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# Chitin Oligosaccharide Synthesis by Rhizobia and Zebrafish Embryos Starts by Glycosyl Transfer to O4 of the Reducing-Terminal Residue<sup>†</sup>

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**ABSTRACT:** Lipochitin oligosaccharides are organogenesis-inducing signal molecules produced by rhizobia to establish the formation of nitrogen-fixing root nodules in leguminous plants. Chitin oligosaccharide biosynthesis by the *Mesorhizobium loti* nodulation protein NodC was studied in vitro using membrane fractions of an *Escherichia coli* strain expressing the cloned *M. loti nodC* gene. The results indicate that prenylpyrophosphate-linked intermediates are not involved in the chitin oligosaccharide synthesis pathway. We observed that, in addition to *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc, NodC also directly incorporates free GlcNAc into chitin oligosaccharides. Further analysis showed that free GlcNAc is used as a primer that is elongated at the nonreducing terminus. The synthetic glycoside *p*-nitrophenyl- $\beta$ -*N*-acetylglucosaminide (pNPGlcNAc) has a free hydroxyl group at C4 but not at C1 and could also be used as an acceptor by NodC, confirming that chain elongation by NodC takes place at the nonreducing-terminal residue. The use of artificial glycosyl acceptors such as pNPGlcNAc has not previously been described for a processive glycosyltransferase. Using this method, we show that also the DG42-directed chitin oligosaccharide synthase activity, present in extracts of zebrafish embryos, is able to initiate chitin oligosaccharide synthesis on pNPGlcNAc. Consequently, chain elongation in chitin oligosaccharide synthesis by *M. loti* NodC and zebrafish DG42 occurs by the transfer of GlcNAc residues from UDP-GlcNAc to O4 of the nonreducing-terminal residue, in contrast to earlier models on the mechanism of processive  $\beta$ -glycosyltransferase reactions.

Rhizobia are soil bacteria from several genera of the *Rhizobiaceae* (1–3) and have the ability to establish a symbiotic relationship with leguminous plants. This symbiosis results in the formation of a new organ, the root nodule (4), in which rhizobia are able to fix atmospheric nitrogen into biologically utilizable ammonia. Essential for the nodulation process is the synthesis and secretion of rhizobial lipo-chitin oligosaccharides, also known as Nod factors, which induce cell division and differentiation in the plant root (5, 6). Nod factors consist of a linear oligomer of  $\beta$ 1 $\rightarrow$ 4 linked *N*-acetyl-D-glucosamine (GlcNAc)<sup>1</sup> residues, which is *N*-acylated on the nonreducing-terminal residue. Most Nod factors contain a chitintetraose or -pentaose backbone, but Nod factors with a di-, tri-, or hexasaccharide backbone have also been detected (7–9).

Heterologous expression of the rhizobial nodulation gene *nodC* in *Escherichia coli* leads to the synthesis of chitin oligosaccharides, indicating that NodC is a chitin oligosaccharide synthase (10, 11). NodC having sequence homology with chitin synthase, which produces a linear polymer of

$\beta$ 1 $\rightarrow$ 4 linked GlcNAc residues, has also been reported (12). The synthesis of chitin has been the subject of intense research over the past few decades [reviewed in references (13, 14)]. As a result, the enzymatic properties of chitin synthase have been thoroughly characterized. However, the direction in which chitin chains are elongated is still unknown. An amino acid sequence similarity between NodC and the *Xenopus leavis* DG42 protein has also been reported (12). The DG42 gene is only expressed during early embryonic development in *Xenopus* and zebrafish (15, 16). The nature of the enzymatic activity of DG42 is controversial. Based on amino acid sequence homology, DG42 is most closely related to enzymes involved in the synthesis of hyaluronic acid (also known as hyaluronan), the polymer of [ $\rightarrow$ GlcNAc  $\beta$ 1 $\rightarrow$ 4 GlcA  $\beta$ 1 $\rightarrow$ 3] disaccharide repeating units (17). There are reports describing that heterologous expression of DG42 in yeast or mammalian cells leads to increased hyaluronic acid-synthase activity (18, 19). However, DG42 protein synthesized in vitro clearly has chitin oligosaccharide synthase activity (20). This chitin oligosaccharide synthase activity has also been shown in embryos and correlates with expression of the DG42 gene (16, 21).

In this paper we report the results of a study of the chitin oligosaccharide biosynthesis pathway, focusing on the initiation and the direction of chain elongation. The data presented show that chain elongation in chitin oligosaccharide synthesis in rhizobial bacteria and in zebrafish embryos takes place at

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<sup>1</sup> Abbreviations: GlcN, D-glucosamine; GlcNol, D-glucosaminitol; GlcNAc, *N*-acetyl-D-glucosamine; pNPGlcNAc, *p*-nitrophenyl- $\beta$ -*N*-acetylglucosaminide.

the O4 position of the nonreducing-terminal residue and provide the first experimental data showing the direction of chain elongation of processive  $\beta$ -glycosyltransferases. In contrast, elongation of hyaluronic acid chains has been reported to proceed in the opposite direction. Our data therefore support the proposed chitin oligosaccharide synthase activity of DG42.

## MATERIALS AND METHODS

**NodC and DG42 Enzyme Preparation.** As a source of NodC protein, the *E. coli* strain BL21(DE3) carrying a plasmid with the *M. loti nodC* gene was used (22). Induction of *nodC* expression, disruption of bacteria by sonication, and the subsequent isolation of the membrane fraction were performed as described (22). Enzyme preparations retained full activity for at least 2 months. Zebrafish (*Danio rerio*) were maintained under standard conditions, embryos were obtained by natural spawning at 28 °C, and the resulting fertilized embryos were dechorionated and lysed as described by Westerfield et al. (23) and Bakkers et al. (21).

