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Identification of a Second Cellulose Synthase Gene (*acsAII*) in *Acetobacter xylinum*

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A second cellulose synthase gene (*acsAII*) coding for a 175-kDa polypeptide that is similar in size and sequence to the *acsAB* gene product has been identified in *Acetobacter xylinum* AY201. Evidence for the presence of this gene was obtained during analysis of *A. xylinum* mutants in which the *acsAB* gene was disrupted (I. M. Saxena, K. Kudlicka, K. Okuda, and R. M. Brown, Jr., J. Bacteriol. 176:5735–5752, 1994). Although these mutants produced no detectable cellulose, they exhibited significant cellulose synthase activity in vitro. The *acsAII* gene was isolated by using an *acsAB* gene fragment as a probe. The *acsAII* gene coded for cellulose synthase activity as determined from sequence analysis and study of mutants in which this gene was disrupted. A mutant in which only the *acsAII* gene was disrupted showed no significant differences in either the in vivo cellulose production or the in vitro cellulose synthase activity compared with wild-type cells. Mutants in which both the *acsAII* and *acsAB* genes were disrupted produced no cellulose in vivo and exhibited negligible cellulose synthase activity in vitro, thus confirming that the cellulose synthase activity observed in the *acsAB* mutants was coded by the *acsAII* gene. These results establish the presence of an additional gene for cellulose synthase expressed in cells of *A. xylinum*, yet this gene is not required for cellulose production when cells are grown under laboratory conditions.

Cellulose biosynthesis is best understood at present for the gram-negative bacterium *Acetobacter xylinum* (23). In addition to knowledge obtained from ultrastructural and biochemical investigations, studies in the past few years have led to the identification of genes that are involved in cellulose production in this bacterium (28–30, 33, 34). Operons carrying the gene(s) for cellulose synthase along with other genes have been described for two strains of *A. xylinum* (28, 34). Although the operons isolated from these two strains are similar, a difference in the number(s) of genes coding for cellulose synthase in these two operons is observed. In the *bcs* operon isolated from *A. xylinum* 1306-3, the *bcsA* and *bcsB* genes are believed to code for two subunits, BcsA and BcsB, of cellulose synthase (16, 34). In *A. xylinum* ATCC 53582, a single gene, *acsAB*, in the *acs* operon has been shown to code for cellulose synthase, which is synthesized as a polypeptide (AcsAB) with a molecular mass of 168 kDa (28). The BcsA and BcsB polypeptides are homologous to the N-terminal half and C-terminal half, respectively, of the AcsAB polypeptide. Among the genes present in the *acs*/*bcs* operon, only *acsAB*/*bcsA* and *bcsB* have clearly defined functions, namely, coding for the cellulose synthase activity. The *acsC*/*bcsC* and *acsD*/*bcsD* genes are required for normal cellulose production in vivo; however, the precise functions of the polypeptides encoded by these genes are not clearly understood at present (28, 34). Recently, two more genes localized upstream of the *acs*/*bcs* operon have been identified, and though the function of one of these genes is not known, it is required for cellulose synthesis in vivo (33). The other gene codes for carboxymethyl cellulose-hydrolyzing activity; however, the biological function of this gene is not known (33). The structural genes for phosphoglucomutase and UDP-glucose pyrophosphorylase, which are involved in the synthesis of the cellulose synthase substrate UDP-glucose, have also been cloned from *A. xylinum* (4, 5).

The function of the *acsAB*/*bcsA* and *bcsB* genes in the polymerization step of cellulose production was confirmed from sequence comparisons as well, in which the cellulose synthase polypeptide sequence was found to show homology with other β-glycosyl transferases (28). Analysis of the homologous sequences by hydrophobic cluster analysis (HCA) showed the presence of two conserved domains in processive transferases, while only one of these domains was present in transferases that added a single sugar residue (27). The identification of these domains will be useful in assigning possible roles for sequences with unknown functions. This may be especially helpful in identifying cellulose synthases from other organisms. Interestingly, open reading frames (ORFs) coding for polypeptides homologous to the *acsAB*/*bcsA*, *bcsB*, and *acsC*/*bcsC* gene products have been identified for *Escherichia coli*, although at present the roles of these ORFs in *E. coli* remain unknown (32).

