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Detection and Separation of *Rhizobium* and *Bradyrhizobium* Nod Metabolites Using Thin-Layer Chromatography

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Using radioactive acetate as a precursor, it was shown that the common *nodABC* genes of *Rhizobium* and *Bradyrhizobium* strains are involved in the production of one or more metabolites that are excreted into the growth medium. A rapid thin-layer chromatography (TLC) system has been developed to separate these so-called Nod metabolites that can then be visualized by autoradiography. Different patterns of Nod metabolites were observed in the tested strains of the cross-inoculation groups of *R. leguminosarum* bv. *viceae*, *R. l.* bv. *trifolii*, *R. meliloti*, and *B. japonicum*. Only Nod metabolites of *R. meliloti* became labeled when radioactive sulphate was present in the medium. The role of the other nodulation genes of *R. l.* bv. *viceae* in the production of the detected Nod metabolites was tested in further detail. In addition to the common *nodABC* genes, the *nodFE* and *nodL* genes are involved in the production of Nod metabolites. In contrast, the chromosomal background did not influence the number of detected Nod metabolites or their mobilities on TLC plates. Nod metabolites could also be produced and excreted in *Escherichia coli* cells in which the appropriate nodulation genes were expressed.

Additional keywords: excreted metabolites, Rhizobial nodulation proteins, signal molecules.

The host-specific interaction between leguminous plants and bacteria of the genera *Rhizobium* and *Bradyrhizobium* results in the formation of nitrogen-fixing root nodules. At least two steps of molecular signaling between plant and bacteria appear to be involved in the determination of host-specific nodulation. In the first step, flavonoids excreted by the plant induce the transcription of bacterial nodulation genes (called *nod* or *nol* genes) (reviewed in Long 1989; Kondorosi 1991). The host-specificity of this induction process involves the bacterial NodD protein that presumably directly interacts with the flavonoids (Horvath *et al.* 1987; Spaink *et al.* 1987b). In the second step, the bacterium, by means of the *nod* genes, produces one or more signals that activate plant genes (Scheres *et al.* 1990) and trigger root responses like root hair curling (van Brussel *et al.* 1986) and nodule meristem induction (Hollingsworth *et al.* 1989; Roche *et al.* 1991; H. P. Spaink *et al.*, 1991b).

In the case of *R. meliloti*, one bacterial host-specific signal, called NodRm1, has been identified as a sulphated lipo-oligosaccharide (N-Acyl N′N″N‴triacetyl-chitotetraose) (Lerouge *et al.* 1990). In *R. leguminosarum* bv. *viceae* at least two signals were partially identified that were lipo-oligosaccharides similar to NodRm1 (Spaink *et al.* 1991). The latter signals differ from NodRm1 in the absence of a sulphate group, the presence of an additional O-acetyl group on the nonreducing end sugar and a different fatty acid substituent (H. P. Spaink *et al.* 1991b). The biochemical role of some of the *nod* gene proteins in the production of these signal molecules has been predicted based on homology with other enzymes (Downie 1989; Bibb *et al.* 1989; Downie *et al.* 1991) or directly shown biochemically (Roche *et al.* 1990; Schwedock and Long 1990; Spaink *et al.* 1991a; Kondorosi 1991). However, for the common NodABC proteins, which are essential for the production of the bacterial signal (van Brussel *et al.* 1986), and for the majority of the other *nod* and *nol* proteins of *Rhizobium* and *Bradyrhizobium* no biochemical function can be proposed.

A major bottle-neck in the biochemical study of the metabolic products of the Nod proteins is the absence of rapid detection methods. In this paper, we describe a general method to detect and separate various compounds whose production requires specific nodulation proteins. This method has been used to detect previously unknown Nod metabolites produced by *Rhizobium* and *Bradyrhizobium* strains. The described methods can be a powerful tool for future purification and characterization of rhizobial signal molecules.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. *R. l.* bv. *viceae* and *R. l.* bv. *trifolii* strains were grown on modified B^- medium (Mannitol, 10 g/l; $MgSO_4 \cdot 7H_2O$, 0.55 g/l; KNO_3, 0.55 g/l; $Ca(NO_3)_2 \cdot 4H_2O$, 1.3 g/l; Fe(III)-NaEDTA, 33 mg/l; Biotin, 0.2 mg/l; Thiamine·HCl, 5 mg/l; trace elements, see below) to which after sterilization potassium phosphate buffer, pH 7.2, was added to a concentration of 1 mM. *R. meliloti* Dangeard was grown on yeast-mannitol (YM) medium

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(Hooykaas *et al.* 1977). *B. japonicum* (Kirchner) Jordan strains were grown on modified Bergersen's minimal medium (BM: K_2HPO_4, 0.23 g/l; $MgSO_4 \cdot 7H_2O$, 0.1 g/l; NH_4NO_3, 0.5 g/l; glycerol, 0.4% v/v; thiamine, 1 mg/ml; biotin, 0.2 mg/ml; trace elements, see below; pH 7.0). A 400-fold concentrated stock solution of trace elements ($MnSO_4 \cdot H_2O$, 0.609 g/l; $ZnSO_4 \cdot 7H_2O$, 0.1 g/l; H_3BO_3, 1.27 g/l; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g/l; $CuSO_4$, 0.04 g/l) was used. Chemicals (highest purity grade) were obtained from Sigma (St Louis, MO). Strains containing plasmids were grown in the presence of the suitable antibiotics (Spaink *et al.* 1987a).

Radioactive labeling of Nod metabolites. Labeling with ^{14}C acetate was done as follows: cells were grown overnight in liquid medium to an OD_{660} value of 0.2 and subsequently diluted to an OD_{660} value of 0.02. 1-^{14}C-labeled acetate (25 μCi) (specific activity 45–60 mCi/mmol, NEC-084H obtained from NEN, Boston, MA) or malonate (specific activity 50 mCi/mmol, NEC-196 obtained from NEN) was added to 1 ml of culture. Appropriate flavonoid inducers (from stock solutions of 2 mg/ml in ethanol) were added when indicated to a concentration of 2 μM. Cells were grown standardly for 18 hr in 10-ml tubes (Greiner B.V., Alphen a/d Rijn, Netherlands) at 28° C with shaking at 300 rpm. Under these conditions, all strains with the LPR5045 chromosomal background were growing at equivalent rates to the same final culture density. In some experiments with strain RBL5560, bacteria were cultured in glass flasks with shaking at 40 rpm. The results from these experiments showed that the level of production and excretion of the Nod metabolites was not influenced by rigorous shaking. Labeling with ^{35}S sulphate was done in the same way except that the cells were grown in liquid YM media in which $MgSO_4$ was omitted. One hundred microcuries of ^{35}S-labeled sodium sulphate (specific activity 602 mCi/mmol, obtained from Amersham Int. plc, Buckinghamshire, U.K.) was added per culture. In the control experiment, cells were grown in the same medium containing 0.2 μM of unlabeled $MgSO_4$ and 25 μCi of ^{14}C acetate.

