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Characterization of a *Rhizobium meliloti ndvB* Mutant and a Symbiotic Revertant that Regains Wild-Type Properties

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Rhizobium meliloti **mutant strain GRT21s, which is generated by heat treatment, was previously reported not to form cyclic β-(1-2)-glucan *in vivo* or *in vitro*. The mutant lacks a 235-kilodalton (kDa) protein that acts as an intermediate in β-(1-2)-glucan synthesis. In this work, we genetically characterized strain GRT21s as a *ndvB* mutant and selected a symbiotic revertant (GRT21sR) that restored β-(1-2)-glucan synthesis as well as other vegetative properties altered in strain GRT21s. Furthermore, by two-dimensional gel electrophoresis, we identified a group of membrane proteins linked to β-(1-2)-glucan synthesis. Our data indicate that NdvB protein and/or cyclic glucans influence proper functioning of such important systems as motility, adaptation to low osmolarity, and nodule formation, as well as alterations in membrane protein composition.**

In *Agrobacterium tumefaciens* (Smith and Townsend) Conn, two chromosomal virulence genes, *chvA* and *chvB*, are believed to be involved in export and synthesis, respectively, of cyclic β-(1-2)-glucan (Zorreguieta and Ugalde 1986; Zorreguieta *et al.* 1988; Cangelosi *et al.* 1989). *chvA* and *chvB* have homologous and functionally interchangeable genes in *Rhizobium*, termed *ndvA* and *ndvB*, respectively (Dylan *et al.* 1986). In *Rhizobium meliloti* Dangeard, the *ndvA* and *ndvB* loci are required for effective nodulation (Dylan *et al.* 1986). The *ndvA* and *ndvB* loci of *R. meliloti* have been recently sequenced and found to encode 67-kDa and 319-kDa proteins, respectively (Stanfield *et al.* 1988; Ielpi *et al.* 1990). *R. meliloti* GRT21s was generated by heat treatment and elicits pseudonodules on alfalfa (Toro and Olivares 1986). Strain GRT21s does not form β-(1-2)-glucan, due to a defect in the formation of the 235-kDa protein (Geremia *et al.* 1987). This mutant strain displays other phenotypic properties, including small colony size and loss of motility; it produces about twice as much exopolysaccharide than that produced by the wild type (Geremia *et al.* 1987). In the present study, strain GRT21s has been genetically characterized as a *ndvB* mutant. The isolation of a symbiotic revertant, GRT21sR, which restored all vegetative properties including β-(1-2)-glucan synthesis and the 235-kDa protein, allowed us to identify a group of membrane proteins linked to β-(1-2)-glucan synthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

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Media, growth conditions, and bacterial matings. Culture media used included TY (0.5% tryptone, 0.3% yeast extract, 2 mM $CaCl_2$) (Beringer 1974), GYM (1 mM glutamate, 0.02% yeast extract, 2.5 mM mannitol, 1 mM K_2HPO_4, pH 6.8, 0.5 mM $CaCl_2$, and 0.5 mM $MgSO_4$) (Ielpi *et al.* 1990), and minimal medium (Robertson *et al.* 1981) for *R. meliloti* and Luria-Bertani medium (Khan *et al.* 1974) for *Escherichia coli* (Migula) Castellani and Chalmers. Transconjugants were selected on either solid or liquid minimal medium containing tetracycline (10 μg/ml). We used the triparental mating protocol (Ditta *et al.* 1980) for bacterial crosses.

Recombinant DNA techniques. Recombinant DNA techniques, Southern blotting, and hybridization were performed following established protocols (Maniatis *et al.* 1982).

Plant testing. Alfalfa plants were grown on nitrogen-free medium and inoculated as described previously (Olivares *et al.* 1980). Nodules were examined between 2 and 6 wk after inoculation. Nitrogen fixation was detected by acetylene reduction (Bedmar and Olivares 1980).