Early on, the analysis of mutants for cellulose biosynthesis had suggested the presence of more than a single gene for cellulose synthase in *A. xylinum*; however, these mutants either were spontaneous or were obtained following nitrosoguanidine treatment (26). Following the identification of a cellulose-synthesizing operon, insertions were obtained in each of the three genes (*acsAB*, *acsC*, and *acsD*) of the *acs* operon, and the resulting mutants were analyzed for cellulose production in vivo and cellulose synthase activity in vitro. Mutants with insertions in the *acsAB* or *acsC* gene showed loss of cellulose production in vivo, yet in vitro cellulose synthase activity was observed in the membrane fraction prepared from these mutants (28). These results provided further evidence for the presence of another gene(s) for cellulose synthase and led to the identification of a second cellulose synthase gene (*acsAII*) in *A. xylinum* AY201. The *acsAII* gene described in this report is homologous to the complete *acsAB* gene, which was originally described as two separate genes (28). Analysis of the *acsAII* gene shows that though it is expressed in *A. xylinum*

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
JM109	F′ *traD36 proAB lacI*^{q}Δ(*lacZ*) M15/e14$^-$(*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*)	35
DH5αMCR	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 supE44* λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Laboratories
A. xylinum		
AY201	Derivative of ATCC 23769	Laboratory stock
AY201-41-34	AY201 *acsAB*::Tn*phoA* Kan^r	28
AY201-95-4-1	AY201 *acsAB*::Tn*phoA* Kan^r	28
AY201-15-3	AY201 *acsAB*::Tn*phoA* Kan^r	28
AY201-95-1-1	AY201 *acsC*::Tn*phoA* Kan^r	28
AY201-895	AY201 *acsAII*::pIS895 Amp^r	This study
AY41-34-895	AY201-41-34 *acsAII*::pIS895 Kan^r Amp^r	This study
AY95-4-1-895	AY201-95-4-1 *acsAII*::pIS895 Kan^r Amp^r	This study
AY15-3-895	AY201-15-3 *acsAII*::pIS895 Kan^r Amp^r	This study
AY95-1-1-895	AY201-95-1-1 *acsAII*::pIS895 Kan^r Amp^r	This study
Plasmids		
pMAL-c	Amp^r; *lacI*^{q} P_{tac} *malE*Δ2-26-fx-*lacZ*α	New England BioLabs
pIS532	Amp^r; 9.5-kb *Hin*dIII fragment from *A. xylinum* ATCC 53582 cloned in pUC18	29
pIS88	Amp^r; 8.2-kb *Hin*dIII fragment from *A. xylinum* AY201 cloned in pUC18	This study
pIS167	Amp^r; 2.49-kb *Sma*I fragment from *A. xylinum* AY201 cloned in pUC19	This study
pIS30	Amp^r; 3.8-kb *Hin*dIII fragment from *A. xylinum* AY201 cloned in pUC18	This study
pIS895	Amp^r; 895-bp *Eco*RI-*Hin*dIII fragment from pIS88 cloned in pUC18	This study
pIS119	Amp^r; 8.2-kb *Hin*dIII fragment from pIS88 cloned in pMAL-c	This study

cells, its role in the production of cellulose, as tested under laboratory conditions, is not known at present.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used in this study.

Reagents and enzymes. Ampicillin, kanamycin, and lysozyme were purchased from Sigma Chemical Co. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and the Sequenase version 2.0 DNA sequencing kit were obtained from United States Biochemical. Isopropyl-β-D-thiogalactopyranoside (IPTG), phenol, restriction endonucleases, and T4 DNA ligase were from Bethesda Research Laboratories. The Prime-a-Gene labeling system was obtained from Promega Corporation. GeneScreen hybridization transfer membrane was purchased from NEN Research Products. [α-^{32}P]dCTP (3,000 Ci/mmol) and α-^{35}S-dATP (1,000 Ci/mmol) were obtained from Amersham Corporation. UDP-[U-^{14}C]glucose (200 mCi/mmol) was obtained from ICN Biochemicals Inc. The Geneclean kit was purchased from BIO 101. The Qiagen plasmid kit was from Qiagen Inc. Celluclast (cellulase) was obtained from Novo Industri. Oligonucleotides used as primers in DNA sequencing were synthesized by Operon Technologies, Inc.

Media and growth conditions. *A. xylinum* strains were grown in SH medium (31) at 28°C either statically or on a rotary shaker. For preparation of *A. xylinum* cells that were not attached to the cellulose product, cellulase (0.1% Celluclast) was added to the culture medium 24 to 48 h before harvesting to digest the cellulose and give a uniform cell suspension. *E. coli* strains were grown in LB medium (15) at 37°C on a rotary shaker. For selection of resistance markers, antibiotics were used at the following concentrations unless indicated otherwise: ampicillin (100 μg/ml) and kanamycin (50 μg/ml).

DNA techniques. Total genomic DNA from *A. xylinum* was isolated by a procedure (18) modified from that of Dhaese et al. (8). Plasmid DNA from 0.5- to 1.0-ml bacterial cultures was isolated according to the screening method of Birnboim (3). Large-scale purified plasmid DNA was prepared according to the procedure supplied with the Qiagen plasmid kit (20). DNA fragments for cloning and preparation of radioactive probes were isolated from gel slices with the reagents and according to the protocol supplied with the Geneclean kit. Restriction enzyme digestions and DNA ligations were performed in the buffer supplied with the enzymes, according to the specifications of the supplier. For Southern hybridization, DNA was transferred from the agarose gel to GeneScreen hybridization transfer membrane essentially as described by Reed and Mann (21). DNA probes were labeled with [α-^{32}P]dCTP with the Prime-a-Gene labeling system which was based on the method of Feinberg and Vogelstein (10). Prehybridization and hybridization of immobilized DNA were done at 65°C in the buffer system described by Church and Gilbert (6). Membranes were washed twice with 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature, and when required, a high-stringency wash was performed in 0.2× SSC only at room temperature. Both strands of the *acsAII* gene were sequenced with DNA fragments from plasmids pIS88, pIS167, and pIS30 cloned in the vectors M13 mp18 and M13 mp19. Sequencing was done according to the dideoxy-chain termination method (24) with the Sequenase version 2.0 kit and single-stranded DNA from M13 mp18 and M13 mp19 derivatives or double-stranded plasmid DNA.

