canter Cremers et al. (1988) that nodulation on *V. hirsuta* is less affected by the absence of *nodIJ* than nodulation on *V. sativa*. Therefore we tested *V. hirsuta* as a host in an experiment with strain RBL5562.pMP2777 (*nodB*, *nodC*).pMP4156 (*nodA*), with plants grown on agar medium. Much more efficient formation of nodules was observed with *V. hirsuta* than with *V. sativa*; almost all plants nodulated and the number of nodules per plant was higher. Compared to wild-type rhizobia, nodulation is still delayed, however, and the number of nodules per plant is lower (Table 1). When plants were grown on liquid medium with roots exposed to the light in the presence of AVG, an inhibitor of ethylene biosynthesis, the results were comparable to those with plants on grown on agar (data not shown).

We have also tested the wild-type nodulating strain RBL5560 harbouring pMP4156 (*nodA*) for nodulation on *V. hirsuta*. The nodulation phenotype was indistinguishable from that of strain RBL5560 (Table 1). The results therefore show that NodA overproduction has no effect on nodulation.

Strain RBL5562.pMP2777 (*nodB*, *nodC*), which does not contain *nodA*, is not nodulating under any of the conditions described above (Table 1 for *V. hirsuta* and data not shown for *V. sativa*). This shows that our test system is indeed completely dependent on the presence of *nodA* for nodulation.

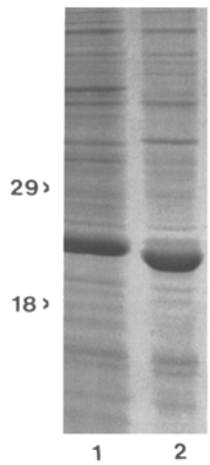

Fig. 2 SDS-PAGE of total cell extracts from *E. coli* strain JM101 harbouring either pMP4150 (*nodA R. l.* bv. *viciae*) or pMP4112 (*nodA* ANU289). The extract was fractionated on a 15% SDS-PAGE gel and proteins were visualized with Coomassie Brilliant Blue. Positions of marker proteins are indicated. Lane 1, JM101.pMP4150 (*nodA R. l.* bv. *viciae*); lane 2, JM101.pMP4112 (*nodA* ANU289)

Complementation by *nodA* of *Bradyrhizobium* sp. (*Parasponia*) ANU289

We have analyzed whether the test system described above is also suited for the functional analysis of *nodA* genes of rhizobia other than *R. l.* bv. *viciae*. Using PCR technology, we constructed the IncP plasmid pMP4116, which contains the *nodA* gene of the distantly related *Bradyrhizobium* sp. ANU289. This construct only differs from plasmid pMP4156 in the nucleotide sequence of the *nodA* gene. As an intermediate cloning step the *nodA* gene of *Bradyrhizobium* sp. ANU289 was cloned into pET9a, leading to plasmid pMP4112 (Fig. 1C). Induction of the T7 promoter of pMP4112 in *E. coli* leads to the same level of NodA protein production as obtained with pMP4150 (*nodA R. l.* bv. *viciae*) (Fig. 2).

The results of nodulation experiments showed that the strain RBL5562.pMP2777 (*nodB*, *nodC*).pMP4116 (*nodA* ANU289), does not nodulate *V. sativa* and *V. hirsuta* under any of the conditions described above (Table 1 for *V. hirsuta* and data not shown for *V. sativa*). To test whether *nodA* of *Bradyrhizobium* sp. ANU289 is harmful for nodulation of *Vicia* by *R. l.* bv. *viciae*, we constructed strain RBL5560.pMP4116 (*nodA* ANU289). This strain shows a nodulation behavior that is indistinguishable from that of strains RBL5560 and RBL5560.pMP4156 (*nodA R. l.* bv. *viciae*), which demonstrates that plasmid pMP4116 (*nodA* ANU289) does not decrease nodulation ability (Table 1).