Isolation of β-(1-2)-glucan. Cell-associated, soluble carbohydrates were isolated from 500-ml cultures of *R. meliloti* grown for 4 days at 30° C in GYM medium containing 20 mM mannitol and 20 mM NaCl (Ielpi *et al.* 1990). Isolation was performed as described previously (Miller *et al.* 1988). Samples were applied to a column of Sephadex G-50 (2.6 × 53 cm), and fractions of 1 ml were collected. Total carbohydrate content was determined by the phenol method (Hanson and Phillips 1981).

Preparation of inner membranes and *in vitro* assay. Inner membranes were prepared as previously described (Osborn and Munson 1984; Zorriegueta *et al.* 1985a). *In vitro* assay of β-(1-2)-glucan synthesis was carried out as described previously (Zorreguieta and Ugalde 1986) at 10° C for 15 min with 0.075 μCi of [UDP-^{14}C]glucose and purified inner membranes (0.2 mg of protein). The reaction was stopped by the addition of 10% trichloroacetic acid. Pellets were subjected to polyacrylamide gel electrophoresis as described (Zorreguieta and Ugalde 1986). For chase experiments, 2 mM nonradioactive UDP-Glc was added after 15 min

of incubation; the reaction was stopped after 15 min. We stopped the synthesis reaction made with inner membranes of *A. tumefaciens* A348 by heating at 100° C for 5 min. The neutral products were recovered as described previously (Zorreguieta and Ugalde 1986).

Chemical treatments. For removing nonsugar substituents, the oligosaccharides recovered from the Sephadex G-50 column were first desalted on a Sephadex G-25 column (15 × 0.8 cm), evaporated to dryness, and then treated with HCl (10 mM) at 100° C for 90 min and with NaOH (0.5 N) for 80 min, as previously described (Iñon de Iannino and Ugalde 1989). Samples were neutralized, desalted, and concentrated. Then, they were subjected to a DEAE-Sephadex A-25 column (6 × 0.8 cm) as described (Iñon de Iannino and Ugalde 1989).

Acid hydrolysis and paper chromatography. Partial acid hydrolysis of neutral oligosaccharides was carried out with HCl (0.5 N) at 100° C for 15–30 min. HCl was removed by evaporation under an air stream, and the hydrolysates were subjected to descending paper chromatography with isopropanol acetic acid water (27:4:9) as solvent. Sugar was detected by the alkaline-silver method (Treveylan *et al.* 1950).

Membrane protein preparation and two-dimensional gel electrophoresis. Cells from 200-ml cultures grown in GYM medium supplemented with 20 mM NaCl were harvested by centrifugation and then resuspended in 10 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% [v/v] 2-mercaptoethanol). Cells were disrupted by sonication, and insoluble material was removed by centrifugation. A membrane fraction was obtained after centrifugation at 178,000 × *g* for 1 hr. Proteins from the membrane fraction were phenol-extracted and solubilized for electrophoresis as described (Hurkman and Tanaka 1987). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% running gel, 5% stacking gel) and isoelectric focusing (IEF) were carried out essentially as described (Cellis *et al.* 1990). First-dimensional gels were loaded with 150 μg of solubilized proteins. After electrophoresis of the second dimension, gels were processed by silver staining.

RESULTS AND DISCUSSION

Genetic complementation of *R. meliloti* GRT21s with the *ndvB* locus. Initial studies on GRT21s demonstrated that this mutant lacks a 235-kDa protein required for synthesis of β-(1-2)-glucan (Geremia *et al.* 1987), which is also true of *A. tumefaciens* mutant *chvB* (Zorreguieta and Ugalde 1986). To determine if the *ndvB* locus was sufficient to complement the symbiotic mutant phenotype of strain GRT21s, we transferred plasmid pMJS1, containing the well-characterized 5′ half of the *ndvB* locus of *R. meliloti* 102F34 (Fig. 1), into mutant strain GRT21s. Transconjugants were tested for their ability to nodulate and fix nitrogen on alfalfa. We found that these transconjugants induced Fix^+ nodules with similar morphology to wild-type nodules. Bacteria were isolated from one of the resulting pink nodules on TY agar containing tetracycline. Plasmid DNA was isolated and back-mobilized into the GRT21s mutant. The transconjugants obtained again were restored for the symbiotic mutant phenotype, which indicates that symbiotic complementation was plasmid-encoded. The 6.2-kb *Eco*RI fragment as contained in pMJS1 (Fig. 1) was used in these complementation assays; this fragment contains over 2 kb of uncharacterized DNA upstream of the *ndvB* locus as reported by Ielpi *et al.* (1990). To rule out the possibility that mutation in GRT21s could be located in that upstream DNA portion, the region of *ndvB* contained in plasmid pMJS1 was

