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# Cyclic β-1,6 -1,3 Glucans Are Synthesized by *Bradyrhizobium japonicum* Bacteroids within Soybean (*Glycine max*) Root Nodules[1]

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**We have previously reported that free-living cultures of *Bradyrhizobium* species produce novel oligosaccharides that are cyclic, contain between 10 and 13 glucose residues, and are linked by β-1,6 and β-1,3 glycosidic bonds (K.J. Miller, R.S. Gore, R. Johnson, A.J. Benesi, V.N. Reinhold [1990] J Bacteriol 172: 136–142). In the present study, we show that these glucans are also synthesized by bacteroids of *Bradyrhizobium japonicum* USDA 110 within *Glycine max* root nodules.**

The cell-surface carbohydrates of bacteria within the Rhizobiaceae family provide important functions during legume nodulation. Such functions are most clearly evident for the rhizobial lipooligosaccharides, which have been shown to elicit root hair deformation, cortical cell division, and nodule organogenesis (refs. in Fisher and Long, 1992; Franssen et al., 1992). Genetic studies have also provided evidence that a second class of rhizobial cell-surface carbohydrate, the exopolysaccharides, is required for nodule development in plants that form indeterminate nodules (refs. in Leigh and Coplin, 1992). Recent studies in our laboratory have focused on a third class of rhizobial-cell-surface carbohydrate, the cyclic β-glucans.

All members of the Rhizobiaceae family examined to date have been found to synthesize periplasmic cyclic β-glucans. The cyclic β-glucans of species of *Agrobacterium* and *Rhizobium* are composed of 17 to 24 Glc residues linked solely by β-1,2 glycosidic bonds. The synthesis of the cyclic β-1,2-glucans is osmotically regulated, and it appears that these molecules provide important functions for the free-living bacterium during hypoosmotic adaptation (Cangelosi et al., 1990; Dylan et al., 1990a). A study by Dylan and co-workers (Dylan et al., 1990b) also indicates that the cyclic β-1,2-glucans may act as signal molecules during legume nodulation.

Although species of *Bradyrhizobium* also synthesize periplasmic cyclic β-glucans, the structure of the bradyrhizobial glucans is different from that of the cyclic β-1,2-glucans. Specifically, the bradyrhizobial glucans are composed of between 10 to 13 Glc residues linked by both β-1,6 and β-1,3 glycosidic bonds (Miller et al., 1990). It is interesting that the biosynthesis of the bradyrhizobial glucans is also osmotically regulated, and we have previously proposed that the bradyrhizobial cyclic β-glucans may represent functional analogs of the cyclic β-1,2-glucans of *Rhizobium* (Miller and Gore, 1992).

Recently, we have reported that microaerobic, nitrogenase-active, continuous cultures of *Bradyrhizobium* sp. strain 32H1 synthesize high levels of cell-associated cyclic β-1,6 -1,3 glucans (Gore and Miller, 1992). Based on this result, we have speculated that bradyrhizobial bacteroids may continue to synthesize cyclic β-1,6 -1,3 glucans within the microaerobic environment of the root nodule. In the present study, we show that *Bradyrhizobium japonicum* USDA 110 bacteroids derived from mature soybean root nodules contain cyclic β-1,6 -1,3 glucans at levels similar to those previously found within free-living cultures of this bacterium.

## MATERIALS AND METHODS

### Bacterial Strain

*Bradyrhizobium japonicum* USDA 110 has been described previously (Miller et al., 1990). Cultures were maintained on YM medium (Miller et al., 1990) containing 1.5% (w/v) Bacto-Agar (Difco Laboratories, Detroit, MI).

### Growth and Inoculation of Soybeans

Soybean seeds (*Glycine max* cv 3385) were provided by F. Leply of the Hoffman Seed Co. (Landisville, PA). Soybean seeds were surface sterilized by soaking in bleach for 10 min. Seeds were then rinsed three times with sterile deionized water, soaked in sterile 0.01 N HCl for 10 min, and rinsed three additional times with sterile deionized water.

Surface-sterilized seeds were placed onto YM medium containing 0.75% (w/v) Bacto-Agar and were allowed to germinate in the dark for 3 d at 30°C. Germinated seedlings with roots 2 to 3 inches long were inoculated with 1 mL of a mid-logarithmic culture of *B. japonicum* USDA 110 (approximately $10^8$ cells). After 1 h of incubation at 30°C in the presence of the bacterial culture, seedlings were planted in sterile sand (3.0 flintshot silica 28–30 mesh; MDC Industries, Philadelphia, PA) within opaque plastic containers. Plants

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Abbreviation: FABMS, fast-atom-bombardment mass spectrometry.

were grown in a greenhouse at ambient temperature and watered with a nutrient solution (Stacey et al., 1980) at the time of inoculation and after 4 weeks of growth. In addition, plants were watered (on average) every other day with tap water.

