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New Osmoregulated β(1-3),β(1-6) Glucosyltransferase(s) in *Azospirillum brasilense*

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A linear β(1-3),β(1-6) glucan was detected in the periplasm of *Azospirillum brasilense* cells growing in a medium of low osmotic strength. This glucan was produced in vitro by purified bacterial inner membranes with UDP-glucose as the sugar donor in the presence of Mg^{2+}. Growth in a high-osmotic-strength medium strongly reduced the amount of this glucan accumulated in the periplasmic space, and the inhibition was associated with a reduction in the enzymatic activity of the β(1-3),β(1-6) glucosyltransferase(s).

Bacteria of the genus *Azospirillum* have been studied extensively because of their effect in promoting plant growth (21). Under certain conditions, they show moderate host specificity, as reflected by their isolation from a wide variety of, but not all, plants. The ability of azospirilla to colonize the rhizosphere, although not fully understood, is presumed to depend on chemotaxis towards root exudates and several other properties determined by bacterial cell surface polysaccharides (10, 21, 25).

In *Rhizobium meliloti*, the role of exopolysaccharides in nodule development has been well established, and the genes necessary for their production (*exo* genes) have been identified and studied (2, 18, 20). Cyclic β(1-2) glucans play a key role in plant interaction in most species of the family *Rhizobiaceae*. The chromosomal regions *chvB* and *chvA* in *Agrobacterium* spp. and *ndvB* and *ndvA* in *Rhizobium* spp. are required for the synthesis and secretion of cyclic β(1-2) glucan (9, 13, 30, 36). Mutants of *R. meliloti* and *Agrobacterium tumefaciens* deficient in the production of cyclic β(1-2) glucan form ineffective empty nodules (12) or are avirulent (27, 36), respectively.

It has been shown that cellular cyclic β(1-2) glucan production in some members of the family *Rhizobiaceae* is osmoregulated (24, 34). In medium of low osmotic strength, *R. meliloti* and *A. tumefaciens* mutants defective in the synthesis of β(1-2) glucans are strongly impaired in their ability to grow, suggesting that the glucan is important for survival under such conditions (34). Growth is restored by raising the osmolarity of the medium (34). On the other hand, in medium of high osmotic strength, the synthesis of glucans is strongly repressed in *A. tumefaciens* (34). In *A. tumefaciens*, the inhibition of the formation of β(1-2) glucan at high osmolarity has been shown to be due to an in vivo inhibition of the activity of β(1-2) glucosyltransferase, since inner membranes prepared from cells grown in medium of high or low osmolarity displayed the same specific activity (34).

Bradyrhizobium japonicum, a soybean-nodulating bacterium, does not produce β(1-2) glucan (14). A cyclic β(1-3),β(1-6) glucan was isolated from this bacterium (23, 28), and the enzymatic synthesis of this compound was studied in vitro (5, 14).

We recently reported that *Azospirillum brasilense* does not synthesize cyclic β(1-2) glucan (1). In this work, we report that *A. brasilense* synthesizes instead a periplasmic linear β(1-3),β(1-6) glucan, the synthesis of which is regulated by the osmotic strength of the growth medium.

MATERIALS AND METHODS

Bacterial strains and media. The *A. brasilense* CdRif ATCC 29710 strain was used in this study (1). Bacteria were grown in LB broth supplemented with rifampin (100 μg/ml).

Medium YL was used to investigate the effect of different osmolytes on the synthesis of glucan. The medium is identical to the YM medium used by Miller (22), but lactate was used as the carbon source instead of glucose. When required, the medium was supplemented with 0.25 M NaCl or 0.5 M mannitol. Medium M9 was used to grow cells for in vivo [^{14}C]succinic acid labeling experiments.

Extraction of cell-associated oligosaccharides. Cells from 100-ml cultures were harvested by centrifugation at 10,000 × *g* for 10 min. Pellets were extracted with 1% trichloroacetic acid (TCA) for 30 min at room temperature as described previously (24). TCA extracts were neutralized with ammonium hydroxide, concentrated, and subjected to gel filtration on Bio-Gel P4 columns as previously described (13). The sugar content was determined by the anthrone-sulfuric acid method (7). The average partition coefficient (K_{av}) was calculated as $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 is the void volume, and V_t is the total volume.

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 125.8 MHz. The samples were dissolved in D_2O at concentrations of approximately 20 mg/ml. Glucans were purified by Bio-Gel P4 chromatography and high-pressure liquid chromatography (HPLC) with a C18 silica column as described before (28).

Preparation of inner membranes. *Azospirillum* cells grown in LB medium were harvested after 24 or 48 h. Inner membranes were prepared by the method described previously (26) with the modifications introduced for *A. tumefaciens* (36).

In vivo synthesis of glucan. Overnight cultures of *A. brasilense* were diluted 1:100 in fresh M9 liquid medium with

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1% succinic acid as a carbon source. After 3 h at 30°C, cells were collected from 1.5 ml of culture medium, and cell pellets were washed twice in M9 medium without succinic acid. Pellets were resuspended in 0.3 ml of M9 medium with 2 μCi of [2,3-^{14}C]succinic acid (56 mCi/mmol) and incubated at 30°C for 3 h. Cells were harvested by centrifugation, washed twice with 1 ml of M9 medium, and extracted with TCA as described above.

In vitro synthesis of glucan. Inner membranes were incubated with UDP-[^{14}C]glucose (0.05 μCi; 285 mCi/mmol)–40 mM $MgCl_2$–50 mM Tris-HCl buffer (pH 8.2) in a total volume of 50 μl. The reaction was carried out at 30°C and stopped, depending on the experiment, by one of the following procedures. In the first method, 300 μl of water was added, and the mixture was then heated at 100°C for 1 min and centrifuged at 5,000 × *g* for 5 min. The supernatants were then subjected to DEAE-Sephadex chromatography as described previously (36). Pellets were resuspended in 10% TCA and filtered through glass microfiber filters (GF/C Whatman) to determine incorporation into insoluble products as described before (36). When partial purification of in vitro-synthesized soluble glucan was required for structural studies, DEAE-Sephadex percolates were subjected to gel chromatography on a Bio-Gel P4 column, which was eluted with 0.15 M amonium acetate in 7% (vol/vol) water–2-propanol. In the second method, 1.0 ml of cold 10% TCA was added, and the mixture was then centrifuged at 5,000 × *g* for 10 min. The pellet was subjected to polyacrylamide gel electrophoresis and fluorography as described before (36). When required, the TCA-insoluble fraction was filtered through glass microfiber filters as in the first method.

Preparation of permeabilized cells and osmotic shock. Cells grown in LB medium were harvested after 24 h and permeabilized by the procedure described previously (31). Osmotic shock was carried out as described before (3) with the following modification: washed pellets were resuspended in 30 mM Tris-HCl buffer (pH 7.5) with 12% mannitol instead of 20% sucrose. Malate dehydrogenase (EC 1.1.1.37) was assayed as described before (6).

Acid hydrolysis, paper chromatography, and paper electrophoresis. Partial and total acid hydrolysis assays were carried out as indicated previously (13). Hydrolysates were subjected to descending paper chromatography on Whatman no. 1 paper (Whatman Inc., Clifton, N.J.) with solvent A (butanol-pyridine-water, 6:4:3) (15) or solvent B (isopropanol-acetic acid-water, 27:4:9) (33). Sugars were detected by the alkaline silver method (32). Paper electrophoresis was carried out with buffer C (2% sodium molybdate [pH 5.0]) for 2 h at 15 V/cm (4) or with buffer D (0.05 M sodium borate [pH 9.2]) (11) for 2 h at 15 V/cm.

