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Genes Required for Cellulose Synthesis in *Agrobacterium tumefaciens*

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A region of the chromosome of *Agrobacterium tumefaciens* 11 kb long containing two operons required for cellulose synthesis and a part of a gene homologous to the *fixR* gene of *Bradyrhizobium japonicum* has been sequenced. One of the cellulose synthesis operons contained a gene (*celA*) homologous to the cellulose synthase (*bscA*) gene of *Acetobacter xylinum*. The same operon also contained a gene (*celC*) homologous to endoglucanase genes from *A. xylinum*, *Cellulomonas uda*, and *Erwinia chrysanthemi*. The middle gene of this operon (*celB*) and both the genes of the other operon required for cellulose synthesis (*celDE*) showed no significant homology to genes contained in the databases. Transposon insertions showed that at least the last gene of each of these operons (*celC* and *celE*) was required for cellulose synthesis in *A. tumefaciens*.

Cellulose, which is made by plants and by some bacteria and fungi, is one of the most abundant biologically produced materials. Despite this fact, relatively little is known about the mechanism of cellulose synthesis (3, 4, 20). The genetics of cellulose synthesis has been examined in two species of bacteria, *Acetobacter xylinum* and *Agrobacterium tumefaciens* (13, 26, 28). The biochemistry of cellulose synthesis in *A. xylinum* has also been studied extensively (20). The DNA sequence of an operon from *A. xylinum* involved in cellulose synthesis has been determined. There are four genes in the operon, of which only the first has a known function, namely, cellulose synthase production (28). Cellulose synthase has also been sequenced from another strain of *A. xylinum* (21). It has been suggested that the product of the second gene may function with cellulose synthase to form a cellulose synthase membrane complex (22).

A. xylinum synthesizes ribbons of cellulose which protrude from one side of the cell and coalesce to form a raft of cellulose which supports the bacteria floating at an air-water interface (2).

In *A. tumefaciens*, cellulose fibrils appear to emerge at random from all sides of the cell surface (15). These fibrils play a role in pathogenesis and serve to bind the bacteria tightly to the plant host cell (13, 14). Mutants which cannot synthesize cellulose are reduced in virulence to 1/10 to 1/1,000 that of the wild-type bacteria (17). In this bacterium, the production of cellulose is stimulated by the presence of plant cells or plant extracts (15).

Cellulose-minus mutants of *A. tumefaciens* were used to obtain the wild-type genes for cellulose synthesis. These genes were sequenced. The results suggest that cellulose synthesis in *A. tumefaciens* involves two genes for which homologous genes are known in *A. xylinum*, although only one of the genes, that for cellulose synthase, is known to function in cellulose synthesis in that bacterium. The other three genes contained in the *cel* operons of *A. tumefaciens* showed no homology to known genes from *A. xylinum*. This may reflect a difference in the mechanisms of cellulose synthesis in these two bacteria.

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MATERIALS AND METHODS

Bacterial strains, growth, and construction of mutants. The bacterial strains and plasmids (including the cellulose-minus mutants of *A. tumefaciens* A6 which were obtained by screening random Tn*5* mutants) used in this research were previously described (13). A library of *A. tumefaciens* NT1 DNA, partially digested with *Sau*3A and cloned in the *Bam*HI site of pCP13 in *Escherichia coli* DH5α, was obtained from S. Farrand. Bacteria were grown in Luria broth or minimal medium as previously described (15). 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). *E. coli* strains were grown at 37°C.

In order to obtain transposon insertions in cloned DNA in *E. coli*, the Tn*3*HoHo1 system described by Stachel et al. (24) was used. Tn*3*HoHo1 is an artificial transposon which carries a β-lactamase gene but lacks a transposase. Plasmids containing transposon insertions were introduced into *A. tumefaciens* by conjugation using pRK2013 as a mobilizing plasmid in triparental matings as previously described (13). Cellulose-minus *A. tumefaciens* strains, in which the wild-type DNA was replaced in the chromosome by DNA containing the transposon insertion, were isolated by the introduction of the plasmid pRK2014, which is incompatible with the plasmid pCP13 which carried the cloned DNA. Transconjugants were selected for gentamicin resistance (carried by pRK2014) and carbenicillin resistance (carried by the transposon) and were screened for loss of tetracycline resistance (carried by pCP13). Bacterial mutants were characterized with respect to their ability to produce cellulose, as previously described (13, 15). This characterization included their ability to fluoresce when grown on plates containing Cellufluor (Polysciences; 20 mg/liter), their ability to form aggregates when grown in medium containing soytone (0.02%), and their ability to form strands which showed fluorescent staining with Cellufluor when examined by fluorescent microscopy. When pCP13.101 was introduced into these mutant strains by conjugation from *E. coli*, the mutants all regained the ability to synthesize cellulose.

