Biochemical Characterization of Avirulent exoC Mutants of Agrobacterium tumefaciens

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The synthesis of periplasmic β(1-2)glucan is required for crown gall tumor formation by Agrobacterium tumefaciens and for effective nodulation of alfalfa by Rhizobium meliloti. The exoC (pscA) gene is required for this synthesis by both bacteria as well as for the synthesis of capsular polysaccharide and normal lipopolysaccharide. We tested the possibility that the pleiotropic ExoC phenotype is due to a defect in the synthesis of an intermediate common to several polysaccharide biosynthetic pathways. Cytoplasmic extracts from wild-type A. tumefaciens and from exoC mutants of A. tumefaciens containing a cloned wild-type exoC gene synthesized in vitro UDP-glucose from glucose, glucose 1-phosphate, and glucose 6-phosphate. Extracts from exoC mutants synthesized UDP-glucose from glucose 1-phosphate but not from glucose or glucose 6-phosphate. Membranes from exoC mutant cells synthesized β(1-2)glucan in vitro when exogenous UDP-glucose was added and contained the 235-kilodalton protein, which has been shown to carry out this synthesis in wild-type cells. We conclude that the inability of exoC mutants to synthesize β(1-2)glucan is due to a deficiency in the activity of the enzyme phosphoglucomutase (EC 2.7.5.1), which in wild-type bacteria converts glucose 6-phosphate to glucose 1-phosphate, an intermediate in the synthesis of UDP-glucose. This interpretation can account for all of the deficiencies in polysaccharide synthesis which have been observed in these mutants.

Polysaccharides synthesized by Agrobacterium tumefaciens and Rhizobium meliloti have been shown to play a role in the interactions of these bacteria with plants (3, 14, 19, 20). Rhizobium spp. form nitrogen-fixing nodules on legumes, while A. tumefaciens induces crown gall tumors on a broad range of dicotyledonous plants. Both genera belong to the family Rhizobiaceae, and aside from their associative properties, they resemble each other in most respects. Both A. tumefaciens and R. meliloti synthesize a neutral or partially substituted cyclic β(1-2)glucan (6). A. tumefaciens strains unable to synthesize or excrete this glucan do not attach normally to plant cells (4, 16, 28, 35). The genes involved in the synthesis and excretion of β(1-2)glucan are in both cases chromosomal and are homologous and functionally interchangeable between the two genera (9). One of them, chvB (ndvB in R. meliloti), codes for a 235-kilodalton (kDa) inner membrane protein which is a protein-glucan intermediate in β(1-2)glucan synthesis (14, 33, 35, 36). The other, chvA (ndvA in R. meliloti), is not required for glucan synthesis but is involved in the secretion or release of the cyclic polysaccharide into the periplasm and extracellular medium (4, 16). The predicted amino acid sequences of the ChvA and NdvA proteins are homologous to those of other bacterial and eucaryotic export proteins (4, 29).

Both A. tumefaciens and R. meliloti also synthesize an identical capsular exopolysaccharide (succinoglycan), and most of the genes involved in this synthesis are functionally interchangeable between the two genera (3). Succinoglycan consists of repeating units of β-linked glucose and galactose, with acidic substitutions (15). A large number of mutants (exo mutants) in both genera which are deficient in succinoglycan synthesis have been obtained and assigned to several genetic complementation groups (3, 20). The exo mutations inactivate the ability of R. meliloti to effectively nodulate alfalfa but do not affect crown gall tumor formation by A. tumefaciens.

One exo locus, exoC (also termed pscA) (23), is required for plant interactions by both genera (3, 20, 23). Mutations in this locus result in the inability to synthesize either β(1-2)glucan or succinoglycan, explaining the requirement for a functional exoC in A. tumefaciens as well as in R. meliloti. At least in the case of R. meliloti, exoC mutants also synthesize an abnormal lipopolysaccharide (19). Moreover, A. tumefaciens exoC mutants are nonmotile (3).

