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Formation in *Rhizobium* and *Agrobacterium* spp. of a 235-Kilodalton Protein Intermediate in β-D(1-2) Glucan Synthesis†

ANGELES ZORREGUIETA AND RODOLFO A. UGALDE*

Instituto de Investigaciones Bioquímicas Fundacion Campomar, Facultad de Ciencias Exactas y Naturales and CONICET, 1405 Buenos Aires, Argentina

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β-D(1-2) Glucan was synthesized by *Agrobacterium* and *Rhizobium* spp. in vitro with enzymes from the internal membranes upon the addition of UDF glucose and Mg^{2+} or Mn^{2+}. An intermediate containing protein and β-D(1-2) glucan was formed during the reaction. It could be precipitated with trichloroacetic acid or separated by polyacrylamide gel electrophoresis under denaturing conditions. After detection with Coomassie blue or a radioactive substrate, the intermediate appeared as a 235-kilodalton protein. The radioactivity could be chased with a nonradioactive substrate. All strains that formed β-D(1-2) glucan in vitro formed the 235-kilodalton protein, whereas avirulent, β-D(1-2) glucan-negative mutants did not synthesize it. Transposon insertions in the *chvB* locus of strains ME2 and ME116 did not alter the virulence of the strains. These strains were able to form β-D(1-2) glucan in vitro and synthesize the 235-kilodalton protein.

Polysaccharides are believed to be involved in the infection process by which *Agrobacterium* spp. produce tumors in dicotyledonous plants and by which *Rhizobium* spp. induce the formation of nitrogen-fixing nodules in legumes. Exopolysaccharide (6, 11), lipopolysaccharide (2, 18), cellulose (13), and β-D(1-2) glucan (1, 16) have been implicated. The last compound is formed by both rhizobia and agrobacteria (9, 14, 19). Its enzymatic synthesis was reported previously (20). In a previous paper (21) evidence was presented indicating that β-D(1-2) glucan is synthesized in *Agrobacterium* spp. through the formation of a protein-containing intermediate as follows: UDP glucose + protein → protein β-D(1-2) glucan + UDP and protein β-D(1-2) glucan → protein + cyclic β-D(1-2) glucan. These studies have now been extended to some *Agrobacterium* mutants that do not form β-D(1-2) glucan and are not infectious (16). Chromosomal avirulent mutants mapped in two different transcriptional units named *chvA* and *chvB* (5). They were defective in plant cell attachment (4, 5), and it was recently shown that *chvB* mutants do not produce β-D(1-2) glucan in vivo (16).

In this paper we report studies on the in vitro synthesis of β-D(1-2) glucan in *Rhizobium meliloti* and in avirulent mutants of *Agrobacterium tumefaciens* and characterize the protein–β-D(1-2) glucan intermediate by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Organisms and culture media. *A. tumefaciens* LBA4001 was obtained from P. J. J. Hooykaas, State University of Leiden, Leiden, The Netherlands (10). All other strains of *A. tumefaciens* studied were described previously (5, 8). *Rhizobium meliloti* 102F51 was obtained from Nitragin Co., Milwaukee, Wis. *Agrobacterium* strains were grown in 0.5% tryptone–0.3% yeast extract medium at 28°C with good aeration for 16 or 20 h (A_{660}, 1.1 to 1.4). When required, kanamycin (100 μg/ml) or carbenicillin (100 μg/ml) was added to the same medium. *Rhizobium* strains were grown in yeast extract-mannitol medium (12) at 28°C until the A_{660} was about 0.7.

Enzyme preparation. Permeabilized cells (17) and inner membranes (15, 21) were prepared as previously described.

