JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 1076–1081 Vol. 177, No. 4
0021-9193/95/$04.00+0

Mechanism of Cellulose Synthesis in *Agrobacterium tumefaciens*

ANN G. MATTHYSSE,*[1] DARBY L. THOMAS,[1]† AND ALAN R. WHITE[2]

Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599,[1] and Department of Botany, North Dakota State University, Fargo, North Dakota 58105[2]

Received 17 February 1994/Accepted 16 December 1994

Extracts of *Agrobacterium tumefaciens* incorporated UDP-[^{14}C]glucose into cellulose. When the extracts were fractionated into membrane and soluble components, neither fraction was able to synthesize cellulose. A combination of the membrane and soluble fractions restored the activity found in the original extracts. Extracts of cellulose-minus mutants showed no significant incorporation of UDP-glucose into cellulose. When mixtures of the extracts were made, the mutants were found to fall into two groups: extracts of mutants from the first group could be combined with extracts of the second group to obtain cellulose synthesis. No synthesis was observed when extracts of mutants from the same group were mixed. The groups of mutants corresponded to the two operons identified in sequencing the *cel* genes (A. G. Matthysse, S. White, and R. Lightfoot. J. Bacteriol. 177:1069–1075, 1995). Extracts of mutants were fractionated into membrane and soluble components, and the fractions were mixed and assayed for the ability to synthesize cellulose. When the membrane fraction from mutants in the *celDE* operon was combined with the soluble fraction from mutants in the *celABC* operon, incorporation of UDP-glucose into cellulose was observed. In order to determine whether lipid-linked intermediates were involved in cellulose synthesis, permeabilized cells were examined for the incorporation of UDP-[^{14}C]glucose into material extractable with organic solvents. No radioactivity was found in the chloroform-methanol extract of mutants in the *celDE* operon, but radioactive material was recovered in the chloroform-methanol extract of mutants in the *celABC* operon. The saccharide component of these compounds was released after mild acid hydrolysis and was found to be mainly glucose for the *celA* insertion mutant and a mixture of cellobiose, cellotriose, and cellotetrose for the *celB* and *celC* insertion mutants. The radioactive compound extracted with chloroform-methanol from the *celC* insertion mutant was incorporated into cellulose by membrane preparations from *celE* mutants, which suggests that this compound is a lipid-linked intermediate in cellulose synthesis.

Cellulose, which is made by plants and by some bacteria and fungi, is one of the most abundant biologically produced materials. It is a relatively simple molecule consisting of a linear chain of β-1,4-glucose residues without any modifications or side chains. Despite these facts, the mechanism of cellulose synthesis is not understood in detail (5, 7, 19). We do not know whether all organisms which make cellulose use similar pathways or whether there exist alternate pathways used by different groups of organisms.

The mechanism of cellulose synthesis has been examined most extensively in *Acetobacter xylinum*. The precursor is UDP-glucose. Cell extracts of the bacteria incorporate UDP-glucose into cellulose (1, 11). An unusual cofactor, cyclic diguanylic acid, is required for cellulose synthesis in this organism. The cofactor can be degraded by a protein contained in the cell extracts, and it has been proposed that the concentration of cyclic diguanylic acid may regulate cellulose synthesis in this bacterium (18). An operon containing four genes involved in cellulose synthesis has been sequenced from one strain of *A. xylinum* (25). The first two genes of this operon have also been identified and sequenced from another strain of *A. xylinum* (21, 22). The product of the first gene appears to be a cellulose synthase which binds UDP-glucose (21). The product of the second gene has been proposed to be involved in the interaction of the enzyme with cyclic diguanylic acid (22). In *A. xylinum*, it is believed that cellulose is synthesized directly from UDP-glucose and that the function of the other genes is to determine the organization or packing of the cellulose chains with respect to each other (19, 22).

Agrobacterium tumefaciens synthesizes cellulose after it has come into contact with host cells. These cellulose fibrils bind the bacteria tightly to the plant cell surface (14). They also cause the formation of large aggregates of bacteria in which only a small fraction of the bacteria are directly bound to the host cell. The majority of the bacteria are held in place by a meshwork of bacterially produced cellulose fibrils (12, 13). Such exopolysaccharides appear to play a role in the interaction of many pathogenic bacteria with host cells and in the interaction of many ecologically important bacteria (including those which form biofilms) with the substrata to which they attach (10, 19).