Reduction with sodium borohydride. Oligosaccharides were dissolved in 300 μl of water, 5 mg of $NaBH_4$ was added, and the mixture was incubated overnight in the dark. To remove Na^+, an excess of Dowex 50W-X8 resin (Bio-Rad Laboratories) was added, and oligosaccharides were recovered by washing the resin with water. Samples were dried several times under an air stream. Methanol was added and evaporated in order to remove boric acid as methyl borate. Reduced oligosaccharides were subjected to total acid hydrolysis with 1 N HCl for 4 h at 100°C. HCl was eliminated by several evaporations under an air stream, and the hydrolysate was subjected to paper electrophoresis with buffer C. The paper strips were cut, and radioactivity was counted with an Omnifluor-toluene solution (New England Nuclear, Boston, Mass.) in a liquid scintillator.

Smith degradation. Oligosaccharides were oxidized for 3 days with 0.04 M sodium periodate at 4°C in the dark (final reaction volume, 200 μl). The excess of periodate was destroyed with 10 μl of ethylene glycol, and 8 mg of sodium borohydride was added after 2 h. Samples were kept in the dark for 16 h. Acetic acid (10%) was added to eliminate the $NaBH_4$ excess, and the samples were chromatographed through a Bio-Gel P2 column to remove salts. Desalted samples were lyophilyzed and subjected to mild acid hydrolysis (0.1 N HCl) at room temperature overnight. HCl was removed by evaporation under an air stream, the hydrolysates were subjected to descending paper chromatography on Whatman no. 1 paper with solvent B, and radioactivity was detected with a radioscanner. For further identification, radioactive products were eluted from the paper strip with water and subjected to different treatments as described in Results.

RESULTS

Cellular polysaccharides accumulated in vivo by *A. brasilense*. Cells were treated with 1% TCA, and the TCA extracts were subjected to gel chromatography on Bio-Gel P4 columns. Sugars were detected by the anthrone-sulfuric acid method as described in Materials and Methods. As shown in Fig. 1A, TCA extracts yielded three main sugar-containing compounds. One compound eluted with the V_0 of the column. Total acid hydrolysis and paper chromatography with solvent A indicated that it is a polysaccharide formed by the monosaccharides glucose, rhamnose, galactose, and fucose, indicating that it might be part of the already described *A. brasilense* exopolysaccharide (data not shown). A second compound eluted from the column with a K_{av} of 0.4. The elution profile of this fraction is heterogeneous, suggesting the presence of a mixture of polysaccharides with different degrees of polymerization. Total acid hydrolysis yielded only glucose, and partial acid hydrolysis followed by paper chromatography of the hydrolysates with solvent B yielded glucose, gentiobiose [glucose β(1-6) glucose], laminaribiose [glucose β(1-3) glucose], and a series of oligosaccharides with increasing degrees of polymerization (data not shown). The third compound that eluted from the column near the total volume (K_{av} of 0.8) was not analyzed. These results suggested that the polysaccharides eluting at a K_{av} of 0.4 are glucans formed mainly by β(1-3),β(1-6) linkages. To further characterize these glucans, cells were incubated with [2,3-^{14}C]succinic acid as the radioactive precursor in order to obtain ^{14}C-labeled glucan. As shown in Fig. 1B, TCA extracts obtained from ^{14}C-labeled cells contained three main radioactive compounds that eluted from the Bio-Gel P4 column at V_0, K_{av} 0.4, and K_{av} 0.8 as the products obtained from unlabeled cells (Fig. 1A).

Total acid hydrolysis of the K_{av} 0.4 glucans labeled in vivo yielded glucose as the only monosaccharide. Partial acid hydrolysis yielded products that, on paper chromatography with solvent B, were identified as glucose, laminaribiose, gentiobiose, and a series of oligosaccharides with increasing degrees of polymerization (Fig. 2A). Further fractionation of the oligosaccharides recovered from the Bio-Gel P4 column, by HPLC with a C18 silica column, resolved three main components, all of which yielded the same partial acid hydrolysis pattern (data not shown). Compounds migrating as gentiobiose and laminaribiose (G and L in Fig. 2A) were eluted with water from the paper strip and further characterized as follows. (i) They were reduced with sodium borohydride and subjected to paper electrophoresis with buffer C. Under these conditions, only (1,3)-linked glucose disaccharides remain at the origin, while all other glucose disaccharides behave as anions. Reduced compound G comigrated with a gentiobiosi-

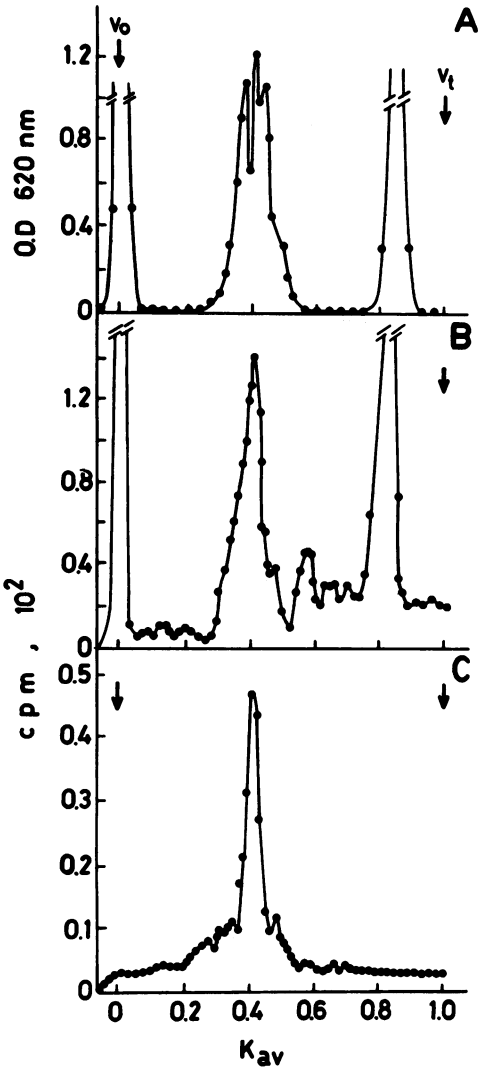

FIG. 1. Bio-Gel P4 chromatography of cellular polysaccharides formed by *A. brasilense*. (A) Polysaccharides accumulated in vivo. Extraction of cellular glucans with TCA, gel chromatography, and assay of carbohydrates were carried out as described in Materials and Methods. (B) Polysaccharides synthesized in vivo. Cells of *A. brasilense* were labeled with [2,3-^{14}C]succinic acid as described in Materials and Methods. TCA extracts were made and gel chromatography was performed as described in the text. (C) Polysaccharides formed by *A. brasilense* inner membranes. Inner membranes were incubated with UDP-[^{14}C]glucose as described in Materials and Methods. Columns were eluted with 0.15 M amonium acetate with 7% (vol/vol) aqueous 2-propanol, and fractions (0.45 ml) were collected. V_0, void volume; V_t, total volume. Radioactivity is expressed in counts per minute. OD, optical density.

