

Rhizobium Nodulation Protein NodC Is an Important Determinant of Chitin Oligosaccharide Chain Length in Nod Factor Biosynthesis

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Synthesis of chitin oligosaccharides by NodC is the first committed step in the biosynthesis of rhizobial lipochitin oligosaccharides (LCOs). The distribution of oligosaccharide chain lengths in LCOs differs between various *Rhizobium* species. We expressed the cloned *nodC* genes of *Rhizobium meliloti*, *R. leguminosarum* bv. *viciae*, and *R. loti* in *Escherichia coli*. The *in vivo* activities of the various NodC proteins differed with respect to the length of the major chitin oligosaccharide produced. The clearest difference was observed between strains with *R. meliloti* and *R. loti* NodC, producing chitintetraose and chitinpentaose, respectively. *In vitro* experiments, using UDP-[¹⁴C]GlcNAc as a precursor, show that this difference reflects intrinsic properties of these NodC proteins and that it is not influenced by the UDP-GlcNAc concentration. Analysis of oligosaccharide chain lengths in LCOs produced by a *R. leguminosarum* bv. *viciae* *nodC* mutant, expressing the three cloned *nodC* genes mentioned above, shows that the difference in oligosaccharide chain length in LCOs of *R. meliloti* and *R. leguminosarum* bv. *viciae* is due only to *nodC*. The exclusive production of LCOs which contain a chitinpentaose backbone by *R. loti* strains is not due to NodC but to end product selection by Nod proteins involved in further modification of the chitin oligosaccharide. These results indicate that *nodC* contributes to the host specificity of *R. meliloti*, a conclusion consistent with the results of several studies which have shown that the lengths of the oligosaccharide backbones of LCOs can strongly influence their activities on host plants.

Bacteria belonging to the genera *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* are able to induce the formation of a new organ, a nodule, on the roots of leguminous plants. The synthesis of signal molecules by these members of the family *Rhizobiaceae* is essential for this nodulation process (reviewed in references 3, 6, and 29). These signal molecules consist of an oligomer of β 1 \rightarrow 4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues which is *N*-acylated at the nonreducing residue and hence are designated lipochitin oligosaccharides (LCOs). The structures of LCOs produced by different rhizobia vary in (i) the presence of additional groups on either the reducing or nonreducing terminus of the chitin oligosaccharide, (ii) the type of acyl chain present on the nonreducing residue, and (iii) the length of the oligosaccharide backbone. The presence of special, highly unsaturated fatty acids as well as of additional substitutions on the sugar backbone has been shown to play a crucial role in the determination of the host specificity of nodulation (recently reviewed in references 26 and 30). Several studies have also shown that the length of the oligosaccharide backbone of LCOs can strongly influence their activity on host plants (1, 8, 11, 27, 35). The chain length of the major oligosaccharide moiety in LCOs differs most between LCOs produced by *Rhizobium meliloti* and *R. loti*. *R. meliloti* LCOs mainly contain a chitintetraose backbone (18, 24, 27), whereas *R. loti* LCOs always have a chitinpentaose backbone (19). Other species, such as *R. leguminosarum* bv. *viciae*, produce LCOs in which both chitintetraose and pentaose can be present (33).

It has been shown that NodC, in the absence of the other Nod proteins, directs the synthesis of intermediates in the

synthesis of LCOs that were characterized as chitin oligosaccharides (10, 34). This conclusion was recently confirmed by the structural analysis of these metabolites by mass spectrometry (16, 22). Factors responsible for the observed differences in oligosaccharide chain lengths are poorly understood. Iñón de Iannino et al. (13) recently proposed that the UDP-GlcNAc concentration is an important factor in the control of chitin oligosaccharide chain length during LCO biosynthesis. In this paper, we report results of a study on the role of the NodC protein and the UDP-GlcNAc concentration in the control of chitin oligosaccharide chain length *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions. Recombinant DNA techniques were performed as described by Sambrook et al. (25). Enzymes were purchased from Pharmacia LKB (Uppsala, Sweden) unless indicated otherwise. The construction of plasmids is illustrated in Fig. 1. The expression vector pET9a (36) was used for expression of *nodC* in *Escherichia coli*, whereas the IncP vector pMP92 (32) was used for expression in *Rhizobium*. *E. coli* strains were routinely grown at 37°C in LC medium (23). Strains carrying pET9a-derived plasmids were grown in the presence of kanamycin (50 μ g ml⁻¹). Strains derived from *E. coli* XL1-blue (Stratagene, La Jolla, Calif.) were grown in the presence of tetracycline (20 μ g ml⁻¹) to ensure maintenance of the F factor. *Rhizobium* strains were grown in YMB or B⁻ medium (12, 38). *R. leguminosarum* bv. *viciae* RBL5622, originally described by Wijffelman et al. (39) as strain RBL607, carries a deletion in the *nodC* gene, which does not affect the expression of downstream *nod* genes (2). IncP plasmids were mobilized to *Rhizobium* by using the helper plasmid pRK2013 as described by Ditta et al. (7). Transconjugants were selected on YMB medium solidified with 1.8% agar and containing rifampin (20 μ g ml⁻¹) and tetracycline (2 μ g ml⁻¹). The resulting strains were routinely grown in the presence of tetracycline.