**Thin-Layer Chromatography (TLC).** Chitin oligosaccharides were analyzed either on silica 60 thin-layer chromatography plates (Merck, Darmstadt, Germany), using 1-butanol/ethanol/water (5:3:2, v/v/v) as the mobile phase, or on NH<sub>2</sub>-TLC plates, using acetonitrile/water (60:40, v/v). *p*-Nitrophenyl-linked chitin oligosaccharides were separated on NH<sub>2</sub>-TLC plates, using acetonitrile/water (70:30, v/v) as the mobile phase. Radioactive compounds were visualized using a Phosphor imaging system in combination with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Standard Chitin Oligosaccharide Synthase (NodC) Assay.** Reaction mixtures for quantitative standard assays contained 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl (pH 7.5), 250  $\mu$ M UDP-*N*-acetyl-D-[U-<sup>14</sup>C]glucosamine (UDP-GlcNAc, final specific activity 10 mCi/mmol, obtained from Amersham International, UK), and approximately 50 ng of membrane protein/ $\mu$ L, in a final volume of 25  $\mu$ L. When higher UDP-GlcNAc concentrations were used, unlabeled UDP-GlcNAc (Sigma) was added to the desired concentration. Incubations were performed at 20 °C for 15 min. Unincorporated UDP-GlcNAc was removed using a 100  $\mu$ L Dowex 1  $\times$  8-400 anion exchange column (Sigma) as described (22). The resulting material was dissolved in 10  $\mu$ L of water of which 1  $\mu$ L was examined on silica 60 TLC plates as described above. The UDP-GlcNAc analogues nikkomycin Z and polyoxin D were purchased from Calbiochem (La Jolla, CA). Chitin oligosaccharide standards were purchased from Seikagaku Kogyo (Tokyo).

**Detection of Chitin Oligosaccharide Synthase Activity Using pNPGlcNAc as Acceptor.** pNPGlcNAc (*p*-nitrophenyl- $\beta$ -*N*-acetylglucosaminide, Sigma) was used to assay NodC activity in the presence of 50  $\mu$ M UDP-[U-<sup>14</sup>C]GlcNAc (final specific activity 25 mCi/mmol), in NodC reaction buffer. After incubation at 20 °C for 1 h, one-tenth of the sample was directly analyzed on NH<sub>2</sub>-TLC plates. Chitinase treatments were performed in 10 mM Tris-HCl buffer (pH 6.8) for 3 h at 37 °C with 1  $\mu$ g of chitinase-63 (24). To isolate the pNP-linked reaction products, reaction mixtures were loaded on a 1 mL octadecyl (C18) column (J. T. Baker, Phillipsburg, NJ). Columns were washed with 3 mL of water, followed by elution of pNP-linked chitin oligosaccharides

with 0.2 mL of methanol. After the eluent was concentrated using evaporation under vacuum, analysis was performed on NH<sub>2</sub>-TLC plates as described above.

Extracts from zebrafish embryos were incubated with pNPGlcNAc for 45 min at room temperature in the presence of 0.3 mM UDP-GlcNAc, 12 mM MgCl<sub>2</sub>, and 30  $\mu$ M Lognac (2-acetamido-2-deoxy-D-glucopyranose-1,5-lactone, a competitive inhibitor of *N*-acetyl- $\beta$ -D-glucosaminidases) (CarboGen, Zürich). Reactions were stopped by the addition of 150  $\mu$ L of water and boiling for 2 min. The tubes were centrifuged and the precipitates washed twice with 100  $\mu$ L of water. The resulting supernatants were combined. After in vitro incubation of zebrafish extracts in the presence of pNPGlcNAc, the products were first purified by chromatography on Sep-Pak C18 cartridges (Waters, Milford, MA) as described by Palcic et al. (25), and subsequently radiolabeled by the fucosyltransferase NodZ as described (21). This analysis (Figure 5A, lane 3) showed that chromatography of the reaction products on Sep-Pak cartridges, in contrast to the C18 cartridges used in the NodC assay (Figure 4B), completely removed all chitin oligosaccharides that were not linked to pNP. For HPLC analysis, 50  $\mu$ L samples were loaded onto a CarboPac-PA1 preparative column using a DX-300 Dionex system (Dionex Corp., Sunnyvale, CA). Isocratic elution using 150 mM NaOH with a flow rate of 3 mL/min according to the suppliers methods was performed. Radioactivity was measured using a Radiomatic 500TR flow scintillation analyzer using Ultima-Flo AP scintillation fluid (Packard Instrument Co., CT) at 3 mL/min. Peak analysis was performed using radiometric FLO-ONE software version 3.01 (Packard Instrument Co.).

**Analysis of Lipid-Linked Saccharides.** Formation of lipid-linked saccharides was investigated using the method described by Reuber and Walker (26), with some modifications. After incubation of 100  $\mu$ L of permeabilized cells in the reaction buffer described for NodC assays containing 0.2  $\mu$ Ci of UDP-[U-<sup>14</sup>C]GlcNAc (251 mCi/mmol, Amersham) for 1 h at room temperature, cells were collected by centrifugation and extracted with chloroform/methanol/water (10:20:0.3, v/v/v). One-tenth of the resulting extract was examined on silica 60 TLC plates as described above. The remaining material was used to quantify the incorporation of radioactivity using liquid scintillation counting. Inhibition of lipid-linked saccharide formation was investigated by adding tunicamycin (Calbiochem, 25  $\mu$ g/mL), amphomycin (200  $\mu$ g/mL), or bacitracin (Calbiochem, 4 mg/mL).