Plasmid constructions. The *A. xylinum* AY201 *Hin*dIII fragment present in pIS88 was cloned in the vector pMAL-c to generate plasmid pIS119 (Fig. 1B) for expression of the *acsAII* gene in *E. coli*. The *malE* gene in this vector has an exact deletion of the signal sequence, leading to cytoplasmic expression of maltose-binding protein fusion proteins. Plasmid pIS895, used for disruption of the *acsAII* gene in wild-type and mutant strains of *A. xylinum* AY201 by plasmid integration, was constructed by cloning an 895-bp *Eco*RI-*Hin*dIII fragment from pIS88 into pUC19 (Fig. 1B).

Genetic manipulations. Preparation and transformation of *E. coli* competent cells were performed according to the procedure of Miller (17). Plasmid DNA was introduced by electroporation in cells of *A. xylinum* for the generation of mutants by plasmid integration. *A. xylinum* cells were prepared essentially as described previously (2), and plasmid DNA was purified by the Qiagen plasmid purification procedure (20). Electroporation was performed in 0.1-cm-gap cuvettes with the Bio-Rad Gene Pulser apparatus attached to a pulse controller set at a field strength of 15 kV/cm and with the capacitor and the resistor set at 25 μF and 200 Ω, respectively. Following electroporation, *A. xylinum* cells were grown in SH medium for 3 h before plating on selective plates.

Enzyme assays. Cellulose synthase activity in membrane preparations of *A. xylinum* was determined according to the procedure of Lin and Brown (13). For enzyme assays, approximately 150 μg of membrane protein was incubated at 30°C for 30 min in a reaction mixture with a final concentration of 20 μM UDP-[^{14}C]glucose and 20 μM cyclic diguanylic acid (c-di-GMP) (gift from J. H. van Boom, Department of Organic Chemistry, Leiden, The Netherlands), and the radioactivity present in the alkali-insoluble product obtained after boiling was used to determine the cellulose synthase activity. Protein concentrations were determined by Peterson's modification of the micro-Lowry method (19) with a protein assay kit (Sigma).

Sequence analysis. Nucleotide and derived protein sequences were analyzed with version 7.0 of the Genetics Computer Group sequence analysis software package (7). Percent identity and percent similarity for predicted amino acid sequences were determined by the BESTFIT program (gap weight and length weight, 3.0 and 0.1, respectively) of the Genetics Computer Group package. Transmembrane segments in the deduced amino acid sequences were predicted by the methods of Eisenberg et al. (9) and Klein et al. (11) with the PC/GENE program (IntelliGenetics, Inc.). A search of databases for sequences homologous to the *acsAII* gene product was performed with the BLASTP program (1).

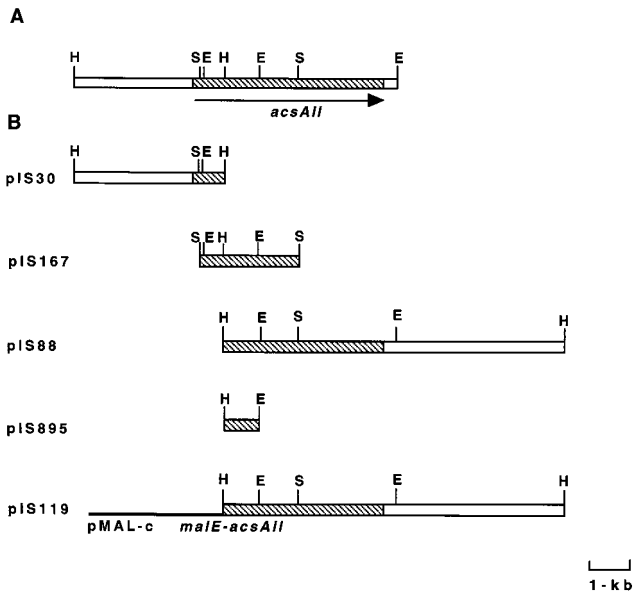

FIG. 1. (A) Sequenced region of the *A. xylinum* AY201 chromosome. The location of the *acsAII* gene is shown. (B) DNA fragments from *A. xylinum* AY201 used in construction of various plasmids. Plasmids pIS30, pIS167, and pIS88 were used to determine the sequence of the region shown in panel A. Plasmid pIS895 was used in the disruption of the *acsAII* gene. Plasmid pIS119 shows the region of *acsAII* expressed in *E. coli*. Abbreviations: S, *Sma*I; E, *Eco*RI; H, *Hin*dIII.

