JOURNAL OF BACTERIOLOGY, May 1989, p. 2842–2849
0021-9193/89/052842-08$02.00/0

Vol. 171, No. 5

Biochemical Characterization of Avirulent *Agrobacterium tumefaciens chvA* Mutants: Synthesis and Excretion of β-(1-2)Glucan†

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Received 12 September 1988/Accepted 16 February 1989

The *chvA* gene product of *Agrobacterium tumefaciens* is required for virulence and attachment of bacteria to plant cells. Three *chvA* mutants were studied. In vivo, they were defective in the synthesis, accumulation, and secretion of β-(1-2)glucan; however, the 235-kilodalton (kDa) protein known to be involved in the synthesis of β-(1-2)glucan (A. Zorreguieta and R. Ugalde, J. Bacteriol. 167:947–951, 1986) was present and active in vitro. Two molecular forms of cyclic β-(1-2)glucan, designated types I and II, were resolved by gel chromatography. Type I β-(1-2)glucan was substituted with nonglycosidic residues, and type II β-(1-2)glucan was nonsubstituted. Wild-type cells accumulated type I β-(1-2)glucan, and *chvA* mutant cells accumulated mainly type II β-(1-2)glucan and a small amount of type I β-(1-2)glucan. Inner membranes of wild-type and *chvA* mutants formed in vitro type II nonsubstituted β-(1-2)glucan. A 75-kDa inner membrane protein is proposed to be the *chvA* gene product. *chvA* mutant inner membranes had increased levels of 235-kDa protein; partial trypsin digestion patterns suggested that the 235-kDa protein (the gene product of the *chvB* region) and the gene product of the *chvA* region form a complex in the inner membrane that is involved in the synthesis, secretion, and modification of β-(1-2)glucan. All of the defects assigned to the *chvA* mutation were restored after complementation with plasmid pCD522 containing the entire *chvA* region.

The integration of a segment of *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid DNA into the plant genome results in crown gall tumor formation on dicotyledonous plants (19, 22). This process requires a number of plasmid Ti and chromosomal virulence genes (9). Chromosomal virulence loci *chvA* and *chvB* are required for attachment of bacteria to plant cells (6, 9). *chvA* and *chvB* have homologous and functionally interchangeable counterparts in *Rhizobium meliloti*, in which they are required for effective nodulation of alfalfa (7, 10).

Avirulent *A. tumefaciens chvB* mutants do not form in vivo (20) or in vitro (30) cyclic β-(1-2)glucan. The same phenotype was described for an *R. meliloti* mutant (10). The *chvB* region, as defined phenotypically, partly encodes a 235-kilodalton (kDa) inner membrane-bound protein required as an intermediate in β-(1-2)glucan synthesis (28, 30, 31). The 235-kDa structural protein gene extends 3.5 kilobases downstream from the phenotypically defined *chvB* region. Mutants with insertions in this 3.5-kilobase DNA region are virulent and form truncated proteins that are active in the β-(1-2)glucan synthesis (28), which demonstrates that only the part of the *chvB* locus necessary for β-(1-2)glucan synthesis is required for effective plant infection. This glucan has been observed only in *Agrobacterium* and *Rhizobium* species (24) and is believed to play a central role in the interaction of these bacteria with plants, since in both genera, mutants affected in synthesis of the glucan are avirulent or form ineffective empty nodules.

Agrobacterium chvA DNA contains a 1.8-kilobase open reading frame coding for a protein similar to an *Escherichia coli* hemolysin export protein (HlyB). The protein also has homology to HisP, MalK, and OppD transport proteins (3, 11). The *R. meliloti ndvA* (the equivalent of *A. tumefaciens chvA*) DNA nucleotide sequence was recently determined, with results very similar to those obtained for the *A. tumefaciens* gene; this finding suggests a very conserved function for the gene products (23). It has been proposed that *ndvA* (23) and *chvA* (3) are involved in the excretion of cyclic β-(1-2)glucan. In this study, we further characterized *A. tumefaciens chvA* mutants at the biochemical level and found that although they have increased levels of the 235-kDa β-(1-2)glucan intermediate protein, they are defective in vivo in the synthesis and secretion of β-(1-2)glucan and accumulate an altered molecular form of cellular β-(1-2) glucan.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *A. tumefaciens* strains and plasmids used are listed in Table 1. Bacteria were grown on tryptone-yeast extract (TY) medium (30). Carbenicillin or kanamycin was added to a final concentration of 100 μg/ml. Merodiploid strains for complementation analysis were obtained by triparental mating as described previously (5). Transconjugants were selected on AB agar medium (6) with the appropriate antibiotic and were purified from *E. coli* by being streaked five times on selective media.

Isolation of β-(1-2)glucan from cells. Cells from 100-ml cultures were harvested by centrifugation at 10,000 × *g* for 10 min. Pellets were extracted with 10% trichloroacetic acid (TCA) for 30 min at room temperature 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.). Columns were eluted with 0.1 M pyridine-acetate buffer (pH 5.5). Fractions of 1.5 ml were collected, and carbohydrates were detected by the anthrone-

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† Dedicated to the memory of Luis F. Leloir, who died on 2 December 1987.

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or phenotype	Reference or source
A. tumefaciens		
A348	Wild type, motile, Vir$^+$ pTiA6NC	9
ME42	Vir$^-$ Cb^r, motile, Tn*3*:: HoHo1 in *chvA* region	6
ME66	Vir$^-$ Cb^r, motile, Tn*3*:: HoHo1 in *chvA* region	6
ME104	Vir$^-$ Cb^r Km^r, motile, Tn*3*::HoHo in *chvA* region	6
NI1	Vir$^+$ Cb^r Km^r, motile, ME42(pCD522)	This study
NI2	Vir$^+$ Cb^r Km^r, motile, ME66(pCD522)	This study
NI3	Vir$^+$ Cb^r Km^r, motile, ME104(pCD522)	This study
Plasmid		
pRK2013	IncP Tra$^+$ Nm^r	5
pCD522	pVK102-*A. tumefaciens chvA* Km^r	6

[a] All *A. tumefaciens* strains have been described previously (6) and have a C58 chromosomal background. Precise map positions of Tn*3*::HoHo1 insertions are shown in Fig. 8. Abbreviations: Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Nm^r, neomycin resistance; Vir$^+$, virulent; Vir$^-$, avirulent.

sulfuric acid method (4). K_{av} was calculated as $V_e - V_0/V_t - V_0$, in which V_e is the elution volume, V_0 is the void volume, and V_t is the total volume.