Analysis of functionality of *nodA* from *Bradyrhizobium* sp. ANU289 in LCO biosynthesis

The activity of *nodA* from *Bradyrhizobium* sp. ANU289 in LCO biosynthesis was analyzed by inducing

Table 1 Nodulation characteristics of different *Rhizobium leguminosarum* bv. *viciae* strains on *Vicia hirsuta*

Strain	*nod* genes present	Days	Nodules/nodulated plant ± S.D.	Percentage nodulated plants[c]
RBL5560	All[a]	11	10 ± 4	80
		18	16 ± 7	100
		26	26 ± 6	100
RBL5562.	*nodDFELMNTO*	11	0	0
pMP2777	*nodBC*	18	0	0
		26	0	0
RBL5562.	*nodDFELMNTO*	11	2 ± 1	40
pMP2777.	*nodBC*	18	2 ± 2	60
pMP4156	*nodA* (*Rv*)[b]	26	3 ± 2	90
RBL5562	*nodDFELMNTO*	11	0	0
pMP2777.	*nodBC*	18	0	0
pMP4116	*nodA* (*B*)[b]	26	0	0
RBL5560.	All[a]	11	6 ± 5	80
pMP4156	*nodA* (*Rv*)[b]	18	15 ± 6	100
		26	24 ± 5	100
RBL5560.	All[a]	11	14 ± 6	80
pMP4116	*nodA* (*B*)[b]	18	20 ± 7	100
		26	24 ± 6	100

[a] All, nodABCIJDFELMNTO
[b] *Rv*, *R. l.* bv. *viciae*
B, *Bradyrhizobium* sp. ANU289
[c] For each strain 10 plants were tested

the *nod* genes of strain RBL5562.pMP2777 (*nodB*, *nodC*).pMP4116 (*nodA* ANU289) with naringenin in the presence of D-[1-^{14}C]glucosamine, followed by analysis of the LCOs using TLC (Fig. 3). Based on the appearance of radiolabelled spots with the same R_f values as those obtained from the strain RBL5562.pMP2777 (*nodB*, *nodC*).pMP4156 (*nodA R. l.* bv. *viciae*) we conclude that *nodA* of *Bradyrhizobium* sp. ANU289 is active in LCO production. Also the chitinase breakdown products of LCOs produced by these strains showed a similar pattern in TLC analysis (data not shown).

LCOs were analyzed in more detail using HPLC. After separation of culture extracts on a reversed phase column, LCOs were detected in three ways: (i) absorbance at 206 nm, (ii) absorbance at 303 nm to detect C18:4 multi-unsaturated fatty acids and (iii) pulsed amperometric detection (PAD). The PAD system is very useful for the detection of LCOs that do not have a specific UV absorbance, because they can be distinguished from non-sugar contaminants that also absorb at 206 nm. We found that the PAD detector has a high sensitivity for LCOs, allowing the detection of amounts as low as 1 μg (data not shown). Comparison of the peaks detected with PAD and absorbance at 206 nm and at 303 nm clearly shows (Fig 4C) the presence of both host-specific (303 nm-absorbing) LCOs and non-specific (206 nm-absorbing) LCOs in extracts from strain RBL5562.pMP2777 (*nodB*, *nodC*).pMP4156 (*nodA R. l.* bv. *viciae*). The amounts of LCOs are low compared to those produced by strain RBL5560.

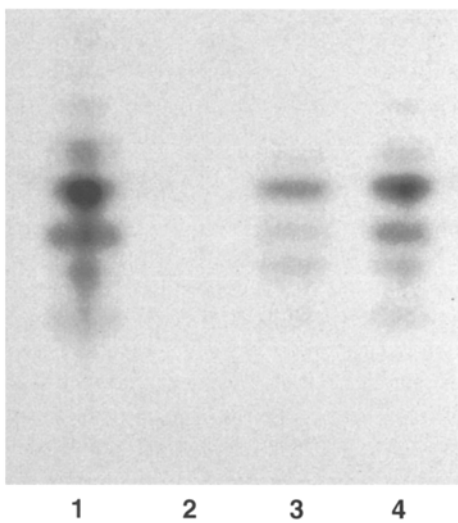

Fig. 3 TLC analysis of *n*-butanol extracts from naringenin-induced and D-[1-^{14}C]glucosamine-labeled rhizobial cultures. Strains analyzed: lane 1, RBL5560; lane 2, RBL5562.pMP2777 (*nodB*, *nodC*); lane 3 RBL5562.pMP2777.pMP4156 (*nodA R. l.* bv. *viciae*); lane 4 RBL5562.pMP2777.pMP4116 (*nodA* ANU289)

Hardly any LCOs with a backbone of four *N*-acetyl glucosamine residues were detected. In extracts from strain RBL5562.pMP2777 (*nodB*, *nodC*).pMP4116 (*nodA* ANU289) non-specific LCOs could be detected, but specific LCOs that absorb at 303 nm were not detected (Fig. 4D).

