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# Biosynthesis and Structure of Cell Associated Glucans in the Slow Growing *Rhizobium loti* Strain NZP 2309

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## Abstract

Slow growing *Rhizobium loti* strain NZP 2309 synthesizes cellular neutral cyclic β(1-6), β(1-3) glucans closely related with the previously described cyclic β(1-6), β(1-3) glucans of *Bradyrhizobium japonicum* USDA 110. This glucan consists of ten (three-1,3 and six-1,6 β-linked glucose units) with a single 6 branched glucose unit with a β-1 linked terminal glucose. The ratio of β(1-6) to β(1-3) linked glucose units is 2:1. Incubation of inner membranes derived from strain NZP 2309 with UDP-[$^{14}$C]glucose led to the incorporation of radioactivity into soluble and TCA-insoluble compounds. A soluble compound was characterized by gel filtration, TLC, mild acid hydrolysis, paper chromatography and Smith degradation as a β(1-3) linked ring of 10 glucose units with a β(1-6) linked branch of two glucoses, similar to that formed *in vitro* by the slow growing strain *B. japonicum* USDA 110 and identical to the glucan synthesized by the *ndv*C mutant of *B. japonicum* AB-1 (Bhagwat et al., 1999). A TCA-insoluble compound labeled after incubation with UDP-[$^{14}$C]glucose was characterized as an insoluble protein having an apparent

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molecular mass of 85 kDa and an associated insoluble laminarin-like glucan. Pulse chase experiments suggest that this protein may be an intermediate or precursor during the synthesis of insoluble laminarin-like glucans, soluble β(1-3), β(1-6) glucans, or both. REP-PCR analysis showed that *R. loti* NZP 2309 and *B. japonicum* USDA 110 are different strains; but according to PCR-RFLPs of the 16SrDNA gene they are closely phylogenetically related. The strains have different host specificity for nodulation.

Keywords: *Rhizobium loti*, cyclic glucan structure, NMR

## 1. Introduction

*Rhizobium* sp. form nitrogen-fixing nodules on legumes, and the related *Agrobacterium* sp. induces crown gall tumors on a broad range of dicotyledonous plants. Both genera are included in the family Rhizobiaceae. Cell-associated glucans are present in bacteria of different genera. In Rhizobiaceae, cyclic β(1-2) glucans play a crucial role in nodule invasion and virulence (Puvanesaraja et al., 1985; Dylan et al., 1986; Geremia et al., 1987). Both chromosomal virulence genes (*chvB* and *chvA*) of *Agrobacterium* sp. (Zorreguieta and Ugalde, 1986; Iñón de Iannino and Ugalde, 1989) and nodule development genes (*ndvB* and *ndvA*) of *Rhizobium* sp. (Dylan et al., 1986; Geremia et al., 1987; Zorreguieta and Ugalde, 1986; Iñón de Iannino and Ugalde, 1989; Stanfield et al., 1988) are involved in the synthesis and secretion of cyclic β(1-2)glucans. The *chvB* gene of *Agrobacterium* and *ndvB* gene of *Rhizobium* encodes a large membrane protein with an apparent molecular mass of 235 kDa (Zorreguieta and Ugalde, 1986). However, as suggested by nucleotide sequence analysis, the molecular mass of the protein encoded by *ndvB* revealed a molecular mass of 319 kDa (Ielpi et al., 1990).

The ChvB protein is thought to be a multifunctional enzyme that catalyses the synthesis of the cyclic β(1-2)glucans (Altabe et al., 1990; Castro et al., 1996). We have proposed that the synthesis is initiated by the glucose residues transferred from UDP-glucose to an unidentified amino acid residue of the 319 kDa inner membrane protein. The polyglucose β(1-2) chain then elongates until it reaches a degree of polymerization adequate to be cyclizised and is released from the intermediate protein (Iñón de Iannino and Ugalde, 1996). The 319 kDa protein is responsible for initiation, elongation and cyclization, and determines the degree of polymerization of the cyclic glucan (Lepek et al., 1990).

The family Rhizobiaceae includes both fast and slow-growing species. In those species producing periplasmic cyclic glucans, a relationship between the type of glucan synthesized and the growth rate is observed (Iñón de Iannino and Ugalde, 1993). Fast growing species such as *R. fredii* produce cell-associated

cyclic β(1-2) glucans (Bhagwat et al., 1992; Iñón de Iannino et al., 1996). Slow growing species such as *Bradyrhizobium japonicum* synthesize cyclic β(1-3), β(1-6) glucans (Miller et al., 1990; Tully et al., 1990; Iñón de Iannino and Ugalde, 1993) and phosphocholine substituted β(1-3), β(1-6) glucans (Rolin et al., 1992). *R. fredii* and *B. japonicum* nodulate soybean and other legumes. Biosynthesis of cyclic glucans with β(1-3), β(1-6) linkages and insoluble laminarin-like glucans were described in *B. japonicum* USDA 110 and *Bradyrhizobium* strains BR4406 and BR8404 isolated from tree legume nodules in Southeast Brazil (Iñón de Iannino and Ugalde, 1993). Proteins of 90 kDa and 100 kDa, respectively, were suggested to be involved in the biosynthesis of these glucans. *Azospirillum brasiliense* accumulates β(1-3), β(1-6) glucans in the periplasm. The biosynthesis of these glucans was examined but an intermediate protein was not identified to participate in their synthesis and no insoluble laminarin-like glucans were produced *in vitro* by purified bacterial inner membranes with UDP-glucose (Altabe et al., 1994).

Two genes related to cyclic glucan biosynthesis have been identified in *B. japonicum*: *ndvC* and *ndvB*, which respectively encode a putative β(1-6) glucosyl transferase, and a putative β(1-3) glucan synthetase (Bhagwat et al., 1996). Mutants of *ndvB* produced no glucans (Bhagwat and Keister, 1995), and the *ndvC* mutant of *B. japonicum* AB-1 synthesized cyclic β-glucans, which lacked β(1-6) glucosyl linkages (Bhagwat et al., 1999). It is currently postulated that β(1-6) chains are assembled upon or simultaneously added to β(1-3) linked chains and in the absence of the latter, no glucan synthesis takes place. This hypothesis is attractive since the *ndvB* mutant (strain AB-14), although carrying a functional copy of the *ndvC* gene, is unable to synthesize glucans. Putative NdvC protein had a region of similarity in the vicinity of a conserved active-site domain of other enzymes involved in β-glucan metabolism, e. g., yeast β-glucan synthases Kre6p and Skn1p (Bhagwat et al., 1996). Using hydrophobic cluster analysis, Montijn et al. (1999) showed that Kre6 and Skn1 share significant similarities to family 16 glycoside hydrolases but not to nucleotide diphospho-sugar glycosyl transferases, indicating that they are glucosyl hydrolases or transglucosylases instead of genuine glucosyltransferases as previously described.

A fast growing strain of *Rhizobium loti* isolated from nodules of *Lotus tenuis* produced cellular cyclic β(1-2) glucans similar to that described in *Agrobacterium tumefaciens* and fast growing strains of *Sinorhizobium meliloti* (formerly known as *Rhizobium meliloti*), *R. fredii* and *R. leguminosarum* (Lepek et al., 1990; York et al., 1987; Hisamatsu et al., 1983; Zevenhuizen et al., 1990). Because slow growing strains of *R. loti* also exist, we have characterized the cellular glucans accumulated by one of these strains (*R. loti* NZP2309) and investigated their synthesis.

Comparative studies by repetitive extragenic palindromic sequence (rep)-PCR genomic fingerprint showed that *R. loti* NZP 2309 and *B. japonicum* USDA 110 are different strains of *Bradyrhizobium*, but according to PCR-RFLPs of the 16S rDNA gene they are closely phylogenetically related (Estrella et al., unpublished data). These strains have different host specificity for nodulation. *R. loti* NZP 2309 forms effective nodules on *Lotus pedunculatus* and *Lotus corniculatus*, and does not form nodules in *Glycine max* cv. Mc Call or Peking. On the other hand *B. japonicum* USDA 110 is specific for the nodulation of *Glycine max* cv. Mc Call or Peking and not effective in the nodulation of *Lotus* spp. (Estrella et al., unpublished data). These results are in agreement with previous studies carried out by Vinuesa et al. (1998). These authors showed by genotypic characterization that *Bradyrhizobium* strains nodulating legumes of the Canary islands are related phylogenetically to *B. japonicum*; however nodulation assays revealed that none of the Canarian isolates nodulated *Glycine max* (Vinuesa et al., 1998).

