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A *Rhizobium meliloti* Mutant That Forms Ineffective Pseudonodules in Alfalfa Produces Exopolysaccharide But Fails to Form β-(1→2) Glucan†

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A mutant of *Rhizobium meliloti* that elicited the formation of inactive nodules in alfalfa was found not to form β-(1→2) glucan in vivo or in vitro. It was nonmotile because it lacks flagella. The 235-kilodalton protein which acts as an intermediate in β-(1→2) glucan synthesis was undetectable in the mutant. These properties of the mutant are common to those of *chvB* mutants of *Agrobacterium tumefaciens*. Exopolysaccharide formation by the *R. meliloti* mutant was about double that by the wild type.

Nitrogen-fixing nodules are formed by the symbiotic association of rhizobia with legumes. The coordinated development of these nodules requires a multistep process in which specific plant and bacterial products are involved (for a review, see references 24 and 25).

In *Rhizobium meliloti* a cluster of *nod* loci is linked to the nitrogenase *nif* loci on a large megaplasmid (3, 4, 12, 14, 16, 19). The function of these genes seems to be common to several species, since mutants are complemented with cloned DNA from different rhizobia without changing plant host specificity (9). Therefore, they are referred to as common *nod* genes. Other loci besides the common genes have been described in *R. meliloti* as being required for specific invasion of the host (14). The function of these genes and their role in nodulation are not understood. The possible role of *Rhizobium* polysaccharides in the nodulation process has been the subject of speculation for a number of years. Further evidence was presented recently when a large set of mutants of *R. meliloti* affected in the synthesis of exopolysaccharide was obtained and found to form ineffective pseudonodules (8, 15). The nodulation step associated with the presence of exopolysaccharide was found to be the invasion rather than the formation of the nodules (8, 15). Exo$^-$ mutants were not able to induce shepherd's crooks and infection threads (15), suggesting that the exopolysaccharide might be involved in the induction of a signal required for the invasion process. It has been reported that rhizobial polysaccharides promote root hair deformation and infection threads (1, 2).

Another polysaccharide involved in infection is of the teichuronic acid type and contains galacturonic acid and galactose; it is found in the more competitive variants of *R. meliloti* (10, 22, 23). It was proposed to be responsible for some surface properties such as reactivity to agglutinin and antisera and phage sensitivity (23). Mutants selected for phage resistance lost competitiveness and the ability to form the polysaccharide (10, 23). More competitive strains were found to nodulate faster than were isogenic mutants that do not produce the anionic polysaccharide, suggesting some role for the latter in the invasion process.

According to recent evidence, β-(1→2) glucan may have a role in *Agrobacterium* infections. Avirulent mutants of *Agrobacterium tumefaciens* affected in the *chvB* locus (5) have been found not to attach to plant cells, to have no flagella, and to be defective in the formation of β-(1→2) glucan (18). These mutants lack a 235-kilodalton (kDa) inner membrane protein required for the synthesis of the glucan (28). No mutants affected in the synthesis of β-(1→2) glucan in *Rhizobium* species have been described until now.

In this paper we describe some relevant biochemical properties of a mutant of *R. meliloti* GRT21s (21). This mutant is generated by heat treatment, grows at the same rate as the parental strain, is prototrophic, and elicits pseudonodules in alfalfa. It has a small-colony phenotype that could be due to the fact that it is nonmotile. The location of the mutation is unknown. No deletions on the megaplasmid are detectable by agarose gel electrophoresis.

MATERIALS AND METHODS

Organism and growth conditions. *R. meliloti* GR4 and GTR21s were previously described (21). Bacteria were grown in yeast extract-mannitol medium (26) at 28°C for 24 or 48 h.

