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Biosynthesis of the Nod Factor Chito-oligosaccharide Backbone in *Rhizobium fredii* is Controlled by the Concentration of UDP-*N*-acetyl-D-glucosamine

Nora Iñón de Iannino,[1] Steven G. Pueppke,[2] and Rodolfo A. Ugalde[1]

[1]Instituto de Investigaciones Bioquímicas, Fundación Campomar, CONICET, Universidad de Buenos Aires.1405, Buenos Aires, Argentina; [2]Department of Plant Pathology, University of Missouri, Columbia, 65211, U.S.A.

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A cell-free system was used to study the biochemical properties of the β(1-4) *N*-acetyl-D-glucosaminyl transferase that forms the Nod factor chito-oligosaccharide backbone in *Rhizobium fredii*. Total membrane fractions prepared from a genistein-induced culture of wild-type *R. fredii* USDA257 incorporated *N*-acetyl-D-glucosamine at the rate of 6.0 nmoles · h^{-1} · mg^{-1} protein into a β(1-4) *N*-acetyl-D-glucosamine pentasaccharide. The Km for the substrate UDP-*N*-acetyl-D-glucosamine was 42 μM. For maximal activity, the reaction required free *N*-acetyl-D-glucosamine that could not be substituted by glucosamine, *N*-acetyl-D-galactosamine, or galactosamine. Chain elongation of the oligosaccharide proceeded toward the nonreducing end. Pulse-chase experiments demonstrated that the reaction follows a single chain mechanism; free chitobiose, chitotriose or chitotetraose were not substrates for elongation and/or initiation. The concentration of UDP-*N*-acetyl-D-glucosamine controlled the degree of polymerization of the chito-oligosaccharide formed in vitro. At low concentration (0.1 μM) the main product was chitobiose and chitotriose, and at concentrations higher than 10 μM, chitopentaose was the main product. Polyoxln D (200 μM) had no effect on the synthesis and or the degree of polymerization of the chito-oligosaccharide formed.

Additional keywords: chitin synthase, Nod C, *nod* gene induction, nodulation.

Bacteria of the genus *Rhizobium* interact with leguminous plants, inducing the formation of root nodules in which the bacteria fix nitrogen symbiotically (Long 1989). Root hair deformation, and sometimes nodule meristems, are elicited by signals secreted by *Rhizobium* and called Nod factors (Kondorosi 1991; Lerouge 1994; Spaink et al. 1991a). These compounds are lipo-oligosaccharides formed by a chito-oligosaccharide backbone with degrees of polymerization ranging from three to five and a fatty acid side chain at the nonreducing end of the oligosaccharide. (Lerouge et al. 1990; Price et al. 1992; Schulze et al. 1992). The nature of this side chain (Demont et al. 1993, 1994; Spaink et al. 1991b) in conjunction with modification of this basic structure with sulfate (Lerouge et al. 1990; Roche et al. 1991), carbamoyl groups or sugars (Mergaert et al. 1993; Price et al. 1992; Sanjuan et al. 1992), methyl groups (Geelen et al. 1993), and *O*-acetyl groups (Firmin et al. 1993) helps to determine the specificity displayed by different rhizobia.

Rhizobium genes required for the synthesis of Nod factors (*nod* genes) have been classified into three categories: the common *nod,* the host-specific *nod,* and the regulatory *nod* genes (Göttfert 1993). The common *nod* genes are structurally conserved and functionally interchangeable between *Rhizobium* species. Inactivation of these genes abolishes the ability to elicit all symbiotic reactions on the plant (Long 1989). The common *nod* genes (*nodABC*) are involved in the synthesis of the lipo-oligosaccharide backbone of Nod factors, whereas the host-specific *nod* genes (sometimes termed *hsn*) are responsible for the modifications of the chito-oligosaccharide basic structure that determine plant specificity (Lerouge 1994). The regulatory *nod* genes are expressed constitutively and their products control the expression of common and host-specific nod genes upon interaction with plant factors (Dénarié et al. 1992; Göttfert 1993).

Protein homology and direct measurement of enzymatic activity indicates that the *nodC* gene encodes a β(1-4) *N*-acetylglucosaminyl transferase that builds up the chito-oligosaccharide chain (Atkinson and Long 1992; Bulawa and Wasco 1991; Debellé et al. 1992; Dougherty and van de Rijn, 1994; Geremia et al. 1994). *nodB* encodes a specific chito-oligosaccharide deacetylase that removes the acetyl group from the *N*-acetylglucosamine residue at the nonreducing end of the chito-oligosaccharide backbone (John et al. 1993). The NodA protein appears to be involved in transferring the acyl chain to the free amino group on the nonreducing glucosamine residue of the chito-oligosaccharide synthesized by NodC and NodB proteins (Lerouge 1994; Röhrig et al. 1994). Host-specific nodulation genes are not present in all rhizobia, and inactivation results in an alteration of host range. This has been correlated with changes in the side groups of Nod factors. The alleles of *nodD* function as regulatory genes, controlling the activities of genes in the other two classes by perceiving flavonoid signals from the host and activating transcription (Long 1989).

Corresponding author's present address: Rodolfo A. Ugalde, Patricias Argentinas 435, 1405 Buenos Aires, Argentina.

The biosynthesis of chitin, a β(1-4) *N*-acetyl-D-glucosaminyl polymer, has been studied in cell-free fungal systems (Glaser and Brown 1957; Keller and Cabib 1971; McMurrough and Bartniki-Garcia 1971; Porter and Jaworski 1966; Plessmann Camargo et al. 1967; Rousset-Hall and Gooday 1975; Ruiz-Herrera et al. 1977; Ryder and Peberdy 1977). Three different chitin synthetases have been described in *Saccharomyces cerevisiae* (Bulawa and Osmond 1990; Bulawa 1993; Orlean 1987; Sburlati and Cabib 1986). All of them are membrane-bound enzymes that use UDP-*N*-acetyl-D-glucosamine as sugar donor. NodC protein shares homology with yeast chitin synthetase (Atkinson and Long 1992; Bulawa and Wasco 1991; Debellé et al. 1992), yet it apparently synthesizes only oligomers and not long polymers. This observation raises interesting questions that can only be solved by studying the kinetics and other properties of the chito-oligosaccharide synthetase of rhizobia. We have carried out a series of experiments on the Kinetics and biochemical properties of chito-oligosaccharide biosynthesis in *R. fredii* strain USDA257, a model symbiont that has both broad host range for nodulation of legume species and cultivar specificity for nodulation of soybean (Keyser et al. 1982; Heron and Pueppke 1984; Krishnan and Pueppke 1994).