## 2. Materials and Methods

*Bacterial strains and media*

*Rhizobium loti* NZP 2309 was obtained from Steven G. Pueppke (Department of Plant Pathology, University of Missouri, Columbia, MO). *B. japonicum* strain USDA 110 was obtained from the United States Department of Agriculture, Beltsville, MD, USA. Strains were grown for 5 days in yeast extract-mannitol medium (AMA) at 28°C with good aeration in a rotary shaker. AMA contains 10 g mannitol, 1 g yeast extract, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.5 g $K_2HPO_4$ per liter.

*Extraction of cell-associated oligosaccharides*

Cells from 100 ml cultures were harvested by centrifugation at 10,000 x g for 10 min. Pellets were extracted with 1% trichroloacetic acid (TCA) for 30 min at room temperature as described previously (Iñón de Iannino and Ugalde, 1993). TCA extracts were neutralized with ammonium hydroxide, concentrated and subjected to gel filtration on Bio-Gel P4 columns as described (Iñón de Iannino and Ugalde, 1989). For preparative purposes 20 liter cultures were extracted with 2% TCA and 10% TCA successively and the neutralized extracts subjected to preparative Bio-Gel P4 chromatography (2 cm x 90 cm) eluted with 15% acetic acid, fractions of 3 ml were collected, and carbohydrates were detected by the anthrone-sulphuric acid method (Dische, 1962). No more than 10 mg of glucose equivalent TCA extracts were run through the column in order to

improve separation. Compounds with similar elution volumes were pooled and lyophilized. Glucans were further purified by HPLC on a $C_{18}$ ODS/2 column (see below).

*Preparation of inner membranes*

*R. loti* cells were harvested after 5 days of culture and the capsule removed by shearing as described previously (Iñón de Iannino and Ugalde, 1993). Decapsulated cells were washed twice with 30 mM TrisHCl buffer (pH 8.0) and inner membranes prepared following the method previously described for other gram-negative bacteria (Osborn and Munson, 1984). PMSF (Phenyl methyl sulfonile fluoride) 2 mM was added prior to shearing with a French Press.

*In vitro synthesis of glucan*

Inner membranes (200 µg protein) were incubated with UDP-[$^{14}C$]glucose (90,000 cpm; 10.5 Gbq/mmol), 10 mM $MgCl_2$, 50 mM Tris hydrochloride buffer (pH 8.2) in a total volume of 50 µl. The reaction was carried out at 10°C, 20°C or 30°C, and stopped depending on the experiment, by the following methods:

A) Addition of 300 µl of water. Samples were heated at 100°C for 1 min, centrifuged at 5,000 x g for 5 min and supernatants subjected to DEAE-Sephadex chromatography as previously described (Zorreguieta and Ugalde, 1986). Pellets were resuspended in 10% TCA and filtered through glass-fiber filters to determine incorporation into insoluble products as described (Zorreguieta and Ugalde, 1986). DEAE-sephadex percolates were subjected to gel chromatography in a Bio-Gel P4 column as previously described (Iñón de Iannino and Ugalde, 1989).

B) Addition of 1 ml of cold 10% TCA. Samples were centrifuged at 5,000 x g for 10 min and pellet subjected to polyacrylamide gel electrophoresis and fluoro-graphy as described (Briones et al., 1997). When required, the TCA pellet was filtered through glass fiber filters as in method A.

For characterization of *in vitro* synthesized glucans the synthesis was carried out at 30°C for 30 min and stopped by method A. When the characterization of *in vitro* soluble glucan was not required we used the inactivation with 10% TCA (Method B).

*High performance liquid chromatography (HPLC)*

Products recovered from the preparative Bio-Gel P4 column (fractions 35 to 42) were subjected to HPLC on a $C_{18}$ ODS2 Pharmacia column (25 cm x 4 mm). After application of each sample the column was washed with water (25 ml)

and the glucans were eluted in isocratic form with 40 ml of 20% methanol. The column was eluted at a flow rate of 0.5 ml $min^{-1}$ at room temperature. Fractions of 1 ml were collected. The glucans eluted in fractions 4 and 5.

*Acid hydrolysis, paper chromatography and thin layer chromatography*

Total and partial acid hydrolysis was carried out as previously indicated (Iñón de Iannino and Ugalde, 1989). Hydrolysates were subjected to descending paper chromatography on Whatman No.1 paper (Whatman, Clifton, NJ, USA) with solvent A [butanol-pyridine-water (6:4:3)] (Jeanes et al., 1951), or solvent B [isopropanol-acetic acid-water (27:4:9)] (Tung and Nordin, 1968). Sugars were detected by the alkaline silver method (Treveylan et al., 1950). TLC (thin layer chromatography) was carried out with silica gel-60 plates (Merck) developed with solvent C [butanol-ethanol-water (5:5:4, by vol.)] as previously indicted (Briones et al., 1997). Sugars on the TLC plates were detected by spraying with 5% sulfuric acid in ethanol and heating for 5 min at 120°C.

*Paper electrophoresis*

Paper electrophoresis was carried out with buffer C (2% sodium molybdate pH 5.0) for 2h at 15 V/cm (Bourne et al., 1961) or buffer D [0.05 M sodium borate (pH 9.2) for 2 h at 15 V/cm].

*Reduction with borohydride*

Oligosaccharides were dissolved in 300 µl of water, 8 mg of $NaBH_4$ was added and the samples incubated overnight in the dark. To remove $Na^+$, an excess of Dowex 50W-X8 resin (Bio-Rad Laboratories, Richmond, CA, USA) was added, and oligosaccharides were recovered by washing the resin with water. Samples were dried several times under an air stream; methanol was added and evaporated in order to remove boric acid as methyl borate. Reduced oligosaccharides were subjected to total acid hydrolysis with HCl 1 M 4 h at 100°C, HCl was eliminated by several evaporations under an air stream and hydrolysate subjected to paper electrophoresis with buffer C.

*Smith degradation*

Oligosaccharides were oxidized for 3 days with 0.04 M sodium periodate at 4°C in the dark (final reaction volume 200 µl). The excess of periodate was destroyed with 10 µl of ethylene glycol and after 2 h, 8 mg of sodium borohydride were added. Samples were kept in the dark for 16 h. Acetic acid

(10%) was added to eliminate the $NaBH_4$ excess and samples were chromatographed through a Bio-Gel P2 column to remove salts. Desalted samples were lyophilized. Products were subjected to mild acid hydrolysis (HCl 0.1 M at room temperature over night) or to total acid hydrolysis (HCl 1 M 4 h, 100°C). HCl was removed by evaporation under an air stream and hydrolysates subjected to gel chromatography on Bio-Gel P2 columns.

*Digestion with laminarinase*

Samples were incubated at 37°C with 20 µg of laminarinase (Sigma EC 3.2.1.6) 5.7 U/mg in 50 mM Na-acetate buffer (pH 5.0).

*DEAE-Sephadex chromatography*

Oligosaccharides were loaded on DEAE-Sephadex A-25 columns (6 by 0.8 cm; Pharmacia Fine Chemicals, Piscataway, NJ) and were batch-eluted with 6 ml of water and with 6 ml portions of an increasing concentration of NaCl as described previously (Iñón de Iannino and Ugalde, 1989).

*Sample preparation for direct amplification of DNA from cell cultures*

Genomic DNA of the bacteria was prepared from liquid cultures harvested at stationary-phase by a standard protocol (Sambrook et al., 1989). The DNA of this preparation was used as template in polymerase chain reactions (PCRs).

*16S rDNA amplification by PCR*

Primers 27f (5'-GAGATTTGATC-CTGGCTCAG) and 1495r (5'-CTACGGCTA CCTTGTTACGA), are derived from conserved regions of the 16S rRNA genes and are capable of amplifying nearly full-length 16S rDNA from many bacterial genera (Grifoni et al., 1995). PCR amplification was carried out by mixing template DNA, i.e., pure DNA (50 µg) or alternatively supernatant of boiled cells (1 to 2 µl), with the polymerase reaction buffer (10 mM Tris-Cl, pH 9.9, 50 mM KCl, 1% Triton X- 500, 1.5 mM $MgCl_2$); 20 µM (each) primers 27f and 1495r; and 2U of Taq DNA polymerase (Promega, Madison, WI). DNA amplification was done in a Minicycler MJ research-Thermoblock, USA, with the following temperature profile: an initial denaturation at 95°C for 3 min; 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 56°C), and extension (2 min at 72°C); and examined by horizontal electrophoresis in 0.9% agarose gel.