Expression of transposon insertions. Transposon Tn*3*HoHo1 can be used as a promoter probe in *A. tumefaciens*, since it contains a promoterless β-galactosidase gene at one end (24). *A. tumefaciens* does not have a β-galactosidase gene. β-Galactosidase activity was measured in *A. tumefaciens* strains carrying a plasmid with a Tn*3*HoHo1 insertion or with a Tn*3*HoHo1 insertion which was located in the bacterial chromosome as a result of marker exchange as described by Stachel et al. (24). Units of enzyme activity were measured and calculated as described by Stachel and Nester (25).

DNA sequencing and analysis of sequence data. DNA fragments to be sequenced were subcloned from pCP13 into pBluescript KS− (Stratagene) by the protocols described by Maniatis et al. (12). Plasmid DNA was purified as previously described (13), and double-stranded sequencing was carried out at the University of North Carolina nucleic acid sequencing facility with a model 373A DNA sequencer (Applied Biosystems) using the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems). In order to sequence across the length of the clone, artificial oligonucleotides 20 to 22 bases long identical to a sequence near the end of the region previously sequenced were synthesized by the University of North Carolina oligonucleotide synthesis facility, purified, and used as primers for further sequencing reactions. Unless otherwise indicated, both strands of the DNA were sequenced. DNA sequences were aligned and analyzed by means of the Genetics Computer Group (GCG) computer analysis programs. BlastX (1, 6) and Blocks (8) were used to search databases for protein

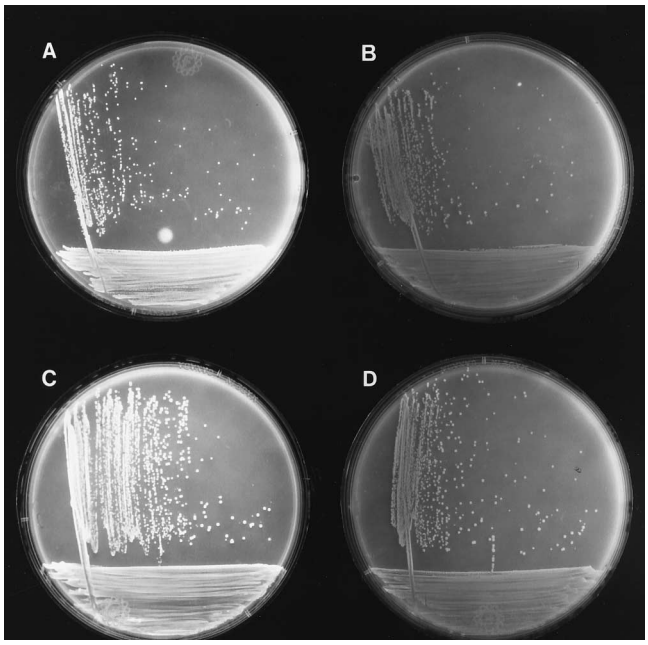

FIG. 1. Complementation of cellulose-minus mutant Cel-1 by various cosmids. Bacteria were grown on Luria agar plates with 0.02% soytone and 0.02% Cellufluor, which stains cellulose and results in colony fluorescence under UV light. The plates were photographed after 3 days of growth under UV light. (A) Strain A6, wild-type cellulose-positive parent strain. (B) Cel-1 cellulose-negative mutant. (C) Cel-1 mutant carrying pDK11. Note the complementation to a cellulose-positive phenotype. (D) Cel-1 mutant carrying pDK11::A4. Note that the transposon insertion has rendered the plasmid ineffective in complementing the mutant and the bacteria are cellulose negative.

amino acid sequences similar to those obtained by translating the DNA sequence. Similar sequences were aligned by using the Pileup program from GCG. Percent identity and homology were calculated with the GCG program Bestfit, using a gap weight of 3.0 and a gap length weight of 0.10. Probabilities that regions of the predicted proteins are located in the membrane were estimated by using the ALOM program for detecting membrane-spanning proteins (10).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported here is L38609.