RESULTS

In vivo formation of Nod factors.

Cultures of *R. fredii* USDA191 and USDA257 were induced with the flavonoid signal genistein (Krishnan and Pueppke 1993) and labeled with sodium [^{14}C]-acetate. Butanol extracts were subjected to thin-layer chromatography (TLC), and products detected by autoradiography. As shown in Figure 1, four products with R_f values corresponding to Nod factors were observed (Spaink et al. 1992). The formation of these products was not observed when wild-type cells were grown in the absence of genistein or when a *nodABC* mutant, 257B3, was grown with or without genistein, thus indicating that they were Nod factors. (Fig. 1A, lanes 1, 3, 5, and 6). The capacity for induction of *nod* genes seemed to be a transient process, since very low accumulation of labeled Nod factors was observed when the induction was carried out with nondividing cells (Fig. 1B, lane 6). It can be seen in Figure 1B lanes 3 and 4 that labeling for 16 h did not increase the amount of labeled Nod factors recovered, confirming the conclusion that the formation of Nod factors is a transient process. These results indicate that for studying the in vitro synthesis of Nod factors, preparations of membranes from cells in the early exponential phase should be used.

In vitro formation of chito-oligosaccharides.

Incubation of membranes prepared from strain USDA257 induced with 2 μM genistein in the presence of UDP-*N*-[^{3}H]-acetyl-D-glucosamine led to the incorporation of radioactivity into water- soluble products. As shown in Table 1, incorporation was only observed with membranes from genistein induced cells, thus suggesting that the water-soluble products could be related to Nod factors. The low levels of incorporation of radioactivity into compounds soluble in the lower phase of chloroform/methanol/water (3:2:1) partitions or into compounds extracted by chloroform/methanol/water (1:2:0.3) from the proteinaceous interphases were not genistein-depen-

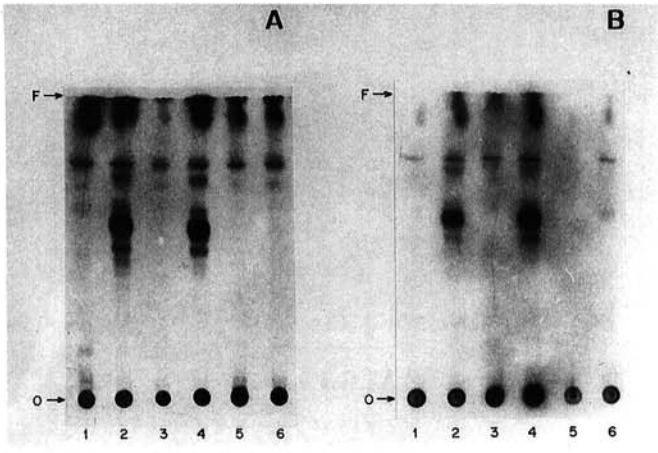

Fig. 1. Thin-layer chromatography (TLC) of *Rhizobium fredii* Nod factors. Cells were grown, induced with genistein and labeled with sodium [^{14}C]-acetate as described in Materials and Methods. Butanolic extracts were subjected to TLC on C18-coated silica gel plates with acetonitrile/water (1:1) as solvent and exposed to X-Omat AR films. **A,** Strain-specificity of the response. Extracts of *R. fredii* USDA191, not induced (lane 1) or induced (lane 2); extracts of strain *R. fredii* USDA257, not induced (lane 3) or induced (lane 4); extracts of strain *R. fredii* 257B3 (*nodABC*-mutant), not induced (lane 5) or induced (lane 6). Cell were induced with genistein at A_{625} = 0.4. **B,** Effect of culture age and labeling time with *R. fredii* U5DA191. Cultures (A_{625} = 0.4) not induced (lane 1) or induced (lane 2) were labeled with sodium [^{14}C]-acetate for 4 h. Cultures (A_{625} = 0.4) not induced (lane 3) or induced (lane 4) were labeled with sodium [^{14}C]-acetate for 16 h. Cultures (A_{625} = 0.7) not induced (lane 5) or induced (lane 6) were labeled with sodium [14C]-acetate for 4 h. (O): origin; (F): solvent front.

Table 1. Solubility characteristic of *N*-acetyl-D-glucosamine incorporated by membranes from *Rhizobium fredii* USDA257[a]

	Incorporation (dpm per hour)			
Flavonoid induction	**Water-soluble phase**	**Lower phase soluble**	**1203 phase soluble**	**Insoluble residue**
No	<40	180	250	<40
Yes	48,600	<40	150	<40

[a] Reactions were carried out by method (ii) as described in Materials and Methods, with a 10-μM final concentration of UDP-*N*-acetyl-D-glucosamine. Incorporation of *N*-[^{3}H]-acetyl-D-glucosamine into the different fractions as dpm per hour was determined by counting in a liquid scintillator with Bray's solution.

Table 2. Effect of the addition of sugars and amino sugars[a]

Additions	**Activitiy (pmol/h)**
40 mM *N*-acetyl-D-glucosamine	546.0
40 mM D-glucosamine	73.7
40 mM *N*-acetyl-D-galactosamine	64.6
40 mM D-galactosamine	141.0
40 mM D-glucose	64.8
40 mM 2-deoxy-D-glucose	111.9
20 mM chitobiose	141.1
20 mM chitotriose	91.0

[a] Reactions were carried out by method (i) as described in Materials and Methods, with 50 μM UDP-*N*-acetyl-D-glucosamine. Control reaction without sugars or amino sugars incorporated 3,993 dpm in 15 min, which corresponbded to 91.0 pmol/h. Most values are the means of at least two determinations.

dent, and so lipid intermediates may not be involved during the formation of the water-soluble products. No accumulation of radioactivity was observed in water-insoluble or organic solvent–insoluble compounds, indicating that insoluble chitinlike products were absent (Table 1). Polyacrylamide gel electrophoresis of the TCA-insoluble fraction revealed that no proteins became labeled with *N*-acetyl-D-glucosamine during the reaction (data not shown). Thus, there is no evidence for the formation of protein intermediates.

Cation requirement and the effect of saccharides.

