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Cell-Associated Oligosaccharides of *Bradyrhizobium* spp.†

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We report the initial characterization of the cell-associated oligosaccharides produced by four *Bradyrhizobium* strains: *Bradyrhizobium japonicum* USDA 110, USDA 94, and ATCC 10324 and *Bradyrhizobium* sp. strain 32H1. The cell-associated oligosaccharides of these strains were found to be composed solely of glucose and were predominantly smaller than the cyclic beta-1,2-glucans produced by *Agrobacterium* and *Rhizobium* species. Linkage studies and nuclear magnetic resonance analyses demonstrated that the bradyrhizobial glucans are linked primarily by beta-1,6 and beta-1,3 glycosidic bonds. Thus, the bradyrhizobia appear to synthesize cell-associated oligosaccharides of structural character substantially different from that of the cyclic beta-1,2-glucans produced by *Agrobacterium* and *Rhizobium* species.

Bacterial genera in the family *Rhizobiaceae* are distinguished by their ability to infect higher plants. In the case of *Rhizobium* and *Bradyrhizobium* species, this infection process leads to a beneficial symbiotic relationship in which nitrogen-fixing nodules develop on the roots of leguminous plants. Plant infection by *Agrobacterium* species, however, results in the production of tumors on susceptible plant hosts. The cell surface carbohydrates of all three genera are believed to play important roles in the plant infection process. These cell surface carbohydrates include extracellular polysaccharides, capsular polysaccharides, lipopolysaccharides, and periplasmic glucans. Recent studies have demonstrated that *Agrobacterium* and *Rhizobium* species synthesize neutral and anionic periplasmic glucans of similar structure (2, 5, 16, 17, 20, 21, 23). In both genera, these periplasmic glucans are composed of a cyclic beta-1,2-glucan backbone containing 17 to 24 glucose residues. In *Agrobacterium tumefaciens*, approximately 50% of the total periplasmic cyclic beta-1,2-glucans are present as neutral, unsubstituted molecules (22). The remaining molecules are substituted with one or more phosphoglycerol moieties (23). In *Rhizobium meliloti*, as much as 90% of the periplasmic cyclic beta-1,2-glucans may be substituted with anionic moieties (21). As in the cyclic glucans of *A. tumefaciens*, the predominant anionic substituent present on the cyclic beta-1,2-glucans of *R. meliloti* 1021 is phosphoglycerol (21).

Recently, studies by Nester and co-workers (9, 26) and Geremia and co-workers (11) have provided evidence for a role for cyclic beta-1,2-glucans in the plant infection process. Specifically, cyclic beta-1,2-glucans have been implicated in the attachment of the bacterial cell to the plant host. Although there have been previous reports that at least two strains of *Bradyrhizobium japonicum* are capable of synthesizing neutral beta-1,2-linked glucans (1, 4), very little characterization of the cell-associated oligosaccharides of *Bradyrhizobium* species has been performed to date. We now report the results of our analyses of the cell-associated oligosaccharides of four *Bradyrhizobium* strains.

(A preliminary report of this work has appeared previously [K. J. Miller and R. S. Gore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, N-92, p. 300].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. japonicum* USDA 110 and USDA 94 were provided by R. F. Griffin of the Nitrogen Fixation and Soybean Genetics Laboratory, Agricultural Research Service, Beltsville, Md. *B. japonicum* ATCC 10324 and *Bradyrhizobium* sp. strain 32H1 (ATCC 33848) were obtained from the American Type Culture Collection, Rockville, Md. *R. meliloti* 1021 was provided by F. M. Ausubel, Harvard Medical School, Boston, Mass., and *A. tumefaciens* C58 was provided by W. S. York, University of Colorado, Boulder. Six-liter cultures of each strain were grown in YM medium (0.4 g of yeast extract, 10 g of mannitol, 0.1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of K_2HPO_4 per liter [pH 7]) at 30°C on a rotary shaker.

Extraction of cell-associated oligosaccharides. Cells were harvested during logarithmic growth at a density of approximately 50 μg of total cell protein per ml. Pellets were washed once with YM salts (0.1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of K_2HPO_4 per liter [pH 7]), and cell-associated oligosaccharides were extracted into a methanol-water phase by a modified Bligh and Dyer extraction procedure as described previously (21).

Column chromatographic analysis of cell-associated oligosaccharides. Aqueous methanol extracts were concentrated to dryness under nitrogen at 37°C and analyzed by gel filtration chromatography on a Sephadex G-50 column (Pharmacia, Inc., Piscataway, N.J.) as described in the legend to Fig. 1. Fractions containing oligosaccharides (eluting in the volume between 25 and 50 ml) were pooled, concentrated, and desalted on a column (1 by 56 cm) of Sephadex G-15 (Pharmacia) with 7% (vol/vol) propanol as the eluant. The desalted samples were then analyzed on a column (1 by 23 cm) of DEAE-cellulose (DE52; Whatman, Inc., Hillsboro, Oreg.). After application of each sample to DEAE-cellulose, the column was washed with 40 ml of 10 mM Tris hydrochloride (pH 8.4) containing 7% (vol/vol) 1-propanol. Next, a 100-ml gradient was applied, beginning with 0 mM KCl and ending with 300 mM KCl in the same buffer.