*Restriction fragment analysis*

The PCR products were digested with restriction enzymes Hinf I, Sau 3AI, TaqI and HhaI (Promega) and resolved by electrophoresis on 2% agarose gels in Tris-Borate-EDTA (Laguerre et al., 1994). A 100 bp ladder (GIBCO BRL, Eggenstein, Germany) was run at both sides and in the central lane of each gel. Gels were stained in an aqueous solution of ethidium bromide. A dendogram was constructed from the distance matrix by using the unweighted pair group method using arithmetic means (UPGMA) (Sokal et al., 1958).

*Repetitive extragenic palindromic sequence (rep) amplification by PCR*

Oligonucleotide primers were based on the highly conserved repetitive extragenic palindromic (REP) repeated DNA element (Versalovic et al., 1991). For this study, primers REP 1R (5'-IIIICgICgICATCIggC-3') and REP 2I (5'-ICgICTTATCIggCCTAC-3') were used. PCR tubes (25 µl) contained 50 pmol of each primer; 50 ng of template DNA; 1.25 mM (each) dATP, dCTP, dGTP and dTTP; 2U of Taq DNA Polymerase (Promega, Madison, Wisconsin); 10% dimethylsulfoxide; 160 µg/ml of bovine serum albumin; 1X Gitchier Buffer (de Bruijn et al., 1996). PCR was performed in an automated Perkin-Elmer thermal cycler, with an initial denaturation step at 95°C for 7 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min and extension at 65°C for 8 min. Twelve microliters samples of amplification products were electrophoresed in a 1.6% agarose gel containing 0.5 x TBE (Tris-boric-EDTA) (de Bruijn et al., 1996) and 0.5 µg of ethidium bromide per ml and photographed under UV light.

*Nodulation test*

Soybean seeds of McCall and Peking cultivars were surface sterilized and germinated on water-agar plates for 2 days as described (Pueppke, 1983). *L. pedunculatus* and *L. corniculatus* var. *hirsutus* were sterilized with sulfuric acid for 15 min, washed with sterilized water several times to eliminate sulfuric acid and hydrated during 24 h. Disinfected seeds were germinated on water-agar plates for 3 days in the dark. Seedlings were planted in autoclaved modified Leonard jars filled with vermiculite and Jensen's nitrogen-free solution (Vincent, 1970). Seedlings were inoculated with 0.2 ml of *Rhizobium* culture ($A_{660}$ nm 0.8) or 0.2 ml AMA medium. After 6 weeks plants were removed, nodules counted and nitrogen fixation evaluated by the acetylene reduction assay as described (Wacek and Brill, 1976). For Lotus spp. 10 plants for each strain were examined. For soybean 3 plants for each strain were tested.

*HPAEC-PAD analyses*

Conditions for high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) were similar to those reported previously (Koizumi et al., 1989). HPAEC-PAD was performed with a Dionex 4500 system, which included a Pulsed Electrochemical Detector (PED; gold working electrode) operated in the integrated amperometry mode (E1 = 0.35 V, 0.48 sec; E2 = 0.95 V, 0.12 sec; E3 = -0.25 V, 0.07 sec; integration: 0.28–0.48 sec), and a pressurized bottle post-column delivery system that added 500 mM sodium hydroxide prior to the detector. A CarboPac PA1 column (4 X 250 mm; Dionex Corp.) and a CarboPac PA Guard column (3 X 25 mm; Dionex Corp.) were utilized. The mobile phase (0.8 ml/min) consisted of a linear gradient of 5–375 mM $CH_3COONa$ in 100 mM NaOH for 90 min (obtained by mixing eluants consisting of 100 mM NaOH and 500 mM $CH_3COONa$/100 mM NaOH) and then up to 500 mM $CH_3COONa$ in 100 mM NaOH at 100 min. Chromatograms thus obtained were analyzed with a Chrom Perfect Direct (Justice Innovations) chromatography data system that included a DT2804 A-D board. Maltrin M-040 Maltodextrin was generously provided by Grain Processing Corp. (Muscatine, IA) and α- and β-cyclodextrins were generously provided by American Maize-Products Co. (Hammond, IN).

*FAB mass spectral analysis*

Mass spectra were obtained with a VGZAB-2SE/FPD magnetic sector mass spectrometer (VG Analytical, Manchester, UK) by fast atom bombardment ionization at 8 kev using a thioglycerol/m-nitrobenzoic acid (MNBA) matrix. An exponential downscan from 4000-100 AMU at a scan rate of 15 sec/decade with a 2 s reset time was used.

*Nuclear magnetic resonance spectroscopy*

$^{1}H$ and $^{13}C$ NMR spectroscopy was performed with a Varian Unity plus 400 MHz spectrometer at 35°C. The samples were exchanged at least twice in $D_2O$, before obtaining the spectra in $D_2O$. The 1D $^{13}C$ spectra were obtained with a 70 degree pulse and 6000–15,000 scans, depending on the quantity of sample available, and a repetition time of 1.8 s. Spectral widths were 25,000 Hz with 32K data points. Linebroadening was 3–5 Hz depending on the level of noise in the spectrum. Chemical shifts were measured relative to external p-dioxane (67.4 ppm).

The HMQC experiments were run in the phase-sensitive mode using the TPPI method with a GARP sequence for $^{13}C$ decoupling (Crousman and Carlson, 1994). Low power presaturation was applied to the residual HDO signal. The proton

spectral width was 1554 Hz (3.7 ppm) and the carbon spectral width was 22,124 Hz with a total data of 256 pts. zero filled to 1024 and processed with a sine bell function, 0.0 Hz linebroadening. The 1H homonuclear 2D COSY experiments were performed on a Varian Unity plus 400 MHz spectrometer with low power presaturation of the water signal.

The two dimensional gradient heteronuclear multiple bond correlation (HMBC) (Bax and Summer, 1986) spectrum of the cyclic glucan was recorded on a Varian Unity Plus 600 MHz spectrometer. The spectrum was collected with 272 transients of 4K complex points in the $^{1}$H direct dimension with the $^{13}$C correlations evolved in 128 increments in the indirect dimension. The $^{1}$H sweep width was 3,001 Hz and the $^{13}$C sweep width was 75 ppm. The HMBC low pass filter delay was set to 3.3 ms to remove one bond $^{13}$C-$^{1}$H correlations with the long range couplings optimized using a 60 ms delay. Data were processed as a 4K by 512 real matrix. The $^{1}$H dimension was multiplied by a 12 Hz exponential and the indirect $^{13}$C dimension was multiplied by a 90 degree shifted sine function. The obtained spectrum was in the absolute value mode.

## 3. Results

Characterization of *Rhizobium loti* cellular glucans. The glucans obtained from *R. loti* NZP 2309, a strain that effectively nodulate *L. corniculatus* var. *hirsutus* and *L. pedunculatus* and do not form nodules on *Glycine max* cv Mc Call and Glycine max cv Peking (Estrella et al., unpublished data), were studied. As it is shown in Fig. 1, TCA extracts of *R. loti* NZP2309 were resolved in a preparative Bio-Gel P4 column into six anthrone-positive compounds. Two percent TCA extracted approximately 70% of the glucans (pools 3 to 5, Fig. 1A), while 10% TCA extracted the remaining 30% (Fig. 1B). However, the profile was very similar in both cases. Thus, 10% TCA is advisable to be used for a complete extraction of glucans.

Total acid hydrolysis and paper chromatography with solvent A, of compounds present in pool 1 (fractions 60 to 66) and pool 2 (fractions 48 to 52) revealed that they contained, besides glucose as the main monosaccharide, two other sugars that were tentatively identified as mannose and fucose. No further studies were carried out on these two pools.

