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Phosphoglycerol Substituents Present on the Cyclic β-1,2-Glucans of *Rhizobium meliloti* 1021 Are Derived from Phosphatidylglycerol†

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The synthesis of periplasmic cyclic β-1,2-glucans is a property unique to species of the family *Rhizobiaceae*. For this reason, it is generally believed that these molecules may play an important role in the plant infection process. In the present study, we determined that the cyclic β-1,2-glucans produced by *Rhizobium meliloti* 1021 were predominantly anionic in character and contained both phosphoglycerol and succinic acid substituents. In addition, we demonstrated that phosphatidylglycerol was the source of the phosphoglycerol substituents present on these oligosaccharides and that greater than 60% of the total phospholipid turnover in this organism involved this substitution reaction.

The interaction of gram-negative bacteria of the genus *Rhizobium* with the roots of certain leguminous plants leads to the development of nitrogen-fixing nodules (5, 15, 28, 37). This interaction has been shown to be species specific and is believed to be mediated through a "signal exchange" mechanism between the two organisms (see reference 15 for a review). Because of their possible role as signal molecules in nodulation, the cell envelope carbohydrates of members of the genus *Rhizobium* have been the subject of much study.

Compelling evidence for a role of cell surface carbohydrates in nodulation has come primarily from studies of rhizobial mutants that are defective in the synthesis of exopolysaccharides (8, 29, 30, 34, 36). Recently, however, attention has focused on the possible role of the periplasmic cyclic β-1,2-glucans in legume nodulation (1, 7, 12–15, 18, 35, 42). These compounds were first identified in *Agrobacterium tumefaciens* by McIntire and co-workers (31) in 1942. Since that time, there have been several analyses of the structure and distribution of these compounds (2, 19–21, 26, 27, 38–41). With regard to distribution, the cyclic β-1,2-glucans appear to be unique to species of the family *Rhizobiaceae*. With regard to structure, it has been demonstrated that the cyclic glucans consist of 17 to 24 glucose residues linked solely by β-1,2 glycosidic bonds. Until 1987, it was believed that the cyclic β-1,2-glucans were strictly unsubstituted and neutral in character. However, most recently, three laboratories have reported the discovery of anionic, substituted forms of these molecules (4, 21, 33).

Initial evidence for a possible role of the cyclic glucans in rhizobial nodulation was reported by Abe et al. (1) and Higashi and Abe (18), who observed that the addition of cyclic β-1,2-glucans to white clover seedlings resulted in the enhancement of infection thread formation and nodule number by *Rhizobium trifolii*. A second line of evidence has come from recent studies by Nester and co-workers (7, 12, 13, 35) and Ugalde and co-workers (14, 42) of mutants of *Agrobacterium tumefaciens* and *Rhizobium meliloti* found to be defective in the synthesis of cyclic β-1,2-glucans. The cyclic β-1,2-glucan-deficient mutants of *A. tumefaciens* are avirulent and attachment defective (7, 35), and the mutants of *R. meliloti* form ineffective nodules (13, 14).

Although the cyclic β-1,2 backbone structure of the rhizobial glucans distinguishes these oligosaccharides from those thus far identified in other bacterial genera, it should be noted that these molecules appear to be closely related to the membrane-derived oligosaccharides of *Escherichia coli* (32, 33). Similarities between these classes of oligosaccharides include (i) intermediate size, (ii) glucose as the sole sugar, (iii) a β-1,2-linked backbone, (iv) periplasmic localization, (v) osmoregulated biosynthesis, and (vi) *sn*-1-phosphoglycerol and succinyl substituents.

In the present study, we have examined the synthesis of cyclic β-1,2-glucans by *R. meliloti* 1021. Our analyses reveal that the cyclic β-1,2-glucans produced by *R. meliloti* 1021 are highly substituted. Both phosphoglycerol and succinic acid substituents were identified. Furthermore, we demonstrate that the phosphoglycerol substituents present on the cyclic β-1,2-glucans of *R. meliloti* 1021 derive from the head group of the membrane phospholipid, phosphatidylglycerol. This metabolic link to phosphatidylglycerol turnover represents yet another significant parallel to membrane-derived oligosaccharide biosynthesis by *E. coli*.

MATERIALS AND METHODS

Organism and culture conditions. *R. meliloti* 1021 was generously provided by F. M. Ausubel, Harvard Medical School, Boston, Mass. Cells were cultured in TY medium (6) and incubated at 30°C on a gyratory shaker (Labline Instruments, Inc., Melrose Park, Ill.). Growth was monitored turbidometrically at 650 nm with a Spectronic 21 spectrophotometer (Bausch and Lomb, Rochester, N.Y.).

Chemicals. [2-^{3}H]Glycerol was purchased from ICN Radiochemicals Inc. (Irvine, Calif.). Phospholipid standards were purchased from Sigma Chemical Co. (St. Louis, Mo.). Deuterium oxide (99.996 atom % D) was purchased from Merck, Sharp and Dohme (St. Louis, Mo.).