Assay. The reaction mixture contained 60 to 90 kcpm of UDP [U-^{14}C]glucose (320 Ci/mol), 50 mM Tris hydrochloride (pH 8.2), 10 mM $MgCl_2$, and enzyme (permeabilized cells or purified inner membranes) in a total volume of 50 μl. The reaction was carried out at 10°C for various times and stopped by heating at 100°C for 1 min. Centrifugation at 1,500 × *g* for 10 min yielded a pellet and a supernatant fluid. The latter was passed through a small DEAE-Sephadex G-25 column (0.6 by 4 cm) and eluted with water. The neutral labeled products recovered in the percolate contained mainly β-D(1-2) glucan. Radioactivity was determined by counting aliquots with Bray solution in a liquid scintillator. The pellet obtained as described above was suspended in 0.5 ml of 5% trichloroacetic acid (TCA) and filtered through a glass microfiber filter (Whatman GF/C). After being washed successively with 5% TCA and methanol, the dry filters were counted with toluene 2,5-diphenyoxazole–dimethyl 1,4-bis(5-phenyloxazolyl)benzene in a scintillator. Alternatively, the reaction was stopped by the addition of 1 ml of TCA as described below for gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One-dimensional polyacrylamide gel electrophoresis was performed on a 5 to 15% polyacrylamide continuous gradient as described previously (7). Samples of inner membranes (0.2 to 0.3 mg of protein) were precipitated with 10% TCA overnight at 5°C, and the pellet obtained by centrifugation was solubilized by heating at 100°C for 3 min in a cracking buffer containing 50 mM Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, 2% mercaptoethanol, 8 M urea, and bromophenol blue.

Fluorographic detection of radioactivity in polyacrylamide gels. Radioactivity in the polyacrylamide gels was detected by the methods described by Bonner and Laskey (3).

HPLC. The neutral reaction products recovered in the percolate of DEAE-Sephadex chromatography were subjected to high-performance liquid chromatography (HPLC) on a Lichrosorb-NH_2 column 104 (250 by 4.6 mm) (Merck & Co., Inc., Rahway, N.J.) eluted with acetonitrile-water

* Corresponding author.

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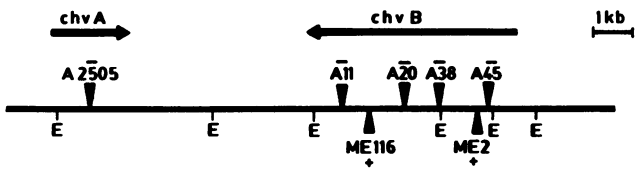

FIG. 1. *Eco*RI (E) restriction map of the virulence region of *A. tumefaciens*. The arrowheads and numbers above the horizontal line show the locations of Tn*5* insertions. The arrowheads below the horizontal line indicate the locations of Tn*3* and Tn*3*-HoHo insertions. +,Virulent; −, avirulent. The data are from reference 5. kb, Kilobases.

(70:30). The flow rate was 1 ml/min at 600 lb/in^2. Fractions of 0.5 ml were collected, and radioactivity was counted in Bray solution in a liquid scintillator.

RESULTS

β-D(1-2) glucan synthesis in different *A. tumefaciens* strains. It has been reported (16) that β-D(1-2) glucan cannot be detected in the cells or in the culture fluid of nonvirulent *chvB* mutants of *A. tumefaciens*. Figure 1 shows the location of different chromosomal insertions in the *chvB* locus. In vitro synthesis was measured with enzymes from such mutants and wild-type strains. Wild-type LBA4001 and A723 incorporated 4.1 and 2.6 nmol of glucose per h per mg of protein, respectively, into the neutral soluble fraction. HPLC of the neutral compounds formed by both strains showed that more than 80% corresponded to β-D(1-2) glucan, according to its retention time, as compared with a well-characterized cyclic β-D(1-2) glucan standard prepared as described previously (20) (retention time, > 30 min). The product obtained was polydisperse, and its degree of polymerization varied from 14 to 25 (20) (Fig. 2a). Mutants A1020, A1011, A1038, and A1045 incorporated <10% glucose into the neutral fraction, as compared with the wild type (A723) (Table 1). When the neutral fractions obtained from the mutants were analyzed by HPLC, only compounds eluting with a retention time of 5 min were detected; no β-D(1-2) glucan was detected (results are shown in Fig. 2c for strain A1020; strains A1011, A1038, and A1045 [data not shown] gave the same results). Transposon insertion-containing strains ME2 and ME116 (Fig. 1), which are virulent, had inner membranes as active as those of the wild-type strain A723 (Table 1), and the reaction products were identical, as judged by HPLC (Fig. 2b and d). Thus, mutations in the *chvB* locus that affect virulence impede the synthesis of β-D(1-2) glucan.

