Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3122–3126, April 1994
Biochemistry

Biosynthesis of lipooligosaccharide nodulation factors: *Rhizobium* NodA protein is involved in N-acylation of the chitooligosaccharide backbone

(nitrogen fixation/common *nod* genes/*Rhizobium meliloti*)

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Contributed by Jeff Schell, December 28, 1993

ABSTRACT **_Rhizobium meliloti_ interacts symbiotically with alfalfa by forming root nodules in which the bacteria fix nitrogen. The _Rhizobium_ nodulation genes _nodABC_ are involved in the synthesis of lipooligosaccharide symbiotic signal molecules, which are mono-_N_-acylated chitooligosaccharides. These bacterial signals elicit nodule organogenesis in roots of legumes. To elucidate the role of the NodA protein in lipooligosaccharide biosynthesis, we prepared a radiolabeled tetrasaccharide precursor carrying an amino group as a potential attachment site for N-acylation at the nonreducing glucosamine residue. Various criteria demonstrate that NodA is involved in the attachment of a fatty acyl chain to this tetrasaccharide precursor, yielding a biologically active nodulation factor.**

Rhizobial nodulation factors represent a class of plant growth regulators involved in the control of root nodule development and possibly also in other plant developmental processes (1). These Nod factors are lipooligosaccharides, basically consisting of a tetra- or pentameric chitooligosaccharide backbone, carrying a fatty acyl chain at the nonreducing end (2–5). The oligosaccharide chain length of the various Nod factors, the structure of their fatty acyl substituents, and other modifications on the chitooligosaccharide backbone determine the response of the host plant (2–9).

Previous findings indicate that the highly conserved *Rhizobium* nodulation genes *nodABC* are sufficient to produce the basic lipooligosaccharide structure (4). Since NodC is homologous to chitin synthase (10) and acts as an *N*-acetylglucosaminyltransferase (11), this protein is involved in the formation of the oligosaccharide backbone. The next step in the assembly of lipooligosaccharides could be the deacetylation of the nonreducing *N*-acetylglucosamine residue of chitooligosaccharides by the NodB protein (12). This step provides a free amino group at the nonreducing terminus of the carbohydrate chain, which is a necessary prerequisite for fatty N-acylation in the pathway of Nod factor synthesis. The nucleotide sequence of the *Rhizobium meliloti nod* genes revealed that the *nodA* stop codon overlaps the *nodB* start codon (13), suggesting a functional coupling between the two genes (14). Therefore, it seemed reasonable to assume that subsequent to deacetylation by NodB, the NodA protein may play a role in N-acylation of the carbohydrate backbone. To test this hypothesis by appropriate acylation assays, we synthesized radiolabeled tetraacetylchitotetraose which was deacetylated at the nonreducing terminus by NodB. Using bacterial cell extracts, we have demonstrated that NodA can utilize this tetrasaccharide precursor as an acyl acceptor to form a biologically active Nod factor.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. The construction of the Nod factor-overproducing strain *R. meliloti* 1021/pEK327 (5) and the *R. meliloti* deletion mutant AK635 (Δ*nodABC*; ref. 15) has been described. *R. meliloti* 1021 mutants carrying Tn*5* insertions in *nodABC* were constructed by conjugating plasmid pKSK5 carrying *R. meliloti* 41 *nodABC* genes with the appropriate Tn*5* insertions (16) into *R. meliloti* 1021. Homogenotes were obtained as described by Kondorosi *et al.* (16). Selected colonies were checked by Southern hybridization. To provide high constitutive *nod* gene expression, pNID6 carrying the regulatory genes *syrM* and *nodD3* was then introduced into the *R. meliloti* 1021 mutants, yielding strains ISV1502 (*nodA*::Tn*5*), ISV1501 (*nodB*::Tn*5*), and ISV1503 (*nodC*::Tn*5*).