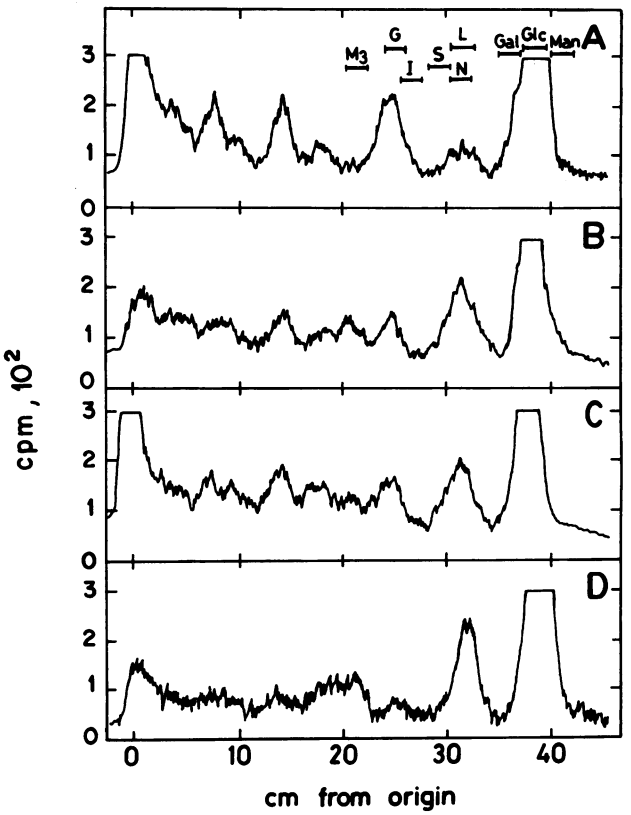

FIG. 2. Paper chromatography of products obtained after partial acid hydrolysis. Neutral soluble glucans eluted from the Bio-Gel P4 column with a K_{av} of 0.4 were subjected to partial acid hydrolysis, and the products were subjected to paper chromatography with solvent B as described in Materials and Methods. Radioactivity was detected with a radioscanner. Internal unlabeled standards: G, gentiobiose; L, laminaribiose; Glc, glucose; Man, mannose; Gal, galactose; N, nigerose; S, sophorose; I, isomaltose; M3, maltotriose. (A through C) Neutral soluble glucans eluted from the Bio-Gel P4 column with a K_{av} of 0.4, formed in vivo by *A. brasilense* (A), in vitro by *A. brasilense* inner membranes (B), and in vitro by *A. brasilense* permeabilized cells (C). (D) Neutral soluble glucans eluted from the Bio-Gel P4 column with a K_{av} of 0.37, formed in vitro by inner membranes of *B. japonicum*.

tol standard, and reduced compound L did not migrate (data not shown). (ii) They were electrophoresed with buffer D under conditions that resolve α and β anomer glucose disaccharides (i.e., gentiobiose from isomaltose and laminaribiose from nigerose). Under these conditions, compound G comigrated with the gentiobiose standard and compound L comigrated with the laminaribiose standard (data not shown). These results confirmed that this glucan contains β1-3 and β1-6 linkages and that both linkages are present in the same glucan, as revealed by the partial acid hydrolysis pattern obtained from HPLC-fractionated glucans. Sephadex DEAE-A25 chromatography of this β(1-3),β(1-6) glucan revealed that it has no charge.

Cellular localization. In order to establish the subcellular localization of the K_{av} 0.4 cell-associated *Azospirillum* glucan, cell pellets were subjected to osmotic shock as described in Materials and Methods. As shown in Fig. 3A, the glucan was recovered from the periplasmic space fluid. The pellet remaining after osmotic treatment did not contain detectable glucan (Fig. 3B). No malate dehydrogenase activity was released into the periplasmic extract, indicating that no cytoplasmic fluid contaminated the periplasmic space fluid. These results indicate that the K_{av} 0.4 glucan accumulated in the periplasmic space.

NMR spectroscopy. The cell-associated glucan recovered from *A. brasilense* was purified on a Bio-Gel P4 column as shown in Fig. 1A. This glucan was further purified on a C18 silica column with 20% methanol as described before (28) and subjected to NMR spectroscopy at room temperature with a Bruker AM-500 spectrometer in D_2O. The 500.1-MHz ^{1}H

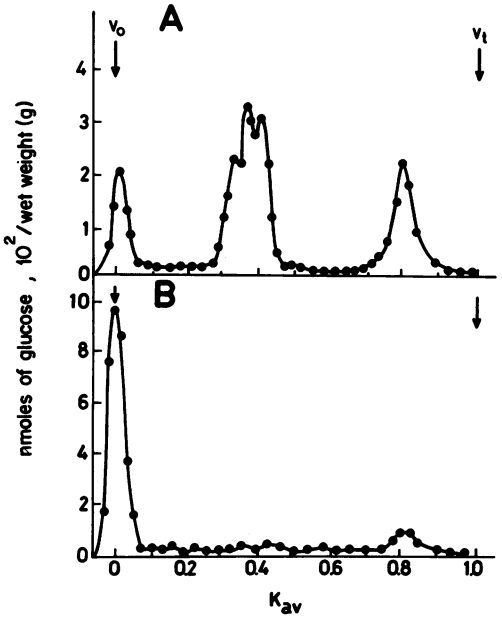

FIG. 3. Cellular localization of *A. brasilense* glucan. Cell pellets were subjected to osmotic shock as described in Materials and Methods. Extracts were applied to a Bio-Gel P4 column and eluted with 0.1 M pyridine–acetate buffer (pH 5.5). Fractions of 1.0 ml were collected. Sugars were determined as described in the legend to Fig. 1. (A) Periplasmic fluid. (B) TCA (10%) extract obtained from the pellet after osmotic shock. Data are 10^2 nmol of glucose per g (wet weight).

NMR spectrum shows a pattern in the sugar region (3.2 to 5.0 ppm) similar to that of a β(1-3),β(1-6) glucan reported by Rolin et al. (28), with resonances observed at δ 4.86 and 4.48 ($J_{1,2}$ = 7.2 Hz) (Fig. 4A).

The 125.8-MHz natural-abundance ^{13}C proton-decoupled spectrum of cell-associated glucans purified by HPLC (Fig. 4B) was similar to that of the *B. japonicum* extracellular β(1-3),β(1-6) glucan reported by Dudman and Jones (8) and to that of the *B. japonicum* cell-associated glucan described by Miller et al. (23). The spectrum also shows some additional signals at higher (15.1 and 35.3 ppm) and lower (183 ppm) fields. The following carbon signals (in parts per million), corresponding to 1,3 and 1,6 linkages, were assigned according to reference 28: 1,3 linkage glucose: C1, 104.05, 103.8; C2, 74.2; C3, 85.4, 85.31; C4, 70.6; C5, 76.6; C6, 61.9; 1,6 linkage glucose: C1, 104.0; C2, 74.10; C3, 76.2; C4, 70.6; C5, 76.7; C6, 61.9, 69.5, 69.9.

However, a difference from the *B. japonicum* cellular glucans is that no choline residues seem to be present in this fraction, since the choline resonance at 55.2 ppm cannot be seen in the spectrum (see Fig. 4B). Although the resonances at 92 to 96 ppm, corresponding to the C_1-reducing glucose, are absent, a carbonyl resonance at 183 ppm is present (data not shown). These results confirm the presence in *A. brasilense* of a β(1-3),β(1-6) cell-associated glucan similar to the cell-associated glucan described for *B. japonicum*.