RESULTS

Identification and characterization of a clone containing *cel* genes from a library of *A. tumefaciens* DNA. We have previously identified and mapped on the bacterial chromosome 5 stable closely linked Tn*5* mutations which fail to synthesize cellulose fibrils (19). In a mixed mating, random clones from a library of *A. tumefaciens* DNA were transferred to one of these mutants, Cel-1. The transconjugants were selected for the ability to grow on minimal medium containing tetracycline. The medium also contained the dye Cellufluor, which causes bacterial colonies synthesizing cellulose to fluoresce blue-white under UV light. The parent strain Cel-1 was dark when examined under UV light in the presence of this dye. Transconjugants which showed blue-white fluorescence were recovered at a frequency of about 0.1%. The cosmid contained in one of these transconjugants was isolated and reintroduced into *E. coli* LE392 by transformation. This clone, pCP13.101, was then introduced by conjugation into each of the cellulose-minus mutant strains. It was able to complement each of these mutations so that they showed fluorescence on Cellufluor plates (Fig. 1).

The two largest *Eco*RI fragments of the inserted DNA of pCP13.101 were subcloned individually into pRK290 (Fig. 1 [5]). Like the original clone, subclone pDK11 was able to complement a Cel-1 mutant. The artificial promoter-probe transposon Tn*3*HoHo1 was introduced into these subclones in *E. coli*. The location and orientation of the transposon insertions were mapped with the restriction enzymes *Eco*RI and *Hin*dIII. The plasmids containing the transposon insertions were introduced into wild-type *A. tumefaciens* C58, and bacteria in which the DNA containing the transposon insertion replaced the wild-type DNA were examined for cellulose synthesis and for the expression of the promoterless β-galactosidase gene contained in the transposon. The results are shown in Fig. 2. One DNA region of about 10 kb appears to be required for cellulose synthesis in *A. tumefaciens*. Insertions to the right of this region did not affect cellulose synthesis. One insertion at the right end of the region, insertion 12, had an intermediate effect on cellulose synthesis. Bacteria carrying this insertion produced a reduced amount of cellulose.

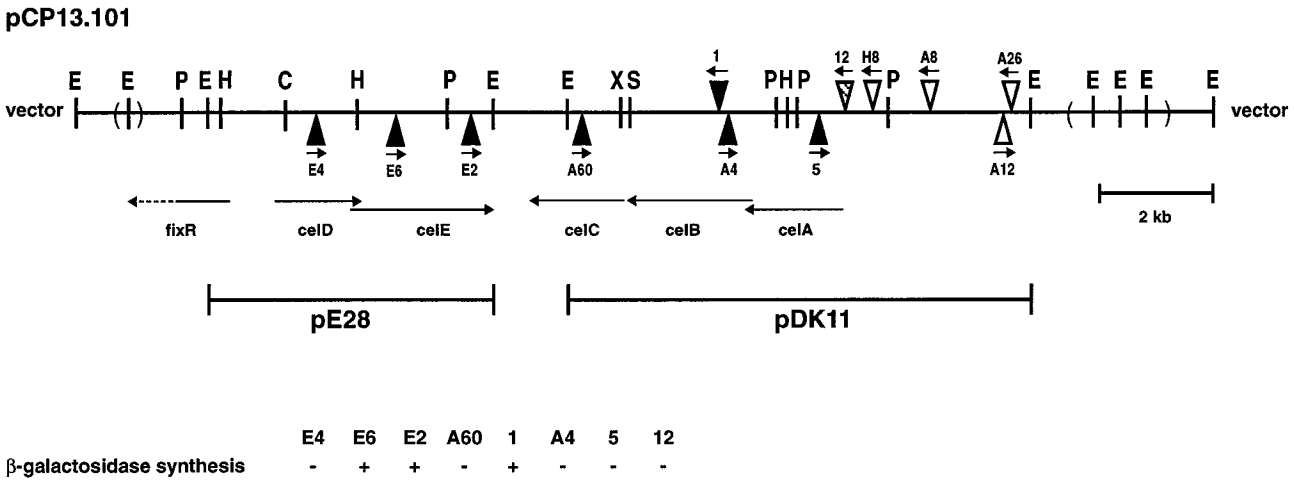

FIG. 2. Map of the cloned DNA in pCP13.101 showing Tn*3*HoHo1 insertions. The positions of Tn*3*HoHo1 insertions which resulted in a cellulose-minus phenotype (filled triangles), insertion 12, which resulted in a reduced level of cellulose synthesis (striped triangle), insertions which did not affect cellulose synthesis (open triangles), and the direction of β-galactosidase transcription (arrows) are indicated. The ability (+) or inability (−) of some of the insertions to synthesize β-galactosidase is shown below the map. 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 location and direction of ORFs are shown by the arrows below the map. The location of the *Eco*RI fragments cloned in pDK11 and pE28 are also shown below the map of the ORFs. Numbers and exact sizes of the fragments enclosed in parentheses were not determined.