**Oligosaccharide Reduction and Hydrolysis.** Sodium borohydride reductions were essentially carried out as described by Leloir et al. (27), with some modifications. A NaBH<sub>4</sub> solution (50 mg/mL) in 0.2% NaOH was freshly prepared, and added to a solution of chitin oligosaccharides to a final concentration of 10 mg/mL, followed by incubation at room temperature for 24 h. A volume of 100  $\mu$ L of Amberlite IR-120 (H<sup>+</sup>) ion-exchange resin (Sigma) was added, and gently mixed for 5 min. The treatment with ion-exchange resin was repeated. The supernatant was then dried under vacuum. To completely remove any remaining boric acid, three sequential evaporations with 1 mL of anhydrous methanol were performed. The residual material was redissolved in water. Oligosaccharides were hydrolyzed in 2 M HCl at 100 °C for 4 h. A volume of 1  $\mu$ L was then analyzed using NH<sub>2</sub>-TLC as described above.

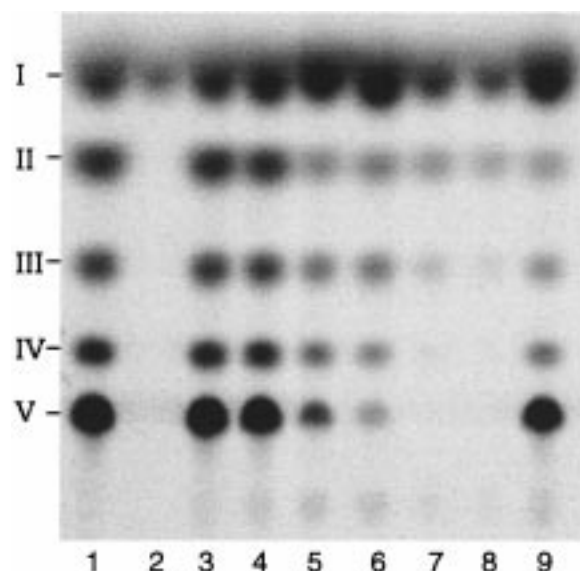


FIGURE 1: Effect of nikkomycin Z on chitin oligosaccharide synthesis by NodC *in vitro*. Reactions were performed with 0.2  $\mu\text{g}$  of membrane protein/ $\mu\text{L}$  and 10  $\mu\text{M}$  UDP-[ $^{14}\text{C}$ ]GlcNAc, followed by extraction, and analysis of chitin oligosaccharides on silica 60 TLC plates as described under Materials and Methods. Membranes were isolated from an *E. coli* strain expressing *M. loti* NodC. Results were visualized by using a PhosphorImager system in combination with the ImageQuant software. Roman numerals I–V indicate the positions of GlcNAc (I) and oligosaccharides (II–V). Lanes 1 and 2 show reaction products of NodC (lane 1) and control membranes (lane 2), in the absence of nikkomycin Z. All other lanes show reaction products of NodC in the presence of the following nikkomycin Z concentrations: lane 3, 0.5  $\mu\text{M}$ ; 4, 1  $\mu\text{M}$ ; 5, 10  $\mu\text{M}$ ; 6, 25  $\mu\text{M}$ ; 7, 100  $\mu\text{M}$ ; 8, 250  $\mu\text{M}$ ; 9, 10  $\mu\text{M}$  nikkomycin Z together with 100  $\mu\text{M}$  UDP-GlcNAc.

## RESULTS

**Initiation of Chitin Oligosaccharide Synthesis by NodC.** Incubation of membranes isolated from an *E. coli* strain expressing the *M. loti nodC* gene, with UDP-D-[ $^{14}\text{C}$ ]GlcNAc, results in the formation of chitin oligosaccharides with a degree of polymerization ranging from 2 to 5 [Figure 1 and (22)]. To investigate whether chitin oligosaccharide synthesis is initiated on a lipid carrier, we determined the incorporation of radiolabeled GlcNAc from UDP-[ $^{14}\text{C}$ ]GlcNAc into the glycolipid fraction. Neither liquid scintillation counting nor TLC analysis of the glycolipid fractions in these experiments showed the synthesis of NodC-dependent lipid-linked saccharides (data not shown). Tunicamycin, amphomycin, and bacitracin inhibit the formation of prenyl pyrophosphate-linked saccharides in bacteria. Bacitracin (150–300  $\mu\text{g}/\text{mL}$ ) inhibits the dephosphorylation of undecaprenyl pyrophosphate by forming a complex with this lipid (28, 29). This dephosphorylation step is required to regenerate undecaprenyl phosphate, the lipid carrier in peptidoglycan biosynthesis. The formation of MurNAc-pentapeptide-pyrophosphoryl-undecaprenyl, another step in bacterial cell wall biosynthesis, has been shown to be sensitive to amphomycin (100  $\mu\text{g}/\text{mL}$ ) (30). Tunicamycin is a compound that is well-known for its inhibition of the transfer of GlcNAc from UDP-GlcNAc to undecaprenyl pyrophosphate in eukaryotic cells. Barr et al. (31) have shown that GlcNAc-pyrophosphoryl-undecaprenol is also an intermediate in the biosynthesis of the enterobacterial common antigen in *E. coli*, and the *in vitro* synthesis of this

intermediate is completely blocked by the presence of 10  $\mu\text{g}/\text{mL}$  tunicamycin. None of these inhibitors, however, affected the formation of chitin oligosaccharides by NodC in a standard assay (data not shown). These results indicate that prenyl phosphate carriers are not required for the synthesis of chitin oligosaccharides by *M. loti* NodC.

**Inhibition of Chitin Oligosaccharide Synthesis by Substrate Analogues.** Nikkomycin Z and polyoxin D are UDP-GlcNAc analogues that, at micromolar concentrations, strongly inhibit the *in vitro* synthesis of chitin polymers by chitin synthase [reviewed in reference (32)]. The presence of 10  $\mu\text{M}$  nikkomycin Z clearly inhibits chitin oligosaccharide formation (Figure 1). At increasing nikkomycin Z concentrations, a decrease in oligosaccharide chain length was observed. In contrast, polyoxin D concentrations up to 0.5 mM did not affect chitin oligosaccharide synthesis at all (data not shown).