Preparation of cyclic β-1,2-glucans. *R. meliloti* 1021 was grown to the mid-logarithmic phase to a cell density of approximately 0.1 mg of total cellular protein per ml. Cells were harvested by centrifugation (5,000 × *g* at 5°C), washed once with YM salts (1.71 mM NaCl, 0.81 mM $MgSO_4$, 2.87 mM K_2HPO_4, pH 7.0), and subjected to a modified Bligh-Dyer extraction procedure (24). Aqueous methanol extracts were concentrated under nitrogen at 37°C or by rotary evaporation. Concentrated extracts were chromatographed

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at room temperature on a column of Sephadex G 50 (Pharmacia Inc., Piscataway, N.J.) as described below.

Oligosaccharide analysis on Sephadex G50. A 6-liter culture of *R. meliloti* 1021 was grown to the mid-logarithmic phase in TY medium at 30°C. Cells were harvested by centrifugation, and the cellular pellet was extracted with chloroform, methanol, and water. The aqueous methanol phase was concentrated to dryness by rotary evaporation. The sample was redissolved in water (2 ml) and applied to a column of Sephadex G50 (1 by 56 cm). The column was eluted at room temperature at a rate of 15 ml/h with 0.15 M ammonium acetate (pH 7.0) containing 7% (vol/vol) propanol. Fractions (1 ml) were collected and assayed for total carbohydrate by the phenol method (16). Purified neutral cyclic β-1,2-glucan standard was prepared from *A. tumefaciens* C58 as described previously (32).

DEAE-cellulose chromatography of oligosaccharides. Sephadex G50 fractions containing oligosaccharides of the same molecular weight as the cyclic β-1,2-glucans (see Fig. 1) were pooled, concentrated, and desalted on a column (1 by 56 cm) of Sephadex G25 (Pharmacia) with 7% propanol as the eluant. The desalted sample was then applied to a column (1 by 23 cm) of DEAE-cellulose (Whatman DE52). The column was first washed with 40 ml of 10 mM Tris hydrochloride (pH 7.4) containing 7% (vol/vol) propanol. Next, a 100-ml gradient was applied, beginning with 0 mM KCl and ending with 200 mM KCl in the same buffer. Elution conditions were similar to those described previously (32, 33). Fractions (2 ml) were collected and assayed for total carbohydrate.

Chemical analysis of the cyclic β-1,2-glucans of *R. meliloti* 1021. Desalted oligosaccharide preparations were subjected to a variety of chemical analyses. Total carbohydrate was determined by the phenol method (16). Glucose content was determined by the glucose oxidase method (Sigma Chemical Co.) after hydrolysis for 4 h in 1.0 M HCl at 100°C. Total phosphorus was determined by the method of Chen et al. (9) after digestion with magnesium nitrate (3). Phosphomonoester content was determined after treatment with alkaline phosphatase as described previously (33). Total organic ester content was determined by the hydroxamic acid method described by Hestrin (17). Succinate was determined by the succinate thiokinase method (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) after treatment with 0.1 M NaOH for 30 min at 37°C. Phosphoglycerol content was determined by using alkaline phosphatase and glycerol kinase after treatment with 0.5 M NaOH (100°C, 80 min) as described previously (33).

NMR. ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were recorded at ambient temperature on a Bruker AM-500 spectrometer operating at 125.76 MHz. The samples were dissolved in D_2O at concentrations of approximately 10 mg/ml in 5-mm NMR tubes. Acetone was used as an internal reference, with the methyl carbons assigned to 29.2 ppm relative to tetramethylsilane. Waltz-16 decoupling was used to minimize sample heating. The 90° pulse width was 5.4 μs, and a typical experiment consisted of 5,000 to 20,000 transients collected with 2 s between 45° pulses.

RESULTS

Cyclic β-1,2-glucans of *R. meliloti* 1021 are highly substituted and predominantly anionic. When methanol extracts of *R. meliloti* 1021 were chromatographed on Sephadex G50, one major peak of oligosaccharide material was detected (Fig. 1). The elution volume of this material corresponded to

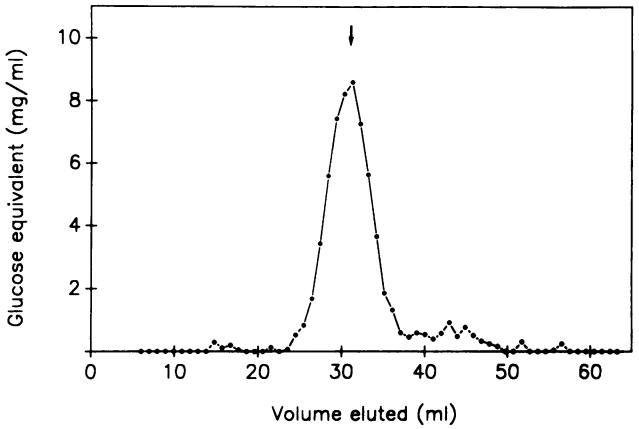

FIG. 1. Oligosaccharide analysis on Sephadex G50. An aqueous methanol extract derived from a 6-liter culture of *R. meliloti* was chromatographed on Sephadex G50 as described in the text. Fractions (1 ml) were collected and assayed for total carbohydrate. The arrow indicates the position expected for the cyclic β-1,2-glucans as determined by calibration with a purified standard.

that of a purified cyclic β-1,2-glucan standard. After further fractionation of this extract on DEAE-cellulose, these oligosaccharides were found to be predominantly anionic in character (Fig. 2), with only 7% eluting as neutral oligosaccharides. In addition to the neutral fraction (F1), five anionic fractions were present (designated F2, F3, F4, F5, and F6). In order to identify further the chemical nature of this total oligosaccharide preparation, a variety of analyses were performed as described below.