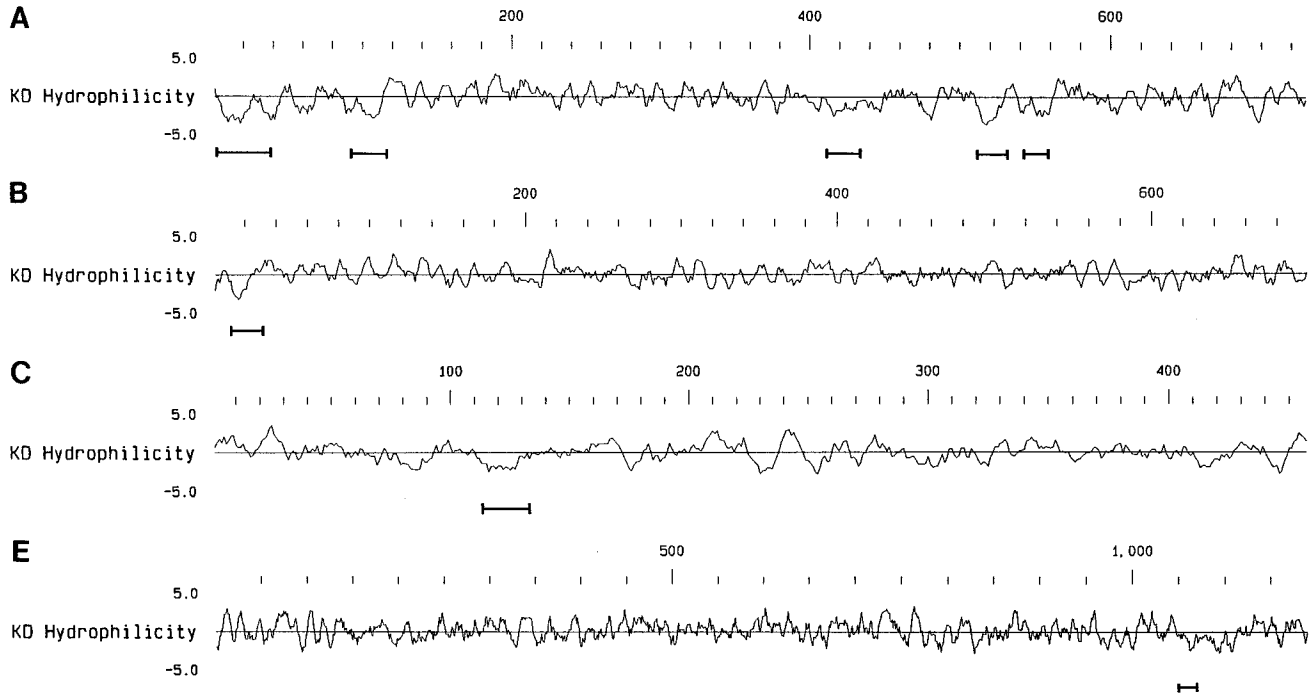

FIG. 3. Hydropathy plot of the predicted protein products of the *celA* (A), *celB* (B), *celC* (C), and *celE* (E) genes. The plots were made by the method of Kyte and Doolittle (KD) (11) and with the programs contained in the GCG package. The domains predicted by ALOM (13) to be localized in the membrane are underlined.

No insertions were recovered on the extreme left end of pCP13.101. However, the DNA sequence data reported below suggest that the left end of the clone contains genes which are not involved in cellulose synthesis.

All of the insertions which resulted in a cellulose-minus phenotype were complemented by pCP13.101. The A60, A4, 1, and 5 insertions were also complemented by pDK11. This result combined with the nucleotide sequence data reported below suggests that the carboxy-terminal portion of the *celC* gene beyond the end of pDK11 is not required for cellulose synthesis.

When β-galactosidase activity was assayed in *A. tumefaciens* carrying those insertions which affected bacterial cellulose synthesis, it was found that the insertions on the right end which read to the left and insertions on the left end, except E4, which read to the right were expressed (Fig. 2). The E4 insertion is located just before the beginning of the open reading frame (ORF) and may be in the vicinity of the promoter for these genes. These results suggest that this region contains two operons required for cellulose synthesis which are oriented facing toward each other.