Total acid hydrolysis, paper chromatography with solvent A and TLC with solvent C carried out on pool 3 (fractions 35 to 42), pool 4 (fractions 23 to 25), pool 5 (fractions 19 to 21) and pool 6 (fractions 9 to 11) revealed that they contained glucose as the only monosaccharide. Partial acid hydrolysis and paper chromatography with solvent B carried out on pools 3, 4 and 5 yielded similar patterns. Glucose, gentiobiose, laminaribiose and a series of oligosaccharides with increasing degree of polymerization and in identical

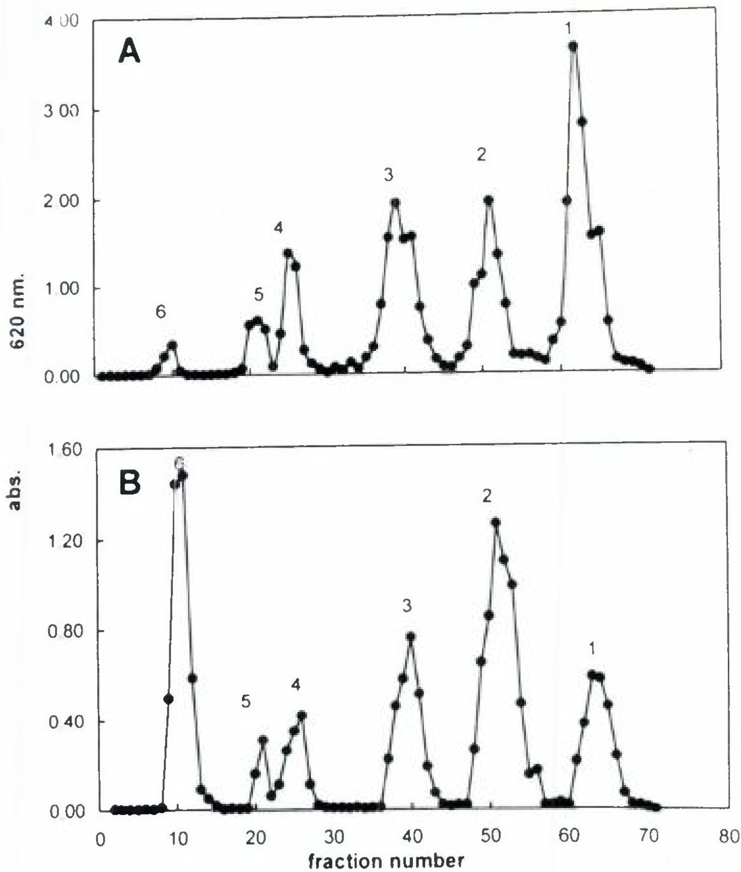


Figure 1. Bio-Gel P4 chromatography of *R. loti* NZP2309 TCA cell extracts. Extraction of cellular glucans with TCA, gel chromatography in preparative column, and assays of carbohydrates were carried out as described in Materials and Methods. A: 2% TCA extract; B: 10% TCA extract.

relations were recovered, thus indicating that they are a closely related family of glucans containing β(1-6), β(1-3) linkages.

Pool 3, pool 4 and pool 5 behaved as neutral glucans upon DEAE-sephadex chromatography. TLC with solvent C of these pools indicated that pool 3 gave a pattern of closely situated bands with moving rates comparable with maltooligosaccharides (DP=11, DP=9 and DP=12), while pools 4 and 5 remained close to the TLC origin. TLC characterization suggested that pool 4 and 5 could be linear glucans. A FAB mass spectrum demonstrated that pool 4 was a linear glucan with DP=14 (M+1=2386) and pool 5 contained a linear

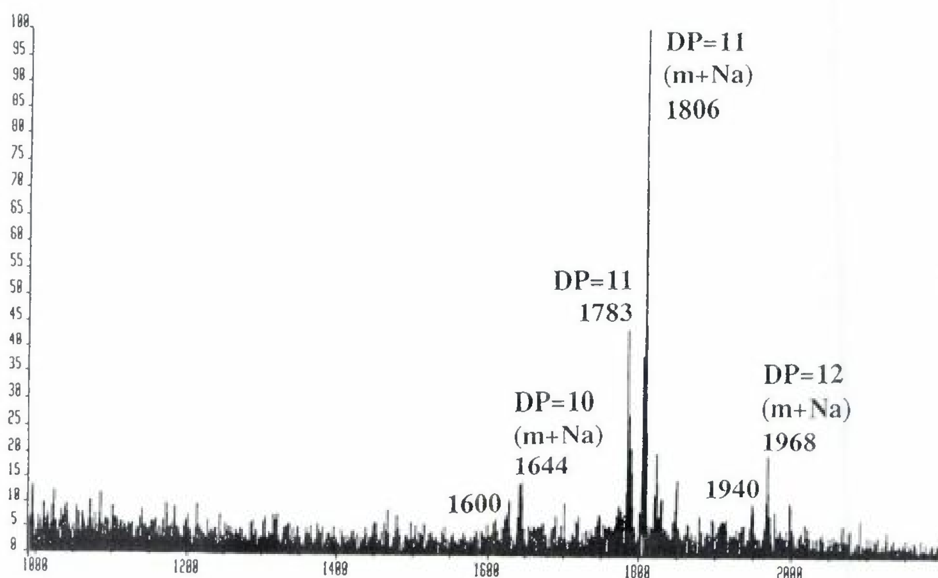


Figure 2. FAB mass spectrum of cyclic glucans derived from *R. loti* NZP 2309. Note that all three glucans exhibit larger molecular ions corresponding to M+Na than M+1. See experimental section for conditions used.

glucan with DP=13 (M+1=2124) and a cyclic glucan with DP=16 (M+1=2593) (data not shown). Further characterization of pool 3 by 1D and 2D-NMR spectroscopy was carried out (see below).

*Structural analysis of the cell-associated glucan*

The compounds recovered in pool 3 from *R. loti* strain NZP 2309 contained only glucose and were desorbed from a $C_{18}$ ODS/2 column with 30% aqueous methanol. The positive ion FAB mass spectrum showed that the compounds are glucans with the composition of approximately 76% DP=11 plus 10% each of DP=12 and DP=10. All the products are cyclic as indicated by their molecular ions (-$H_2O$). (M+1=1783, M+Na=1806; M+1 =1946, M+ Na =1968; M+1=1622; M+Na =1644) (see Fig. 2).

High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex column shows that the major component (Fig. 3A) has a slightly shorter retention time (44.7 min) than the *B. japonicum* cyclic β(1-3), β(1-6) glucan (45.28 min) (Fig. 3B) DP=12 M+1=1946 described previously (Rolin et al., 1992).

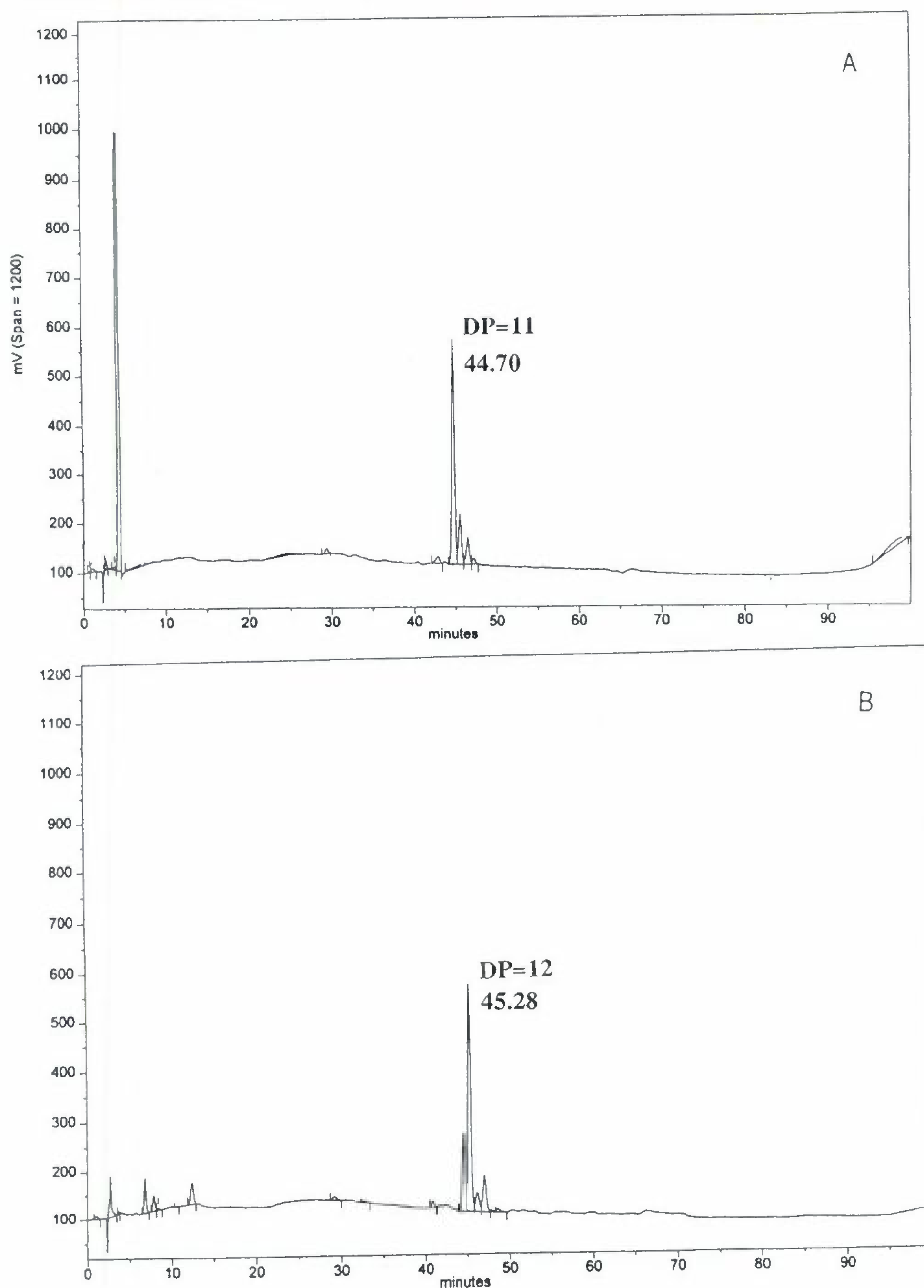


Figure 3. HPAEC-PAD separation of gluco-oligosaccharides. A: HPAEC profile of *R. loti* NZP 2309 cyclic glucans; B: profile of cyclic glucans derived from *B. japonicum* USDA 110. See experimental section for chromatographic conditions.