As shown in Table 1, it was determined that glucose could account for the total carbohydrate present in the oligosaccharides extracted from *R. meliloti* 1021. Consistent with the predominantly anionic character revealed by chromatography on DEAE-cellulose, these glucans were found to be highly substituted with phosphoryl moieties. A total-phosphorus determination revealed that the phosphorus-to-glucose ratio for these glucans was approximately 0.11 (Table 1). Treatment of the glucans with alkaline phosphatase did not result in the release of detectable levels of

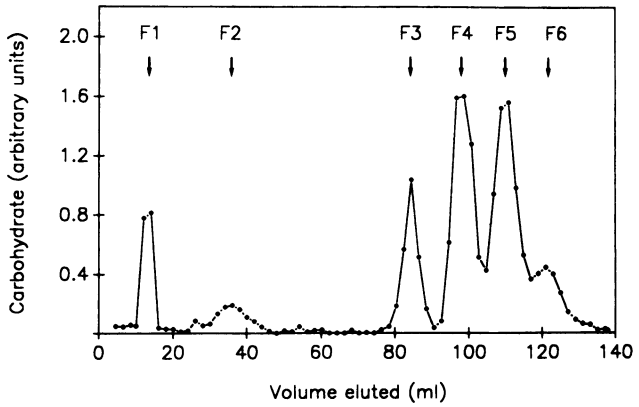

FIG. 2. DEAE-cellulose chromatography of oligosaccharides. A desalted preparation of *R. meliloti* 1021 oligosaccharides was chromatographed on DEAE-cellulose as described in the text. Fractions (2 ml) were collected and assayed for total carbohydrate. Results are expressed in arbitrary units. F1 through F6 mark the peaks of six major oligosaccharide subfractions as described in the text.

TABLE 1. Analysis of oligosaccharides from *R. meliloti* 1021[a]

Component	Concn (μmol/ml)
Total carbohydrate (glucose equivalent)	20.0
Total glucose	19.6
Total phosphorus	2.2
Phosphate monoester	0.0
Phosphate monoester released after alkaline hydrolysis	2.2
Phosphoglycerol released after alkaline hydrolysis	1.6
Total organic ester	1.4
Total succinate	0.5

[a] Oligosaccharides were extracted from a 6-liter culture of *R. meliloti* 1021 and chromatographed on a column of Sephadex G50. The material was subsequently desalted on a column of Sephadex G25 and subjected to a variety of chemical determinations as described in Materials and Methods. Results are expressed as micromoles per milliliter of the desalted oligosaccharide preparation.

inorganic phosphorus. Thus, the phosphoryl moieties present on the rhizobial glucans are not present as phosphomonoesters. If, however, the glucan fraction was first subjected to conditions of alkaline hydrolysis (0.5 M NaOH, 100°C, 80 min), all of the phosphorus was released in the form of phosphomonoester (Table 1). These results therefore indicate that all of the phosphoryl moieties within the glucan fraction of *R. meliloti* 1021 are phosphodiester in character. Further analysis of these phosphoryl substituents was performed by using alkaline phosphatase and glycerol kinase after treatment with 0.5 M NaOH (100°C, 80 min). The results of these analyses indicated that phosphoglycerol accounted for approximately 73% of the total phosphodiester moieties present within the glucans of this organism (Table 1).

In addition to phosphoglycerol substituents, the presence of organic ester substituents was also demonstrated. As shown in Table 1, it was determined that there were 0.07 organic ester substituents per glucose residue. Consistent with the anionic nature of these glucans, additional analyses indicated that succinic acid could account for 36% of the total organic ester substituent present.

In order to determine whether the anionic glucans of *R. meliloti* 1021 represented substituted forms of the cyclic β-1,2-glucans, the preparations were examined by NMR spectroscopy. The NMR spectrum of purified, neutral cyclic β-1,2-glucan standard is shown in Fig. 3A. The spectrum obtained for this standard was essentially identical to that reported previously (11). Based on previous analysis by Dell (11), peaks at 60.2, 68.3, 81.8, and 101.6 ppm were assigned to the C-6, C-4, C-2, and C-1 resonances of the glucosyl residues of these molecules. Although Dell (11) could not unambiguously distinguish the assignments for C-3 and C-5 of the neutral glucans, studies of glycerophosphorylated cyclic β-1,2-glucans produced by *Rhizobium* sp. strain NGR234 (4) indicate that the peaks at 75 and 75.9 ppm correspond to the C-3 and C-5 resonances, respectively. As is characteristic of the NMR spectra of the neutral cyclic β-1,2-glucans, multiple resonances for the C-1, C-2, C-3, and C-4 carbon atoms were observed. Dell (11) has proposed that these multiple resonances derive from the range of sizes of these cyclic molecules (17 to 24 glucose residues) due to the slightly different angles between adjacent glucosyl residues. Also characteristic of the NMR spectra of these unbranched, cyclic glucans was the absence of signals at 92 to 96 ppm. Signals in this region of the NMR spectrum correspond to the C-1 resonances of reducing glucose residues (4, 11). Furthermore, the absence of a signal at approximately 73 to 74 ppm indicates that all C-2 carbons are involved in glycosidic bonds (11).