Nucleotide sequence accession number. The DNA sequence described in this paper has been assigned GenBank accession no. U15957.

RESULTS

Cloning and sequencing of the *acsAII* gene from *A. xylinum* AY201. To determine if the other gene(s) for cellulose synthase was homologous to the *acsAB* gene, a 1.4-kb *Eco*RI fragment from pIS532 (29) carrying part of the *A. xylinum* ATCC 53582 *acsAB* gene was used as a probe in Southern hybridizations with DNA from *A. xylinum* AY201 and ATCC 53582. As shown in Fig. 2A (lane 1), the *acsAB* gene probe hybridized with two *Hin*dIII fragments from *A. xylinum* AY201. Hybridization of the probe DNA with the 9.5-kb *Hin*dIII fragment showed an intense signal, while hybridization of the probe with the 8.2-kb *Hin*dIII fragment resulted in a weak signal. A hybridization pattern identical to the one observed for *A. xylinum* AY201 was obtained when DNA from *A. xylinum* ATCC 53582 was probed in the same way (data not shown), confirming that the genomes of these two strains contain similar DNA sequences (28). The 9.5-kb *Hin*dIII fragment carries the complete *acsAB* gene and part of the *acsC* gene, and this fragment was initially cloned for isolation of the cellulose synthase gene from *A. xylinum* ATCC 53582 (29). The weak signal following hybridization of the *acsAB* probe with the 8.2-kb *Hin*dIII fragments from *A. xylinum* AY201 and ATCC 53582 suggested the presence of a DNA sequence homologous to that of the *acsAB* gene on these fragments. An *A. xylinum* mutant, AY201::pIS44 (25), in which the *acsAB* gene was disrupted by integration of a plasmid carrying a part of the *acsAB* gene, was also analyzed by Southern hybridization. Hybridization of the *acsAB* gene probe with fragments from the mutant showed a weak signal with the 8.2-kb *Hin*dIII fragment and intense signals from two new *Hin*dIII fragments (7.7 and 7.2 kb) instead of the 9.5-kb *Hin*dIII fragment (Fig. 2A, lane 2), confirming the disruption of the *acsAB* gene in this mutant and the presence of a homologous DNA sequence on the 8.2-kb *Hin*dIII fragment.

The 8.2-kb *Hin*dIII fragment from *A. xylinum* AY201 was cloned in the vector pUC18, and a recombinant plasmid, pIS88, carrying this DNA fragment, was obtained (Fig. 1B). Sequencing of DNA fragments from this plasmid showed the presence of a large ORF. The deduced amino acid sequence of the polypeptide encoded by this reading frame showed similarities with the sequence of the cellulose synthase (AcsAB polypeptide) from *A. xylinum* ATCC 53582 (28) and with the sequences of the BcsA and BcsB polypeptides of *A. xylinum* 1306-3 (34). Since the DNA sequence coding for the start of this polypeptide was not present on the 8.2-kb *Hin*dIII fragment, two more DNA fragments were cloned from *A. xylinum* AY201, resulting in plasmids pIS167 and pIS30 (Fig. 1B). The location of the *acsAII* gene in the sequenced region is shown in Fig. 1A, and the DNA sequence of the *acsAII* gene is shown in Fig. 3. The *acsAII* coding region begins at position 371 and ends at position 5158 of the nucleotide sequence, coding for a polypeptide of 1,596 amino acid residues with a predicted molecular mass of 175 kDa (Fig. 3). Sequencing of DNA at the two ends of the *acsAII* gene has not led to the identification of any other gene at present, although an ORF extending from position 5265 to the end of the DNA sequence shown in Fig. 3 has been detected.

The presence of DNA fragments homologous to the *A. xylinum* AY201 *acsAII* gene was investigated for other strains of *A. xylinum* by Southern hybridization. When the 895-bp *Eco*RI-*Hin*dIII fragment from *A. xylinum* AY201, containing a region of the *acsAII* gene cloned in pIS895 (Fig. 1B), was used as a probe, *Hin*dIII fragments of 9.5 and 8.2 kb (Fig. 2B, lane 3) and *Sma*I fragments of 2.5 kb and approximately 15 kb (Fig. 2B, lane 4) were observed in DNA digests of *A. xylinum* ATCC 53582. These DNA fragments were similar to the *Hin*dIII and *Sma*I fragments (Fig. 2B, lanes 1 and 2, respectively) observed following hybridization of DNA from *A. xylinum* AY201. At least two *Hin*dIII fragments in each of the *A. xylinum* strains ATCC 23768, ATCC 12733, and NQ1 showed hybridization with the *A. xylinum* AY201 *acsAII* gene (data not shown). As observed for hybridizations with *A. xylinum* AY201 and ATCC 53582, one fragment exhibited an intense signal and the other fragment(s) showed a weak signal. These observations provide evidence that a second cellulose synthase gene, one that is more closely related to the *A. xylinum* AY201 *acsAII* gene and is present on the DNA fragment showing an intense signal, also exists in other strains of *A. xylinum*.