Acid hydrolysis and paper chromatography. Partial acid hydrolysis of the glucan was carried out in 0.5 N HCl at 100°C for 20 to 30 min; total acid hydrolysis was carried out in 1 N HCl at 100°C for 4 h. HCl was removed by evaporation under an air stream, and the 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]) or solvent B (isopropanol-acetic acid-water [27:4:9]). Sugars were detected by the alkaline-silver method (26).

DEAE-Sephadex chromatography. Materials applied to DEAE-Sephadex A-25 columns (6 by 0.8 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) were batch eluted with 6 ml of water and with 6 ml each of 10, 50, 100, 200, and 500 mM NaCl. Fractions of 1.5 ml were collected.

Chemical treatments. β-(1-2)Glucan recovered from the Bio-Gel P4 columns was successively treated with HCl (10 mM) at 100°C for 90 min and with NaOH (0.5 M) at 100°C for 80 min as previously described for removing polysaccharide nonsugar substituents (14, 18).

In vivo synthesis of β-(1-2)glucan. Overnight cultures (1.5 ml) grown in yeast extract-mannitol medium (27) were centrifuged (15 min at 2,200 × *g*), and cells were washed once with 5 ml of M9 medium minus glucose (15). Cells suspended in 0.5 ml of M9 medium minus glucose but containing 30 μCi of [^{14}C]glucose (381 mCi/mmol) were incubated for 10 min at 28°C. Cells and supernatants were separated by centrifugation (2 min in an Eppendorf centrifuge), and cell pellets were extracted with 10% TCA for 30 min at room temperature. TCA extracts and supernatants were subjected to gel chromatography on Bio-Gel P4 columns as described above for the isolation of β-(1-2)glucan.

Preparation of inner membranes and in vitro β-(1-2)glucan synthesis. Inner membranes were purified as described previously (31). In vitro synthesis of β-(1-2)glucan, polyacrylamide gel electrophoresis of inner membrane proteins, and fluorography were carried out as described previously (30).

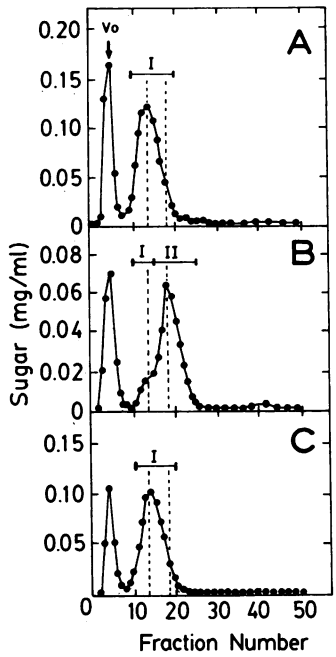

FIG. 1. Bio-Gel P4 chromatography of 10% TCA cell extracts. TCA extraction, gel chromatography, and assays of carbohydrates were carried out as described in Materials and Methods. (A) Wild-type strain A348; (B) *chvA* mutant strain ME42; (C) strain NI1. I and II, Fractions pooled to obtain type I and type II glucans, respectively; dashed vertical lines indicate the eluting positions of the compounds.

Incubation with Glusulase and β-galactosidase assay. Glusulase treatments were carried out as described previously (29). Assay of β-galactosidase was performed as described elsewhere (16), in the presence of 0.1% sodium deoxycholate, using purified inner membranes (0.3 mg of protein) washed twice with 30 mM Tris hydrochloride buffer (pH 8.2)–3 mM EDTA.

Trypsin digestion. *A. tumefaciens* inner membranes (0.2 mg of protein) were partially digested with 0.02 mg of trypsin (Sigma Chemical Co., St. Louis, Mo.) in 50 mM Tris hydrochloride buffer (pH 8.5)–10 mM $MgCl_2$. After incubation for 5 min at 10°C, reactions were stopped by addition of 0.02 mg of soybean trypsin inhibitor (Sigma). UDP-[U-^{14}C]glucose (90,000 cpm; 285 mCi/mmol) was then added;

TABLE 2. Accumulation of cellular β-(1-2)glucan[a]

Strain	Cellular glucan[b]		
	Type I	Type II	Total
A348	6.4	NP[c]	6.4
ME42	0.8	2.9	3.7
ME104	0.6	2.4	3.0
NI1	5.9	NP	5.9
NI3	6.5	NP	6.5

[a] Strains were grown in 100-ml cultures, and cell pellets were extracted with 10% TCA as described in Materials and Methods.

[b] Accumulation of cellular glucan recovered from a Bio-Gel P4 column with a K_{av} of 0.20 (type I) or 0.25 (type II) (Fig. 1), expressed as milligrams of glucose equivalent per gram (wet weight) of cells.

[c] NP, Not present. Under the conditions used, no detectable type II glucan was observed in strains A348 and NI1 (see legend to Fig. 1).

