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Biosynthesis of *Rhizobium meliloti* lipooligosaccharide Nod factors: NodA is required for an N-acyltransferase activity

(nodulation/*nod* genes)

E. MORREY ATKINSON*, MONICA M. PALCIC†, OLE HINDSGAUL†, AND SHARON R. LONG*

*Department of Biological Sciences, Stanford University, Stanford, CA 94305; and †Department of Chemistry, University of Alberta, Edmonton, AB Canada T6G 2G2

Contributed by Sharon R. Long, April 20, 1994

ABSTRACT ***Rhizobium*** **bacteria synthesize N-acylated β-1,4-*N*-acetylglucosamine lipooligosaccharides, called Nod factors, which act as morphogenic signal molecules to legume roots during development of nitrogen-fixing nodules. The biosynthesis of Nod factors is genetically dependent upon the nodulation (*nod*) genes, including the common *nod* genes *nodABC*. We used the *Rhizobium meliloti* NodH sulfotransferase to prepare ^{35}S-labeled oligosaccharides which served as metabolic tracers for Nod enzyme activities. This approach provides a general method for following chitooligosaccharide modifications. We found *nodAB*-dependent conversion of *N*-acetylchitotetraose (chitotetraose) monosulfate into hydrophobic compounds which by chromatographic and chemical tests were equivalent to acylated Nod factors. Sequential incubation of labeled intermediates with *Escherichia coli* containing either NodA or NodB showed that NodB was required before NodA during Nod factor biosynthesis. The acylation activity was sensitive to oligosaccharide chain length, with chitotetraose serving as a better substrate than chitobiose or chitotriose. We constructed a putative Nod factor intermediate, GlcN-β1,4-(GlcNAc)$_3$, by enzymatic synthesis and labeled it by NodH-mediated sulfation to create a specific metabolic probe. Acylation of this oligosaccharide required only NodA. These results confirm previous reports that NodB is an N-deacetylase and suggest that NodA is an N-acyltransferase.**

In the symbiotic relationship between *Rhizobium* bacteria and leguminous host plants, a set of molecular signals is exchanged (1–3). The rhizobia have a set of *nod* genes that are required for the establishment of the root nodule symbiosis. These genes are grouped into three categories: common *nod* genes, required for nodulation and conserved in all of the nodulating bacteria; host-specific genes, which vary according to the bacterial cross-inoculation group; and regulatory *nod* genes, which activate *nod* gene expression, often in conjunction with plant-derived flavonoid inducers. The common and host-specific *nod* genes specify the production of signal molecules, the lipooligosaccharide Nod factors. The common *nod* genes are necessary for Nod factor production, while the host-specific *nod* genes are required for structural modifications which impart specificity to the various factors.

The Nod factors of diverse rhizobia share certain structural features (2, 4–8). All are β-1,4-*N*-acetyl-D-glucosamine (GlcNAc) oligosaccharides with various N-linked fatty acyl groups replacing the acetyl group on the nonreducing sugar residue. *Rhizobium meliloti* Nod factors also carry a 6-O sulfate moiety on the reducing terminus (4) (Fig. 1). The presence of the sulfate group requires the *nodH* gene in *R. meliloti* (9) and is a strong determinant of the specificity of the *R. meliloti* factors (9, 10). The *nodH* gene encodes an O-sulfotransferase which can specifically transfer a sulfate to the reducing-end GlcNAc of a GlcNAc oligosaccharide (ref. 9 and unpublished data).