Table 1. Strains and plasmids used in this study

Strain designation	Relevant characteristics	Source or reference
Rhizobium meliloti		
GR4	Wild-type strain	This study
GRT21s	GR4 derivative by heat treatment, deficient in β-(1-2)-glucan synthesis, lacking the 235-kDA protein	Toro and Olivares 1986, Geremia *et al.* 1987
GRT21sR	Symbiotic revertant of GRT21s	This study
Agrobacterium tumefaciens		
A348	*A. tumefaciens* A136 containing PTiA6NC	Garfinkel and Nester 1980
Escherichia coli		
HB101	*E. coli* K12-*E. coli* B hybrid	Boyer and Rulland-Dussoix 1969
DH5α	*recA1, endA1, ϕ80d, lacZ, dm15*	Bethesda Research Lab, Gaithersburg, MD
Plasmid		
pRK290	Tc^r, *oriT* from RK2, broad host range vector	Ditta *et al.* 1980
pRK2013	Km^r, *rep*, ColE1	Ditta *et al.* 1980
pRK290.112	Km^r, *rep*, ColE1 pRK290 clone carrying *ndvA* and *ndvB* of *R. meliloti* 102F34	Dylan *et al.* 1986
pMJS1	pRK290 clone carrying *ndvB* as a 6.2-kb *Eco*RI fragment of *R. meliloti* 102F34	This study
pMJS2	pUC18 clone carrying *ndvB* as a 6.6-kb *Eco*RI fragment of *R. meliloti* GR4	This study
pMJS100	pLAFR3 clone carrying a 4.2-kb *Eco*RI-*Bgl*II fragment of pMJS1	This study
pRmIL1,2,3,4,5	Cosmid clones based on pLAFR1, carrying the *ndv* region of *R. meliloti* GR4	This study
pRmMJS100,200,300	Cosmid clones based on pLAFRI, carrying the *ndv* region of *R. meliloti* GRT21sR	This study

disrupted by deletion of the smaller 2-kb *Eco*RI-*Bgl*II fragment as contained in pMJS100 (Fig. 1). The symbiotic mutant phenotype of *R. meliloti* GRT21s was not complemented by pMJS100, which indicates, therefore, that genetic complementation of mutant GRT21s depends on *ndvB*.

To determine if the genetic complementation of mutant GRT21s by plasmid pMJS1 led to the presence of an active truncated NdvB protein or restoration of the 235-kDa β-(1-2)-glucan intermediate protein, we prepared and incubated inner membrane fractions of GR4 and GRT21s(pMJS1), as described in Materials and Methods. The 235-kDa β-(1-2)-glucan intermediate protein was visible by Coomassie blue staining (Fig. 2A). Fluorography of the gel showed that GRT21s(pMJS1) had a labeled protein indistinguishable from the GR4 235-kDa β-(1-2)-glucan intermediate protein (Fig. 2B). On addition of nonlabeled UDP-Glc, the radioactivity decreased, showing that the membranes were active in β-(1-2)-glucan synthesis. Data suggest that positive complementation of GRT21s mutant with plasmid pMJS1 could be due to homologous recombination, which restores the integrity of the gene.

Isolation of symbiotic revertants of mutant strain GRT21s. An overnight cell culture of GRT21s mutant (10 ml; 10^9 cells per milliliter) was used to inoculate alfalfa plants (100 tubes; three plants per tube). After 4 wk of inoculation, all nodules induced by strain GRT21s had a characteristic pseudonodule external morphology. Interestingly, after 6 wk of inoculation, some pink nodules appeared. Bacteria were recovered from two pink nodules, each isolated from a separate plant, and then retested on alfalfa plants. One of the two isolated bacteria, GRT21sR, induced nitrogen-fixing nodules in all plants inoculated, and, therefore, it was studied further.