The formation of water-soluble compounds depended on the presence of divalent cations in the reaction mixture, since 10 mM EDTA completely abolished the incorporation. Highest level of activity was obtained in the presence of 10 mM of $MgCl_2$, but it could be partially substituted by 10 mM of $CaCl_2$ (40%) or 10 mM of $MnCl_2$ (27%).

A variety of sugars and amino sugars were tested for their effect on the incorporation of radioactivity into water-soluble compounds (Table 2). The presence of 40 mM *N*-acetyl-D-glucosamine stimulated the incorporation of radioactivity between 6- and 10-fold (Fig. 2, Table 2). The addition of D-glucosamine, *N*-acetyl-D-galactosamine, and D-glucose inhibited from 20 to 30%; D-galactosamine, 2-deoxi-D-glucose, chitobiose stimulated from 20 to 55%, and chitotriose had no effect on the formation of the water-soluble compound. These results indicated that *N*-acetyl-D-glucosamine is the most effective activator of the reaction.

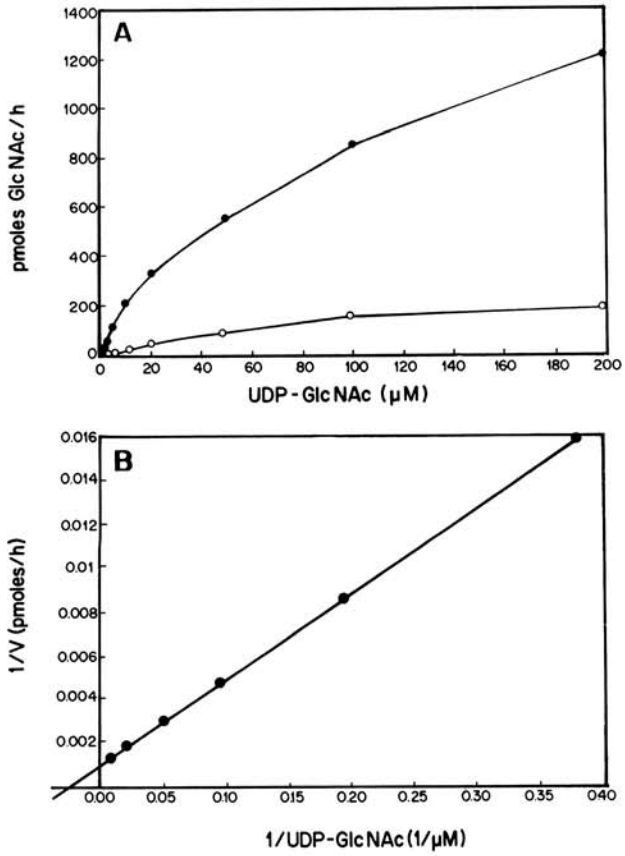

Fig. 2. Synthesis of chito-oligosaccharide at different concentrations of UDP-*N*-acetyl-D-glucosamine. **A,** open circles: no addition of *N*-acetyl-D-glucosamine; closed circles: with 40 mM *N*-acetyl-D-glucosamine. Panel B shows the reciprocal plot of the reaction carried out with 40 mM *N*-acetyl-D-glucosamine.

Characterization of water-soluble products.

Total membranes prepared from genistein-induced cells of strain USDA257 were incubated with UDP-[^{14}C]-*N*-acetyl-D-glucosamine (final concentration 14 μM), in the presence of 40 mM *N*-acetyl-D-glucosamine and 6 mM $MgCl_2$. Radioactivity incorporated into water-soluble products was subjected to TLC and chromatography with Bio-Gel P2. As shown in Figure 3, five radioactive compounds were resolved by both chromatographic systems. Most of the radioactivity was incorporated into compounds that eluted from the Bio-Gel P2 column with an elution volume corresponding to those of oligosaccharides with a degree of polymerization lower than 10 glucose residues (Iñón de Iannino and Ugalde 1993). (Fig. 3A, compound V). TLC revealed that radioactivity was incorporated into four compounds that migrated as *N*-acetyl-D-glucosamine, *N,N'*diacetylchitobiose, *N,N',N''*triacetyl-chitotriose, and *N,N',N'',N'''*tetraacetylchitotetraose standards. A fifth reaction product migrated more slowly than the last-mentioned standard. Compounds I to V were recovered from the Bio-Gel P2 column (Fig. 3A) and chromatographed on TLC. Compound I migrated as *N*-acetyl-D-glucosamine, and compounds II, III, and IV as the disaccharide, trisaccharide and tetrasaccharide, respectively, formed from the same monosaccharide (data not shown). The standard for the corresponding pentasaccharide is not commercially available, but the degree of polymerization of the available standards

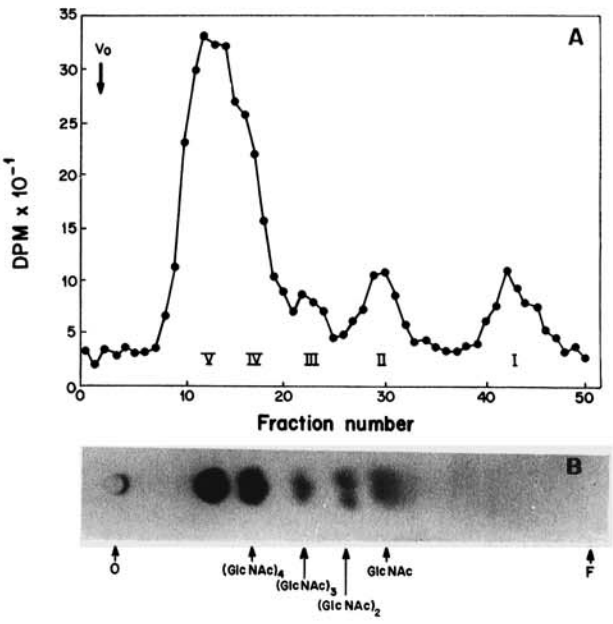

Fig. 3. Bio-Gel P2 and thin layer chromatography of products formed in vitro. Reactions were carried out as described in Materials and Methods. Radioactivity incorporated into water-soluble products was divided into two aliquots, which were concentrated and subjected to **(A)** Bio-Gel P2 chromatography or **(B)** thin-layer chromatography. Fractions from the Bio-Gel P2 column were pooled for further analysis: 40 to 45 (pool I); 28 to 32 (pool II); 20 to 25 (pool III); 14 to 19 (pool IV); 9 to 13 (pool V). Standards: GlcNAc, *N*-acetyl-D-glucosamine; $(GlcNAc)_2$, *N,N'*diacetylchitobiose; $(GlcNAc)_3$, *N,N',N''*triacetychitotriose; $(GlcNAc)_4$ *N,N', N'',N'''*tetraacetylchitotetraose.

showed a linear relation with the logarithm of their respective elution volumes from Bio-Gel P2 or distances migrated on TLC. The fifth reaction product behaved in both systems as expected for a pentasaccharide.