Compositional analysis of cell-associated oligosaccharides. Oligosaccharide preparations were subjected to a variety of

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chemical analyses. Total carbohydrate was measured by the phenol method (14). Glucose content was measured by the glucose oxidase method (Sigma Chemical Co., St. Louis, Mo.) after hydrolysis for 4 h in 1.0 M HCl at 100°C. Galactose content was measured with galactose oxidase after hydrolysis for 4 h in 1.0 M HCl at 100°C (23). Total phosphorus was measured as P_i after digestion with magnesium nitrate (21). Succinate was measured by the succinate thiokinase method (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) after samples were treated with 0.1 M NaOH for 30 min at 37°C (21). Reducing sugars were measured by the Nelson-Somogyi method (29). Total monosaccharide compositional analysis of oligosaccharides derived from *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 was performed by gas-liquid chromatography after methanolysis, acetylation, and silylation reactions (3).

FABMS. Mass spectra of oligosaccharide samples derived from *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 were recorded on a VG-ZAB-SE double-focusing instrument (VG Analytical Ltd., Manchester, United Kingdom) under conditions similar to those described previously (23). For some analyses, samples were treated with sodium borohydride before examination by fast-atom-bombardment mass spectrometry (FABMS). In these experiments, 500-μg (glucose equivalent) samples were incubated with 5 mg of sodium borohydride for 30 min at room temperature. After incubation, excess sodium borohydride was destroyed by the addition of glacial acetic acid. The samples were then desalted by chromatography on Sephadex G-15 and subsequently analyzed by FABMS.

NMR analysis. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 25°C on an AM-500 spectrometer (Bruker Instruments Inc., Billerica, Mass.) operating at 125.76 MHz. The samples were dissolved in D_2O at concentrations of approximately 10 mg/ml in 5-mm NMR tubes. 1,4-Dioxane was used as an internal reference (67.6 ppm relative to tetramethylsilane). Selected samples were referenced without internal dioxane because the reference frequency proved to be sample independent. Waltz 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.

Glycosidic-linkage analysis. A glycosidic-linkage analysis was performed on the cell-associated oligosaccharides of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1. Samples were permethylated, subjected to acid hydrolysis, acetylated, and subsequently analyzed by gas chromatography (15).

SFC. Supercritical fluid chromatography (SFC) was carried out with a model 501b chromatograph (Lee Scientific, Inc., Salt Lake City, Utah) with flame ionization detection. Permethylated cyclic glucans were injected onto a cyanopropyl column (Lee Scientific) with a pneumatically actuated submicroliter internal loop injection valve (Valco Instrument Co., Houston, Tex.). SFC-grade carbon dioxide (Scott Gases, Plumsteadville, Pa.) was used as the mobile phase. The pressure was held at 200 atm (1 atm = 101.29 kPa) for 10 min and then linearly increased to 415 atm at a rate of 3 atm/min. The oven temperature was initially held at 120°C for 20 min and was then increased to 180°C at a rate of 1°C/min. The flow restrictor has been described previously (12).

SFC-mass spectrometry. The design and operating conditions for interfacing a capillary SFC and a magnetic-sector mass spectrometer have been described previously (27). Ammonia was used as the reagent gas.

RESULTS

Cell-associated oligosaccharides of *Bradyrhizobium* spp. are neutral glucans smaller than the cyclic beta-1,2-glucans of *Rhizobium* and *Agrobacterium* spp. The examination of aqueous methanol cell extracts by chromatography on Sephadex G-50 revealed the presence of cell-associated oligosaccharides in all four *Bradyrhizobium* strains (Fig. 1). Although the levels of the cell-associated oligosaccharides of the bradyrhizobia were similar to those produced by *R. meliloti* 1021 and *A. tumefaciens* C58 (Fig. 1; Table 1), the elution volumes on Sephadex G-50 indicated that the bradyrhizobial oligosaccharides were predominantly smaller than the cyclic beta-1,2-glucans. Also, the amounts of cell-associated oligosaccharide extracted from cultures of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 were found to be more variable than the amounts extracted from cultures of *A. tumefaciens* C58 (Table 1). Because the amount of cell-associated oligosaccharide is expressed relative to the wet weight of cellular pellets, it is likely that the range of values observed resulted from the variable yet significant amounts of capsular material associated with the bradyrhizobial cellular pellets. Significant quantities of capsular material were not observed in cellular pellets of *A. tumefaciens* C58 or *R. meliloti* 1021.

A second major difference between the cell-associated oligosaccharides of *Bradyrhizobium* spp. and the cyclic beta-1,2-glucans of *Rhizobium* and *Agrobacterium* spp. was revealed after further fractionation of these compounds on DEAE-cellulose. The cell-associated oligosaccharides of all four *Bradyrhizobium* strains were found to elute in the void volume (data not shown), which is indicative of an unsubstituted, neutral character. This is in contrast to the highly anionic character of the cyclic beta-1,2-glucans produced by *R. meliloti* 1021 (21) and *A. tumefaciens* C58 (22, 23).