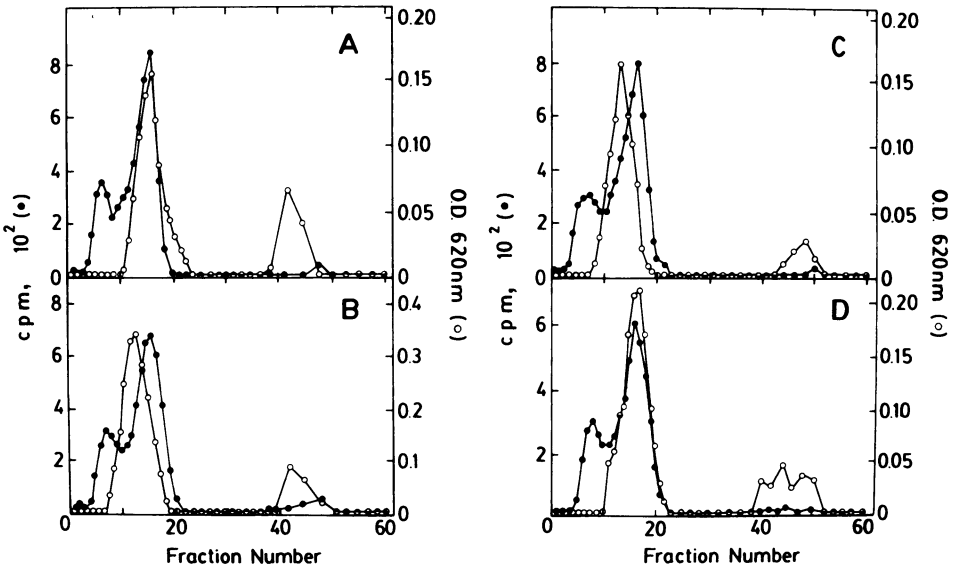

FIG. 2. Bio-Gel P4 chromatography of β-(1-2)glucans accumulated in vivo by *A. tumefaciens*. β-(1-2)Glucans (●) were obtained after incubation of wild-type inner membranes with UDP-[^{14}C]glucose as described in the legend to Fig. 5. ○, Type II β-(1-2)glucan from mutant strain ME42 (A); type I β-(1-2)glucan from wild-type strain A348 (B); type I β-(1-2)glucan from mutant strain ME42 (C); type I β-(1-2)glucan from mutant strain ME42 (D) subjected to alkaline and acid treatments as described in Materials and Methods. O.D., Optical density.

after 10 min at 10°C, reactions were stopped by addition of 0.5 ml of 10% TCA. The precipitates were subjected to polyacrylamide gel electrophoresis and fluorography as described elsewhere (30). Control assays were carried out with addition of soybean trypsin inhibitor before trypsin treatment. In some experiments, inner membranes were incubated with UDP-[U-^{14}C]glucose (285 mCi/mmol) as described above, and inner membrane proteins were solubilized with 1% sodium dodecyl sulfate before trypsin treatment.

RESULTS

It was recently reported that one *R. meliloti ndvA* mutant, equivalent to *A. tumefaciens chvA* mutants (7, 23), neither secreted nor accumulated cellular β-(1-2)glucan; however, it was previously reported that an *A. tumefaciens chvA* mutant produced β-(1-2)glucan (20). To clarify this controversy, we studied the accumulation, secretion, and in vivo and in vitro synthesis of β-(1-2)glucan in three *chvA* mutants.

Accumulation of cellular β-(1-2)glucan. Cell pellets were extracted with 10% TCA as described in Materials and Methods, and neutralized TCA extracts were subjected to gel chromatography on Bio-Gel P4 columns. TCA extracts of *A. tumefaciens* A348 (wild-type strain), ME42 (*chvA* mutant), or NI1 (ME42 complemented with plasmid pCD522 [Table 1]) contained polysaccharides eluting in the void volume and in the partial included volume of the column (Fig. 1). Total acid hydrolysis of the void volume polysaccharide yielded glucose and galactose, which indicated that these polysaccharides probably were succinoglucans produced by *A. tumefaciens* (25). Polysaccharides recovered in the partial included volume were characterized as β-(1-2)glucans (see below). Cells of *A. tumefaciens* A348 and NI1 contained β-(1-2)glucan that eluted from the Bio-Gel P4 column with a K_{av} of 0.20 (Fig. 1A and C). On the other hand, *A. tumefaciens* ME42 contained primarily a different molecular form of β-(1-2)glucan that eluted from the column with a K_{av} of 0.25 and a small amount of β-(1-2)glucan with a K_{av} of 0.20 (Fig. 1B). β-(1-2)Glucan eluting from the column with K_{av} values of 0.20 and 0.25 will be referred to as type I and type II β-(1-2)glucan, respectively. The amount of type I β-(1-2)glucan recovered from *chvA* mutant cells was approximately 10% of that recovered from wild-type cells (Table 2). The total amount of β-(1-2)glucan (type I plus type II) accumulated in *chvA* mutant cells was approximately 50% of the amount accumulated in wild-type cells (Table 2). The same results were obtained with *A. tumefaciens chvA* mutants ME104 and ME66 (data not shown). Complementation of *chvA* mutants with plasmid pCD522, containing a 9.5-kilobase DNA fragment spanning the entire phenotypically defined *chvA* region (6), restored the accumulation of type I β-(1-2)glucan to wild-type levels and abolished the accumulation of type II β-(1-2)glucan (Table 2).

Characterization of cellular glucans. Total acid hydrolysis of cellular type I β-(1-2)glucans accumulated by *A. tumefaciens* A348, ME42, and ME104 yielded only glucose on paper chromatography with solvent A; partial acid hydrolysis on paper chromatography with solvent B yielded glucose, sophorose, and a series of oligosaccharides with higher degrees of polymerization, as expected for β-(1-2)glucan degradation products (29). DEAE-Sephadex chromatography of cellular type I β-(1-2)glucans showed that 80% were anionic glucans that eluted from the column with 50, 100, and 200 mM NaCl and that 20% were neutral glucans. On the other hand, 93% of cellular type II β-(1-2)glucan accumulated by *chvA* mutants was neutral and 7% was a highly charged compound that eluted from the DEAE-Sephadex column with 500 mM NaCl. Total and partial acid hydrolysis of this highly charged compound revealed that it was not β-(1-2)glucan.