Isolation of β-(1→2) glucan from cells. Cells from a 1-liter culture were harvested by centrifugation at 5,000 × *g* for 20 min and washed once with Tris hydrochloride (0.03 M) buffer (pH 8.2), and the pellet was extracted with trichloroacetic acid (TCA) as described previously (17). TCA extracts were neutralized with ammonium hydroxide, concentrated, and subjected to gel filtration on Bio-Gel P4 columns (78 by 1.8 cm) (Bio-Rad Laboratories, Richmond, Calif.). The columns were eluted with pyridine-acetate (0.1 M) buffer (pH 5.5), fractions of 1 ml were collected, and carbohydrates were detected by the phenol-sulfuric acid method (4). Polysaccharides eluting with K_{AV} between 0.12 and 0.27 were pooled, concentrated, and subjected to anion-exchange chromatography on Dowex AG2 × 10 (Serva, Heidelberg, Federal Republic of Germany) columns equilibrated with

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TABLE 1. Properties of the exopolysaccharide formed by wild-type GR4 and mutant GRT21s of *R. meliloti*

Strain	Amt of:			K_{AV}[a]	Amt of substituent (mol/repeating unit)[b]:	
	Exopolysaccharide (mg [dry wt]/mg of cell protein)	Glucose[c]	Galactose[c]		*O*-Acetyl	Pyruvic acid
GR4	0.78	++++	+	0.16	0.8	1.37
GRT21s	1.53	++++	+	0.16	0.9	1.48

[a] Determined by gel chromatography as described in Materials and Methods.
[b] Moles of substituent per 8 mol of sugar (glucose equivalents determined by the phenol-sulfuric acid method).
[c] Determined by paper chromatography of the total acid hydrolysate of purified exopolysaccharide. No other sugars were detected.

pyridine-acetate (0.01 M) buffer (pH 5.5). The columns were washed with the equilibration buffer until no phenol-sulfuric acid-positive material was detected. The eluate contained the neutral polysaccharides. Anionic polysaccharides were recovered from the column after it was washed with 1 M acetic acid. Both fractions were concentrated under vacuum, and total carbohydrates were estimated by the phenol-sulfuric acid method.

Isolation of polysaccharides from the culture supernatant. Culture supernatants obtained by centrifugation at 5,000 × *g* for 20 min were concentrated to one-fifth the original 1-liter volume under vacuum. Three volumes of ethanol were added, and the exopolysaccharide was wound around a glass rod. Short fibers not adhered to the glass rod were collected by centrifugation at 13,000 × *g* for 30 min. The supernatant after ethanol precipitation was dried under vacuum, dissolved in water, and precipitated overnight at 4°C with 10 volumes of ethanol. The precipitate was recovered by centrifugation at 17,000 × *g* for 20 min, dissolved in water, ultrafiltered three times through Amicon YM 2 membranes (Amicon Corp., Danvers, Mass.), and concentrated to 2 ml. The concentrate was chromatographed on Bio-Gel P4 and Dowex AG2 × 10 as described above for the isolation of β-(1→2) glucan from cells.

Preparation of inner membranes and in vitro assay. Inner membranes were prepared from 24-h cultures as previously described (30). The in vitro assay of β-(1→2) glucan synthesis and the formation of the protein intermediate were as described previously (29). UDP-[U-^{14}C]glucose (200 to 300 Ci/mol) was prepared as described previously (27). Polyacrylamide gel electrophoresis was carried out as previously described (29).

Acid hydrolysis and paper chromatography. Partial acid hydrolysis was carried out in 0.5 N HCl at 100°C for 20 to 30 min, and total acid hydrolysis was carried out in 1 N HCl at 100°C for 4 h. After the acid treatments, HCl was removed by evaporation under vacuum (four times), and samples were subjected to descending paper chromatography on Whatman no. 1 paper (Whatman Ltd., Clifton, N.J.) with solvent A (butanol, pyridine, water [6:4:3]) or solvent B (isopropanol, acetic acid, water [27:4:9]). Sugars were localized by the alkaline-silver method.

Characterization of the exopolysaccharide. Exopolysaccharide was recovered from the culture supernatant by ethanol precipitation as described above; after three reprecipitations, it was lyophilized and weighed. Dried exopolysaccharide (1 mg) was dissolved in water and chromatographed on a Bio-Gel A5m column (44 by 1 cm) (Bio-Rad) equilibrated with pyridine-acetate (0.1 M) buffer (pH 5.5) at a flow rate of 0.12 ml/min. Fractions of 2 ml were collected, and the carbohydrates were detected by the phenol-sulfuric acid method. Fractions containing the exopolysaccharide were pooled, concentrated, and subjected to total acid hydrolysis as described above. Pyruvic acid and *O*-acetyl groups were determined as described previously (6, 20).