Preparation of Cell Extracts. *Rhizobium* strains were grown in M9 salts (17) supplemented with 0.2% Casamino acids, 0.4% glycerol, and 5 μM luteolin as *nod* gene inducer. Late-logarithmic-phase cultures ($OD_{600} \approx 0.8$) were centrifuged and cells were washed in cold 0.1 M Mops (pH 7.6) prior to storage at −70°C. Frozen cells were resuspended in 0.1 M Mops (pH 7.6) supplemented with leupeptin (2 μg/ml) and α_2-macroglobulin (1 unit/ml). The bacterial suspension was diluted in the same buffer to a final concentration corresponding to 30 times an OD_{600} of 1. Cells were ruptured by sonication (Branson Sonifier 250, 3 min, output setting 4), and the resulting extracts were frozen in aliquots in liquid nitrogen and stored at −70°C until use.

Preparation and Purification of β-1,4-Tri-*N*-[^{14}C]acetyl-D-glucosamine Tetrasaccharide. Five milligrams of β-1,4-D-glucosamine tetrasaccharide ($GlcN_4$; Seikagaku, Tokyo) was dissolved in 150 μl of water containing 15 μl methanol and 150 μl of AG1-X8 anion-exchange resin (bicarbonate form, Bio-Rad). The mixture was stirred on ice while 1.7 μl of [1-^{14}C]-acetic anhydride (0.25 mCi, Sigma) was added. Radiolabeling was allowed to proceed on ice for 90 min and then the reaction was chased for 60 min by adding 5 μl of nonradioactive acetic anhydride. The solution was passed through an AG50W-X4 cation-exchange minicolumn (hydrogen form, Bio-Rad). Column effluent and washings containing the radiolabeled tetraacetylchitotetraose were concentrated and applied to an Aminex HPX-42 HPLC column (18). Purified ^{14}C-labeled tetraacetylchitotetraose (≈3 mg) was deacetylated at the nonreducing end by using recombinant NodB protein essentially as described (12). The heat-inactivated incubation mixture containing tri-*N*-[^{14}C]acetylglucosamine tetrasaccharide was loaded onto a 2-ml column of AG50W-X4 (hydrogen form). The column was washed free of salts and nondeacetylated tetrasaccharide with 10 ml of water, and the product

Abbreviation: Had, root hair deformation.

was eluted with two 1.0-ml washes of 1.5% NH_4OH (pH ≈11.5). The eluate was collected and lyophilized.

Assay for Acylation of β-1,4-Tri-*N*-[^{14}C]acetyl-D-glucosamine Tetrasaccharide. To 1.0 ml of bacterial cell extract containing 0.1 M Mops (pH 7.6) and protease inhibitors, 20 μl of 10% (vol/vol) Triton X-100 and 0.1 μCi (3.7 kBq) of tri-*N*-[^{14}C]acetylglucosamine tetrasaccharide were added. The mixture was incubated at 30°C for 2–3 hr with gentle agitation. Lipid-linked compounds were extracted with 600 μl of water-saturated 1-butanol for 1 hr by vigorous vortexing. The butanol phase was dried and the material was suspended in 60% acetonitrile at 56°C. After centrifugation the supernatant was dried and the pellet was dissolved in 10 μl of TLC mobile phase. Compounds were analyzed by TLC on silica gel 60 plates (0.25 mm; Merck) with 1-butanol/ethanol/water (50:30:20) as solvent. Carbohydrate-containing compounds were visualized either by spraying the plates with diphenylamine/aniline/orthophosphoric acid reagent (19) or by autoradiography.

Isolation of the Acylated [^{14}C]Glucosamine Tetrasaccharide Product. A large-scale reaction (5 ml) with *R. meliloti* 1021/*nodB*::Tn*5* cell extract was performed essentially under the conditions described above. After extraction, the butanol extract was concentrated and analyzed by HPLC on a C_{18} reversed-phase column (1.0 cm × 25 cm, Ultrasphere ODS; Beckman). The compounds were separated at a flow rate of 2.0 ml/min with a 30-min linear acetonitrile gradient (30–80%), followed by a wash with 80% acetonitrile for 10 min. The HPLC was connected to a variable-wavelength detector and an on-line Berthold radioactivity monitor. The radioactive peak 2 (see Fig. 4) corresponding to the retention time of NodRm-IV was collected and analyzed.