PCR amplification and cloning of *nodC* genes. Total DNA was isolated from *R. meliloti* 1021 (21) and *R. loti* E1R (19). The primers used for amplifying *R. meliloti* and *R. loti* *nodC* were based on the nucleotide sequences reported by Jacobs et al. (15) and Collins-Emerson et al. (5), respectively. The *nodC* genes were amplified in a 100- μ l PCR mixture containing 25 pmol of each appropriate primer (Isogen Bioscience, Maarsse, The Netherlands), 0.1 μ g of *Rhizobium* DNA as the template, 1 U of *Pfu* polymerase (Stratagene), and the buffer supplied by the manufacturer. Amplifications were performed by using a Robo-Cycler (Stratagene) for 25 cycles. Each cycle consisted of 1 min of denaturation

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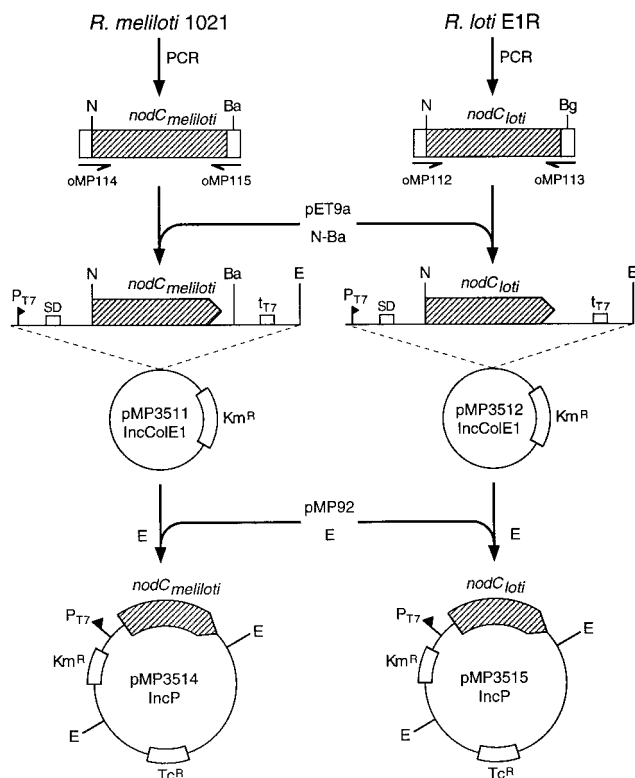


FIG. 1. Cloning of *R. meliloti* and *R. loti* *nodC* genes. Using total DNA of *R. meliloti* 1021 (21) and *R. loti* E1R (19) as templates, *nodC* genes were amplified by PCR. Due to ligation of *Bgl*II ends to *Bam*HI ends in the construction of plasmid pMP3512, these restriction sites were lost. For expression of *nodC* in *Rhizobium*, plasmids pMP3511 and pMP3512 were inserted as *Eco*RI fragments into the IncP broad-host-range vector pMP92 (32). In all plasmids, *nodC* is preceded by the Shine-Dalgarno sequence (ribosome binding site) of pET9a, and expression of the genes is under the control of the T7 promoter. Abbreviations: N, *Nde*I; Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; SD, Shine-Dalgarno sequence; P_{T7}, T7 promoter; t_{T7}, T7 transcription terminator; Km^R, kanamycin resistance; Tc^R, tetracycline resistance.

at 95°C, 2 min of primer annealing at 55°C, and primer extension for 1 min at 72°C. The upstream primers (omp112 [5'-TGACATCATATGAACCTGTTTGCACAGCCAGTACG-3'] for *R. loti* and omp114 [5'-AGAACATATGTACC TGCTTGACACAACCAGCAC-3'] for *R. meliloti*) provide a *Nde*I restriction site, whereas the downstream primers (omp113 [5'-CTCTTAGACTCTGATC GATTGACTATTGCTTTCGCT-3'] for *R. loti* and omp115 [5'-TTTCCAGG GATCCTACTGTCCTCGCCGCT-3'] for *R. meliloti*) provide *Bgl*II and *Bam*HI sites, respectively. These restriction sites allowed the insertion of amplified sequences in the translational start site of the *E. coli* expression vector pET9a (36). Plasmids were maintained in the *E. coli* XL1-blue.