Sequence analysis of the AcsAII polypeptide. The AcsAII polypeptide is predicted to be an integral membrane protein as determined by sequence analysis. According to the method of Eisenberg et al. (9), 13 membrane-associated helices were predicted in the AcsAII polypeptide, of which 8 were tentatively classified as transmembrane. Sequence analysis by the method of Klein et al. (11) predicted 10 transmembrane segments in the AcsAII polypeptide, with 9 of these segments present in the N-terminal half and a single transmembrane segment at the C-terminal end of the polypeptide. A search of the protein databases showed that AcsAII shared extensive sequence similarity with the known *A. xylinum* cellulose synthase sequences (28, 34). The AcsAII and AcsAB sequences aligned over their entire lengths (68.7% similarity, 47.3% identity), while AcsAII residues 2 to 743 aligned with residues 4 to 754 of the BcsA polypeptide (73.4% similarity, 54.2% identity) and residues 748 to 1592 aligned with residues 4 to 802 of the BcsB polypeptide (65.1% similarity, 40.4% identity). AcsAII also showed strong sequence similarity to the predicted F692 and F779 polypeptides of *E. coli* (32), with AcsAII residues 59 to 784 aligning with residues 5 to 692 of F692 (66.2% similarity,

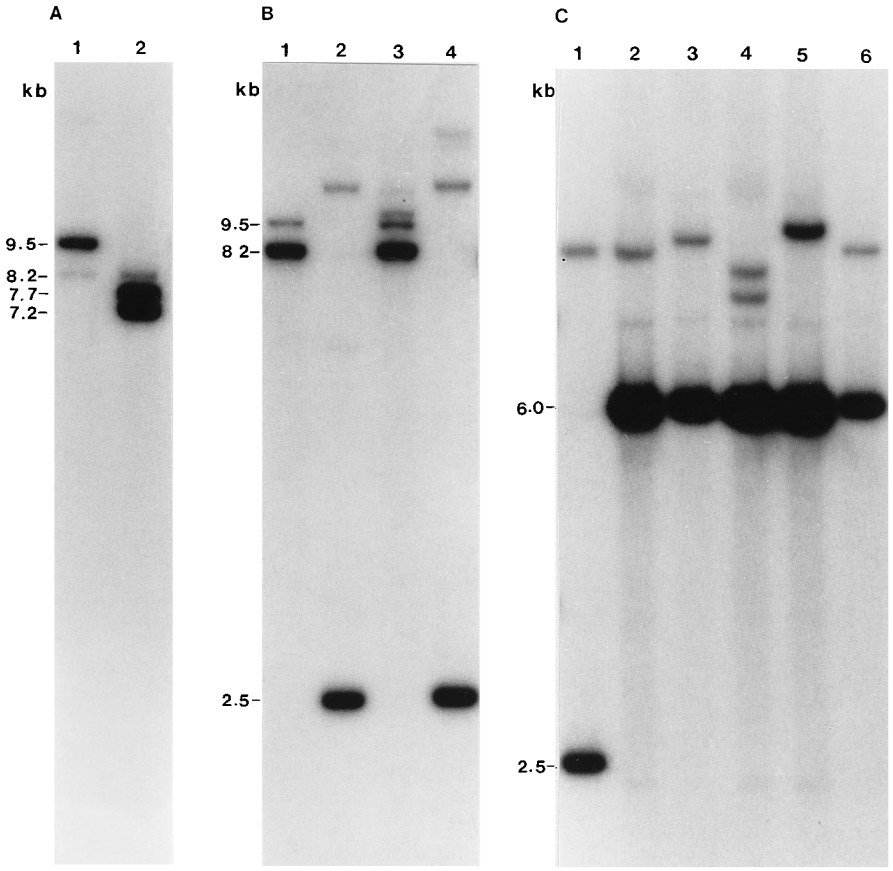

FIG. 2. (A) *Hin*dIII-cleaved DNA from wild-type *A. xylinum* AY201 (lane 1) and mutant AY201::pIS44 (lane 2) hybridized with a ^{32}P-labeled 1.4-kb *Eco*RI fragment from the *acsAB* gene. (B) DNA from *A. xylinum* AY201 (lanes 1 and 2) and ATCC 53582 (lanes 3 and 4) cleaved with *Hin*dIII (lanes 1 and 3) and *Sma*I (lanes 2 and 4) hybridized to a ^{32}P-labeled 895-bp *Eco*RI-*Hin*dIII fragment from the *acsAII* gene. (C) *Sma*I-cleaved DNA from *A. xylinum* AY201 (wild type) (lane 1), AY201-895 (lane 2), AY15-3-895 (lane 3), AY41-34-895 (lane 4), AY95-1-1-895 (lane 5), and AY95-4-1-895 (lane 6) hybridized to a ^{32}P-labeled 895-bp *Eco*RI-*Hin*dIII fragment from the *acsAII* gene.