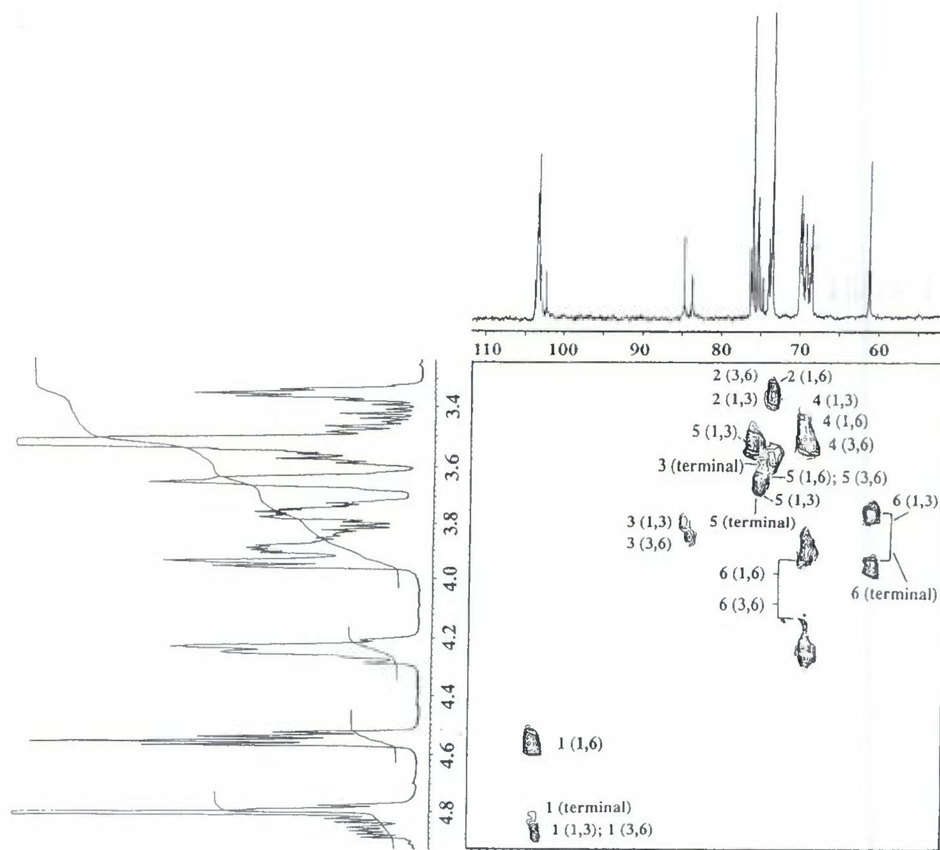


Figure 4. 2D HMQC spectrum of cyclic glucans derived from *R. loti* NZP 2309. See experimental section for details on conditions used.

The 2D HMQC spectrum (Fig. 4) displayed all eleven C1 resonances (11 glucose units based on FAB mass spectral data) centered at 103.5 ppm, three C3 glycosidically-linked carbon resonances (ratio of 2:1:1) at 84.0, 84.2 and 85.0 ppm, respectively indicating that four of the eleven glucose units are C3 linked. Resonances for C3 and C5 carbons of a non-reducing terminal glucose residue were observed at 76.3 ppm and 77.0 ppm (intensity of one carbon each), respectively, indicating that this cyclic structure contains a single branch off the ring. Resonances corresponding to the C3 and C6 of the 3,6 linked glucose branch are observed at 85.0 and 70.5 ppm, respectively. Additionally the resonances of four non-glycosylated C6 carbons (one assigned to the terminal glucose residue and three to the 1,3 linked glucose units within the ring are observed at 61.5 ppm (Table 1). The chemical shifts and correlations of the corresponding protons were verified with a $^{1}H$ 2D COSY experiment (see Table 1).

Table 1. $^1H$ and $^{13}C$ chemical shift compilation for β-1,3; 1,6 cyclic glucan

| Resonance assignment | $^1H$ (ppm) | # of protons | $^{13}C$ (ppm) | # of carbons |
|---|---|---|---|---|
| 1(1,3); 1(3,6) | 4.70; 4.75; 4.81; 4.83 | 4 | 103.0 | 4 |
| 1(1,6) | 4.42; 4.43; 4.44 | 7 | 104.0 | 7 |
| 1(terminal) | 4.44 | nr | 103.3 | 1 |
| 2(1,3) | 3.51; 3.52; 3.54 | nr | 73.8 | nr |
| 2(1,6) | 3.27; 3.31; 3.35 | nr | 73.8 | nr |
| 2(3,6) | 3.55 | nr | 73.8 | nr |
| 2(terminal) | 3.32 | nr | 73.4 | nr |
| 3(1,3) | 3.72; 3.80 | nr | 84.0; 84.2; 85.0 | 2:1:1 |
| 3(1,6) | 3.49 | nr | 76.3 | nr |
| 3(3,6) | 3.50 | nr | 73.6 | nr |
| 3(terminal) | 3.58 | nr | 75.0 | 1 |
| 4(1,3) | 3.54 | nr | 69.9; 70.18; 70.3 | nr |
| 4(1,6) | 3.50 | nr | 70.0 | nr |
| 4(3,6) | 3.42 | nr | 70.2 | nr |
| 4(terminal) | 3.38 | nr | 70.3 | nr |
| 5(1,3) | 3.63 | nr | 75.5 | nr |
| 5(1,6) | 3.65 | nr | 76.3 | nr |
| 5(3,6) | 3.70 | nr | 76.8 | 1 |
| 5(terminal) | 3.68 | nr | 76.8 | 1 |
| 6(1,3) | 3.70; 3.90 | nr | 61.50 | 3 |
| 6(1,6) | 3.83; 4.20 | 6 | 69.50 | nr |
| 6(3,6) | 3.87; 4.20 | 1 | 70.50 | 1 |
| 6(terminal) | 3.70; 3.90 | nr | 61.50 | 1 |

nr = not resolved.

The 2D HMBC spectrum confirmed a three bond correlation between the C3 terminal glucose resonance at 76.6 ppm and H1 terminal glucose resonance at 4.53 ppm. In addition the H3 terminal glucose resonance at 3.52 ppm is correlated with the C1 terminal glucose resonance at 104.1 ppm and this in turn was found to be correlated with the C6,6'proton resonances of the branching point glucose resonating at 3.87 and 4.22 ppm.

Within the ring, the branching C6,6' resonance at 70.50 ppm correlated with the H4 of the 3,6 substituted glucose at 3.55 ppm which correlates with the C3(3,6) at 85.0 ppm and this correlated with the C1(3,6) at 4.83 ppm. This in turn connects with C3(1,3) glucose at 85 ppm. No other correlation could be made beyond this point, because a residual background due to water protons obscured this portion of the spectrum (data not shown).