Synthesis of β-1,4-*N*-Hexanoyl- and *N*-Hexadecanoyl-tri-*N*-[^{14}C]acetyl-D-glucosamine Tetrasaccharides. Tri-*N*-[^{14}C]acetylglucosamine tetrasaccharide was acylated with caproic ($C_{6:0}$) or palmitic ($C_{16:0}$) anhydrides (both from Sigma) by incubation of the oligosaccharide (0.2 μCi) in 50 μl of water, 100 μl of methanol, and 500 μl of tetrahydrofuran with 5 mg of the appropriate anhydride. The reaction was allowed to proceed for 16 hr at room temperature. After evaporation of the solvent the residue was extracted with 400 μl of 80% acetonitrile at 56°C. Radiolabeled lipooligosaccharides carrying C_6 or C_{16} fatty acids were obtained by HPLC purification on a preparative C_{18} reversed-phase column.

Other Procedures. SDS/PAGE was performed in 12% polyacrylamide gels. Proteins were transferred to nitrocellulose and the membranes were incubated with rabbit anti-NodA antibodies (20). Bound antibodies were localized with gold-conjugated anti-rabbit IgG (Bio-Rad).

NodRm-IV(S) was a gift from M. Schultze (Gif-sur-Yvette, France). This alfalfa-specific nodulation factor (2, 5) was desulfated by mild methanolysis as described (21). Root hair deformation activity (Had activity) was tested in various dilutions on vetch (*Vicia sativa* subsp. *nigra*) as described by Schultze *et al.* (5). Radiolabeled TLC reference compounds were prepared by re-N-acetylation of a glucosamine oligosaccharide mixture (monomer to hexamer; Seikagaku, Tokyo) with [1-^{14}C]acetic anhydride by the procedure described above.

For enzymatic hydrolysis of the HPLC-purified acylated [^{14}C]glucosamine tetrasaccharide product (see peak 2 in Fig. 4), recombinant chitinase from *Serratia marcescens* (22) was used as described (12). Degradation products were analyzed by TLC and autoradiography.

RESULTS

Overproduction of NodA in *R. meliloti*. Since Nod proteins are synthesized in *R. meliloti* wild-type strains at relatively low levels, we used NodA-overproducing strains for the preparation of cell extracts. To allow constitutive high-level *nod* gene expression, we used *R. meliloti* 1021/pEK327 (5) and mutant strains carrying Tn*5* insertions in individual *nodABC* genes and containing a multicopy plasmid with the regulatory genes *nodD3* and *syrM*. The synthesis of NodA in these overproducing strains was analyzed by immunoblotting. A Tn*5* insertion in *nodA* (Fig. 1, lane 2) prevented the synthesis of the NodA protein, whereas transposon insertions in the *nodB* and *nodC* genes did not affect the synthesis of NodA (lanes 3 and 4). The *R. meliloti nodA*::Tn*5* mutant was used as a negative control in the subsequent studies.

Use of a Tetrasaccharide Precursor as an Acyl Acceptor. A defined substrate was synthesized in order to provide evidence that NodA is involved in the attachment of the fatty acyl chain to the NodB-released free amino group at the terminal nonreducing glucosamine residue of the chitooligosaccharide backbone. For this purpose, radiolabeled N-acetylated glucosamine tetrasaccharide was prepared (see *Materials and Methods*) and then was treated with the deacetylase NodB. The resulting β-1,4-tri-*N*-[^{14}C]acetyl-D-glucosamine tetrasaccharide was purified and used as acceptor in an assay for N-acyltransferase activity. This specific labeled substrate was incubated with *Rhizobium* cell extracts as enzyme source and lipid donor. To test the stability of the deacetylated tetrasaccharide in bacterial cell extracts, the modified oligosaccharide was incubated overnight with *Rhizobium* extracts not containing the NodA protein. TLC analysis of the reaction products showed that the deacetylated tetrasaccharide was not affected by degradation (Fig. 2, lane 2), whereas the nondeacetylated tetraacetylchitotetraose was partially degraded to the monosaccharide *N*-acetylglucosamine (Fig. 2, lane 3). The same result was obtained when extracts from luteolin-induced or noninduced cells of the *R. meliloti* deletion mutant AK635 (Δ*nodABC*) were used (data not shown). This indicates that an endogenous *N*-acetylglucosaminidase of *R. meliloti* can remove *N*-acetylglucosamine residues from chitooligosaccharides but that deacetylation at the nonreducing terminus by NodB protects these molecules from hydrolysis. Further studies will be necessary to determine whether or not this particular glycosidase plays a role in determining the proper chain length of the chitooligosaccharide backbone in Nod factor synthesis.