MATERIALS AND METHODS

Bacterial strains, growth, and construction of mutants. *A. tumefaciens* C58 is a wild-type strain from the laboratory collection. A1045 is a *chvB* mutant of C58 obtained from E. Nester. Derivatives of strain C58 which contain transposon insertions that result in a cellulose-minus phenotype have been described by Matthysse et al. (15). The positions of the insertions are shown in Fig. 1. The insertions were introduced into the chromosome of strain A1045 as described by Matthysse et al. (15). Bacteria were grown in Luria broth or minimal medium as previously described (12). Antibiotics were used at the following concentrations: neomycin at 20 mg/liter in liquid media and 60 mg/liter in agar media, carbenicillin at 50 mg/liter, gentamicin at 50 mg/liter, and tetracycline at 10 mg/liter. *A. tumefaciens* strains were grown at room temperature (approximately 25°C). *Escherichia coli* strains were grown at 37°C.

Preparation of permeabilized cells, cell extracts, and cell fractions. Bacteria were grown to stationary phase in Luria broth containing 0.02% soytone (Difco) to maximize the production of cellulose by wild-type strains and the accumulation of intermediates by mutants. Bacteria were harvested by centrifugation at 10,000 × *g* and washed in 50 mM Tris-HCl, pH 7.5. Permeabilized cells were prepared by the method of Reuber and Walker (17). The bacteria were resus-

* Corresponding author. Phone: (919) 962-6941. Fax: (919) 962-1625. Electronic mail address: matthysse@unc.edu.

† Present address: Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, TX 77030.

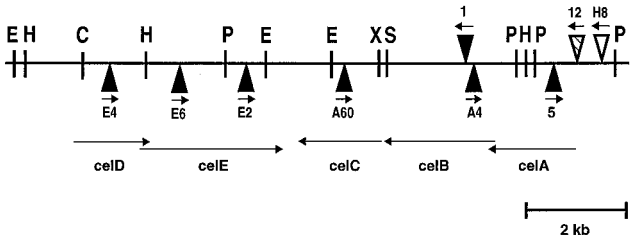

FIG. 1. Map of the cosmid clone containing *cel* genes from *A. tumefaciens* C58. The positions of Tn*3*HoHo1 insertions which resulted in a cellulose-minus phenotype (filled triangles), insertion 12, which resulted in a reduced level of cellulose synthesis (striped triangle), insertion H8 (open triangle), which had no effect on cellulose synthesis, and the direction of β-galactosidase transcription (arrows) are indicated. Restriction enzyme sites are indicated as follows: E, *Eco*RI; C, *Cla*I; P, *Pst*I; B, *Bam*HI, H, *Hin*dIII; S, *Sac*I; and X, *Xba*I. The locations and directions of open reading frames are shown by the long arrows below the map (15).

pended in 70 mM Tris-HCl, pH 8.2, at 1/100 of the original volume of culture. They were then frozen and thawed three times and were stored frozen at −70°C until use. For the preparation of cell extracts, a modification of the method of Aloni et al. (1) was used. The bacteria were resuspended in 1/20 of the original culture volume in ice-cold 50 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–1 mM EDTA (TME) and were disrupted by sonication in a Branson sonifier, with bursts of 5 s alternating with 5-s intervals for 5 min. The unbroken cells were removed by centrifugation at 10,000 × *g* for 2 min. The supernatant was frozen and stored at −70°C for use as a whole cell extract. For the preparation of cell membrane and soluble fractions, the cell extract was centrifuged at 150,000 × *g* for 90 min. The supernatant was used as the soluble fraction. The pellet was resuspended in TME and used as the membrane fraction. Both fractions were stored frozen at −70°C.

Measurement of cellulose synthesis. *A. tumefaciens* synthesizes at least two polysaccharides for which UDP-glucose is a precursor, cellulose and β-1,2-D-glucan (9). The latter compound is water soluble and did not directly interfere with measurements of cellulose synthesis. However, in some extracts the two reactions appeared to compete for UDP-glucose, so unless otherwise noted all measurements of cellulose synthesis were carried out with *A. tumefaciens* Tn*5* mutant strain A1045, which lacks the enzyme for the synthesis of β-1,2-D-glucan from UDP-glucose (16, 26). All of the cellulose-minus mutants were examined after the *cel* mutation had been introduced into the A1045 genetic background.