DNA sequence and homologies of the *celABC* operon. The nucleotide sequence of 11 kb of pCP13.101 from the *Eco*RI site near the left end of the clone to the *Pst*I site just beyond transposon insertion 12 (Fig. 2) was determined. The directions of ORFs identified in the base sequence agreed with the directions expected from examination of the expression of β-galactosidase from Tn*3*HoHo1 insertions. Three ORFs oriented from right to left were identified, beginning 710 bp inside the *Pst*I site towards the right end of the clone. The first ORF encodes a polypeptide of 729 amino acids. No obvious Shine-Dalgarno sequence was seen upstream of the first ATG in the ORF. When a hydrophilicity profile of this protein was examined, there appeared to be five membrane-spanning domains (Fig. 3). The protein was identified by BlastX search (1, 6) as showing homology with the cellulose synthase genes sequenced from two strains of *A. xylinum* (20a [GenBank number U15957], 21, 28). A Pileup comparison of the four genes is shown in Fig. 4. The amino acid sequence encoded by *celA* showed 30% identity and 53% homology with the amino acid sequence of the *A. xylinum* BscA gene, 32% identity and 55% homology with the amino acid sequence of the *A. xylinum* cellulose synthase gene, and 32% identity and 55% homology with the amino acid sequence of the *A. xylinum* AscAII gene. The homology was greatest in the middle of the proteins, suggesting that this is where the active site is located. In addition, the *celA* peptide and the other cellulose synthases showed significant homology with a hypothetical 78.6-kDa protein of unknown function from an intergenic region of *E. coli* (23). The significance of this homology is not known.

The second ORF, *celB*, begins immediately after the first ORF, with overlapping initiation and termination codons (ATGA) for the two ORFs suggesting that the synthesis of the two proteins is coupled. *celB* encodes a putative protein of 698 amino acids. A hydrophilicity profile showed one possible membrane-spanning region near the amino-terminal end (Fig. 3). This region may act as a signal sequence. The putative protein encoded by the *celB* gene showed no significant homology to any protein in the GenBank, Swiss protein, or EMBL databases when a BlastX search was used. The protein may be involved in the reactions of lipid-linked intermediates in cellulose biosynthesis (16).

The third ORF, *celC*, begins 71 bp downstream from the stop codon at the end of *celB*. A possible Shine-Dalgarno sequence is located 4 bp upstream from the ATG which represents the putative start of the ORF, **GAGG**GGGT**ATG** (both sequences are shown in boldface type). The protein encoded is 342 amino acids long and contains one possible membrane-spanning domain (Fig. 3). The amino acid sequence showed significant homology to several β-endoglucanases in a BlastX