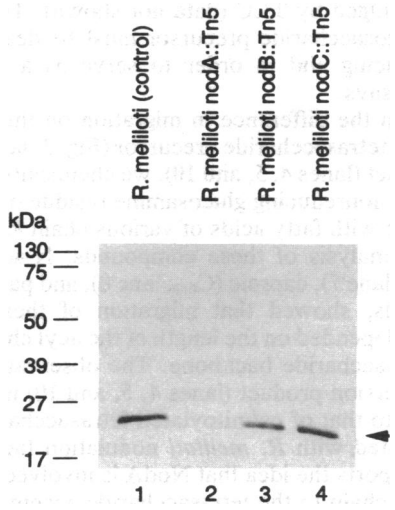

FIG. 1. Synthesis of NodA protein in mutants of *R. meliloti* 1021 carrying Tn*5* insertions in the *nod* genes as indicated. Lane 1, *R. meliloti* 1021/pEK327 (control); lane 2, ISV1502; lane 3, ISV1501; lane 4; ISV1503. Bacterial cell extracts were analyzed by SDS/PAGE and immunoblotting. Arrowhead marks the position of NodA. Molecular size markers are indicated at left.

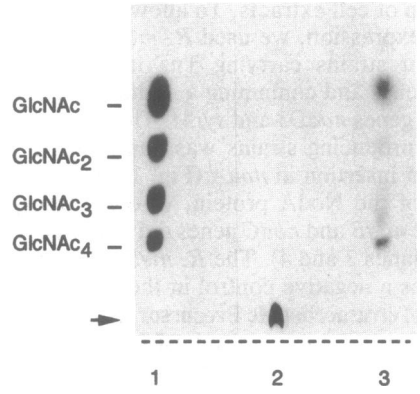

FIG. 2. Stability of NodB-treated chitotetraose in cell extracts of *R. meliloti* 1021/*nodA*::Tn*5*. Incubations of bacterial cell extracts with 400 μg of NodB-treated tetraacetylchitotetraose (lane 2) and the same amount of untreated tetrasaccharide (lane 3) were performed in 50 mM Mops, pH 7.6/0.05% Tween 80 at 30°C for 16 hr. Products were analyzed by TLC and carbohydrates were detected on the plate by spraying with the diphenylamine/aniline/orthophosphoric acid reagent (19). Reference compounds (lane 1): *N*-acetylglucosamine (GlcNAc), diacetylchitobiose ($GlcNAc_2$), triacetylchitotriose ($GlcNAc_3$), and tetraacetylchitotetraose ($GlcNAc_4$). Arrow indicates position of deacetylated tetrasaccharide; broken line marks the origin of sample application.