For cell extracts and fractions, the procedure used to measure the incorporation of UDP-[^{14}C]glucose into cellulose was based on that of Aloni et al. (1). The reaction mixtures contained TME (pH 7.5), 1 mM $CaCl_2$, 30 μM UDP-glucose, and 1 μCi of UDP-[^{14}C]glucose per ml. Reactions after incubation at 25°C for 20 min were stopped by the addition of 25 volumes of 0.5 M NaOH–0.05 M $NaBH_4$ containing 1 mg of cellulose (Sigmacell) per ml as a carrier. The mixtures were heated at 65°C for 20 min, and the precipitate was collected by filtration onto GF/C glass filter papers and was washed three times with water and three times with ethanol. The filter papers were dried, and radioactivity was measured in a liquid scintillation counter.

For permeabilized cells, reaction mixtures contained permeabilized cells, 12 mM $MgCl_2$, and a total of 0.02 to 0.05 μCi of UDP-[^{14}C]glucose. The procedure was modified from that of Reuber and Walker (17). The mixture was incubated for 30 min at 10°C. The reaction was stopped by the addition of 5 volumes of ice-cold 70 mM Tris-HCl (pH 8.3)–10 mM EDTA. The cells and insoluble material were collected by centrifugation at 10,000 × *g* for 2 min and were washed two times with cold 70 mM Tris-HCl (pH 8.3). The level of incorporation of UDP-glucose into cellulose was determined by the same procedure (base hydrolysis and filtration) as described above for the cell extracts and fractions.

Methylation linkage analysis of reaction products. Samples were derivatized for glycosyl linkage analysis by methylation with *n*-butyllithium (6). Reaction products dried over P205 were dissolved in 0.2 to 0.5 ml of dimethyl sulfoxide, and *n*-butyllithium followed by methyl iodide was added slowly. Methylated samples were extracted with chloroform and washed with water, and the chloroform phase was evaporated to dryness. The samples were fully hydrolyzed with 2 M trifluoroacetic acid at 121°C for 1 h and were reduced with $NaBD_4$. Per-O-methylated alditols were acetylated with acetic anhydride and 1-methylimidazole as a catalyst (3).

Partially methylated alditol acetates were analyzed for incorporation of [^{14}C]glucose by the method of White et al. (23, 24). Radiolabelled partially methylated alditol acetates were dissolved in 100 μl of acetone, and the radioactivity of 1 μl of this sample was determined. An aliquot containing 60 to 300 kBq of radioactivity was evaporated to dryness, redissolved in 5 μl of acetone, and introduced by a 3-min splitless injection on an SP-2330 glass capillary column (30 m by 0.75 mm; Supelco Inc., Bellefonte, Pa.) at 170°C. After a 3-min delay, the column temperature was raised by 4°C/min to 240°C. Column effluents were split (10:90) between a flame ionization detector and a radiogas proportional counter (Radiomatic Flo-One\Beta, model GCR Radiochromatography detector; Radiomatic Instruments and Chemical Co., Meriden, Conn.). Radioactive peaks were identified by comparing peaks and retention times with injections of the same samples on a Hewlett-Packard gas chromatography mass spectrometer with a 30-m SP-2330 fused silica capillary column and a similar temperature program. Proportions of ^{14}C incorporated into different glycosyl linkages were determined by calculation of percent area of identified peaks from integrations of the detector response.

Isolation of intermediates and thin-layer chromatography. The procedure for isolation of possible lipid-linked intermediates was that of Reuber and Walker (17). After washing of the permeabilized cells which had been incubated with UDP-[^{14}C]glucose, the cell pellet was extracted twice with chloroform-methanol-water (1:2:0.3). The radioactivity in 5% of the extract was determined, and the remainder was dried in a Speed Vac (Savant). The pellet was dissolved in 10 μl of water. To remove the lipid from the carbohydrate, the extract was hydrolyzed with 0.01 M trifluoroacetic acid at 95°C for 20 min. The extract was then neutralized with 1 M NH_4OH. For some samples, the extract was treated with alkaline phosphatase as described by Reuber and Walker (17). The extract was then extracted with 5 volumes of chloroform-methanol (1:2), and the water fraction was dried in a Speed Vac and dissolved in water. The sample was then applied to thin-layer chromatography plates. Some extracts were digested with cellulase (Worthington P or PB) or with laminarinase (Sigma).