We present here evidence that exoC mutants lack phosphoglucomutase (EC 2.7.5.1), which carries out one step in the biosynthesis of UDP-glucose, a substrate in the biosynthesis of β(1-2)glucan, succinoglycan, and the saccharide portion of lipopolysaccharide. Other steps in the pathway for β(1-2)glucan synthesis appear to be intact in these mutants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. Bacterial strains were grown in Luria broth at 28°C. When required, media were supplemented with chloramphenicol (20 μg/ml) and neomycin (100 μg/ml). Transconjugants were obtained by the triparental mating procedure with Escherichia coli (pRK2073) as the helper plasmid (21). Luria agar plates containing 0.02% Cellulfluor White (Polysciences, Inc., Warrington, Pa.) were used for the detection of the Exo phenotype (11, 24). Cellulfluor White-Luria medium supplemented with galactose (0.2%) or galactose plus glucose (0.2%) and M9 minimal liquid medium (22) supplemented with galactose (0.2%) or glucose (0.2%) were used.

Determination of capsular exopolysaccharide content in culture supernatants. A. tumefaciens strains were grown in 250 ml of Luria broth for 24 h. Cells were harvested by
centrifugation (6,000 × g, 15 min). Supernatants were concentrated sixfold, and the exopolysaccharide content was determined after precipitation with 3 volumes of ethanol as described previously (14).

Isolation of cellular β(1-2)glucan. Cells from 100-ml cultures were extracted with trichloroacetic acid as described previously (25). Total glucan eluting from a Bio-Gel P2 column with a Kᵥₑ between 0.12 and 0.27 was quantitated by the anthrone-sulfuric acid method (7). Kᵥₑ was calculated as (Vₑ − Vₒ)/(Vₑ − Vᵢ), where Vₑ is the elution volume, Vₒ is the voided volume, and Vᵢ is the total volume.

Preparation of inner membranes and cytosolic fractions. Cells of an exponential-phase culture (1 liter) were harvested by centrifugation and suspended in 10 ml of 30 mM Tris hydrochloride buffer (pH 8.0)−20% (w/v) sucrose−10 mM EDTA, 10 mg of lysozyme (from chicken egg white; Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was incubated for 45 min at 0°C and centrifuged for 10 min at 3,000 × g. The pellet was suspended in 10 ml of 30 mM Tris hydrochloride buffer (pH 8.0)−20% (w/v) sucrose−10 mM MgCl₂−10 mg of DNase I (bovine pancreas type IV; Sigma) and disrupted with a French press. Total membranes were pelleted at 100,000 × g for 5 h. The supernatant was recovered as a cytosolic fraction, and the pellet was separated into inner and outer membranes as described previously (36).

In vitro assays for β(1-2)glucan synthesis. The assays for in vitro β(1-2)glucan synthesis and labeling of the 235-kDa β(1-2)glucan–protein intermediate were described previously (35, 36). Inner membranes (200 µg of total protein) were incubated with 400,000 cpm of UDP-[U-¹⁴C]glucose (specific activity, 285.1 µCi/µmol) (12) for 10 min at 10°C. The chase experiment was carried out for 90 min with 16 mM unlabeled UDP-glucose. The reaction was stopped by heating at 100°C for 3 min; 300 µl of water was added, and the mixture was centrifuged at 1,000 × g for 10 min. The supernatant was chromatographed with a DEAE-Sephadex A-25 column (0.5 by 3 cm), and the neutral fraction was recovered. The linear or cyclic structure of β(1-2)glucan formed in vitro was determined by incubation with Glusulase (Endo Laboratories Inc., Du Pont) as previously described (34). The insoluble fraction was washed once with 10% trichloroacetic acid, and the precipitate was subjected to polyacrylamide gel electrophoresis. Protein staining and fluorography were carried out as described previously (35).