These results showed that cellular type II β-(1-2)glucans accumulated by *chvA* mutants were nonmodified neutral glucans and that type I β-(1-2)glucan had a different degree of modification with anionic substituents. To verify these findings, type I β-(1-2)glucans were subjected to alkaline and acid treatments known to remove nonsugar substituents,

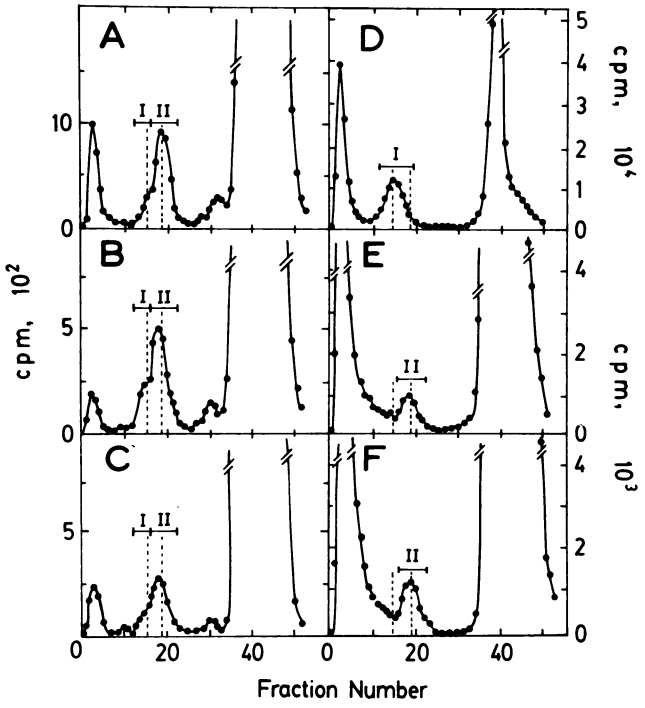

FIG. 3. Bio-Gel P4 chromatography of [^{14}C]glucose-labeled polysaccharides obtained in vivo. TCA cell extracts and supernatants were obtained as described in Materials and Methods and subjected to gel chromatography as described in the legend to Fig. 1. (A to C) Polysaccharides recovered from 10% TCA cell extracts; (D to F) polysaccharides in culture supernatants. (A and D) Wild-type strain A348; (B and E) mutant strain ME42; (C and F) mutant strain ME104. For other details, see the legend to Fig. 1.

such as *sn-1* phosphoglycerol (1, 18), succinate (13), methyl malonate (13), or pyruvate (12), that have been reported to be present in β-(1-2)glucan. Type II β-(1-2)glucan eluted from the Bio-Gel P4 column with the same K_{av} as did the most abundant β-(1-2)glucan obtained in vitro with purified inner membranes (Fig. 2A). Figures 2B and C show the elution profiles of type I β-(1-2)glucan recovered from strains A348 and ME42, respectively. ME42 type I β-(1-2)glucan treated with alkali and acid yielded a type II β-(1-2)glucan, as judged by its elution volume from the Bio-Gel P4 column (Fig. 2D). DEAE-Sephadex chromatography of this treated type I β-(1-2)glucan showed that 100% became neutral. Alkaline and acid treatments did not break the β-(1-2)-glycosidic linkage because the sensitivity to Glusulase did not change after these treatments (29). On the other hand, type II β-(1-2)glucan recovered from the ME42 mutant and treated in the same way did not change its elution volume from the Bio-Gel P4 column. Thus, type I and type II β-(1-2)glucans differed in modification with nonsugar substituents that could be removed by alkaline and acid treatments; in addition, type II β-(1-2)glucan was indistinguishable by Bio-Gel P4 chromatography from β-(1-2)glucan obtained in vitro with purified inner membranes.

In vivo synthesis of β-(1-2)glucan. To study the rate of synthesis of β-(1-2)glucan, growing cultures were incubated for short periods of time with [^{14}C]glucose as described in Materials and Methods. After 10 min of incubation, cells were spun down, the supernatants were recovered, and the cells were extracted with 10% TCA. Supernatants and TCA extracts were subjected to gel chromatography on Bio-Gel P4 columns (Fig. 3).

TABLE 3. Rate of synthesis of β-(1-2)glucan by *A. tumefaciens*[a]

Strain	Synthesis (cpm/mg of protein per min)[b]					
	Type I			Type II		
	Cellular	Extracellular	Total	Cellular	Extracellular	Total
A348	550	62,000	62,550	1,100	NP	1,100
ME42	600	NP	600	870	3,000	3,870
ME104	370	NP	370	840	3,500	4,340

[a] Growing cultures were incubated with [^{14}C]glucose as described in Materials and Methods. Radioactivity recovered from culture supernatants (extracellular) or from 10% TCA cell extracts (cellular) was subjected to Bio-Gel P4 chromatography (see legend to Fig. 3). Radioactivity was pooled, concentrated, and subjected to DEAE-Sephadex chromatography as described in Materials and Methods.

[b] Calculated after subtracting the radioactivity that eluted from the DEAE-Sephadex column with 0.5 M NaCl. NP, Not present.

TCA extracts of *A. tumefaciens* wild-type strain A348 and mutant strains ME42 and ME104 contained type II β-(1-2)glucan and a small amount of type I β-(1-2)glucan (Fig. 3A to C). The wild-type A348 culture supernatant contained type I β-(1-2)glucan; remarkably, no type II β-(1-2)glucan was observed (Fig. 3D). On the other hand, mutant supernatants did not contain type I β-(1-2)glucan (Fig. 3E and F), but a small amount of type II β-(1-2)glucan was detected. The rate of synthesis of the two types of β-(1-2)glucan is shown in Table 3. Wild-type cells formed type I β-(1-2)glucan at a rate 100 times higher than that of *chvA* mutants of strain ME42 or ME104. In addition to having a low rate of synthesis, the mutants were totally defective in secretion into the culture supernatant of type I β-(1-2)glucan (Table 3 and Fig. 3). Thus, the synthesis of type I β-(1-2)glucan was strongly reduced in *chvA* mutants, but an increase in the rate of synthesis of type II was observed (Table 3).