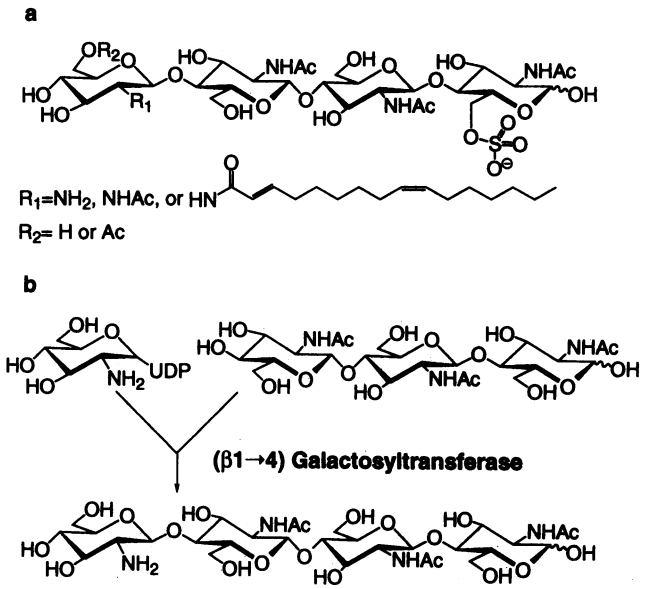

FIG. 1. Structure of substrates and products produced in this study. (*a*) Substrates for the acylation reaction were chitotetraose (R_1 = NHAc, R_2 = H) or GlcN-β1,4-(GlcNAc)$_3$ (R_1 = NH_2, R_2 = H) [^{35}S]monosulfate. Oligosaccharides were 6-*O*-[^{35}S]sulfated by NodH and used in acylation assays. Products of the *Rhizobium* assays included compounds which co-migrated with purified lipooligosaccharide standards NodRm-IV(S) (R_1 = C16:2 acyl group, R_2 = H) and NodRm-IV(Ac,S) (R_1 = C16:2 acyl group, R_2 = Ac). (*b*) GlcN-β1,4-(GlcNAc)$_3$ was prepared enzymatically with bovine β-1,4-galactosyltransferase from UDP-glucosamine and chitotriose.

The common *nod* genes were the first to be identified, because they are absolutely required for nodulation. Their central role in Nod factor production and in nodulation indicates that they encode unique and important functions. This study addresses the function of *nodA* and *nodB*, the first two genes in the common *nod* operon. It has been reported that *nodB* encodes an N-deacetylase that removes the nonreducing-end acetyl from a GlcNAc oligosaccharide (11). Here we report data that support this conclusion, and we show that *nodA* and *nodB* are required for an N-acyltransferase activity, which we detected using oligosaccharide substrates labeled with ^{35}S by NodH-mediated sulfation. This activity would account for the acylation seen on the various rhizobial Nod factors. The activity is sensitive to oligosaccharide chain length, preferentially acting upon chitotetraose. To separate the activities of the deacetylase and acyltransferase, we synthesized an N-deacetylated substrate, GlcN-β1,4-(GlcNAc)$_3$. Use of this substrate obviates the need for *nodB*

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Abbreviation: ACP, acyl carrier protein.

in the acylation process, showing that only *nodA* is required for derivatization of the free amino group.

MATERIALS AND METHODS

Bacterial Cultures. *R. meliloti* strains were grown in TY medium at 30°C under antibiotic selection to an OD_{600} of 1.0–1.2. Transposon Tn*5* insertion strains were grown in medium with neomycin (50 μg/ml). We used 3 μM luteolin and plasmid pRmE65, which expresses high levels of NodD3 protein (12), to maximize *nod* gene expression. *Escherichia coli* cells were grown in ACH medium (13) with ampicillin (50 μg/ml) at 30°C to an OD_{600} of 1.0–1.2.