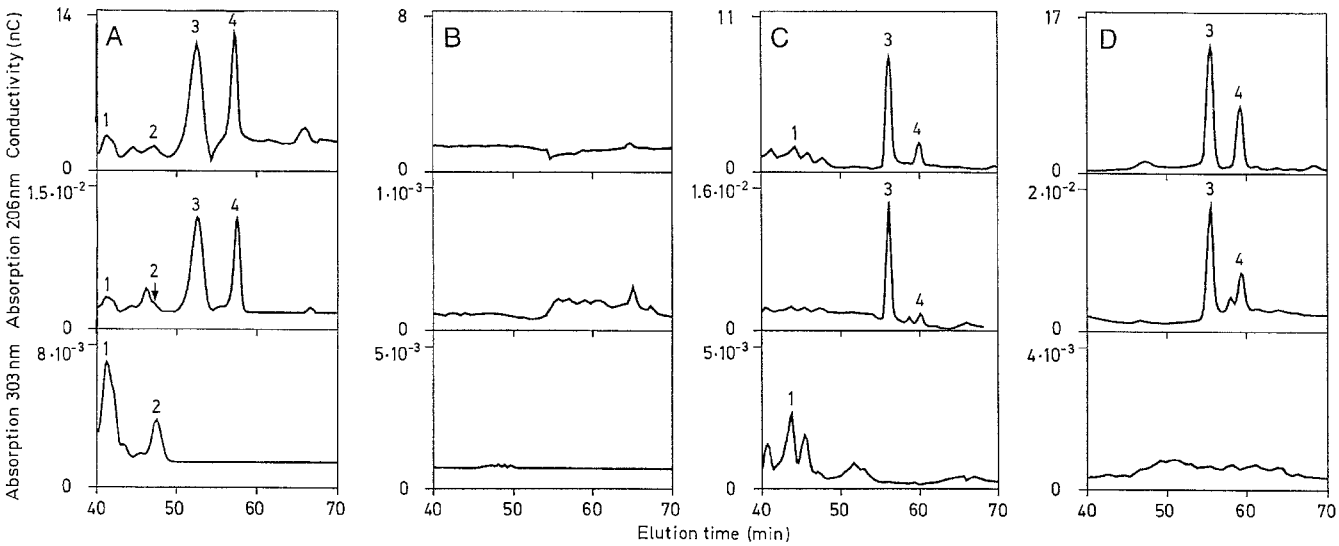

Fig. 4A–D HPLC analysis of *n*-butanol extracts from naringenin-induced rhizobial cultures. For each run PAD and absorption profiles at 206 nm and 303 nm are recorded; different scales are used in different parts of the Figure as indicated. Strains analyzed: **A** RBL5560. **B** RBL5562.pMP2777 (*nodB, nodC*). **C** RBL5562.pMP2777.pMP4156 (*nodA R. l.* bv. *viciae*). **D** RBL5562.pMP2777.pMP4116 (*nodA* ANU289). Peaks are numbered as follows: 1, NodRlv-V(C18:4,Ac); 2, NodRlv-IV(C18:4,Ac); 3, NodRlv-V(C18:1,Ac); 4, NodRlv-IV(C18:1,Ac). The ratios of different LCOs produced by strain RBL5560 can deviate from data published previously due to changes in growth conditions and isolation procedures

Discussion

A system has been developed to test *nodA* function in vivo. With this new genetic system we confirmed the notion that *nodA* is essential for nodulation of *Vicia* by *R. l.* bv. *viciae*. Although *nodA* was expected to be required for nodulation based on data from experiments in which was shown that *nodA* is essential for LCO biosynthesis (Atkinson et al. 1994; Röhrig et al. 1994; Spaink et al. 1994), previously, this could not be shown directly due to the lack of a suitable biological test system.