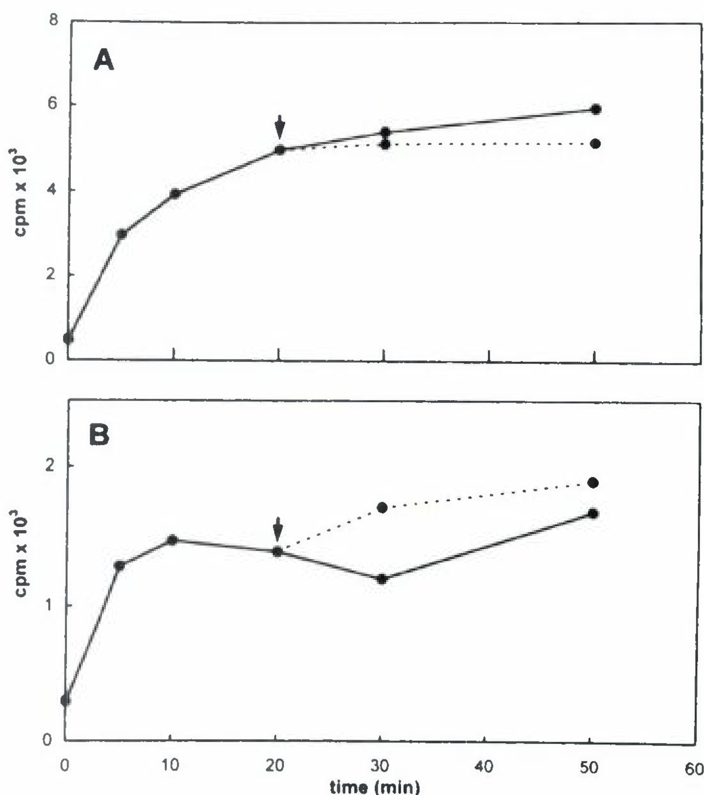


Figure 5. *In vitro* glucan synthesis by inner membranes of *R. loti* NZP 2309. Purified inner membranes were incubated with UDP-[$^{14}$C]glucose at 20°C as described in Materials and Methods. A: Incorporation of [$^{14}$C] glucose into the soluble fraction. B: Incorporation of [$^{14}$C]glucose into the insoluble fraction (10% TCA precipitate). Symbols: (- - -), incorporation after addition of 2 mM non radioactive UDP-glucose (arrow, indicates time of addition) ; (—), control (no addition of UDP-glucose).

*Synthesis in vitro*

*Rhizobium loti* NZP 2309 inner membranes incubated with UDP-[$^{14}$C] glucose at 20°C as described in Materials and Methods led to the incorporation of radioactivity into soluble and 10% TCA-insoluble compounds. The kinetics of the reaction is shown in Fig. 5. The addition of unlabelled UDP-glucose at 20 min stopped the incorporation of radioactivity into soluble compounds and an increment in the amount of radioactivity recovered in the TCA-insoluble fraction was observed. Soluble products can be resolved on a Bio-Gel P4

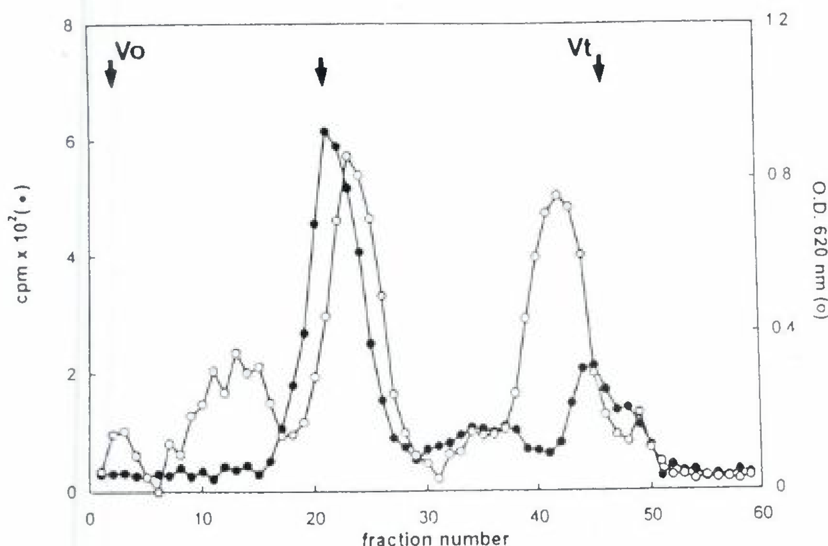


Figure 6. Comparison of glucans accumulated *in vivo* and synthesized *in vitro* by *R. loti* NZP 2309. Glucans synthesized *in vitro* at 30°C as described in Materials and Methods (method A) (o) and cellular glucans extracted with TCA (o), were subjected to molecular gel filtration chromatography in a Bio-Gel P4 analytical column. Arrow indicates elution position of β-(1-3), β-(1-6) glucans formed *in vivo* by *B. japonicum* USDA 110.

analytical column as compounds eluting with an elution volume slightly smaller than cellular glucans recovered in pool 3 and similar to those glucans obtained *in vitro* with inner membranes of *B. japonicum* USDA 110 (Fig. 6). When the reaction was carried out at 10°C, compounds with higher degree of polymerization that eluted between fractions 1 to 11 were formed (Fig. 7). This behavior was also observed with *B. japonicum* inner membranes and compounds were identified by laminarinase digestion as glucans structurally related to laminarin (Iñón de Iannino et al., 1993).

*Characterization of soluble glucans: Linkage composition*

Soluble products obtained after incubation of inner membranes with UDP-[$^{14}$C]glucose at 30°C (see Materials and Methods) were recovered from the Bio-Gel P4 analytical column (Fig. 6 fractions 18 to 26) and subjected to total and partial acid hydrolysis and paper chromatography with solvent A or B, respectively. Total acid hydrolysis yielded only glucose on paper chromatography with solvent A (Fig. 8A) and partial acid hydrolysis and paper chromatography with solvent B yielded glucose, laminaribiose [glucose

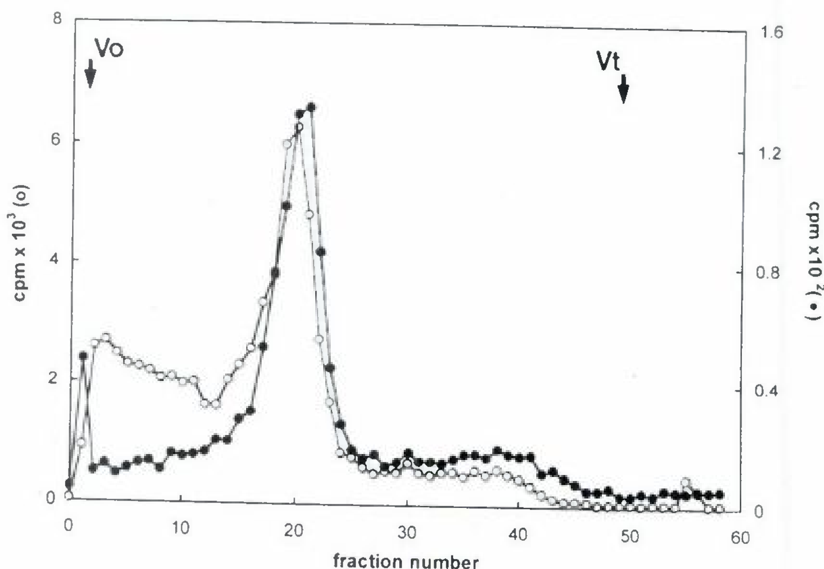


Figure 7. Comparison of glucans formed *in vitro* by *R. loti* NZP2309 at two different temperatures. Purified inner membranes were incubated with UDP-[$^{14}$C]glucose ($1.92\times10^6$ cpm) at 10°C (o) or with UDP-[$^{14}$C]glucose ($2.7\times10^5$ cpm) at 30°C (o) and the reaction was stopped as described in Materials and Methods (method A). The supernatants were subjected to DEAE-Sephadex chromatography and Bio-Gel P4 columns as previously described by Iñón de Iannino and Ugalde (1989). Vo is the void vol., Vt is the total vol.

β(1-3) glucose], gentiobiose [glucose β(1-6) glucose] and a series of oligosaccharides with increasing degree of polymerization (Fig. 8B). Compounds migrating as laminaribiose ($L_2$) and gentiobiose ($G_2$) were eluted from the paper strip with water, reduced with sodium borohydride and subjected to paper electrophoresis with buffer C. Under these conditions, only (1,3)-linked glucose disaccharides remain at the origin, while all other glucose disaccharides behave as anions. Reduced compound $G_2$ comigrated with a gentiobiositol standard, while reduced compound $L_2$ did not. α and β anomer glucose disaccharides are resolved by electrophoresis with buffer D (i.e., gentiobiose from isomaltose and laminaribiose from nigerose). Under these conditions, compound $G_2$ comigrates with the gentiobiose standard and compound $L_2$ comigrates with the laminaribiose standard. These results confirmed that this glucan contains β(1-3) and β(1-6) linkages.