A compositional analysis of the cell-associated oligosaccharides of the bradyrhizobial strains was performed, and it was determined that glucose could account for all of the carbohydrate present in the oligosaccharides derived from all four strains. A more detailed analysis of the cell-associated oligosaccharides of *B. japonicum* USDA 110 revealed an absence of detectable reducing sugars within the preparation. Further analysis of this sample demonstrated the presence of low levels of succinic acid and phosphorus (0.01 and 0.03 mol of succinic acid and phosphorus, respectively, per mol of glucose). Gas chromatographic analysis of the oligosaccharide preparations derived from *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 after methanolysis, re-N-acetylation, and silylation reactions confirmed that glucose was the only monosaccharide present in both preparations. It should be noted that no mannose, galactose, or rhamnose was detected in these oligosaccharide preparations, indicating the absence of contaminating extracellular polysaccharide material (6, 7, 18, 19, 24, 25).

The molecular weight distribution of the glucans of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 was determined by negative-ion FABMS as described in Materials and Methods. This analysis revealed the presence of two major deprotonated molecules, $[M - H]^-$, with masses of 1,781 and 1,943 daltons in both glucan preparations (Fig. 2). These molecular ion species are 18 daltons lower in mass than would be expected for unsubstituted, linear glucans composed of 11 and 12 glucose residues,

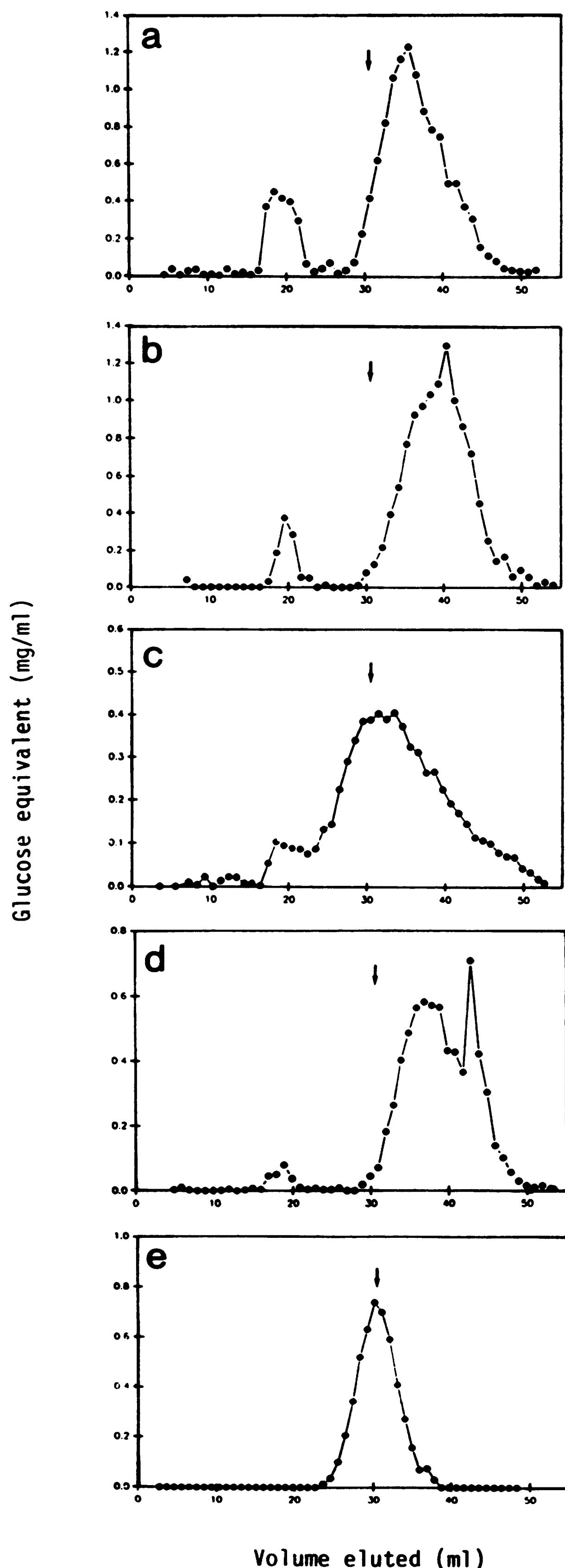

FIG. 1. Oligosaccharide analysis on Sephadex G-50. Aqueous methanol extracts derived from 6-liter cultures of four *Bradyrhizobium* strains and *R. meliloti* 1021 were concentrated and applied to a column (1 by 56 cm) of Sephadex G-50. The column was eluted at room temperature at a rate of 15 ml/h with 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 (14). The arrow indicates the position expected for cyclic beta-1,2-glucans as determined by calibration with a purified standard derived from *A. tumefaciens* C58. Results are expressed as milligrams of equivalent glucose per milliliter of eluant and are normalized per gram (wet weight) of cells. (a) *B. japonicum* USDA 110; (b) *Bradyrhizobium* sp. strain 32H1; (c) *B. japonicum* USDA 94; (d) *B. japonicum* ATCC 10324; (e) *R. meliloti* 1021.

TABLE 1. Cell-associated oligosaccharide content of *Bradyrhizobium* spp., *A. tumefaciens*, and *R. meliloti*

Strain	Oligosaccharide content (mg/g [wet wt])[a]
B. japonicum USDA 110	11.4[b]
Bradyrhizobium sp. strain 32H1 (ATCC 33848)	11.4[c]
B. japonicum USDA 94	6.1
B. japonicum ATCC 10324	6.6
R. meliloti 1021	4.9
A. tumefaciens C58	8.9[c]

[a] Aqueous methanol cell extracts were applied to a column of Sephadex G-50 as described in the legend to Fig. 1. Oligosaccharides represent the total carbohydrate eluting from the column in the volume between 25 and 50 ml. Results are expressed as milligrams of equivalent glucose normalized per gram (wet weight) of cells.