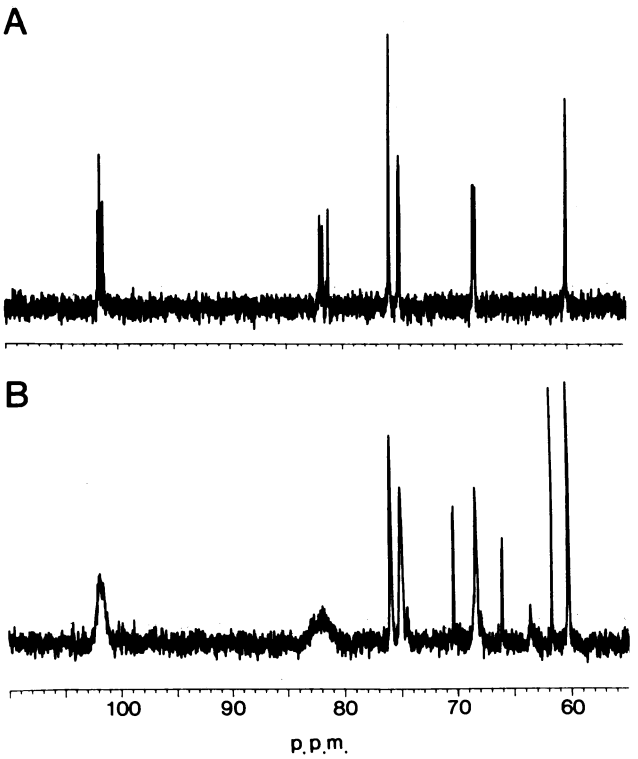

FIG. 3. ^{13}C-NMR spectrum of the anionic cyclic β-1,2-glucans of *R. meliloti* 1021. (A) Neutral, unsubstituted cyclic β-1,2-glucan standard was prepared as described previously (32). The sample was dissolved in deuterium oxide at a concentration of 14 mg/ml (glucose equivalent). (B) Anionic cyclic β-1,2-glucan subfraction F4 of *R. meliloti* 1021 was purified by chromatography on DEAE-cellulose as described in the text. After desalting by chromatography on Sephadex G25 (see Materials and Methods), the sample was dissolved in deuterium oxide at a concentration of 7 mg/ml (glucose equivalent).

When an NMR spectrum of the anionic glucan preparation of *R. meliloti* 1021 was examined and compared with that of the standard, similarities were immediately apparent (Fig. 3B). Furthermore, the spectrum was strikingly similar to that reported previously by Batley and co-workers (4) for the glycerophosphorylated cyclic β-1,2-glucans of *Rhizobium* sp. strain NGR234. As shown in Fig. 3B, there were no detectable peaks at 92 to 96 ppm, consistent with an unbranched, cyclic backbone structure. In addition, four signals not present in the spectrum for the neutral cyclic glucan standard were observed (61.8, 66.1, 70.4, and 63.6 ppm). Based on our determination that phosphoglycerol is a major substituent of these glucans and also on previous NMR analyses by Batley and co-workers (4), these peaks were assigned, respectively, to the C-3, C-1, and C-2 resonances of *sn*-1-phosphoglycerol substituents and to the C-6 resonance of glucose residues bearing these substituents. Other similarities between the NMR spectrum of the anionic cyclic β-1,2-glucans isolated from *R. meliloti* 1021 and the spectra reported by Batley and co-workers (4) include broad resonances for C-1 and C-2 of the glucosyl residues, as well as the appearance of two small shoulder peaks approximately 0.5 ppm upfield from the C-4 glucosyl resonance and 1.4 ppm upfield from the C-5 glucosyl resonance. These peaks were assigned to the C-4 and C-5 resonances of the substituted