Characterization of [^{14}C]glucose-labeled β-(1-2)glucan. Total and partial acid hydrolysis, DEAE-Sephadex chromatography, and degradation with Glusulase were carried out on type I and type II β-(1-2)glucans obtained in vivo after incubation with [^{14}C]glucose. Wild-type and mutant type I β-(1-2)glucans showed no difference. Neutral type II β-(1-2)glucans obtained from wild-type and *chvA* mutant strains were contaminated with a highly charged compound

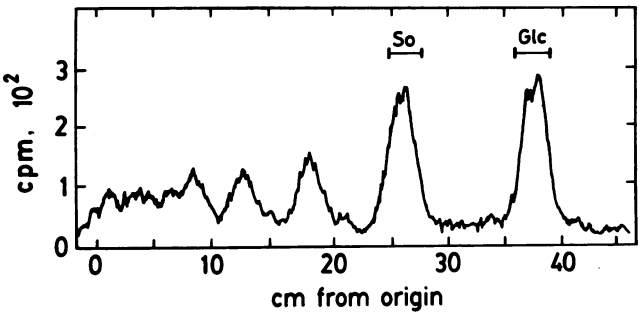

FIG. 4. Paper chromatography of partial acid hydrolysis. Neutral type II β-(1-2)glucan labeled in vivo with [^{14}C]glucose was subjected to partial acid hydrolysis, and the products were subjected to paper chromatography in solvent B as described in Materials and Methods. Radioactivity was detected in a radioscanner. So, Sophorose; Glc, glucose. Type I β-(1-2)glucan labeled in vivo with [^{14}C]glucose and subjected to the same treatment showed a similar profile.

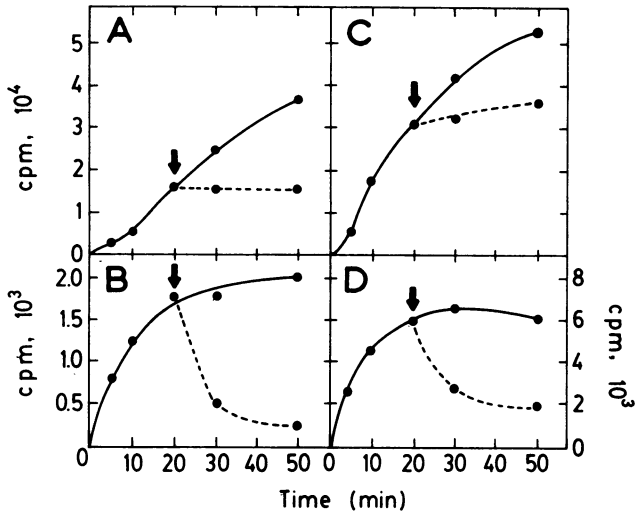

FIG. 5. β-(1-2)Glucan synthesis by inner membranes of *A. tumefaciens* wild-type and *chvA* mutant strains. The experiment was carried out as described in Materials and Methods. (A and C) Incorporation of [^{14}C]glucose into β-(1-2)glucan (neutral fraction) of strains A348 (wild type) and ME104 (*chvA* mutant), respectively; (B and D) incorporation of [^{14}C]glucose into the β-(1-2)glucan protein intermediate (5% TCA precipitate). Symbols: – – –, incorporation after addition of 2 mM nonradioactive UDP-glucose (↓); ——, control (no addition of UDP-glucose).

that eluted from DEAE-Sephadex columns with 0.5 M NaCl; total and partial acid hydrolysis of the highly charged compound showed that it was not β-(1-2)glucan. Neutral glucans contained only [^{14}C]glucose, as shown by total acid hydrolysis, and after partial acid hydrolysis yielded [^{14}C]glucose-, [^{14}C]sophorose-, and a series of [^{14}C]glucose-labeled oligosaccharides with a higher degree of polymerization on paper chromatography with solvent B, as expected for β-(1-2)glucan degradation products (Fig. 4). Neutral glucans were resistant to degradation with Glusulase, which suggested that they had a cyclic structure. Glusulase was active in releasing glucose from partially acid-hydrolyzed β-(1-2)glucan, whereas native β-(1-2)glucan was insensitive to this effect (29).

In vitro synthesis of β-(1-2)glucans. Inner membranes of *A. tumefaciens* wild-type strain A348 and *chvA* mutant strains ME42, ME66, and ME104 were purified as described previously (30). In vitro synthesis of β-(1-2)glucan was carried out as described in Materials and Methods. Wild-type and mutant strains formed β-(1-2)glucan in vitro (Fig. 5). Mutant strain ME104 incorporated [^{14}C]glucose into β-(1-2)glucan at a rate three times higher than that of the wild-type strain, and three times more [^{14}C]glucose was incorporated into the TCA-insoluble glucoprotein (Fig. 5). The same results were obtained with strains ME42 and ME66 (data not shown). Complementation of mutant strains with plasmid pCD522