**Processive Nature of Chain Elongation in Chitin Oligosaccharide Synthesis.** Incubation of NodC preparations with UDP-[ $^{14}\text{C}$ ]GlcNAc results in the formation of chitinobiose, chitintriose, chitintetraose, and chitinpentaose (Figure 1), indicating that chitin oligosaccharide synthesis occurs by the sequential addition of monosaccharides to a growing chain. We investigated whether free chitin oligosaccharides are intermediates in chitin oligosaccharide synthesis by adding chitin oligosaccharides ranging in length from chitinobiose to chitinpentaose to NodC reaction mixtures, followed by analysis of radiolabeled chitin oligosaccharides using TLC. This analysis showed that the addition of an excess amount of any of these chitin oligosaccharides (25 mM) to a reaction mixture containing 10  $\mu\text{M}$  UDP-GlcNAc had no effect on the chitin oligosaccharide synthase activity of NodC (data not shown). Moreover, no change in the distribution of radioactivity in chitin oligosaccharides of different chain lengths was observed. We therefore conclude that NodC does not use free chitin oligosaccharides as acceptors.

**Direction of Chain Elongation in Chitin Oligosaccharide Synthesis by NodC.** Since GlcNAc stimulates the synthesis of chitin oligosaccharides by NodC (22), we investigated the possibility that GlcNAc may act as the primer in this reaction. When NodC preparations were incubated with [ $^{14}\text{C}$ ]GlcNAc and unlabeled UDP-GlcNAc, incorporation of radioactivity into chitin oligosaccharides was observed (Figure 2A, lane 1). Incorporation of [ $^{14}\text{C}$ ]GlcNAc into chitin oligosaccharides was not observed when UDP-GlcNAc was omitted from the reaction mixture (Figure 2A, lane 2). Moreover, synthesis of UDP-GlcNAc from GlcNAc could not be detected when control membranes were incubated with [ $^{14}\text{C}$ ]GlcNAc (data not shown). We therefore conclude that GlcNAc is directly incorporated into chitin oligosaccharides, and that this does not occur via conversion into UDP-GlcNAc. To determine the position of the incorporated GlcNAc in the oligosaccharides, [ $^{14}\text{C}$ ]GlcNAc-labeled chitinpentaose was purified from a TLC plate. The resulting preparation was subjected to reduction with sodium borohydride. After complete removal of sodium borohydride, the oligosaccharide was fully hydrolyzed. This procedure results in the formation of glucosaminitol (GlcNol) from the reduced terminal residue of the original oligosaccharide, and glucosamine (GlcN) from the nonreducing-terminal and backbone residues. When purified [ $^{14}\text{C}$ ]GlcNAc-labeled chitinpentaose was treated this way,  $^{14}\text{C}$  label was only incorporated into GlcNol (Figure 2B). This shows that the [ $^{14}\text{C}$ ]GlcNAc

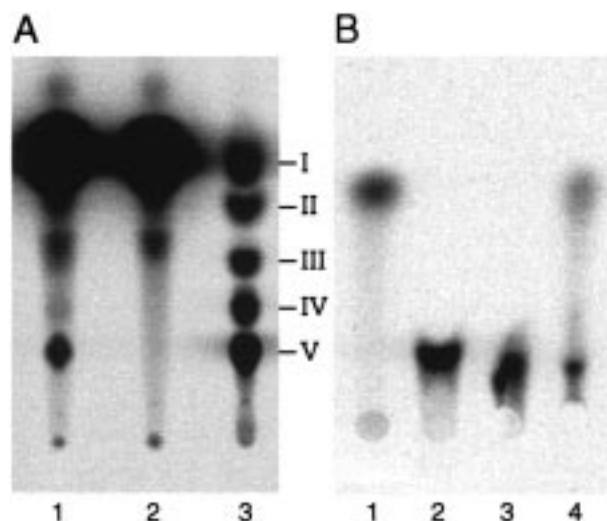


FIGURE 2: Incorporation of free GlcNAc into chitin oligosaccharides by NodC. (A) TLC analysis of GlcNAc incorporation into chitin oligosaccharides by NodC. Reactions were performed with 2  $\mu$ g/ $\mu$ L membrane protein, 1 mM GlcNAc, and 1 mM UDP-GlcNAc, as indicated below. Reaction products were extracted and analyzed on silica 60 TLC plates. Lanes show reaction products formed in the presence of: 1, [ $^{14}$ C]GlcNAc and UDP-GlcNAc; 2, [ $^{14}$ C]-GlcNAc alone; 3, GlcNAc and UDP-[ $^{14}$ C]GlcNAc. Roman numerals indicate the position of GlcNAc (I) and chitin oligosaccharides (II–IV). (B) [ $^{14}$ C]GlcNAc-labeled chitin pentaose was isolated from a TLC plate (panel A, lane 1), reduced with NaBH<sub>4</sub>, and subsequently hydrolyzed, yielding monosaccharides and a monosaccharide alditol. Reaction products were analyzed on NH<sub>2</sub>-TLC. Lanes represent: 1, [ $^{14}$ C]GlcN; 2, [ $^{14}$ C]GlcNol, prepared by NaBH<sub>4</sub> reduction of [ $^{14}$ C]GlcN; 3, reduced and hydrolyzed [ $^{14}$ C]GlcNAc-labeled chitin pentaose; 4, reduced and hydrolyzed chitinbiose, synthesized from UDP-[ $^{14}$ C]GlcNAc in the absence of free GlcNAc. This last sample was prepared before the reducing agent was completely removed before the reduced oligosaccharides were hydrolyzed. The two spots in lane 4 have a comparable intensity, judging from the ImageQuant software. This confirms that after hydrolysis of the disaccharide, no further reduction of the resulting monosaccharides occurred.

is exclusively incorporated into chitin oligosaccharides as the reducing-terminal residue.