Effect of osmolytes on glucan accumulation. It has been reported that osmolytes affect the formation of other cellular glucans (16, 34). Accordingly, the effect of different osmolytes on glucan synthesis by *A. brasilense* was studied. As shown in Fig. 5, 500 mM mannitol or 250 mM NaCl strongly inhibited the accumulation of this periplasmic glucan.

The amount of cellular glucan accumulated by *A. brasilense* cells grown in medium of 151 mOsM was 6.3 μmol of glucose equivalent per g (wet weight) (Fig. 5A). The addition of 500 mM mannitol (Fig. 5B) or 250 mM NaCl (Fig. 5C) reduced the amount to 1.9 and 1.5 μmol of glucose per g (wet weight), respectively. These results show that the synthesis of *A. brasilense* glucan is osmoregulated, as was reported for *E. coli* membrane-derived oligosaccharides (16) and *A. tumefaciens* β(1-2) glucan (34).

In vitro synthesis. The biosynthesis of *A. brasilense* β(1-3),β(1-6) glucan was studied in vitro by using different enzyme preparations and labeled UDP-glucose as the sugar donor. Inner membranes incorporated 10-fold more label than outer membranes; the amounts of glucan synthesized by the enzyme from permeabilized cells, total membranes, inner membranes, and outer membranes were 0.060, 0.193, 0.994, and 0.065 nmol of glucose per mg of protein per h, respectively. The low incorporation by outer membranes is probably due to contamination of this fraction with inner membranes, as determined by protein polyacrylamide gel electrophoresis (data not shown).

In addition to the incorporation of radioactivity into a soluble glucan (Fig. 6A), a small amount of radioactivity was incorporated into TCA-insoluble compounds (Fig. 6B).

In order to establish the presence of possible TCA-insoluble intermediates during the synthesis of this glucan, a pulse-chase experiment was carried out. As shown in Fig. 6B, TCA-insoluble products did not behave as intermediates, since the radioactivity incorporated in this fraction did not decrease after a 50-min chase. Polyacrylamide gel electrophoresis and fluorography of the gel showed no accumulation of ^{14}C-labeled proteins during the reaction (Fig. 7).

The formation of a soluble glucan required the presence of divalent cations, since the addition of 10 mM EDTA totally abolished the formation of these products. Activity was restored by addition of $MgCl_2$, $MnCl_2$, or $CaCl_2$. However, the highest activity was obtained with 40 mM $MgCl_2$. UDP-[^{14}C]glucose was the only sugar donor. The addition of ADP-[^{14}C]glucose did not result in the formation of the β(1-3),β(1-6) glucan. However, the formation of glycogen was observed with this sugar nucleotide (data not shown).

The effect of the addition of disaccharides was also studied. Gentiobiose (20 mM) stimulated the incorporation of radioactivity into the soluble fraction by 40%, the same concentration of laminaribiose stimulated it by 27%, and sophorose stimulated it by 18%; no effect was observed with nigerose. The formation of a soluble glucan and TCA-insoluble products was strongly dependent on the incubation temperature, the optimum one for both reactions being 30°C (data not shown).

Characterization of in vitro-synthesized soluble glucans. The soluble product obtained after incubation of inner membranes or permeabilized cells with UDP-[^{14}C]glucose was subjected to Bio-Gel P4 and Bio-Gel P2 chromatography. As shown in Fig. 1C, the main product formed in vitro eluted from the Bio-Gel P4 column with a K_{av} of 0.40. When this product was subjected to Bio-Gel P2 chromatography, it eluted from the column with a slightly higher elution volume than the glucan formed in vivo (Fig. 8A), suggesting that it had a lower degree of polymerization. Total acid hydrolysis and paper chromatography with solvent A yielded glucose as the only monosaccharide present. Partial acid hydrolysis and paper chromatography with solvent A yielded products that migrated as glucose, laminaribiose, gentiobiose, and a series of oligosaccharides with increasing degrees of polymerization (Fig. 2B). Compounds with the mobility of laminaribiose and gentiobiose were subjected to further characterization by paper electrophoresis as described above for the glucans labeled in vivo, and

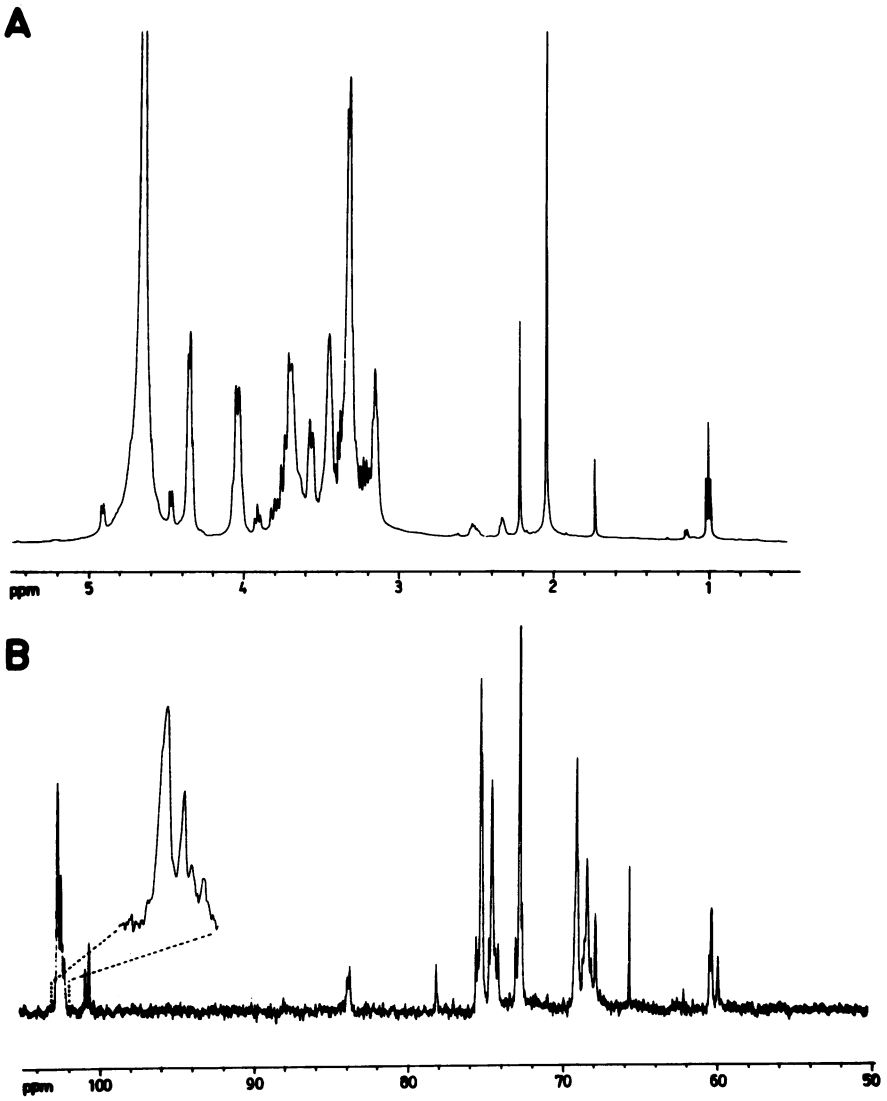

FIG. 4. NMR spectra of *A. brasilense* glucan in D_2O. (A) ^1H NMR spectrum in the region from 32 to 50 ppm. The 4.86- and 4.48-ppm signals are visible. (B) ^{13}C NMR spectrum. Inlet expansion of the anomeric carbon atom (C-1) showing the signal 1→6, 104.0 ppm, and 1→3, 103.8 ppm.

their identities as laminaribiose and gentiobiose were confirmed.