Characterization of chito-oligosaccharide.

Compounds recovered from the Bio-Gel P2 column as mono-, di-, tri-, tetra-, and pentamers of *N*-acetyl-D-glucosamine were subjected to several chemical and enzymatic treatments. Strong acid hydrolysis (2 N HCl, 4 h, 100°C) and paper chromatography of the resulting products yielded D-glucosamine as the only monosaccharide. Exhaustive digestion with chitinase yielded, on paper chromatography with solvent B and TLC with solvent A, *N*-acetyl-D-glucosamine as the only monosaccharide. When treatment with chitinase was carried out under controlled conditions (1 h with 0.001 U of enzyme), mainly *N,N'*diacetylchitobiose and *N*-acetyl-D-glucosamine were recovered (data not shown). Paper electro-

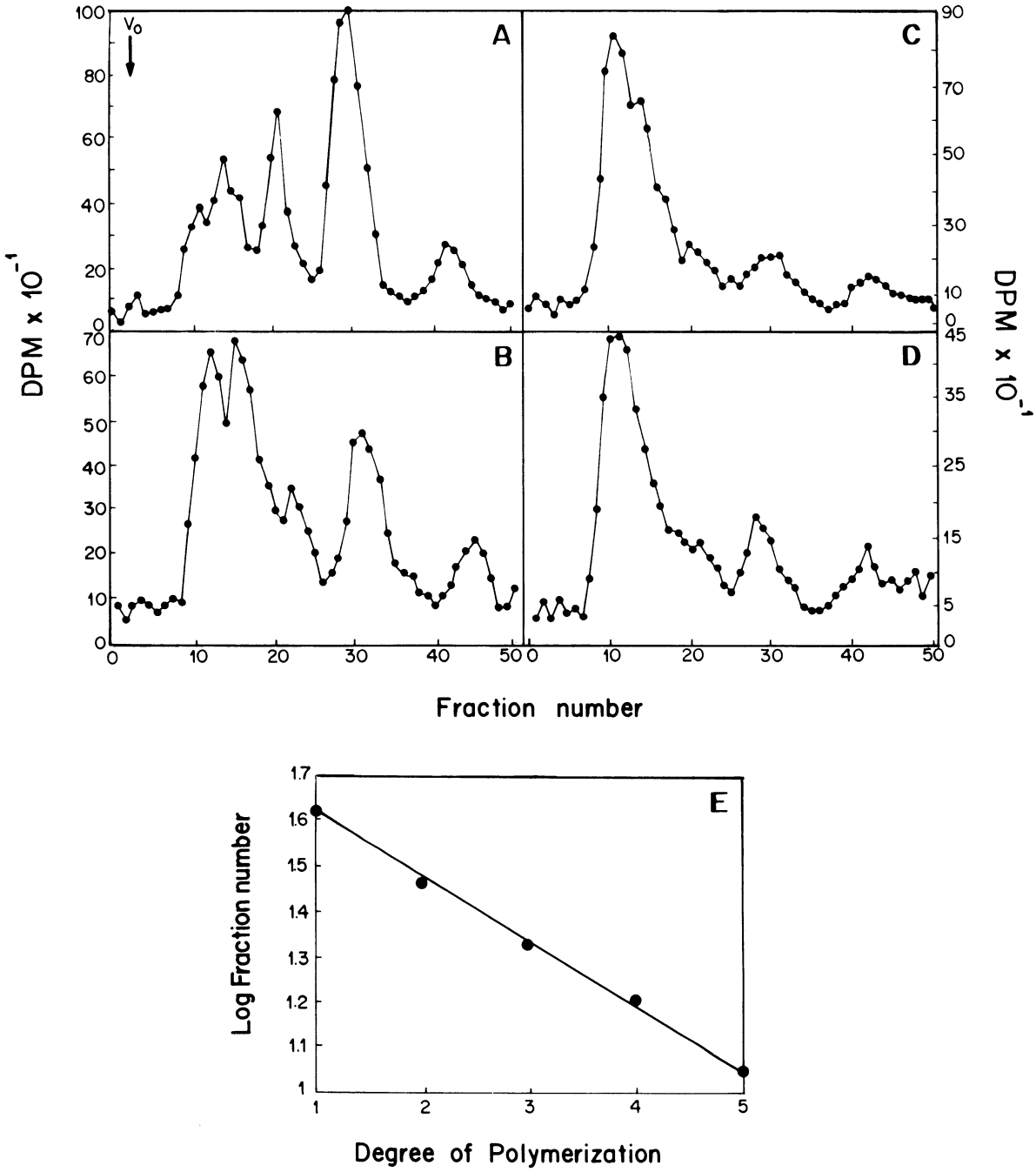

Fig. 4. Bio-Gel P2 chromatography of products formed at different UDP-*N*-acetyl-D-glucosamine concentrations. Incubations were carried out with 0.2 μCi of UDP-*N*-acetyl-[^{3}H]-D-glucosamine and different concentrations of nonlabeled UDP-*N*-acetyl-D-glucosamine as described in Materials and Methods (method [i]). **A** to **D,** the profiles obtained with 0.1, 1, 10, and 100 μM UDP-*N*-acetyl-D-glucosamine, respectively. **E,** the plot of the logarithm of the fraction number vs. degree of polymerization. Bio-Gel P2 column (116 × I cm) was eluted with 0.1 M pyridine-acetate buffer, pH 5.5, fractions of 1 ml were collected and radioactivity quantitated in 200-μl aliquots in Bray's solution.

phoresis with buffer D revealed that all compounds were neutral. This indicated that no deacetylation occurred under the conditions used for synthesis.

Kinetics of chito-oligosaccharide formation and effect of polyoxin D.