Thin-layer chromatography was carried out by the method of Doner (8), using silica gel or cellulose flexible sheets (Baker-flex). The solvent was *n*-butanol–pyridine–water (8:5:4). The plates were run two or three times as described by Doner (8). Nonradioactive carbohydrate standards were detected by spraying the chromatogram with a mixture of 4 ml of aniline, 4 g of diphenylamine, 30 ml of 85% H_3PO_4, and 200 ml of acetone. The chromatogram was heated briefly in a 90°C oven to develop the color. Radioactive compounds run in separate lanes from the standards were detected by cutting the chromatogram into pieces and measuring the radioactivity in a liquid scintillation counter.

RESULTS

Conditions for incorporation of UDP-[^{14}C]glucose into cellulose. The effects of various conditions on the incorporation by bacterial extracts of UDP-[^{14}C]glucose into material which was insoluble in base, water, and ethanol were examined. The incorporation was linear with time for 30 min or more. The rate of incorporation was higher at 37 than at 25°C. However, since *A. tumefaciens* does not grow at 37°C, except as we have noted, all measurements were made at 25°C. No difference was seen between the incorporation by extracts of wild-type C58 bacteria and those of the *chvB* mutant A1045, except that the results were more reproducible in the A1045 strain (presumably because of the lack of the competing reaction for the incorporation of UDP-glucose into β-1,2-D-glucan). The reaction was not significantly affected by the addition of ATP, GTP, or cellobiose. The pH optimum for the reaction was between 7.0 and 8.0. Incorporation was reduced at pH 8.5 and 6.5. No incorporation was observed when the bacterial extract was boiled before use (Table 1). Cyclic diguanylic acid is required for in vitro cellulose synthesis by extracts of *A. xylinum* (20). The effect of cyclic diguanylic acid on incorporation of UDP-glucose into insoluble material by extracts of *A. tumefaciens* strain A1045 was examined. No effect was seen, although the same preparation of cyclic diguanylic acid was effective in increasing the incorporation by extracts of *A. xylinum* ATCC 53582.

In order to examine the identity of the base-, water-, and ethanol-insoluble material into which UDP-[^{14}C]glucose was incorporated by extracts of *A. tumefaciens*, the insoluble material was collected by centrifugation in an Eppendorf centrifuge tube and was digested with enzymes or dilute acid and the products were examined by thin-layer chromatography. When the material was digested by cellulase or 1 M HCl, radioactivity was recovered in cellobiose and glucose after short digestion times. After longer digestion times (overnight) all of the radioactivity was recovered in glucose. No soluble radioactive material was recovered after digestion of the product by laminarinase.

TABLE 1. Incorporation of UDP-[^{14}C]glucose into base-insoluble material by extracts of *A. tumefaciens*

Source of extract	Addition(s) to reaction mixture[a]	pmol of UDP-glucose incorporated (± SD)[b]
C58	None	140 ± 50
A1045[c]	None	110 ± 15
A1045	2 mM cellobiose	130 ± 15
A1045	200 μM ATP	100 ± 20
A1045	200 μM GTP	70 ± 20
A1045	Cyclic diguanylic acid[d]	100 ± 15
A1045	200 mM GTP, 2mM $CaCl_2$, excess supernatant[e]	90 ± 15
A1045	pH 8.5[f]	30 ± 10
A1045	pH 6.0[f]	50 ± 15
C58, boiled before use	None	<10

[a] The reaction mixture contained 10 mM $MgCl_2$, 75 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM $CaCl_2$, 30 μM UDP-glucose, and a cell extract from 10^8 bacteria.

[b] The reactions were carried out at 37°C for 10 min. About half of the UDP-glucose incorporated at 37°C was incorporated at 25°C. Values are the means ± the standard deviations of a minimum of three independent measurements.

[c] C58::Tn*5* *chvB* mutant.

[d] Prepared according to the method of Ross et al. (18) from *A. xylinum*. The same preparation yielded a 10-fold increase in incorporation by *A. xylinum* extracts.

[e] Additional supernatant from the preparation of the membrane fraction from 10^8 cells was added.

[f] Tris-HCl (75 mM) made to the appropriate pH.