Vegetative properties of strain GRT21sR. Reversion is not unusual in *ndv* mutants; *R. meliloti ndv* mutants obtained by Tn*5* insertion were able to induce some nitrogen-fixing nodules on alfalfa (Dylan *et al.* 1990). In a previous study, pseudorevertants, which maintained the Tn*5* insertion, were selected. However, these pseudorevertants were not restored for any vegetative property, including periplasmic β-(1-2)-glucan. To determine if strain GRT21sR had behavior similar to that of symbiotic pseudorevertants isolated previously by Dylan *et al.* (1990), we examined different vegetative properties such as adaptation to low osmolarity, motility, and periplasmic β-(1-2)-glucan synthesis. As shown in Figure 3A, mutant strain GRT21s was slower than the wild type in adapting to the low osmolarity medium as described for other *ndvB* mutants (Dylan *et al.* 1990). However, strain GRT21sR showed osmoadapting behavior similar to that of the wild type. Mutant strain GRT21s was reported to be nonmotile when tested on semisolid medium (Geremia *et al.* 1987). Figure 3B shows that strain GRT21sR exhibits motility similar to that of the wild-type strain GR4. Finally, we analyzed the presence of β-(1-2)-glucan in cell extracts from strains GR4, GRT21s, and GRT21sR. As shown in Figure 4, strain GRT21sR showed a profile of hexose-containing polysaccharide similar to that of the wild-type strain GR4. The major peak, containing hexose-positive material from

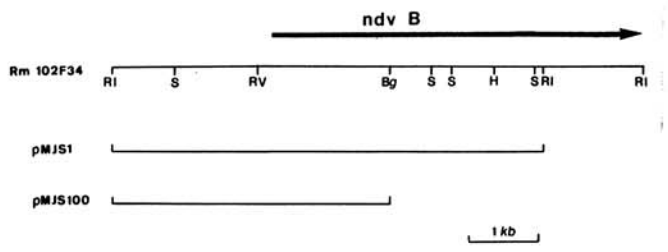

Fig. 1. Map of the *ndvB* region of *Rhizobium meliloti* 102F34. **A,** Restriction map of the *ndvB* locus of *R. meliloti* 102F34. The horizontal arrow indicates the location of a large open reading frame, according to Ielpi *et al.* (1990). Restriction fragments cloned in pMJS1 and pMJS100 are also shown.

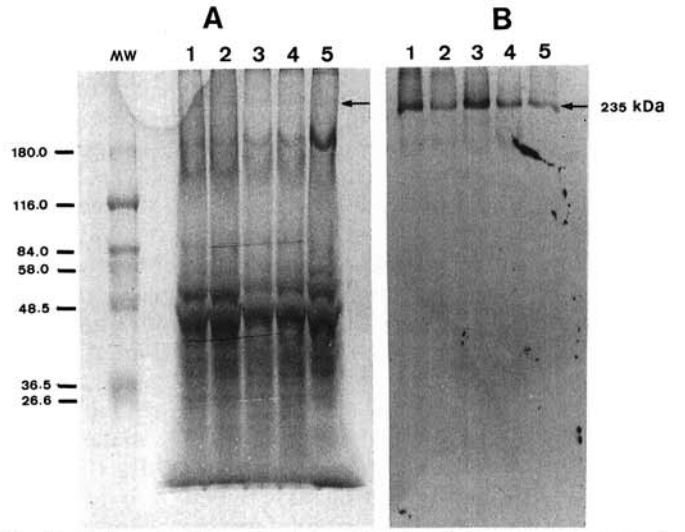

Fig. 2. Polyacrylamide gel electrophoresis of inner membranes of *Rhizobium meliloti* incubated with [UDP-^{14}C]glucose. **A,** Protein was stained with Coomassie blue; **B,** radioactivity was detected by fluorography. Standard of molecular weight is indicated by MW; numbers indicate relative molecular weight in kilodaltons. Lanes 1 and 2, *R. meliloti* GRT21sR inner membranes; 3 and 4, *R. meliloti* GRT21s (pMJS1); and 5, *R. meliloti* GR4 inner membranes. Pulse (1 and 3) and chase (2 and 4) experiments were performed as described in text. The arrows indicate the location of the 235-kDa protein.