Sulfation of Chitooligosaccharides. Radioactive oligosaccharide was prepared as follows: $Na_2[^{35}S]SO_4$ (2.5 mCi, 43 Ci/mg of S, ICN; 1 Ci = 37 GBq), 20 mM ATP, 3 mM GTP, inorganic pyrophosphatase (4 units; Sigma), partially purified adenosine-5′-phosphosulfate kinase (10 μg of protein), and yeast ATP sulfurylase (12 units; Sigma) were incubated in buffer [50 mM Tris, pH 8.0/30 mM KCl/5 mM $MgCl_2$/1 mM EDTA/10% (vol/vol) glycerol] at 30°C for 1 hr to produce 3′-phosphoadenosine 5′-phosphosulfate (PAPS). The products were analyzed by TLC on PEI-cellulose (14), and the labeled material was >95% [^{35}S]PAPS. Roughly 200 μCi of this material was incubated with 400 μg of chitotetraose (Seikagaku Kogyo, Tokyo) and 1 mg of partially purified NodH in buffer for 16 hr. The reaction mixture was boiled and dialyzed (molecular weight cutoff, 500; Spectrum) against 2 liters of distilled water. The chitotetraose [^{35}S]monosulfate was purified on a Mono Q anion-exchange column (Pharmacia) with ammonium acetate solvent, and purified fractions were lyophilized. Final activity of the substrate was 5 μCi/μl (≈4 pmol/μl). ^{35}S-labeled chitotriose and chitobiose were prepared in parallel with chitotetraose as described above, but the reactions were scaled down by a factor of 10. Sulfation was confirmed on TLC with PEI-cellulose plates and 0.9 M LiCl as the mobile phase.

***In Vivo* Preparation of ^{35}S-labeled Nod Factors.** For labeled Nod factors, a 500-ml *R. meliloti* culture (1021/pE65) was grown to saturation in RDM medium (15) supplemented with 3 μM luteolin in the presence of 500 μCi of inorganic [^{35}S]sulfate. The culture supernatant was extracted in batch with Bio-Beads SM16 (Bio-Rad). The labeled material was eluted with 80% methanol. Labeled compounds corresponded to purified Nod factor standards by reversed-phase HPLC and TLC analysis.

Assays with Permeabilized *R. meliloti*. Wild-type (1021/pRmE65), *nolI*::Tn*5* (TJ164/pRmE65), *nodN*::Tn*5* (TJ2A3/pRmE65), *nodA*::Tn*5* (TJ1A3/pRmE65), *nodB*::Tn*5* (TJ2B2/pRmE65), and *nodC*::Tn*5* (TJ170/pRmE65) *R. meliloti* (16) were assayed with 5 μCi of chitotetraose [^{35}S]monosulfate in 100-μl aliquots by the method of Reuber and Walker (17). Briefly, cells were washed twice with ice-cold 70 mM Tris (pH 8.2) and resuspended in 0.01 volume of cold 70 mM Tris, pH 8.2/2.5 mM EDTA. $MgCl_2$ was added to 5 mM, followed by 5 μCi of chitotetraose [^{35}S]monosulfate. The cells were frozen and thawed three times in liquid nitrogen. The assay mixture was incubated for 2 hr at 15°C. The reaction was stopped with 0.5 ml of 70 mM Tris, pH 7.2/2.5 mM EDTA. The solid material was pelleted in a microcentrifuge and the pellet was extracted twice with 100 μl of chloroform/methanol/water, 10:20:3 (vol/vol), and the extract was dried. Reactions were normalized to cell density (OD_{600}).

Assays with Permeabilized *E. coli*. *E. coli* strain HB101 was transformed with plasmids expressing *nodA* and *nodB* (pE40), *nodA* (pE45), or *nodB* (pE41) or with the expression vector alone (pAD10) (13). Cells were assayed as described for *Rhizobium* assays. For sequential analysis, permeabilized cells were incubated with 10 μCi of chitotetraose [^{35}S]monosulfate and disrupted after 2 hr by sonication for 10 min followed with boiling for 10 min, repeated three times each. The particulate material was pelleted in a microcentrifuge, and the cooled supernatant was used to suspend the secondary test cells. This mixture was incubated at 15°C for 2 hr, and the products were extracted and analyzed by TLC. For mild alkaline hydrolysis, reaction products were treated with concentrated NH_4OH/1-propanol, 1:1, at 40°C for 16 hr. The hydrolysis products were extracted with 1-butanol and resolved by TLC.

Chromatographic Analysis of Assay Products. For TLC analysis, dried extracts were suspended in 3 μl of chloroform/methanol/water, 10:20:3, and applied directly to 10-cm silica-gel 60 high-performance TLC plates (Merck). The plates were developed in chloroform/methanol/water/acetic acid, 25:15:4:2, for 8–9 cm. The plates were dried and exposed to x-ray film.