The same products were obtained by using permeabilized cells as the enzyme source (Fig. 2C). It can be seen that the profile was similar to that obtained after partial hydrolysis of the ^{14}C-labeled β(1-3),β(1-6) glucan obtained in vitro from inner membranes of *B. japonicum* (Fig. 2D), although a higher proportion of laminaribiose seems to be present in *B. japonicum* glucan. A comparison of the partial acid hydrolysis profiles of glucan formed in vitro and in vivo revealed that the in vitro-synthesized glucan is enriched in (β1-3) linkages (Fig. 2A and B).

Linear structure. The presence of a reducing terminal glucose was determined by subjecting 60,000 cpm of the K_{av} 0.40 glucan obtained in vivo or in vitro to $NaBH_4$ reduction. Total acid hydrolysis and identification of the resulting products by paper electrophoresis with buffer C showed that the glucan formed in vivo yielded between 4 and 6% D-sorbitol. The product obtained in vitro yielded 9 to 11% D-sorbitol under conditions in which *A. tumefaciens* and *B. japonicum* cyclic glucans yielded no detectable D-sorbitol, suggesting the presence of a free reducing terminus (Fig. 9).

Degree of polymerization. In order to determine the degree of polymerization of the glucan formed in vitro, it was subjected to partial acid hydrolysis and chromatography on a Bio-Gel P2 column. As shown in Fig. 8, a series of oligosaccharides with increasing degrees of polymerization were resolved (Fig. 8B). A plot of the degree of polymerization versus the log of the elution volume (fraction number) showed a linear function (Fig. 8C). The glucan formed in vitro eluted from the column with an elution volume that corresponded to an apparent degree of polymerization of approximately 9 glucose units (compound II in Fig. 8C). The glucan formed in vivo (Fig. 8A and compound I in Fig. 8B and C) eluted with a volume that corresponds to 10 glucose residues.

Smith degradation. Periodate oxidation and $NaBH_4$ reduction were carried out on the K_{av} 0.4 glucan formed in vivo. The treatment released approximately 10% of the radioactivity as a compound recovered from the Bio-Gel P2 column close to the total volume. Most of the radioactivity was recovered as a

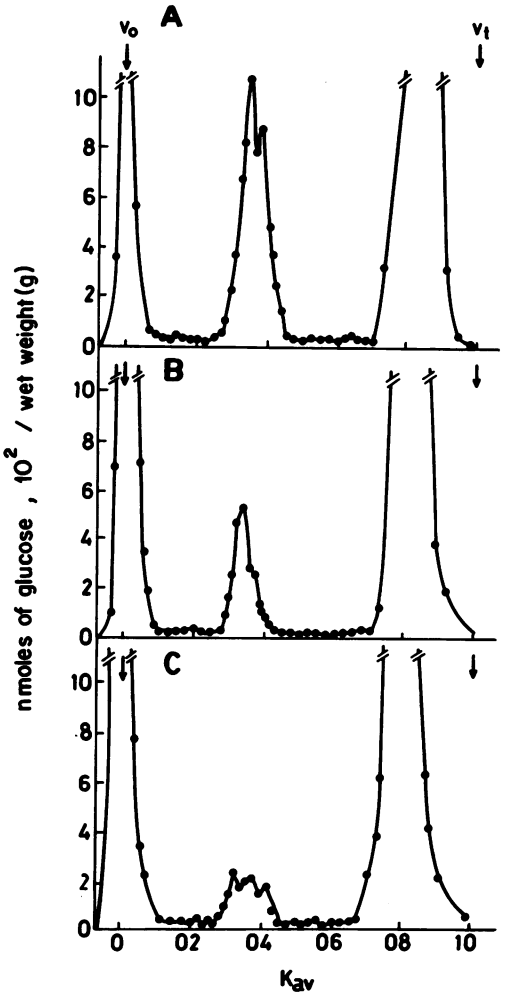

FIG. 5. Osmoregulation of *A. brasilense* periplasmic glucan synthesis. TCA extracts obtained from *A. brasilense* grown on LB medium (A) or LB medium supplemented with 0.5 M mannitol (B) or 0.25 M NaCl (C) were chromatographed on a Bio-Gel P4 column and eluted with 0.1 M pyridine–acetate buffer (pH 5.5). Fractions of 1.0 ml were collected. Sugars were detected as described in the legend to Fig. 1. Data are 10^2 nmol of glucose per g (wet weight).

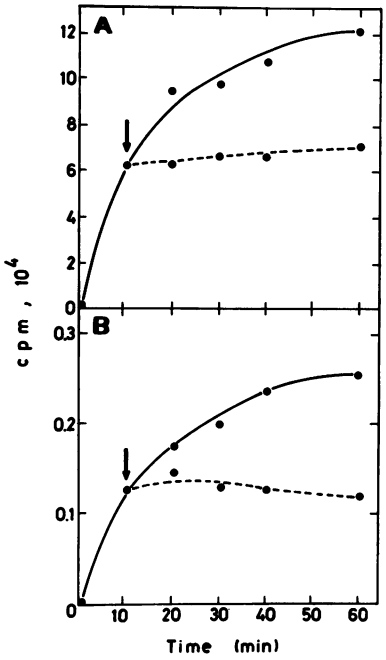

FIG. 6. Glucan synthesis by inner membranes of *A. brasilense*. Inner membranes were incubated with UDP-[^{14}C]glucose as described in Materials and Methods. Incorporation of [^{14}C]glucose into the soluble fraction (A) and the insoluble fraction (10% TCA precipitate) (B). ---, incorporation of [^{14}C]glucose after addition of 2 mM nonradioactive UDP-glucose; arrows indicate the time of addition of nonradioactive UDP-glucose.

product that eluted from the column with the same elution volume as the untreated control. This product was recovered from the column and subjected to mild acid hydrolysis and paper chromatography with solvent B as described in Materials and Methods. Glycerol and two other compounds were recovered (Fig. 11A).

Compound a in Fig. 11A was eluted from the paper strip with water and subjected to gel chromatography in order to determine the degree of polymerization. As shown in Fig. 11C, the compound eluted from the Bio-Gel P2 column with a lower elution volume than the tetrasaccharide stachiose, in a position between the tetrasaccharide and the pentasaccharide of the calibration plot shown in Fig. 8C.

The compound was subjected to partial acid hydrolysis and paper chromatography with solvent B. As shown in Fig. 11B, glycerol, glucose, laminaribiose, and gentiobiose were recovered. Thus, the compound is a glycerol-substituted tetrasaccharide formed by β1-3 and β1-6 linkages, demonstrating that both linkages are part of the same molecule of glucan.

Compound b was eluted from the paper strip with water and subjected to total acid hydrolysis and paper chromatography with solvent B. Glucose and glycerol were recovered (data not shown). Compound b is therefore glucose substituted with glycerol.