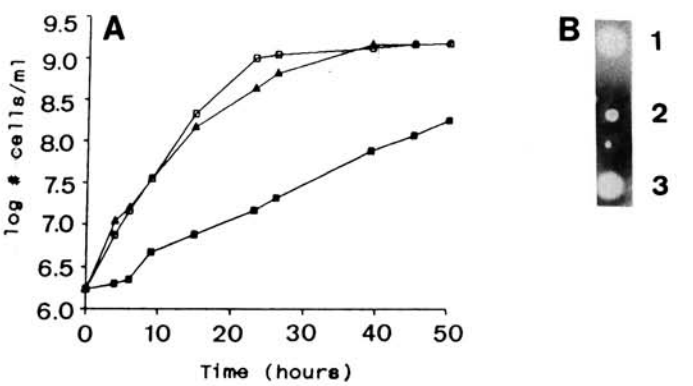

Fig. 3. Vegetative properties of symbiotic revertant GRT21sR. **A,** Ability of symbiotic revertant GRT21sR to adapt to low osmolarity. Bacteria were grown in GYM medium with 100 mM NaCl and diluted back in low osmolarity GYM medium as described (Dylan *et al.* 1990). □ = Wild-type strain GR41; ■ = mutant GRT21s; △ = revertant GRT21sR. **B,** Restoration of motility in symbiotic revertant GRT21sR. Bacteria grown in GYM medium containing 100 mM NaCl were spotted onto the surface of soft agar motility plates as described (Dylan *et al.* 1990).

GRT21sR, was further characterized after the nonsugar substituents were removed by partial acid hydrolysis and paper chromatographic analysis. The major peak yielded a pattern identical to that of neutral-labeled β-(1-2)-glucan of *Agrobacterium* (Zorreguieta *et al.* 1985b), indicating that it was β-(1-2)-glucan. As can be seen in Figure 4, this peak was absent in mutant GRT21s as previously reported (Geremia *et al.* 1987). Furthermore, the revertant strain GRT21sR recovered the 235-kDa protein as it is shown in Figure 2B. We conclude that this symbiotic revertant strain is different from both motile and symbiotic pseudorevertants previously described by Dylan *et al.* (1990).

Isolation of the second mutation in *R. meliloti* strain GRT21sR. To isolate the second mutation that occurs in strain GRT21sR, we constructed its genomic library in pLAFRI. This library was transferred in mass into mutant strain GRT21s, and transconjugants were inoculated on alfalfa plants. Six weeks after inoculation, bacteria were isolated from pink nodules. Cosmids were isolated, and restriction analysis showed three different overlapping cosmids, pRmMJS100, 200, and 300. Interestingly, these cosmids showed restriction patterns similar to those obtained when merodiploid complementation of mutant GRT21s was carried out with the wild-type cosmid bank, cosmid clones pRmIL1, 3, and 4 (see Fig. 5A,C). Furthermore, it was demonstrated by Southern blot hybridization that all three cosmids contained the *ndvB* locus (Fig. 5B). A restriction map of the *ndv* region of wild-type strain GR4 is shown in Figure 5C. The previous results indicate that strain GRT21sR has a functional *ndv* region and suggest that the second mutation occurred in the same *ndvB* locus.

Identification of membrane proteins linked to β-(1-2)-glucan synthesis by two-dimensional electrophoresis. In *E. coli*, synthesis of membrane-derived oligosaccharides (MDO) is osmoregulated (Kennedy 1982); this also occurs in *A. tumefaciens* (Miller *et al.* 1986). Furthermore, it has been shown that adaptation to osmotic stress in *E. coli* and other enteric bacteria alters the amount of several specific membrane proteins (Barron *et al.* 1986). Symbiotic revertant strain GRT21sR, originated by a second mutation at the *ndvB* locus in mutant GRT21s, recovered all vegetative properties including the ability to synthesize β-(1-2)-glucan. To determine if the former ability was associated with a change of the membrane proteins profile, we performed two-dimensional gel electrophoresis of total membrane proteins from wild-type strain GR4, mutant GRT21s, and revertant GRT21sR. The 235-kDa protein was not observed under the experimental conditions used, probably because of the low amount of the former protein