Table 1. Bacteria and plasmids used in this study

Strains/ plasmids	Relevant characteristics	Source or reference
RBL5560	LPR5045 containing Sym plasmid pJB5JI	Spaink *et al.* 1987a
RBL1	Wild type *Rhizobium leguminosarum* bv. *viceae*	van Brussel *et al.* 1982
ANU843	Wild type *R. l.* bv. *trifolii*	Djordjevic *et al.* 1985
0403	Wild type *R. l.* bv. *trifolii*	Gardiol and Dazzo 1986
2011	Wild type *R. meliloti*, Sm^r	Debellé and Sharma 1986
USDA110	Wild type *Bradyrhizobium japonicum*	Nieuwkoop *et al.* 1987
USDA135	Wild type *B. japonicum*	USDA, Beltsville, MD
LPR5045	*R. l.* bv. *trifolii* RCR5, Rif^r, cured of its Sym plasmid	Hooykaas *et al.* 1981
RBL5505	LPR5045, Str^r	Priem and Wijffelman 1984
RBL5515	LPR5045, Spc^r	Priem and Wijffelman 1984
ANU845	*R. l.* bv. *trifolii* ANU843 cured of its Sym plasmid	Djordjevic *et al.* 1985
RBL1391	*R. l.* bv. *viceae* 248 Rif^r, cured of its Sym plasmid	Priem and Wijffelman 1984
GMI766	*R. meliloti* 2011 deletion of Sym plasmid (*nod-nifA*)	Faucher *et al.* 1988
8401	*R. l.* bv. *phaseoli* cured of its Sym plasmid	Lamb *et al.* 1982
RBL610	RBL5505 pRL1JI, *nodA*::Tn*5*	Wijffelman *et al.* 1985
RBL611	RBL5505 pRL1JI, *nodB*::Tn*5*	Wijffelman *et al.* 1985
RBL607	RBL5505 pRL1JI::Tn*5*, *nodC7* (a nonpolar deletion in *nodC*)	Wijffelman *et al.* 1985
RBL5729	RBL5505 pRL1JI, *nodI*::Tn*5*	Schlaman *et al.* 1990
RBL5734	RBL5505 pRL1JI, *nodJ*::Tn*5*	Schlaman *et al.* 1990
RBL618	RBL5505 pRL1JI, *nodF*::Tn*5*	Wijffelman *et al.* 1985
RBL601	RBL5505 pRL1JI, *nodE*::Tn*5*	Wijffelman *et al.* 1985
RBL5793	RBL5515 pRL1JI, *nodL*::Tn*5PhoA*	Canter Cremers *et al.* 1989
RBL5794	RBL5515 pRL1JI, *nodM*::Tn*5PhoA*	Canter Cremers *et al.* 1989
RBL5795	RBL5515 pRL1JI, *nodT*::Tn*5PhoA*	Canter Cremers *et al.* 1989
RBL5797	RBL5560 *nodO*::Tn*5*	de Maagd *et al.* 1989
ANU252	ANU843 *nodA*::Tn*5*	Djordjevic *et al.* 1985
GMI5382	2011 *nodA*::Tn*5*	Faucher *et al.* 1988
AN122	USDA 110 *nodC*::Tn*5*	Nieuwkoop *et al.* 1987
BL21 (DE3)	*Escherichia coli* containing T7 polymerase gene under control of *lac*-UV5 promoter	Studier *et al.* 1990
Plasmids		
pJB5JI	*R. l.* bv. *viceae* pRL1JI (mep::Tn*5*)	Johnston *et al.* 1978
pRtRF101	Cloned *nod* region of *R. l.* bv. *trifolii* in IncP1	Fisher *et al.* 1985
pMP280	Cloned *nodD* gene of *R. l.* bv. *viceae* in IncP1	Spaink *et al.* 1987b
pMP604	Cloned hybrid gene *nod604* (FITA) in IncP1	Spaink *et al.* 1989b
pMP1715	*nodFE* genes of *R. l.* bv. *viceae* under control of *E. coli lac* promoter, Cb^r	Geiger *et al.* 1991
pMP248	*nodABC* genes of *R. l.* bv. *viceae* under control of *E. coli lac* promoter, Cm^r	This work
pMP1255	IncQ plasmid containing *R. l.* bv. *viceae nodFE* genes	Geiger *et al.* 1991
pMP3401	*nodL* gene of *R. l.* bv. *viceae* under expression of T7 promoter, Km^r	This work

Nod metabolites extraction. Partition coefficients (K, as defined by Conway and Ito [1984]) in various solvent systems were calculated after performing TLC analysis on equal amounts of the separated phases and subsequent comparison of the radioactivity of the spots of interest in a scintillation counter. Standard n-butanol extraction was done as follows: cultures were transferred to Eppendorf tubes and centrifuged for 15 min at 18,000 rpm. The supernatant fluid was transferred back to the original tube in which the culture had been grown. Water-saturated n-butanol (0.5 ml) was added, and the tubes were shaken vigorously for 1 hr. The tubes were centrifuged for 1 min at 3,000 rpm. The n-butanol phase was removed and evaporated in Eppendorf tubes in a speed-vac evaporator. The dry matter was taken up in 50 μl of water-saturated n-butanol. Unless indicated otherwise, 1 μl of each sample was applied to a TLC plate. Samples were stored at +4° C.

TLC analysis. Silica gel 60 TLC plates were obtained from EM Merck (Darmstadt, Germany). Reverse phase C18-coated silica (ODS: 100% octadecyl silanization) plates were obtained from Sigma. ODS plates from E. M. Merck gave slightly less separation of the Nod metabolites. TLC plates were exposed to X-ray film Kodak X-omat AR (obtained from Sigma) for a 48-hr period unless indicated otherwise. The Nod metabolites produced by strain RBL5560 were extracted from the reverse phase plate layer as follows: the silica layer of the thoroughly dried ODS TLC plates containing the labeled compounds were scraped from the glass plates. Nod metabolites were extracted from ODS silica by rigorous shaking with 1 ml of solvent V for 2 hr and subsequent centrifugation. Extracted material was dried in a speed-vac concentrator and taken up in 50 μl of n-butanol saturated with water. Analysis of the resulting samples with silica TLC using solvent II showed the presence of radiolabeled contaminants that were comigrating in the ODS silica TLC system. These contaminants, which were equally well produced in the noninduced situation, could be pre-extracted by washing the collected ODS silica for 30 min, first with 1 ml of n-butanol and then with 1 ml of ethanol (100%) by rigorous shaking and subsequent centrifugation. Subsequently, the Nod metabolites that were quantitatively retained on the ODS silica were extracted using solvent V.

Used solvent systems were: I, acetonitrile/water/acetic acid = 47.5:47.5:5; II, n-butanol/acetic acid/water = 6:2:2; III, chloroform/methanol = 1:1; ethylacetate/ethanol/15%(NH_4)acetate (pH = 9.6 upper layer) = 9:4:8; IV, toluene/chloroform/aceton = 40:25:35; V, acetonitrile/water = 1:1. Solvents I and V were always prepared fresh, because slight variations in the concentration of acetonitrile