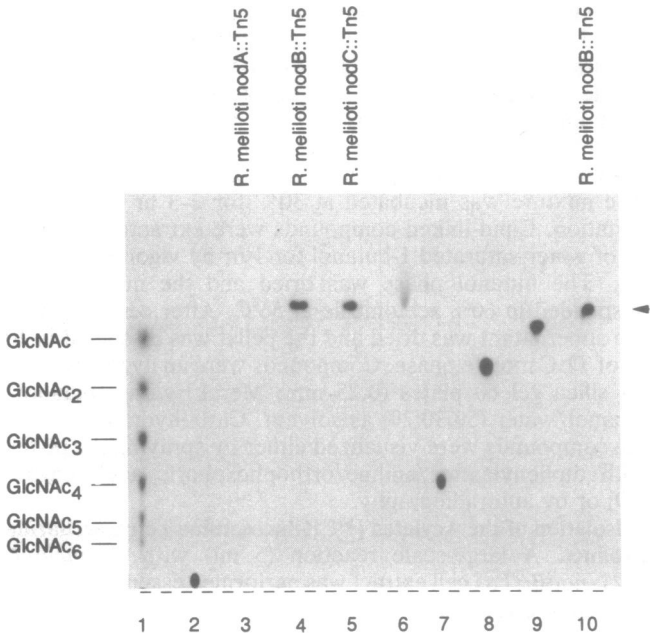

FIG. 3. Conversion of ^{14}C-labeled triacetylglucosamine tetrasaccharide to hydrophobic compounds. Reference compounds: lane 1, ^{14}C-labeled GlcNAc to hexaacetylchitohexaose ($GlcNAc_6$); lane 2, radiolabeled triacetylglucosamine tetrasaccharide used as substrate in the acylation assays. ^{14}C-labeled substrate was incubated with cell extracts of *R. meliloti* mutants carrying Tn*5* insertions in the *nod* genes as indicated (lanes 3–5 and 10). Assay conditions and extraction of hydrophobic compounds are described in *Materials and Methods*. *R. meliloti* nodulation factor NodRm-IV ($C_{16:2}$, S) served as control (lane 6). This lipooligosaccharide was visualized as described in Fig. 2. Tri-*N*-[^{14}C]acetylglucosamine tetrasaccharide was acylated with acetic (lane 7), caproic (lane 8), and palmitic (lane 9) acids, respectively. Samples (2 μl) were spotted on a silica gel 60 plate which was developed and subjected to autoradiography. Arrowhead marks position of acylated tetrasaccharide derived from the action of NodA.

NodA Converts the Tetrasaccharide Precursor to a Hydrophobic Compound. When the radiolabeled deacetylated tetrasaccharide was incubated with cell extracts of *R. meliloti* containing the NodA protein, it was converted to a hydrophobic compound (Fig. 3, lanes 4 and 5) that migrated as a single radioactive spot on the TLC plate at a rate comparable to that of *R. meliloti* nodulation factor (lane 6). This radiolabeled hydrophobic compound was isolated from the incubation mixture prior to TLC analysis by butanol extraction, a procedure which is also used for the isolation of lipooligosaccharide Nod factors from bacterial culture media (2). In reaction mixtures containing extracts from *R. meliloti* cells in which *nodA* was not expressed due to a Tn*5* insertion, no formation of the hydrophobic product was observed (Fig. 3, lane 3). Furthermore, when radiolabeled nondeacetylated tetraacetylchitotetraose was used as a substrate in the assay mixture containing NodA, no hydrophobic compound was formed as judged by TLC (data not shown). This indicates that the oligosaccharide precursor must be deacetylated at the nonreducing end in order to serve as a substrate in acylation assays.

To explain the difference in migration on the silica plate between the tetrasaccharide precursor (Fig. 3, lane 2) and the NodA product (lanes 4, 5, and 10), we chemically N-acylated the terminal nonreducing glucosamine residue of the precursor molecule with fatty acids of various chain lengths (lanes 7–9). TLC analysis of these compounds, N-acylated with acetic ($C_{2:0}$; lane 7), caproic ($C_{6:0}$; lane 8), and palmitic ($C_{16:0}$; lane 9) acids, showed that migration of these synthetic substances depended on the length of the acyl chain attached to the tetrasaccharide backbone. The observation that the NodA conversion product (lanes 4, 5, and 10) migrated at a rate similar to that of palmitoylated tetrasaccharide (lane 9) and comigrated with *R. meliloti* nodulation factor (lane 6) strongly supports the idea that NodA is involved in transferring an acyl chain to the tetrasaccharide acceptor.

The NodA Product Comigrates on a Reversed-Phase HPLC Column with *R. meliloti* Nod Factor and Is Biologically Active. To identify the radiolabeled NodA product, a large-scale reaction was conducted as described in *Materials and Methods*. The product of the enzyme reaction was characterized by reversed-phase HPLC (Fig. 4). The elution profile of the butanol-extracted incubation mixture yielded two radioactive peaks (Fig. 4*B*, peaks 1 and 2) which were coeluted with sulfated and nonsulfated *R. meliloti* lipooligosaccharides, respectively (Fig. 4*A*). HPLC analysis also showed that the major radiolabeled compound (peak 2) exhibited a hydrophobicity similar to that of the synthetic $C_{16:0}$ acylated tetrasaccharide standard (arrowhead in Fig. 4*A*). No radioactivity signal was obtained when the reaction mixture contained cell extracts from *R. meliloti* carrying the transposon Tn*5* in *nodA* (data not shown). From these data we conclude that NodA converts the tetrasaccharide precursor to a hydrophobic compound which shows the same chromatographic properties on HPLC as the *R. meliloti* NodRm-IV lipooligosaccharide. Furthermore, the HPLC fraction corresponding to the nonsulfated NodRm-IV factor (peak 2, Fig. 4*B*) was tested for Had activity in different concentrations on vetch. In this bioassay the purified NodA product was able to elicit Had activity. It has been shown that tetraacetylchitotetraose is inactive in the Had bioassay (4), indicating that acylation of the chitooligosaccharide backbone with a specific fatty acid substituent is essential for biological activity (1). These data indicate that the NodA protein acted as an acyltransferase in these assays and transferred a specific long-chain fatty acid to the terminal nonreducing glucosamine residue of the tetrasaccharide precursor, resulting in the formation of a biologically active Nod factor.