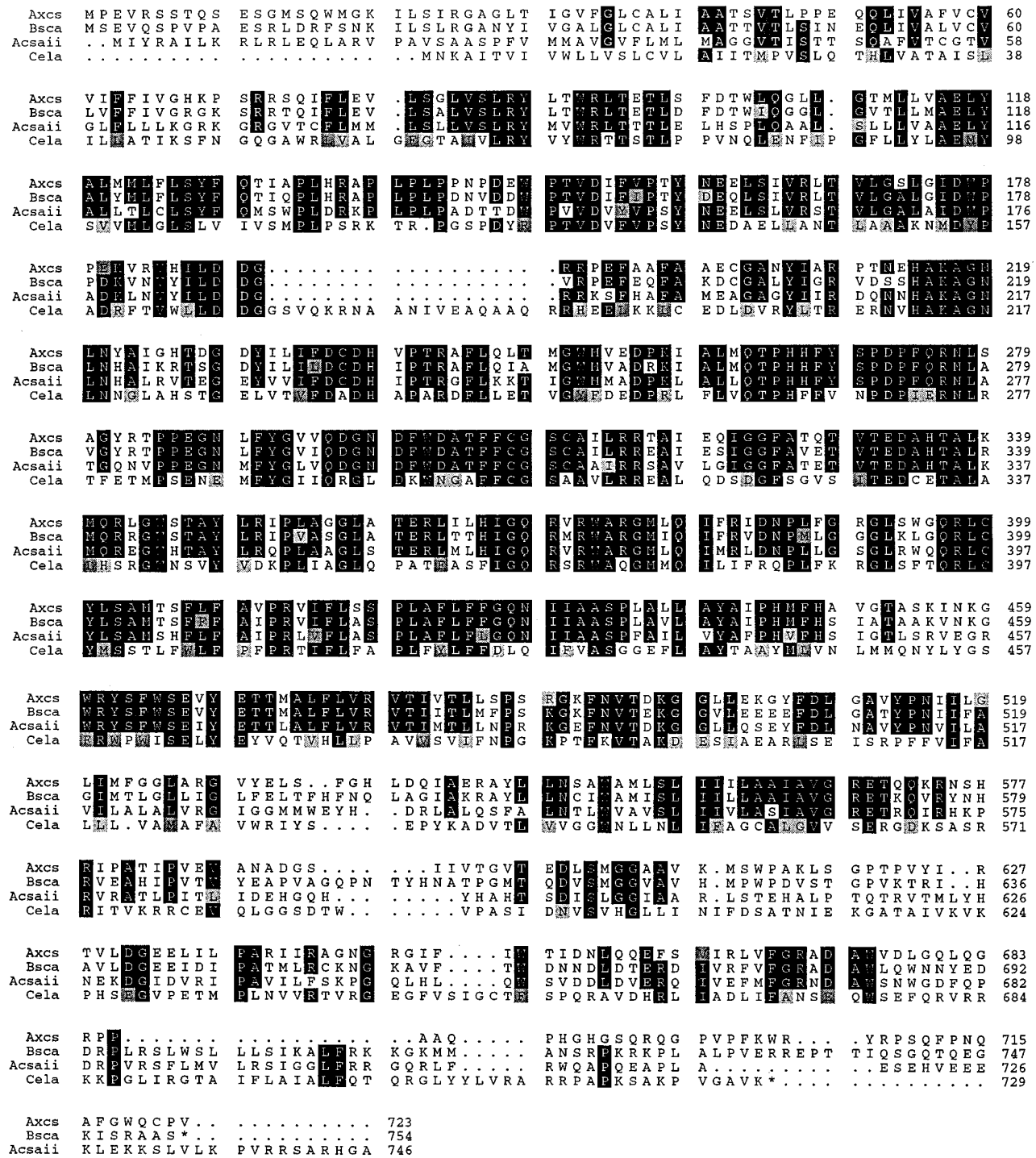

FIG. 4. Homology between *celA* from *A. tumefaciens* (Cela) and the *bscA* (Bsca) (29), *acsAII* (GenBank sequence number U159570) (Acsaii), and cellulose synthase (Axcs [2]) genes from *A. xylinum*. Solid boxes with reverse type indicate identical amino acids, and shaded boxes indicate similar amino acids. The sequence of *acsAII* continues beyond that shown for a total length of 1,596 amino acids. Sequence position numbers are indicated on the right.

search (Fig. 5). The closest homologies were to a 1,4-β-D-glucanohydrolase EngX from *A. xylinum* (GenBank number M96060 [26]) with 35% identity and 62% similarity, to an endoglucanase from *Cellulomonas uda* (18) with 39% identity and 60% similarity, and to an endoglucanase Y from *Erwinia chrysanthemi* (7) with 34% identity and 57% homology. These last two genes are involved in the degradation of cellulose. The role of the *engX* gene in *A. xylinum* is unknown. It is found in an operon adjacent to the operon containing the cellulose synthase gene. In *A. tumefaciens*, *celC* is the last gene in an operon which begins with the cellulose synthase gene (*celA*). The *celC* gene appears to be required for cellulose synthesis,