### Nitrogenase Assay

Nitrogenase activity within isolated root nodules was measured by the acetylene reduction assay previously described (Gore and Miller, 1992).

### Bacteroid Isolation

Plants were harvested between 6 and 9 weeks after inoculation, and nodules were removed by twisting. Bacteroid isolation was based on the method described by Reibach and co-workers (Reibach et al., 1981). Nodules were placed into a chilled mortar containing buffer A (0.15 M NaCl, 50 mM $KH_2PO_4$, pH 7.6) at 5°C. Five milliliters of buffer A were added per g of fresh nodule. Nodules were gently ground using a pestle. The crude material was filtered through four layers of cheesecloth, and the nodule paste was reground to isolate additional bacteroids. The crude filtrate was centrifuged at 6000*g* for 15 min at 5°C. The pellet was resuspended in 0.8 mL of buffer A per g of original nodule mass. Self-generating Percoll gradients were made by mixing 24.5 mL of Percoll (Sigma) with 7.0 mL of deionized water and 3.5 mL of buffer B (0.5 M $KH_2PO_4$, 1.5 M NaCl [pH 7.6]). Four milliliters of the resuspended crude extract were gently layered on top of the Percoll solution. The extract and Percoll mixture were centrifuged at 43,000*g* for 50 min at 5°C.

Bacteroids were removed from the gradient using a 60-mL syringe and a 16-gauge needle and were examined by phase contrast microscopy. Bacteroids were washed free of Percoll by dilution with 5 volumes of buffer A, followed by centrifugation at 10,000*g* for 15 min at 5°C. Pellets were washed one additional time using buffer A, and the final bacteroid pellets were frozen at −20°C until the time of methanol extraction (see below).

### Analysis of Bacteroid-Associated Oligosaccharides

Oligosaccharides were extracted from washed bacteroid preparations into a methanol-water phase by a modified Bligh and Dyer extraction procedure (Miller et al., 1990). Aqueous methanol extracts were concentrated to dryness under nitrogen at 37°C and analyzed by gel filtration chromatography and ion-exchange chromatography as previously described (Miller et al., 1990). NMR analysis and FABMS were performed as previously described (Miller et al., 1990; Gore and Miller, 1992). Total carbohydrate content and Glc content were measured as previously described (Miller et al., 1990).

## RESULTS AND DISCUSSION

At the time of harvest, each plant was found to have, on average, 100 root nodules. The total fresh weight of nodules per plant was found to be approximately 1.6 g, and the acetylene reduction assay revealed a nitrogenase level of approximately 0.29 nmol of ethylene produced per min per mg of nodule. This value is in good agreement with those previously reported by others (Hardy et al., 1973).

When nodule extracts were centrifuged within Percoll gradients, a diffuse bacteroid band was readily identified. Aqueous methanol extracts of purified bacteroid preparations were found to contain oligosaccharides of size similar to the cyclic β-1,6 -1,3 glucans previously identified within free-living cultures of this bacterium (Miller et al., 1990). As shown in Figure 1, A, B, and C, the major oligosaccharide fraction detected within bacteroid extracts was found to elute in the position expected for the cyclic β-1,6 -1,3 glucans on three different gel-filtration chromatography media (Sephadex G-50, G-25, and G-15). When this oligosaccharide fraction was further analyzed by ion-exchange chromatography on DEAE-cellulose (Fig. 1D), greater than 80% was found to be neutral in character. This result is also consistent with our previous studies, which have revealed that the cell-associated cyclic β-1,6 -1,3 glucans are predominantly neutral in character and do not bind to DEAE-cellulose (Miller et al., 1990).