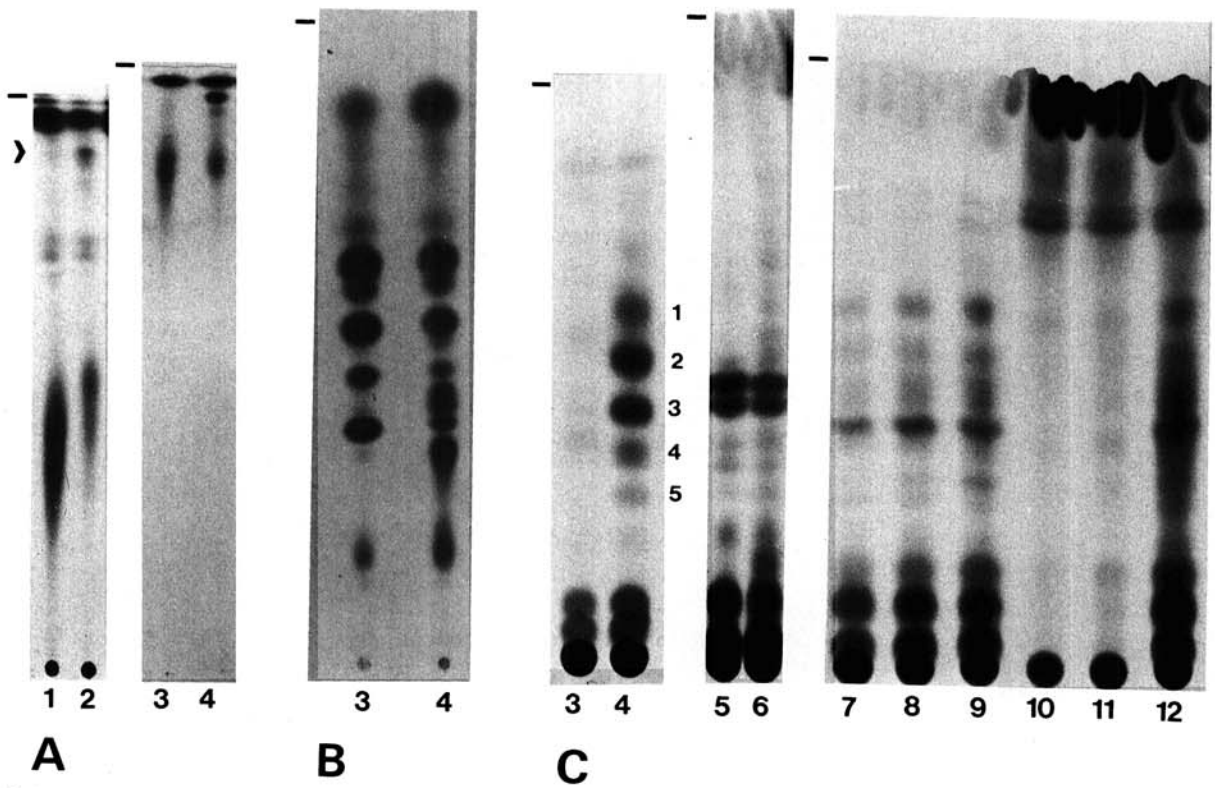

Fig. 1. Thin-layer chromatography (TLC) analysis of radiolabeled compounds produced by *Rhizobium leguminosarum* bv. *viceae* strain RBL5560. **A,** silica TLC using solvent system I (3-wk exposure); **B,** silica TLC using solvent system II; **C,** ODS (octadecyl silanization) silica using solvent system V. Cells were grown on medium containing ^{14}C acetate unless indicated otherwise. The solvent front is indicated with a dash. Samples are: 1, growth medium of uninduced cells; 2, growth medium of naringenin-induced cells; 3, n-butanol extract of growth medium of uninduced cells; 4, n-butanol extract of growth medium of naringenin-induced cells; 5, n-butanol extract of uninduced cell pellets; 6, n-butanol extract of naringenin-induced cell pellets; 7, 8, and 9, n-butanol extract of growth medium of cells 2, 4, and 8 hr after induction with naringenin, respectively; 10, 11, and 12, n-butanol extract of growth medium of cells grown in the presence of ^{14}C-labeled malonate 2, 4, and 8 hr after induction with naringenin, respectively.

results in large differences in Rf values of the Nod metabolites. Using standard conditions (Stahl 1969), the variation in Rf values of the Nod metabolites was within 2.8%. Solvents (highest grade) were obtained from J. T. Baker B.V. (Deventer, The Netherlands).

RESULTS

Detection, isolation, and separation of extracellular Nod metabolites. The growth medium of *R. l.* bv. *viceae* strain RBL5560 was investigated for the presence of flavonoid-inducible compounds by radiolabeling the cells with ^{14}C acetate with the subsequent analysis on silica TLC plates. One flavonoid-inducible spot was detected on silica TLC plates using solvent system I as the mobile phase (Fig. 1A). For the compound(s) in this spot, partition coefficients with the organic solvents chloroform, methylene chloride, butylacetate, ethylacetate, and n-butanol were estimated using TLC analysis. n-Butanol was the only solvent with a high partition coefficient (Ku/l, larger than 30) and therefore could be used to efficiently extract the flavonoid-inducible compound(s) (Fig. 1A). The n-butanol extracts were also tested on silica TLC with other solvent systems. The use of solvent system II showed that more than three flavonoid-inducible compounds were present in the growth medium (Fig. 1B) that were comigrating in solvent system I as shown by two-dimensional chromatography (data not shown). The other solvent systems tested (III, IV, and V) in one-dimensional and two-dimensional runs (using solvent system I in the first dimension) on Silica TLC did not indicate other flavonoid-inducible compounds (data not shown). The n-butanol extracts were also tested in reverse phase (ODS) TLC using solvent systems I–V. The best separation was obtained with solvent system V, which showed that at least five flavonoid-inducible compounds are present in the growth medium of RBL5560 (Fig. 1C). The radioactive compounds contained in the spots numbered 1–5 could be isolated from TLC plates by extraction from the scraped silica layer (see Materials and Methods). Testing of the isolated spots showed that they correspond with the flavonoid-inducible spots previously detected on silica TLC in Figure 1A and B (data not shown).

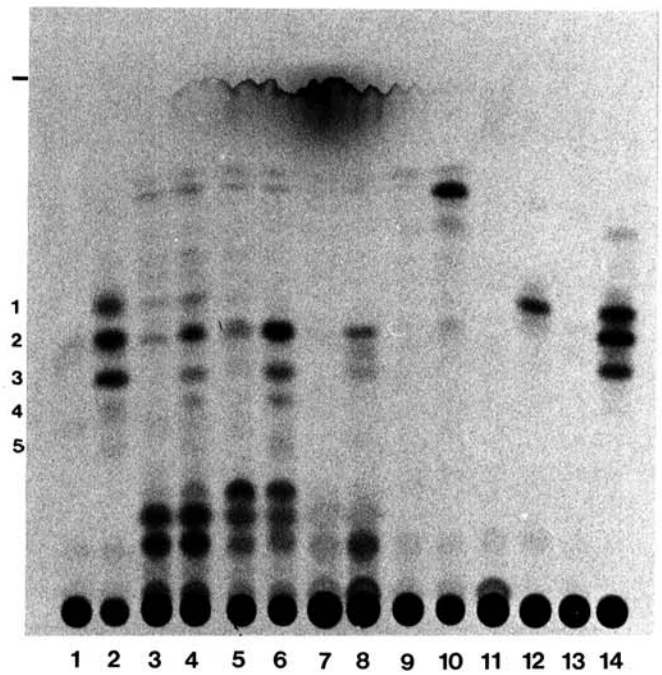

Fig. 2. Thin-layer chromatography (TLC) analysis of radiolabeled compounds produced by various wild type strains. The culture medium of cells grown in the presence of ^{14}C-labeled acetate was extracted with n-butanol. The resulting samples were applied to ODS (octadecyl silanization) silica plates and chromatographed in solvent system V. The plate was exposed to X-ray film for 1 wk. Samples are: 1, strain RBL5560; 2, strain RBL5560 grown in the presence of naringenin; 3, strain RBL1; 4, strain RBL1 grown in the presence of naringenin; 5, strain ANU843; 6, strain ANU843 grown in the presence of naringenin; 7, strain 0403; 8, strain 0403 grown in the presence of naringenin; 9, strain 2011.pMP280; 10, strain 2011.pMP280 grown in the presense of naringenin; 11, strain USDA110; 12, strain USDA110 grown in the presence of genistein; 13, strain USDA135; 14, strain USDA135 grown in the presence of genistein.