Enzymatic Degradation of the Reaction Product. The fraction which comigrated with the Nod Rm-IV lipooligosaccharide (peak 2 in Fig. 4*B*) was pooled and digested with chitinase from *S. marcescens*. TLC analysis of the degradation prod-

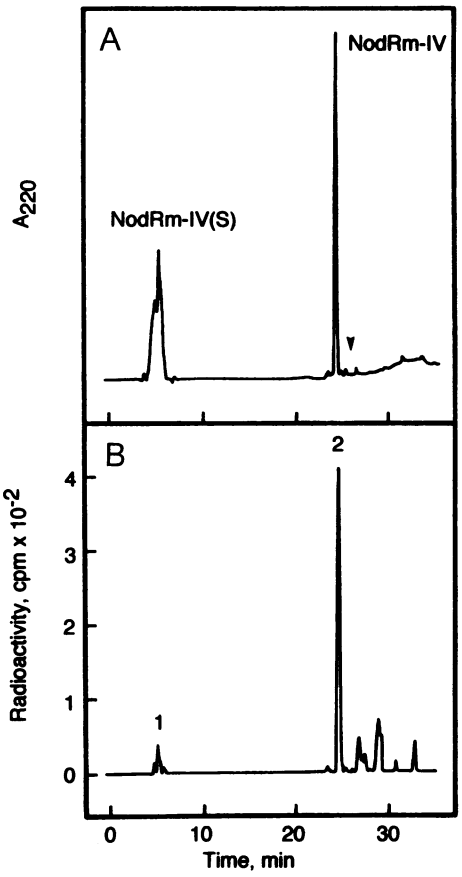

FIG. 4. Reversed-phase HPLC of acyl-transfer products formed from tri-*N*-[^{14}C]acetylglucosamine tetrasaccharide. Compounds were separated on a C_{18}-reversed phase column (1.0 cm × 25 cm, Ultrasphere ODS) and eluted with a linear acetonitrile gradient (30–80%) at a flow rate of 2.0 ml/min. (*A*) Elution profile of reference compounds monitored at 220 nm. NodRm-IV(S), β-1,4-linked 6-*O*-sulfated *N*-($C_{16:2}$)acyl-tri-*N*-acetyl-D-glucosamine tetrasaccharide; NodRm-IV, nonsulfated $C_{16:2}$ acylated tetrasaccharide; arrowhead indicates the position of β-1,4-*N*-hexadecanoyl-tri-*N*-acetyl-D-glucosamine tetrasaccharide. (*B*) Analysis of products synthesized from NodB-treated chitotetraose by the action of NodA. Incubation of the radiolabeled oligosaccharide substrate with cell extracts containing NodA protein and subsequent extraction of the acylated products is described in *Materials and Methods*. The chromatogram shows radioactivity detected by an on-line radioactivity monitor.

ucts showed that the NodA-acylated tetrasaccharide was completely hydrolyzed to radiolabeled *N*-acetylglucosamine, minor amounts of diacetylchitobiose, and lipid-linked di- or trisaccharides (Fig. 5, lane 3). The di- or trisaccharides bearing a lipid moiety migrated on the silica plate at a faster rate than the nondegraded NodA product (Fig. 5, lane 2). These findings confirmed the presence of a β-1,4 linkage between N-acetylglucosamine residues in the NodA-acylated tetrasaccharide and the presence of an acyl substituent at the nonreducing end of the remaining radiolabeled carbohydrate chain.