TLC. Chitin oligosaccharides and LCOs were analyzed on a silica 60 thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany), using *n*-butanol-ethanol-water (5:3:2, vol/vol/vol) as the mobile phase. Results were visualized and quantified by using a PhosphorImager system in combination with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Isolation and analysis of LCOs. Logarithmic-phase *Rhizobium* strains growing in B⁻ medium were diluted to an *A*₆₂₀ of 0.1. One milliliter of this culture was added to a mixture of naringenin (final concentration of 1.5 μM; Sigma, St. Louis, Mo.), to induce expression of the *nod* genes, and 0.2 μCi of D-[1-¹⁴C]glucosamine (GlcN; 50 mCi mmol⁻¹; Amersham International, Amersham, England). After incubation for 4 h at 28°C, LCOs were extracted from the culture with 1 ml of water-saturated *n*-butanol as described previously (28). The butanol phase was dried under vacuum and dissolved in 10 μl of acetonitrile-water (1:1, vol/vol). A volume of 2 μl of this sample was analyzed by TLC as described above.

Analysis of NodC activity in vivo. *E. coli* XL1-blue strains, carrying either a *nodC*-containing plasmid or the vector pET9a without an insert, were grown overnight at 37°C in LC medium in the presence of kanamycin (50 μg ml⁻¹), diluted 1:100 in fresh medium, and grown to an *A*₆₂₀ of between 0.1 and 0.2. To 1 ml of these cultures, 0.2 μCi of [1-¹⁴C]GlcN was added, and *nodC* expression was induced by infection with phage mgP1-2, encoding the T7 RNA polymerase

(37). After various periods of incubation at 28°C, bacteria were collected by centrifugation and extracted with 200 μl of chloroform-water (1:1, vol/vol). The aqueous phase was dried, and the residue was dissolved in 10 μl of water. A volume of 1 μl of this sample was analyzed by TLC as described above.

Preparation of cell extracts and membrane fractions. pET9a-derived plasmids carrying a *nodC* gene were introduced into *E. coli* BL21(DE3) (36). Bacteria were grown to an *A*₆₂₀ of 0.4, at which stage *nodC* expression was induced by the addition of isopropylthiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After induction for 2 h at 28°C, bacteria were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5) at 1/20 of the original culture volume. Bacteria were disrupted by sonication at 4°C in a Branson model 250 Sonifier, using 15 pulses of 0.9 s, corresponding to an output of approximately 75 W, with 0.1-s intervals. Unbroken cells were removed by centrifugation at 4,000 × *g* for 10 min. Membrane fractions were isolated from these cell extracts by centrifugation at 100,000 × *g* for 2 h. The membrane pellet was resuspended in 50 mM Tris-HCl (pH 7.5). Protein concentrations in membrane preparations were determined by using the Coomassie protein assay reagent (Pierce, Rockford, Ill.) according to the manufacturer's guidelines. Membrane fractions were stored at -80°C at protein concentrations of between 10 and 25 mg ml⁻¹.

Analysis of NodC activity in vitro. Unless indicated otherwise, membrane preparations were assayed for chitin oligosaccharide synthase activity in a 50-μl reaction mixture containing 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 10 μM UDP-[U-¹⁴C]GlcNAc (216 to 232 mCi mmol⁻¹; Amersham International), and 25 μg of membrane protein. When higher UDP-GlcNAc concentrations were used, unlabelled UDP-GlcNAc (Sigma) was added to the desired concentration. Incubations were performed at 20°C for 15 min. The reaction was stopped by boiling for 2 min. After the addition of 200 μl of water, the mixture was centrifuged for 10 min at 13,000 rpm in a microcentrifuge, and the supernatant fluid was loaded onto a 100-μl Dowex 1X8-400 anion-exchange column (Sigma) to remove unincorporated UDP-GlcNAc. Columns were washed with 200 μl of water. The flowthrough and wash fractions were combined and dried under vacuum. The resulting pellet was dissolved in 10 μl of water, and a sample of 1 μl was analyzed by TLC as described above. The amount of [¹⁴C]GlcNAc that can be incorporated in an oligosaccharide depends on the number of GlcNAc residues. Therefore, spot intensities of different chitin oligosaccharides were corrected for differences in chain length before the relative amount of each oligosaccharide was determined.