Replacement of the *nodA* gene of *R. l.* bv. *viciae* by that of *Bradyrhizobium* sp. ANU289 results in a nodulation-minus phenotype on *Vicia* (Table 1). This result seems to be in conflict with observations that *nodA* mutants of *R. meliloti* and *R. trifolii* could be complemented by DNA fragments of the symbiotic region of heterologous rhizobia (Kondorosi et al 1984; Fisher et al. 1985; Marvel et al. 1985). These complementing DNA fragments contained several genes besides *nodA*. To test whether *nodA* of *Bradyrhizobium* sp. ANU289 is functional in this system, in which all other genes are derived from *R. l.* bv. *viciae*, we have analyzed the production of LCOs in our test system. The results show that *nodA* of *Bradyrhizobium* sp. ANU289 is active in the biosynthesis of LCOs (Fig. 3). A more detailed analysis of the LCOs produced by this strain revealed that none of the LCO peaks detected absorbs at 303 nm (Fig. 4D), reflecting the absence of the multi-unsaturated fatty acyl moiety which is normally present in the LCOs of *R. l.* bv. *viciae* (Fig. 4A, Spaink et al. 1991).

The strain harbouring *nodA* of *Bradyrhizobium* sp. ANU289 produces the same range of LCOs as a *nodE* mutant of *R. l.* bv. *viciae*; it therefore appears to be a phenocopy of a *nodE* mutant. However, unlike the *nodE* mutant, this strain cannot nodulate *V. hirsuta*. Perhaps the perturbation of the normal LCO profile brought about by the uncoordinated expression of the nodulation genes reduces nodulation to such an extent that further impairment of LCO structure is not tolerated by *V. hirsuta*. On *T. subterraneum*, which is exceptional in that it can be nodulated by both *R. leguminosarum* biovars *viciae* and *trifolii* (Canter Cremers et al. 1989), both the strain harbouring *nodA* of *R. l.* bv. *viciae* and the strain harbouring *nodA* of *Bradyrhizobium* sp. ANU289 showed an equal ability to induce pseudonodules.

It has been shown that biosynthesis of the multi-unsaturated C18:4 fatty acid is not dependent upon NodA (Geiger et al. 1994). Recent results show that NodA is necessary for the transfer of the acyl chain to the oligosaccharide backbone (Atkinson et al. 1994; Röhrig et al. 1994). Therefore, the most likely explanation for our results is that *nodA* of *Bradyrhizobium* sp. ANU289 is unable to transfer the specific C18:4 fatty acid and, as a result, only LCOs containing common fatty acids are produced. From the biological and biochemical data presented in this paper we conclude that the notion that *nodA* is a common *nod* gene should be revised.

It is not yet known to which donor the fatty acids are attached during transfer to the chitin oligosaccharide backbone by NodA, but acyl carrier proteins are good candidates. The synthesis of the multi-unsaturated fatty

acid is dependent on the presence of the NodF and NodE proteins, which are homologous to acyl carrier proteins and β-keto acyl synthases, respectively (Shearman et al. 1986; Bibb et al. 1989; Spaink et al. 1991; Geiger et al. 1994). NodA might use acyl-NodF as the donor for the multi-unsaturated fatty acid. NodF and NodE have not been found in rhizobia that produce LCOs with a common fatty acid, such as *Bradyrhizobium* bacteria. In these cases NodA might use the acylated housekeeping ACP as the sole donor of fatty acids. The inability of *Bradyrhizobium* sp. ANU289 NodA to transfer multi-unsaturated C18:4 fatty acids might be caused by its inability to recognize the specialized acyl carrier protein NodF. Alternatively, NodA of *R. l.* bv. *viciae* may be specialized for the transfer of multi-unsaturated fatty acids, whereas they are not recognized by NodA of *Bradyrhizobium* sp. ANU289.

We propose that the presence of the highly unsaturated fatty acid on the LCO is specified at two levels. The NodF and NodE proteins determine the biosynthesis of the host-specific fatty acid, after which the NodA protein determines which fatty acid is transferred to the chitin oligosaccharide backbone.

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