Cellular and Molecular Biology 42 (5), 617-629
Printed in France.

0145-5680/96
1996 Cell. mol. Biol.

CHARACTERIZATION OF THE BIOSYNTHESIS OF β(1-2) CYCLIC GLUCAN IN *R. FREDII*. β(1-2) GLUCAN HAS NO APPARENT ROLE IN NODULE INVASION OF MC CALL AND PEKING SOYBEAN CULTIVARS

Nora IÑÓN DE IANNINO, Gabriel BRIONES, Gabriel KREIMAN and Rodolfo UGALDE[*]

[*] Instituto de Investigaciones Bioquímicas, Fundación Campomar, CONICET, y
Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,
Av. Patricias Argentinas 435, 1405 Buenos Aires, Argentina

Accepted March 29, 1996

Abstract - Three wild type strains of *Rhizobium fredii*, USDA 191, USDA 257 and HH 303, do not synthesize *in vivo* or *in vitro* β(1-3), β(1-6) cyclic glucans, all strains form *in vitro* and *in vivo* cyclic β(1-2) glucans. Approximately 80% of the recovered *R. fredii* cellular cyclic β(1-2) glucans were anionic and the substituent was identified as phosphoglycero-. Inner membranes prepared from these *R. fredii* strains have a β(1-2) glucan-intermediate-protein with apparent molecular mass undistinguishable from *Agrobacterium tumefaciens* β(1-2) glucan intermediate protein. Studies of the degree of polymerization of the oligosaccharides recovered from the protein-intermediate after short pulse incubations with UDP-^{14}C-glucose suggested that the rate limiting step in the biosynthesis of cyclic glucan is cyclization. Kinetic studies revealed that the K_m for UDP glucose was 0. 33 mM. No difference was detected between the K_m for initiation/elongation and cyclization reactions. Nodulation studies of a *ndvB R. fredii* mutant with Mc Call and Peking soybean cultivars, revealed that β(1-2) glucans do not seem to be required for normal nodule invasion of these soybean cultivars.

Key words: ndvB, cyclic glucan, *Rhizobium fredii*, soybean

INTRODUCTION

Bacteria of the family *Rhizobiaceae* form nitrogen fixing nodules in legume roots. Bacterial polysaccharides are recognized to be important for this process (Carlson *et al.*, 1987; Finan *et al.*, 1985; Geremía *et al.*, 1987; Maier and Brill, 1978; Puvanesarajah *et al.*, 1985; Stacey *et al.*, 1991; Truchet *et al.*, 1991). In *R. meliloti*, β(1-2) cyclic glucans were described to be required for effective nodule invasion (Dylan *et al.*, 1986; Geremía *et al.*, 1987).

In *Rhizobium* spp and *Agrobacterium* spp the synthesis of cyclic β(1-2) glucans proceeds through a 235 kDa inner membrane intermediate protein (Zorreguieta and Ugalde, 1986). We proposed that glucose residues are transferred from UDP-glucose to an unidentified amino acid residue of the 235 kDa inner membranes protein, that the polyglucose β(1-2) chain elongates until it reaches a degree of polymerization ranging between 17 to 25 glucose units and that it then cyclizises and is released from the 235 KDa intermediate protein (Zorreguieta and Ugalde, 1986). The intermediate protein is responsible for initiation, elongation and cyclization, and it determines the degree of polymerization of the cyclic glucan (Altabe *et al.*, 1990; Lepek *et al.*, 1990). Some years ago publi-

shed kinetic studies suggested that the size distribution of cyclic β(1-2) glucans depends on competition between elongation and cyclization reactions (Williamson *et al.*, 1992).

Bradyrhizobium japonicum and *R. fredii* nodulate soybean and other legumes; *B. japonicum* does not form cyclic β(1-2) glucans, but β(1-3), β(1-6) cyclic glucans are accumulated in the periplasmic space (Miller *et al.*, 1990; Rolin *et al.*, 1992; Tully *et al.*, 1990). In *B. japonicum*, a 90 kDa protein was described to participate as an intermediate in the synthesis of cyclic β(1-3), β(1-6) glucan (Iñón de Iannino and Ugalde, 1993), suggesting a common mechanism for the synthesis of cyclic glucans through intermediate proteins. DNA homology between *Rhizobium ndv*B and *Agrobacterium chv*B regions was described (Dylan *et al.*, 1986). An *R. fredii ndv*B mutant was obtained by site-directed mutagenesis of an *R. fredii* cosmid that complemented an *R. meliloti ndv*B mutant (Bhagwat *et al.*, 1992). This *ndv*B mutant was described to induce only pseudonodules on soybean (*Glycine Max cv. Williams*) (Bhagwat *et al.*, 1992), even though Ko and Gayda (1990) had earlier shown that *R. fredii* mutants, lacking the ability to form exopolysaccharides and glucans, nevertheless retained normal nodulating ability on soybean. *chv*B in *Agrobacterium* sp. and *ndv*B in *Rhizobium* sp. encode the 235 kDa intermediate protein. Membranes prepared from the *R. fredii ndv*B mutant do not have the 235 kDa intermediate protein and do not form β(1-2) glucan *in vitro* (Bhagwat *et al.*, 1992). Because of the importance assigned by Bhagwat *et al.* (1992) to cyclic glucans in nodule development and the contradictory results of Ko and Gaida (1990), we decided to carry out nodulation experiments with a *ndv*B mutant of strain HH303 in soybean cultivars Peking and Mc Call. Here we report the results of the biosynthesis and properties of cyclic β(1-2) glucans of *R. fredii* strains USDA191, USDA257 and HH303. We show that the *ndv*B mutant of strain HH303, obtained by Bhagwat *et al.* (1992) which lacks the 235 kDa protein and is unable to form β(1-2) cyclic glucan, induces active, nitrogen-fixing nodules on the advanced soybean cultivar Mc Call and the primitive cultivar Peking.

MATERIALS AND METHODS

Bacterial Strains and Media

Rhizobium fredii USDA191 and USDA257 were kindly provided by P. Van Berkum and S.G. Pueppke (from Soybean & Alfalfa Res. Lab. USDA, Beltsville, Maryland and Dept. of Plant Pathology, Univ. of Missouri, Columbia, respectively. Strain HH303 and *ndv*B Rf19 mutant was kindly provided by D.L. Kiester (from Soybean & Alfalfa Res. Lab. USDA, Beltsville, Maryland). *Agrobacterium tumefaciens* A348 was provided by E.W. Nester (from Dept. of Microbiology and Immunology, Univ. of Washington, Seattle, Washington). *R. fredii* strain NI01 was isolated from active nodules obtained from plants inoculated with *ndv*B mutant Rf19. *Rhizobium* strains were grown for 2 days in yeast extract-mannitol (AMA) medium (Iñón de Iannino and Ugalde, 1993) at 28°C in a rotary shaker. For *in vivo* labeling experiments *R. fredii* strains were grown on gluconate-mannitol medium (Bhuvaneswari *et al.*, 1977). *Agrobacterium* strains were grown for 1 day in tryptone-yeast extract (TY) media (Iñón de Iannino and Ugalde, 1989). When required, kanamycin 500 μg/ml (AMA-agar) or 100 μg/ml (AMA-broth) was added.