RESULTS

Formation of the exopolysaccharide. It has been reported previously that *R. meliloti* mutants which do not form exopolysaccharide form pseudonodules in alfalfa (8, 15). Wild-type parental strain GR4 and mutant strain GRT21s were grown on LB agar with 0.02% Calcofluor (Tinopal DMS-X; CIBA-GEIGY, Buenos Aires, Argentina), and fluorescence was observed under epi-illumination at 366 nm. Both strains were bright blue; however, the colonies from mutant GRT21s were more brilliant, as though more fluorescent material were being produced. Since fluorescence was found to be correlated in *R. meliloti* with the production of the galactose- and glucose-containing exopolysaccharide (8), culture supernatants from the wild type and the mutant were precipitated with ethanol, and the amount of exopolysaccharide produced was determined. The wild type yielded 0.78 mg of exopolysaccharide per mg of cell protein, and the mutant yielded 1.53 mg. This difference could account for the higher fluorescence observed in LB-Calcofluor plates. The exopolysaccharides recovered from the wild type and mutant were compared for molecular weight, sugar composition, and *O*-acetyl and pyruvic acid substituents (Table 1). No difference was detected between the strains, except that the mutant produced almost twice the amount of exopolysaccharide.

Absence of β-(1→2) glucan in *R. meliloti* GRT21s cells and culture supernatant fluid. *R. meliloti* forms, besides exopolysaccharide, β-(1→2) glucan, which can be recovered from the periplasmic space and the culture fluid. The amount recovered from cells under normal culture conditions can account for 0.5% of the cell weight. *A. tumefaciens* also forms β-(1→2) glucan, but avirulent mutants that do not form it have been isolated (5, 18). To detect β-(1→2) glucan in the cells and culture fluid of *R. meliloti*, we subjected TCA extracts from wild-type and mutant cells to gel chromatography on Bio-Gel P4 columns as described in Materials and Methods. A polysaccharide that eluted from the column with a K_{AV} of 0.18 was present in the wild-type cells but absent in the mutant cells (Fig. 1). For identification of this polysaccharide, fractions with a K_{AV} between 0.12 and 0.27 obtained from the wild-type strain were pooled, concentrated, and chromatographed on Dowex AG2 × 10 as described in Materials and Methods. Ninety-five percent of the polysaccharide recovered from this pool was neutral. The neutral fraction was concentrated and subjected to total and partial acid hydrolyses. After paper chromatography of the total acid hydrolysate in solvent A, glucose was the only sugar detected. Paper chromatography of the partial acid hydrolysate in solvent B yielded glucose, sophorose, and a series of

homologous oligosaccharides that had a higher degree of polymerization and that corresponded to those obtained after partial acid hydrolysis of a well-characterized, ^{14}C-labeled β-(1→2) glucan obtained in vitro (28). Therefore, the wild type formed β-(1→2) glucan, but mutant GRT21s did not.

In vitro synthesis of β-(1→2) glucan. Inner membranes of the wild type incorporated 4.12 pmol of glucose per min per mg of protein into neutral compounds, and those of mutant GRT21s incorporated 0.15 pmol. The small amount of radioactivity incorporated into neutral compounds by the inner membranes of the mutant was found to elute in high-pressure liquid chromatography (HPLC) with a retention time of 5 min, but no radioactivity was detected in the position that corresponds to β-(1→2) glucan (retention time, longer than 25 min; Fig. 2). Thus, mutant GRT21s failed to form β-(1→2) glucan when tested in vitro. HPLC of the neutral compounds formed by the inner membranes of the wild type showed that the degree of polymerization of β-(1→2) glucan was higher than that of β-(1→2) glucan formed by the inner membranes of *A. tumefaciens* (Fig. 2). These compounds were characterized as β-(1→2) glucans by partial acid hydrolysis (data not shown). The higher degree of polymerization of the most abundant species of *R. meliloti* GR4 β-(1→2) glucan as compared with that of *Agrobacterium* sp. β-(1→2) glucan was previously observed with two different strains of *A. tumefaciens* and *R. meliloti* (11, 13, 28).