DISCUSSION

All rhizobia studied so far produce nodulation factors which are fatty N-acylated chitooligosaccharides (1). Our current studies were initiated to determine the steps involved in the attachment of the fatty acyl substituent to the chitooligosaccharide backbone. It seemed reasonable to assume that the N-acylated glucosamine oligosaccharide signals might be synthesized by the enzymatic acylation of a free amino group released by NodB at the nonreducing terminus of the oligo-

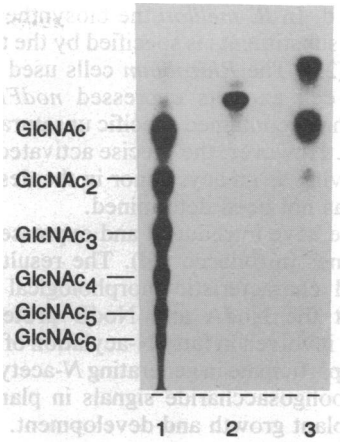

FIG. 5. TLC of radiolabeled products derived from the action of chitinase on acylated tri-*N*-[^{14}C]acetylglucosamine tetrasaccharide. Lane 1, reference compounds (see Fig. 3); lane 2, NodA-acylated tetrasaccharide isolated by HPLC (peak 2 in Fig. 4*B*); lane 3, digestion of the compound shown in lane 2 with chitinase from *S. marcescens*. For other details see Fig. 3.

saccharide backbone (1, 12). To demonstrate that acyl-group transfer was catalyzed by NodA, we synthesized a radiolabeled tetrasaccharide precursor carrying an amino group as a potential attachment site for N-acylation at the nonreducing glucosamine residue.

An acyl-transfer reaction was carried out with this specific substrate as acyl acceptor and rhizobial cell extracts as a source of enzyme and lipid donors. Our data show that cell extracts containing the NodA protein were able to convert this tetrasaccharide precursor into a butanol-extractable hydrophobic compound that comigrated on the TLC plate with *R. meliloti* lipooligosaccharide (Fig. 3, lanes 4–6). Moreover, this NodA conversion product migrated on a silica gel plate at a rate comparable to that of a tetrasaccharide bearing a palmitoyl substituent at the terminal glucosamine residue (Fig. 3, lanes 9 and 10).

The hydrophobic NodA product was retained by a C_{18} reversed-phase HPLC column and exhibited the same retention time as nonsulfated *R. meliloti* nodulation factor NodRm-IV ($C_{16:2}$; Fig. 4). To demonstrate that the hydrophobic product had the characteristic β-1,4 linkage of lipooligosaccharides, the HPLC-purified compound was digested with chitinase and yielded the expected degradation pattern (Fig. 5). Finally, the HPLC fraction that was coeluted with NodRm-IV was shown to elicit deformation of root hairs on vetch, which can be observed only with N-acylated chitooligosaccharides carrying a correct structure of the lipid moiety (4). The various criteria shown here demonstrate that NodA is involved in the incorporation of a fatty acyl chain into the synthetic tetrasaccharide acceptor to yield biologically active nodulation factor. Nevertheless, a computer search (GenBank and EMBL data libraries) revealed no significant homology of NodA to other N-acyltransferases.

We therefore propose that fatty N-acylation of an oligosaccharide backbone in Nod factor biosynthesis proceeds in two steps: (*i*) removal of the acetyl group at the nonreducing *N*-acetylglucosamine residue from a chitooligosaccharide precursor molecule by NodB and (*ii*) NodA-catalyzed attachment of a fatty acyl chain to the free amino group. One major sharp peak was obtained by HPLC fractionation of the NodA product that was coeluted with nonsulfated NodRm-IV ($C_{16:2}$; Fig. 4), which suggests a considerable acyl-chain specificity of the NodA transferase. Whether or not the terminal nonreducing glucosamine residue of the tetrasaccharide precursor was N-acylated with an unsaturated $C_{16:2}$ fatty acid carrying two (Δ2, 9) double bonds (2, 5, 6) remains

to be elucidated. In *R. meliloti* the biosynthesis of unsaturated fatty acid substituents is specified by the two host-range genes *nodFE* (23). The *Rhizobium* cells used for the preparation of the cell extracts expressed *nodFE* so that the extracts must have contained specific unsaturated fatty acids as acyl donors. However, the precise activated form of these fatty acids serving as an acyl donor in the described N-acylation assay has not been determined.