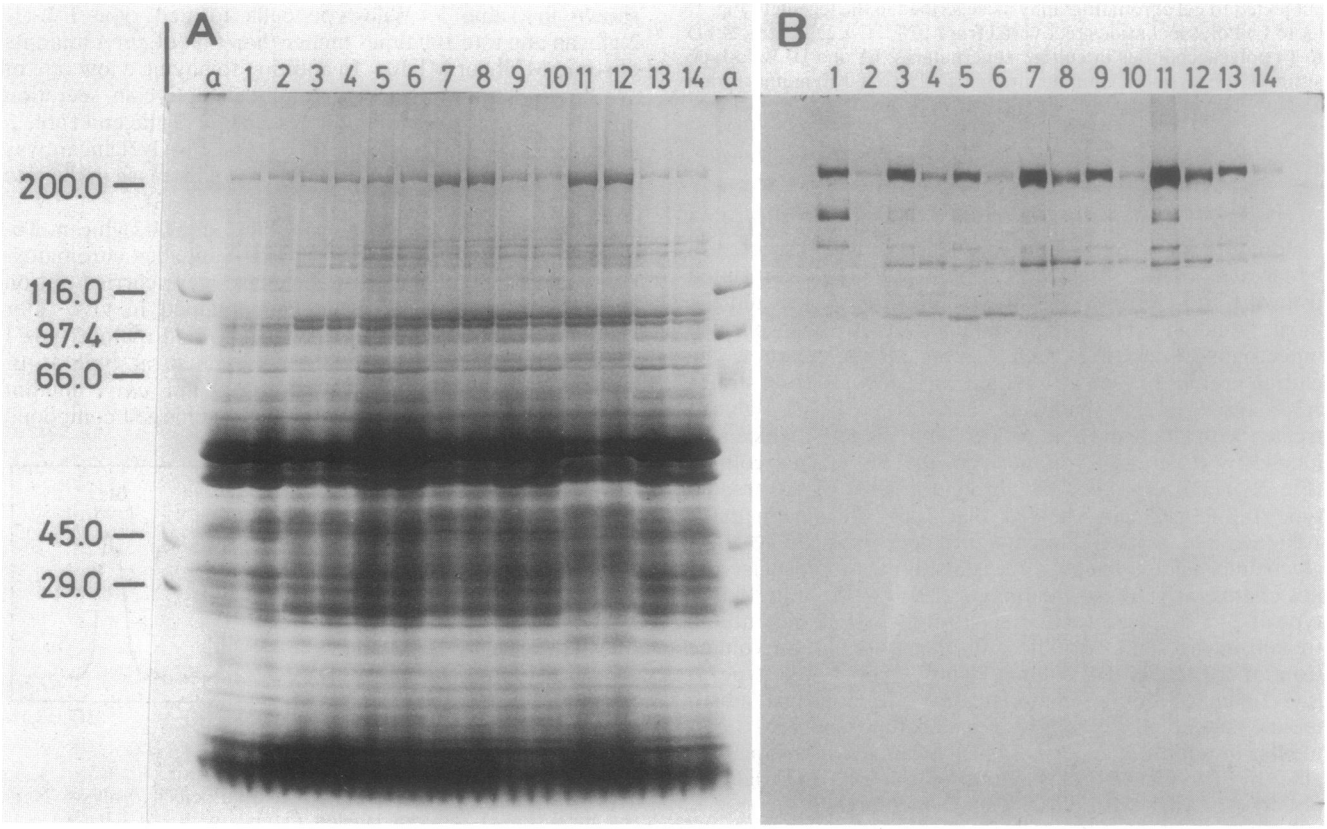

FIG. 6. Polyacrylamide gel electrophoresis of inner membranes of *A. tumefaciens* incubated with UDP-[^{14}C]glucose. Inner membranes (0.2 mg of protein) were incubated with UDP-[^{14}C]glucose, the reactions were stopped by addition of 10% TCA, and the precipitates were subjected to gel electrophoresis as described in Materials and Methods. Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). For a chase experiment (even-numbered lanes), 2 mM nonradioactive UDP-glucose was added after a 15-min incubation, and the reaction was stopped after 15 min. Lanes: 1 and 2, A348; 3 and 4, ME42; 5 and 6, NI1; 7 and 8, ME104; 9 and 10, NI3; 11 and 12, ME66; 13 and 14, NI2. Numbers on the left indicate molecular masses of standards (in kilodaltons).

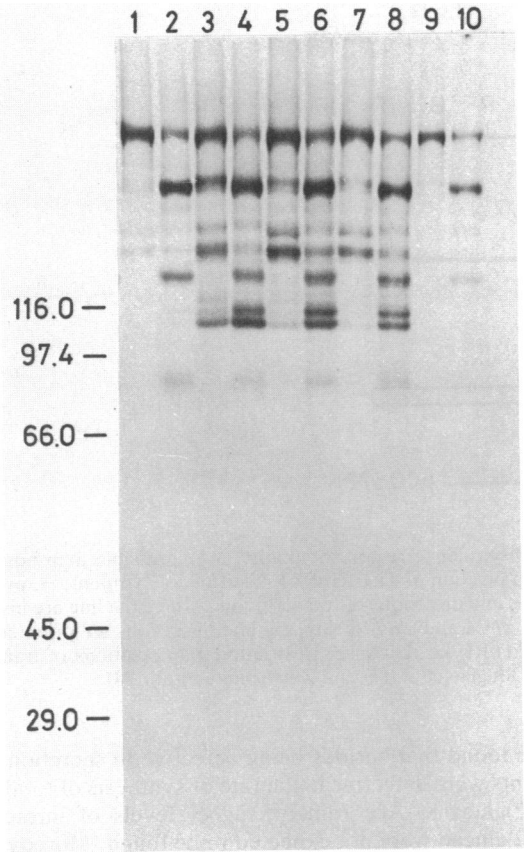

FIG. 7. Polyacrylamide gel electrophoresis of trypsin-treated inner membranes of *A. tumefaciens*. Inner membranes (0.2 mg of protein) were partially digested with trypsin and incubated with UDP-[^{14}C]glucose as described in Materials and Methods. The reactions were stopped by addition of TCA, and the precipitates were subjected to gel electrophoresis as described by Zorreguieta and Ugalde (30). Radioactivity was detected by fluorography. Lanes: 1 and 2, A348; 3 and 4, ME42; 5 and 6, ME66; 7 and 8, ME104; 9 and 10, NI1; 2, 4, 6, 8, and 10, digested with trypsin; 1, 3, 5, 7, and 9, controls (soybean trypsin inhibitor added before trypsin). Numbers on the left indicate molecular masses of standards (in kilodaltons).