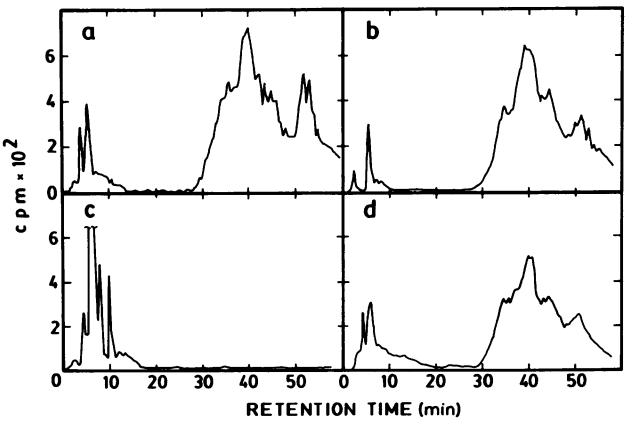

FIG. 2. HPLC of the neutral soluble fractions formed by different strains of *A. tumefaciens*. Neutral soluble fractions (about 4 × 10^4 cpm) formed by inner membranes of *A. tumefaciens* A723 (a), ME2 (b), and ME116 (d) and that (about 6 × 10^3 cpm) formed by inner membranes of mutant A1020 (c) were analyzed by HPLC as described in Materials and Methods.

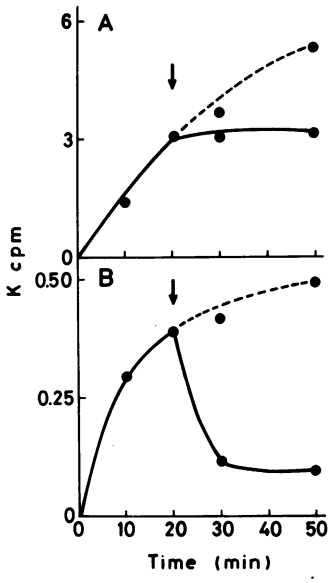

FIG. 3. β-D(1-2) glucan synthesis by inner membranes of *R. meliloti*. The experiment was carried out as described in Materials and Methods. (A) Incorporation of glucose into β-D(1-2) glucan (neutral fraction). (B) Incorporation of glucose into the protein–β-D(1-2) glucan intermediate (5% TCA precipitate). ————, incorporation after the addition of 2 mM nonradioactive UDP glucose (arrow). - - - -, control (no addition of UDP glucose).

β-D(1-2) glucan synthesis in *R. meliloti*. Permeabilized cells of *R. meliloti* also formed β-D(1-2) glucan in vitro (20). Purified inner membranes of *R. meliloti* 102F51 were used to characterize further the biosynthesis of β-D(1-2) glucan in this organism. The incorporation of glucose into the soluble fraction and the 5% TCA precipitate is shown in Fig. 3. After the addition of nonlabeled UDP glucose (2 mM), the radioactivity in the TCA precipitate decreased very rapidly at first and very slowly thereafter. The incorporation of radioactivity into the soluble fraction after the addition of UDP glucose

TABLE 1. Incorporation of glucose into the neutral soluble fraction by different strains of *A. tumefaciens*[a]

Strain	Virulence[b]	Attachment[b]	Glucose incorporated (nmol/h per mg of protein)
A1011 *chvB*::Tn*5*	−	−	0.2
A1020 *chvB*::Tn*5*	−	−	0.2
A1038 *chvB*::Tn*5*	−	−	0.2
A1045 *chvB*::Tn*5*	−	−	0.2
ME2 *chvB*::Tn*3*	+	+	3.6
ME116 *chvB*-Tn*3*::HoHo	+	+	3.7
A723 (wild type)	+	+	2.6
LBA4001 (wild type)	+	+	4.1

[a] The reaction was carried out for 60 min as described in Materials and Methods with 0.2 to 0.3 mg of protein from purified inner membranes.

[b] The virulence and attachment ability of the different strains were previously reported (4, 5).