*Cyclic structure of in vitro products*

The absence of reducing terminal glucose was determined by subjecting 50,000

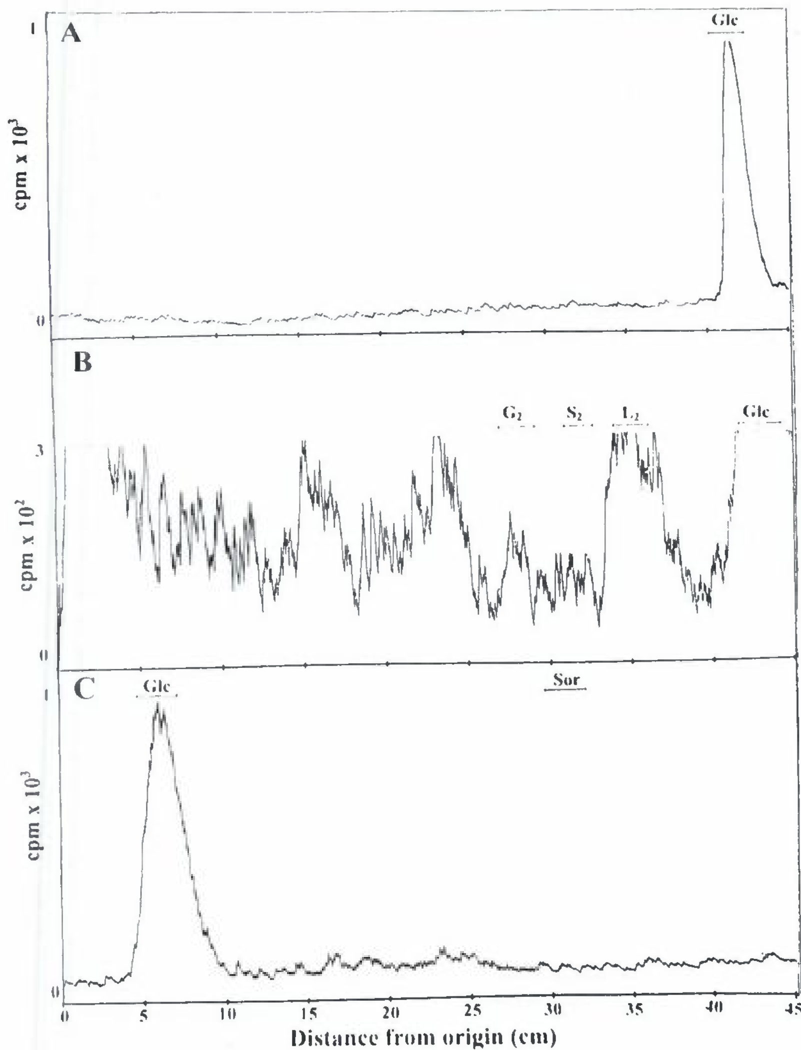


Figure 8. Paper chromatography and electrophoresis of total and partial acid hydrolysis of glucans obtained *in vitro*. Neutral soluble glucans eluted from Bio-Gel P4 analytical column (fractions 17 to 23) were subjected to total acid hydrolysis or partial acid hydrolysis and the products were subjected to paper chromatography with solvent A (panel A) or solvent B (panel B). Paper electrophoresis of total acid hydrolysis products after reduction with sodium borohydryde (panel C). The radioactivity was detected with a radioscanner. The sugar standards were detected as described (Trevelyan et al., 1950). Glc, glucose; $G_2$, geniobiose; $S_2$, sophorose; $L_2$, laminaribiose; Sor, sorbitol.

cpm of Bio-Gel P4 purified glucan to $NaBH_4$ reduction, total acid hydrolysis and identification of the resulting products by paper electrophoresis with buffer C. All the radioactivity was recovered as glucose and no sorbitol was detected, thus suggesting that no free reducing terminus was present (Fig. 8C).

*Smith degradation*

Periodate oxidation, $NaBH_4$ reduction and mild acid hydrolysis were carried out on glucans obtained after incubation of inner membranes with UDP-[$^{14}$C]glucose at 30°C and recovered from the Bio-Gel P4 column (Fig. 6 fractions 18 to 26) as described in Materials and Methods. The treatment released approximately 4.8% of the radioactivity as a compound that was recovered from a Bio-Gel P2 column near the total volume and identified by paper chromatography with solvent B as glycerol. Most of the radioactivity was recovered as a glucan that eluted from the Bio-Gel P2 column with the same elution volume as that of the non treated original glucan. A new round of oxidation, reduction, mild acid treatment and Bio-Gel P2 chromatography liberated approximately 5.8% of radioactivity that eluted near the total volume of the column. By using the same methodology described above, glycerol was identified as the saccharide present in this fraction. Most of radioactivity, however, was recovered with approximately the same elution volume as the original glucan. A third round of oxidation, reduction and mild acid treatment did not release detectable radioactivity, nor was a change in the elution volume of the glucan evident. These results indicated the absence of reducing terminal glucose. Resistance to periodate oxidation suggested the absence of vicinal hydroxyl group as would be expected for a β(1-3) linked glucan. Total acid hydrolysis of the three times treated glucan did not yield D-glucitol indicating the absence of reducing terminal glucose. All these results suggested that this glucan has a cyclic β(1-3) structure with a degree of polymerization of approximately 11 or 12 and a side branch of at least one β(1-3) linked glucose residue.

*Characterization of TCA-insoluble products obtained in vitro: Polyacrylamide gel electrophoresis*

To identify the radioactive compound(s) associated with protein(s) in the TCA-insoluble fraction, the reaction was stopped with 1 ml of 10% TCA, centrifuged and the precipitate divided in two parts. One was subjected to partial acid hydrolysis and the other to SDS-polyacrylamide gel electrophoresis with or without prior laminarinase treatment. Partial acid hydrolysis and paper chromatography of the hydrolysates revealed the

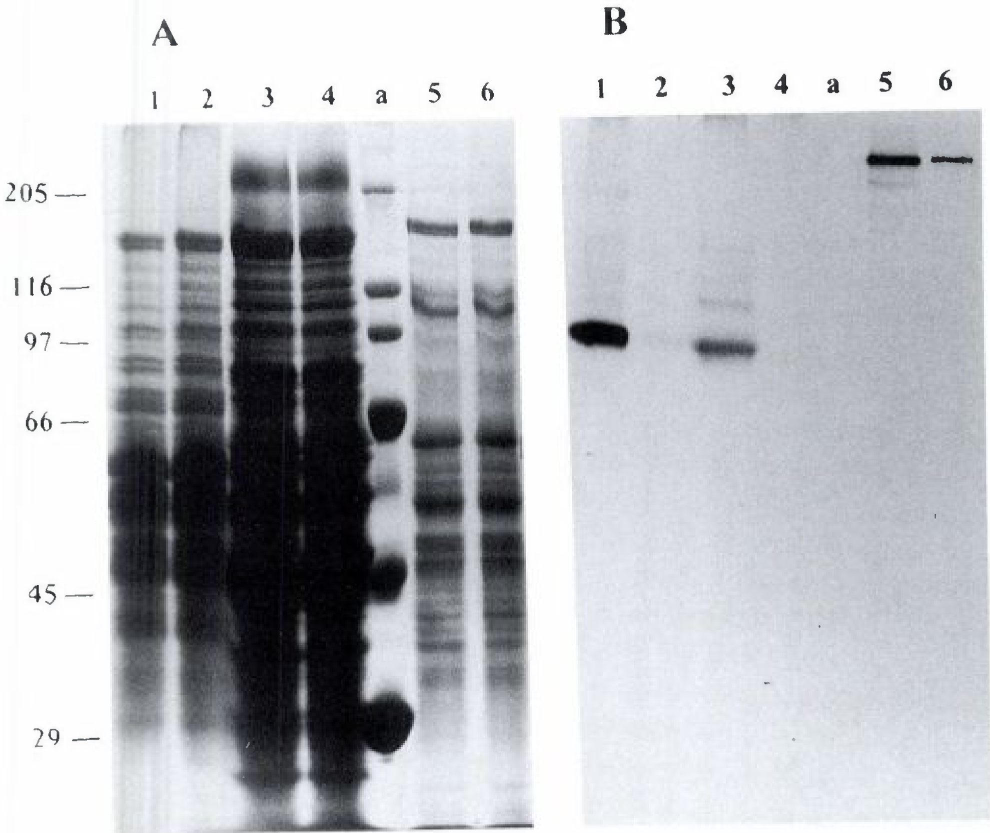


Figure 9. Polyacrylamide gel electrophoresis of inner membranes incubated with UDP-[$^{14}$C]glucose. Inner membranes (0.2 mg of protein) were incubated with UDP-[$^{14}$C]glucose, the reaction was stopped by 10% TCA and precipitates were subjected to gel electrophoresis as described in Materials and methods. Proteins were stained with Coomassie blue (A) and radioactivity was detected by fluorography (B). For a chase experiment (even lanes), 2 mM of non radioactive UDP-Glc were added after 15 min of incubation, and the reaction was stopped after 15 min. Lanes 1 and 2 *B. japonicum* USDA 110;.3 and 4 *R. loti* NZP 2309; 5 and 6 *R. loti* NZP2037; lane a size standards, sizes are shown at the left (in kilodaltons).