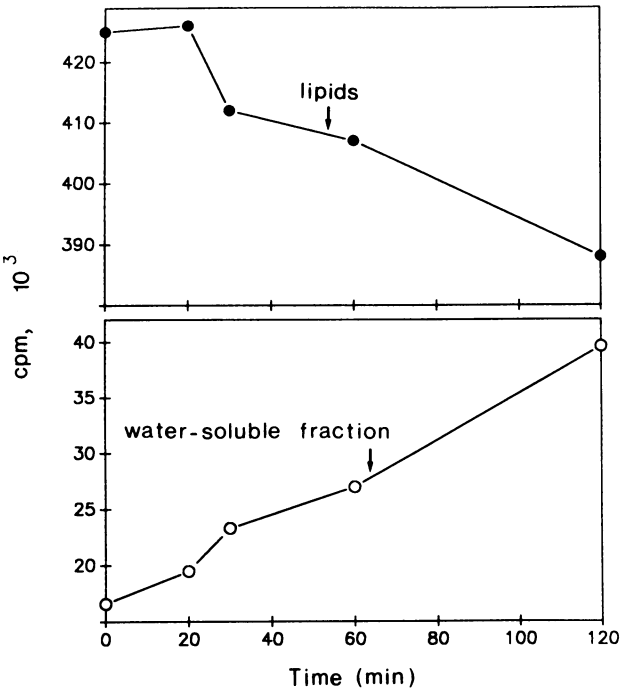

FIG. 4. Pulse-chase experiment using [2-^{3}H]glycerol. A 5-ml culture of *R. meliloti* 1021 in TY medium was radiolabeled by using [2-^{3}H]glycerol at a specific activity of 1.6×10^4 cpm/nmol as described in the text. Under chase conditions, portions were removed at 0, 20, 30, 60, and 120 min and examined for radioactivity in both the lipid (chloroform phase) and water-soluble (aqueous-methanol phase) fractions. At each time point, 1-ml samples were removed to tubes containing 2.5 ml of methanol (containing 0.1 N HCl), 1.25 ml of chloroform (preequilibrated with 1 M $MgCl_2$), and approximately 2 mg of carrier lipid. The contents of the tubes were mixed, and the tubes were allowed to sit for at least 30 min at room temperature. Next, 0.625 ml of chloroform and 0.625 ml of 1 M $MgCl_2$ were added. The two phases were separated by centrifugation (1,790 × *g*), and portions from both phases were assayed for radioactivity by using Liquiscint scintillation solution (National Diagnostics, Manville, N.J.) and a Beckman LS1701 liquid scintillation system (Beckman Instruments, Fullerton, Calif.).

glucose residues (4). In addition to these features, the ^{31}P-induced splitting of the C-1 and C-2 resonances of *sn*-1-phosphoglycerol substituents was observed to be similar to that reported previously by Batley et al. (4).

Thus, the results of the above chemical determinations and NMR analyses indicate that *R. meliloti* 1021 synthesizes highly substituted forms of the cyclic β-1,2-glucans with *sn*-1-phosphoglycerol as the major substituent.

Phosphoglycerol substituents present on the cyclic β-1,2-glucans are derived from phosphatidylglycerol. The presence of cyclic β-1,2-glucans substituted with *sn*-1-phosphoglycerol has been demonstrated for *A. tumefaciens* (33) and *Rhizobium* sp. strain NGR234 (4). Furthermore, the noncyclic membrane-derived oligosaccharides of *E. coli* have also been shown to contain phosphoglycerol substituents (25). In the case of these membrane-derived oligosaccharides, it has been shown that phosphatidylglycerol is the source of the phosphoglycerol substituents (22, 23). It was therefore of interest to determine whether phosphatidylglycerol was likewise the source of the phosphoglycerol substituents present on the cyclic β-1,2-glucans of *R. meliloti* 1021. A pulse-chase experiment was performed in order to evaluate this possibility.

A 5-ml logarithmic-phase culture of *R. meliloti* 1021 in TY medium was radiolabeled by the addition of 0.25 μmol of [2-^{3}H]glycerol containing a total of 4×10^6 cpm. After 60 min at 30°C with gentle shaking, the cells were harvested by filtration (0.22-μm-pore size filter; Millipore Corp., Bedford, Mass.). The cells were washed with 20 ml of unlabeled TY medium containing 50 μM glycerol and then suspended in 15 ml of unlabeled TY medium containing 50 μM glycerol. During the chase period, samples were withdrawn and analyzed for radioactivity in both the lipid and nonlipid cell fractions (Fig. 4). At the start of the chase, it was determined that 90% of the total cell-associated radioactivity was present within the lipid fraction of the cells (Fig. 4). Over the course of 2 h, there was a continuous loss of count from the lipid fraction (Fig. 4, top panel). Concomitant with this loss, there was a continuous increase in radioactivity detected within the nonlipid, water-soluble fraction of these cells (Fig. 4, bottom panel). The continuous increase of counts within the water-soluble fraction during chase conditions indicated that the radiolabeled phospholipids represented the precursor pool. Other possible precursors such as free [^{3}H]glycerol or [^{3}H]glycerol phosphate would be subject to efficient chase under the conditions of the experiment.

In order to identify the nature of the radiolabeled phospholipid precursor, lipid extracts were analyzed by thin-layer chromatography. Extracts were applied to silica gel 60–aluminum-backed plates (E. Merck, Darmstadt, Federal Republic of Germany) and developed in one dimension with chloroform-acetone-methanol-acetic acid-water (60:80:20:20:10). With this system, it was possible to separate phosphatidylglycerol from both phosphatidylethanolamine and cardiolipin. As shown in Table 2, greater than 74% of the loss of label from the phospholipid fraction of *R. meliloti* 1021 could be attributed to the turnover of phosphatidylglycerol. Although there was a significant loss of label from phosphatidylethanolamine, approximately 50% of this loss could be attributed to the synthesis of phosphatidylcholine by these cells (Table 2).