restored incorporation of [^{14}C]glucose into the β-(1-2)glucan protein intermediate to wild-type levels (data not shown). The behavior of the TCA-insoluble protein intermediate of the mutant strains was very similar to that obtained with wild-type cells after a chase with nonlabeled UDP-glucose (Fig. 5B and D). This result indicates that the mutant protein intermediate was active in the synthesis of β-(1-2)glucan. β-(1-2)glucans obtained in vitro were subjected to gel chromatography on a Bio-Gel P4 column. The most abundant β-(1-2)glucan obtained in vitro eluted with a K_{av} of 0.25 (Fig. 2A), which corresponded to the elution volumes of type II β-(1-2)glucan and of type I β-(1-2)glucan after removal of nonglucosidic substituent groups (Fig. 2D). This result indicated that a nonsubstituted β-(1-2)glucan was formed in vitro. Wild-type and mutant membranes formed in vitro a small proportion of β-(1-2)glucans that eluted from the column with a K_{av} of 0.09; these glucans were not observed to be formed in vivo. Total and partial acid hydrolyses of the in vitro products showed that they were β-(1-2)glucans. In addition, they were resistant to degradation by Glusulase, which suggested that they had a cyclic structure (data not shown). The same results were obtained with *chvA* mutant strains ME42 and ME66 (data not shown). These results indicate that wild-type and *chvA* mutants formed in vitro the same nonmodified β-(1-2)glucan (type II) which, in vivo, was accumulated by *chvA* mutants and not by wild-type cells.

Polyacrylamide gel electrophoresis of inner membrane proteins. Inner membranes incubated with UDP-[^{14}C]glucose were subjected to polyacrylamide gel electrophoresis as described previously (30). Inner membranes of *chvA* mutant strains ME104 and ME66 contained higher levels of 235-kDa protein than did wild-type cells (Fig. 6A, lanes 1, 2, 7, 8, 11, and 12); with inner membranes of strain ME42, the difference was less apparent (lanes 3 and 4). In other preparations of ME42 inner membranes, however, the increment in the amount of 235-kDa protein was comparable to that observed in strains ME66 and ME104 (data not shown). The increased level of 235-kDa protein corresponded to a higher accumulation of [^{14}C]glucose in the intermediate and to a higher rate of synthesis of β-(1-2)glucan (Fig. 5 and 6). Complementation of mutants with plasmid pCD522 restored to wild-type levels the amount of 235-kDa protein (Fig. 6A, lanes 5, 6, 9, 10, 13, and 14). Mutant inner membranes had reduced amounts of a 75-kDa protein (lanes 3, 4, 7, 8, 11, and 12). Complementation with plasmid pCD522 restored the amount of this protein to wild-type levels (lanes 5, 6, 9, 10, 13, and 14).

Partial trypsin digestion of the β-(1-2)glucan protein intermediate. Wild-type inner membranes that were first partially digested with trypsin and then incubated with UDP-[^{14}C]glucose (see Materials and Methods) showed a reduction in the amount of radioactive 235-kDa protein, with the accumulation of three polypeptides of 170, 130, and 90 kDa (Fig. 7, lanes 1 and 2). Inner membranes of strains ME42, ME66, and ME104 subjected to the same trypsin treatment yielded, in addition to the three polypeptides observed in the assays with wild-type membranes, two polypeptides of 112 and 106 kDa. In the wild-type membranes, the latter two polypeptides were weakly visible (Fig. 7, lanes 3 to 8). Complementation of strain ME42 with plasmid pCD522 (strain NI1) restored the wild-type trypsin degradation pattern (lanes 9 and 10). The same results were obtained with strains NI2 and NI3 (data not shown). Thus, the *chvA* mutation was responsible for the observed change in the trypsin digestion pattern.

β-Galactosidase fusion. Strains ME42, ME104, and ME66 are *chvA* mutant strains obtained by Tn*3*::HoHo1 insertions that can create *lac* fusions (Fig. 8). In strain ME42, the insertion of the *lacZ* gene is oriented in the same direction of transcription as is *chvA*, whereas in strains ME104 and ME66 the orientation is in the opposite direction (Fig. 8). Inner membranes of strains A348, ME42, ME104, and ME66 were assayed for β-galactosidase activity as described in Materials and Methods. Strain ME42 contained 12.4 U of β-galactosidase per mg of protein, six times higher than the activity obtained with wild-type inner membranes. Inner membranes of strains ME66 and ME104 contained less than 0.05 U of β-galactosidase per mg of protein. These results indicated that in strain ME42, a fusion protein with β-galactosidase activity generated as a result of the Tn*3*::HoHo1 insertion was inserted into the inner membrane, which suggests that the *chvA* product may be an inner membrane protein.

DISCUSSION

We have demonstrated that *A. tumefaciens chvA* mutants are defective in the synthesis and secretion of β-(1-2)glucan