Extraction of the pelleted cells with n-butanol showed that the flavonoid-inducible compounds did not accumulate on the cell surface (Fig. 1C). This result was confirmed by the analysis of the samples from the pelleted cells with silica TLC using solvents I and II. The compounds 1–5 were detectable in the growth medium within 2 hr after induction with naringenin (Fig. 1C). Spots with identical mobility were also detected after growing the cells with ^{14}C-labeled malonate instead of acetate, although they appeared later in a time course (Fig. 1C). Because the use of radioactive malonate had no advantage and because it is very expensive, acetate was used subsequently to analyze the presence of flavonoid-inducible compounds. The detected radiolabeled products are apparently not the translational products of the *nod* genes themselves (data not shown) and therefore are referred to here as Nod metabolites.

Comparison of Nod metabolites in various wild type *Rhizobium* and *Bradyrhizobium* strains. Using the above reverse phase TLC system, the growth medium of several wild type *Rhizobium* and *Bradyrhizobium* strains was analyzed for the presence of flavonoid-inducible compounds using radiolabeled acetate. As a control, *R. l.* bv. *viceae* strain RBL5560 was used. The results (Fig. 2) show that the *R. l.* bv. *viceae* strain RBL1 (lanes 3 and 4), the *R. l.* bv. *trifolii* strains ANU843 (lanes 5 and 6) and 0403 (lanes 7 and 8) and the *B. japonicum* strain USDA135 (lanes 13 and 14) excrete several flavonoid-inducible compounds. In *B. japonicum* strain USDA110, one major genistein-inducible spot was detected (Fig. 2, lanes 11 and 12). As in the case of *R. leguminosarum,* this *B. japonicum* flavonoid-inducible compound had a high partition coefficient (Ku/l, larger than 30) with n-butanol but with none of the other tested solvents. No luteolin-inducible compounds were detected in *R. meliloti* wild type strain 2011 using the standard procedure. However, after introduction of plasmid pMP280, containing the cloned *nodD* gene of *R. l.* bv. *viceae* strain RBL5560, one naringenin-inducible spot was detected in the growth medium (Fig. 2, lanes 9 and 10). This flavonoid-inducible compound migrates much faster, and therefore is more hydrophilic than any of the detected flavonoid-inducible compounds produced by the other rhizobia.

It has been reported that the signal molecule NodRm1

produced by *R. meliloti* strain 2011 contains a sulphate group (Lerouge *et al.* 1990). After growing *R. meliloti* strain 2011 (containing pMP280) in the presence of ^{35}S-labeled sulphate, one naringenin-inducible spot was detected after TLC (Fig. 3, lane 16). This spot, presumably the reported compound NodRm1, migrates identically to the ^{14}C-labeled spot detected in Figure 2. We also tested whether flavonoid-inducible compounds of other *Rhizobium* and *Bradyrhizobium* strains could be detected after labeling with radioactive sulphate. The results (Fig. 3A) show that in none of the tested strains a sulphate-labeled flavonoid-inducible compound was detectable. Also chromatography on silica TLC plates using solvent II did not reveal such compounds in the case of *B. japonicum*, whereas inducible spots in the control with ^{14}C-labeling are clearly visible (Fig. 3B). Uptake studies (legend to Fig. 3) indicate that ^{35}S-labeled sulphate is taken up in the cells as efficiently as in *R. meliloti* strain 2011.pMP280. The control experiment in which the same cultures were grown under identical culture conditions with ^{14}C-labeled acetate showed that the previously detected flavonoid-inducible compounds are still produced (Fig. 3A). In conclusion, it is very unlikely that the observed flavonoid-inducible compounds (Fig. 2) of the tested strains other than *R. meliloti* contain a group that is derived from sulphate. This could explain the more hydrophobic nature of these compounds compared to the flavonoid-inducible compound of *R. meliloti* strain 2011.

Nod metabolites secreted by nodulation mutants. The observation that rhizobia produce and excrete flavonoid-inducible compounds suggests that the *nod* genes are involved in the synthesis of these compounds. *Rhizobium* strain RBL5560 containing the *nodD604* gene which directs flavonoid independent transcription activation (FITA) of the *nod* genes (Spaink *et al.* 1989b) was tested for the production of Nod metabolites. The results (Fig. 4A, lanes 1 and 2) show that Nod metabolites can also be produced