For HPLC analysis, assay products were extracted with 0.5 ml of 1-butanol, dried under vacuum, and suspended in 20% acetonitrile/0.1% trifluoroacetic acid. This suspension was injected onto a Beckman 421 series HPLC equipped with a C_{18} reverse-phase column (250 mm × 4.6 mm i.d.) at 1 ml/min. The compounds were eluted with a shallow gradient from 20% to 35% acetonitrile. Fractions of 1 ml each were collected and radioactive disintegrations were counted for 10 min in 5 ml of Ecolume scintillation cocktail in a Beckman LS6000IC scintillation counter, while Nod factor standards were detected by UV absorbance at 215 nm.

Preparation and Assay of GlcN-β1,4-(GlcNAc)$_3$. GlcN-β1,4-(GlcNAc)$_3$ was prepared from UDP-glucosamine and chitotriose by using bovine galactosyltransferase (18) as follows: commercial chitotriose (5.4 mg) was incubated with synthetic UDP-glucosamine (9.1 mg) and galactosyltransferase (5 units; Sigma) in 1.5 ml of buffer (25 mM Mes, pH 7.4/5 mM $MnCl_2$) at 37°C for 48 hr; 2.5 units of enzyme and 4 units of enzyme were added at two 24-hr intervals, for a total incubation time of 96 hr. TLC of the reaction mixture indicated conversion of the starting chitotriose (R_f 0.56) to a 20–30% yield of slower-migrating ninhydrin-positive material (R_f 0.40). The crude product was purified on a Bio-Gel P2 column (Bio-Rad) with aqueous 10% ethanol and then on a Partisil PAC HPLC column (Waters) with acetonitrile/water, 4:1. The structure of the oligosaccharide was confirmed by ^{1}H NMR analysis (500 MHz, ^2H_2O) and fast-atom bombardment MS. The oligosaccharide was sulfated at a 10-fold lower scale.

RESULTS

Incorporation of Chitotetraose Monosulfate into Nod Factors. To investigate the role of the *nod* genes in Nod factor synthesis, we employed a radiolabeled oligosaccharide substrate to trace the enzymatic steps in the pathway. The *R. meliloti* NodH protein is a chitooligosaccharide 6-*O*-sulfotransferase enzyme (unpublished data) and in this study we used NodH to label chitotetraose or its mono-*N*-deacetylated derivative (Fig. 1) with [^{35}S]sulfate in order to follow the synthesis of Nod factors in an *in vitro* assay. We compared wild-type bacteria with mutant *R. meliloti* carrying deletions of or Tn*5* insertions in various *nod* genes (Fig. 2). The permeabilized-cell-assay products were analyzed by TLC and HPLC using purified Nod factors as chromatographic standards.

We observed the modification of chitotetraose [^{35}S]monosulfate into several species that comigrated with ^{35}S-labeled Nod factors in TLC (Fig. 3). This reaction depended on *nodA* and *nodB* but not on other *nod* genes, including *nodC*. The picomolar scale of the reactions precluded direct spectroscopic analysis, so we chose cochromatography with structurally characterized Nod factor standards to verify the identity of the products. The compounds were analyzed by

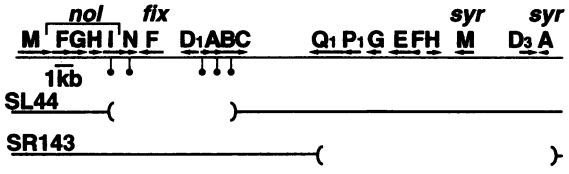

FIG. 2. Genetic map of the *R. meliloti nod* region. Map shows the relevant deletions and Tn*5* insertions (↓) used in this study. Deletion SL44 removes the common *nod* genes and part of the *nol* operon (12). Deletion SR143 removes the host-specific *nod* genes (19).