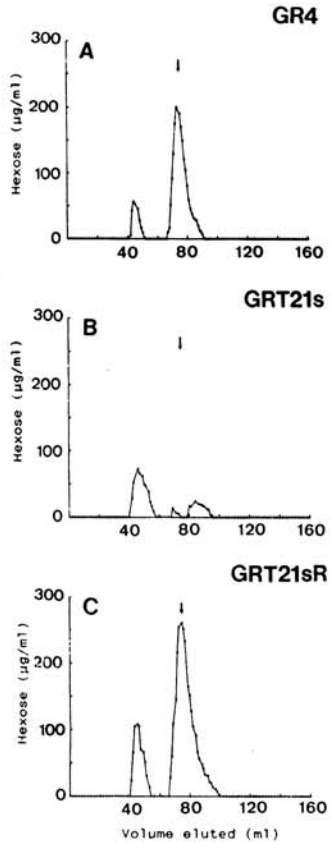

Fig. 4. Production of periplasmic oligosaccharides. **A,** The wild-type strain GR4; **B,** mutant GRT21s; and **C,** revertant GRT21sR. The arrows indicate the position of β-(1-2)-glucan. Void volume (V_o) = 45 ml and total volume (V_t) = 158 ml. Results are normalized per gram (wet weight) of cells.

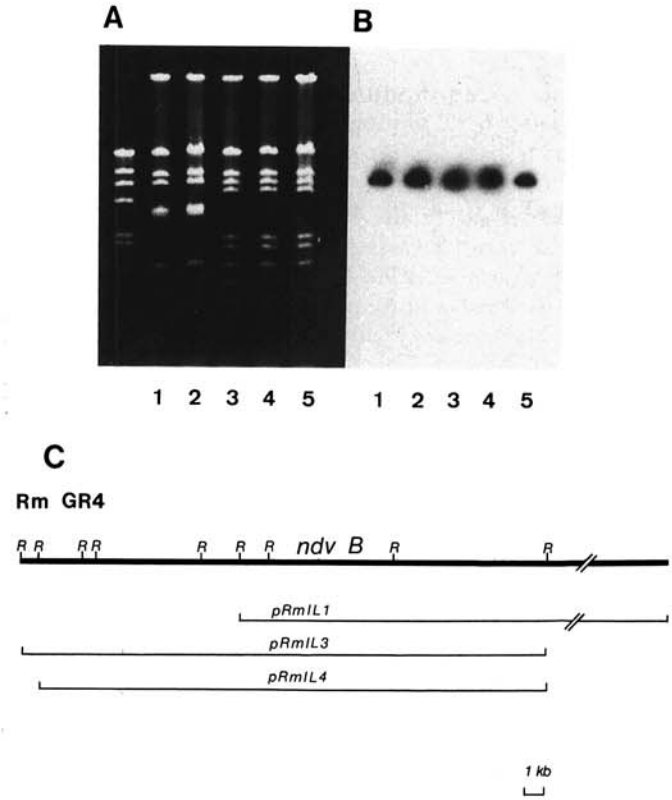

Fig. 5. Identification and characterization of cosmid clones carrying the *ndvB* locus of *Rhizobium meliloti* GR4 and revertant strain GRT21sR. **A,** *Eco*RI restriction analysis of complementing cosmids of mutant strain GRT21s. Molecular weight marker (λ-*Hin*dIII) is shown at the left of lane 1. **B,** The corresponding Southern blot hybridization with a *ndvB* probe is shown. Lane 1, pRmIL1; 2, pRmIL2; 3, pRmIL3; 4, pRmIL4; and 5, pRmIL5. **C,** *Eco*RI restriction map of the *ndvA* and *ndvB* regions of *R. meliloti* GR4. The broken segments denote additional DNA fragments of unknown arrangement. Cosmid clones, pRmMJS100, 200, and 300 carrying the *ndvB* locus of revertant strain GRT21sR were identical to cosmid clones isolated from wild-type strain GR4.