RESULTS

Cloning and expression of different *nodC* genes in *E. coli*. To investigate the activities of different NodC proteins, we cloned the *nodC* genes of *R. meliloti* and *R. loti* by using PCR as shown in Fig. 1. These genes were cloned into the *E. coli* expression vector pET9a, leading to constructs which are comparable to a previously described plasmid carrying the cloned *R. leguminosarum* bv. *viciae* *nodC* (31). These plasmids were introduced into *E. coli*, resulting in an isogenic set of strains differing only in the origin of the *nodC* gene. The expression of *nodC* in these strains was induced in the presence of [1-¹⁴C]GlcN in order to label the chitin oligosaccharides produced by the different NodC proteins. TLC analysis of radiolabelled chitin oligosaccharides (Fig. 2) showed that the chain length of the major chitin oligosaccharide produced depends on the origin of the *nodC* gene. *R. meliloti* NodC mainly produced chitintetraose, whereas the major product of *R. loti* NodC was chitinpentaose. Expression of the *R. leguminosarum* bv. *viciae* *nodC* led to the production of a mixture of chitintetraose and pentaose as the major products. The same results were obtained when the experiments were repeated with two independent PCR clones of each *nodC* gene. These results show that the in vivo activities of NodC proteins from these three *Rhizobium* species differ clearly with respect to the length of the major oligosaccharide produced.

Influence of NodC on the chitin oligosaccharide chain length in LCOs. The results presented above suggest that the differences in the lengths of the oligosaccharide backbones of LCOs produced by different rhizobia are due to differences in the NodC protein. To test this hypothesis, *nodC* genes of *R. meliloti*, *R. leguminosarum* bv. *viciae*, and *R. loti* were inserted in a broad-host-range vector (Fig. 1) and introduced in a *R. leguminosarum* bv. *viciae* strain which carries a nonpolar deletion in the *nodC* gene (2, 39). After induction of the *nod* genes

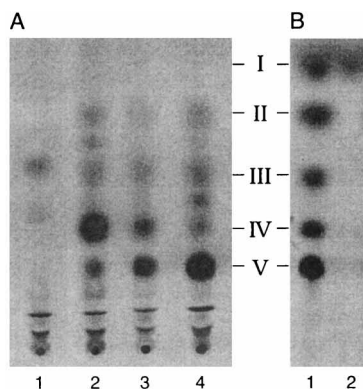


FIG. 2. TLC analysis of chitin-oligosaccharides produced by NodC in vivo and in vitro. Chitin-oligosaccharides were analyzed on silica 60 TLC plates. Results were visualized by using a PhosphorImager system in combination with ImageQuant software (Molecular Dynamics). The positions of GlcNAc (I) and chitin oligosaccharides ranging from chitinbiose (II) to chitinpentose (V) are indicated between panel A and B. (A) Chitin oligosaccharides extracted from *E. coli* strains expressing different *nodC* genes. Lanes 1 to 4 represent extracts from XL1/pET9a (control), XL1/pMP3511 (*R. meliloti nodC*), XL1/pMP2065 (*R. leguminosarum* bv. *viciae nodC*), and XL1/pMP3512 (*R. loti nodC*), respectively. (B) Chitin oligosaccharides synthesized in vitro by membrane preparations containing *R. loti* NodC in the presence of UDP-[^{14}C]GlcNAc. Membrane preparations are derived from *E. coli* strains. Lanes 1 and 2 represent reaction products of membranes of XL1/pMP3512 (*R. loti nodC*) and control membranes, respectively.

in the presence of [^{14}C]GlcN, LCOs were extracted with butanol and quantitatively analyzed by using a TLC system in which the LCOs of *R. leguminosarum* bv. *viciae* are separated on the basis of the lengths of their oligosaccharide backbones (11). Expression of *R. leguminosarum* bv. *viciae nodC* or *R. meliloti nodC* led to the synthesis of lipochitin tetrasaccharides and pentasaccharides in ratios of 30:70 and 72:28, respectively (Fig. 3). These ratios correspond to those reported for strains containing the wild-type *R. leguminosarum* bv. *viciae* or *R. meliloti nod* genes (18, 24, 27, 33), indicating that the difference in chitin oligosaccharide chain length in LCOs produced by *R. leguminosarum* bv. *viciae* and *R. meliloti* is due only to the difference between their NodC proteins. The *R. leguminosarum* bv. *viciae nodC* deletion mutant complemented with *R. loti nodC* produced slightly more chitinpentose-containing LCOs than the strain expressing *R. leguminosarum* bv. *viciae nodC*, resulting in a lipochitin tetraose/pentaose ratio of 24:76. This result indicates that the absence of lipochitin tetrasaccharides in *R. loti* is not determined by NodC but is due to other factors.