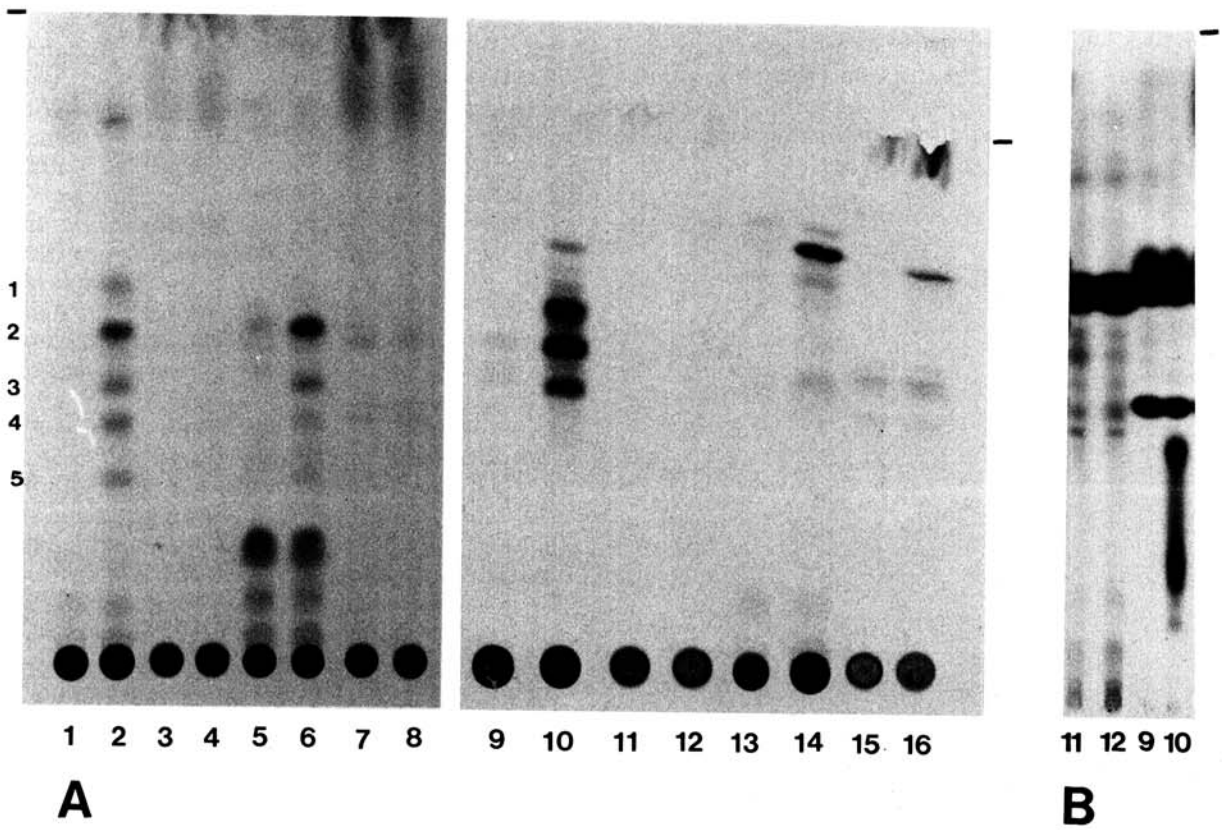

Fig. 3. Thin-layer chromatography (TLC) analysis of culture medium of cells grown in the presence of ^{35}S-labeled sulphate. As a control, the cells of each strain were also grown under identical conditions in the presence of radiolabeled acetate instead of sulphate. The uptake of radioactive label by the cells was determined and is indicated in percentage of total added label for each tested strain. In **A**, the resulting samples were applied to ODS (octadecyl silanization) silica TLC plates and chromatographed in solvent system V. In **B**, the samples were applied to silica TLC plates and chromatographed in solvent II (the exposure time to X-ray film was 8 wk). Samples are: 1, *Rhizobium leguminosarum* bv. *viceae* strain RBL5560 grown in the presence of ^{14}C-labeled acetate, uptake 23%; 2, strain RBL5560 grown in the presence of ^{14}C-labeled acetate and naringenin, uptake 25%; 3, strain RBL5560 grown in the presence of ^{35}S-labeled sulphate, uptake 4%; 4, strain RBL5560 grown in the presence of ^{35}S-labeled sulphate and naringenin, uptake 5%; 5, *R. l.* bv. *trifolii* strain ANU843 grown in the presence of ^{14}C-labeled acetate, uptake 11%; 6, strain ANU843 grown in the presence of ^{14}C-labeled acetate and naringenin, uptake 12%; 7, strain ANU843 grown in the presence of ^{35}S-labeled sulphate, uptake 8%; 8, strain ANU843 grown in the presence of ^{35}S-labeled sulphate and naringenin, uptake 10%; 9, *Bradyrhizobium japonicum* strain USDA135 grown in the presence of ^{14}C-labeled acetate, uptake not determined; 10, strain USDA135 grown in the presence of ^{14}C-labeled acetate and genistein, uptake 4%; 11, strain USDA135 grown in the presence of ^{35}S-labeled sulphate, uptake 2%; 12, strain USDA135 grown in the presence of ^{35}S-labeled sulphate and genistein, uptake 2%; 13, *R. meliloti* strain 2011.pMP280 grown in the presence of ^{14}C-labeled acetate, uptake 7%; 14, strain 2011.pMP280 grown in the presence of ^{14}C-labeled acetate and naringenin, uptake 5%; 15, strain 2011.pMP280 grown in the presence of ^{35}S-labeled sulphate, uptake 1%; 16, strain 2011.pMP280 grown in the presence of ^{35}S-labeled sulphate and naringenin, uptake 1%.

in the absence of flavonoids and confirms a role of the *nod* genes in their production. We tested mutations in the common Nod genes of several *Rhizobium* and *Bradyrhizobium* strains (Fig. 4A). The results show that in strains containing Tn*5* insertions in the *nodABC* operon the Nod metabolites (Fig. 2) are not observed. Because Tn*5* mutations prevent normal transcription of the downstream genes of an operon, a nonpolar deletion in the *nodC* gene was used to confirm the requirement of the *nodC* gene of *R. l.* bv. *viceae* for the production of the Nod metabolites (Fig. 4A, lanes 7 and 8).

R. l. bv. *viceae* strain RBL5560 was further investigated for the role of the other nodulation genes in the synthesis of the compounds represented by the spots 1–5 (Fig. 1B,C). Mutations in the *nodI*, *nodJ*, *nodM*, *nodT*, and *nodO* genes had no observable effect on the pattern of Nod metabolites (Fig. 4B). This result was confirmed by the analysis of these strains using the silica TLC system with solvent II. A significant quantitative effect is observed in the case of the *nodM* mutant (Fig. 4B, lanes 23 and 24), which produced approximately threefold less of the Nod metabolites as judged by counting of the radioactivity of the isolated spots. The pattern of radioactive spots is different in strains containing mutations in the *nodF* or *nodE* genes in that the inducible spot number 1 is not detectable and that spot 2 is relatively less intense (Fig. 4C, lanes 27–30). This difference is even more apparent after TLC using methanol in the second dimension, which separates the Nod metabolites from weak, noninducible, background spots (data not shown). This result indicates a role of the NodF and NodE proteins in the production or excretion of at least two Nod metabolites. Mutation of the *nodL* gene results in Nod metabolites with a different mobility to that of wild type compounds (Fig. 4C, lanes 31 and 32). Moreover, these compounds were isolated from the spent growth medium in five- to ten-fold lower quantities than the wild type Nod metabolites as estimated by counting of the radioactivity of the isolated spots.

Investigation of the influence of the chromosomal background on Nod metabolite production. The TLC patterns of Nod metabolites produced by *R. l.* bvs. *viceae* and *trifolii* are very similar. However, the most hydrophilic compound of *R. l.* bv. *viceae* represented by spot 1 (Fig. 1C) is not detected in the growth medium of *R. l.* bv. *trifolii* strain ANU843 (Fig. 2, lanes 5 and 6). Furthermore, less Nod metabolites were isolated from strain ANU843. To investigate the influence of the chromosomal background on these differences, the cloned nodulation genes of *R. l.* bv. *trifolii* strain ANU843, present on plasmid pRtrRF101 (containing all known *nod* genes of this strain; Fisher *et al.* 1985), were introduced in various *Rhizobium* strains that had been cured of their Sym plasmids or in which the *nod* genes had been deleted. The pattern of Nod metabolites produced by all strains containing pRtRF101 were similar to that of the wild type in TLC (Fig. 5A). However, with the strains containing the cloned *R. l.* bv. *trifolii nod* genes, larger quantities of the Nod metabolites were present in the growth medium.

Several *E. coli* strains were constructed in which the Nod proteins are expressed under control of the *lac*