TABLE 2. Incorporation of UDP-glucose into cellulose by bacterial extracts[a]

Bacterial strain(s)	Fraction(s)	pmol of UDP-[^{14}C]glucose incorporated (± SD)
A1045	Whole cell extract	70 ± 7
A1045	Membrane	5 ± 1
A1045	Soluble	<1
A1045	Membrane and soluble	60 ± 8
A1045::E2 (*celE*)	Whole cell extract	7 ± 1
A1045::E4 (*celD*)	Whole cell extract	8 ± 3
A1045::A4 (*celB*)	Whole cell extract	<1
A1045::1 (*celB*)	Whole cell extract	<1
A1045::A60 (*celC*)	Whole cell extract	<1
A1045::E2 (*celE*) and A1045::A4 (*celB*)	Whole cell extracts	70 ± 10
A1045::E2 (*celE*) and A1045::A60 (*celC*)	Whole cell extracts	65 ± 8
A1045::A4 (*celB*) and A1045::A60 (*celC*)	Whole cell extracts	<1
A1045::E2 (*celE*) and A1045::A4 (*celB*)	Soluble (E2) and membrane (A4)	<1
A1045::E2 (*celE*) and A1045::A4 (*celB*)	Membrane (E2) and soluble (A4)	75 ± 7

[a] The reaction conditions were the same as those described in Table 1 and its footnotes.

The product into which [^{14}C]glucose was incorporated by permeabilized cells of *A. tumefaciens* A1045 was further characterized by methylation linkage analysis. Of the glucose recovered, 55% was 4-linked glucose and 45% was terminal glucose. The terminal glucose may include both ends of chains, glucose bound to other molecules, and unincorporated UDP-glucose carried along during the purification. No 2-, 3-, or 6-linked glucose was detected.

Cellulose synthesis by cell fractions and by mutants. When extracts of strain A1045 were separated into membrane and soluble fractions, both fractions showed much reduced incorporation of UDP-[^{14}C]glucose into insoluble material when compared with incorporation by the unfractionated extract (Table 2). When the membrane and soluble fractions were combined, most of the activity of the original extract was restored. Extracts of cellulose-minus mutants did not incorporate UDP-glucose into cellulose. However, when mixtures of the extracts from different mutants were made, the extracts were found to fall into two groups. When extracts of mutants carrying the E2, E4, or E6 Tn*3*HoHo1 insertions in the *celDE* genes were mixed with extracts of mutants carrying the *celC*A60, *celB*A4, *celB*1, or *celA*5 insertions, levels of incorporation of UDP-glucose were almost as high as for extracts of wild-type cells. When extracts of mutants from within a single group were mixed, no increase in the level of incorporation over that obtained with single extracts was observed. When the extracts of the mutant cells were fractionated into membrane and soluble components and these fractions were combined, it was found that the membrane fraction from the *celE*E2 or the *celD*E4 insertion combined with the soluble fraction from the *celB*A4 insertion showed incorporation of UDP-glucose into insoluble material. The reverse combination yielded no incorporation (Table 2).

Intermediates in cellulose synthesis. These results suggested that the gene products from the *celE*E2 and the *celD*E4 insertion region (the *celD* and *celE* gene products) were found in the soluble fraction and that the products from the *celB*A4 insertion region (the *celB* and *celC* gene products) were found in the membrane fraction. They also suggested that it was possible that there were intermediates in cellulose synthesis. In order to test the possibility that lipid intermediates were involved in cellulose synthesis, permeabilized cells of the various mutants were incubated with UDP-[^{14}C]glucose and then were extracted with chloroform-methanol-water. The radioactivity in the organic solvent extract was measured. Small amounts of radioactivity (50 to 100 cpm) were found in extracts of A1045. No significant radioactivity was found in extracts of the *celE*E2, *celD*E4, and *celE*E6 insertion mutants. Extracts of the *celB*1, *celC*A60, and *celA*5 insertion mutants all showed radioactivity in the extract (Table 3). The labelled saccharide from these extracts was hydrolyzed from the lipid carrier, treated with alkaline phosphatase, and characterized by thin-layer chromatography. Most of the radioactivity was found in glucose in the *celA*5 insertion mutant. In the *celB*1 and *celC*A60 insertion mutants, the radioactivity comigrated with the cellobiose, cellotriose, and cellotetrose standards (Fig. 2).