Extraction of Cell-associated Oligosaccharides

Cells from 1.0 l cultures were harvested by centrifugation at 10,000 g for 20 min. Pellets were extracted with 1% trichloroacetic acid (TCA) for 30 min. at room temperature as described previously (Miller *et al.*, 1986). TCA extracts were neutralized with ammonium hydroxide, concentrated and subjected to gel filtration on Bio-Gel P4 columns as described previously (Iñón de Iannino and Ugalde, 1989). Neutralized and concentrated *R. fredii* TCA extracts were precipitated with 3 vol. ethanol to remove exopolysaccharides prior to column chromatography on Bio-Gel P4. When indicated, 30,000 to 40,000 cpm of cyclic β(1-2) glucan, prepared *in vitro* as indicated above, were added as internal standard. Fractions of 1.5 ml were collected. Carbohydrates were detected in aliquots of 200 μl by the anthrone-sulfuric method (Dische, 1962) and radioactivity counted with Bray's solution in a liquid scintillation counter.

Preparation of Inner Membranes and in vitro β(1-2) Glucan Synthesis

Inner membranes were prepared following the method described by Osborn and Munson (1984) with modifications (Iñón de Iannino and Ugalde, 1989). When inner membranes were prepared from *R. fredii* strains, phenylmethylsulfonylfluoride (PMSF) 2 mM was added prior to shearing with a French Press. *In vitro* synthesis of β(1-2) glucan, polyacrylamide gel electrophoresis of inner membrane proteins and fluorography were carried out as described previously (Iñón de Iannino and Ugalde, 1989). The apparent K_m for UDP-glucose was determined by plotting inverse of velocity versus inverse of substrate concentration (Lineweaver-Burk plot).

DEAE-Sephadex Chromatography and Reduction with Sodium Borohydride Reduction
They were carried out as described previously (Iñón de Iannino and Ugalde, 1989).

Acid Hydrolysis, Paper Chromatography and Paper Electrophoresis
Partial acid hydrolysis of glucans was carried out with 0.5 N HCl at 100°C for 20 min.; total acid hydrolysis was carried out with 1 N HCl at 100°C for 4 hrs. HCl was removed by evaporation under an air stream, and the hydrolysates were subjected to descending paper chromatography on Whatman number 1 paper (Whatman, Clifton, NJ) with solvent A [butanol-pyridine-water (6:4:3)] or solvent B [isopropanol-acetic acid-water (27:4:9)]. Paper electrophoresis was carried out with buffer C (1.2 M pyridinium acetate, pH 6. 5) for 2 hrs. at 1,000 volts or buffer D (2% sodium molybdate, pH 5.0) for 2 hrs. at 15 V/cm. Sugars were detected by the alkaline-silver method (Trevelyan *et al.*, 1950). Compounds containing phosphorus were detected by Burrows reagent (Burrows *et al.*, 1952).

Chemical Treatments
β(1-2) glucans from the Bio-Gel P4 columns were submitted to different treatments: a) 10 mM HCl at 100°C for 90 min. to remove pyruvic and a ketoglutaric acid substituents (Koepsell and Sharpe, 1952). b) 0.1 N NaOH for 30 min. at 37°C to eliminate succinate or malonate substitutents (Miller *et al.*, 1988). c) 0.3 N NaOH, for 80 min. at 100°C to remove phosphoglycerol residues in phosphodiester linkage (Miller *et al.*, 1987).

Proteolysis
Washed TCA precipitates (50,000 cpm) (obtained after incubation of inner membranes with UDP-^{14}C-Glc as described above), were treated at 37°C with 2 mg of Type XIV protease from *Streptomyces griseus* (Sigma, St. Louis, MO) with 100 mM TRIS HCl (pH 7) and 10 mM Cl_2Ca in a total vol. of 1 ml. After 48 hrs. of incubation, 1 mg of protease was added and incubation was continued for 8 days. TCA (10%) was added to stop the reaction, glycopeptides recovered from the supernatant after centrifugation. To remove TCA, supernatants were washed several times with ethyl ether and evaporated under a stream of nitrogen to eliminate ether. Glycopeptides labelled with ^{14}C-glucose were subjected to paper electrophoresis with 5% formic acid (v/v), eluted from the paper strip and chromatographied in Bio-Gel P4 columns as described above. Cyclic β(1-2) glucan labelled with ^{3}H glucose, obtained after incubating inner membranes with UDP-^{3}H glucose, was added as internal standard.

In vivo Labeling of β(1-2) Glucan with ^{32}P-Orthophosphate
Four ml of a culture of *R. fredii* USDA257, which had been grown overnight in a defined gluconate-mannitol medium without vitamins (Bhuvaneswari *et al.*, 1977), were labelled with 50 μCi of disodium ^{32}P-orthophosphate (Atomic National Comision, Argentina) by incubating for 1 day at 28°C. Cells were harvested by centrifugation (4 min. at 14,000 rpm) in an Eppendorf centrifuge. Cell pellets were extracted with 1% TCA for 30 min. at room temperature and TCA extracts subjected to gel chromatography on a Bio-Gel P4 column as described above for the isolation of β(1-2) glucan. The equivalent to 400 μg of glucose of this glucan was added to the column as internal standard. Radioactivity was determined by counting in a liquid scintillation counter and sugars determined by the sulfuric acid anthrone method (Dische, 1962). Fractions containing ^{32}P and sugars were pooled, concentrated and submitted to alkaline treatment (0.5 M NaOH at 100°C for 80 min.). After hydrolysis samples were diluted with water and neutralized with Bio-Rad AG50W-X8 cation exchange resin. The neutralized hydrolysate was then submitted to paper electrophoresis with buffer C as indicated above and radioactivity detected with a radioscanner. Phosphorus and phosphoglycerol were detected with Burrows reagent (Burrows *et al.*, 1952).

Nodulation Test
Seeds were surface sterilized and pregerminated on water-agar plates (Pueppke, 1983). Two days old seedlings were planted in autoclaved modified Leonard jars filled with vermiculite and Jensen's N-free solution (Vincent, 1970). Seedlings were dipped into a 2-days old *Rhizobium* culture before planting. After 6 weeks plants were removed, nodules counted and nitrogen fixation evaluated by the acetylene reduction assay as described (Wanek and Brill, 1976).

Nodules were processed for light and electron microscopy as described by Parkhurst *et al.* (1979). Sections were cut on a Servall MT-2B Ultramicrotome and examined in an electron microscopy Zeiss EM 109 turbo.

Root nodules were removed from the roots of plants using a scalpel blade and sterilizated by immersing in 95% ethanol for 30 sec., soaked in acidified mercuric chloride (1% $HgCl_2$ in 0.06 N HCl) for two min. and rinsed with sterile distilled water. Bacteria were recovered from sterilized nodules after crushing and plating on AMA-agar medium with the appropriate antibiotic. Surface contamination of the nodules was tested by rolling the nodules on AMA-agar medium. No contaminants were detected before crushing.