As shown in Figure 2, formation of chito-oligosaccharides followed Michaelis-Menten kinetics, with a Km for UDP-*N*-acetyl-D-glucosamine of 42 μM and a Vmax of 6 to 6.5 nmoles of *N*-acetyl-D-glucosamine incorporated per hour per milligram of protein. As described above, the formation of chito-oligosaccharide was stimulated by the addition of free *N*-acetyl-D-glucosamine, which could not be substituted by other amino sugars. As shown in Figure 2, in the absence of *N*-acetyl-D-glucosamine the reaction proceeded at very low rates, even at substrate concentrations of 200 μM. *N*-Acetyl-D-glucosamine was thus essential for the reaction to proceed. The addition of a final concentration of 200 μM polyoxin D had no effect on the synthesis or the degree of polymerization of the chito-oligosaccharide formed from 100 μM UDP-*N*-acetyl-D-glucosamine, thus indicating that β(1-4) *N*-acetyl-D-glucosaminyl transferase is insensitive to this fungal chitin synthetase inhibitor.

Effect of substrate concentration on the formation of chito-oligosaccharides.

The products formed at 0.1, 1, 10, and 100 μM UDP-*N*-acetyl-D-glucosamine were analyzed by Bio-Gel P2 chroma-

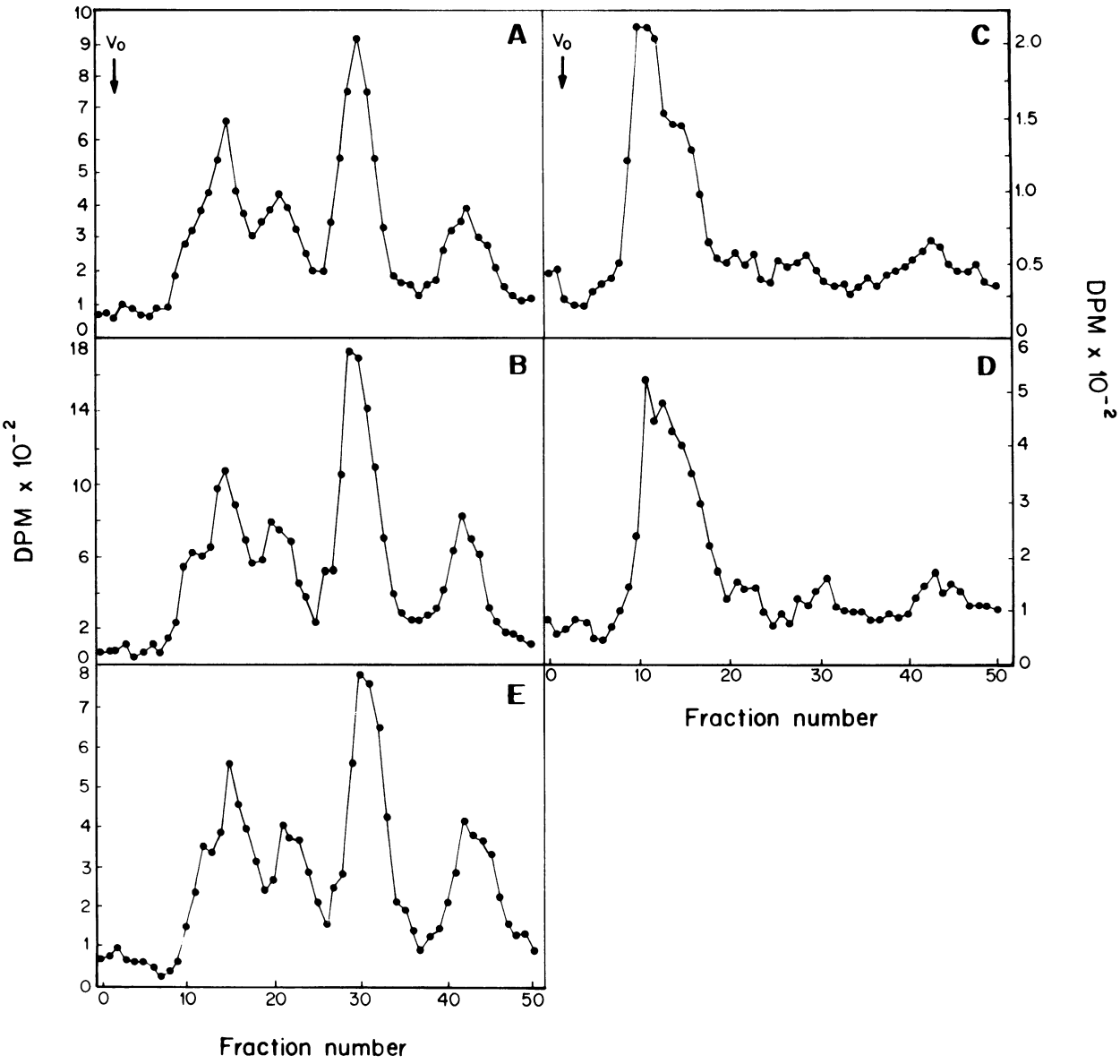

Fig. 5. Bio-Gel PS chromatography of products formed in a pulse.chase experiment. Membranes were incubated with 0.2 μCi of UDP-*N*-acetyl [^{3}H]-D-glucosamine with the addition of nonlabeled UDP-*N*-acetyl-D-glucosamine to reach the appropriate substrate concentration as described in Materials and Methods. DEAE-Sephadex A-25 eluates were concentrated and subjected to chromatography on Bio-Gel P2 columns as described in the legend to Figure 4. **A** and **B,** incubation with 0.1 μM UDP-*N*-acetyl-D-glucosamine for 5 and 20 min, respectively. **C** and **D,** incubation with 100 μM UDP-*N*-acetyl-D-glucosamine for 5 and 20 min, respectively. **E,** incubation with 0.1 μM UDP-*N*-acetyl-D-glucosamine for 5 min followed by a chase of 15 min with 100 μM UDP-*N*-acetyl-D-glucosamine.

tography. As shown in Figure 4, the degree of polymerization of the chito-oligosaccharide products depended on the substrate concentration. At 0.1 μM UDP-*N*-acetyl-D-glucosamine, most of the radioactivity accumulated as chitobiose (Fig. 4A); at 100 μM of substrate the most abundant product was chitopentaose (Fig. 4D), while at 1 and 10 μM UDP-*N*-acetyl-D-glucosamine, varying amounts of chitobiose, chitotriose, chitotetrose, and chitopentaose were formed. The products belonged to a homologous series of compounds with different degrees of polymerization, as concluded from the linear relation observed by plotting the logarithm of the elution volume versus the degree of polymerization of the products (Fig. 4E). The identification of *N*-acetyl-D-glucosamine, chitobiose, chitotriose, and chitotetraose was determined by TLC using commercial standards.