By comparing the radioactivity incorporated into chitin-tetraose and -pentaose in identical incubation mixtures in which either free GlcNAc or UDP-GlcNAc was radiolabeled, we determined the percentage of these oligosaccharides that were synthesized using free GlcNAc as the acceptor (Figure 3A) using the formula:

$$\% = 100 \times n \times [G/(G + \text{UDPG})]$$

in which % = the percentage of oligosaccharide molecules that is synthesized using GlcNAc as acceptor,  $n$  = the number of GlcNAc residues per oligosaccharide molecule,  $G$  = the amount of incorporated free GlcNAc, and UDPG = the amount of incorporated GlcNAc from UDP-GlcNAc. The latter two parameters were calculated by dividing the quantified spots by the specific activity of the radiolabeled sugar used in the enzyme reaction.

At a GlcNAc concentration of 25 mM, all chitin-tetraose and -pentaose molecules are synthesized using GlcNAc as acceptor. The presence of GlcNAc at these concentrations stimulates chitin oligosaccharide synthesis approximately 2-fold (Figure 3B), and no changes in oligosaccharide chain length are observed (Figure 3C). These results can only be

explained by a mechanism in which GlcNAc is used as primer for the synthesis of chitin oligosaccharides (Figure 6). Consequently, chitin oligosaccharide synthesis by NodC proceeds by addition of GlcNAc residues to O4 of the nonreducing-terminal residue.

**Elongation of *p*-Nitrophenyl-GlcNAc by NodC.** *p*-Nitrophenyl- $\beta$ -*N*-acetylglucosaminide (pNPGlcNAc) consists of a *p*-nitrophenyl group linked to C1 of GlcNAc in the  $\beta$ -anomeric configuration. pNPGlcNAc was added to NodC reaction mixtures at various concentrations. In addition to free chitin oligosaccharides, four relatively hydrophobic compounds were produced as might be expected for pNP-linked structures (Figure 4A). All these compounds are chitinase-degradable, confirming that they contain chitin oligosaccharides. The pNP-linked chitin oligosaccharides could readily be purified from the reaction mixture using an octadecyl (C18) cartridge. TLC analysis of the resulting preparation (Figure 4B) indicates that in addition to pNP-linked chitin oligosaccharides up to pNP-chitin pentaose, pNP-chitin hexaose is also formed. The synthesis of pNP-linked chitin oligosaccharides increased as the pNPGlcNAc concentration was increased up to 1 mM (Figure 4A, lanes 1–5). Concomitantly, the synthesis of free chitin oligosaccharides decreased. At pNPGlcNAc concentrations higher than 1 mM, both the synthesis of pNP-linked chitin oligosaccharides and the formation of free chitin oligosaccharides decreased (Figure 4A, lanes 1–5), but the length of the oligosaccharides that are formed remains unaltered. These data again show that chitin oligosaccharide chain elongation by NodC takes place at the nonreducing-terminal residue of a growing chain.

**Synthesis of *p*NP-Linked Chitin Oligosaccharides by Zebrafish Embryos.** Extracts of late gastrula zebrafish embryos have been reported to synthesize chitin oligosaccharides using UDP-GlcNAc as precursor (16, 21). To investigate whether the direction of chain elongation in this system is the same as that identified for NodC, extracts of late gastrula (10 h) zebrafish embryos were incubated with UDP-GlcNAc and pNPGlcNAc. Reaction products were purified using a reversed-phase (Sep-Pak) cartridge and were subsequently radiolabeled with GDP-[ $^3$ H]fucose, using the fucosyltransferase NodZ (33). This indirect method for the detection of chitin oligosaccharides is more sensitive than direct labeling with UDP-[ $^{14}$ C]GlcNAc (21). TLC analysis of the fucosylated products (Figure 5A) clearly shows the presence of a compound that migrates as a fucosylated pNP-chitin tetraose standard. Chitinase degradation of this reaction product results in the formation of a degradation product that migrates as a fucosylated pNP-chitinbiose. Together, these data show that the chitin oligosaccharide synthase in extracts of the zebrafish embryos can use pNPGlcNAc as acceptor to synthesize pNP-chitin tetraose. Addition of an antiserum that specifically inhibits the chitin oligosaccharide synthase activity of the DG42 protein (16) reduced the formation of pNP-linked chitin oligosaccharides to less than 5% of the control level observed when a control serum was added (Figure 5B). We therefore conclude that synthesis of pNP-linked chitin oligosaccharides in this system is due to the action of DG42. This confirms the conclusions of two previous reports on the role of DG42 in chitin oligosaccharide synthesis (20, 21). Increasing the pNPGlcNAc concentration above 0.25 mM led to a decrease in pNP-linked chitin