RESULTS

Characterization of cellular Glucans

Cells of *R. fredii* strains grown in AMA medium for 48 hrs. at 28°C were extracted with TCA as described in Materials and Methods. Gel chromatography on Bio-Gel P4 columns showed that *R. fredii* strains USDA191 and USDA257 had two main sugar containing compounds (Figs. 1A, 1C). After total acid hydrolysis and paper chromatography with solvent A polysaccharides eluting in fractions 7 to 15 yielded glucose as the only monosaccharide. After partial acid hydrolysis and paper

chromatography with solvent B, this glucan yielded glucose, sophorose and a homologous series of oligosaccharides with increasing degree of polymerization, as expected for β(1-2) glucan degradation products (data not shown). These results confirmed that these strains contained cellular β(1-2) glucans. The small molecular weight compound eluting in the total vol. of the columns (fractions 38 to 45) was not characterized. Cellular β(1-2) glucans recovered from Bio-Gel P4 columns were subjected to DEAE-Sephadex chromatography. A small fraction (17%) percolated through the column, thus indicating chemical neutrality, but most of the glucan eluted from the column as negatively-charged molecules. In *R. fredii* USDA 191 20% eluted with 10 mM NaCl, 25% with 50 mM NaCl, 29% with 100 mM NaCl and 6% with 500 mM NaCl. A similar distribution between charged an neutral glucans was observed in strains USDA 257 and HH303. The pattern was similar to that observed previously with *R. loti* β(1-2) cyclic glucans (Lepek *et al.*, 1990).

Cyclic β(1-2) glucans of *Rhizobiaceae* can be substituted with anionic non glycosidic residues (Batley *et al.*, 1987; Hisamatzu *et al.*, 1987; Miller *et al.*, 1987). In order to identify the charged substituent present in *R. fredii* glucans, cellular β(1-2) glucans were recovered from Bio-Gel P4 columns, subjected to different chemical treatments and the products analyzed by chromatography on DEAE-Sephadex (data not shown) and Bio-Gel P4 (Fig. 1). Treatment with 10 mM HCl for 90 min. at 100°C, which is known to release pyruvate

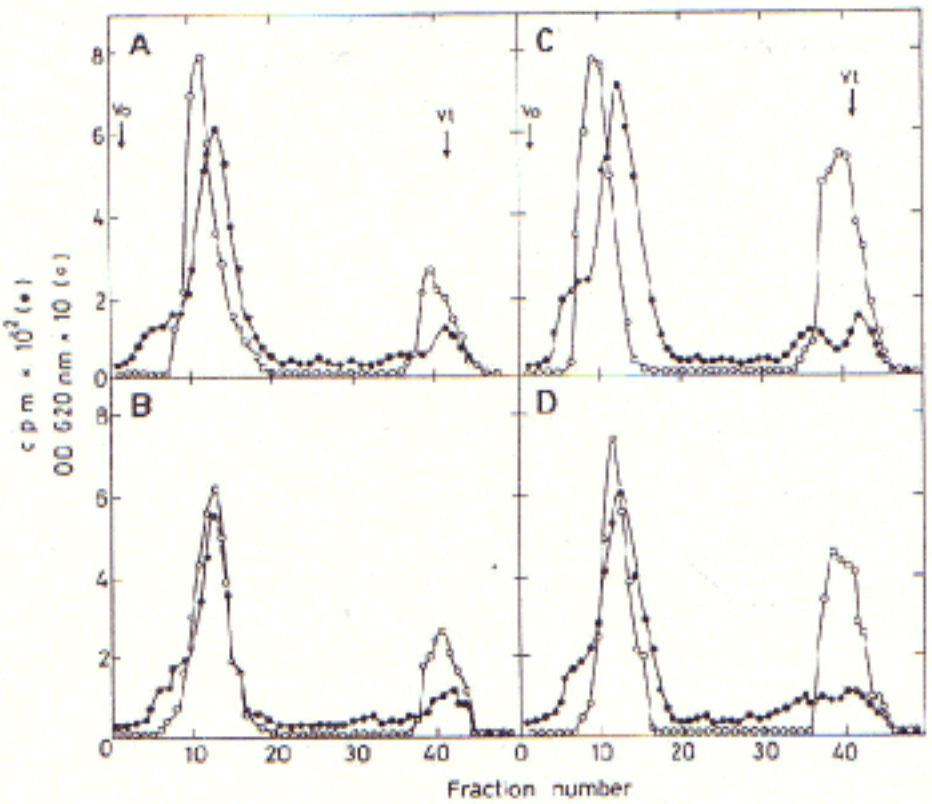

Fig. 1 *Bio-Gel P4 chromatography of β(1-2) glucans accumulated in vivo by R. fredii strains.* A) o, *R. fredii* USDA191 cellular glucans accumulated *in vivo*; •, glucans formed *in vitro*. B) o, *R. fredii* USDA191 cellular glucans accumulated *in vivo* after alkaline treatment (0.5 N NaOH, 80 min., 100°C); •, glucans formed *in vitro*. C) o, *R. fredii* USDA257 cellular glucans accumulated *in vivo*; •, glucans formed *in vitro*. D) o, *R. fredii* USDA257 cellular glucans accumulated *in vivo* after alkaline treatment (0.5 N NaOH, 80 min., 100°C); •, glucans formed *in vitro*. Carbohydrate assays and counting of radioactivity were carried out as described in Materials and Methods. Bio-Gel P4 columns (78 x 1.8 cm) were eluted with 0.1 M pyridine-acetate buffer (pH 5.5). Fractions of 1.5 ml were collected Vo is the void vol. ; Vt is the total vol.

(Koepsell and Sharpe 1952), or treatment with 0.1 M NaOH for 30 min. at 37°C, which is known to release succinate and malonate (Miller *et al.*, 1988), did not yield neutral glucans. On the other hand, treatment with 0.5 M NaOH for 80 min. at 100°C, which is known to release phosphoglycerol (Kennedy *et al.*, 1976), yielded neutral unsubstituted glucans (Figs. 1B, 1D), suggesting that in *A. tumefaciens* and *R. meliloti*, *R. fredii* β(1-2) glucans are substituted with phosphoglycerol residues.

In order to confirm the presence of phosphoglycerol in *R. fredii* β(1-2)glucans were labelled *in vivo* with ^{32}P-phosphate as described in Materials and Methods. ^{32}P-labelled TCA extracts were subjected to chromatography on a Bio-Gel P4 column and 400 µg of glucose equivalents of non-labelled β(1-2) glucan were added as internal standard. Fractions containing ^{32}P and glucan, as detected by the anthrone-sulfuric acid method, were pooled concentrated and subjected to alkaline treatment under conditions described to release phosphoglycerol residues. After treatment, samples were neutralized with the cationic form of Bio-Rad AG50W-X8 resin and the resulting products subjected to paper electrophoresis with buffer C. Radioactivity was detected with a radiochromatogram scanner (Packard model 7201) and phosphate developed with Burrows reagent. ^{32}P-labelled phosphoglycerol and inorganic phosphorous were recovered (data not shown), thus confirming the presence of phosphoglycerol as a charged substituent in *R. fredii* β(1-2) glucans.