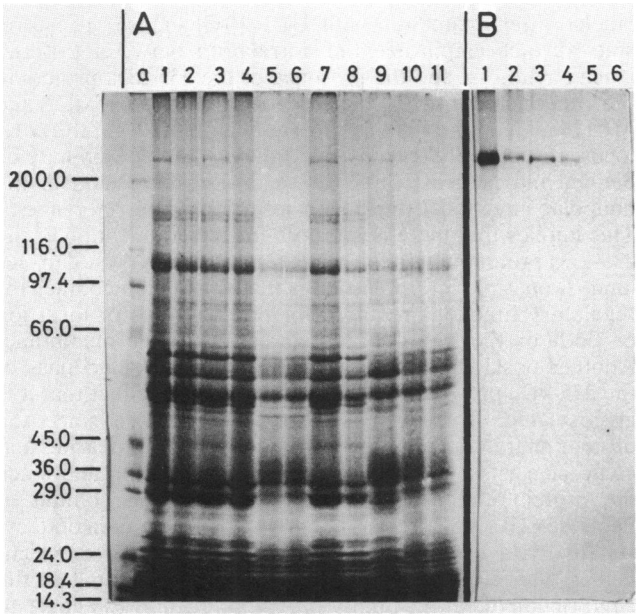

FIG. 4. Polyacrylamide gel electrophoresis of inner membranes of *A. tumefaciens* incubated with UDP [^{14}C]glucose. Inner membranes (0.22 mg of protein) were incubated with UDP [^{14}C]glucose, the reaction was stopped with TCA, and the precipitate was subjected to gel electrophoresis as described in Materials and Methods. Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). Lanes 1 to 4 correspond to a chase experiment. After 10 min of incubation, 2 mM nonradioactive UDP glucose was added, and the reaction was stopped at 0 min (B, lane 1), 5 min (B, lane 2), 15 min (B, lane 3) and 35 min (B, lane 4). Lanes 5 and 6 correspond to inner membranes from mutants A1011 and A1045, respectively. Lanes 7, 8, and 9 correspond to three different preparations of *A. tumefaciens* LBA4001 inner membranes not incubated with UDP [^{14}C]glucose. Lanes 10 and 11 correspond to inner membranes from mutants A1011 and A1045, respectively, not incubated with UDP [^{14}C]glucose. Numbers indicate the molecular masses (in kilodaltons) of standards (lane a).

slowed down but still increased. This behavior is as expected for a reaction intermediate and product and was very similar to that obtained with the inner membranes of *A. tumefaciens* (21).

Identification of the protein–β-D(1-2) glucan intermediate. The 10% TCA precipitate obtained after 10 min of incubation of inner membranes of *A. tumefaciens* LBA4001 under the conditions described in Materials and Methods was subjected to polyacrylamide gel electrophoresis, followed by an analysis of radioactivity by fluorography. A radioactive band of approximately 235 kilodaltons (kDa) was detected (Fig. 4). The position of the radioactive band corresponded to that of a protein detected by Coomassie blue staining (Fig. 4). As described previously (21), when the TCA precipitate was subjected to extensive proteolysis, the radioactivity was recovered in a glycopeptide containing an oligosaccharide which was identified as β-D(1-2) glucan. A chase experiment is shown in Fig. 4. After the addition of nonlabeled UDP glucose (2 mM), the radioactivity of the 235-kDa protein decreased rapidly as a function of time. Thus, as previously described for the radioactivity of the 5% TCA precipitate (21), the labeled product of the 235-kDa protein behaved as an intermediate. The 235-kDa protein was searched for in the purified inner membranes of different strains. Inner membranes prepared from avirulent *A. tumefaciens* A1011, A1020, A1038, and A1045 contained no detectable amounts of the 235-kDa protein after being stained either with Coomassie blue (Fig. 4 and 5) or by the silver staining procedure (data not shown). Other differences observable between LBA4001 and mutants A1011 and A1045 (see the proteins around 25, 27, and 50 kDa, Fig. 4) were not observed in experiments in which those mutants were compared with the parental strain A723 (data not shown). No other major difference was observed in the proteins of the inner membranes among mutants A1020 and A1038 and the wild type strain A723 (Fig. 5). After incubation with UDP [^{14}C]glucose, no radioactivity was found in the position that corresponds to the 235-kDa protein or any other protein (Fig. 4 and 5). On the other hand, inner membranes from strains ME2 and ME116, which form β-D(1-2) glucan, had the same levels of the 235-kDa protein as did the wild-type strain. Thus, mutations in the *chvB* locus that lead to avirulence were defective in the in vitro synthesis of β-D(1-2) glucan, probably owing to the fact that they lacked the 235-kDa protein on which the β-D(1-2) glucan intermediate is built up.