Compositional analysis of the neutral oligosaccharide fraction derived from *B. japonicum* USDA 110 bacteroids revealed that Glc accounted for greater than 99% of the total carbohydrate content. Consistent with this, NMR analysis revealed that the bacteroid oligosaccharides were composed of Glc residues linked by β-1,6 and β-1,3 glycosidic bonds (data not shown). In fact, the NMR spectra of these glucans were found to be essentially identical to spectra previously obtained for the cell-associated cyclic β-1,6 -1,3 glucans present within free-living cultures of this bacterium (Miller et al., 1990). Further analysis of the bacteroid-associated glucans by FABMS revealed the presence of two major species within these preparations. Specifically, negative-ion FABMS analysis revealed the presence of two major deprotonated molecules, $[M-H]^-$, with masses of 1781 and 1943 D (data not shown). These two molecular ion species correspond to cyclic glucans containing 11 and 12 Glc residues and are the same two major molecular ion species previously detected within cell-associated cyclic β-1,6 -1,3 glucan preparations derived from free-living cultures of this bacterium (Miller et al., 1990).

The results presented above reveal that cyclic β-1,6 -1,3 glucans are synthesized by bacteroids of *B. japonicum* USDA 110 within soybean nodules. It is interesting that the bacteroid-associated glucan content was found to correspond to approximately 0.1 mg/mg of total bacteroid protein, and we have previously shown the cell-associated cyclic β-1,6 -1,3 glucan content of free-living cultures (aerobic and nitrogenase-active, microaerobic) of *Bradyrhizobium* species to range between 0.04 and 0.13 mg/mg of total cellular protein (Gore and Miller, 1992; Miller and Gore, 1992). Because the size, shape, and protein content of bacteroids and free-living cells of *B. japonicum* are very similar (Paau et al., 1979; refs. in Werner, 1992), these values may be compared directly. In a recent study, Rolin and co-workers (Rolin et al., 1992) have also reported the presence of cyclic β-1,6 -1,3 glucans within soybean nodules infected with *B. japonicum* USDA 110. It is noted, however, that the glucans identified by Rolin et al. were not isolated from purified bacteroid preparations. Furthermore, these researchers did not quantitate the levels of the cyclic β-glucans within nodule extracts. In the present