C_{18} HPLC using a stringent gradient which clearly differentiates between lipooligosaccharides containing minor structural differences, such as NodRm-IV(S), from its 6-O-acetylated cognate, NodRm-IV(Ac,S) (Fig. 4*a*, peaks A and B, respectively). Assay extract from a wild-type strain contained several labeled species (Fig. 4*b*), and two prominent products had retention times identical to the NodRm-IV(S) (peak A) and NodRm-IV(Ac,S) (peak B) factors. Extract from a *nodA*::Tn*5* strain contained no labeled compounds retained on the column (Fig. 4*c*). We further confirmed the sensitivity of the assay, and the acyl nature of the modification that converts chitotetraose to a hydrophobic compound, by analysis of products from a *nodFE* mutant strain which should cause a known structural modification in the acyl group of the Nod factor (20). Deletion strain SR143 (19), which lacks the host-specific genes *nodH*, *nodFEG*, and *nodPQ* (Fig. 2), does produce common *nod* gene-specific products (asterisks) but fails to produce compounds with the same retention as the two Nod factor standards (Fig. 4*d*). The *nodH* and *nodPQ* sulfurylation defects should have no effect, as the precursor in the assay reaction is already sulfated (9, 14, 19). Since *nodFE*$^-$ *R. meliloti* produce lipooligosaccharides with altered acyl substitutions, *nodFE* genes are proposed to play a direct role in the synthesis of the specific acyl groups (8, 20). NodF is probably a specialized acyl carrier protein (ACP) (21, 22), and NodE most likely corresponds to a specialized condensing enzyme (20, 23). Our observation of alternative hydrophobic products in *nodAB*$^+$*nodFE*$^-$ *R. meliloti* is therefore consistent with the conclusion that NodA and NodB carry out acylation but that the structure of the acyl group is determined by NodF and NodE. This analysis indicates that wild-type cells incorporate chitotetraose [^{35}S]monosulfate into Nod factor in a *nodA*-dependent manner.

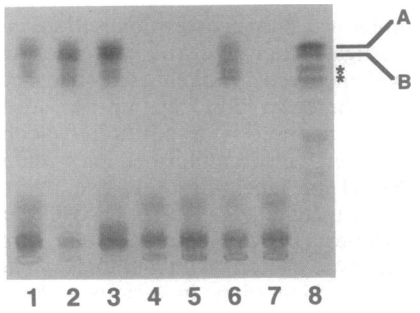

FIG. 3. TLC analysis of ^{35}S-labeled *R. meliloti* assay products. Silica-gel TLC lanes correspond to acylation of chitotetraose [^{35}S]monosulfate by wild-type *R. meliloti* (lane 1), *nolI*::Tn*5* (lane 2), *nodN*::Tn*5* (lane 3), *nodA*::Tn*5* (lane 4), *nodB*::Tn*5* (lane 5), *nodC*::Tn*5* (lane 6) (16), deletion SL44 (lane 7), or *in vivo* labeled Nod factors (lane 8). The acylation activity was absent only in the *nodA*::Tn*5*, *nodB*::Tn*5*, and common *nod* gene deletion strain. In lane 8, the NodRm-IV(Ac,S) (A, R_f 0.64) and NodRm-IV(S) (B, R_f 0.61) factors align with major products of the positive reactions. Asterisks indicate putative lipooligosaccharide species, varying possibly by specific acyl group or degree of polymerization.