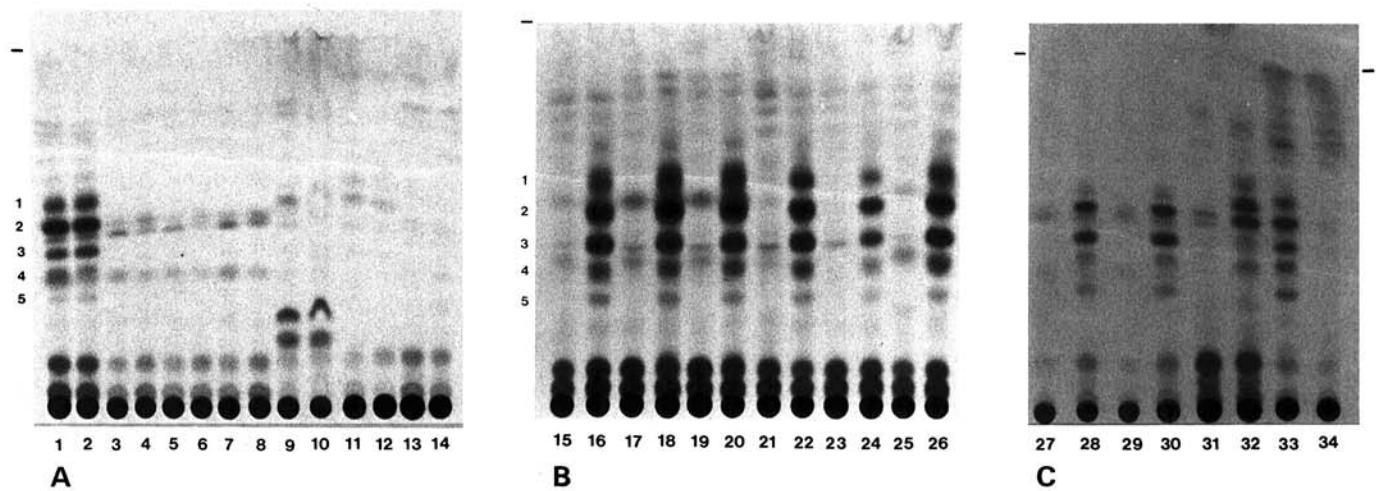

Fig. 4. Thin-layer chromatography (TLC) analysis of radiolabeled compounds produced by various *nod* mutants. The culture medium of cells grown in the presence of ^{14}C-labeled acetate was extracted with n-butanol. The resulting samples were applied to ODS (octadecyl silanization) silica plates and chromatographed in solvent system V. In **A**, the TLC plate was exposed to X-ray film for 2 wk; in **B**, for 3 wk; and in **C**, for 1 wk. Samples are: 1, RBL5560.pMP604 (FITA *nodD*); 2, RBL5560.pMP604 grown in the presence of naringenin; 3, RBL610 (*nodA*::Tn*5*); 4, RBL610 grown in the presence of naringenin; 5, RBL611 (*nodB*::Tn*5*); 6, RBL611 grown in the presence of naringenin; 7, RBL607 (*nodC7*); 8, RBL607 grown in the presence of naringenin; 9, ANU252 (ANU843 *nodA*::Tn*5*); 10, ANU252 grown in the presence of naringenin; 11, *Rhizobium meliloti* GMI5382(*nodA*::Tn*5*).pMP280 (cloned *nodD* of *R. leguminosarum* bv. *viceae*); 12, GMI5382.pMP280 grown in the presence of naringenin; 13, *Bradyrhizobium japonicum* AN122 (*nodC*::Tn*5*); 14, AN122 grown in the presence of genistein; 15, wild type *R. l.* bv. *viceae* RBL5560; 16, RBL5560 grown in the presence of naringenin; 17, RBL5729 (*nodI*::Tn*5*); 18, RBL5729 grown in the presence of naringenin; 19, RBL5734 (*nodJ*::Tn*5*); 20, RBL5734 grown in the presence of naringenin; 21, RBL5795 (*NodT*::Tn*5PhoA*); 22, RBL5795 grown in the presence of naringenin; 23, RBL5794 (*nodM*::Tn*5PhoA*); 24, RBL5794 grown in the presence of naringenin; 25, RBL5797 (*nodO*::Tn*5*); 26, RBL5797 grown in the presence of naringenin; 27, RBL618 (*nodF*::Tn*5*); 28, RBL618 grown in the presence of naringenin; 29, RBL601 (*nodE*::Tn*5*); 30, RBL601 grown in the presence of naringenin; 31, RBL5793 (*nodL*::Tn*5*), relatively 10 times more sample applied; 32, RBL5793 grown in the presence of naringenin, relatively 10 times more sample applied; 33, RBL5560 grown in the presence of naringenin; 34, RBL5560.

promoter. In these strains the expression of Nod proteins can be regulated by the addition of lactose as was confirmed by immunological detection of the produced NodA, NodF, NodE, and NodL proteins (data not shown). Radioactive labeling with ^{14}C acetate showed that these *E. coli* strains also produce several inducible compounds in the growth medium (Fig. 5B). These metabolites that migrate in TLC similar to the wild type Nod metabolites were isolated in much higher amounts than the Nod metabolites produced by *Rhizobium* itself. In the absence of the NodABC products, no inducible metabolites were observed (Fig. 5B, lanes 1–4).

DISCUSSION

In this paper, a rapid method is described to detect and separate Nod metabolites produced by *Rhizobium* and *Bradyrhizobium* strains. This method has been used to compare several rhizobial strains, to study the role of the *nod* genes in metabolite production, and to investigate the role of the chromosomal background in their production. This method is also currently being used as a test system in the purification and further characterization of the detected Nod metabolites. Some of the excreted Nod metabolites of *R. l.* bv. *viceae*, *R. l.* bv. *trifolii*, and *Bradyrhizobium* represented by the flavonoid-inducible spots in Figure 2 have recently been purified and appear to have strong biological effects on the roots of the host plants (Spaink *et al.* 1991; and H. P. Spaink *et al.*, unpublished). These results confirm the role of the detected Nod metabolites as molecules involved in microbe-plant signaling.

In several tested rhizobial strains more than one Nod metabolite could be detected. Considering the observed difference in the two tested *B. japonicum* strains (Fig. 2, lanes 12 and 14), the phenomenon of the production of multiple Nod metabolites is apparently not a characteristic feature for each cross-inoculation group. Furthermore, the