In vitro assays for UDP-glucose synthesis. UDP-glucose synthesis by cytosolic fractions (150 µg of protein) was carried out in reaction mixtures containing 50 mM Tris hydrochloride (pH 7.5), 8 mM MgCl₂, 20 mM UTP, the cytosolic sample, and the appropriate substrate in a final volume of 50 µl. The substrates were [U-¹⁴C]glucose 1-phosphate (specific activity, 322.0 µCi/µmol; Du Pont, NEN Research Products, Boston, Mass.) (60,000 cpn), [U-¹⁴C]glucose 6-phosphate (specific activity, 285.0 µCi/µmol) (12) (60,000 cpn), or [U-¹⁴C]glucose (specific activity, 300.0 µCi/µmol; ARC Inc., St. Louis, Mo.) (180,000 cpn). When [¹⁴C]glucose 6-phosphate or [¹⁴C]glucose was used as the substrate, the incubation mixture was supplemented with 2 mM glucose 1,6-biphosphate (Sigma) as a cofactor. With [¹⁴C]glucose, 10 mM ATP was also added. The reactions were carried out at 37°C for 30 min and stopped by the addition of 100 µl of ethanol. The supernatants obtained after centrifugation at 1,000 × g for 5 min were spotted onto Whatman no. 1 paper and subjected to descending chromatography with solvent A (ethanol−1 M ammonium acetate [pH 3.8] (5:2)) (27). Radioactivity on chromatograms was located with a model 7201 radiochromatogram scanner (Packard Instrument Co., Inc., Rockville, Md.). Compounds with mobilities similar to that of the UDP-glucose standard (sugar nucleotides plus sugar biphosphates) were eluted from the paper strip with water and chromatographed with solvent B (ethanol−1 M ammonium acetate [pH 7.4] (5:2)) (27), in which sugar biphosphates migrate more slowly than sugar nucleotides. Compounds with mobilities similar to that of glucose 6-phosphate in solvent B were eluted, hydrolyzed with alkaline phosphatase (E. coli type III-S; Sigma) (12), and chromatographed with solvent C (1-butanol−pyridine−water [6:4:3]) (17) to analyze the hexoses present. Compounds with mobilities similar to that of glucose 6-phosphate in solvent A were eluted, a sample was hydrolyzed for 10 min at 100°C with 10 mM HCl, and the products were subjected to chromatography with solvent C. Under these conditions, hexose 1-phosphate was hydrolyzed to hexose and phosphate, but hexose 6-phosphate was resistant and remained at the origin of the chromatogram. Hexose 6-phosphate was eluted, hydrolyzed with alkaline phosphatase (12), and chromatographed with solvent C to identify the hexose.

After elution from chromatograms, the radioactivity of each compound (from one experiment) was counted, and the relative percentages were calculated and referred to the total radioactivity recovered in the first chromatogram (solvent A).

The UDP-glucose standard was observed under UV light, and sugar phosphate and hexose standards were developed by the methods of Burrows et al. (2) and Trevelyan et al. (30; alkaline silver method), respectively.

RESULTS

In vivo synthesis of cellular β(1-2)glucan and extracellular succinoglycan. Previous work (3) demonstrated that culture media obtained from A. tumefaciens exoC mutants do not contain β(1-2)glucan. However, no studies were carried out on the cellular accumulation of β(1-2)glucan in these mutants. We obtained trichloroacetic acid extracts from wild-type and mutant cells and submitted them to Bio-Gel P₄ chromatography. Extracts from the mutant cells (A5129 and A5503) contained less than 1% of the wild-type level of antrhine-positive material eluting at the position corresponding to β(1-2)glucan (Kᵥₑ, 0.2) (14). Plasmid pFC6251 containing the exoC region restored the accumulation of cellular β(1-2)glucan to 0.66 mg of glucose equivalents per g of wet cell weight, which is comparable to the accumulation obtained with wild-type strain A348 (1.08 mg/g). These results are similar to those observed by Leigh and Lee (19) for R. meliloti exoC mutants and showed that in A. tumefaciens exoC is required for the in vivo synthesis of cellular

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<th>Strain or plasmid</th>
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<td>pFC6251</td>
<td>pVK102 (Km⁺)</td>
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TABLE 2. In vitro production of β(1-2)-glucan and accumulation of intermediates

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<th>Protein intermediate accumulation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β(1-2) Glucan production&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Counts per minute per milligram of protein in the 10% trichloroacetic acid-insoluble fraction.
<sup>b</sup> Picomoles of glucose per minute per milligram of protein recovered in the neutral fraction (DEAE-Sephadex A-25 eluate) from supernatants from incubations.
<sup>c</sup> Incubation of inner membranes with 400,000 cpm of UDP-[14C]glucose for 10 min at 10°C.
<sup>d</sup> Incubation as described in footnote c followed by the addition of 16 mM nonlabeled UDP-glucose and further incubation for 90 min.

as well as extracellular β(1-2)glucan. As shown previously (3, 19, 23), the mutants produced no detectable extracellular polysaccharide of any type. Plasmid pFC6251 restored the synthesis of succinoglycan to levels comparable to those in the wild-type strain.