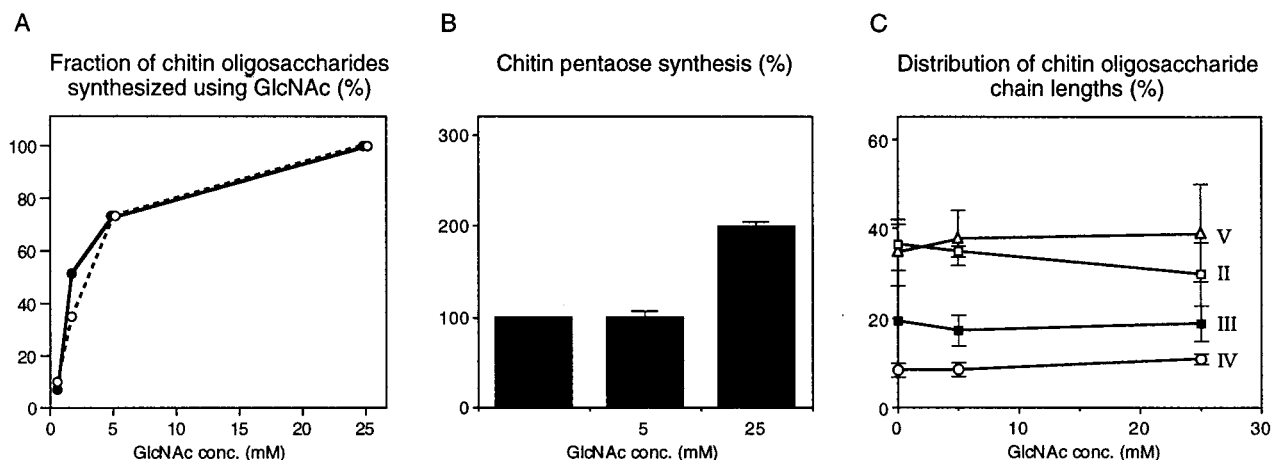


FIGURE 3: Analysis of chitin oligosaccharides produced at various GlcNAc concentrations. All reactions were performed in a standard reaction mixture containing 10  $\mu$ M UDP-GlcNAc. Chitin oligosaccharides were analyzed on silica 60 TLC plates. Results were visualized and quantified by using a PhosphorImager system in combination with the ImageQuant software. (A) The fraction of chitin tetraose (—) and pentaose (- - -) molecules synthesized using free GlcNAc as acceptor was determined by comparing the radioactivity incorporated into chitin tetraose and pentaose by NodC in parallel incubation mixtures in which either free GlcNAc or UDP-GlcNAc was radiolabeled. For example, when 100% of chitin pentaose is synthesized using GlcNAc as acceptor, the ratio between GlcNAc residues from the GlcNAc acceptor and those originating from UDP-GlcNAc is 0.25 (1:4); when 50% of the pentaose molecules is synthesized this way, the ratio is 0.11 (1:9). (B) and (C) are derived from the results of the same incubations. (B) shows the effect of addition of free GlcNAc on the amount of chitin pentaose by NodC, whereas the relative amount of each oligosaccharide produced in these reaction mixtures is shown in (C). The relative amount of each oligosaccharide was determined after correction of spot intensities for differences in chain length. Roman numerals indicate the oligosaccharide chain length. Error bars represent the deviation between two independent experiments.

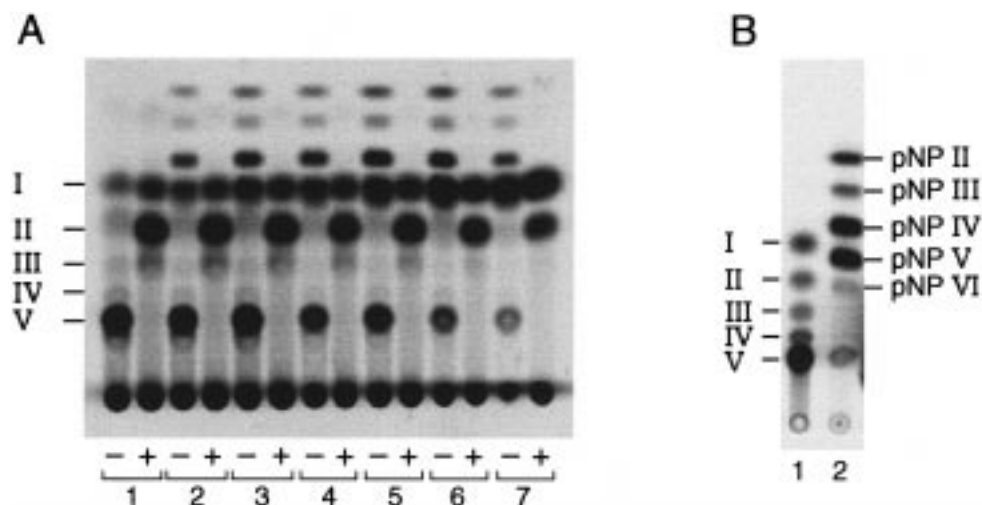


FIGURE 4: TLC analysis of pNP-linked chitin oligosaccharides synthesized by NodC. (A) Reaction products of NodC synthesized in the presence of 50  $\mu$ M UDP-[U- $^{14}$ C]GlcNAc and various pNPGlcNAc concentrations were analyzed on NH<sub>2</sub>-TLC plates as described under Materials and Methods. Samples were loaded onto the TLC plate directly (-), or after incubation with chitinase (+). Incubations were performed with the following pNPGlcNAc concentrations: 1, control reaction without pNPGlcNAc; 2, 0.1 mM; 3, 0.25 mM; 4, 0.5 mM; 5, 1 mM; 6, 2 mM; 7, 4 mM. Roman numerals indicate the position of GlcNAc (I) and chitin oligosaccharides (II–IV). (B) Purification of pNP-linked chitin oligosaccharides on a reversed-phase (C18) cartridge. Lane 1 shows chitin oligosaccharides synthesized by NodC in a control reaction lacking pNPGlcNAc. A sample of the pNP-linked chitin oligosaccharides synthesized in the presence of 1 mM pNPGlcNAc and eluted from the reverse-phase (C18) cartridge is shown in lane 2. Roman numerals indicate the length of chitin oligosaccharides, linked to pNP.