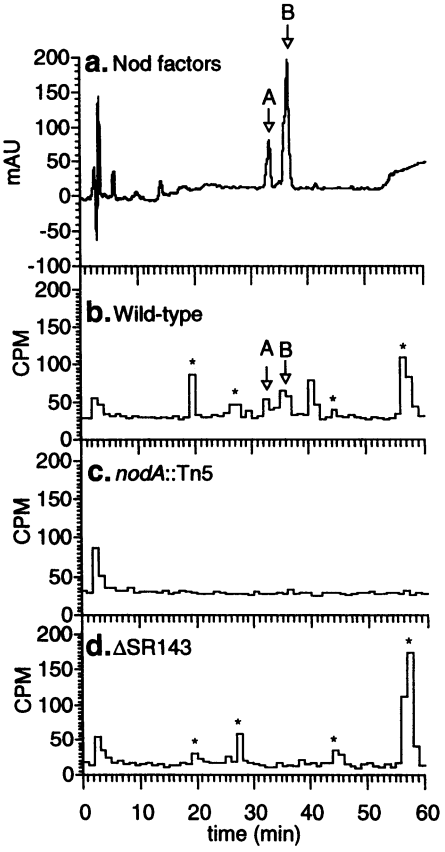

FIG. 4. HPLC analysis of reaction products. Products of acylation assays performed on wild-type *R. meliloti* (*b*), a *nodA*::Tn*5* insertion strain (*c*), and SR143, a host-specific-deletion strain (*d*) (19), were resolved on a C_{18} HPLC column. The wild-type reaction extract was coinjected with purified Nod factor standards (*a*). Two principal products of the wild-type reaction (*b*, peaks A and B) were coeluted with the Nod factors, NodRm-IV(S) (32.5 min) and NodRm-IV(Ac,S) (36.0 min), respectively. Asterisks indicate *nodA*-dependent peaks that do not correspond to NodRm-IV(Ac,S) or NodRm-IV(S) and have not been structurally characterized. Direct ^{35}S labeling is more sensitive than the UV absorption usually employed to detect Nod factors (4, 6–8), and these peaks may correspond to related lipooligosaccharides. mAU, absorbance milliunits.

***E. coli* Expressing *nod* Genes.** To separate the functions of NodA and NodB, we assayed these proteins in *E. coli*. Cells expressing both NodA and NodB converted chitotetraose [^{35}S]monosulfate to a hydrophobic compound (Fig. 5*a*, lane 2), whereas cells expressing either protein alone lacked this activity (Fig. 5*a*, lanes 3 and 4). The relative mobility on TLC of the observed product from *E. coli* differed from that of the *R. meliloti* products (R_f 0.30 vs. R_f 0.61–0.64), suggesting that the hydrophobic product carries a different acyl group, as expected from the fact that *E. coli* does not have *nodFE*. In the absence of a known standard to use for chromatographic analysis, we tested the nature of the linkage by mild alkaline treatment of the product. We found that it was not sensitive to mild alkaline hydrolysis (data not shown), from which we infer that the acylation is N- rather than O-linked.

The *E. coli* expression system also allowed us to address the order of reactions in acylation by means of sequential incubations in *E. coli* extracts expressing either NodA or NodB. Assay products extracted from a NodA reaction and subsequently incubated with a NodB extract yielded no hydrophobic products (Fig. 5*a*, lane 5). Assay products from a NodB reaction added to a NodA extract produced the hydrophobic oligosaccharide derivatives (Fig. 5*a*, lane 6).

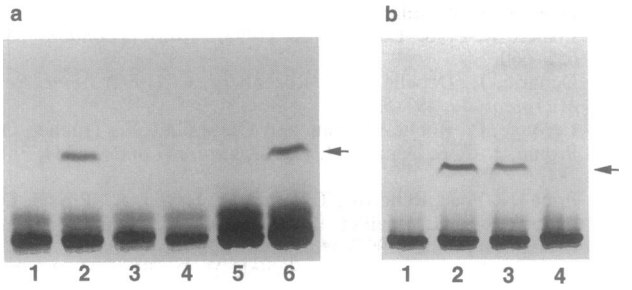

FIG. 5. TLC analysis of permeabilized *E. coli* expressing *nod* genes. (*a*) Acylation of chitotetraose [^{35}S]monosulfate by *E. coli* expressing no *nod* genes (lane 1), *nodAB* (lane 2), *nodA* (lane 3), or *nodB* (lane 4). Products extracted from sequential incubations with NodA followed by NodB, and with NodB followed by NodA, are shown in lanes 5 and 6, respectively. Hydrophobic products are indicated with an arrow (R_f 0.30). (*b*) Acylation assays were performed with [^{35}S]-GlcN-β-1,4-(GlcNAc)$_3$ in *E. coli* expressing *nod* genes. Lanes are the same as in *a*.

This suggests that the acylation of chitotetraose monosulfate requires NodB prior to NodA and that the two enzymes do not obligatorily act as a complex. The action of NodB on the substrate produces an intermediate which can then be derivatized by the NodA permeabilized-cell extract.