[b] Average of determinations from three different culture preparations. Range, 5.0 to 23.1 mg/g (wet weight) of cells.

[c] Average of determinations from two different culture preparations. Ranges, 5.6 to 17.1 mg/g (wet weight) of cells for *Bradyrhizobium* sp. strain 32H1 and 7.8 to 9.9 mg/g (wet weight) of cells for *A. tumefaciens* C58.

respectively. As discussed below, the most likely explanation for these 18-mass-unit discrepancies is that these glucans are cyclic. In addition to the two major ions, there are two less abundant deprotonated molecular ions with masses of 1,619 and 2,105 daltons, which correspond to those expected for cyclic glucans composed of 10 and 13 glucose residues, respectively. Thus, an envelope of cyclic glucans of composition Glc_n, where $n = 10$ to 13, is observed.

The detection of two major and two minor species by negative-ion FABMS is consistent with the results obtained by SFC with flame ionization detection and by SFC coupled with mass spectrometry. The SFC chromatograms of the permethylated glucans from both strains contained two minor peaks and two major peaks (Fig. 3). The molecular weights of these four components were determined by SFC-mass spectrometry with ammonia as the chemical ionization reagent gas. The masses of the observed molecular ion species, $[M + NH_4]^+$, corresponded to those expected for permethylated cyclic glucans composed of 10 to 13 glucose residues (data not shown).

Glucans of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 are linked primarily by beta-1,6 and beta-1,3 glycosidic bonds. ^{13}C-NMR analysis of the cell-associated glucans of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 revealed the presence of beta glycosidic linkages and the apparent absence of alpha glycosidic linkages in these preparations. The NMR spectra of the bradyrhizobial glucans, however, were substantially different from the spectrum obtained for the neutral cyclic beta-1,2-glucan standard. The NMR spectra of both bradyrhizobial glucan

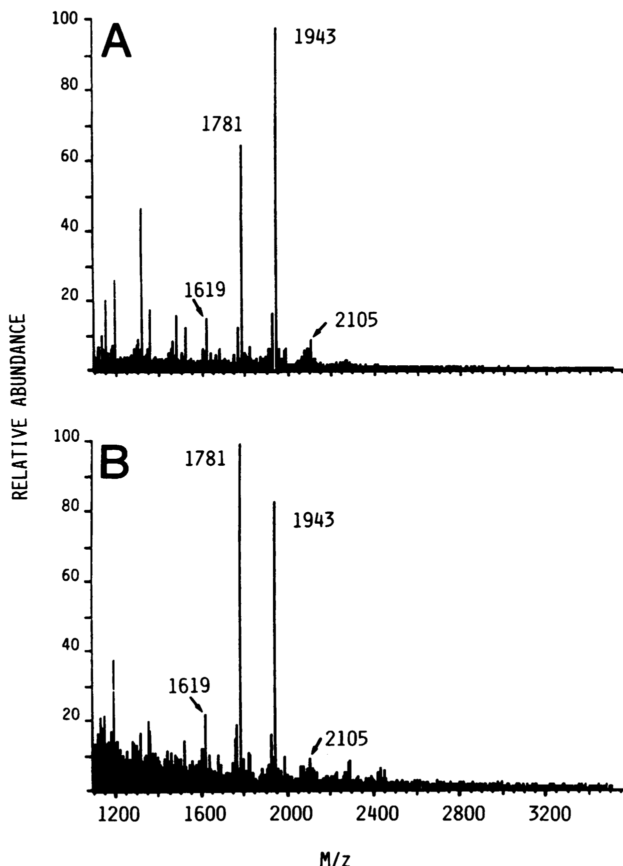

FIG. 2. Negative-ion FABMS of cell-associated oligosaccharides of *B. japonicum* USDA 110 (A) and *Bradyrhizobium* sp. strain 32H1 (B). The mass spectra were recorded as described in Materials and Methods. The *m/z* values are reported as the nominal masses of the deprotonated molecules, $[M - H]^-$.