44.1% identity) and residues 750 to 1586 aligning with residues 1 to 778 of F779 (55.5% similarity, 32.1% identity).

Sequence conservation has been observed in a globular region from a number of β-glycosyl transferases, and two domains were identified in the processive enzymes, including cellulose synthase, by hydrophobic cluster analysis (27). Analysis of the AcsAII sequence by hydrophobic cluster analysis showed the presence of these two domains, and they were also observed in the F692 polypeptide of *E. coli* (32). The two domains, A and B, are shown in a multiple alignment of the globular region from the known cellulose synthase sequences (AcsAB, BcsA, and AcsAII) and the *E. coli* F692 sequence (Fig. 4). On the basis of their strict conservation in the β-glycosyl transferases that were analyzed (27), three Asp residues and the sequence motif QXXRW in cellulose synthases (Fig. 4, shown in boldface) are proposed to be involved in the catalytic activity and processivity, respectively.

Cellulose synthase activity in mutants of *A. xylinum* AY201 that have disruption of the *acsAII* gene. To determine if *acsAII* contributes to cellulose synthase activity, the *acsAII* gene in wild-type and *acsAB* mutants of *A. xylinum* AY201 was disrupted by integration of plasmid pIS895 in the genome, and disruption was confirmed by Southern hybridization (Fig. 2C). Mutant AY201-895, in which only the *acsAII* gene was disrupted by insertion of pIS895, formed a normal cellulose pellicle, and the in vitro cellulose synthase activity in this mutant was found to be comparable to the wild-type activity (Table 2). *A. xylinum* AY201 mutants in which both the *acsAII* and the *acs* operon genes were disrupted showed no cellulose production in vivo but exhibited reduced levels of in vitro cellulose synthase activity. The level of activity in these mutants correlated with the site of disruption in the *acs* operon. In mutants AY41-34-895 and AY95-4-1-895, in which synthesis of the AcsAII polypeptide was disrupted, only the N-terminal 411 and 853 residues, respectively, of the AcsAB polypeptide were synthesized as AcsAB-PhoA fusion proteins. These fusion proteins probably were sufficient for the low levels of cellulose synthase activity observed in the membrane fractions obtained from these mutants (Table 2). A much larger region of the AcsAB (residues 1 to 1326) was synthesized as an AcsAB-PhoA fusion protein in mutant AY15-3-895, and this strain showed a relatively higher level of cellulose synthase activity (Table 2). Mutant AY95-1-1-895 synthesized the complete AcsAB polypeptide and an AcsC-PhoA fusion protein, and therefore the level of cellulose synthase activity in this mutant was found to be much higher than those of the other mutants (Table 2). These results show that negligible cellulose synthase activity is observed when an incomplete AcsAB polypeptide and no AcsAII polypeptide is synthesized. The observed cellulose synthase activity probably is contributed by the incomplete AcsAB-PhoA protein, the presence of which has been