Synthesis in vitro

Inner membranes of *R. fredii* USDA 191 and USDA 257 incubated with UDP-^{14}C-glucose (90,000

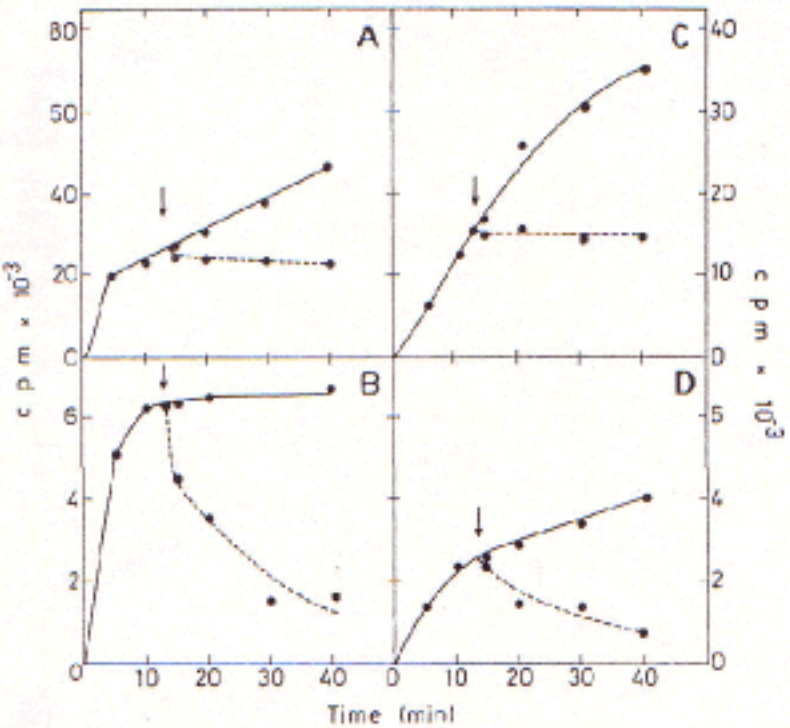

Fig. 2 *In vitro synthesis of β(1-2) glucan by R. fredii inner membranes.* The experiment was carried out as described in Materials and Methods. A) Incorporation of ^{14}C-glucose into β(1-2) glucan by inner membranes of *R. fredii* USDA191; B) Incorporation of ^{14}C-glucose into TCA-insoluble intermediates by inner membranes of *R. fredii* USDA191; C) Incorporation of ^{14}C-glucose into cyclic β(1-2) glucan by inner membranes of *R. fredii* USDA257; D) Incorporation of ^{14}C-glucose into TCA-insoluble intermediate by inner membranes of *R. fredii* USDA257. Symbols: •– – –•, incorporation after addition of 2 mM non-radioactive UDP-glucose (arrow, indicates time of addition); •——•, control (no addition of UDP-glucose).

cpm; 10.5 GBq/mM), led to the incorporation of ^{14}C-glucose into soluble and TCA-insoluble compounds (Fig. 2). The apparent K_m for UDP-glucose, determined from Lineweaver-Burk plots was 0.33 mM for the soluble product and 0.22 mM for the insoluble product. Pulse-chase experiments showed that TCA-insoluble compounds behaved as intermediates of soluble products; similar results were obtained with strain HH303 (data not shown). TCA-insoluble products were subjected to SDS polyacrylamide gel electrophoresis and fluorography as described previously (Zorreguieta and Ugalde, 1986). A 235 kDa protein undistinguishable from the *A. tumefaciens* β(1-2) glucan intermediate protein was observed (Fig. 3). With inner membranes prepared from strains USDA191 and USDA257. The presence of labelled proteins was observed with apparent molecular mass higher than *A. tumefaciens* intermediate protein (Fig. 3, lanes 7-10). These higher molecular mass proteins were not allways observed. Therefore, we attributed them to the formation of dimers not completely disrupted by the cracking treatment. Radioactivity of the 235 kDa protein decreased rapidly after chasing with non-labelled UDP-glucose, thus indicating that it behaves as an intermediate (Fig. 3B).

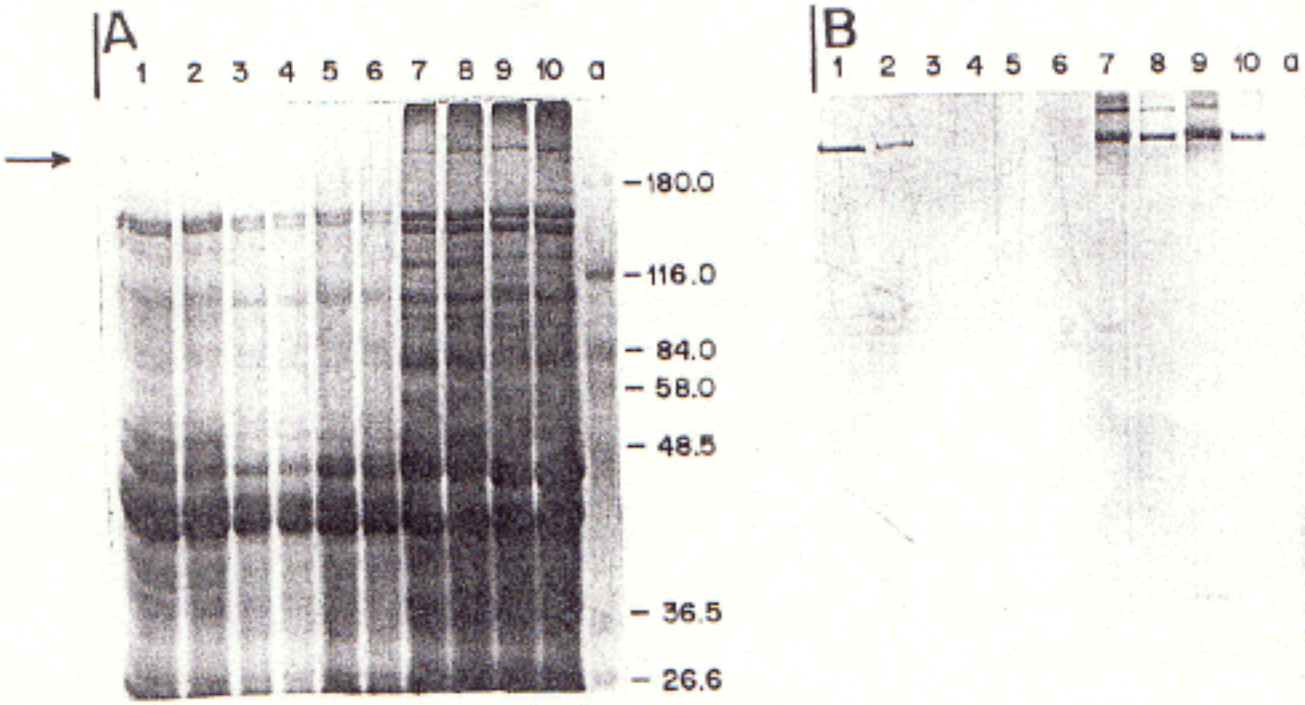

Fig. 3 *Polyacrylamide gel electrophoresis of inner membranes of R. fredii strains.* Inner membranes were incubated with UDP-^{14}C-glucose at 12°C. Reactions were stopped by addition of 10% TCA, and precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as indicated in Materials and Methods. Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). For a chase experiment (even-numbered lanes), 2 mM non-radioactive UDP-glucose was added after a 10 min. incubation, and the reaction was stopped after 10 min. Lanes 1 and 2: Strain HH303; lanes 3 and 4: Strain ndvB Rf19; lanes 5 and 6: strain NI01; lanes 7 and 8: strain USDA191; lanes 9 and 10 strain USDA257. a: molecular weight standards. Numbers on the left indicate molecular masses of standards (in kDa). The arrow indicates the migration position of the *A. tumefaciens* 235 kDa intermediate protein.