The selective loss of radioactivity from phosphatidylglycerol in these cells is consistent with the transfer of phosphoglycerol (from the head group of this lipid) to the cyclic

TABLE 2. Metabolic turnover of the phospholipids of *R. meliloti* 1021 during a pulse-chase experiment with [2-^{3}H]glycerol[a]

Fraction	^{3}H (10^3 cpm)			Net loss or gain
	0 min	60 min	120 min	
Total phospholipid[b]	427.2	408.9	390.2	−37.0
Phosphatidylglycerol	228.1	208.6	200.6	−27.5
Phosphatidylethanolamine[c]	131.9	134.9	120.2	−11.7
Phosphatidylcholine	41.8	44.2	47.6	+5.8
Cardiolipin	25.2	21.7	21.8	−3.4
Water-soluble fraction[d]	16.6	27.0	39.6	+23.0

[a] *R. meliloti* 1021 in TY medium was radiolabeled with [2-^{3}H]glycerol as described in the legend to Fig. 4. Portions were removed at 0, 60, and 120 min and examined for total radioactivity in both the lipid (chloroform phase) and water-soluble (aqueous methanol phase) fractions. In addition, the radioactivity associated with individual phospholipid species was determined after thin-layer chromatography.

[b] A portion of the chloroform phase was counted for radioactivity. Results represent radioactivity present in the total phospholipid extracts of these cells.

[c] The thin-layer chromatography system used for phospholipid separation was not capable of resolving all of the phosphatidylethanolamine derivatives. The results represent the combined radioactivity present within phosphatidylethanolamine, phosphatidyl-*N*-methylethanolamine, and phosphatidyl-*N*,*N*-dimethylethanolamine.

[d] A portion of the aqueous methanol phase was counted for radioactivity. Results represent the radioactivity present within the total water-soluble fraction of these cells.

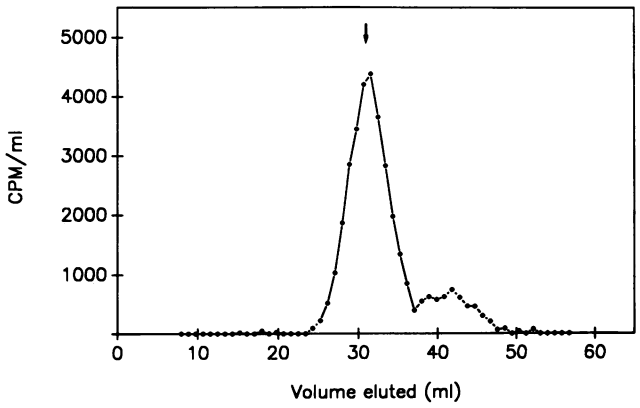

FIG. 5. Sephadex G50 analysis of ^{3}H-labeled water-soluble products synthesized by *R. meliloti* 1021 during a pulse-chase experiment using [2-^{3}H]glycerol. An 8-ml culture of *R. meliloti* 1021 in TY medium was radiolabeled by the addition of 0.4 μmol of [2-^{3}H]glycerol containing a total of 6.5×10^6 cpm. Conditions were similar to those described in the legend to Fig. 4; however, cells were subsequently incubated in unlabeled medium for a total of 240 min. The cell culture was extracted for water-soluble radioactive products as described in the legend to Fig. 4. After 240 min, approximately 5×10^4 cpm was extracted into the aqueous-methanol phase. Of this, a portion containing 4×10^4 cpm was concentrated under nitrogen and subsequently applied to a column of Sephadex G50. Chromatography conditions were similar to those described in the legend to Fig. 1. Fractions (1 ml) were collected, and portions were counted for radioactivity. Arrow, Position expected for the cyclic β-1,2-glucans.

β-1,2-glucans of this organism. Thus, the nature of the water-soluble radiolabeled products which accumulated during chase conditions was investigated. A portion of the radiolabeled water-soluble fraction was concentrated under nitrogen and applied to a Sephadex G50 column under the conditions described above. As shown in Fig. 5, one major peak of radioactivity was detected and eluted at precisely the position of the cyclic β-1,2-glucan standard. To provide further evidence that this peak represented ^{3}H-cyclic β-1,2-glucans, the corresponding fractions were pooled, desalted, and subfractionated further on a column of DEAE-cellulose. Approximately 25 mg (glucose equivalent) of unlabeled carrier cyclic β-1,2-glucans was added to the radiolabeled material. As shown in Fig. 6, the DEAE-cellulose profile of the radiolabeled material was nearly identical to that of the carrier cyclic β-1,2-glucan preparation. Cyclic β-1,2-glucan subfractions F2 through F6 were identified in the radiolabeled preparation and were present at relative proportions similar to those present within the carrier cyclic β-1,2-glucans. The only significant difference between the two preparations was the lack of radiolabeled fraction F1. However, because fraction F1 probably represents unsubstituted cyclic β-1,2-glucans (32, 33), it would be expected that this fraction would not become radiolabeled during chase conditions.