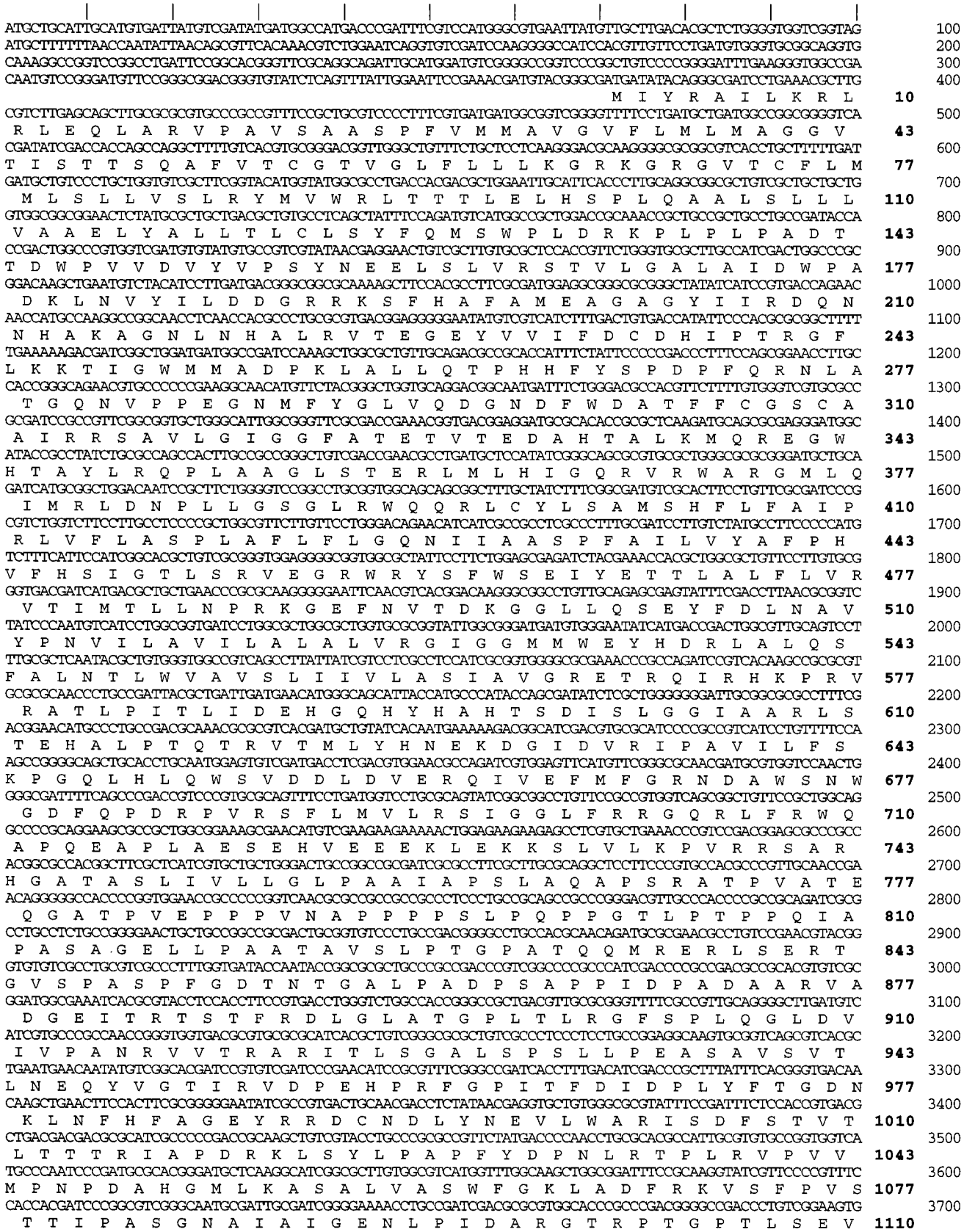

FIG. 3. DNA sequence of a region of the *A. xylinum* AY201 chromosome showing *acsAII* along with the deduced amino acid sequence.