The 235-kDa protein was also searched for in inner membranes of *R. meliloti*, known to synthesize β-D(1-2) glucan, and in *Escherichia coli*, which does not form this polysaccharide. The 235-kDa protein was detected in *R. meliloti* by Coomassie blue staining, and when these inner membranes were incubated with UDP [^{14}C]glucose and subjected to polyacrylamide gel electrophoresis, the 235-kDa protein became labeled (Fig. 5). On the other hand, the 235-kDa protein was not present in the inner membranes of *E. coli*, and after incubation with UDP [^{14}C]glucose, no radioactivity was detected in the position that corresponds to the 235-kDa

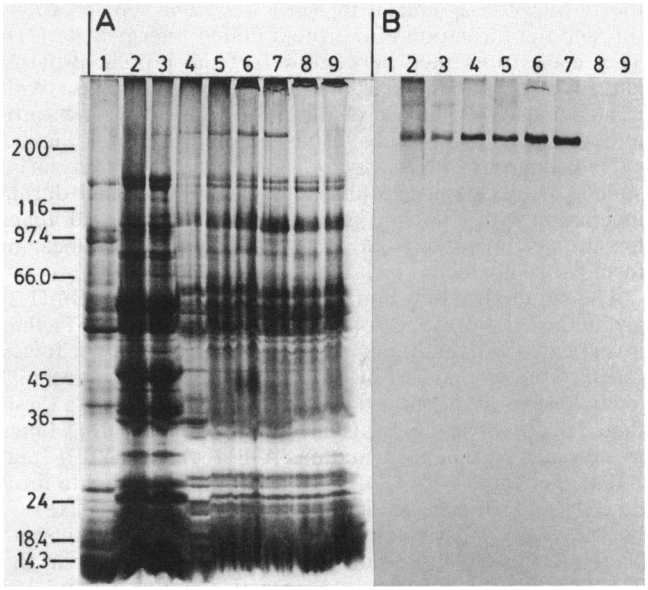

FIG. 5. Polyacrylamide gel electrophoresis of inner membranes of *R. meliloti*, *E. coli*, and *A. tumefaciens* incubated with UDP [^{14}C]glucose. Inner membranes (0.2 to 0.3 mg of protein) were incubated for 10 to 20 min with UDP [^{14}C]glucose and subjected to polyacrylamide gel electrophoresis as described in the legend to Fig. 4. Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). Lanes: 1, *E. coli*; 2 and 3, *R. meliloti*; 4, *A. tumefaciens* LBA4001; 5, *A. tumefaciens* A723; 6, strain ME116; 7, strain ME2; 8, *A. tumefaciens* A1038; 9, *A. tumefaciens* A1020. Numbers indicate the molecular masses (in kilodaltons) of standards.

protein or in any other protein (Fig. 5). These results further emphasize the role of the 235-kDa inner membrane protein in the synthesis of β-D(1-2) glucan.

In experiments in which the formation of the protein–β-D(1-2) glucan intermediate was measured after incubation with UDP [^{14}C]glucose, it was observed that the inner membranes of nonvirulent strains A1011 and A1045 formed a certain amount of a radioactive TCA-insoluble compound. This substance was formed more slowly than was the protein–β-D(1-2) glucan intermediate obtained with wild-type inner membranes. It increased for about 1 h at 10°C and faster at 30°C. In contrast, the protein–β-D(1-2) glucan intermediate formed rapidly, reached the maximum, and then decreased. Another difference was that while the protein–β-D(1-2) glucan intermediate decreased upon the addition of nonlabeled UDP glucose (21), the compound formed by the mutants remained constant after a chase with UDP glucose.

On the other hand, the products of partial acid hydrolysis of the labeled TCA-insoluble compounds accumulated by the mutants were different from those of the wild type.The protein–β-D(1-2) glucan intermediate obtained from the wild-type strain produced glucose, sophorose, and higher homologs on paper chromatography (21), while galactose, a small amount of glucose, and slower-running compounds were recovered from mutants. Thus, it seems that the radioactive TCA-insoluble compounds formed by the mutants were not related to β-D(1-2) glucan synthesis. They were not studied further.