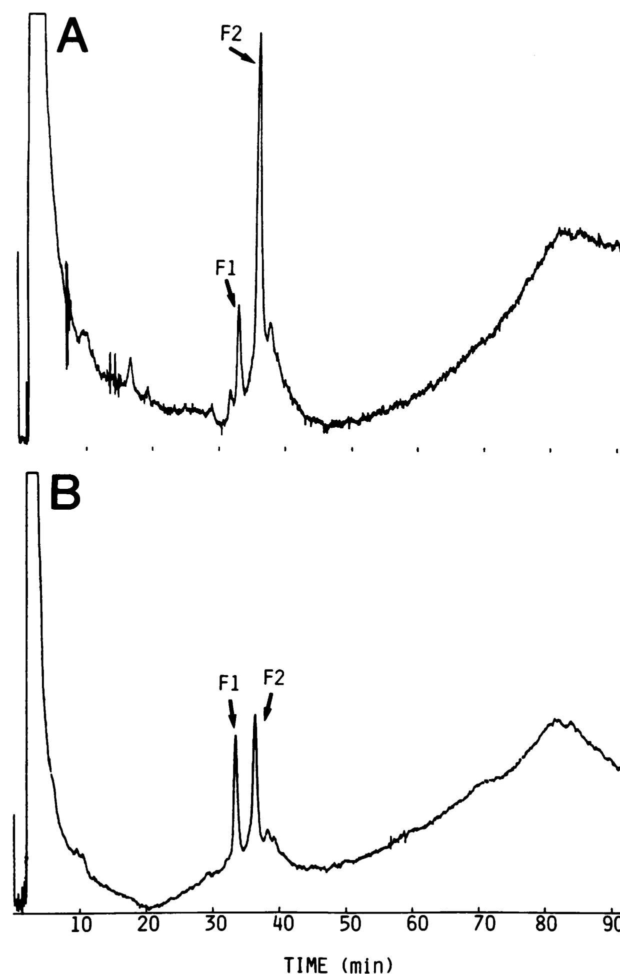

FIG. 3. SFC analysis. Cell-associated oligosaccharides from *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 were examined by SFC as described in Materials and Methods. (A) *B. japonicum* USDA 110; (B) *Bradyrhizobium* sp. strain 32H1. F1 and F2 mark the peaks of the two major components present in both preparations.

preparations contained C-1 resonance peaks clustered near 104 ppm (Fig. 4A and B), while those of the cyclic beta-1,2-glucan standard clustered near 103 ppm (Fig. 4C). Both shifts are indicative of beta glycosidic linkages (30). The NMR spectra of the bradyrhizobial glucans displayed a higher degree of complexity near 70 ppm than did the tightly clustered set of C-4 resonances observed for the neutral cyclic beta-1,2-glucan standard. The additional peaks near 70 ppm are indicative of the presence of beta-1,6 linkages and represent the resonances for both C-4 and C-6 (beta-1,6) carbons (30). A further difference between the NMR spectra of the bradyrhizobial glucans and that of the neutral cyclic beta-1,2-glucan standard occurs near 85 ppm. While the NMR spectrum of the cyclic beta-1,2-glucan standard contains resonances near 83 ppm, the NMR spectra of the bradyrhizobial glucans contain resonances shifted further downfield near 85 ppm. The resonances near 83 ppm can be assigned to C-2 in beta-1,2 glycosidic linkages (30), while those near 85 ppm can be assigned to C-3 in beta-1,3 glycosidic linkages (30). Further evidence that beta-1,2 glycosidic linkages are minor or absent in the bradyrhizobial glucan preparations is derived from the presence of major peaks at approximately 74 ppm. Peaks near 74 ppm are indicative of C-2 carbons not involved in glycosidic linkages (5, 30) and are clearly absent in the NMR spectrum of the cyclic beta-1,2-glucan standard (Fig. 4C).

For both of the bradyrhizobial glucans and the neutral cyclic glucan standard, the resonances near 77.5 and 76.5 ppm may be assigned to C-5 and C-3 carbons, respectively, that are not involved in glycosidic linkages (21). These assignments were confirmed by a CH-correlated two-dimensional NMR experiment with the neutral cyclic beta-1,2-glucan standard.

Several unassignable resonances are present in the spectra of the bradyrhizobial glucans. A major resonance at 55 ppm is present in both bradyrhizobial spectra, although it is much more intense in the spectrum of *Bradyrhizobium* sp. strain 32H1 (Fig. 4B). The chemical shift of this resonance is outside the range of shifts expected for glucan carbons; therefore, it may represent a side group or a contaminant in the preparation. Additional unassignable resonances present within the ^{13}C-NMR spectrum of *Bradyrhizobium* sp. strain 32H1 include those at 57.5, 60.5, 64, and 67 ppm. The intense, narrow resonance at 64 ppm has a considerably shorter correlation time than that of the glucan carbons and may possibly represent a substituent carbon. Alternatively, it is possible that this resonance (as well as the other unassignable resonances) results from a contaminant within the preparation. It is interesting to note that a small reso-