Absence of the 235-kDa protein. It has been previously reported that avirulent mutants of *A. tumefaciens* that do not form β-(1→2) glucan lack a 235-kDa inner membrane protein (29). Wild-type and mutant inner membranes were analyzed for the presence of the 235-kDa protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Materials and Methods. Inner membranes of mutant cells had no 235-kDa protein detectable by Coomassie blue (Fig. 3A, lanes 3 and 4). Inner membranes were incubated with UDP-[U-^{14}C]glucose under conditions that led to the accumulation of β-(1→2) glucan on the 235-kDa protein intermediate (29). With mutant inner membranes, no radioactivity was detected in the position that corresponds to the 235-kDa protein or to any other protein (Fig. 3B, lanes 3 and 4). In addition, radioactivity accumulated in the 235-kDa protein by wild-type inner membranes decreased rapidly after a chase with 2 mM nonlabeled UDP-glucose (Fig. 3B, lanes 1 and 2), as expected for an intermediate. These results indicate that mutant GRT21s does not form β-(1→2) glucan, owing to a defect in the formation of the 235-kDa protein, which has been found previously to be required for the synthesis of the polysaccharide (29).

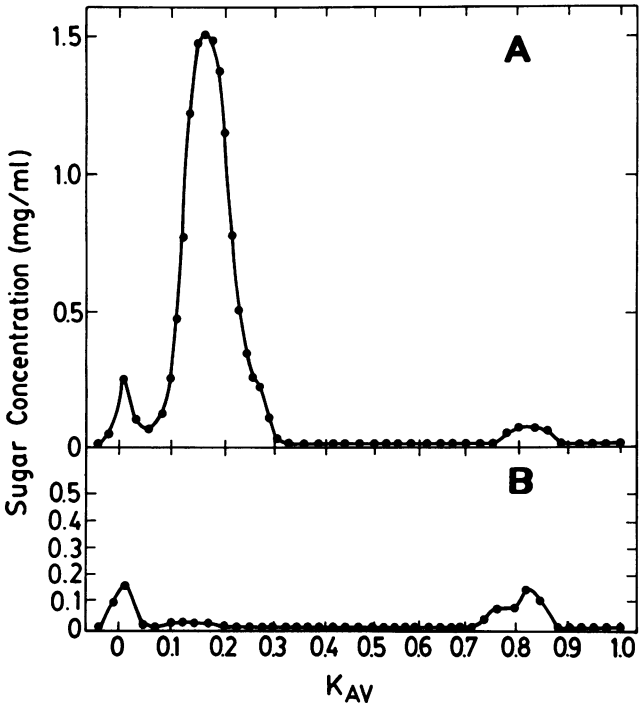

FIG. 1. Chromatography of TCA extracts from *R. meliloti* GR4 (A) and GRT21s (B). TCA extracts were prepared and chromatographed on Bio-Gel P4 columns as described in Materials and Methods.