In order to test the role of the lipid-linked compound in cellulose synthesis, the radioactive organic solvent extracts from the *celB*1 and *celC*A60 insertion mutants were dried before being dissolved in water. They were then used to replace UDP-[^{14}C]glucose in a reaction mixture for cellulose synthesis with membrane preparations of the *celD*E4 insertion

TABLE 3. Radioactivity recovered in the organic solvent extract of permeabilized cells incubated with UDP-[^{14}C]glucose[a]

Bacterial strain	Radioactivity (cpm ± SD) recovered in chloroform-methanol extract
A1045::E2 (*celE*)	<20
A1045::5 (*celA*)	430 ± 80
A1045::1 (*celB*)	210 ± 50
A1045::A60 (*celC*)	180 ± 60

[a] The reaction conditions were the same as those described in Table 1 and its footnotes.

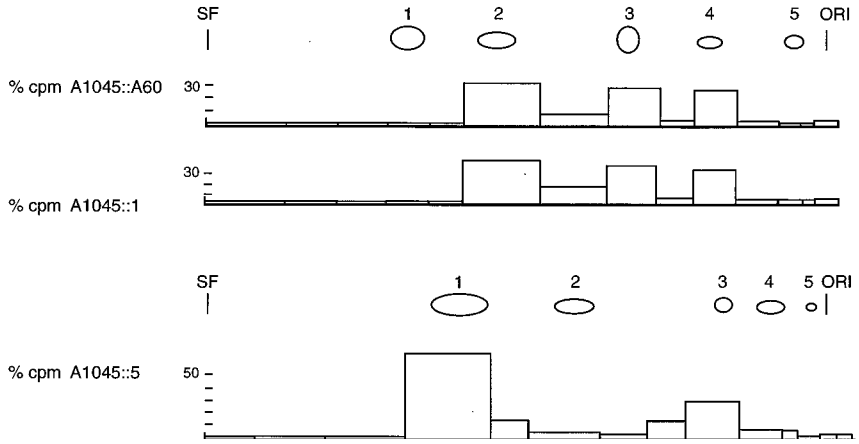

FIG. 2. Thin-layer chromatogram of sugars released by acid hydrolysis of the organic solvent extract of permeabilized cells incubated with UDP-[^{14}C]glucose. The products of the *celA*5, *celB*1, and *celC*A60 Tn*3*HoHo1 insertion mutants are shown. Carbohydrate standards were run in a separate lane before being stained. The standards used were glucose (1), cellobiose (2), cellotriose (3), cellotetrose (4), and cellopentose (5). A tracing of their positions is shown above the graph of the remainder of the chromatogram, which was cut up and counted in a liquid scintillation counter. The percent of the total counts per minute found in each peak is given, since the total count-per-minute value was determined by the amount of sample loaded. The totals ranged from 300 to 800 cpm in different loadings of the samples. SF, solvent front; ORI, origin.

mutant and of cellulose-positive parent strain A1045. When the extract of the *celB*1 insertion mutant was used, no significant radioactivity was recovered in a base- and ethanol-insoluble product. When the extract of the *celB*A60 insertion mutant was used, most of the radioactivity included in the reaction mixture was recovered in a base- and ethanol-insoluble product. The addition of unlabelled UDP-glucose to the reaction mixture had no effect on the incorporation of the intermediate by the membrane preparation of the *celE*E2 mutant (Table 4). These results suggest that although the saccharide moiety of the radioactive lipid-linked compound isolated for the *celC*A60 and *celB*1 insertion mutants appears to be the same, the complete compounds are not the same. The possibility that one of these compounds was phosphorylated was examined by comparing the results of thin-layer chromatography of extracts which had been acid hydrolyzed and were dephosphorylated before they were analyzed with results for extracts that were not dephosphorylated. No difference between extracts of the *celB*1 and *celC*A60 insertion mutants that were dephosphorylated and extracts that were not dephosphorylated was observed which suggests that the difference between the two compounds may reside in the lipid moiety.

TABLE 4. Incorporation of radioactive intermediates into cellulose

Source of radioactive intermediate[a]	Addition(s) to reaction mixture[b]	Radioactivity incorporated (cpm ± SD)[c]
A1045::1 (*celB*)	A1045::E4 (*celD*) membrane fraction	<10
A1045::A60 (*celC*)	A1045::E4 (*celD*) membrane fraction	410 ± 50
A1045::A60 (*celC*)	A1405::E4 (*celD*) membrane fraction, 20 μM UDP-glucose	400 ± 60
A1045::A60 (*celC*)	A1045::E4 (*celD*) soluble fraction	<20
A1045::A60 (*celC*)	A1045::1 (*celB*) whole cell extract	<20

[a] Radioactive intermediates were extracted from permeabilized cells incubated with UDP-[^{14}C]glucose. Between 500 and 700 cpm of radioactivity were added to the reaction.