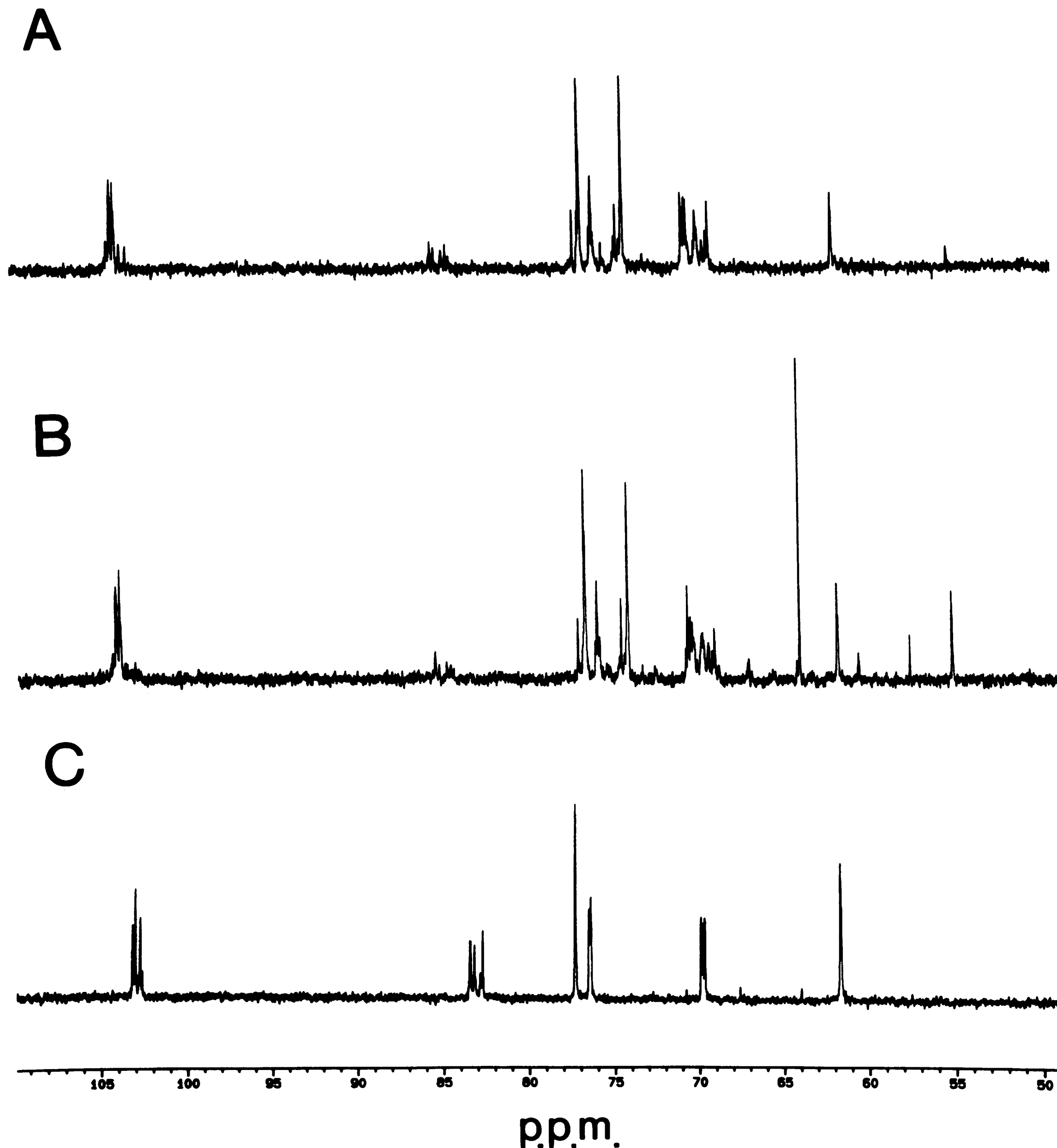

FIG. 4. ^{13}C-NMR analysis of cell-associated oligosaccharides. NMR spectra were recorded as described in Materials and Methods. (A) *B. japonicum* USDA 110; (B) *Bradyrhizobium* sp. strain 32H1; (C) neutral cyclic beta-1,2-glucan standard prepared from *A. tumefaciens* C58.

nance, also at 64 ppm, is present in the spectrum of the neutral cyclic beta-1,2-glucan standard.

Characteristic of the NMR spectra of all three glucan preparations is the presence of irregular multiple resonances for all carbons (except for that of nonglycosidic C-6 near 62 ppm). The presence of multiplets in the ^{13}C-NMR spectrum for the neutral cyclic beta-1,2-glucan standard has previously been observed by Dell and co-workers (5), who concluded that multiple resonances result from different torsion angles between adjacent glucosyl residues in cyclic glucan molecules of various sizes (e.g., 17 to 24 glucose residues). It is possible that similar differences in torsion angles are present in the bradyrhizobial glucans.

As described above, the ^{13}C-NMR spectra of the glucans of both *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 contain features indicative of the presence of beta-1,6 and beta-1,3 glycosidic linkages. The presence of 1,6 and 1,3 glycosidic linkages within these preparations was confirmed by gas chromatographic analysis after permethylation, acid hydrolysis, and acetylation. Both glucan preparations were composed almost entirely of glucose linked by 1,6 (58.9 and 61.8% for *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1, respectively, as determined from peak areas in the gas chromatographic profile) and 1,3 (35.0 and 38.2% for *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1, respectively) glycosidic bonds. It should be noted, however, that minor amounts of 1,2 glycosidic linkages (6.1%) were also detected in the glucan preparation extracted from *B. japonicum* USDA 110.

Oligosaccharides of *B. japonicum* USDA 110 and *Brady-*

rhizobium sp. strain 32H1 are cyclic glucans. The FABMS and SFC-mass spectrometry analyses described above have provided strong evidence that the glucans of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 are cyclic in structure. As described above, the molecular ion distributions of both nonderivatized and permethylated oligosaccharide preparations corresponded to those of cyclic glucans composed of 10 to 13 glucose residues. Indeed, it may be noted that FABMS analysis has previously provided critical evidence for the cyclic structure of the beta-1,2-glucans of *Rhizobium* and *Agrobacterium* spp. (5).

Consistent with a cyclic structure for these molecules is the fact that no reducing-sugar residues were detected in the oligosaccharide preparation derived from *B. japonicum* USDA 110. Additional evidence for the absence of reducing sugars within these preparations was obtained from NMR analysis. There was an absence of resonances at 92 to 96 ppm in both bradyrhizobial glucan preparations (Fig. 4). Signals in this region have been shown to correspond to the C-1 resonances of reducing-glucose residues (5).