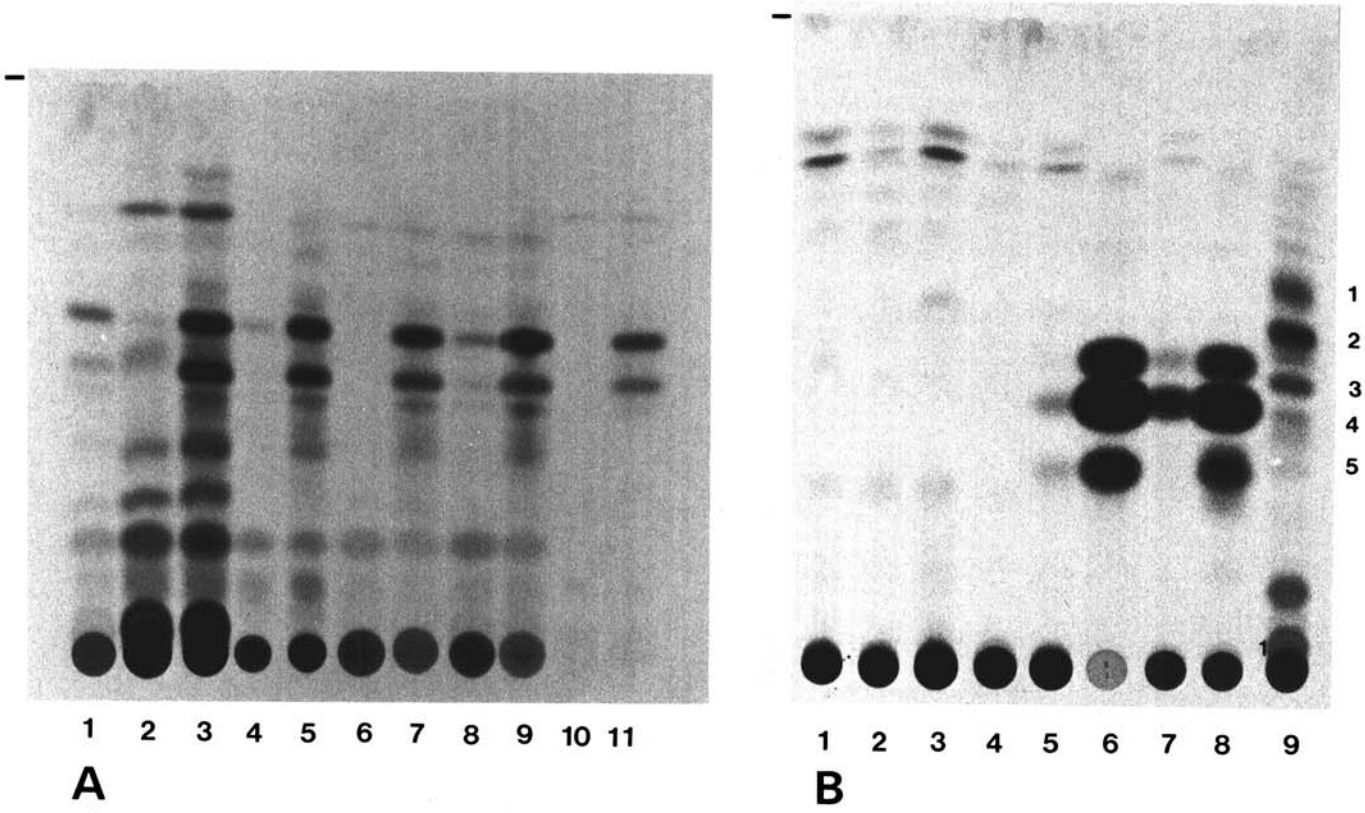

Fig. 5. Thin-layer chromatography (TLC) analysis of Nod metabolites produced by cloned *nod* genes in various chromosomal backgrounds. In **A,** various cured *Rhizobium* strains harbouring plasmid pRtRF101 (containing the *nod* region of *R. leguminosarum* bv. *trifolii*) are analyzed. In **B,** *Escherichia coli* strains containing cloned *nod* genes under control of the *lacZ* promoter are analyzed. In **B,** the TLC plates were exposed to X-ray film for 8 hr. The spent culture medium of cells grown in the presence of ^{14}C-labeled acetate was extracted with n-butanol. The resulting samples were applied to ODS (octadecyl silanization) silica plates and chromatographed in solvent system V. A samples are: 1, wild type *R. l.* bv. *trifolii* strain ANU843 grown in the presence of naringenin; 2, strain ANU845.pRtRF101; 3, strain ANU845.pRt101 grown in the presence of naringenin; 4, strain LPR5045.pRtRF101; 5, strain LPR5045.pRtRF101 grown in the presence of naringenin; 6, strain RBL1391.pRtRF101; 7, strain RBL1391.pRt101 grown in the presence of naringenin; 8, strain 8401.pRtRF101; 9, strain 8401.pRtRF101 grown in the presence of naringenin; 10, strain GMI766.pRtRF101; 11, strain GMI766.pRtRF101 grown in the presence of naringenin. B samples are: 1, *Escherichia coli* BL21(DE3).pMP3401 grown in the presence of glucose; 2, *E. coli* BL21(DE3).pMP3401 grown in the presence of lactose; 3, *E. coli* BL21(DE3).pMP3401.pMP1715 grown in the presence of glucose; 4, *E. coli* BL21(DE3).pMP3401.pMP1715 grown in the presence of lactose; 5, *E. coli* BL21(DE3).pMP248.pMP3401 grown in the presence of glucose; 6, *E. coli* BL21(DE3).pMP248.pMP3401 grown in the presence of lactose; 7, *E. coli* BL21(DE3).pMP248.pMP1715.pMP3401 grown in the presence of glucose; 8, *E. coli* BL21(DE3).pMP248.pMP1715.pMP3401 grown in the presence of lactose; 9, *R. l.* bv. *viceae* RBL5560 grown in the presence of naringenin.

number of Nod metabolites produced is also not linked to the chromosomal background as shown by the apparent production of the *R. l.* bv. *trifolii* Nod metabolites in all tested chromosomal backgrounds including *R. meliloti*. The situation in *R. l.* bv. *viceae*, which has been subject to further studies, indicates that the detected Nod metabolites are not merely the result of the breakdown of one single metabolic product. First, in a time course of Nod metabolite production (Fig. 1C) the relative amount of the detected Nod metabolites does not change. Second, recent results indicate that the compound represented by spot 3 (Fig. 1C) has significantly different chemical characteristics than the compound represented by spot 1 (H. P. Spaink *et al.*, 1991b). Because bioassays indicate that the Nod metabolites represented by spots 1–5 (Fig. 1C) of strain RBL5560 are biologically active in root hair deformation assays (Spaink *et al.* 1991; H. P. Spaink *et al.*, unpublished) the multiplicity of Nod metabolites could have important biological relevance.

Only the detected *R. meliloti* compound could be labeled using radioactive sulphate. In the case of *R. meliloti* overproduction of the Nod proteins by introducing multiple copies of the *nodD* gene of *R. l.* bv. *viceae* was necessary to detect Nod metabolite production. This is probably due to the extremely low level of expression of the *nod* genes in strain 2011 compared to other *Rhizobium* strains (Spaink *et al.* 1989a). The detected compound is presumably the reported signal molecule NodRm1. The presence of the sulphate group on NodRm1 could explain the more hydrophilic character as compared to the Nod metabolites of other rhizobia.

The results of the tested *nod* gene mutants (Fig. 4A) indicate that at least NodC protein is essential for the production or excretion of the detected Nod metabolites. In *R. l.* bv. *viceae* RBL5560, the host specificity-determining NodE protein, which is produced by the *nodFEL* operon, also plays a role in the production of Nod metabolites. It has been shown that NodF protein contains a 4′phosphopantetheine prosthetic group that is indicative for a function in the production of a fatty acyl chain (Geiger *et al.* 1991). However, Nod metabolites are still produced in the absence of the NodF and NodE proteins (Fig. 4C, lanes 27–30). These compounds are very hydrophobic and do contain a lipid moiety like the wild type Nod metabolites (Spaink *et al.* 1991a; H. P. Spaink *et al.*, 1991b). The NodL protein appears to be involved in the production of the O-acetyl moiety on Nod metabolites of *R. l.* bv. *viceae* (Spaink *et al.* 1991). Results with the mutant (Fig. 4C, lane 32) are consistent with this function in that the pattern of produced Nod metabolites is different from that of the wild type. In addition to its function in modification of Nod metabolites, NodL also appears to be important for the amount of Nod metabolites present in the bacterial growth medium. Further study should indicate whether this is the result of a difference in production or excretion of Nod metabolites. Mutations in the other *nod* genes do not seem to be essential for the production and excretion of Nod metabolites. In the case of NodM, this is surprising because it has been suggested to function in the synthesis of glucosamine, an obvious precursor of Nod metabolites (Downie *et al.* 1991; Kondorosi 1991). However, it is possible that a chromosomal counterpart is partially compensating for the loss of NodM function in the case of *R. l.* bv. *viceae* (Downie *et al.* 1991). Supporting this notion is our observation that in a *nodM* mutant strain quantitatively less Nod metabolites are produced. In the case of *nodO,* a role in the production of secondary metabolites was not expected because it codes for a secreted protein that is homologous to the haemolysin of *E. coli* (de Maagd *et al.* 1989; Economou *et al.* 1990). There are no indications for the function of the other *nod* genes, like *nodT*, which do not influence the spectrum of detected Nod metabolites. It is very well possible that factors that are produced by such genes are not extracted by any of the tested solvents because these extractions are only useful for metabolites that have hydrophobic characteristics.

Production of Nod metabolites is also detected in *E. coli* expressing the *nod* genes. This result is consistent with earlier reports showing rhizobial signal production in *E. coli* strains containing cloned *nod* genes (Downie *et al.* 1985; Banfalvi and Kondorosi 1989). Surprisingly, the detected level of production and excretion is much higher than in *Rhizobium* itself. The study of Nod metabolite production in *E. coli* would have the advantage of the availability of temperature-sensitive mutants disturbed in essential metabolic pathways necessary for Nod metabolite production.