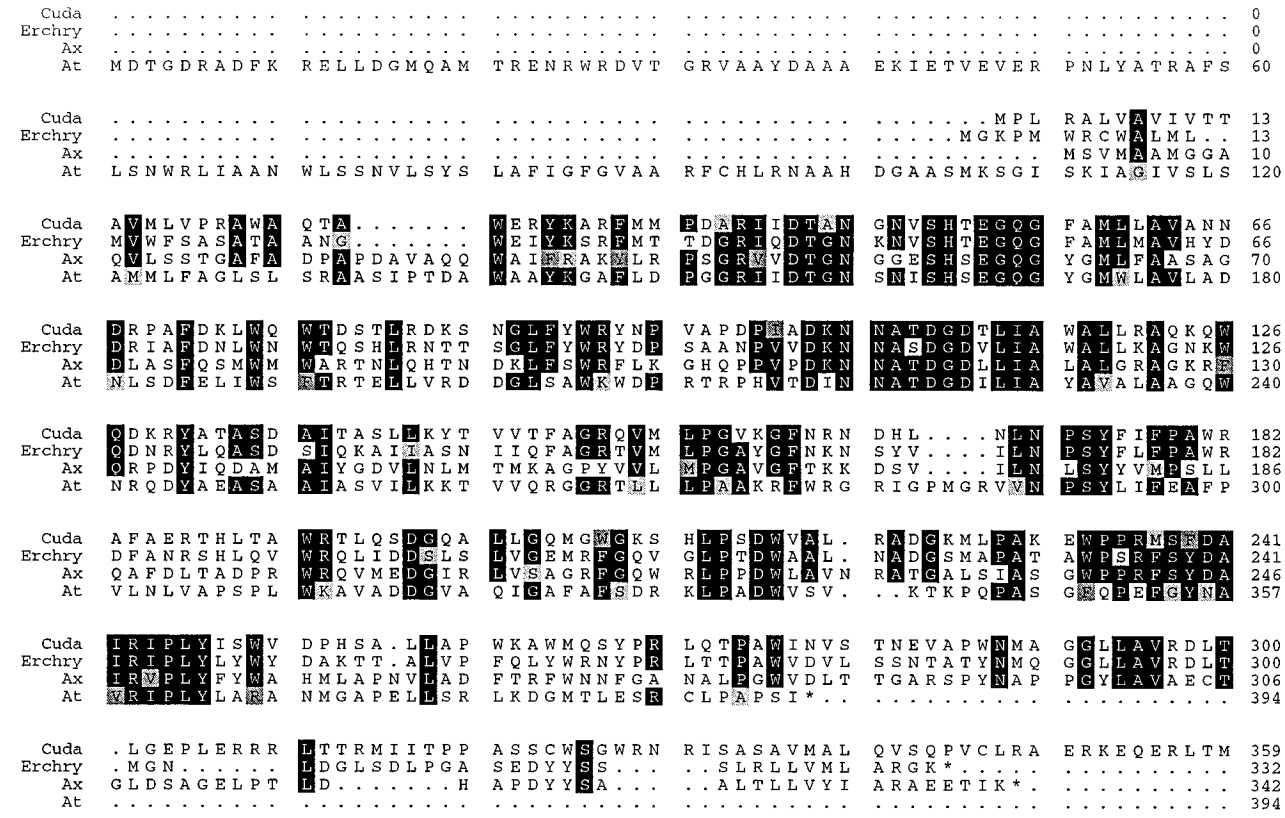

FIG. 5. Homology between endoglucanase genes from *C. uda* (Cuda), *E. chrysanthemi* (Erchry), *A. xylinum engX* (Ax), and *A. tumefaciens celC* (At). Solid boxes with reverse type indicate identical amino acids, and shaded boxes indicate similar amino acids. Sequence position numbers are indicated on the right.

since bacterial mutants carrying transposon insertion A60 which is located in *celC* (the last gene in this operon) were unable to synthesize cellulose (Fig. 2). Data from in vitro cellulose synthesis experiments suggest that the protein is a membrane protein involved in the transfer of cellulose oligomers from a lipid carrier to the growing cellulose chain (16).

DNA sequence of the *celDE* operon. Transposon insertions in two ORFs located to the left of the *celABC* operon result in the failure of the bacteria to synthesize cellulose (Fig. 2). The direction of transcription of these ORFs is in the opposite direction from that of the *celABC* genes and towards those genes. The deduced amino acid sequence for the *celD* ORF contains 584 amino acids. A possible Shine-Dalgarno sequence is located 7 bp upstream of the putative ATG initiation codon, **GGAGG**GGCAAGA**ATG** (both sequences are shown in boldface type). The termination codon of the *celD* ORF overlaps the putative initiation codon of the *celE* ORF with the sequence ATGA. The deduced amino acid sequence for the *celE* ORF contains 1,189 amino acids. Neither of these amino acid sequences showed any significant homology to any sequence in the databases in a BlastX search. They also showed no significant amino acid blocks of known function in a Blocks search. Hydrophilicity profiles suggest that the predicted *celD* gene product is a soluble protein. The predicted *celE* gene product has one possible membrane-spanning domain near the carboxy-terminal end (Fig. 3). Data from in vitro cellulose synthesis experiments suggest that these proteins are involved in the synthesis of lipid intermediates in the production of cellulose (16).

Identification and DNA sequence of a *fixR*-like gene. The DNA sequence region to the left of the *celDE* operon showed a sequence which had a high degree of homology to the FixR gene of *Bradyrhizobium japonicum* (27). This gene reads in the opposite direction from the *celDE* operon. A possible Shine-Dalgarno sequence is located 7 bp upstream of the putative ATG start codon, **GGAG**TTCTCAT**ATG** (both sequences are shown in boldface type). A Pileup alignment of the FixR gene and this gene is shown in Fig. 6. The amino acid sequences of the two proteins showed 56% identity and 70% homology for the region of the *A. tumefaciens* gene sequenced. Both this gene and the *B. rhizobium* FixR protein gene showed homology to alcohol dehydrogenases in a Blast search. A Blocks search showed that both the *A. tumefaciens* and *B. japonicum* genes exhibited homology to all three amino acid blocks conserved in alcohol dehydrogenases, with a spacing between the blocks similar to that found in alcohol dehydrogenases. These three regions are underlined in Fig. 6. As noted by Hennecke (9), *fixR* showed homology to many NAD-dependent dehydrogenases. The *fixR* gene is not required for nitrogen fixation in *B. japonicum*. Since it seems unlikely that alcohol dehydrogenase is involved in cellulose synthesis, this gene was not studied further and the remainder of the gene sequence was not determined.