GlcN-β1,4-(GlcNAc)$_3$ as a Substrate. We sought to establish more specifically the function of NodA as opposed to NodB. The results of John *et al.* (11) suggest that NodB functions as an N-deacetylase. If so, preincubation of chitotetraose monosulfate with a NodB extract should produce GlcN-β1,4-(GlcNAc)$_3$, in which the nonreducing-end residue lacks the N-acetyl group. We synthesized the oligosaccharide GlcN-β1,4-(GlcNAc)$_3$ by utilizing the loose substrate specificity of galactosyltransferase (18) to add glucosamine to the nonreducing end of chitotriose (Fig. 1). This produced the desired GlcN-β1,4-(GlcNAc)$_3$, which we then purified and [^{35}S]sulfate-labeled by means of NodH. Using this substrate in the permeabilized-cell assay of *E. coli* expressing NodA or NodB, we found that NodA alone was now necessary and sufficient for conversion of the precursor into the hydrophobic product (Fig. 5*b*, lane 3). Preincubation with NodB was unnecessary, and NodB alone had no detectable effect (Fig. 5*b*, lane 4). These data support the conclusion that NodB removes an N-acetyl group, allowing the transfer of the acyl group by NodA.

GlcNAc Oligosaccharides as Substrates for Acylation. We tested the ability of different-length chitooligosaccharides to serve as substrates in the acylation assay. NodB has been shown to deacetylate different GlcNAc chain lengths, including chitobiose, chitotriose, and chitotetraose (11). NodH can transfer sulfate to the reducing end of GlcNAc oligosaccharides up to six sugar units in length (D. Ehrhardt and S.R.L., unpublished data). To test NodA specificity, we used NodH to create [^{35}S]sulfate-labeled chitobiose, chitotriose, and chitotetraose as model substrates. Fig. 6 shows TLC analysis of permeabilized *E. coli* expressing NodA and NodB using these oligosaccharides as substrates. Only the chitotetraose was derivatized to a detectable level (lane 3); neither chitobiose nor chitotriose was acylated (lanes 1 and 2). This indicates that NodA is sensitive to oligosaccharide chain length and may determine the oligosaccharide chain-length specificity of the acylation reaction.

DISCUSSION

The *nodABC* genes are referred to as common *nod* genes because they are found in all rhizobia studied so far and are functionally interchangeable between species. Nod factors, which cause nodule-like reactions on specific host plants (24),

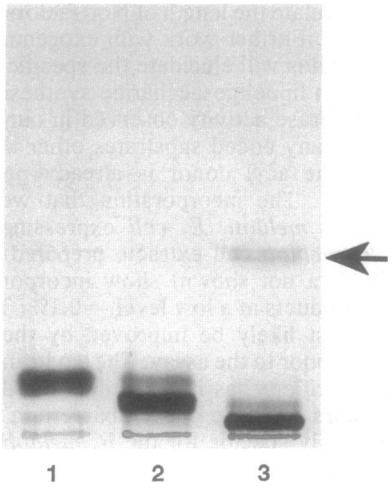

FIG. 6. TLC analysis of different-length oligosaccharide substrates. ^{35}S-labeled chitobiose (lane 1), chitotriose (lane 2), and chitotetraose (lane 3) were used as substrates for permeabilized *E. coli* expressing NodA and NodB (HB101/pE40). Only the reaction with ^{35}S-labeled chitotetraose showed a detectable amount of product (arrow, R_f 0.31).

are synthesized by *Rhizobium* only if *nodABC* are present (4), which indicates a central role of lipooligosaccharides in the nodulation process. The conservation of the *nodABC* operon predicts that the outcome of NodABC activity would also be conserved in the Nod factor structures. Homology suggests that NodC is a β-1,4-synthase (25, 26), and it is clear that the β-1,4 glycosidic linkage is conserved in the Nod factors (1, 2). The *nodA* and *nodB* genes most likely specify similarly conserved domains in the lipooligosaccharide structure. Comparison of various Nod factor structures indicates that N-acylation is a similarity between the different lipooligosaccharides, even if the nature of the acyl group varies widely; NodA and NodB could be involved in the creation of this linkage.