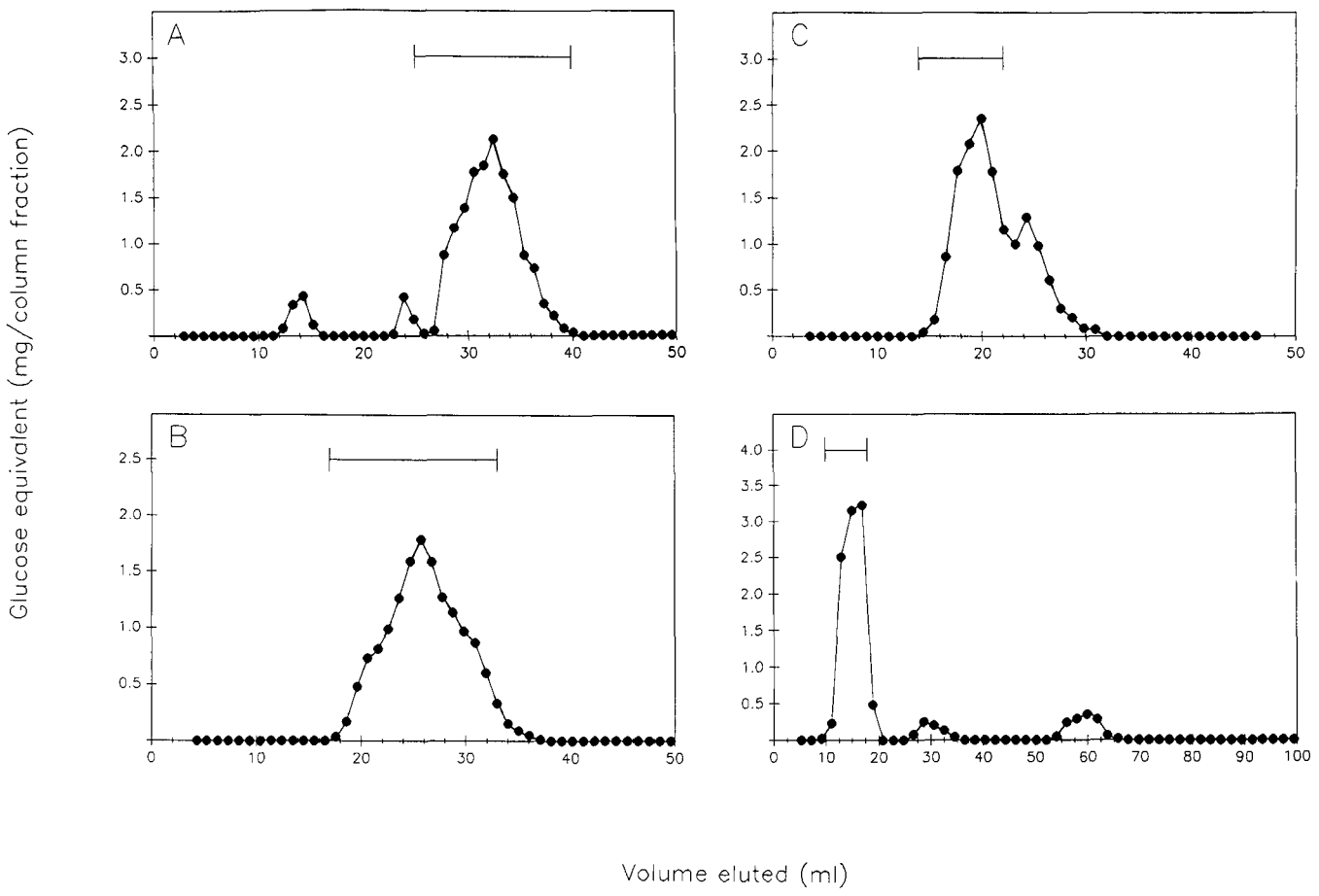


**Figure 1.** Analysis of bacteroid-associated oligosaccharides by gel filtration and ion-exchange column chromatography. Oligosaccharides were extracted from purified bacteroid preparations as described in the text. A, Sephadex G-50 analysis. Extracts were applied to a Sephadex G-50 column (1 × 56 cm), which was eluted at room temperature at a rate of 15 mL/h with 0.15 M ammonium acetate (pH 7) containing 7% (v/v) propanol. One-milliliter fractions were collected. Oligosaccharides eluting between 25 and 40 mL (marked by bracket) were pooled, concentrated, and further fractionated on a Sephadex G-25 column. B, Sephadex G-25 analysis. Oligosaccharides from Sephadex G-50 were further fractionated using a Sephadex G-25 column (1 × 56 cm). The column was eluted using the same conditions described in A. One-milliliter fractions were collected. Material eluting between 17 and 33 mL (marked by bracket) was pooled, concentrated, and further fractionated on a Sephadex G-15 column. C, Sephadex G-15 analysis. Oligosaccharides from Sephadex G-25 were further fractionated using a Sephadex G-15 column (1 × 56 cm). The column was eluted with 7% (v/v) propanol at room temperature at a rate of 15 mL/h. One-milliliter fractions were collected. Material eluting between 14 and 22 mL (marked by bracket) was pooled, concentrated, and further fractionated using a DEAE-cellulose column. D, DEAE-cellulose analysis. Oligosaccharides from Sephadex G-15 were further fractionated using a DEAE-cellulose column (1 × 23 cm). The column was eluted at room temperature at a flow rate of 12 mL/h. Two-milliliter fractions were collected. The column was first eluted using 40 mL of 10 mM Tris-HCl buffer (pH 8.4) containing 7% (v/v) propanol. Next, the column was eluted with the same buffer containing 300 mM KCl. Neutral material eluting between 10 and 18 mL (marked by bracket) was pooled and used for NMR and FABMS analyses. Fractions from all of the above columns were assayed for total carbohydrate by the phenol method as described in the text. Results are expressed as mg of Glc equivalent per column fraction and are normalized to 100 mg of total bacteroid protein. In addition to designating material pooled from each column, the elution volumes marked by the brackets in A–D represent those expected for the cyclic β-1,6 -1,3 glucans based on prior chromatographic analyses with cell-associated cyclic β-1,6 -1,3 glucans obtained from free-living cultures of *B. japonicum* USDA 110.

study, we have shown that the cyclic β-1,6 -1,3 glucans are, in fact, associated with *B. japonicum* bacteroids and that the levels within bacteroids are similar to those found within free-living cultures of this bacterium.

The presence of high levels of cyclic β-1,6 -1,3 glucans within *B. japonicum* USDA 110 bacteroids isolated from mature soybean root nodules is intriguing and suggests that these glucans may provide functions during the later stages of legume nodulation. This would appear to be in contrast with the roles for rhizobial exopolysaccharides, whose levels are dramatically reduced in the bacteroid state (Dixon, 1964; Roth and Stacey, 1989). The functions for the cyclic glucans

within bacteroids remain unclear; however, it is possible that they may act as signal molecules or may be involved in bacteroid osmoregulation. The identification of mutants of *B. japonicum* defective for the biosynthesis of these glucans should provide an important future approach toward understanding the functions of these molecules.

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