Cell Surface Carbohydrates of Microaerobic, Nitrogenase-Active, Continuous Cultures of *Bradyrhizobium* sp. Strain 32H1

RICHARD S. GORE[1] AND KAREN J. MILLER[1,2,3]*

Graduate Programs in Genetics[1] and Plant Physiology[2] and Department of Food Science,[3]
The Pennsylvania State University, University Park, Pennsylvania 16802

A continuous culture system was developed to examine the cell surface carbohydrates of *Bradyrhizobium* sp. strain 32H1. When cultures were shifted from aerobic to microaerobic growth conditions, nitrogenase activity was induced and extracellular polysaccharide levels were greatly reduced; however, the levels of cell-associated cyclic beta-1,6 -1,3 glucans were found to be essentially unchanged.

Members of the genera *Rhizobium* and *Bradyrhizobium* are distinguished from other species by their ability to infect leguminous plants. This infection process leads to the development of nitrogen-fixing nodules on the roots of the legume host. Within these nodules, the bacteria are released intracellularly and differentiate into nitrogen-fixing forms termed "bacteroids."

The cell surface carbohydrates of these bacteria are believed to provide functions during the plant infection process. These carbohydrates include extracellular polysaccharides, capsular polysaccharides, lipopolysaccharides, and oligosaccharides. Studies in our laboratory have focused upon a class of rhizobial oligosaccharides, the cyclic beta glucans, and we have shown that the cyclic beta glucans of bradyrhizobial species are different in structure than those synthesized by *Rhizobium* species. Specifically, the cyclic beta glucans produced by *Rhizobium* species contain 17 to 24 glucose residues linked solely by beta-1,2 glycosidic linkages, while the bradyrhizobial cyclic beta glucans are smaller (10 to 13 glucose residues) and are linked by both beta-1,6 and beta-1,3 glycosidic linkages (6, 7).

The differentiation of bacterium to bacteroid has been found to be associated with numerous changes in cell metabolism, a general increase in cell volume, and an alteration in cell surface carbohydrate composition. With regard to cell surface carbohydrate composition, it appears that biosynthesis of extracellular polysaccharide, capsular polysaccharide, and lipopolysaccharide is greatly reduced in the bacteroid state (2, 4, 8, 10–12, 14, 15), suggesting, perhaps, that these cell surface carbohydrates provide functions primarily during the early stages of legume nodulation. In order to gain a further understanding of the cell surface carbohydrate changes which occur during bacteroid differentiation, we have taken advantage of the fact that strains of *Bradyrhizobium* are capable of bacteroid differentiation ex planta during microaerobic growth. In the present study, we have utilized a continuous culture system to examine the effects of microaerobic growth conditions on the biosynthesis of cell-associated cyclic beta-1,6 -1,3 glucans by *Bradyrhizobium* sp. strain 32H1.

Continuous culture conditions. *Bradyrhizobium* sp. strain 32H1 has been previously described (7). Cells were grown in continuous culture with a BioFlo III fermentor (total volume, 3.3 liters) (New Brunswick Scientific Co., Edison, N.J.). Continuous culturing was performed under both aerobic (sparging with air) and microaerobic (sparging with a mixture of 1% [vol/vol] air in nitrogen) conditions at 30°C in a 2.5-liter working volume. During aerobic growth, fresh medium was added at a rate of between 80 and 110 ml/h (dilution rate [D] values of between 0.032 and 0.044 h^{-1}), and during microaerobic growth, fresh medium was added at a rate of 24 ml/h ($D = 0.010$ h^{-1}). A defined medium based on that described by Ludwig and Signer (5) was used and contained 17 mM succinate, 20 mM arabinose, 0.5 to 5 mM glutamic acid, 7.5 mM KH_2PO_4, 0.8 mM $MgSO_4$, 36 μM $FeSO_4$, 0.057 μM $(NH_4)_6Mo_7O_{24}$, and 0.05 mM $CaCl_2$ at pH 6.3.