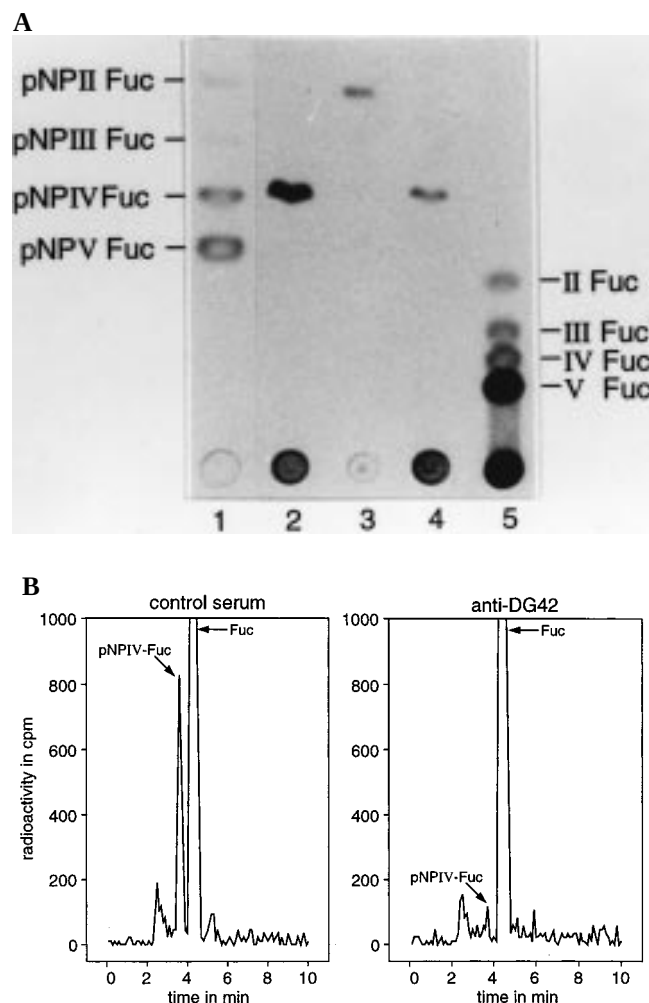
oligosaccharide formation, but just as we observed with NodC, this had no effect on the length of the oligosaccharides that were produced (Figure 5A). We can therefore conclude that DG42 can use pNPGlcNAc as a primer for the synthesis of pNP-linked chitin oligosaccharides in the same way that NodC does.

## DISCUSSION

In view of recent reports suggesting that chitin oligosaccharides or chitin oligosaccharide derivatives may play a general role in developmental processes in plants and animals

(16, 20, 21, 34, 35), defining the biosynthesis pathway of chitin oligosaccharides is becoming increasingly important. Here, we present the results of an *in vitro* study in which we have studied both the mechanism of initiation and the direction of chain elongation in chitin oligosaccharide biosynthesis by NodC.

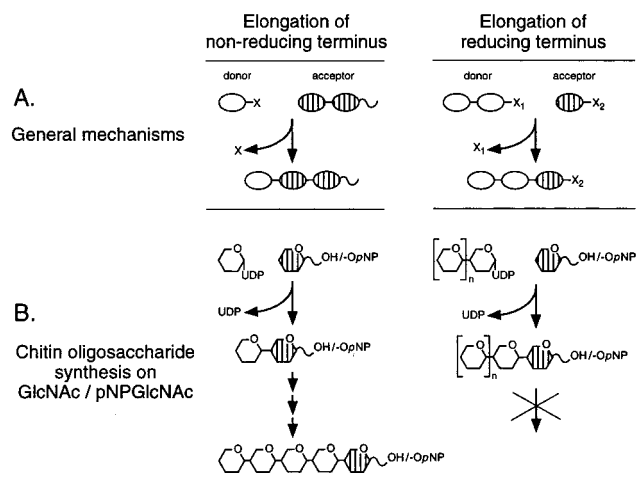
Bacterial polysaccharide synthesis often involves lipid-linked intermediates (36). However, NodC-dependent lipid-linked saccharides were not detected using an extraction procedure that has been used to show the existence of lipid intermediates in the synthesis of bacterial cellulose (37),



**FIGURE 5:** Chromatographic analysis of pNP-linked chitin synthesized by zebrafish embryo extracts. (A) pNP-linked chitin oligosaccharides were purified from reaction mixtures using a reversed-phase (Sep-Pak) cartridge, radiolabeled with [ $^3\text{H}$ ]fucose (Fuc) by the activity of the fucosyltransferase NodZ, and analyzed on  $\text{NH}_2$ -TLC plates. Lanes 1 and 5 show fucosylated pNP-linked chitin oligosaccharides and fucosylated chitin oligosaccharide standards, respectively. The other lanes show the reaction products of zebrafish embryo extracts, after purification on Sep-Pak (C18) cartridges and subsequent fucosylation by NodZ. Lanes: 2, reaction product in the presence of 0.25 mM pNPGlcNAc; 3, a sample of the product obtained by chitinase treatment of the reaction product shown in lane 2; 4, reaction product in the presence of 1 mM pNPGlcNAc. Roman numerals indicate the length of the chitin oligosaccharides in the standards. (B) shows HPLC chromatograms of reaction products formed by zebrafish embryo extracts in the presence of a control serum, or an anti-DG42 serum, which specifically inhibits the enzymatic activity of the DG42 protein (16, 21). The peaks at 3.6 and 4.4 min represent fucosylated pNP-chitintetraose (pNP-IV-Fuc) and free fucose (Fuc), respectively. This was determined by using fucosylated pNP-chitintetraose standard and free fucose as reference compounds.

exopolysaccharides (26), and crustacean chitin (38). In addition, known inhibitors of prenyl pyrophosphate-linked saccharide formation do not affect the chitin oligosaccharide synthase activity of NodC. We therefore conclude that such lipid-linked intermediates are not involved in the synthesis of chitin oligosaccharides by *M. loti* NodC. This conclusion is consistent with the results that were recently obtained with *Azorhizobium caulinodans* NodC (11).