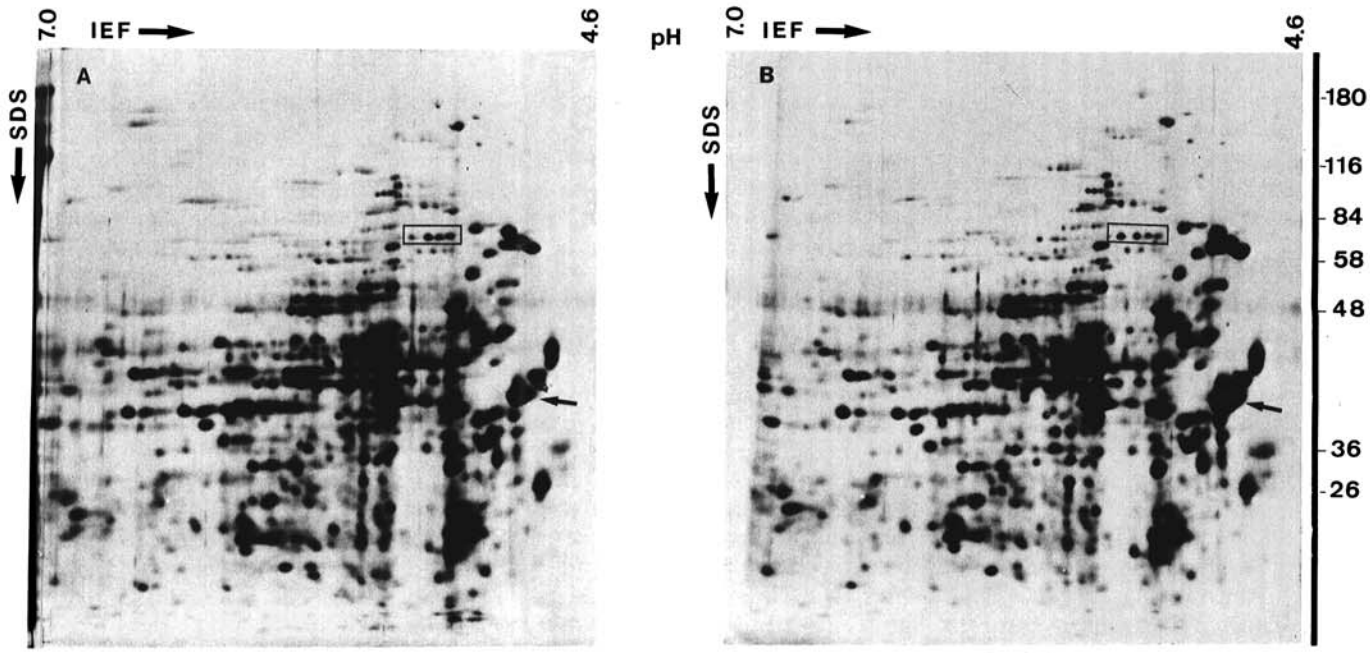

Fig. 6. Analysis of total membrane proteins by two-dimensional gel electrophoresis. **A,** Membrane proteins from mutant GRT21s; **B,** membrane proteins from symbiotic revertant strain GRT21s. Major changes are indicated by a panel and an arrow. Molecular masses in kilodaltons are indicated to the right.

in the total membrane fraction. Consistent variations observed are indicated in Figure 6. Former changes affected essentially a group of polypeptides with similar molecular weight, approximately 70 kDa and with pI ranging from 5.40 to 5.65. Another variation involved a polypeptide of 40 kDa and with pI of 4.90. Wild-type strain GR4 showed a ratio of the former proteins similar to that of revertant strain GRT21sR (data not shown). At this time, the nature of these proteins and the functional significance of these alterations in protein composition are unknown. However, these variations associated with either the NdvB protein and/or β-(1-2)-glucan synthesis may be essential for the cell to recognize and respond to changes in environmental conditions.

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