Precultures (200 ml) of *Bradyrhizobium* sp. strain 32H1 were grown in defined medium on a rotary shaker at 30°C for 4 to 7 days. The preculture was added to the fermentor through an inoculation line by using a peristaltic pump. Initially, the culture was grown in batch mode within the fermentor for approximately 4 days, after which the fermentor was switched to continuous culture mode.

Nitrogenase assay. Nitrogenase activity was measured by the acetylene reduction method (13). Samples (10 ml) from the continuous culture were withdrawn directly into a syringe from a sampling line through a Luer-Lok fitting. Each sample was then added to a serum bottle (total volume, 119 ml) that had previously been flushed with sterile nitrogen gas. Acetylene gas (4.4 ml) and air (2.2 ml) were also added to each serum bottle. The headspace gas was analyzed for the presence of ethylene by using a model 3300 gas chromatograph (Varian Instruments Group, Sunnyvale, Calif.) with a flame ionization detector. The injector, column, and detector temperatures were 125, 40, and 190°C, respectively. Helium was used as the carrier gas at a flow rate of 20 ml/min. Analysis was performed by using a stainless steel Porapak T column (one-eighth in. by 3 ft [ca. 0.3 by 91.4 cm]) (Supelco, Inc., Bellefonte, Pa.).

Analysis of glucans and polysaccharides. Cell-associated cyclic beta-1,6 -1,3 glucans were extracted from cells by using a previously described methanol extraction procedure (7). Extracts were assayed for glucan content by gel filtration chromatography on Sephadex G50 and Sephadex G25 and by ion-exchange chromatography on DEAE-cellulose as previously described (7). The amount of extracellular
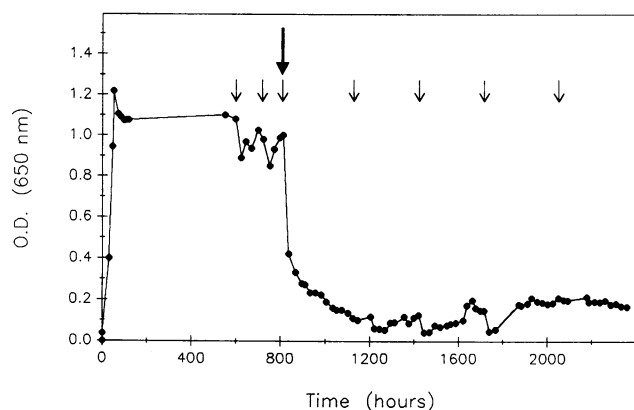
---

* Corresponding author.

FIG. 1. The effect of dissolved oxygen concentration on the cell density of continuous cultures of *Bradyrhizobium* sp. strain 32H1. At the start of the experiment, the fermentor was operated in batch mode. After 120 h, the fermentor was operated in continuous culture mode. During the first 810 h, the culture was grown aerobically (sparged with air at a rate of 500 ml/min; $D = 0.043 \text{ h}^{-1}$). After 810 h (large arrow), the culture was shifted to microaerobic conditions (sparged with 1% [vol/vol] air in nitrogen at a rate of 500 ml/min; $D = 0.010 \text{ h}^{-1}$). The glutamate concentration was maintained constant at 2 mM. At 600, 720, 810, 1,130, 1,425, 1,715, and 2,050 h (small arrows), 1-liter samples were removed from the fermentor. Immediately after sample removal, 1 liter of sterile medium was added to the fermentor to restore the total volume to 2.5 liters. The slight, temporary reduction in optical density (O.D.) values observed at each of these time points results from the removal of each 1-liter sample. Some of the 1-liter samples removed from the fermentor (i.e., those removed at 600, 810, 1,425, 1,715, and 2,050 h) were used for determinations of nitrogenase activity, extracellular polysaccharide content, and cell-associated oligosaccharide content (as reported in Table 1).

polysaccharide within culture supernatants was determined as total carbohydrate precipitated with ethanol (final concentration, 75% [vol/vol]). Total carbohydrate was measured by the phenol method (7).

Nuclear magnetic resonance and fast-atom-bombardment mass spectrometry analyses. Nuclear magnetic resonance and fast-atom-bombardment mass spectrometry experiments were performed as previously described (7).