[b] The reaction mixture contained TME buffer, 2 mM $CaCl_2$, and the indicated fraction from 10^8 bacteria.

[c] Incorporation of radioactivity into cellulose was determined as the counts per minute which were insoluble in water and ethanol after hydrolysis at 65°C in 0.5 M NaOH–0.05 M $NaBH_4$ for 20 min.

DISCUSSION

Cell extracts and permeabilized cells of wild-type *A. tumefaciens* incorporated UDP-[^{14}C]glucose into material which was not hydrolyzed by treatment with 0.5 M NaOH at 65°C in the presence of $NaBH_4$ and was insoluble in water and ethanol. Analysis of the product by enzymatic and acid digestion suggested that it was cellulose. Methylation analysis of the radioactive product produced by permeabilized cells of strain A1045 revealed only terminal and 4-linked glucose residues. This incorporation appeared to be similar to that observed by Amikam and Benziman using membrane preparations of *A. tumefaciens* (2). No incorporation was obtained when the membrane or soluble preparation was boiled prior to addition to the UDP-glucose reaction mixture. The addition of cyclic diguanylic acid to the reaction mixture had no effect on cellulose synthesis by extracts of *A. tumefaciens* A1045, although this compound stimulates in vitro cellulose synthesis in extracts of *A. xylinum* (20) and stimulates cellulose synthesis in extracts of *A. tumefaciens* C58 pTiGV3851 (2). We observed that our preparations of cyclic diguanylic acid stimulated incorporation of UDP-[^{14}C]glucose into cellulose by extracts of *A. xylinum*, as reported by Ross et al. (18). The reason for this difference between *A. tumefaciens* A1045 and C58pGV3851 and *A. xylinum* in their response to cyclic diguanylic acid is not clear. It is possible that this cofactor is required in *A. tumefaciens* and that our extracts contain such large amounts of it that we can observe no effect with additional amounts. It is also possible that the mechanisms of cellulose synthesis differ in the two organisms. A third possibility is that cyclic diguanylic acid is a regulatory molecule in *A. xylinum* (22) and that cellulose synthesis is regulated differently in *A. tumefaciens*. It is thought that cyclic diguanylic acid binds to the *bscB* gene product of *A. xylinum* (22). A gene homologous to *bscB* was not detected in the sequence of the operons required for cellulose synthesis in *A. tumefaciens* (15).

Extracts of cellulose-minus mutant bacteria did not incorporate UDP-[^{14}C]glucose into cellulose. However, when mix-

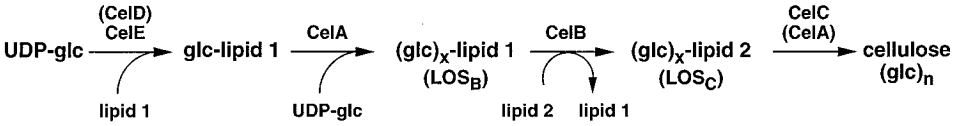

FIG. 3. Model for reactions involved in synthesis of cellulose from UDP-glucose in *A. tumefaciens*. The gene products required for each reaction are indicated above the arrows. When the polar nature of tranposon insertions precludes the determination of the involvement of a particular gene product in a given step, the gene is shown in parentheses. In the reaction catalyzed by the *celB* gene product, the data are insufficient to distinguish between a modification of the first lipid and the transfer to a different lipid carrier. LOS, lipid-linked oligosaccharide. The value of x can be 2, 3, or 4; and that of n greater than 6.

tures of extracts from cellulose mutant strains were made, the mutants fell into two groups. When extracts of mutants of the first group (the *celE*E2, *celD*E4, or *celE*E6 Tn*3*HoHo1 insertion mutants) were mixed with extracts of the second group (the *celC*A60, *celB*A4, *celB*1, or *celA*5 Tn*3*HoHo1 insertion mutants), the mixture of extracts was able to incorporate UDP-glucose into cellulose. However, mixtures of extracts of mutants belonging to the same group were unable to incorporate UDP-glucose into cellulose. These results fit with the results of the DNA sequencing (15) which place the two groups of mutants in two separate operons, *celABC* and *celDE* (Fig. 1).