presence of glucose, laminaribiose and β(1-3) oligomers. SDS polyacrylamide gel electrophoresis and fluorography of the gel showed that part of the radioactivity was associated with a compound that migrates as a 85 kDa protein (Fig. 9). Most of the radioactivity, however, was retained at the top of the gel. Laminarinase treatment reduced the radioactivity associated with

this material, thus indicating that in the TCA-insoluble fraction two types of glucose containing compounds were present, i.e. a glucoprotein and an insoluble β(1-3) glucan (laminarin-like glucan). When a chase experiment with 2 mM non labeled UDP-glucose was carried out and the TCA insoluble fraction submitted to SDS-polyacrylamide gel electrophoresis it was observed that the radioactivity associated with the 85 kDa protein was no longer detected. No change nor a small increment of the radioactivity associated with insoluble products retained at the top of the gel was observed. These results suggest that the 85 kDa protein may be an intermediate or precursor during the synthesis of soluble β(1-3), β(1-6) glucans, insoluble β(1-3) laminarin-like glucans or both, although at this stage it is not possible to distinguish between these possibilities.

## 4. Discussion

We have identified in *R. loti* NZP 2309 the presence of a cyclic β(1-3), β(1-6) glucan closely related with the previously described cyclic β(1-3), β(1-6) glucan of *B. japonicum* USDA 110 (Rolin et al., 1992). REP-PCR analysis showed that *R. loti* NZP 2309 and *B. japonicum* USDA 110 are different strains of *Bradyrhizobium*, but according to PCR-RFLPs of the 16S rDNA gene they are closely phylogenetically related (unpublished data).

Bhagwat et al. (1996) showed that *B. japonicum* carrying a Tn5 insertion in locus *ndvC* resulted in a strain that form altered cyclic β-glucans composed almost entirely of β(1-3) glycosyl linkages. This mutant was severely impaired in symbiotic interaction with soybean (*Glycine max*) plants. They suggested that the structure of the β-glucans is important for a successful symbiotic interaction. Cyclic β(1-6), β(1-3) glucans of *R. loti* NZP 2309 have structural similarities with cyclic β(1-6), β(1-3) glucans of *B. japonicum* USDA 110.

FAB spectral analysis of the major glucan of slow growing *R. loti* strain NZP 2309 showed that it produces a cyclic glucan with a DP of 11 glucose units. Integration of both the $^{13}C$ and $^{1}H$ 1D spectra verify that all 11 anomeric carbons and protons are located at 103–104 and 4.42–4.83 ppm, respectively. A 2D HMQC spectrum demonstrated that this cyclic glucan is branched at the C-6 position of a β(1-3) linked glucose and the ratio of the β(1-6) to β(1-3) linked glucose units [excluding the (3-6) and terminal glucose units] was 2:1, respectively.

Further 2D NMR analysis of this structure in an HMBC experiment established that this molecule has a 10 membered glucose unit ring with a single terminal glucose attached at a C-6 position of a β(1-3) linked glucose. This was verified by tracing the three bond correlations from the terminal C-1 glucose resonances at 76.6 ppm to the corresponding anomeric proton of the

terminal glucose at 4.53 ppm. This resonance was then shown to have a cross-peak with the branch C6,6' carbon resonance at 70.50. A similar correlation was made starting with H3 resonance of the terminal glucose at 3.52 ppm (see Results section). The arrangement of the three other β(1-3) glucose and six β(1-6) units within the ring could not be determined because of severe overlap of a number of proton resonances in the range of 4.75 ppm due to the residual protons in the $D_2O$ solvent.

Preliminary studies indicated that linear β(1-6), β(1-3) linked glucans (pools 4 and 5) closely related with the cyclic form (pool 3) are accumulated in *R. loti* NZP 2309. Similar results were observed with *B. japonicum* USDA 110 (Iñón de Iannino et al., unpublished results). This finding, together with the similarity of the putative *ndvC* translation product with enzymes involved in β-glucan metabolism like endo β(1-3) glucanases or transglucosylases, suggests that linear β(1-6) β(1-3) linked glucans may be precursors or abortive forms in the synthesis of the cyclic β(1-6) β(1-3) linked glucans.

Incubation of inner membranes of *R. loti* NZP 2309 with UDP-[$^{14}$C]glucose resulted in the formation of a cyclic β(1-3) glucan with a β(1-6) linked branch of two glucose residues, similar to that previously described to be formed by *B. japonicum* USDA 110 inner membranes (Iñón de Iannino and Ugalde, 1993) and identical to glucan synthesized *in vivo* by the *B. japonicum* AB-1 *ndvC* mutant (Bhagwat et al., 1999). Pulse-chase experiments suggested that a protein of 85 kDa was involved in the *in vitro* synthesis of soluble β(1-3), β(1-6) glucans, insoluble β(1-3) laminarin-like glucans or both. These results are similar to that obtained with *B. japonicum* USDA 110 (Iñón de Iannino and Ugalde, 1993). In *A. brasilense*, the synthesis of periplasmic β(1-3), β(1-6) glucans was studied, however an intermediate protein was not identified to be involved in the synthesis and no insoluble laminarine-like glucans were produced *in vitro* by purified bacterial inner membranes incubated with UDP-[$^{14}$C]glucose (Altabe et al., 1994). These results suggest that the *B. japonicum* USDA 110 90 kDa and the *R. loti* NZP 2309 85 kDa glycoproteins may be intermediates or precursors during the synthesis of laminarin rather than being involved in the synthesis of cyclic β(1-3), β(1-6) glucans. A protein glucan intermediate was suggested although not firmly established in paramylon synthesis, an insoluble polysaccharide consisting of β(1-3) linked D-glucose residues present in the alga *Euglena gracilis* (Tomos and Northcote, 1978).

Recently the structure of a glucan isolated from a *R. meliloti ndvB* mutant carrying a locus specifying β(1-3), β(1-6) glucan synthesis from *B. japonicum* USDA 110 was characterized. This recombinant strain produced a new cyclic glucan composed only of β(1-3) linkages in a ring of DP 10 and a C-6 linked branch composed of a β(1-3) linear glucan chain (Pfeffer et al., 1996) identical to the glucan synthesized *in vitro* by *R. loti* NZP 2309. It was suggested that locus *ndvC* is not expressed efficiently in the *R. meliloti* genetic background

(Bhagwat et al., 1996) and that the product of this locus may control or influence the synthesis of β(1-6) linkages. Glucans with different ratios of β(1-6) and β(1-3) linkages have been described, and this suggests that the pathway for the synthesis of cyclic β(1-6), β(1-3) glucans may be complex. Our results show that *Bradyrhizobium* strains *B. japonicum* USDA 110 and *R. loti* NZP 2309 synthesized closely related β(1-6), β(1-3) glucans by similar mechanisms. The glucans formed *in vitro* have a different structure than those accumulated *in vivo*, having a different ratio of β(1-3) to β(1-6) linkages (the *in vitro*-synthesized glucans are enriched in β(1-3) linkages). It is possible that *in vitro* the putative β(1-6) glucosyl transferase does not work properly since the ratio β(1-3) to β(1-6) linkages obtained *in vitro* is similar to the glucan obtained *in vivo* with a *ndvC B. japonicum* mutant (Bhagwat et al., 1996). Another possibility is that the *ndvC* locus codes for a soluble enzyme that is not present in the membrane fraction used in the *in vitro* preparation.

Two mechanisms may be involved in the synthesis of cyclic β(1-6), β(1-3) glucans. In one of them β(1-6) linkages may be introduced by transglucosylation in a performed cyclic β(1-3) structure by the putative NdvC enzyme. This mechanism may explain the formation of cyclic β(1-3) glucans in a null *ndvC* mutant (Bhagwat et al., 1999). Our findings that *in vitro* a cyclic β(1-3) glucan is formed without β(1-6) linkages may also be explained by postulating that *in vitro* the NdvC enzyme is not functional. However, according to the current available experimental data we can not rule out a mechanism in which β(1-3) and β(1-6) linkages are constructed as linear glucans prior to cyclization.

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