Influence of UDP-GlcNAc concentration on chitin oligosaccharide chain length in vitro. To set up a system to study the synthesis of chitin oligosaccharides by NodC in vitro, membrane fractions were prepared from *E. coli* BL21(DE3), expressing the *R. loti nodC* gene. Incubation of these membrane fractions with UDP-[^{14}C]GlcNAc resulted in the *nodC*-dependent synthesis of chitin oligosaccharides with a degree of polymerization of 2 to 5 (Fig. 2B). The same results were obtained for cell extracts (data not shown). Neither the amount nor the chain length of chitin oligosaccharides produced in this in vitro system could be influenced by varying the pH in the range of 6.5 to 8.5. The presence of 10 mM EDTA completely prevented the synthesis of chitin oligosaccharides. Addition of 20 mM Mg^{2+} restored chitin oligosaccharide synthase activity. The use of Co^{2+} , instead of Mg^{2+} , has been reported to stimulate the activity of some chitin synthases (4). The addition of 20 mM Co^{2+} to membrane preparations in the presence of 10 mM EDTA, however, did not result in the synthesis of chitin oligosaccharides by NodC. The addition of 25 mM GlcNAc,

which is known to stimulate the synthesis of chitin polymers in vitro, increased the production of chitin oligosaccharides approximately twofold. To investigate the role of the UDP-GlcNAc concentration in the control of chitin oligosaccharide chain length, we incubated *R. loti* NodC preparations at a membrane protein concentration of $0.04 \mu\text{g } \mu\text{l}^{-1}$, with various concentrations of UDP-[^{14}C]GlcNAc. The oligosaccharides produced were analyzed on TLC plates and quantified (Fig. 4A). Varying the UDP-GlcNAc concentration had no effect on the length of the oligosaccharides produced, since at each tested concentration approximately 75% of the oligosaccharides consisted of chitinpentose (Fig. 4A, right panel). This result seems to contradict those reported by Iñón de Iannino et al. (13). These authors described that varying the UDP-GlcNAc concentration affected the chain length of chitin oligosaccharides produced by NodC-containing membrane preparations of *R. fredii*. These experiments were performed under conditions similar to those used for ours. The only major difference is the amount of NodC protein used in the reaction, since Iñón de Iannino et al. used 100 times more membrane protein than we did. When we increased the membrane protein concentration in the reaction mixture from 0.04 to $0.2 \mu\text{g } \mu\text{l}^{-1}$, the relative amount of chitinpentose decreased at UDP-GlcNAc concentrations below $250 \mu\text{M}$ (Fig. 4, middle panel). A further increase in the protein concentration to $1 \mu\text{g } \mu\text{l}^{-1}$ led to a reduction in the relative amount of chitinpentose at all UDP-GlcNAc concentrations below 1 mM (Fig. 4, left panel). This influence of UDP-GlcNAc concentration on chitin oligosaccharide chain length at high protein concentrations could be due to substrate limitation, leading to premature chain termination. To investigate this possibility, we determined the total incorporation of GlcNAc from radiolabelled UDP-GlcNAc into chitin oligosaccharides in the experiments described above (Fig. 5). At 1 mM UDP-GlcNAc, a nearly

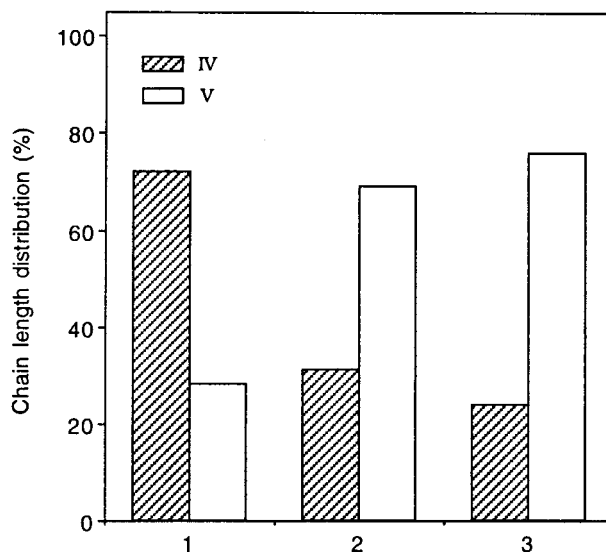


FIG. 3. Chitin oligosaccharide chain lengths in LCOs of recombinant *Rhizobium* strains. The *nodC* genes of *R. meliloti*, *R. leguminosarum* bv. *viciae*, and *R. loti* were introduced in an *R. leguminosarum* bv. *viciae nodC* deletion mutant. Expression of *nod* genes was induced in the presence of [^{14}C]GlcN, LCOs were extracted from these cultures, and samples were analyzed on silica 60 TLC plates. Strains used: 1, RBL5622/pMP3514 (*R. meliloti nodC*); 2, RBL5622/pMP2707 (*R. leguminosarum* bv. *viciae nodC*); 3, RBL5622/pMP3515 (*R. loti nodC*). Plasmid pMP2707 has been described by Spaink et al. (34). Open and hatched bars represent lipochitin tetrasaccharides and pentasaccharides, respectively. Values are the means of two independent experiments.