Pulse-chase experiment.

As shown in the previous section, the concentration of UDP-*N*-acetyl-D-glucosamine in the reaction mixture determines the degree of polymerization of the chito-oligosaccharides products. This suggests that the degree of polymerization of the products could be controlled by the endogenous pool of UDP-*N*-acetyl-D-glucosamine. In order to determine if the chito-oligosaccharides formed at different substrate concentrations were intermediates or final products, a pulse-chase experiment was carried out. Figure 5A shows the Bio-Gel P2 profile of the products formed after a pulse of 5 min with 0.1 μM UDP-*N*-acetyl-D-glucosamine. It can be seen that there was no change in the profile after a chase of 15 min with 100 μM UDP-*N*-acetyl-D-glucosamine (Fig. 5E). This indicated that chitobiose, chitotriose, and chitotetraose, accumulated during the pulse, were not free intermediates in the synthesis of chitopentaose formed at 100 μM UDP-*N*-acetyl-D-glucosamine (Fig. 5C and D). It is interesting that the products formed during the course of the reaction carried out with 0.1 or 100 μM UDP-*N*-acetyl-D-glucosamine did not change, indicating that chitobiose was not a free intermediate for the synthesis of chitopentaose or vice versa. (Fig. 5A–D).

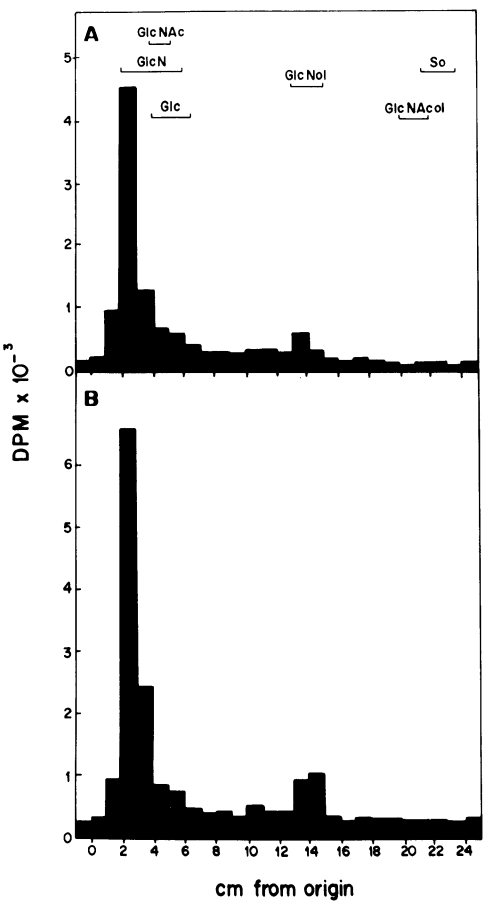

Fig. 6. Characterization of reducing ends. Diacetylchitobiose and tetraacetylchitotetraose labeled in vitro with [^{3}H]-*N*-acetyl-D-glucosamine were recovered from a Bio-Gel P2 column and subjected to reduction with sodium borohydride followed by strong acid hydrolysis as described in Materials and Methods. Paper electrophoresis was carried out with buffer C at 15 V/cm. **A,** *N,N′,N″,N‴*tetraacetylchitotetraose. **B,** *N,N′*diacetylchitobiose. Standards: Glc, glucose ; GlcN, glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; So, sorbitol; GlcNol, glucosaminitol; GlcNAcol, *N*-acetyl-D-glucosaminitol.

Chain elongation.

Chitobiose and chitotetraose obtained after incubation of membranes with UDP-[^{3}H]-*N*-acetyl-D-glucosamine and unlabeled *N*-acetyl-D-glucosamine, were recovered from a Bio-Gel P2 column and subjected to $NaBH_4$ reduction in order to study if the disaccharide and the tetrasaccharide contain radioactive [^{3}H]-*N*-acetyl-D-glucosamine at the reducing end. As shown in Figure 6, from both compounds most of the radioactivity was recovered as [^{3}H]-glucosamine after strong acid hydrolysis (2 N HCl for 4 h at 100°C) due to deacetylation of *N*-acetyl-D-glucosamine during the hydrolisis. No [^{3}H]-glucosaminitol was recovered, thus indicating that unlabeled *N*-acetyl-D-glucosamine was present at the reducing end. These results demostrate that the chito-oligosaccharide is elongated by the addition of *N*-acetyl-D-[^{3}H]-glucosamine from UDP-*N*-acetyl-D-[^{3}H]-glucosamine to the nonreducing end of an unlabeled *N*-acetyl-D-glucosamine, probably provided by the free *N*-acetyl-D-glucosamine present in the reaction mixture.

DISCUSSION

The synthesis of Nod factors in rhizobia is controlled at the level of transcription of a series of *nod* genes, which are only expressed after induction by plant factors. In *R. fredii,* one of the plant factors that control expression of *nod* genes is the isoflavone genistein (Kosslak et al. 1987; Krishnan and Pueppke 1993).

The structure of Nod factors in *R. fredii* was recently described (Bec-Ferté et al. 1994). They consist, as do the Nod factors from other rhizobia, of a β(1-4) *N*-acetyl-D-glucosamine backbone containing three to five sugar residues substituted with vaccenic acid and methyl fucose. It is believed that the biosynthesis of all Nod factors is initiated by the assembly of the chito-oligosaccharide backbone, which is subsequently decorated or modified by different substituents. It was demonstrated that the biosynthesis of the chito-oligosaccharide backbone of *Azorhizobium caulinodans* is carried out by the NodC protein (Geremia et al. 1994).

The ability of genistein to induce the biosynthesis of Nod factors in *R. fredii* appeared to be a transient process that diminished as cultures aged. It was observed that at the beginning of the exponential phase of growth (A_{625} = 0.2 to 0.4),

genistein was active as inducer, but that older cultures (A_{625} > 0.70) responded very poorly to the inducer. These results suggest that there is a narrow time window during culture growth in which genes were coordinately induced to form mature Nod factors. This behavior led us to prepare *R. fredii* membranes for in vitro studies from cells of young cultures induced with genistein. These conditions allowed the study of the in vitro formation of the chito-oligosaccharide Nod factor backbone with wild-type strains and made it unnecessary to use over-expressing strains. As *nodABC* is an operon encoding three proteins, over-expressing strains might introduce artifacts due to an imbalance of the amounts of the three proteins.