Recently, we have introduced and expressed the *R. meliloti nodAB* genes in tobacco (24). The resulting transgenic plants showed characteristic morphological abnormalities indicating that the NodA and NodB proteins, which in *Rhizobium* are involved in fatty N-acylation of chitooligosaccharides, may participate in generating *N*-acetylglucosamine-containing lipooligosaccharide signals in plants that somehow regulate plant growth and development.

We thank Michael Schultze, Institut des Sciences Végétales, Centre National dé la Recherche Scientifique, for providing sulfated NodRm-IV factor. We are grateful to Ton Bisseling (University of Wageningen, Wageningen, The Netherlands) for the supply of *V. sativa* seeds and to Kurt Stüber for computer analysis. We thank Bob Masterson for critical reading of the manuscript and Maret Kalda for photographic work. This work was supported by grants from the European Union (H.R.) and from Bundesministerium für Forschung und Technologie (J. Schmidt).

1. Dénarié, J. & Cullimore, J. (1993) *Cell* **74**, 951–954.
2. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C. & Dénarié, J. (1990) *Nature (London)* **344**, 781–784.
3. Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J. C. & Dénarié, J. (1991) *Nature (London)* **351**, 670–673.
4. 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. & Lugtenberg, B. J. J. (1991) *Nature (London)* **354**, 125–130.
5. Schultze, M., Quiclet-Sire, B., Kondorosi, E., Virelizier, H., Glushka, J. N., Endre, G., Géro, S. D. & Kondorosi, A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 192–196.
6. Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J. & Promé, J. C. (1991) *Cell* **67**, 1131–1143.
7. Price, N. P. J., Relic, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Dénarié, J., Promé, J. C. & Broughton, W. J. (1992) *Mol. Microbiol.* **6**, 3575–3584.
8. Sanjuan, J., Carlson, R. W., Spaink, H. P., Bhat, U. R., Barbour, W. M., Glushka, J. & Stacey, G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8789–8793.
9. Mergaert, P., Van Montagu, M., Promé, J. C. & Holsters, M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1551–1555.
10. Bulawa, C. E. & Wasco, W. (1991) *Nature (London)* **353**, 710.
11. Geremia, R. A., Mergaert, P., Geelen, D., Van Montagu, M. & Holsters, M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, in press.
12. John, M., Röhrig, H., Schmidt, J., Wieneke, U. & Schell, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 625–629.
13. Török, I., Kondorosi, E., Stepkowski, T., Posfai, J. & Kondorosi, A. (1984) *Nucleic Acids Res.* **12**, 9509–9524.
14. Schmidt, J., Wingender, R., John, M., Wieneke, U. & Schell, J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8578–8582.
15. Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. & Kondorosi, A. (1981) *Mol. Gen. Genet.* **184**, 318–325.
16. Kondorosi, E., Banfalvi, Z. & Kondorosi, A. (1984) *Mol. Gen. Genet.* **193**, 445–452.
17. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 3369–3373.
18. Schmidt, J., John, M. & Wandrey, C. (1981) *J. Chromatogr.* **213**, 151–155.
19. Walkley, J. W. & Tillmann, J. (1977) *J. Chromatogr.* **132**, 172–174.
20. Schmidt, J., John, M., Wieneke, U., Krüssmann, H.-D. & Schell, J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9581–9585.
21. Roche, P., Lerouge, P., Ponthus, C. & Promé, J. C. (1991) *J. Biol. Chem.* **266**, 10933–10940.
22. Shapira, R., Ordentlich, A., Chet, I. & Oppenheim, A. B. (1989) *Phytopathology* **79**, 1246–1249.
23. Demont, N., Debellé, F., Aurelle, H., Dénarié, J. & Promé, J. C. (1993) *J. Biol. Chem.* **268**, 20134–20142.
24. Schmidt, J., Röhrig, H., John, M., Wieneke, U., Stacey, G., Koncz, C. & Schell, J. (1993) *Plant J.* **4**, 651–658.