Soluble products recovered from DEAE-Sephadex percolates were subjected to Bio-Gel P4 chromatography as shown in fig. 1. Glucans formed *in vitro* eluted from the column with a greater elution vol. than glucans recovered from cells *in vivo* (compared Figs. 1A vs 1C, 1B vs 1D); however, when cellular glucans were subjected to alkaline treatment to remove phosphoglycerol, *in vitro* and *in vivo* products eluted from the column with the same vol. The same results were obtained with cellular glucans recovered from strain HH303. These results indicated that neutral glucans formed *in vitro* by inner membranes are identical to cellular anionic glucan accumulated *in vivo*, except that the latter were substituted with phosphoglycerol (Figs. 1B, 1D).

Characterization of Glucan formed in vitro

Neutral glucans formed *in vitro* were recovered from Bio-Gel P4 columns (Fig. 1, fractions 7 to 17) and subjected to total and partial acid hydrolysis. Total acid hydrolysis and paper chromatography of the hydrolyzates with solvent A yielded glucose as the only monosaccharide (Fig. 4). Partial acid hydrolysis and paper chromatography with solvent B yielded glucose, sophorose and a homologous series of oligosaccharides with increasing degrees of polymerization (Fig. 4). In order to determine if these molecules were cyclic, 35,000 cpm of the glucan recovered from Bio-Gel P4 columns were subjected to sodium borohydride reduction as described in Materials and Methods. After reduction

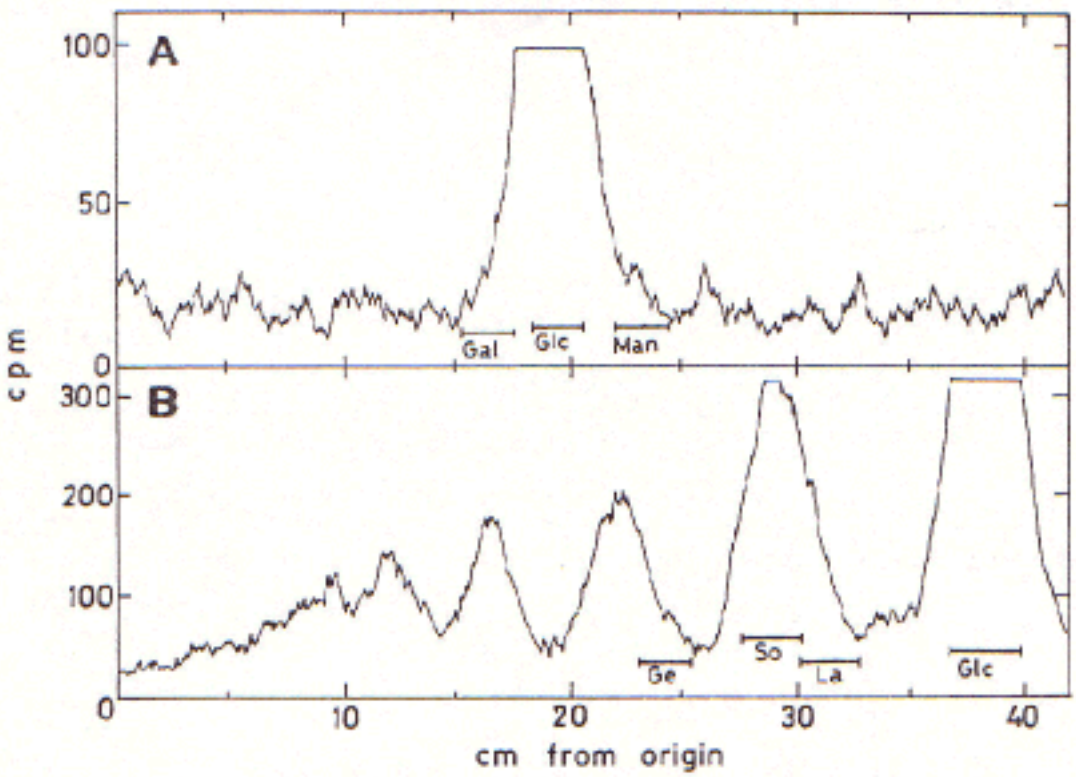

Fig. 4 *Characterization of cyclic β(1-2) glucan formed in vitro.* *In vitro* synthesis of cyclic β(1-2) glucan was carried out with inner membranes of *R. fredii* USDA257 as described in Materials and Methods. Glucan was purified by Bio-Gel P4 chromatography and subjected to total or partial acid hydrolysis as described in Materials and Methods. **A)** Descending paper chromatography of total acid hydrolysis products. The chromatogram was developed with solvent A. **B)** Descending paper chromatography of partial acid hydrolysis products. Chromatograms were developed with solvent B. Standards: Gal: galactose; Glc: glucose; Man: mannose; Ge: gentiobiose; So: sophorose; La: laminaribiose

and total acid hydrolysis, no sorbitol could be detected after paper electrophoresis with buffer D, indicating that the glucan had no free reducing end (data not shown). Thus *R. fredii* inner membranes incubated with UDP-glucose formed a neutral non-substituted cyclic β(1-2) glucan *in vitro*.

Characterization of Glycopeptides

In *R. fredii* a 235 kDa β(1-2) glucan protein intermediate was identified by polyacrylamide gel electrophoresis as shown in figs. 3A and 3B. The intermediate protein had the same apparent molecular mass as the *A. tumefaciens* 235 kDa intermediate protein. It was described previously that ^{14}C-glucose-labelled glucopeptides can be obtained after extensive protease treatment of the 235 kDa intermediate protein (Zorreguieta *et al.*, 1985). It was well established that only one amino acid remains attached to the reducing end of the oligosaccharide after this proteolytic treatment (Yamashita *et al.*, 1978). Thus the elution vol. from a Bio-Gel P4 column is determined by the degree of polymerization of the polyglucose chain originally linked to the 235 kDa protein. As shown in fig. 5, glycopeptides prepared and purified as described in Materials and Methods from *R. fredii* USDA191, USDA257 and *A. tumefaciens* A348 were subjected to Bio-Gel P4 chromatography. Although not completely resolved, three main glycopeptides with oligosaccharides of different degree of polymerization were observed in these three strains (Figs. 5A-5C). It can be observed that the apparent degree of polymerization of the oligosaccharides recovered as glycopeptides are in all cases bigger than the cyclic glucan formed by each strain. This indicates that: a) the oligosaccharides accumulated before releasing as cyclic glucan have a degree of polymerization higher than the final product, and b) no intermediates with lower degree of polymerization than the final product (cyclic glucan) were accumulated on the intermediate protein, so suggesting that the rate limiting step in the biosynthesis of cyclic β(1-2) glucans might be a cyclization.