Examination of the activities of membrane and soluble fractions of the mutants suggested that the activity of the products of the *celABC* operon was localized in the membrane fraction and the activity of the products of the *celDE* operon was localized in the soluble fraction. This result agrees with the predicted localization of the gene products on the basis of their hydrophilicity profiles (15).

Lipid intermediates are known to be involved in the synthesis of several bacterial exopolysaccharides (4, 10, 17). In order to examine the possibility that a lipid intermediate was involved in cellulose synthesis, the incorporation of radioactivity from UDP-[^{14}C]glucose into material extractable by organic solvents from permeabilized cells of each of the Tn*3*HoHo1 mutants was examined. Mutants in the *celDE* operon did not show any such incorporation. Mutants in the *celABC* operon did incorporate radioactivity into organic-solvent-extractable material. When the putative lipid intermediate was acid hydrolyzed and the saccharide portion was examined, most of the radioactivity from insertion mutant *celA*5 was found in glucose, while the radioactivity from the *celB*1 and *celC*A60 insertion mutants was found in cellobiose, cellotriose, and cellotetrose. The intermediate extracted by organic solvents from the *celC*A60 mutant could be incorporated into cellulose by extracts of the *celE*E2 insertion mutant. The intermediate from the *celB*1 insertion mutant could not be incorporated into cellulose by these extracts. These results suggest that the product of the *celA* gene (cellulose synthase) is required for the formation of cellobiose, cellotriose, and cellotetrose lipid-linked intermediates from UDP-glucose and that the intermediates accumulated by the *celBC* and *celC* mutants are not the same.

The results of these experiments and the results of the DNA sequencing (15) can be combined to make a hypothetical pathway for the process of cellulose synthesis in *A. tumefaciens*. This model pathway is shown in Fig. 3; in it, UDP-glucose is first attached to a lipid by the product of the *celE* gene to form a lipid intermediate. The product of the *celD* gene may also be involved in this reaction, but because of the polarity of transposon mutants our data do not allow us to determine whether *celD* is required for this reaction. Both analysis of the predicted amino acid sequences of the *celD* and *celE* gene products and the in vitro cellulose synthesis reactions suggest that these proteins are found in the soluble cell fraction, presumably in the cytoplasm. The lipid-glucose intermediate in the cytoplasm would then have additional glucose moieties added by the *celA* gene product which has homology to the cellulose synthase gene from *A. xylinum* (15). The product of this reaction which accumulates in *celB* mutants has 2 to 4 glucose residues linked to the lipid intermediate. It is possible that the additional glucose residues beyond cellobiose present on the intermediates which accumulate in *celB* and *celC* mutants represent additions to the lipid-linked cellobiose made by the cellulose synthase when further reactions of the lipid-linked intermediates are blocked and thus that lipid-linked cellotriose and cellotetrose are not normal intermediates in cellulose synthesis. The *celB* gene product converts this lipid-linked intermediate into another lipid-linked intermediate without altering the glucose residues attached. This process may involve a reaction with the first lipid which alters its structure or the transfer of the glucose chain to a different lipid. This new lipid-linked intermediate (LOS_c) accumulates in *celC* mutants. The relation of these lipid-linked intermediates to transport across the plasma membrane is unknown. The last intermediate (LOS_c) can be incorporated into cellulose by membrane fractions from wild-type or *celD*E4 insertion mutant cells. This incorporation requires the *celC* gene product. The *celC* gene product is homologous to several bacterial 1,4-endoglucanases. During cellulose synthesis, the *celC* gene product may act as a transferase rather than as an endoglucanase. Because *celC* is the last gene in the *celABC* operon, we are unable to determine whether the *celB* and *celA* gene products are also required for the incorporation of this last intermediate into cellulose. In in vitro cellulose synthesis, the activity of the *celABC* gene products appears to be found in the membrane fraction. This agrees with the results of analysis of the predicted amino acid sequences of these proteins.

ACKNOWLEDGMENTS

We thank Sally White and Richard Lightfoot for help with some of the experiments.

This research was supported by grants MCB-8916586 and MCB-9405844 from the National Science Foundation.

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