In vitro synthesis of β(1-2)glucan. The inability of exoC mutants to synthesize β(1-2)glucan may have been due to a block in the transfer of glucose from UDP-glucose to the glucan or in an earlier step. To discern these possibilities, we tested the ability of purified inner membranes from exoC mutants to synthesize β(1-2)glucan in vitro in the presence of exogenously added UDP-glucose. Mutant inner membranes were active in the in vitro synthesis of β(1-2) [14C]glucan from UDP-[14C]glucose (Table 2), although prolonged incubation (data not shown) or chase conditions (Table 2) were needed. This glucan was eluted from a Bio-Gel P4 column at the same position as was wild-type β(1-2)glucan (Fig. 1). Moreover, no degradation of the glucan was observed after treatment with Glusulase; it was previously reported that Glusulase digested only linear glucans (34). Thus, mutant inner membranes were active in vitro in the formation of a cyclic β(1-2)glucan which was indistinguishable from that synthesized by the wild-type strain.

Presence and activity of the 235-kDa inner membrane protein. To determine if exoC mutants contain normal levels of the chvB-encoded 235-kDa inner membrane protein and whether this protein is active in forming the protein-glucan intermediate (33, 35, 36), we incubated purified inner membranes (200 μg of total protein) with UDP-[14C]glucose and submitted them to denaturing polyacrylamide gel electrophoresis and fluorography. After being stained with Coomassie blue, the 235-kDa protein (which migrated very close to the position of the 200-kDa molecular mass marker) was visible in similar amounts in mutant and wild-type strains (Fig. 2A). Figure 2B and Table 2 show that the accumulation of [14C]glucose in the intermediate was higher in exoC mutants than in wild-type or complemented strains. After a chase with unlabeled UDP-glucose under the conditions described in Materials and Methods, the decrease in the radioactivity of the protein-glucan intermediate was less in the mutants than in the wild-type or complemented strains.

As shown in Table 2, Chase experiments under previously described conditions (20 min with 2 mM UDP-glucose) (36) did not result in a significant dilution of radioactivity in the protein-glucan intermediate of exoC mutants (data not shown). Moreover, under these conditions the mutant's protein incorporated more than 10-fold more [14C]glucose than did the wild-type or complemented strain protein (data not shown). These results and the data on in vitro β(1-2)glucan synthesis indicated that exoC mutant's contain a 235-kDa inner membrane protein which is active in forming the protein-glucan intermediate, although some differences can be observed with respect to the wild type.

In vitro synthesis of UDP-glucose. Since exoC mutants synthesized β(1-2)glucan in vitro from exogenously added UDP-glucose, their inability to synthesize β(1-2)glucan in vivo may have been due to an inability to synthesize UDP-glucose. To test this possibility, we incubated cytosolic extracts from mutant and wild-type cells with three different substrates for UDP-glucose synthesis. The products of these incubations were identified by paper chromatography as described in Materials and Methods.

When [14C]glucose 1-phosphate was used as the substrate, UDP-[14C]glucose was synthesized by extracts from all strains (Table 3). With exoC mutant extracts, glucose 1-phosphate was totally converted to UDP-glucose. With wild-type and complemented strain extracts, 77 and 43% respectively, of the substrate was converted to UDP-glucose. Other labeled products were also observed (Table 3).
These fructose stopped glucose. On medium to 10,000-fold by [14C]glucose. This enzyme catalyzes the reaction glucose 1-phosphate ⇌ glucose 6-phosphate. It has been purified from different sources and requires glucose, 1,6-bisphosphate as a cofactor (18, 26). UDP-glucose is an intermediate in the synthesis of succinoglycan and β(1-2)glucan, both of which are absent in the mutants (3, 19). R. meliloti exoC mutants are also defective in the saccharide component of lipopolysaccharide (19), which contains glucose and galactose (32). Presumably, A. tumefaciens exoC mutants also have this defect. The inability to synthesize UDP-glucose because of a lack of phosphoglucomutase activity could account for all of the known defects in polysaccharide synthesis by exoC mutants.