Nodulation Studies

Soybean plants of the cultivars Mc Call and Peking

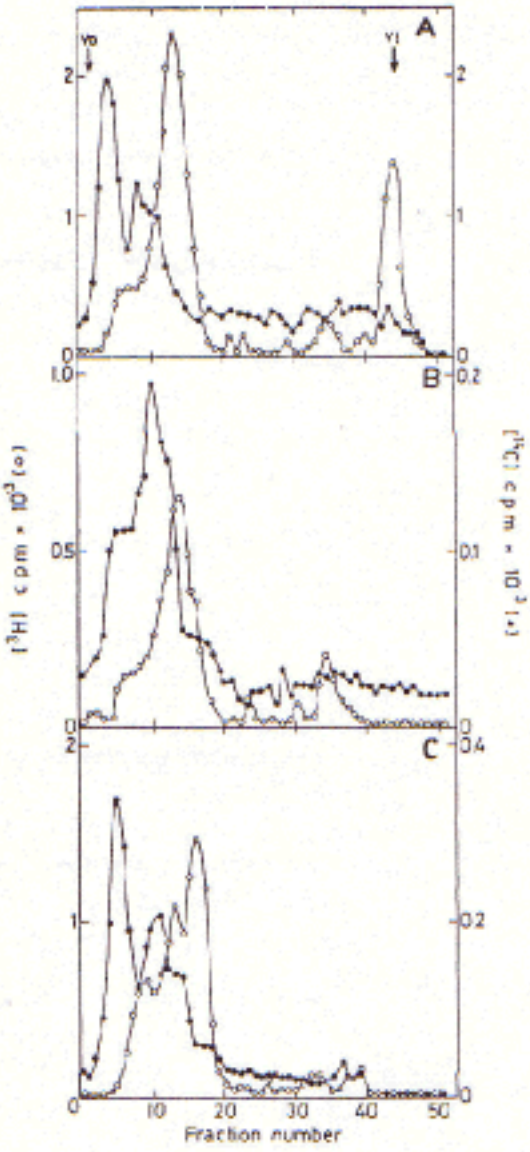

Fig. 5 *Bio-Gel P4 chromatography of ^{14}C-glucose-labelled glycopeptides.* ^{14}C-glucose-labelled peptides (•) were obtained and purified as described in Materials and Methods. o show the elution profile of ^{3}H-glucose cyclic β(1-2) glucan obtained *in vitro*. A) *R. fredii* USDA 191; B) *R. fredii* USDA 257; C) *A. tumefaciens* A 348. Bio-Gel P4 column (78 by 1.8 cm) was eluted with 0.1 M pyridine-acetate buffer (pH 5.5). Fractions of 1.5 ml were collected. Radioactivity was determined by counting with Bray solution in a liquid scintillator.

were inoculated with *R. fredii* strain HH303, strain Rf19 (*ndvB*: Tn5; Bhagwat *et al.*, 1992) and strain NI01, a clone retrieved from nodules of plants inoculated with strain Rf19. Modified Leonard jars with three seedlings each were inoculated with cells from 2 days old cultures and the experiment was carried out in duplicate. As shown in table 1 no statistical difference (difference of means, 95% confidence) was observed in acetylene reduction activity of plants inoculated with either strain or the reisolate. Thus the *R. fredii* Rf19 strain with a Tn5 insertion in the *ndvB* locus yielded active nitrogen fixing nodules. Moreover, electron microscopy revealed that nodules induced by strain Rf19 (Figs. 6D-6F), were undistinguishable from those induced by the wild-type strain HH303 (Figs. 6A-6C). Both strains formed normal infection threads, it can be observed that the mutant strain Rf19 is normally released from the infection thread into the plant cell (Fig. 6E arrow). The only difference observed was that wild type bacteroids are rounded by an electron dense layer (Fig. 6C), whereas this was absent in bacteroids of the Rf19 mutant strain (Fig. 6F).

Nodules were surface sterilized, bacteria recovered and characterized as described in Materials and Methods. Clones resistant to kanamycin, negative for β(1-2) glucan production and lacking the inner membrane 235 KDa protein were recovered from all nodules of plants inoculated with strains Rf19 or NI01. Thus *R. fredii* mutants mapping in the gene encoding the 235 β(1-2) intermediate protein are affected in the synthesis of β(1-2) glucans but induce normal nodules in Mc Call and Peking soybean cultivar and so that they should not be called *ndvB* mutants.

Table 1 *Total acetylene reduction activity of nodulated soybean plants*

R. fredii Strain	Soybean Lines	
	Peking	Mc Call
	Acetylene Reduction Activity (μMol Ethylene. hrs.$^{-1}$. Plant^{-1})	
HH303	0.59	1.10
Rf19[a]	0.67	0.86
NI01[b]	0.56	0.55

Acetylene activity was determined as described in Materials and Methods. [a]*ndvB* mutant obtained by Tn5 mutagenesis of strain HH303 by Bhagwat *et al.* (1992). [b]Strain Rf19 recovered from nodules.

DISCUSSION

Production and secretion of cyclic β(1-2) glucan is required in *R. meliloti* for effective nodule initiation (Dylan *et al.*, 1986; Geremía *et al.*, 1987). Although the mechanism of action is still unknown, cyclic glucan may be a bacterial signal that prevents evoking a plant defence mechanisms, or it may be important for delivering hydrophobic signal molecules occluded inside their hydrophobic ring cavity to the plant. It is a matter of speculation that being cyclic would give to the glucan the ability to resist the action of glucosidases, so that the half life time in the rhizosphere or inside the plant may be lengthened. Soybean plants are nodulated by *Bradyrhizobium japonicum* and *Rhizobium fredii*. *B. japonicum* does not form cyclic β(1-2) glucan, but accumulates a cyclic β(1-3) and β(1-6) glucan, described in this bacterium (Iñón de Iannino and Ugalde, 1989). So far, no role has been assigned to this cyclic glucan in nodule invasion. On the other hand, *R. fredii* forms cyclic β(1-2) glucan instead of β(1-3) and β(1-6) glucans.

There are contradictory results on the role of the *R. fredii* β(1-2) glucan in nodulation. Ko and Gaida (1990) reported that a pleiotropic *R. fredii* USDA 191 mutant is unable to form neutral glucan and exopolysaccharide formed normal nodules on *Glycine max* (cv Peking). On the other hand, Bhagwat *et al.* (1992) communicated that a *R. fredii* HH303 *ndvB* mutant, that does not form β(1-2) glucan induced empty ineffective nodules on cv Williams. Our results showed that ndvB Rf19 mutant obtained by Bhagwat *et al.* formed active nodules on Mc Call and Peking cultivars. Moreover clones isolated from these nodules remained inactive in the synthesis of β(1-2) cyclic