The in vitro synthesis of chito-oligosaccharide required UDP-*N*-acetyl-D-glucosamine, $MgCl_2$, and *N*-acetyl-D-glucosamine, but lipid or protein intermediates could not be detected.

The concentration of UDP-*N*-acetyl-D-glucosamine determined the degree of polymerization of the chito-oligosaccharide under our experimental conditions. At low substrate concentrations (0.1 μM), the main product was the disaccharide. This compound did not serve as a substrate for further elongation once it was released from the active site of the enzyme, as confirmed during a pulse-chase experiment. At 10 μM or higher concentrations of UDP-*N*-acetyl-D-glucosamine, the main product was chitopentaose, which has the degree of polymerization of the most abundant chito-oligosaccharide present in the mature *R. fredii* Nod factor (Bec-Ferté et al. 1994). The possibility exists that the synthesis occured through a single chain mechanism and that the enzyme affinity for UDP-*N*-acetyl-D-glucosamine is modified by substrate concentration.

The effect of UDP-*N*-acetyl-D-glucosamine concentration on the degree of polymerization of the chito-oligosaccharide formed in vitro was not observed in a previous paper (Geremia et al. 1994) in which the authors used an *nodABC* over-expressing strain; another difference from their results was that we did not observe deacetylation of the chito-oligosaccharide formed in vitro. This was not unexpected as the product of *nodB* gene, responsible for the deacetylating activity, (John et al. 1993) is a soluble protein and a membrane preparation was used in the present work.

Our results suggest one possible additional mechanism for the control of the formation of Nod signals. This is reminiscent of the well-known fact that the accumulation of glycogen in bacteria is controlled by the level of available ADP-D-glucose. The level of the sugar nucleotide is in turn regulated by ADP-glucose synthetase, an enzyme under allosteric control by several metabolites (Preiss et al. 1984). We hypothesize that the endogenous pool of UDP-*N*-acetyl-D-glucosamine may be under transcriptional and/or similar allosteric controls. Although it remains to be established whether induction with genistein has any effect on the endogenous pool of UDP-*N*-acetyl-D-glucosamine, it is interesting to note that the *nodM* gene product was found recently to be a glucosamine synthetase (Baev et al. 1991). This enzyme forms glucosamine-6-P from fructose-6-P and glutamine. Glucosamine-6-P is an obligate precursor in the formation of *N*-acetylglucosamine-1-phosphate, the immediate precursor of UDP-*N*-acetyl-D-glucosamine (Davidson 1966).

The synthesis of the chito-oligosaccharide Nod factor backbone was stimulated from six to 10 times by free *N*-acetyl-D-glucosamine, which is used as activator of the enzyme and probably as a primer of the reaction. Accordingly, it seems apparent that for the synthesis to proceed, an endogenous pool of free *N*-acetyl-D-glucosamine must be generated within the cells. Since both compounds are also required for building up cell wall components, one possible mechanism to increase the available pools of the sugar nucleotide and the amino sugar would be to have a duplicate set of genes, as it has been demonstrated for glucosamine syntethase in *R. meliloti* (Baev et al. 1991) and *R. leguminosarum* bv. *viciae* (Marie et al. 1992). The *nodC* gene product is highly conserved (53 to 70% amino acid identity) in different rhizobia (Debellé et al. 1992), and *R. meliloti* NodC protein shares 30% identity over 200 amino acids with the eukaryotic chitin synthetase Call (Debellé et al. 1992), and 32% identity over 401 amino acids with hyaluronate synthase (Dougherty and van de Rijn 1994). The region of strongest homology between Cal1 and NodC is also the most conserved one among different yeast chitin synthetases (Debellé et al. 1992). Protein homology studies showed that chitin synthetases have N-terminal and C-terminal regions that are not homologous to NodC protein. It is reasonable, then, that NodC protein will behave differently than fungal β(1-4) *N*-acetyl-D-glucosaminyltransferases. Although most fungal chitin synthetases are synthesized as zymogens subject to proteolytic activation, the *nodC* product under our assay conditions seems not to have required proteolytic activation, and so resembles Cal1 chitin synthetase. The Km for UDP-*N*-acetyl-D-glucosamine of the *nodC* product was one order of magnitude lower (42 μM) than for fungal chitin synthetases (500 to 1,200 μM) (McMurrough and Bartnicki-Garcia 1971; Porter and Jaworski 1966; Ruiz-Herrera et al. 1977; Ryder and Peberdy 1977).

The *nodC* product forms β(1-4)-acetyl-D-glucosaminyl polymers with a maximum degree of polymerization of five, and not insoluble chitin, which is the main product of all fungal β(1-4)-acetyl-D-glucosaminyl transferases. Our data support the idea that the NodC protein is a modified chitin synthetase designed for the formation of β(1-4) *N*-acetyl-D-glucosaminyl oligomers with no more than five *N*-acetyl-D-glucosamine residues.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Rhizobium fredii strains USDA257 and USDA191 have been described (Heron and Pueppke 1984); and USDA 257B3 contains a deletion and lacks *nodABC* (Heron et al. 1989; Krishnan and Pueppke 1991). *Rhizobium* strains were grown in mannitol-extract medium (AMA) (Iñón de Iannino and Ugalde 1993) at 28° C on a rotary shaker.

Induction of *nod* genes and preparation of membranes.