Further evidence for a cyclic structure was obtained by FABMS analysis after treatment of the bradyrhizobial oligosaccharides with sodium borohydride. The molecular ion distributions of the cell-associated oligosaccharides of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 after treatment with sodium borohydride were indistinguishable from those obtained from untreated preparations (data not shown). Had there been reducing sugars present within these oligosaccharide preparations, the molecular ion distributions would have been shifted by 2 mass units.

Although an absence of nonreducing terminal glucose residues would provide additional evidence for a cyclic structure for the bradyrhizobial oligosaccharides, such an absence would be possible only if there were no branches present in these molecules. Thus, a cyclic, branched glucan should contain nonreducing terminal glucose residues but should not contain reducing terminal glucose residues. Preliminary results indicate that there are branches within the bradyrhizobial oligosaccharides because nonreducing terminal glucose residues were detected (data not shown). Therefore, the cell-associated glucans of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 appear to be cyclic, branched molecules.

DISCUSSION

The present study demonstrates that the structure of the cell-associated oligosaccharides of *Bradyrhizobium* spp. is fundamentally different from that of the cyclic beta-1,2-glucans of *Agrobacterium* and *Rhizobium* spp. Although the bradyrhizobial glucans also appear to be cyclic in structure, these molecules are significantly smaller than the cyclic beta-1,2-glucans and are composed principally of beta-1,6 and beta-1,3 glycosidic linkages. Furthermore, the bradyrhizobial glucans appear to be unsubstituted and neutral in character, whereas the glucans of *Agrobacterium* and *Rhizobium* spp. are highly anionic (21–23).

It is possible that the cell-associated bradyrhizobial glucans described in the present study are structurally related to the extracellular glucans of *B. japonicum* 3I1b71a that have been previously described (8). Like the cell-associated glucans of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1, the extracellular glucans of *B. japonicum* 3I1b71a are composed of beta-1,3 and beta-1,6 glycosidic linkages. However, it should be noted that the extracellular glucans of strain 3I1b71a appear to be larger (molecular weight determined to be approximately 4,500 by gel filtration chromatography) than the glucans described here (8).

The apparent lack of cyclic beta-1,2-glucans in cell extracts of *Bradyrhizobium* spp. is of interest in view of the widespread occurrence of these molecules among *Agrobacterium* and *Rhizobium* species (2, 5, 16, 17, 20, 21, 23). However, the possibility that the cell-associated glucans of *Bradyrhizobium* spp. play a role in plant infection similar to that played by the periplasmic cyclic beta-1,2-glucans should be considered. Thus, it is of interest that the bradyrhizobial glucans also appear to be cyclic in character. Perhaps the cyclic nature of these cell-associated glucans is related to their function during plant infection. In this regard, it is possible that a cyclic glucan is more resistant than a linear glucan to enzymatic cleavage. Such increased resistance to enzymatic cleavage has been noted for other cyclic oligosaccharides (10). Thus, a cyclic glucan may persist in the plant infection environment for a longer period than the corresponding linear glucan of similar size. This may be of particular significance in view of the signaling role that oligosaccharides are believed to play in plant development and defense (13, 28).

The glycosidic linkage composition of the bradyrhizobial glucans represents an additional consideration relevant to their possible roles as signaling molecules in the nodulation process. It is, therefore, particularly intriguing that these glucans appear to share structural similarities with the beta-glucan phytoalexin elicitors synthesized by fungal plant pathogens (see reference 13 for a review).

The results of the present study provide evidence that *Bradyrhizobium* species synthesize cyclic, branched glucans composed of 10 to 13 glucose residues linked by beta-1,6 and beta-1,3 glycosidic bonds. Additional studies in progress are aimed at further elucidating the structure and cellular location of these unusual glucans.

ACKNOWLEDGMENT

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ADDENDUM IN PROOF

Recently, Rolin and co-workers (D. B. Rolin, P. E. Pfeffer, S. F. Osman, B. S. Swergold, and F. Kappler, Abstr. 12th North Am. Symbiotic Nitrogen Fixation Conf., 1989, P-51, p. 113) have described a choline phosphate-substituted glucan isolated from *B. japonicum* USDA 110. This substituted glucan was shown to contain beta-1,3 and beta-1,6 glycosidic linkages; therefore, it is possible that this substituted glucan is similar in structure to the glucans described in the present study.

LITERATURE CITED

1. **Amemura, A., M. Hisamatsu, H. Mitani, and T. Harada.** 1983. Cyclic (1-2)-beta-D-glucan and the octasaccharide repeating units of extracellular acidic polysaccharides produced by *Rhizobium*. Carbohydr. Res. **114**:277–285.
2. **Batley, M., J. W. Redmond, S. P. Djordjevic, and B. G. Rolfe.** 1987. Characterization of glycerophosphorylated cyclic beta-1,2-glucans from a fast-growing *Rhizobium* species. Biochim. Biophys. Acta **901**:119–126.
3. **Chaplin, M. F.** 1986. Monosaccharides, p. 1–36. *In* M. F. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis: a practical approach. IRL Press, Inc., Washington, D.C.
4. **Dedonder, R. A., and W. Z. Hassid.** 1964. The enzymatic synthesis of a (beta-1,2)-linked glucan by an extract of *Rhizobium japonicum*. Biochim. Biophys. Acta **90**:239–248.