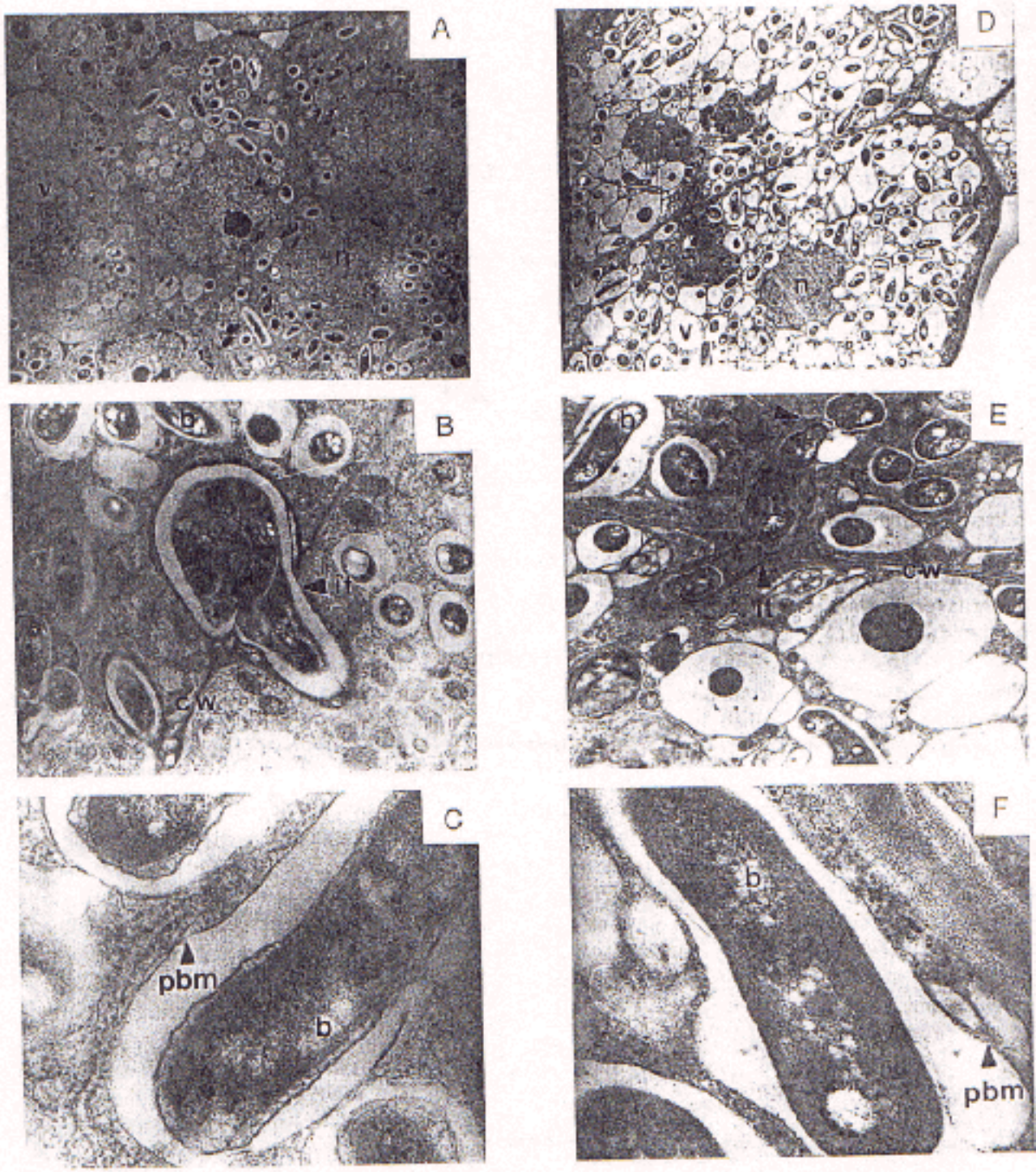

Fig. 6 *Nodulation studies.* Soybean cv. McCall was inoculated with *R. fredii* HH303 or *R. fredii* Rf19 *ndvB* mutant as described in Materials and Methods. **A-C)** Electron microscopy of nodules formed by *R. fredii* HH303 wild type strain. **D-F)** Electron microscopy of nodules formed by *R. fredii* Rf19 *ndvB* mutant strain. Mature nodules were processed for electron microscopy according to Materials and Methods. n: plant cell nucleus; cw: cell wall; b: bacteroid; pbm: peribacteroid membrane; it: infection thread; v: vacuole. Magn. **A, D)** x 3,000, **B, E)** x 12,000, **C, F)** x 50,000

glucan. These results are in agreement with those obtained by Ko and Gaida (1990) with a mutant derived from *R. fredii* USDA 191. We characterized the biosynthesis and structure of cyclic β(1-2) glucans formed by *R. fredii* USDA 191 and HH303, strains that nodulate primititive (Peking) and improved (McCall) soybean cultivars, and strain USDA 257 that nodulates only primitive (Peking) soybean cultivars. No difference was detected among these strains, so that it is unlikely that β(1-2) glucans plays any role in nodule invasion and or specificity.

Polyacrylamide gel electrophoresis of *R. fredii* inner membranes revealed the presence of a 235 kDa β(1-2) glucan intermediate protein; no 90 kDa β(1-3), β(1-6) glucan intermediate was observed. The β(1-2) glucan intermediate proteins of all *R. fredii* strains studied had a molecular mass undistinguishable from *A. tumefaciens* intermediate protein, suggesting that in this species, β(1-2) glucan intermediate proteins are highly conserved.

Since the first demonstration in *A. tumefaciens* and *R. meliloti* that the synthesis of cyclic β(1-2) glucans occurs with the participation of membrane-bound protein intermediates, different authors described the presence of intermediate proteins in different rhizobia. Bhagwat and Keister (1992) communicated that inner membranes of *R. fredii* USDA 205 and HH303 contained a protein similar to *R. meliloti* 235 kDa protein, which is the intermediate in the synthesis of cyclic β(1-2) glucan. These authors showed that incubation of *R. fredii* inner membranes with UDP-^{14}C-Glc led to the incorporation of radioactivity into neutral compounds; however no characterization of these products was provided, and no studies on the formation of glucans *in vivo* was carried out.

Studies on the characterization of glucans formed by *R. fredii* strains with different nodulation specificity showed that β(1-2) cyclic glucans were substituted with phosphoglycerol and that no β(1-3), β(1-6) cyclic glucans could be detected. The degree of polymerization and the net negative charge of *R. fredii* USDA 191 and USDA 257 and HH303 glucans are very similar and all three have a higher degree of polymerization and a higher net charge than *A. tumefaciens* glucans. The cyclic unsubstituted glucans formed *in vitro* by *R. fredii* USDA 191 and USDA 257 also have a degree of polymerization higher than *A. tumefaciens*. The fact that *R. fredii* cyclic glucans are *in vivo* substituted with phosphoglycerol suggested that they were secreted into the periplasmic space, where the substitution takes place (Iñón de Iannino and Ugalde, 1989).

In order to further characterize the protein intermediates formed during the biosynthesis in *R. fredii*, glycopeptides were obtained from the 235 kDa intermediate protein and the degree of polymerization determined by gel filtration. It was observed that oligosaccharides having the size of the final product were accumulated on the protein, thus indicating that during the biosynthesis cyclization might be the rate limiting step.

Acknowledgments - This work was supported by Grants from Consejo Nacional de Investigaciones Cientificas and Técnicas and Secretaría de Ciencia y Técnica de la Universidad de Buenos Aires, Argentina. We acknowledge Susana Raffo and María de los Angeles Curtó (from CONICET) for preparing UDP-^{14}C-glucose and Dra Sara Maldonado for electron microscopy studies. We thank Dr. S.G. Pueppke for useful suggestions.

REFERENCES

Altabe, S., Iñón de Iannino, N., de Mendoza, D. and Ugalde, R.A., Expression of *Agrobacterium tumefaciens chvB* virulence region in *Azospirillum* spp. *J. Bacteriol.* 1990, 172: 2563-2567.

Batley, M., Redmond, J.W., Djordjevic, S.P. and Rolfe, B.G., Characterization of glycerophosphorylated cyclic β(1-2) glucans from fast-growing *Rhizobium* species. *Biochim. biophys. Acta* 1987, 901: 119-126.

Bhagwat, A.A. and Keister, D.L., Synthesis of β glucans by *Bradyrhizobium japonicum* and *Rhizobium fredii*. *Can. J. Microbiol.* 1992, 38: 510-514.