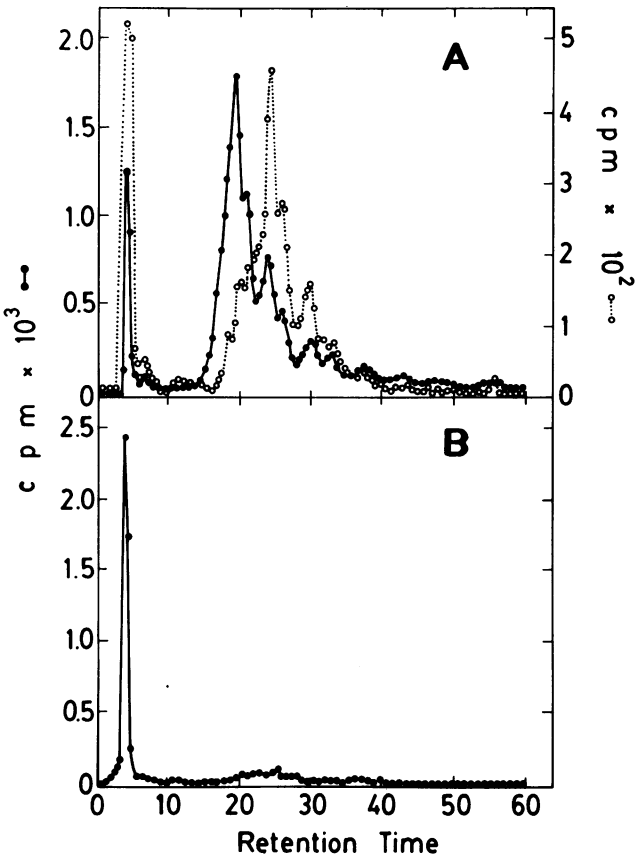

FIG. 2. HPLC of neutral compounds formed in vitro by inner membranes of *R. meliloti* GR4 and GRT21s. In vitro synthesis and HPLC were carried out as described previously (29). (A) The dotted line represents wild-type GR4 (^3H; 10,000 cpm), and the solid line represents *A. tumefaciens* A723 (^{14}C; 11,000 cpm). (B) Mutant GRT21s (^{14}C; 6,000 cpm).

Studies on motility. Avirulent mutants in the *chvB* locus of *A. tumefaciens* are nonmotile, owing to the lack of flagella (5), and are affected in the synthesis of β-(1→2) glucan (18). Motility was investigated in mutant GRT21s grown on semisolid medium; GRT21S was found to be nonmotile (Fig. 4). The same result was obtained when motility was observed under phase-contrast microscopy. Electron microscopy of wild-type and mutant cells showed that the mutant had no flagella. Thus, mutant GRT21s is very similar phenotypically to *chvB* mutants of *A. tumefaciens*.

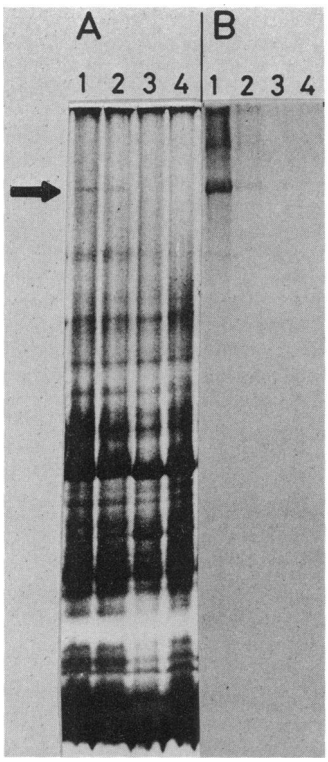

FIG. 3. Polyacrylamide gel electrophoresis of wild-type GR4 and mutant GRT21s inner membranes of *R. meliloti* incubated with UDP-[U-^{14}C]glucose. Lanes: 1, pulse, wild type; 2, pulse chase, wild type; 3, pulse, mutant; 4, pulse-chase, mutant. The pulse consisted of incubating inner membranes (0.2 mg of protein) for 10 min with UDP-[U-^{14}C]glucose. The chase was a subsequent incubation for 20 min with 2 mM UDP-glucose. The reaction was stopped with TCA, and the precipitate was subjected to electrophoresis as described previously (29). Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). The arrow indicates the position of the 235-kDa protein of *A. tumefaciens*.

DISCUSSION

Studies on *R. meliloti* GRT21s are of considerable interest and may help to clarify some aspects of the nodulation process and the role of the polysaccharides. This mutant, generated by heat treatment, forms white, empty, ineffective nodules in alfalfa and induces curling but no infection threads. The bacteria are found in the intercellular spaces but not in the root cortex (21).

When the mutant was grown on agar plates containing Calcofluor, the colonies appeared even brighter than those of the wild-type parental strain. This result is in contrast to what is observed with Exo$^-$ mutants (8), which are dark, although otherwise their nodulating phenotype is very similar to that of GRT21s. Quantitative estimation of the exopolysaccharide formed by the cells showed that mutant GRT21s produced about twice as much as the wild type. However, molecular weight, sugar composition, and amount of substituents were the same. The overproduction of exopolysaccharide by the mutant may have been due to an increased availability of endogenous UDP-glucose, the donor for the synthesis of glucan and exopolysaccharide. It was observed that *chvB* mutants of *A. tumefaciens* also produced more exopolysaccharide; furthermore, wild-type cells grown in a medium of high osmolarity that inhibits the synthesis of β-(1→2) glucan (17) also overproduced exopolysaccharide (unpublished results).