Nitrogenase activity is induced when continuous cultures of *Bradyrhizobium* sp. strain 32H1 are shifted from aerobic to microaerobic growth conditions. Under aerobic continuous culture conditions, the growth of *Bradyrhizobium* sp. strain 32H1 was found to be nitrogen limited at glutamate concentrations below 4 mM. However, when cells were shifted to microaerobic conditions, oxygen became the limiting factor for growth (Fig. 1).

When samples were removed from the continuous culture system and assayed for nitrogenase activity, no activity within aerobic cultures was detected. However, high levels of nitrogenase activity within samples removed from microaerobic continuous cultures were detected (Table 1).

Extracellular polysaccharide levels are dramatically reduced when cells are shifted to microaerobic continuous culture conditions. Concomitant with the induction of nitrogenase activity, a shift to microaerobic growth conditions resulted in a dramatic decrease in the levels of extracellular polysaccharides within continuous cultures of *Bradyrhizobium* sp. strain 32H1. As shown in Table 1, extracellular polysaccharide levels were approximately sevenfold lower in microaerobic cultures than in aerobic cultures. Similar

TABLE 1. Nitrogenase activity, extracellular polysaccharide levels, and cell-associated oligosaccharide levels within continuous cultures of *Bradyrhizobium* sp. strain 32H1

Sparging conditions (time [h] samples removed from continuous culture)[a]	Nitrogenase activity (nmol of ethylene produced/h/mg of total cellular protein)[b]	Extracellular polysaccharides (mg of glucose equivalent/mg of total cellular protein)[c]	Cell-associated oligosaccharides (mg of glucose equivalent/mg of total cellular protein)[d]
Aerobic (600)	None detected	0.65	0.13
Aerobic (810)	None detected	0.62	
Microaerobic (1,425)	1,115		
Microaerobic (1,715)	422	0.10	0.07
Microaerobic (2,050)	650	0.08	0.09

[a] Aerobic, sparging with air; microaerobic, sparging with 1% (vol/vol) air in nitrogen. The samples were obtained from the same continuous culture experiment shown in Fig. 1.
[b] Samples were assayed for nitrogenase activity by the acetylene reduction method described in the text. Activity was measured during the first 8 h of the assay.
[c] Extracellular polysaccharides are defined as total carbohydrate within culture supernatants precipitated with ethanol (75%, vol/vol).
[d] Cell-associated oligosaccharide content was determined after gel filtration chromatography on Sephadex G50 and Sephadex G25 as described in the text.

results have previously been reported by Tully and Terry (12), Agarwal and Keister (1), and Gober and Kashket (3) for microaerobic broth cultures of various bradyrhizobial strains.

Cell-associated cyclic beta-1,6 -1,3 glucan levels are similar in aerobic and microaerobic cultures of *Bradyrhizobium* sp. strain 32H1. In contrast to the decrease in extracellular polysaccharide levels detected when continuous cultures of *Bradyrhizobium* sp. strain 32H1 were shifted from aerobic to microaerobic growth conditions, the cell-associated cyclic beta-1,6 -1,3 glucan content of the cultures was found to remain relatively constant. As shown in Table 1, the amount of cell-associated oligosaccharides corresponded to approximately 0.1 mg of glucose equivalent per mg of total cellular protein under both growth conditions. When cell-associated oligosaccharide preparations from both aerobic and microaerobic cultures were further analyzed by DEAE-cellulose chromatography, between 72 and 84% of the total oligosaccharide material was found to consist of neutral glucans (a result similar to that reported previously [7]). Furthermore, when purified neutral glucan preparations from both aerobic and microaerobic cultures were examined by nuclear magnetic resonance spectroscopy, the spectra were essentially identical, and both glucans were found to contain glucose linked by beta-1,6 and beta-1,3 glycosidic bonds (data not shown). The spectra were also found to be essentially identical to those previously obtained for the cell-associated cyclic beta-1,6 -1,3 glucans of *Bradyrhizobium* sp. strain 32H1 cultured in YM medium (7). Further analysis by fast-atom-bombardment mass spectrometry revealed two major glucan species in both aerobic and microaerobic neutral glucan preparations. These glucan species corresponded to cyclic glucans containing 11 and 12 glucose residues, which is also consistent with results we have previously reported (7).