DISCUSSION

We previously provided evidence indicating that the synthesis of β-D(1-2) glucan in *Agrobacterium* spp. proceeds through the formation of a protein-bound intermediate (21). The results presented here show that the protein–β-D(1-2) glucan intermediate is a 235-kDa protein. A chase with nonlabeled UDP glucose of the pulse-labeled 235-kDa protein showed that it behaves as an intermediate. Similar events occurred with *R. meliloti*. The 235-kDa protein can be stained with Coomassie blue and becomes labeled during incubation with UDP [^{14}C]glucose. On the other hand, inner membranes prepared from *E. coli*, a bacterium that does not form β-D(1-2) glucan, lack the 235-kDa protein.

The parallelism between the ability to synthesize β-D(1-2) glucan and the presence of the 235-kDa protein was further investigated with avirulent mutants of *A. tumefaciens*. It was recently shown that Tn*5* insertions in the *chvB* chromosomal locus lead to avirulent, attachment-defective mutants (5, 8) that do not produce β-D(1-2) glucan in vivo (16). Inner membranes purified from mutants A1011, A1020, A1038, and A1045 incubated with UDP [^{14}C]glucose were found to form no β-D(1-2) glucan, as judged by the results of HPLC. Polyacrylamide gel electrophoresis of the inner membrane proteins showed that none of the mutants contain the 235-kDa protein. Furthermore, no radioactivity accumulated in the position that corresponds to that of the 235-kDa protein or in any other protein after incubation with UDP [^{14}C]glucose.

Two transposon insertions in the *chvB* region produced a wild-type virulence response (5) (Fig. 1, strains ME2 and ME116). Inner membranes prepared from strain ME2 or ME116 incorporated [^{14}C]glucose into β-D(1-2) glucan at rates comparable to those of the wild-type strain. Polyacrylamide gel electrophoresis of the inner membrane proteins showed that the 235-kDa protein was present and became labeled after incubation with UDP [^{14}C]glucose. These results further emphasize the correlation between β-D(1-2) glucan synthesis and the presence of the 235-kDa protein in the inner membranes. The insertions in strains ME2 and ME116 map on the right half (5′) and left half (3′) of the *chvB* locus, respectively (Fig. 5); accordingly, the operon may be divided into at least three genes. For a discussion of the nonpolar effects of transposon insertions, see reference 5. This implies that there is no room for the codification of the 235-kDa protein within the *chvB* region, which is 5 kilobases long. A possible interpretation is that this region codifies for regulatory functions required for the synthesis or insertion or both of the 235-kDa protein in the inner membrane. Another possibility is that the apparent molecular mass of the 235-kDa protein is an artifact, owing to the fact that it is glycosylated. This seems to be less probable since the protein migrated as a sharp band without any smearing that could suggest a variable degree of glycosylation and since the protein eluted in the void volume of a column of Sephadex G-200 with a buffer containing 1% sodium dodecyl sulfate (data not shown), suggesting a molecular weight higher than 200,000. Chromosomal mutations lying in the *chvB* region affect the ability of *A. tumefaciens* to adhere to plants, with loss of virulence. These mutations also result in an inability to form β-D(1-2) glucan in vivo and in vitro and to synthesize the 235-kDa protein intermediate. The mutants are also nonmotile because they no longer have flagella (4) and are no longer able to conjugatively transfer plasmid pAgK84 (A. Kerrs, unpublished results). Thus, *chvB* mutants are pleitropic. If this region codifies for positive regulators, as suggested above, a complex phenotype would be expected. These regulators may control the expression of a set of genes required for infection, among which β-D(1-2) glucan may play a role. The 235-kDa protein may contain the necessary enzyme activities for β-D(1-2) glucan synthesis or may act only as an acceptor for the elongation of the polyglucose chain. In all the mutants, the only difference detected in their inner membranes was the absence of the 235-kDa protein; however, in the region of lower-molecular-weight proteins, the gel was too crowded to draw firm conclusions. Further experiments will be required to establish if some other proteins are required for the synthesis of β-D(1-2) glucan.

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