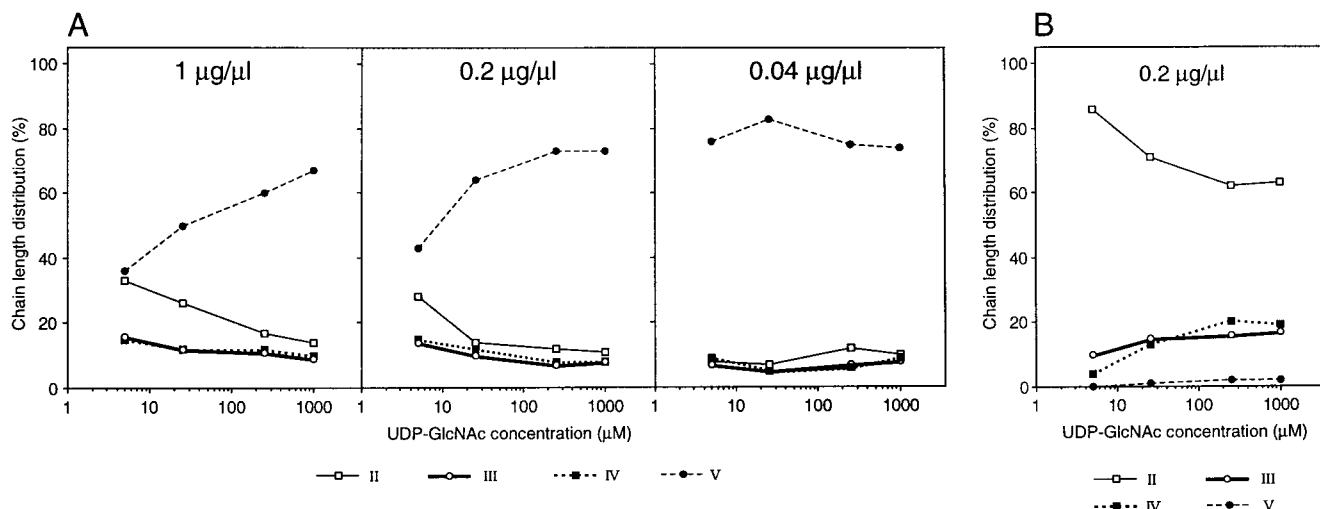


FIG. 4. Chitin oligosaccharide chain length analysis after synthesis *in vitro*. Chitin oligosaccharides were synthesized from membrane preparations containing *R. loti* NodC (A) or *R. meliloti* NodC (B), in the presence of various concentrations of UDP-[U- 14 C]GlcNAc, and analyzed on silica 60 TLC plates. Results were visualized and quantified by using a PhosphorImager system. The relative amount of each oligosaccharide was determined after correction of spot intensities for differences in chain length. Membrane protein concentrations are indicated at the top of each panel.

linear relationship between the protein concentration and the level of chitin oligosaccharide synthesis was observed, leading to the conclusion that the production of chitin oligosaccharides at 1 mM UDP-GlcNAc was not limited by the substrate concentration but limited only by the amount of NodC. However, at decreasing UDP-GlcNAc concentrations, this linearity between the amount of membrane protein and the level of chitin oligosaccharide synthesis was gradually lost. This loss of linearity represents rate limitation of chitin oligosaccharide synthesis by insufficient UDP-GlcNAc concentrations and correlates well with the reduction in oligosaccharide chain length shown in Fig. 4A. We therefore conclude that the effect of low UDP-GlcNAc concentrations and high enzyme concentrations on chitin oligosaccharide chain length is a result of premature chain termination due to limiting substrate concentrations. The chitinpentaose/tetraose ratios produced *in vitro* by NodC at saturating UDP-GlcNAc concentrations are the same as the ratios observed in *E. coli* *in vivo* (Fig. 2), indicating that the substrate concentration *in vivo* was not limiting chitin oligosaccharide production.

Comparison between *in vitro* activities of *R. meliloti* and *R. loti* NodC. The length of the major chitin oligosaccharides produced after expression of *nodC* in *E. coli* differs most clearly between the strains expressing *R. meliloti* and *R. loti nodC* (Fig. 2). We therefore investigated the differences in activity between these NodC proteins in more detail *in vitro*. Chitin oligosaccharides were analyzed after synthesis at various UDP-GlcNAc concentrations as described above. When membrane proteins from an *R. meliloti nodC*-expressing strain were used at a concentration of $0.2 \mu\text{g } \mu\text{l}^{-1}$, increasing UDP-GlcNAc concentrations predominantly led to an increase in the relative amount of chitin tetraose (Fig. 4B). The relative amount of chitin biosis produced by *R. meliloti* NodC was between three- and sixfold higher than the relative amount produced by *R. loti* NodC. As found with *R. loti* NodC, the chitinpentaose/tetraose ratios at saturating UDP-GlcNAc concentrations are the same as the ratio observed *in vivo* (Fig. 2).