The results of the above experiments indicate that the biosynthesis of the cyclic β-1,2-glucans of *R. meliloti* 1021 is linked to the metabolic turnover of phosphatidylglycerol within this organism. From these data, it was expected that phosphoglycerol would represent the radiolabeled moiety associated with the ^{3}H-cyclic β-1,2-glucans produced during the pulse-chase experiments. In order to identify the radiolabeled moiety, the ^{3}H-cyclic β-1,2-glucan preparation was subjected to conditions of alkaline hydrolysis (0.5 M NaOH, 100°C, 80 min). Such treatment will result in the complete release of intact phosphoglycerol substituents (33); however, a mixture of both α- and β-phosphoglycerol products will result due to the formation of a cyclic intermediate upon alkali-mediated bond breakage (25). Consistent with the

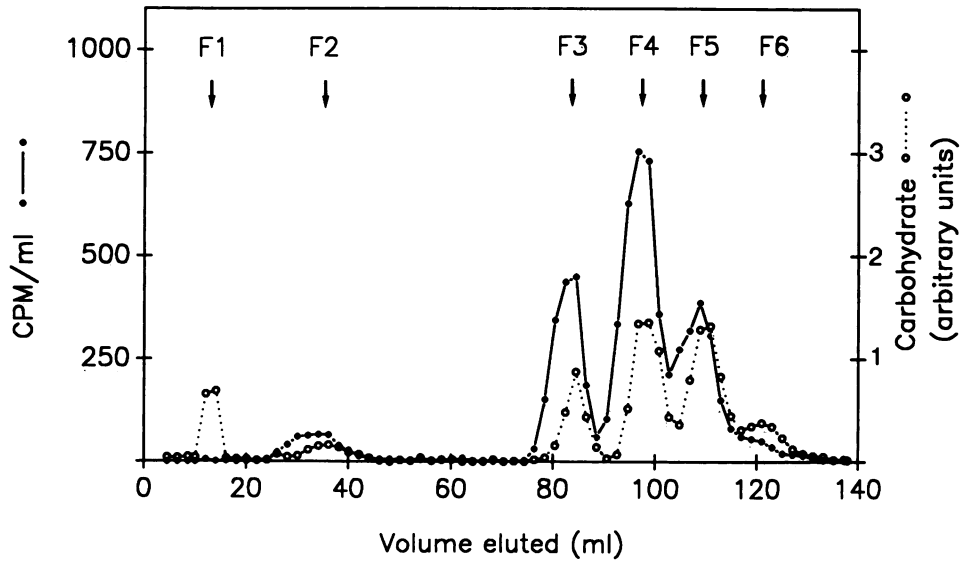

FIG. 6. DEAE-cellulose chromatography of ^{3}H-labeled water-soluble products synthesized by *R. meliloti* 1021 during a pulse-chase experiment using [2-^{3}H]glycerol. ^{3}H-labeled water-soluble material produced by *R. meliloti* 1021 during the pulse-chase experiment described in the legend to Fig. 5 was further characterized by ion-exchange chromatography on DEAE-cellulose. Radioactive material (15,000 cpm) which eluted from Sephadex G50 at a volume expected for the cyclic β-1,2-glucans was pooled and concentrated under a stream of nitrogen. Twenty-five milligrams (glucose equivalent) of carrier cyclic β-1,2-glucans (prepared from *R. meliloti* 1021 as described in the legend to Fig. 1) was added, and the combined sample was desalted by chromatography on Sephadex G25 as described in the text. After being desalted, the combined sample was chromatographed on DEAE-cellulose under the conditions described in the legend to Fig. 2. Each fraction (2 ml) was assayed for total radioactivity and for total carbohydrate. F1 through F6, Peaks of six major oligosaccharide subfractions as described in the text (also see Fig. 2).