Regulation by osmolytes. To characterize the inhibition of periplasmic glucan synthesis by osmolytes (Fig. 5), we determined the glucosyltransferase activities in total membranes obtained from cells growing in medium of low or high osmolarity. Inner membranes prepared from cells grown on LB in the presence of 250 mM NaCl showed an 85% reduction in UDPglucose-β(1-3),β(1-6)-glucosyltransferase activities compared with the activities of inner membranes prepared from cells grown on LB medium (Fig. 10).

Inner membranes were prepared from *A. brasilense* SA1, a recombinant strain that expresses the osmolyte-insensitive β(1-2) glucosyltransferase of *A. tumefaciens* (1, 34). In cells grown on LB medium supplemented or not with 250 mM NaCl, no difference in the activity of β(1-2) glucosyltransferase was detected (data not shown). These results indicate that high-osmolarity repression of the activity of the wild-type *A. brasilense* β(1-3),β(1-6) glucosyltransferase(s) is not a nonspecific effect that inhibits all membrane glucosyltransferases.

DISCUSSION

R. meliloti, *A. tumefaciens*, and *B. japonicum* accumulate cyclic glucans in the periplasmic space (23, 24, 27). A key role in the bacterium-plant interaction was assigned to *R. meliloti* and *A. tumefaciens* cyclic β(1-2) glucans. A cyclic β(1-3),β(1-6) glucan from *B. japonicum* was recently described, although its role in relation to plant interaction is still unknown (14, 23).

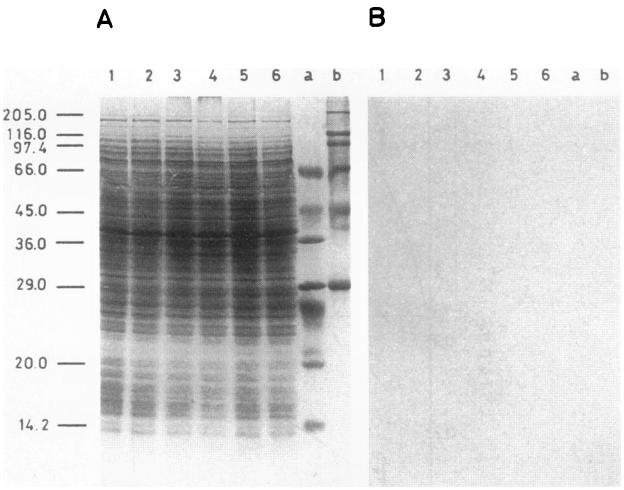

FIG. 7. Polyacrylamide gel electrophoresis of inner membranes of *A. brasilense*. Inner membranes (0.2 mg of protein) were incubated with UDP-[^{14}C]glucose, the reaction was stopped at different times by the addition of 10% TCA, and precipitates were subjected to gel electrophoresis as described in Materials and Methods. Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). Lanes 1, 2, 3, and 5, 0, 10, 20, and 60 min of incubation, respectively. Lanes 4 and 6, chase experiment in which, after 10 min of incubation with UDP-[^{14}C]glucose, 2 mM nonradioactive UDP-glucose was added, and incubation was continued for 10 and 50 min, respectively. Lanes a and b, size standards; sizes are shown at the left (in kilodaltons).

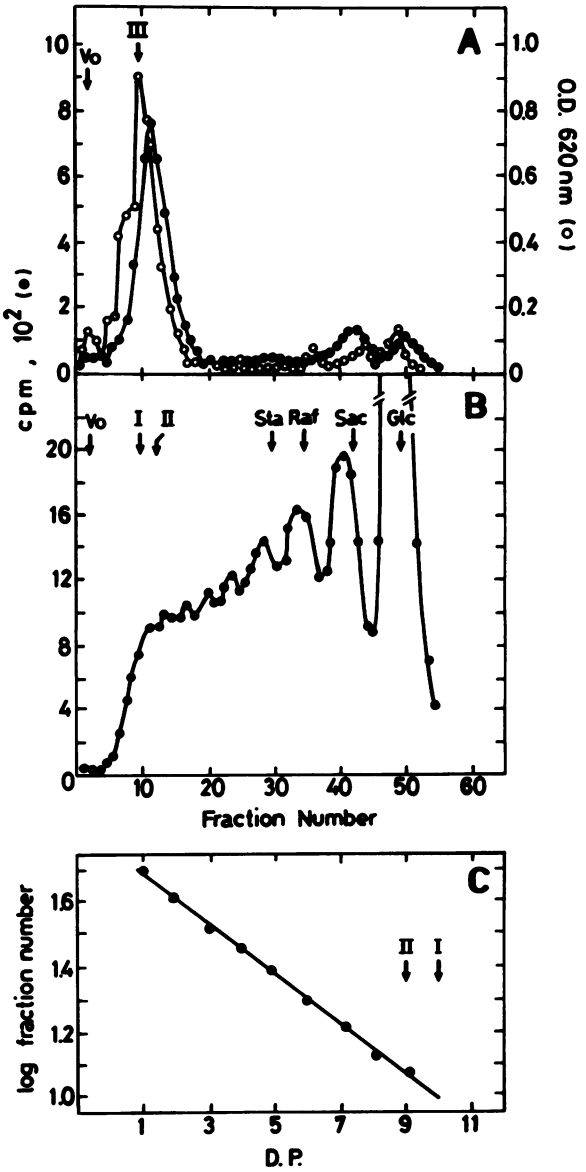

FIG. 8. Bio-Gel P2 chromatography. Neutral soluble glucan was subjected to Bio-Gel P2 chromatography before and after partial acid hydrolysis as described in Materials and Methods. (A) Elution profile before partial acid hydrolysis. Open circles, glucan recovered from cells by extraction with 10% TCA; solid circles, glucan formed in vitro with inner membranes incubated with UDP-[^{14}C]glucose. (B) Elution profile of products after partial acid hydrolysis. (C) Plot of degree of polymerization (D.P.) versus log of fraction number. I, elution position of cellular glucans recovered in vivo; II, elution position of glucan formed in vitro; III, elution position of the cyclic β(1-3),β(1-6) glucan formed in vitro by inner membranes of *B. japonicum* USDA 110. Products were filtered on a Bio-Gel P2 column (116 by 1 cm) eluted with 0.1 M pyridine–acetate buffer (pH 5.5). Fractions of 1 ml were collected, and radioactivity was determined by counting in a liquid scintillator. Internal standards of unlabeled sugars: Glc, glucose; Sac, sucrose; Raf, raffinose; Sta, stachiose.

It has been reported that soil bacteria of the genus *Azospirillum* colonize nonleguminous roots (8, 25). However, as far as we know, none of the gene products involved in this interaction have been described.

We report here that *A. brasilense* produces a β(1-3),β(1-6) glucan similar to that found in *B. japonicum*. However, the *Azospirillum* glucan is not cyclic, since after ^{14}C-labeled glucan synthesized in vivo or in vitro was subjected to sodium borohydride reduction, [^{14}C]sorbitol was recovered. The amount of labeled sorbitol recovered from the in vivo-labeled glucan was lower than expected from its degree of polymerization (4 to 6% instead of 10%). It is possible that during the 3 h of labeling with [^{14}C]succinic acid, label was not incorporated into the glucan uniformly, and thus some glucan molecules might have no [^{14}C]glucose at the reducing end. On the other hand, the in vitro-synthesized glucan obtained after incubation of inner membranes with UDP-[^{14}C]glucose has the expected 10% [^{14}C]sorbitol. It is worth noting that, under in vitro conditions that led to the formation of cyclic glucans by inner membranes of *A. tumefaciens* (35), *R. meliloti* (12), *Rhizobium loti* (19), and *B. japonicum* (14), *A. brasilense* inner membranes formed open linear structures. Moreover, it was not possible to detect any intermediate [^{14}C]glucose-labeled protein, which has been observed in all the other systems that form cyclic glucans (12, 14, 19, 35).