A comparison between the relative amounts of chitin tetraose and pentaose produced by the *R. meliloti* and *R. loti* NodC proteins *in vitro* shows that the chitinpentaose/tetraose

ratio differs approximately 100-fold between the two NodC proteins, independent of the UDP-GlcNAc concentration (Fig. 6). In conclusion, our results show that the NodC proteins of *R. meliloti* and *R. loti* are clearly different from each other with respect to the length of the chitin oligosaccharides produced, both *in vivo* and *in vitro*.

DISCUSSION

The nodulation protein NodC has been shown to direct the synthesis of the chitin oligosaccharide backbones of *Rhizobium* LCOs (10, 16, 22, 34). The length of each of these chitin oligosaccharide backbones is usually restricted to four or five GlcNAc residues. To investigate the possible role of NodC in the control of chitin oligosaccharide chain length in LCO biosynthesis, we expressed the cloned *nodC* genes of *R. meliloti*,

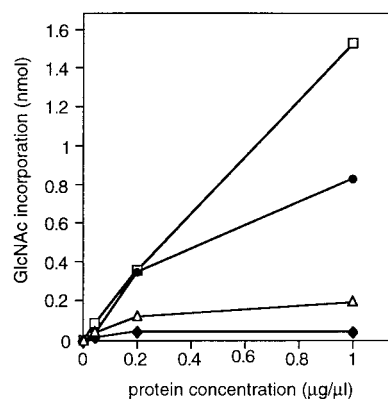


FIG. 5. Chitin oligosaccharide formation at different concentrations of NodC. Total chitin oligosaccharide formation in the experiment described in the legend to Fig. 4 was determined by quantitatively comparing the spot intensities to that of [14 C]GlcNAc standards, spotted on a silica 60 TLC plate. All intensities were within the linear range of detection. Chitin oligosaccharide synthesis is expressed as the amount of GlcNAc incorporated from UDP-GlcNAc into chitin oligosaccharides. Graphs represents oligosaccharide formation at UDP-GlcNAc concentrations of $5 \mu\text{M}$ (\blacklozenge), $25 \mu\text{M}$ (\triangle), $250 \mu\text{M}$ (\bullet), and 1 mM (\square).

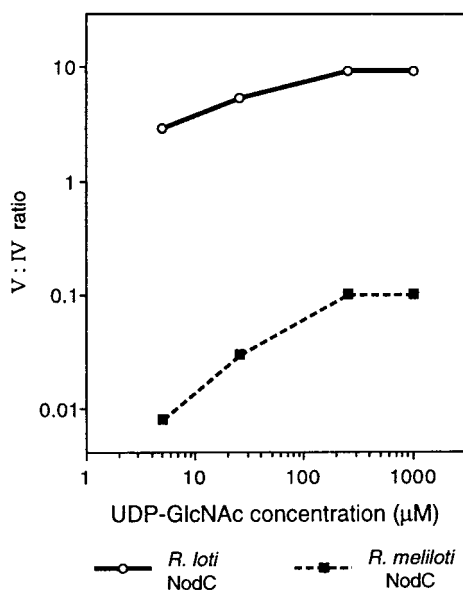


FIG. 6. Analysis of chitin pentaose/tetraose ratios after in vitro chitin oligosaccharide synthesis by NodC. After synthesis at various concentrations of UDP-[U-¹⁴C]GlcNAc, chitin oligosaccharides were analyzed on silica 60 TLC plates. Results were visualized by using a PhosphorImager system. The ratio between the amount of chitin pentaose and chitin tetraose produced was determined after correction of spot intensities for differences in chain length.

R. leguminosarum bv. *viciae*, and *R. loti* in *E. coli*. Analysis of the chitin oligosaccharides produced by these strains showed that the NodC proteins of *R. meliloti*, *R. leguminosarum* bv. *viciae*, and *R. loti* clearly differ from each other with respect to the length of the major oligosaccharide produced (Fig. 2). The major products were a chitin tetraose in case of *R. meliloti* NodC and chitin pentaose in case of *R. loti* NodC. No oligosaccharides longer than chitin pentaose were detected. These results show that in the absence of other Nod proteins, the difference in chitin oligosaccharide chain length is determined only by NodC. Our result also indicates that the UDP-GlcNAc concentration does not play an important role in chitin oligosaccharide chain length control in vivo, which seems to be in contrast to a report by Iñón de Iannino et al. (13). These authors studied the synthesis of chitin oligosaccharides by *R. fredii* NodC in vitro. They observed a reduction in chitin oligosaccharide chain length at low UDP-GlcNAc concentrations and concluded that chitin oligosaccharide chain length in LCO biosynthesis is controlled by the UDP-GlcNAc concentration. However, we observed an effect of low UDP-GlcNAc concentrations on chitin oligosaccharide chain length only when very high amounts of protein were used (Fig. 4A). Quantification of total chitin oligosaccharide production in these experiments showed that at low UDP-GlcNAc concentrations, chitin oligosaccharide formation did not increase linearly with increasing enzyme concentration, showing that chitin oligosaccharide production was limited by the concentration of UDP-GlcNAc (Fig. 5). The limitation of chitin oligosaccharide synthesis by the substrate concentration was found to correlate well with the reduction in oligosaccharide chain length. These results indicate that the reduction in chitin oligosaccharide chain length at low UDP-GlcNAc concentrations and high NodC concentration as described by Iñón de Iannino et al. (13) is a result of premature chain termination due to the limiting substrate concentration, rather than a result of a specific mechanism of oligosaccharide chain length control. Moreover, the