The inability of A. tumefaciens exoC mutants to attach to plant cells and form tumors is probably due to their inability to synthesize cyclic β(1-2)glucan (3). Inner membranes from exoC mutants incubated with UDP-[14C]glucose formed normal cyclic β(1-2)glucan in vitro. Thus, when the intermediate (UDP-glucose) is supplied, glucan synthesis proceeds and the mutation does not appear to have an effect. This hypothesis is consistent with the proposal that a block at the level of phosphoglucomutase and not at a later step in glucan synthesis is the biochemical basis for the inability of the mutants to synthesize β(1-2)glucan. Whether the mutants have defects in later steps in the biosynthesis of other polysaccharides and whether these defects are relevant to tumorigenesis remain to be determined. Our data do not indicate whether exoC is the structural gene for phosphoglucomutase or is required indirectly (perhaps as a positive regulator) for the expression of phosphoglucomutase activity.

The metabolic pathway shown in Fig. 3 was proposed for E. coli and yeasts, and our results suggest that it is partially valid for Agrobacterium spp. It appears that the Leloir pathway, which converts galactose to UDP-galactose by two different reactions (utilizing UTP or UDP-glucose as the substrate), is not present in Agrobacterium spp., based on the following results. (i) Incubation of crude extracts of wild-type or exoC mutant strains with [14C]galactose did not yield glucose, glucose phosphate, galactose phosphate, UDP-glucose, or UDP-galactose (data not shown). (ii) exoC mutants grown on Luria medium with galactose or galactose plus glucose did not synthesize Cellulfluor White-binding succinoglycan. (iii) exoC mutants grew on minimal medium with galactose as the sole carbon source and did not synthesize Cellulfluor White-binding succinoglycan or β(1-2)glucan under these conditions. These results suggest that in Agrobacterium spp. an alternative pathway for the utilization of galactose must be present so that the bacteria can grow on this carbon source. An alternative pathway has been observed in Pseudomonas saccharophila (5). Further work is necessary to determine if this pathway is present in Agrobacterium spp.

The Entner-Doudoroff pathway and the pentose cycle have been proposed to be the main pathways for glucose

**FIG. 2.** Polycrylamide gel electrophoresis of A. tumefaciens inner membrane proteins. Inner membranes (0.2 mg of protein) were incubated with UDP-[14C]glucose for 10 min. The reaction was stopped by heating, the mixture was washed with trichloroacetic acid, and the precipitate was subjected to gel electrophoresis as described previously (35). Proteins were stained with Coomassie blue (A), and radioactivity was detected by fluorography (B). Lanes: 1, 3, and 5 correspond to chase experiments in which, after 10 min of incubation with UDP-[14C]glucose, 1 mM nonradioactive UDP-glucose was added and incubation was continued for 90 min. Lanes: 2, 4, 6, and 8 correspond to chase experiments in which, after 10 min of incubation with UDP-[14C]glucose, 16 mM nonradioactive UDP-glucose was added and incubation was continued for 90 min. Lanes: 1 and 2, wild-type strain A348; 3 and 4, complemented strain A5503(PFC6251); 5 and 6, exoC mutant A5503; 7 and 8, exoC mutant A5129. Numbers at left indicate molecular mass standards in kilodaltons. Incubation of exoC mutant extracts with [14C]glucose or [14C]glucose 6-phosphate did not produce detectable amounts of UDP-glucose, but labeled fructose 6-phosphate, glucose 6-phosphate, and fructose 1,6-bisphosphate were detected (Table 3). With extracts from wild-type and complemented strains, UDP-glucose was obtained, along with fructose 1,6-bisphosphate, glucose 1,6-bisphosphate, fructose 6-phosphate, glucose 6-phosphate, and glucose 1-phosphate. These results are consistent with an inactive phosphoglucomutase in exoC mutants.