DISCUSSION

A region of the *A. tumefaciens* chromosome containing genes required for cellulose synthesis has been sequenced. Five ORFs organized into two operons which appeared to be involved in cellulose production were identified. Transposon insertions to the right of these operons had no effect on cellulose synthesis. The ORF located immediately to the left of these

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At  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . M A V P F M L  L T G A S   12
Bj  M G L D L P N D N L  I R G P L P E A H L  D R L V D A V N A R  V D R G E P K V M L  L T G A S   45

At  R G I G H A T V K L  F Q E K G W R I L T  V S R Q P F S E E .  C R W P S A R E S H  I Q A D L   56
Bj  R G I G H A T A K L  F S E A G W R I I S  C A R Q P F D G E R  C P W E A G N D D H  F Q V D L   90

At  E D L S R I D D L A  D E V R S R L P E G  K L D A L V C N A G  I S P K G D G G S R  L G V V D  101
Bj  G D H R M L P R A I  T E V K K R L A G A  P L H A L V N N A G  V S P K T P T G D R  M T S L T  135

At  T T A D I W T R V M  N V N L I S T A L L  A R A L L P E L E V  A K G S I V N V T S  I V G S R  146
Bj  T S T D T W M R V F  H L N L V A P I L L  A Q G L F D E L R A  A S G S I V N V T S  I A G S R  180

At  V H P F A G V A Y V  A S K A A L A A L T  R E L A H E F G P R  . . . . . . . . . .  . . . . .  176
Bj  V H P F A G S A Y A  T S K A A L A S L T  R E L A H D Y A P H  G I R V N A I A P G  E I R T D  225

At  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .  176
Bj  M L S P D A E A R V  V A S I P L R R V G  T P D E V A K V I F  F L C S D A A S Y V  T G A E V  270

At  . . . . . . . . .176
Bj  P I N G G Q H L278
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FIG. 6. Homology between *fixR* from *B. japonicum* (Bj) and an unidentified ORF at the left end of pCP13.101 from *A. tumefaciens* (At). Solid boxes with reverse type indicate identical amino acids. Sequence position numbers are indicated on the right. The positions of three amino acid blocks conserved in alcohol dehydrogenases are underlined.

operons appeared by homology to code for an alcohol dehydrogenase of unknown function.

Because the mutants we have examined were all transposon mutants, they would all be expected to be polar mutations. Thus, the absence of cellulose synthesis in the various mutants allows us to conclude only that the last genes in each of the two operons, *celC* and *celE*, are required for cellulose synthesis in *A. tumefaciens.*

One ORF, *celA*, showed homology to the cellulose synthase (21) and *bscA* (28) genes from *A. xylinum*. The product of this gene in *A. xylinum* is involved in the elongation of the glucan chain of cellulose. The *celA* gene product may play a similar role in *A. tumefaciens*. No homology to the other genes in the *bcsABCD* operon of *A. xylinum* was found. This difference may reflect the very different geometries of the cellulose production in these two species of bacteria. *A. xylinum* elaborates a sheet of cellulose fibrils seen in the electron microscope as emerging from one side of the bacterium (2). *A. tumefaciens* elaborates individual small fibrils seen in the scanning electron microscope as emerging from random locations on the cell surface (15).

One other ORF from *A. tumefaciens cel* genes showed homology to a gene from *A. xylinum*: the *celC* gene is homologous to endoglucanases found in several bacteria, including *engX* from *A. xylinum* (26). The role of *engX*, if any, in cellulose synthesis in *A. xylinum* is unknown. In *A. tumefaciens*, transposon insertions in *celC* block cellulose synthesis. Data from in vitro cellulose synthesis experiments suggest a role for the *celC* gene product in the hydrolysis of glucose oligomers from a lipid-linked intermediate (16). No significant homologies to *celB*, *celD*, or *celE* were found in the GenBank, Swiss protein, or EMBL databases. Data from in vitro cellulose synthesis experiments suggest that the *celE* gene product and, possibly, the *celD* gene product are required for the synthesis of a lipid-linked intermediate in cellulose synthesis. The *celB* gene product appears on the basis of findings from similar experiments to be involved in the conversion of one lipid-linked intermediate to another (16).

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