^{13}C NMR spectra revealed no signals at 92 to 96 ppm, suggesting the absence of a free reducing glucose; however, a carbonyl signal is visible at 183 ppm, suggesting that the laking of the signal at 92 to 96 ppm might be due to the presence of a substituted reducing terminal glucose.

From the available data, we postulate that the *A. brasilense* cell-associated β(1-3),β(1-6) glucan is linear.

Another difference from the *B. japonicum* β(1-3),β(1-6) glucan is the absence in the NMR spectrum of the 55.2-ppm signal assigned to choline (28), a nonglycosidic substituent present in *B. japonicum* cellular glucan.

In *B. japonicum*, a 90-kDa inner membrane protein was

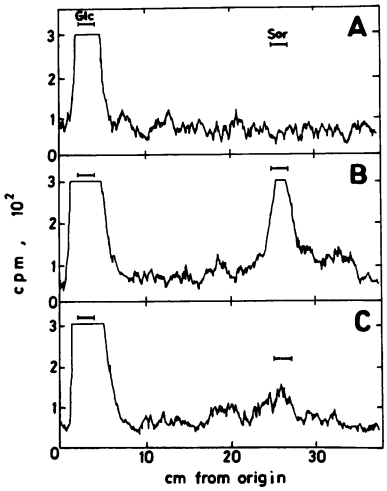

FIG. 9. Paper electrophoresis of total acid hydrolysis products after reduction with sodium borohydride. Approximately 60,000 cpm of glucan formed in vitro by inner membranes of *A. brasilense* was subjected to reduction with $NaBH_4$ followed by total acid hydrolysis as described in Materials and Methods. Products were subjected to paper electrophoresis with buffer C, and radioactivity was detected with a radioscanner. (A) Total acid hydrolysis of *A. brasilense* glucan without reduction with $NaBH_4$. (B) Total acid hydrolysis of *A. brasilense* glucan after reduction with $NaBH_4$. (C) Total acid hydrolysis of *A. tumefaciens* glucan after reduction with $NaBH_4$. Standards: Glc, glucose; Sor, sorbitol.

described as being an intermediate in the synthesis of the β(1-3),β(1-6) glucan (14); this protein may play the equivalent role of intermediate assigned to the 235-kDa protein involved in *A. tumefaciens*, *R. meliloti*, and *R. loti* cyclic β(1-2) glucan synthesis (12, 19, 36). Our results indicated that no intermediate protein participates in the synthesis of the linear β(1-3),β(1-6) glucan in *A. brasilense*, suggesting that the participation of an intermediate protein is restricted to the synthesis of cyclic glucans.

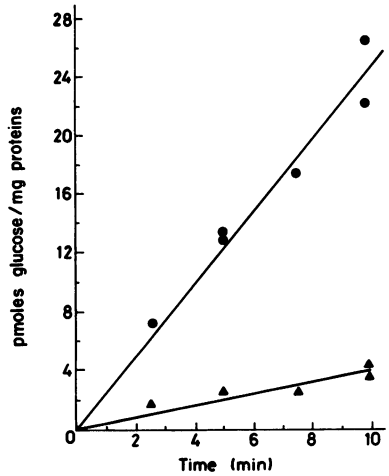

FIG. 10. Glucosyltransferase activity of *A. brasilense* grown in medium of low or high osmolarity. Membranes prepared from cells grown on LB (●) or LB with 0.25 M NaCl (▲) were incubated with UDP-[^{14}C]glucose. Formation of soluble glucan was determined as described in Materials and Methods.

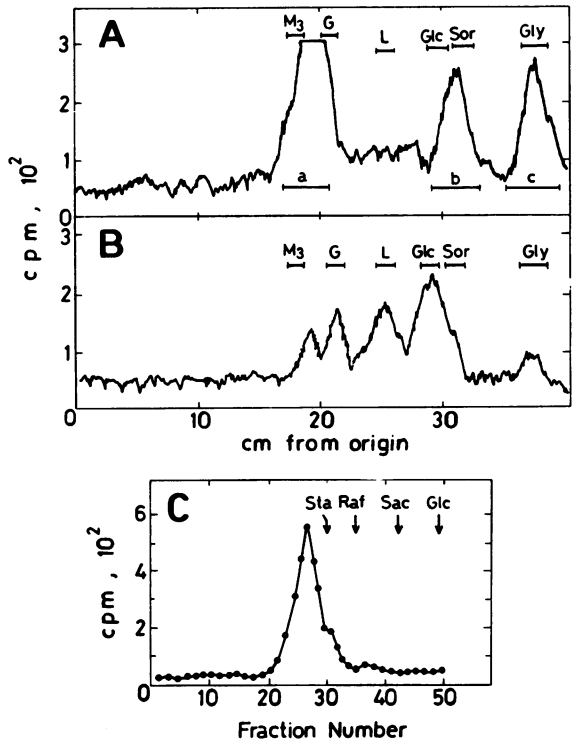

FIG. 11. Periodate oxidation of β(1-3),β(1-6) glucan formed in vivo by *A. brasilense*. ^{14}C-labeled glucan formed in vivo by *A. brasilense* was purified by Bio-Gel P4 chromatography and subjected to periodate oxidation as described in Materials and Methods. (A) Paper chromatography with solvent B of products recovered after mild acid hydrolysis (see Materials and Methods). (B) Paper chromatography with solvent B of product a from panel A after partial acid hydrolysis. (C) Bio-Gel P2 chromatography of compound a. Standards: M_3, maltotriose; G, gentiobiose; L, laminaribiose; Glc, glucose; Sor, sorbitol; Gly, glycerol; Sac, sucrose; Raf, raffinose; Sta, stachiose.

The *Azospirillum* β(1-3),β(1-6) glucosyltransferase(s) is an inner-membrane-bound protein(s), requiring Mg^{2+} and UDP-glucose as a sugar donor, with an optimum temperature of 30°C. Although no protein intermediate was found to be formed during the in vitro synthesis of the *Azospirillum* glucan, the accumulation of glucose-containing organic soluble products was observed (data not shown). Further work will be required to establish the participation of lipid intermediates in the synthesis of this *Azospirillum* glucan.

Our results also show that the *A. brasilense* glucan is localized in the periplasmic space, like the membrane-derived oligosaccharides of *E. coli* (16), the cyclic β(1-2) glucans of *A. tumefaciens* (24), and the cyclic β(1-3),β(1-6) glucan of *B. japonicum* (23).