It was recently reported that avirulent *A. tumefaciens* mutants fail to form β-(1→2) glucan (18). The mutations map on a chromosomal locus named *chvB* (5). Biochemical characterization of *chvB* mutants showed that they fail to form a 235-kDa inner membrane protein intermediate required for the synthesis of β-(1→2) glucan (29). The 235-kDa protein is also present in inner membranes of *R. meliloti* (29). Recently, *R. meliloti* site-directed transposon insertion mutants in the *chvB*-homologous region were obtained. The region was named *ndvB* (7). One of these mutants has the same nodulation phenotype as GRT21s. It produces exopolysaccharide, but the formation of β-(1→2) glucan, the presence of flagella, and the 235-kDa protein were not studied (7).

β-(1→2) glucan could not be detected in the cells or in the culture fluid of mutant GRT21s. To further characterize the failure of mutant cells to form β-(1→2) glucan, we studied its in vitro synthesis. Inner membranes prepared from the mutant did not form β-(1→2) glucan in vitro, while wild-type inner membranes formed β-(1→2) glucans whose most abundant species eluted from the HPLC column with a retention time that corresponded to a higher degree of polymerization than that in *A. tumefaciens*. This result agrees with previous reports in which β-(1→2) glucans from *A. tumefaciens* and other strains of *R. meliloti* were compared (11, 13, 28). These results showed that mutant cells have some alteration in the enzyme(s) required for the synthesis of β-(1→2) glucan. It was previously reported that *A. tumefaciens chvB* mutants that fail to form β-(1→2) glucan do not have the 235-kDa inner membrane protein (29). This protein appears to be required for the synthesis of the polysaccharide because glucan chains are built up on it before being cyclized and released (29, 30). No 235-kDa protein was detectable in the mutant cells. Furthermore, when inner membranes of mutant cells were incubated with UDP-[U-^{14}C]glucose under conditions that led to the accumulation of the β-(1→2) glucan protein intermediate in the wild type, no radioactivity was found in the position that corresponds to the 235-kDa protein or any other protein. As previously described (29), the radioactivity accumulated in this protein behaves as an intermediate, since upon chase with nonlabeled UDP-glucose, the radioactivity decreases rapidly. We conclude that *R. meliloti* GRT21s does not form β-(1→2) glucan because its inner membranes are devoid of the 235-kDa

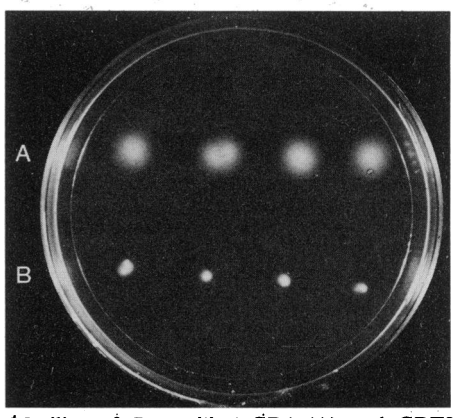

FIG. 4. Motility of *R. meliloti* GR4 (A) and GRT21s (B) in semisolid agar plates. Different clones were grown for 48 h at 28°C in yeast extract-mannitol medium with 0.3% agar.

protein, which is essential for the synthesis of the polysaccharide. Thus, *R. meliloti* GRT21s is very similar to *chvB* mutants of *A. tumefaciens*. The latter are avirulent, presumably because of defective attachment (5). Although this *R. meliloti* mutant elicits the formation of nodules, it is defective in invading the cortical cells. Its phenotype might be related to the Vir$^-$ phenotype of *chvB A. tumefaciens* mutants. Furthermore, like *chvB* mutants, GRT21s is nonmotile, owing to the lack of flagella. We conclude that the function(s) affected in mutant GRT21s leads to the same phenotype as that of the *chvB* mutants of *A. tumefaciens*; i.e., they do not form β-(1→2) glucan in vivo or in vitro, they do not have the 235-kDa inner membrane protein, and they have no flagella. Genetic and molecular analyses are in progress to establish whether this is an *R. meliloti* equivalent to the *ndvB* mutants recently described (7).

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