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LITERATURE CITED

Banfalvi, Z., and Kondorosi, A. 1989. Production of root hair deformation factors by *Rhizobium meliloti* nodulation genes in *Escherichia coli*: *HsnD* (NodH) is involved in the plant host-specific modification of the NodABC factor. Plant Mol. Biol. 13:1-12.

Bibb, M. J., Biro, S., Motamedi, H., Collins, J. F., and Hutchinson, C. R. 1989. Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcm1* genes provide key information about the enzymology of polyketide antibiotic biosynthesis. EMBO J. 9:2727-2736.

Canter Cremers, H. C. J., Spaink, H. P., Wijfjes, A. H. M., Pees, E., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. 1989. Additional nodulation genes on the Sym plasmid of *R. leguminosarum* biovar *viceae*. Plant Mol. Biol. 13:163-174.

Conway, W. D., and Ito, Y. 1984. Evaluation of nonaqueous solvent systems for countercurrent chromatography using an HPLC assay to determine partition coefficients of a mixture of compounds. J. Liq. Chromatogr. 7:291-302.

de Maagd, R. A., Wijfjes, A. H. M., Spaink, H. P., Ruiz-Sainz, J. E., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. 1989. *nodO*, a new *nod* gene of the *Rhizobium leguminosarum* biovar *viciae* Sym plasmid pRL1JI, encodes a secreted protein. J. Bacteriol. 171:6764-6770.

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

Djordjevic, M. A., Schofield, P. R., and Rolfe, B. G. 1985. Tn*5* mutagenesis of *R. trifolii* host-specific nodulation genes result in mutants with altered host-range ability. Mol. Gen. Genet. 200:463-471.

Downie, J. A. 1989. The *nodL* gene from *Rhizobium leguminosarum* is homologous to the acetyl transferases encoded by *lacA* and *cysE*. Mol. Microbiol. 3:1649-1651.

Downie, J. A., Knight, C. D., Johnston, A. W. B., and Rossen, L. 1985. Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. Mol. Gen. Genet. 198:255-262.

Downie, J. A., Marie, C., Scheu, A. K., Firmin, J. L., Wilson, K. E., Davies, A. E., Cubo, T. M., Mavridou, A., Johnston, A. W. B., and Economou, A. 1991. Genetic and biochemical studies on the nodulation genes of *Rhizobium leguminosarum* bv. *viciae*. Pages 134-141 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Economou, A., Hamilton, W. D. O., Johnston, A. W. B., and Downie, J. A. 1990. The *Rhizobium* nodulation gene *nodO* encodes a Ca^{2+} binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J. 9:349-354.

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

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

Gardiol, A. E., and Dazzo, F. B. 1986. Biosynthesis of *Rhizobium trifolii* capsular polysaccharide: Enzymatic transfer of pyruvate substitutions into lipid-bound saccharide intermediates. J. Bacteriol. 168:1459-1462.

Geiger, O., Spaink, H. P., and Kennedy, E. P. 1991. Isolation of *Rhizobium leguminosarum* NodF nodulation protein. NodF carries a 4′-phosphopantetheine prosthetic group. J. Bacteriol. 173:2872-2878.

Hollingsworth, R., Squartini, A., Philip-Hollingsworth, S., and Dazzo, F. 1989. Root hair deforming and nodule initiating factors from *Rhizobium trifolii*. Pages 387-393 in: Signal Molecules in Plants and Plant-Microbe Interactions. NATO ASS series, Vol. H36. B. J. J. Lugtenberg, ed. Springer-Verlag, Berlin.

Hooykaas, P. J. J., Klapwijk, P. M., Nuti, M. P., Schilperoort, R. A., and Rörsch, A. 1977. Transfer of the *Agrobacterium* Ti plasmid to avirulent agrobacteria and to rhizobia ex planta. J. Gen. Microbiol. 98:477-484.

Hooykaas, P. J. J., van Brussel, A. A. N., den Dulk-Ras, H., van Slogteren, G. M. S., and Schilperoort, R. A. 1981. Sym plasmid of *Rhizobium trifolii* expressed in different Rhizobial species and *Agrobacterium tumefaciens*. Nature (London) 291:351-353.

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

Johnston, A. W. B., Beynon, J. L., Buchanon-Wollaston, A. V., Setchell, S. M., Hirsch, P. R., and Beringer, J. E. 1978. High frequency transfer of nodulation ability between strains and species of *Rhizobium*. Nature (London) 276:634-636.

Kondorosi, A. 1991. Overview on genetics of nodule induction: Factors controlling nodule induction by *Rhizobium meliloti*. Pages 111-118 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Lamb, J. W., Hombrecher, G., and Johnston, A. W. B. 1982. Plasmid-determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli*. Mol. Gen. Genet. 186:449-452.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature (London) 344:781-784.

Long, S. R. 1989. *Rhizobium*-legume nodulation: Life together in the underground. Cell 56:203-214.

Nieuwkoop, A. J., Banfalvi, Z., Deshmane, N., Gerhold, D., Schell, M. G., Sirotkin, K. M., and Stacey, G. 1987. A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J. Bacteriol. 169:2631-2638.

Priem, W. J. E., and Wijffelman, C. A. 1984. Selection of strains cured of the *Rhizobium leguminosarum* Sym plasmid pRL1JI by using small bacteriocin. FEMS Microbiol. Lett. 25:247-251.

Roche, P., Lerouge, P., Prome, J. C., Faucher, C., Vasse, J., Maillet, F., Camut, S., De Billy, F., Dénarié, J., and Truchet, G. 1991. NodRm1, a sulphated lipo-oligosaccharide signal of *Rhizobium meliloti* elicits hair deformation, cortical cell division and nodule organogenesis on alfalfa roots. Pages 119-126 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.-M., Gloudemans, T., Van Kammen, A., and Bisseling, T. 1990. The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. Cell 60:281-294.

Schlaman, H. R. M., Okker, R. J. H., and Lugtenberg, B. J. J. 1990. Subcellular localization of the *Rhizobium leguminosarum nodI* gene product. J. Bacteriol. 172:5486-5489.

Schwedock, J., and Long, S. R. 1990. ATP sulphurylase activity of the *nodP* and *nodQ* gene products of *Rhizobium meliloti*. Nature (London) 348:644-647.

Spaink, H. P., Geiger, O., Sheeley, D. M., van Brussel, A. A. N., York, W. S., Reinhold, V. N., Lugtenberg, B. J. J., and Kennedy, E. P. 1991a. The biochemical function of the *Rhizobium leguminosarum* proteins involved in the production of host specific signal molecules. Pages 142-149 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987a. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. 9:27-39.

Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Tak, T., Goosen-de Roo, L., Pees, E., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1989b. Symbiotic properties of Rhizobia containing a flavonoid-independent hybrid *nodD* product. J. Bacteriol. 171:4045-4053.

Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991b. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature (London) 354:125-130.

Spaink, H. P., Weinman, J., Djordjevic, M. A., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. 1989a. Genetic analysis and cellular localization of the *Rhizobium* host specificity-determining NodE protein. EMBO J. 8:2811-2818.

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

Stahl, E. 1969. Thin-layer chromatography: A Laboratory Handbook. 2nd ed. Springer-Verlag, New York.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorf, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Meth. Enzymol. Vol. 185:60-89.

van Brussel, A. A. N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C. A. 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other Rhizobia and Agrobacteria harbouring a leguminosarum plasmid. Plant Sci. Lett. 27:317-325.

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

Wijffelman, C. A., Pees, E., van Brussel, A. A. N., Okker, R. J. H., and Lugtenberg, B. J. J. 1985. Genetic and functional analysis of the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Arch. Microbiol. 143:225-232.