5. **Dell, A., W. S. York, M. McNeil, A. G. Darvill, and P. Albersheim.** 1983. The cyclic structure of beta-D-(1-2)-linked D-glucans secreted by *Rhizobia* and *Agrobacteria*. Carbohydr. Res. **117**:185–200.
6. **Dudman, W. F.** 1976. The extracellular polysaccharides of *Rhizobium japonicum*: compositional studies. Carbohydr. Res. **46**:97–110.
7. **Dudman, W. F.** 1978. Structural studies of the extracellular polysaccharides of *Rhizobium japonicum* strains 71A, CC708, and CB1795. Carbohydr. Res. **66**:9–23.
8. **Dudman, W. F., and A. J. Jones.** 1980. The extracellular glucans of *Rhizobium japonicum* strain 3I1b71a. Carbohydr. Res. **84**: 358–364.
9. **Dylan, I., L. Ielpi, S. Stanfield, L. Kashyap, C. Douglas, M. Yanofsky, E. Nester, D. R. Helinski, and G. Ditta.** 1986. *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA **83**:4403–4407.
10. **French, D.** 1957. The Schardinger dextrins. Adv. Carbohydr. Chem. **12**:189–260.
11. **Geremia, R. A., S. Carvaignac, A. Zorreguieta, N. Toro, J. Olivares, and R. A. Ugalde.** 1987. A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β-(1→2) glucan. J. Bacteriol. **169**:880–884.
12. **Guthrie, E. J., and H. E. Schwartz.** 1986. Integral pressure restrictor for capillary SFC. J. Chromatogr. Sci. **24**:236–240.
13. **Halverson, L. J., and G. Stacey.** 1986. Signal exchange in plant-microbe interactions. Microbiol. Rev. **50**:192–225.
14. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
15. **Harris, P. J., R. J. Henry, A. B. Blakeney, and B. A. Stone.** 1984. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. Carbohydr. Res. **127**:59–73.
16. **Hisamatsu, M., A. Amemura, K. Koizumi, T. Utamura, and Y. Okada.** 1983. Structural studies on cyclic (1-2)-beta-D-glucans (cyclosophoraose) produced by *Agrobacterium* and *Rhizobium*. Carbohydr. Res. **121**:31–40.
17. **Hisamatsu, T., T. Yamada, T. Higashiura, and M. Ikeda.** 1987. The production of acidic, O-acylated cyclosophorans [cyclic (1-2)-beta-D-glucans] by *Agrobacterium* and *Rhizobium* species. Carbohydr. Res. **163**:115–122.
18. **Hollingsworth, R., E. Smith, and M. H. Ahmad.** 1985. Chemical composition of extracellular polysaccharides of cowpea rhizobia. Arch. Microbiol. **142**:18–20.
19. **Kennedy, L. D., and R. W. Bailey.** 1976. Monomethyl sugars in extracellular polysaccharides from slow-growing *Rhizobia*. Carbohydr. Res. **49**:451–454.
20. **Koizumi, K., Y. Okada, S. Horiyama, and T. Utamura.** 1983. Separation of cyclic (1-2)-beta-D-glucans (cyclosophoraose) produced by agrobacteria and rhizobia, and determination of the degree of polymerization by high-performance liquid chromatography. J. Chromatogr. **265**:89–96.
21. **Miller, K. J., R. S. Gore, and A. J. Benesi.** 1988. Phosphoglycerol substituents present on the cyclic beta-1,2-glucans of *Rhizobium meliloti* 1021 are derived from phosphatidylglycerol. J. Bacteriol. **170**:4569–4575.
22. **Miller, K. J., E. P. Kennedy, and V. N. Reinhold.** 1986. Osmotic adaptation by gram-negative bacteria: possible role for periplasmic oligosaccharides. Science **231**:48–51.
23. **Miller, K. J., V. N. Reinhold, A. C. Weissborn, and E. P. Kennedy.** 1987. Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with *sn*-1-phosphoglycerol residues. Biochim. Biophys. Acta **901**:112–118.
24. **Mort, A. J., and W. D. Bauer.** 1980. Composition of the capsular and extracellular polysaccharides of *Rhizobium japonicum*. Plant Physiol. **66**:158–163.
25. **Mort, A. J., and W. D. Bauer.** 1982. Application of two new methods for cleavage of polysaccharides into specific oligosaccharide fragments. J. Biol. Chem. **257**:1870–1875.
26. **Puvanesarajah, V., F. M. Schell, G. Stacey, C. J. Douglas, and E. W. Nester.** 1985. Role for 2-linked-β-D-glucan in the virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **164**:102–106.
27. **Reinhold, V. N., D. M. Sheeley, J. Kuei, and G.-R. Her.** 1988. Analysis of high molecular weight samples on a double-focusing magnetic sector instrument by supercritical fluid chromatography/mass spectrometry. Anal. Chem. **60**:2719–2722.
28. **Ryan, C. A.** 1987. Oligosaccharide signalling in plants. Annu. Rev. Cell Biol. **3**:295–317.
29. **Spiro, R. G.** 1966. Analysis of sugars found in glycoproteins. Methods Enzymol. **8**:3–25.
30. **Usui, T., N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto.** 1973. ^{13}C nuclear magnetic resonance spectra of glucobioses, glucotrioses, and glucans. J. Chem. Soc. Perkin Trans. I. **1**:2425–2432.