In this paper we report two observations that show that chitin oligosaccharide synthesis by NodC proceeds by the



**FIGURE 6:** Mechanisms of chain elongation in polysaccharide and oligosaccharide biosynthesis. (A) The two possible mechanisms of chain elongation described in the literature. Several examples of each mechanism have appeared (36, 46). In a mechanism of elongation from the nonreducing terminus, monosaccharides from an activated donor are added to the nonreducing-terminal residue of a growing chain. In the other mechanism, a preexisting activated chain is transferred to an activated monosaccharide. The net result is elongation of the growing chain from the reducing terminus. (B) shows how the two elongation mechanisms can lead to the incorporation of free GlcNAc or pNPGlcNAc as the reducing-terminal residue of chitin oligosaccharides. In the case of elongation of the nonreducing terminus (left panel), GlcNAc/pNPGlcNAc acts as initial acceptor or primer. When elongation takes place at the reducing end, however (right panel), GlcNAc/pNPGlcNAc can only be the last residue added to the growing chain. The resulting oligosaccharide no longer carries UDP as the activating group on its reducing terminus. Since this reducing-terminal activator is essential for further elongation in this mechanism, incorporation of GlcNAc/pNPGlcNAc leads to premature chain termination and thus to an increase in the formation of short oligosaccharides.

addition of GlcNAc residues to the nonreducing-terminal residue of a growing chain. First, we show that free [ $^{14}\text{C}$ ]-GlcNAc is directly incorporated into chitin oligosaccharides by NodC without being first converted into UDP-GlcNAc (Figure 2A). Chemical analysis of chitinpentaose into which free [ $^{14}\text{C}$ ]GlcNAc had been incorporated by NodC showed that the GlcNAc was exclusively incorporated as the reducing-terminal residue. If elongation of chitin oligosaccharides takes place at the reducing terminus, such a direct incorporation of free GlcNAc leads to premature chain termination, resulting in an increase in the formation of oligosaccharides that are shorter than the natural end-product chitinpentaose (Figure 6). Instead we have observed that GlcNAc stimulates chitin oligosaccharide synthase activity and does not affect oligosaccharide chain length, even when GlcNAc incorporation into chitintetraose and -pentaose molecules is 100%. NodC can therefore only have incorporated GlcNAc into chitin oligosaccharides by using it as a primer, elongating it by glycosyl transfer to O4. The second observation defining the direction of chain elongation is that NodC is capable of using the synthetic glycoside *p*-nitrophenyl- $\beta$ -*N*-acetylglucosaminide (pNPGlcNAc) as an acceptor, producing pNP-linked chitin oligosaccharides. Since pNPGlcNAc has a free hydroxyl group at C4 but not at C1, chain elongation must have taken place at the nonreducing-terminal residue.

We have observed that chitin oligosaccharides ranging from chitinbiose to chitintetraose are incapable of acting as

efficient primers. These results indicate that chitin oligosaccharide synthesis by NodC occurs by a processive mechanism: in such a mechanism, successive addition of saccharide units to a growing chain only proceeds while the enzyme remains continuously in close contact with the oligosaccharide; dissociated oligosaccharides are not further elongated.

Stimulation of the enzymatic activity of NodC by free GlcNAc was only observed at GlcNAc concentrations at which all chitin oligosaccharides produced were synthesized using the added GlcNAc as a primer. This correlation suggests that the use of GlcNAc as primer is involved in the stimulation mechanism. Free GlcNAc is also known to stimulate the *in vitro* synthesis of chitin polymers by fungal chitin synthases. In some cases, GlcNAc appears to be incorporated directly into chitin (39, 40) in a manner similar to that which we report here for NodC. Our present results raise the possibility that GlcNAc can also be used as a primer in chitin synthesis. Considering the similarity between NodC and chitin synthase with respect to amino acid sequence, predicted secondary structure, membrane localization of the two proteins, structural similarity of the enzymatic products, and stimulation of both enzymes by GlcNAc (12, 41–43) (M. Horsch, University of Zürich, personal communication), our results from NodC might suggest that the elongation of chitin polymers also occurs at the nonreducing terminus.

The biosynthesis of  $\beta$ -polysaccharides has been proposed to proceed via a mechanism in which two monosaccharide residues are added to the reducing terminus of a growing chain in a single catalytic event (41). Such a mechanism results in the addition of disaccharide units to a growing chain. NodC, however, produces all oligosaccharide chain lengths ranging from chitinbiose to the end-product chitin-pentaose. Our results therefore suggest that NodC does not use this mechanism.

The use of pNPGlcNAc as a primer for chitin oligosaccharide synthesis allows the rapid purification of reaction products using reversed-phase cartridges. With this novel method for the detection of chitin oligosaccharide synthase activity we show that the chitin oligosaccharide synthase activity which has been reported to be present in late gastrula zebrafish embryos (16, 21) is also able to use pNPGlcNAc as an acceptor. Addition of an antiserum that specifically interacts with DG42 and inhibits chitin oligosaccharide synthesis (16, 21) blocked the chitin oligosaccharide synthase activity forming the pNP-linked chitin-tetraose. We therefore conclude that the DG42 protein can use pNPGlcNAc to synthesize pNP-linked chitin oligosaccharides. Incorporation of pNPGlcNAc into pNP-linked chitin oligosaccharides by DG42 does not shift the synthesis toward the formation of short oligosaccharides, as was also not the case with NodC. Consequently, chain elongation in chitin oligosaccharide synthesis by the rhizobial NodC protein and zebrafish DG42 is comparable. The nature of the enzymatic activity of DG42 is currently a matter of debate (44). There are investigators who have shown a DG42-dependent chitin oligosaccharide synthase activity similar to what we report here (16, 20, 21), whereas others have concluded that DG42 is involved in hyaluronic acid synthesis (18, 19). Interestingly, elongation of growing hyaluronic acid chains in mammalian cells has been reported to take place at the reducing-terminal residue (45), which is opposite to what we show here for the DG42-dependent formation of chitin oligosaccharides by zebrafish

embryos. It seems unlikely that DG42 can catalyze both these reactions. Our data therefore support the notion that DG42 encodes a chitin oligosaccharide synthase, rather than a hyaluronic acid synthase. The use of pNP-linked saccharides as acceptors provides a novel approach to investigate the direction of chain elongation in polysaccharide synthesis and the substrate specificity of polysaccharide synthases which may help to further address the apparent discrepancy regarding the biochemical function of DG42.

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