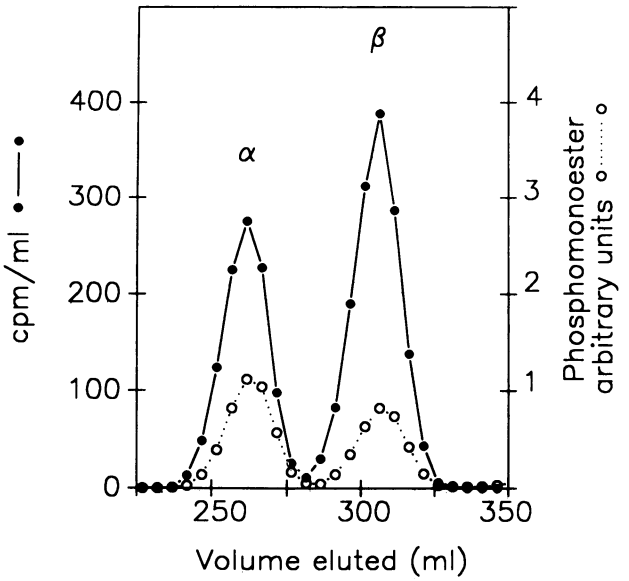

FIG. 7. Identification of [^{3}H]phosphoglycerol substituents on the cyclic β-1,2-glucans produced by *R. meliloti* 1021 during a pulse-chase experiment using [2-^{3}H]glycerol. ^{3}H-labeled cyclic β-1,2-glucans were prepared from *R. meliloti* 1021 as described in the legend to Fig. 5. A sample containing approximately 15,000 cpm of ^{3}H was desalted by chromatography on Sephadex G25. After desalting, 12.5 μmol each of α- and β-phosphoglycerol were added to the sample. The combined sample was then subjected to conditions of alkaline hydrolysis (0.5 M NaOH at 100°C for 80 min). The products of alkaline hydrolysis were chromatographed onto a column (1 by 51 cm) of Dowex-1-acetate (Bio-Rad Laboratories, Richmond, Calif.) by using the potassium acetate gradient system previously described (33). Fractions (5 ml) were collected and assayed for total radioactivity and for phosphomonoester as previously described (33). The figure indicates the region of the chromatogram in which α- and β-phosphoglycerol were eluted.

expected results, examination of the radiolabeled products of alkaline hydrolysis by chromatography on Dowex-1–acetate revealed two major products. The elution volumes for these two radiolabeled products were found to correspond precisely with the elution volumes of added α- and β-phosphoglycerol standards (Fig. 7). Thus, [^{3}H]phosphoglycerol was found to account for all of the radioactivity associated with the ^{3}H-cyclic β-1,2-glucan fraction generated in the pulse-chase experiments.

DISCUSSION

In the present study, we have demonstrated that the cyclic β-1,2-glucans of *R. meliloti* 1021 are highly substituted, anionic molecules containing on average 2.2 phosphodiester substituents and 1.4 organic ester substituents per molecule. In fact, only 7% of the total extractable cyclic β-1,2-glucan fraction from this organism was found to be neutral in character. In addition, it was determined that phosphoglycerol could account for 73% of the total phosphoryl moieties and that succinic acid could account for 36% of the total organic ester substituents.

The presence of phosphoglycerol substituents on the cyclic β-1,2-glucans of the *Rhizobiaceae* was first demonstrated in *A. tumefaciens* C58 (33) and in *Rhizobium* sp. strain NGR234 (4). From these studies, it was predicted that phosphatidylglycerol was the likely source of these substituents (33). This prediction was based on the stereochemistry of the phosphoglycerol substituent (*sn*-1) as well as on previous studies performed by Kennedy and co-workers (22, 23), in which phosphatidylglycerol was demonstrated to be the source of phosphoglycerol substituents present on the membrane-derived oligosaccharides of *E. coli*.

Using pulse-chase experiments in the present work, we have demonstrated that phosphatidylglycerol turnover is indeed linked to the biosynthesis of the glycerophosphorylated cyclic β-1,2-glucans of *R. meliloti* 1021. Furthermore, we have determined that over 60% of the total turnover of the membrane phospholipid of this organism is linked to the biosynthesis of these oligosaccharides. Thus, the similarity between the cyclic β-1,2-glucans of the *Rhizobiaceae* and the membrane-derived oligosaccharides of the enteric bacteria appears to be even more dramatic than originally noted (32, 33).

The presence of highly substituted forms of the cyclic β-1,2-glucans may have significant implications for the possible function of these molecules in the plant infection process. For example, it is generally believed that the structure of rhizobial exopolysaccharides may be a determining factor in host specificity (see references 10, 15, and 37 for a review). In this regard, it is noteworthy that mutants of *R. meliloti* 1021 defective for expolysaccharide succinylation were recently reported to form ineffective nodules on alfalfa (29). Thus, it should be considered that the degree of substitution of the cyclic β-1,2-glucans may provide a determinant for host specificity or effective nodulation. It is, perhaps, relevant that significant differences in the degree of substitution of cyclic β-1,2-glucans apparently occur among different species of the *Rhizobiaceae*. For example, the cyclic β-1,2-glucans of *A. tumefaciens* C58 have been found to either be present as neutral, unsubstituted glucans or to contain only phosphoglycerol substituents (33). In contrast, *Rhizobium trifolii* ANU843 appears to synthesize only neutral, unsubstituted cyclic β-1,2-glucans (4). Further studies have shown that certain strains of *Agrobacterium radiobacter*, *Rhizobium phaseoli*, and *Rhizobium trifolii* synthesize cyclic β-1,2-glucans containing methylmalonic or succinic acid substituents (21). The present study indicates that the cyclic β-1,2-glucans of *R. meliloti* 1021 represent the most highly substituted forms thus far reported.

To date, the strongest evidence for a role for the cyclic β-1,2-glucans in plant infection and legume nodulation comes from studies of mutants defective for the biosynthesis of these compounds (7, 12–14, 35, 42). Cyclic glucan-deficient mutants of *A. tumefaciens* have been found to be avirulent and attachment defective (7, 35). Cyclic glucan-deficient mutants of *R. meliloti* have been shown to form ineffective nodules (13, 14). However, in the above studies, the cyclic glucan-deficient mutants appear to be pleiotropic in character, with apparent defects in motility and other cell surface-associated phenotypes. From these studies and the present work, it is clear that additional mutants defective for cyclic β-1,2-glucan biosynthesis must be isolated in order to clarify further the roles of these glucans and their substituents in plant infection and nodulation.

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