To replace an N-acetyl group with an N-fatty acyl group, the acetyl moiety must first be removed. NodB has been shown to have N-deacetylase activity (11) as well as homology to fungal deacetylase genes (27). Our results confirm this observation: acylation of the oligosaccharide requires NodB first (Fig. 5*a*). NodB is not required if the acetyl group is absent–i.e., the nonreducing sugar in the oligosaccharide is glucosamine (Fig. 5*b*). NodB therefore prepares the amino-group target for the acyltransferase. The subsequent step in lipooligosaccharide acylation would be the actual addition of the lipid to the oligosaccharide. NodA is the logical candidate for this process. Our data strongly support this conclusion: NodA is required for the activity (Fig. 3), and transfer of NodA alone to *E. coli* transfers the acylation activity (Fig. 5*b*).

The exact sequence of the steps in Nod factor synthesis and modification remains to be determined, as well as the *in vivo* nature and location of the intermediates. Our data indicate that the action of NodA follows that of NodC, and polymerized GlcNAc must therefore be available for the acylation reaction, either in the cytoplasm or in the membrane (28, 29). Nod enzymes may possibly act as a complex *in vivo*, as has been suggested based on localization (28) and the overlap of NodA and NodB open reading frames in *R. meliloti* (30). That NodA and NodB do not need to be present in the same extract indicates that these proteins do not obligatorily act as a complex. Nod factors vary from three to five GlcNAc residues in length (1), and our data indicate that the acylation activity we observe is sensitive to oligosaccharide chain length. If NodA determines this specificity, it

could serve to regulate the length of Nod factors produced by different rhizobia. Further work with exogenous substrates and purified proteins will elucidate the specific mechanisms of the reactions in lipooligosaccharide synthesis.

The acyltransferase activity observed in our experiments does not require any added substrates other than an oligosaccharide, so the acyl donor is already present in the bacterial extracts. The incorporation that we observe in permeabilized *R. meliloti, E. coli* expressing NodA and NodB, and *R. meliloti* cell extracts prepared in a French pressure cell (data not shown) show incorporation of the substrate into products at a low level, ≈0.1%. This incorporation would most likely be improved by the addition of exogenous acyl donor to the assay. The acyltransferase must have a loose specificity, as it can utilize the different acyl groups and donors available in each bacterium and is therefore not necessarily specific for the *R. meliloti* C16:2 acyl-ACP; it can apparently use cellular pools of acyl donor to some extent. This is consistent with the observation of heterogeneity in acyl side chains on *R. meliloti* factors (31). The endogenous substrate could be derived from available *Rhizobium* and *E. coli* acyl-ACP, the presumptive acyl donor in the reaction. NodF is most likely an ACP (21, 22); it may serve as the primary acyl donor in Nod factor synthesis.

We found that two major products of our assays with *R. meliloti* cells are equivalent to purified Nod factor standards by HPLC analysis. Other hydrophobic derivatives of chitotetraose monosulfate were also seen (Fig. 4 *b* and *c*); these may result from degradation, polymerization, acetylation, or alternative acylation, producing various compounds reported as Nod factors in *R. meliloti* (7, 9). Mutations in the SR143 deletion strain known to affect the nature of the lipooligosaccharide acyl group (20) changed the chromatographic behavior of the sulfated products detected in our assay; this supports the conclusion that the wild-type products (Fig. 4, peaks A and B) carry the correct Nod factor acyl group.

This study provides a framework from which to study and manipulate the synthesis of this symbiotic signal molecule. The techniques reported here should be of use to researchers studying various carbohydrates, and the use of NodH for *in vitro* sulfation should prove a general approach to direct demonstration of modifications to various β-1,4-GlcNAc oligosaccharides. *In vitro* use of common as well as host-specific Nod enzymes should lead to the synthesis of tailored molecules to test Nod factor specificity and activity in understanding the development of the root nodule.

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