For the induction of nod genes, 10 ml from a 2-day-old culture (A_{625} = 2.0) was transferred into 1 L of AMA medium, allowed to grow to A_{625} = 0.2 before adding genistein to a final concentration of 2 μM, and further incubated for 18 h at 28°C. Cells were harvested by centrifugation at 8,000 × *g* for 20 min, pellets resuspended in 20 ml of buffer A (20% sucrose [w/w], 50 mM Tris-HCl, pH 8, 10 mM EDTA), and cells incubated with 200 μg of lysozyme per milliliter during 40 min in an ice-water bath. Cells were then centrifuged at 12,000 × *g* for 30 min. Cell pellets were resuspended in 20 ml

of buffer B (50 mM Tris-HCl pH 8, 3 mM EDTA) and DNAse (100 μg) and 10 mM $MgCl_2$ was added. Cells were disrupted by passing them three times through a French press at 18,000 psi. Unbroken cells were removed by centrifugation at 3,000 × *g* for 15 min. Total membranes were recovered from the 3,000 × *g* supernatants by sedimentation at 150,000 × *g* for 4 h. They were resuspended in buffer B at a concentration of 20 to 40 mg of protein per milliliter. The enzymatic preparations were stored at –20°C until used. Protein concentrations were determined by the Lowry method with bovine serum albumin as standard. All chemicals were analytical grade and from Sigma, and radioactive compounds were purchased from New England Nuclear (Boston).

Enzymatic assays.

Incubation mixtures contained 50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 40 mM *N*-acetyl-D-glucosamine, 0.2 μCi of UDP-*N*-acetyl-D-glucosamine [glucosamine-6-^{3}H (N)] (30 Ci/mmol) or 0.2 μCi of UDP-*N*-acetyl-D-glucosamine [glucosamine-^{14}C(U)] (200.4 mCi/mmol) and membranes (200 μg protein) in a total volume of 50 μl. The reactions were carried out at 30° C for different time periods in 0.5-ml Eppendorf tubes, and stopped by one of three methods:

(i) Tubes were heated at 100°C for 2 min, 300 μl of water was added and tubes were centrifuged in an Eppendorf centrifuge at 14,000 rpm for 4 min. Supernatants were recovered and pellets washed with 200 μl of water and centrifuged as before. Both supernatants were pooled and applied to a 0.8-ml DEAE-Sephadex A-25 anion-exchange column equilibrated with water. Columns were washed three times with 0.5 ml of water. Unbound radioactivity was recovered, concentrated under an air stream, and subjected to alternative chromatographic procedures as described below.

(ii) Methanol (100 μl) was added and the mixtures were subjected to Folch partition: 10 μl of carrier protein (mouse liver microsomes, 80 mg of protein/ml) and 150 μl of chloroform were successively added. Tubes were then vigorously vortexed and centrifuged in an Eppendorf centrifuge for 4 min at 14,000 rpm. Upper and lower phases were recovered and the interphases extracted twice by the addition 50 μl of water, 100 μl of methanol, and 150 μl of chloroform. The mixtures were vortexed and centrifuged as before. The lower phases were pooled, washed twice with 200 μl of upper-phase solvent (chloroform/methanol/water, 1:16:16), and retained as lower phase soluble. The upper phases were pooled and chromatographed through a DEAE-Sephadex A-25 column as described above. The fractions in the void volume were recovered as water soluble phase. The proteinaceous interphases were extracted three times with 100 μl of chloroform/methanol/water (1:2:0.3) and the extracts pooled as 1203 phase soluble. The remaining interphases were called insoluble residue. Radioactivity recovered in all fractions was counted in a liquid scintillator counter with Bray's solution.

(iii) TCA (0.5 ml of a 10% aqueous solution) was added, the tubes were centrifuged at 14,000 rpm for 2 min, and the pellets subjected to polyacrylamide gel electrophoresis and fluorography as described (Iñón de Iannino and Ugalde 1989).

Bio-Gel P2 chromatography.

Radioactive products recovered from DEAE-Sephadex A-25 were subjected to gel filtration on a Bio-Gel P2 column (116 × 1 cm). Elution was performed with 0.1 M pyridine-acetate buffer, pH 5.5. Fractions of 1 ml were collected and radioactivity quantitated in 200-μl aliquots in Bray's solution.

Thin layer chromatography.

Samples were spotted onto silica gel 60 TLC plates (Merck) and developed with solvent A, 1-butanol/ethanol/ water (5:3:2). Radioactive products were detected by exposing plates for 1 to 2 weeks to Kodak X-Omat AR film. *N*-acetyl-D-glucosamine (GlcNAc), *N,N'*-diacetylchitobiose $(GlcNAc)_2$, *N,N',N''*-triacetylchitotriose $(GlcNAc)_3$, and *N,N',N'',N'''*-tetraacetylchitotetraose $(GlcNAc)_4$ obtained from Sigma were used as standards and developed by spraying plates with the diphenylamine-aniline-orthophosphoric acid reagent (Walkley and Tillman 1977).

Sodium borohydride reductions.

Sodium borohydride reductions were carried out as described previously (Leloir et al. 1973).

Paper electrophoresis and paper chromatography.

Paper electrophoresis was carried out with buffer C (2% sodium molybdate, pH 5.0) (Bourne et al. 1961) or with buffer D (5% formic acid, pH 2.0) for 2 h at 15 V/cm. Descending paper chromatography was carried out on Whatman #1 paper with solvent B (butanol/pyridine/water, 6:4:3). Radioactivity was measured by cutting 1-cm paper strips and counting them in a liquid scintillator with a toluene-omnifluor solution. Sugars were detected by the alkaline silver method (Trevelyan et al. 1950).

Digestion with chitinase.

Digestion with chitinase was carried out by incubating samples for different time periods with 0.001 U of chitinase from *Serratia marcescens* (Sigma) in 50 mM sodium phosphate buffer, pH 6.0, at 25°C.

In vivo labeling of Nod factors.

Rhizobium fredii strains were grown in AMA medium (10 ml) to A_{625} = 0.2 to 0.4. One-milliliter samples were withdrawn, centrifuged in 1.5-ml Eppendorf tubes, and pellets resuspended in 0.5 ml of gluconate-mannitol medium without vitamins (Bhuvaneswari et al. 1977) with the addition of 2 μM genistein and 2 μCi of sodium [^{14}C]-acetate (45 to 60 mCi/mM) (Spaink et al. 1992). After 3 to 4 h of incubation at 28°C, cultures were centrifuged in an Eppendorf centrifuge at 14,000 rpm for 5 min. Supernatants were extracted with 0.5 ml of 1-butanol saturated with water. The butanolic phases were recovered and dried under a nitrogen stream. Substances were dissolved in 50 μl of 1-butanol saturated with water, and 2-μl samples were spotted on TLC plates (reverse phase C18-coated silica, ODS 100% octadecylsilanization) obtained from Sigma. Chromatograms were developed with solvent C (acetonitrile/water 1:1) and exposed to X-Omat AR films (Kodak) for 1 week.

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