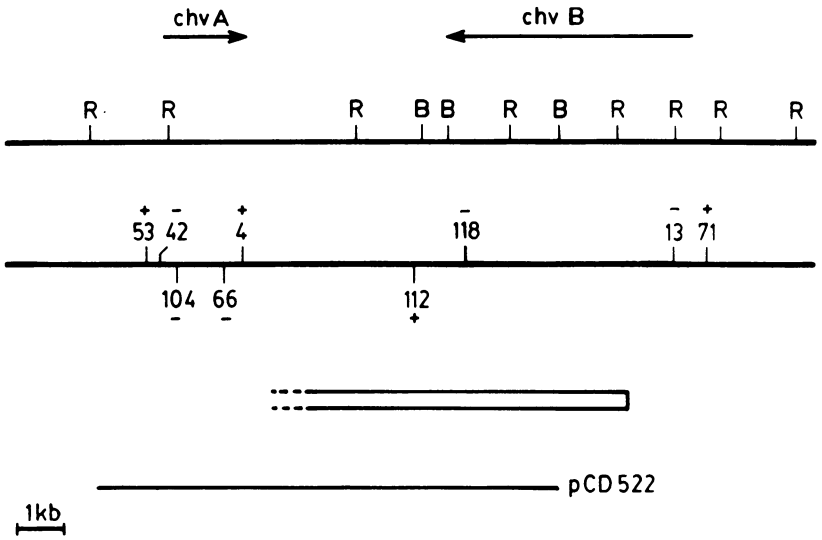

FIG. 8. Restriction endonuclease map of the *A. tumefaciens* chromosomal virulence region. Symbols: ▭, probable map position of the structural gene for the 235-kDa β-(1-2)glucan protein intermediate; |, map position of Tn*3*::HoHo1 insertion; +, virulent; −, avirulent; →, ←, positions and sizes of *chvA* and *chvB* as defined by virulence phenotypes and directions of transcription. Above the line are insertions in which the *lacZ* gene is oriented in the same direction of transcription as are *chvA* and *chvB*; below the line, insertions are oriented in the opposite direction. Plasmid pCD522 is shown at the bottom. B, *Bam*HI; R, *Eco*RI; kb, kilobase. Restriction map positions of transposon insertions and probable location of the 235-kDa protein gene are taken from Douglas et al. (6) and Zorreguieta et al. (28).

and accumulate an altered molecular form of cellular β-(1-2)glucan. We previously reported that avirulent mutants mapping in a nearby locus (*chvB*) were negative in β-(1-2)glucan synthesis because of the absence of a 235-kDa β-(1-2)glucan protein intermediate (30).

In this paper, we have shown that *chvA* mutant inner membranes contained higher amounts of a 235-kDa intermediate protein than did wild-type membranes. Accordingly, mutant inner membranes formed β-(1-2)glucan in vitro at a higher rate than did wild-type membranes. However, in vivo cellular accumulation of β-(1-2)glucan was reduced by approximately 50%. Moreover, mutant cells accumulated primarily a different molecular form of β-(1-2)glucan (type II) that corresponded to a nonsubstituted form of the polysaccharide. Wild-type cells accumulated substituted β-(1-2)glucan (type I). Removal of nonsugar substituents from type I β-(1-2)glucan yielded type II, which suggested that type I is a substituted form of type II. The accumulation by *chvA* mutants of nonsubstituted β-(1-2)glucan may be due either to a defect in the enzymes or substrates required for the modifying reaction or to a defect in the translocation of β-(1-2)glucan into the periplasmic space, where the modifying reactions might take place. It was proposed for the membrane-derived oligosaccharides of *E. coli* that modification of the polysaccharide with nonglycosidic substituents takes place in the periplasmic space (2).

Recently, Stanfield et al. (23) reported that an *R. meliloti ndvA* mutant, equivalent to *Agrobacterium chvA* mutants, contained the 235-kDa β-(1-2)glucan protein intermediate but neither secreted nor accumulated β-(1-2)glucan in vivo. Although these authors proposed, on the basis of protein homology with other export proteins, that *ndvA* is required for secretion of β-(1-2)glucan, they did not rule out the possibility that the mutant was defective in the synthesis of β-(1-2)glucan (23). If in vivo synthesis is not affected but secretion is abolished, one would expect to observe higher levels of intracellular β-(1-2)glucan accumulated, which were not found.

We found that besides being defective in secretion, *chvA* mutants were defective in the rate of synthesis of total cyclic β-(1-2)glucans. Accordingly, higher levels of intracellular β-(1-2)glucan were not expected to be found. Moreover, the mutants accumulated a nonmodified form of β-(1-2)glucan that we suggest is the precursor of the modified wild-type β-(1-2)glucan. Complementation of mutants with plasmid pCD522 restored virulence and accumulation of type I β-(1-2)glucan, which indicated that both characteristics were associated with the mutations. In vitro, wild-type and *chvA* mutant inner membranes formed nonmodified cyclic β-(1-2)glucan that was identical to the nonmodified β-(1-2)glucan accumulated in vivo by *chvA* mutants. These results suggest that the components required for synthesis of modified wild-type β-(1-2)glucan are missing or inactive in vitro, which reproduces the effect observed in vivo in the mutants.

The ChvA protein was proposed to be a membrane protein on the basis of its hydropathy plot (predominantly hydrophobic in the N-terminal end) and high homology to other membrane-bound transport proteins (3). We showed that inner membranes of *chvA* mutants were defective in a protein with an apparent molecular size of 75 kDa, similar to that predicted by sequencing data (3). Furthermore, inner membranes of *chvA* mutant strain ME42 contained β-galactosidase activity, probably as a result of the formation of a fusion protein induced by the insertion of the Tn*3*::HoHo1 transposon (21) in the N-terminal end of the gene (Fig. 8).

Since the *chvA* gene product is probably an inner membrane-bound protein, it may form a complex with the 235-kDa protein. As a result, absence of the ChvA protein may be responsible for the different patterns of tryptic fragments observed in *chvA* mutants. It was reported that other transport proteins highly homologous to the ChvA protein also form complexes with proteins encoded in the same operon (8). Additional work will be required to firmly establish the interaction in the inner membrane of the ChvA protein with the 235-kDa protein.

ACKNOWLEDGMENTS

This work was supported by grant PID 3-007400/85 from Consejo Nacional de Investigaciones Cientificas y Tecnicas (Argentina). The authors are researchers of the National Research Council (Argentina).

We acknowledge Susana Raffo and Marta Bravo for preparing UDP-[^{14}C]glucose and Silvia Curutchet for word processing and corrections of the manuscript. *Agrobacterium* mutants were kindly provided by E. W. Nester. We thank E. W. Nester and G. Cangelosi for critical reading of the manuscript and useful suggestions.

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