Bhagwat, A.A., Tully, R.E. and Keister, D.L., Isolation and characterization of an *ndvB* locus from *Rhizobium fredii*. *Mol. Microbiol.* 1992, 6: 2159-2165.

Bhavaneswari, T.V., Pueppke, S.G. and Bauer, W.D., Role

of lectins in plant-microorganism interactions. *Plant Physiol.* 1977, **60**: 486-491.

Burrows, S., Grylls, F.S.M. and Harrison, J.S., Paper chromatography of phosphoric esters. *Nature* 1952, **170**: 800-801.

Carlson, R.W., Kalembasa, S., Turowski, D., Pachori, P. and Noel, K.D., Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. *J. Bacteriol.* 1987, **169**: 4923-4928.

Dische, Z., General color reactions. *Meth. Carbohydr. Chem.* 1962, **1**: 478-492.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E.W., Helinski, D.R. and Ditta, G., *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *Proc. natn. Acad. Sci.* 1986, **83**: 4403-4407.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.R., Deegan, S., Walker, G.C. and Signer, E.R., Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. *Cell* 1985, **40**: 869-877.

Geremía, R.A., Cavaignac, S., Zorreguieta, A., Toro, N., Olivares, J. and Ugalde, R.A., A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β(1-2) glucan. *J. Bacteriol.* 1987, **169**: 880-884.

Hisamatsu, M., Yamada, T., Higashiura, T. and Ikeda, M., The production of acidic O-acetylated cyclosophorans (cyclic β(1,2)-D-glucans) by *Agrobacterium* and *Rhizobium* species. *Carbohydr. Res.* 1987, **163**: 115-122.

Iñón de Iannino, N. and Ugalde, R.A., Biochemical characterization of a virulent *Agrobacterium tumefaciens chvA* mutants: synthesis and excretion of β(1-2) glucan. *J. Bacteriol.* 1989, **171**: 2842-2849.

Iñón de Iannino, N. and Ugalde, R.A., Biosynthesis of cyclic β(1-3), β(1-6) glucan in *Bradyrhizobium* spp. *Arch. Microbiol.* 1993, **159**: 30-38.

Kennedy, E.P., Rumley, M.K., Schulman, H. and van Golde, L.M.G., Identification of sn-glycero-1-phosphate and phospho-ethanolamine residues linked to the membrane-derived oligosaccharide of *Escherichia coli*. *J. biol. Chem.* 1976, **251**: 4208-4213.

Ko, Y.H. and Gayda, R., Nodule formation in soybeans by exopolysaccharide mutants of *Rhizobium fredii* USDA 191. *J. gen. Microbiol.* 1990, **136**: 105-113.

Koepsell, H.J. and Sharpe, E.S., Microdetermination of pyruvic and alfa ketoglutaric acids. *Arch. Biochem. Biophys.* 1952, **38**: 443-449.

Lepek, V., Navarro de Navarro, Y. and Ugalde, R.A., Synthesis of β(1-2) glucan in *Rhizobium loti*. *Arch. Microbiol.* 1990, **155**: 35-41.

Maier, R.J. and Brill, W.J., Involvement of *Rhizobium japonicum* O antigen in soybean nodulation. *J. Bacteriol.* 1978, **133**: 1295-1299.

Miller, K.J., Gore, R.S. and Benesi, A.J., Phosphoglycerol substituents present on the cyclic β(1,2) glucans of *Rhizobium meliloti* 1021 are derived from phosphatidylglycerol. *J. Bacteriol.* 1988, **170**: 4569-4575.

Miller, K.J., Gore, R.S., Johnson, R., Benesi, A.J. and Reinhold V.N., Cell-associated oligosaccharides of *Bradyrhizobium* spp. *J. Bacteriol.* 1990, **172**: 136-142.

Miller, K.J., Kennedy, E.P. and Reinhold, V.N., Osmotic adaptation by Gram-negative bacteria: possible role for periplasmic oligosaccharides. *Science* 1986, **231**: 48-51.

Miller, K.J., Reinhold, V.N., Weissborn, A.C. and Kennedy, E.P., Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with sn-1-phosphoglycerol residues. *Biochim. biophys. Acta* 1987, **901**: 112-118.

Osborn, M. and Munson, R., Separation of inner (cytoplasmic) and outer membranes of Gram-negative bacteria. *Meth. Enzymol.* 1984, **31A**: 642-653.

Pankhurst, C.E., Craig, A.S. and Jones, W.T., Effectiveness of *Lotus* root nodules. *J. exp. Bot.* 1979, **119**: 1085-1093.

Pueppke, S.G., *Rhizobium* infection threads in root hairs of *Glycine max* (L.) Merr., *Glycine soja* Sieb and Zucc. and *Vigna unguiculata* (L.) Walp. *Can. J. Microbiol.* 1983, **29**: 69-76.

Puvanesarajah, V., Schell, F.M., Stacey, G., Douglas, C.J. and Nester, E.W., Role for 2-linked β-D-glucan in the virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* 1985, **164**: 102-106.

Rolin, D.B., Pfeffer, P.E. and Osman, S.F., Structural studies of a phosphocholine substituted β(1-3), β(1-6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. *Biochim. biophys. Acta* 1992, **1116**: 215-225.

Stacey, G., Jae-Seong, S., Roth, L.E., Bhagya Lakshmi, S.K. and Carlson, R.W., A lipopolysaccharide mutant of *Bradyrhizobium japonicum* that uncouples plant from bacterial differentiation. ???? 1991, **4**: 332-340.

Trevelyan, W.E., Procter, D.P. and Harrison, J.S., Detection of sugar on paper chromatography. *Nature* 1950, **166**: 444-445.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.C. and Dénarié, J., Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. *Nature* 1991, **351**: 670-673.

Tully, R.E., Keister, D.L. and Gross, K.C., Fractionation of the β-linked glucans of *Bradyrhizobium japonicum* and their response to osmotic potential. *Appl. environm. Microbiol.* 1990, **56**: 1518-1522.

Vincent, J.M., *A Manual for practical Study of root-nodule Bacteria*. Blackwell, Oxford, 1970, pp. 90-121.

Wacek, T.J. and Brill, W.J., Simple rapid assay for screening nitrogen fixing ability in soybean. *Crop. Sci.* 1976, **16**: 519-522.

Williamson, G., Damani, K., Devenney, P., Faulds, C.B., Morris, V.J. and Stevens, B.J.H., Mechanism of action of cyclic β(1,2) glucan synthetase from *Agrobacterium tumefaciens*: competition between cyclization and elongation reactions. *J. Bacteriol.* 1992, **174**: 7941-7947.

Yamashita, K., Tachibana, Y. and Kobata, A., The structures of the galactose-containing sugar chains of ovalbumin.

J. biol. Chem. 1978, **253**: 3862-3869.
Zorreguieta, A. and Ugalde, R.A., Formation in *Rhizobium* and *Agrobacterium* spp of a 235-kilodalton protein intermediate in β-D(1-2) glucan synthesis. *J. Bacteriol.* 1986, **167**: 947-951.
Zorreguieta, A., Ugalde, R.A. and Leloir, L.F., An intermediate in cyclic β(1-2) glucan biosynthesis. *Biochem. biophys. Res. Commun.* 1985, **126**: 352-357.