High osmolarity inhibited the in vivo accumulation of the β(1-3),β(1-6) glucan of *A. brasilense*. This conserved response suggests that there might be close parallels between the mechanisms that these organisms employ to regulate the response to osmotic stress. It has been reported that the glucosyltransferase responsible for the synthesis of β(1-2) glucan was not repressed by high osmolarity in *R. meliloti* and *A. tumefaciens* (34). Rumley et al. (29) showed that glucosyltransferase activity in *E. coli* membrane-derived oligosaccharides is also not repressed by the osmolarity of the medium in which the cells have been grown; in this way, the *E. coli* transferase resembles the *A. tumefaciens* transferase. However,

the *Azospirillum* UDPglucose-β(1-3),β(1-6)-glucosyltransferase activity of membranes from cells grown in medium of high osmolarity was about 85% lower than that of membranes prepared from cells grown in regular medium. Thus, this is the first report of the activity of a glucosyltransferase being reduced in membranes of cells grown in medium of high osmolarity.

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The contributions of Silvia G. Altabe and Nora Iñón de Iannino to this work are equivalent.

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REFERENCES

1. **Altabe, S. G., N. Iñón de Iannino, D. de Mendoza, and R. A. Ugalde.** 1990. Expression of the *Agrobacterium tumefaciens chvB* virulence region in *Azospirillum* spp. J. Bacteriol. **172:**2563–2567.
2. **Amman, P., M. McNeil, L. Franzen, A. G. Darvill, and P. Albersheim.** 1981. Structural elucidation, using HPLC-MS and GLC-MS, of the acidic polysaccharide secreted by *Rhizobium meliloti* strain 1021. Carbohydr. Res. **95:**263–283.
3. **Baumann, M., H. Simon, K. H. Schneider, H. J. Danneel, U. Küster, and F. Giffhorn.** 1989. Susceptibility of *Rhodobacter sphaeroides* to β-lactam antibiotics: isolation and characterization of a periplasmic β-lactamase (cephalosporinase). J. Bacteriol. **171:**308–313.
4. **Bourne, E. J., D. H. Huston, and H. Weigel.** 1961. Complexes between molybdate and acyclic polyhydroxy-compounds. J. Chem. Soc. (London) Part I, p. 35–38.
5. **Cohen, J. L., and K. J. Miller.** 1991. A novel membrane-bound glucosyltransferase from *Bradyrhizobium japonicum*. J. Bacteriol. **173:**4271–4276.
6. **Courtright, J. B., and U. Henning.** 1970. Malate dehydrogenase mutants in *Escherichia coli* K-12. J. Bacteriol. **102:**722–728.
7. **Dische, Z.** 1962. General color reations. Methods Carbohydr. Chem. **1:**478–492.
8. **Dudman, W. F., and A. Jones.** 1980. The extracellular glucans of *Rhizobium japonicum* strain 311b 71. Carbohydr. Res. **84:**358–364.
9. **Dylan, T., L. Ielpi, S. Stanfield, L. Kashyap, C. J. Douglas, M. Yanofsky, E. W. 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. **Elmerich, C.** 1984. Molecular biology and ecology of diazotrophs associated with non-leguminous plants. Bio/Technology **2:**967–978.
11. **Foster, A. B.** 1957. Zone electrophoresis of carbohydrates. Adv. Carbohydr. Chem. **12:**81–115.
12. **Geremia, R. A., S. Cavaignac, 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.
13. **Iñón de Iannino, N., and R. A. Ugalde.** 1989. Biochemical characterization of avirulent *Agrobacterium tumefaciens chvA* mutants: synthesis and excretion of β(1-2) glucan. J. Bacteriol. **171:**2842–2849.
14. **Iñón de Iannino, N., and R. A. Ugalde.** 1993. Biosynthesis of cyclic β(*1-3*),β(*1-6*) glucan in *Bradyrhizobium* spp. Arch. Microbiol. **159:**30–38.
15. **Jeanes, A., C. S. Wise, and R. J. Dimler.** 1951. Improved techniques in paper chromatography of carbohydrates. Anal. Chem. **23:**415–420.
16. **Kennedy, E. P.** 1982. Osmotic regulation and biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79:**1092–1095.
17. **Lacroix, J. M., I. Loubens, M. Tempte, B. Menichi, and J. P. Bohin.** 1991. The *mdoA* locus of *Escherichia coli* consists of an operon under osmotic control. Mol. Microbiol. **5:**1745–1753.
18. **Leigh, J. A., E. R. Signer, and G. C. Walker.** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82:**6231–6235.
19. **Lepek, V., Y. Navarro de Navarro, and R. U. Ugalde.** 1990. Synthesis of β(1-2) glucan in *Rhizobium loti*. Arch. Microbiol. **155:**35–41.
20. **Long, S., J. W. Reed, J. Himawan, and G. C. Walker.** 1988. Genetic analysis of a cluster of genes required for the synthesis of the Calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. J. Bacteriol. **170:**4231–4248.
21. **Michiels, K., J. Vanderleyden, and A. Van Gool.** 1989. *Azospirillum*-plant root associations: a review. Biol. Fertil. Soils **8:**356–365.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **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.
24. **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.
25. **Okon, Y.** 1985. *Azospirillum* as a potential inoculant for agriculture. Trends Biotechnol. **3:**223–228.
26. **Osborn, M. J., and R. Munson.** 1984. Separation of inner (cytoplasmic) and outer membranes of gram negative bacteria. Methods Enzymol. **31A:**642–653.
27. **Puvanesarajah, V., F. M. Schell, G. Stacey, C. J. Douglas, and E. W. Nester.** 1985. A role for 2-linked β-D-glucan in the virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **164:**102–106.
28. **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 β-*(1,3);(1,6)* macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. Biochim. Biophys. Acta **1116:**215–225.
29. **Rumley, M. K., H. Therisod, A. C. Weissborn, and E. P. Kennedy.** 1992. Mechanism of regulation of the biosynthesis of membrane derived oligosaccharide in E. coli. J. Biol. Chem. **267:**11806–11810.
30. **Stanfield, S. W., L. Ielpi, D. O'Brochta, D. R. Helinski, and G. S. Ditta.** 1988. The *ndvA* gene product of *Rhizobium meliloti* is required for β(1-2) glucan production and has homology to the ATP-binding export protein HlyB. J. Bacteriol. **170:**3523–3530.
31. **Tolmasky, M. E., R. J. Staneloni, R. A. Ugalde, and L. F. Leloir.** 1980. Lipid bound sugars in Rhizobium meliloti. Arch. Biochem. Biophys. **203:**358–364.
32. **Trevelyan, W. F., D. P. Procter, and J. S. Harrison.** 1950. Detection of sugar on paper chromatograms. Nature (London) **166:**444–445.
33. **Tung, K. K., and J. H. Nordin.** 1968. Structure of the tetrasaccharide produced by the hydrolysis of nigeran by the enzyme mycodextranase. Biochim. Biophys. Acta **158:**154–156.
34. **Zorreguieta, A., S. Cavaignac, R. A. Geremia, and R. A. Ugalde.** 1990. Osmotic regulation of β(1-2) glucan synthesis in members of the family *Rhizobiaceae*. J. Bacteriol. **172:**4701–4704.
35. **Zorreguieta, A., R. A. Geremia, S. Cavaignac, G. Cangelosi, E. W. Nester, and R. A. Ugalde.** 1988. Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. Mol. Plant-Microbe Interact. **1:**121–127.
36. **Zorreguieta, A., and R. A. Ugalde.** 1986. Formation in *Rhizobium* and *Agrobacterium* spp. of a 235-kilodalton protein intermediate in β-D(1-2) glucan synthesis. J. Bacteriol. **167:**947–951.