chitin pentaose/tetraose ratio produced in vitro by *R. loti* and *R. meliloti* NodC differed approximately 100-fold at every UDP-GlcNAc concentration tested. Therefore, the difference in the intrinsic properties of the NodC proteins must be the major determinant of chitin oligosaccharide chain length.

The chitin oligosaccharide chain lengths produced by *E. coli* strains expressing different *nodC* genes closely resemble the lengths of the oligosaccharides in LCOs produced by the *Rhizobium* species from which these *nodC* genes originate. Therefore, we investigated the role of NodC in the control of chitin oligosaccharide chain length in LCO biosynthesis. The cloned *nodC* genes of *R. meliloti*, *R. leguminosarum* bv. *viciae*, and *R. loti* were introduced into a *R. leguminosarum* bv. *viciae* strain carrying a nonpolar deletion in the *nodC* gene. Expression of the *R. leguminosarum* bv. *viciae* or *R. meliloti* *nodC* in the *R. leguminosarum* bv. *viciae* *nodC* deletion mutant resulted in the synthesis of lipochitin tetrasaccharides and lipochitin pentasaccharides in ratios which correspond to those reported for the respective *Rhizobium* species (18, 24, 27, 33). This finding shows that the difference in chitin oligosaccharide chain length in the LCOs of *R. meliloti* and *R. leguminosarum* bv. *viciae* is mainly, if not entirely, due to differences in NodC. *R. loti* strains produce only lipochitin pentaoses, not lipochitin tetraose (19). Expression of *R. loti* *nodC* in the *R. leguminosarum* bv. *viciae* *nodC* mutant led to an increase in lipochitin pentasaccharide production but did not abolish the synthesis of lipochitin tetrasaccharides (Fig. 3). The production of LCOs which always contain a chitin pentaose backbone by *R. loti* strains therefore seems to be due to the selective acylation of chitin pentaose molecules. The results reported by Jabbouri et al. (14) suggest that a factor responsible for the absence of chitin tetraose backbones in *R. loti* LCOs may be NodS, since these authors showed that introduction of the *nodS* gene from *Rhizobium* strain NGR234 into *R. fredii* USDA257 results in the production of only N-methylated lipochitin pentasaccharides, whereas the wild-type *R. fredii* strain also produces lipochitin tri- and tetrasaccharides.

The narrow range of oligosaccharide chain lengths in LCOs indicates that the size of the sugar backbone is important for the efficient recognition of LCOs by host plants. Several investigators have shown that this is indeed the case (1, 8, 11, 27, 35). *R. fredii* lipochitin tetrasaccharides, for instance, are 3 orders of magnitude more active in inducing root hair deformations on natural host plants than the lipochitin trisaccharides. Surprisingly, in this system the lipochitin trisaccharides are 100-fold more active than the lipochitin pentasaccharides (1). *R. meliloti* lipochitin tetrasaccharides are between 10- and 100-fold more active in inducing root hair deformation, membrane depolarization, and meristematic activity than lipochitin pentasaccharides, depending on the host plant used (8, 27). In this report, we show that the high production of lipochitin tetrasaccharides by *R. meliloti* is due to the special properties of the *R. meliloti* NodC protein. We therefore suggest that *nodC* contributes to the host specificity of *R. meliloti*. The *nodC* gene, together with the *nodAB* genes, was originally classified as common since introduction of *nod* regions containing *nodC* from several rhizobia into *R. meliloti* and *R. leguminosarum* bv. *trifolii* *nod* mutants could restore nodulation on natural host plants (9, 17, 20). However, a recent report by Ritsema et al. (23) showed that the substrate specificity of NodA is essential for the synthesis of host-specific LCOs in *R. leguminosarum* bv. *viciae*. These findings, together with our results, suggest that not only the so-called host-specific Nod proteins but also the common Nod proteins involved in the synthesis of LCOs contribute to the optimal production of host specific LCOs. There-

fore, the classification of nodulation genes as being either common or host specific has to be revised.

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