**Growth on galactose.** When grown on Cellulfluor White-Luria agar plates supplemented with 0.2% galactose, A. tumefaciens exoC mutant A5129 remained dark under UV light, indicating that the synthesis of succinoglycan was not restored by growth on galactose (data not shown). Growth of wild-type and exoC mutant bacteria appeared to be faster on this medium than on the same medium supplemented with glucose. Similar results were obtained with M9 minimal medium supplemented with glucose or galactose. The ability to synthesize β(1-2)glucan was not restored to the mutants by growth on minimal medium supplemented with galactose. On this medium, the wild-type strain produced 5.1 mg of glucose equivalents of cellular β(1-2)glucan per g of wet-weight cells. Moreover, exoC mutants were avirulent whether grown on glucose or galactose.

**DISCUSSION**

We have demonstrated that A. tumefaciens exoC mutants are unable to synthesize UDP-glucose in vitro from glucose or glucose 6-phosphate. However, the synthesis of UDP-glucose occurred when glucose 1-phosphate was supplied as the substrate. These results indicate that in the mutants, the enzyme phosphoglucomutase (EC 2.7.5.1) is inactive or is missing (Fig. 3). This enzyme catalyzes the reaction glucose 1-phosphate ⇌ glucose 6-phosphate. It has been purified from different sources and requires glucose, 1,6-bisphosphate as a cofactor (18, 26). UDP-glucose is an intermediate in the synthesis of succinoglycan and β(1-2)glucan, both of which are absent in the mutants (3, 19). R. meliloti exoC mutants are also defective in the saccharide component of lipopolysaccharide (19), which contains glucose and galactose (32). Presumably, A. tumefaciens exoC mutants also have this defect. The inability to synthesize UDP-glucose because of a lack of phosphoglucomutase activity could account for all of the known defects in polysaccharide synthesis by exoC mutants.

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metabolism in Agrobacterium spp. (1, 31). The Embden-Meyerhof-Parnas pathway has been proposed to be nonfunctional, and the key enzyme phosphofructokinase (EC 2.7.1.11) has been proposed to be nonactive (1) or to have very low activity (31). Under our assay conditions, the formation of fructose 6-phosphate and fructose 1,6-bisphosphate was detected, indicating that this enzyme is present. Apart from phosphoglucomutase activity, the only other biochemical difference we observed between wild-type and exoC mutant strains was in the labeling of the 235-kDa protein-glucan intermediate. After incubation of mutant inner membranes with UDP-[14C]glucose, the amount of radioactive intermediate accumulated was approximately 10-fold higher in the mutants than in the wild type. Although the

<table>
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<th>Strain</th>
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<td>Glucose 1,6-bisphosphate</td>
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<td>A348</td>
<td>Glucose</td>
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</tbody>
</table>

* Incubations were carried out with cytosolic fractions as described in Materials and Methods. Amounts recovered are expressed as counts per minute per milligram of protein (percentages of total radioactivity recovered). —, Not determined. ND, None detected (less than 100 cpm; confirmed by three different assays). Total amounts (percentages) of hexose bisphosphate produced by strains A348, A5129(pFC6251), and A5503(pFC6251) with glucose 1-phosphate as the substrate were 13,600 (3.5), 17,600 (7.2), and 17,200 (7.5), respectively. The total amount (percentage) of hexose 6-phosphate produced by strain A5503(pFC6251) with glucose 6-phosphate as the substrate was 55,600 (12.4).

![Figure 3](image-url)
radioactivity decreased upon a chase with nonlabeled UDP-glucose, it remained higher than in the wild type. The simplest interpretation for these results is that the 235-kDa protein from the mutant is free of nascent glucan chains because of the lack of UDP-glucose. When UDP-glucose was added in vitro, the reaction proceeded with no iso- topel dilution with preformed β(1-2)glucan chains, resulting in higher radioactivity on the protein. One explanation for the results obtained after the chase is that the innermost portion of the glucan linked to the protein may not cycle during synthesis, which would result in a nonchase percentage of the radioactivity on the protein from exoC mutants. Further work will be required to firmly establish this possibility.

It is interesting that A. tumefaciens exoC mutants, which we characterized as lacking phosphoglucomutase activity, are not motile (3). pgm mutants of Bacillus licheniformis have also been reported to be nonmotile (10). Like exoC mutants, chvB mutants are defective in β(1-2)glucan synthesis (8, 14) and are nonmotile. These results suggest that there may be a relationship between this polysaccharide and motility in A. tumefaciens. We cannot rule out the possibility that the lack of a glucan in B. licheniformis may also affect motility.

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