While the results of the present study confirm that the induction of nitrogenase activity within microaerobic cultures of a *Bradyrhizobium* sp. is associated with a dramatic reduction in extracellular polysaccharide levels, the levels of cell-associated cyclic beta-1,6 -1,3 glucans remain essentially unchanged. Because cyclic beta-1,6 -1,3 glucan syn-

thesis is not repressed in nitrogenase-active microaerobic cultures, it is possible that these glucans are synthesized by bacteroids within the root nodule. In this regard, we note recent studies that reveal the presence of cyclic beta-1,6 -1,3 glucans within bradyrhizobial bacteroids isolated from soybean nodules (3a, 9).

REFERENCES

1. **Agarwal, A. K., and D. L. Keister.** 1983. Physiology of ex planta nitrogenase activity in *Rhizobium japonicum*. Appl. Environ. Microbiol. **45:**1592–1601.

2. **Dixon, R. O. D.** 1964. The structure of infection threads, bacteria and bacteroids in pea and clover root nodules. Arch. Mikrobiol. **48:**166–178.

3. **Gober, J. W., and E. R. Kashket.** 1987. K$^+$ regulates bacteroid-associated functions of *Bradyrhizobium*. Proc. Natl. Acad. Sci. USA **84:**4650–4654.

3a.**Gore, R. S., and K. J. Miller.** Unpublished data.

4. **Gray, J. X., R. A. de Maagd, B. G. Rolfe, A. W. B. Johnston, and B. J. J. Lugtenberg.** 1992. The role of the *Rhizobium* cell surface during symbiosis, p. 359–376. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communications. CRC Press, Inc., Boca Raton, Fla.

5. **Ludwig, R. A., and E. R. Signer.** 1977. Glutamine synthetase and control of nitrogen fixation in *Rhizobium*. Nature (London) **267:**245–248.

6. **Miller, K. J., and R. S. Gore.** 1992. Cyclic beta-1,6 -1,3 glucans

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

8. **Reuber, T. L., J. W. Reed, J. Glazebrook, A. Urzainqui, and G. C. Walker.** 1991. Analyses of the roles of *R. meliloti* exopolysaccharides in nodulation, p. 182–188. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publ., Dordrecht, The Netherlands.

9. **Rolin, D. B., P. E. Pfeffer, S. F. Osman, B. S. Szwergold, F. Kappler, and A. J. Benesi.** 1992. Structural studies of a phosphocholine substituted beta-(1,3);(1,6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. Biochim. Biophys. Acta **1116:**215–225.

10. **Roth, L. E., and G. Stacey.** 1989. Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. Eur. J. Cell Biol. **49:**13–23.

11. **Roth, L. E., and G. Stacey.** 1991. *Rhizobium*-legume symbiosis, p. 255–301. *In* M. Dworkin (ed.), Microbial cell-cell interactions. American Society for Microbiology, Washington, D.C.

12. **Tully, R. E., and M. E. Terry.** 1985. Decreased exopolysaccharide synthesis by anaerobic and symbiotic cells of *Bradyrhizobium japonicum*. Plant Physiol. **79:**445–450.

13. **Turner, G. L., and A. H. Gibson.** 1980. Measurement of nitrogen fixation by indirect means, p. 111–138. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley and Sons Ltd., New York.

14. **van Brussel, A. A. N., K. Planque, and A. Quispel.** 1977. The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. J. Gen. Microbiol. **101:**51–56.

15. **Vance, C. P.** 1983. *Rhizobium* infection and nodulation: a beneficial plant disease? Annu. Rev. Microbiol. **37:**399–424.

of *Bradyrhizobium*: functional analogs of the cyclic beta-1,2-glucans of *Rhizobium*? Curr. Microbiol. **24:**101–104.