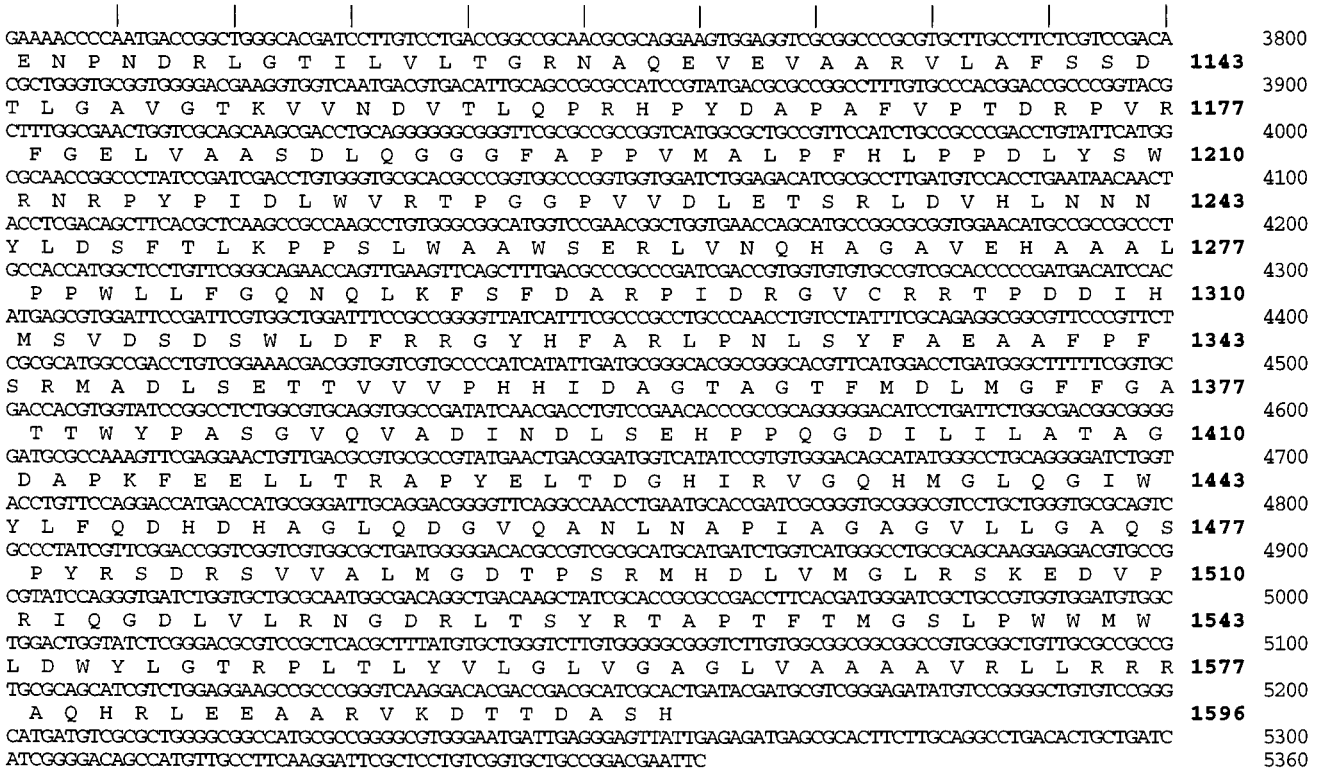

FIG. 3—*Continued.*

demonstrated by the alkaline phosphatase activity in the mutant strains (28).

Expression of the *acsAII* gene in *E. coli*. For expression of the *A. xylinum acsAII* gene in *E. coli*, the 8.2-kb *Hin*dIII fragment of *A. xylinum* AY201 present in pIS88 was cloned into the *E. coli* expression vector pMAL-c to generate pIS119 (Fig. 1B). The *acsAII* gene in pIS119 was fused to the *malE* gene under the control of a *tac* promoter for the synthesis of a maltose-binding protein-AcsAII fusion protein. The region of *acsAII* present in pIS119 codes for amino acid residues 192 to 1596 of the AcsAII polypeptide. Total proteins from *E. coli* DH5αMCR cells carrying plasmid pIS119 were analyzed by

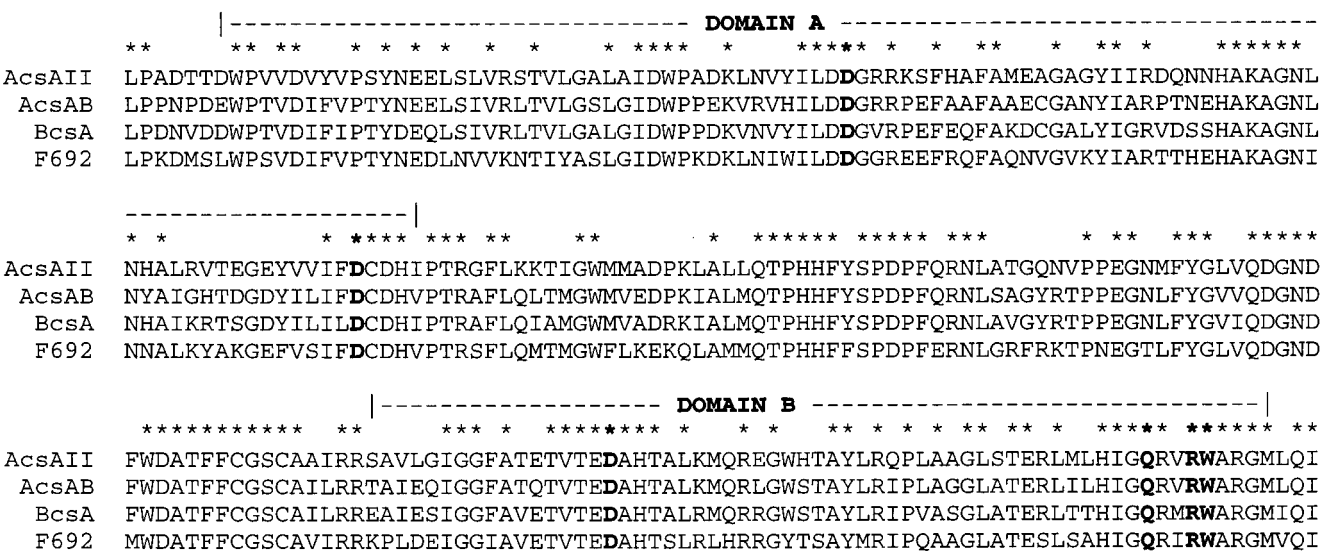

FIG. 4. Multiple alignment of part of the globular region of *A. xylinum* AcsAII (residues 139 to 378), AcsAB (residues 141 to 380), and BcsA (residues 141 to 380) polypeptides and the *E. coli* F692 (residues 85 to 324) polypeptide, showing location of domains A and B that were identified in processive β-glycosyl transferases (27). Residues conserved in all four sequences shown here are marked with asterisks, and residues that are conserved in other β-glycosyl transferases as well (27) are shown in boldface.

TABLE 2. Cellulose synthase activities in *A. xylinum* membrane fractions

Strain	Genotype	Location of Tn*phoA* insertion in *acs* operon	Cellulose synthase sp act[a]
AY201 (wild type)	*acsAB*$^+$ *acsAII*$^+$		1,159.50
AY201-895	*acsAB*$^+$ *acsAII*		932.84
AY41-34-895	*acsAB acsAII*	*acsAB*	33.55
AY95-4-1-895	*acsAB acsAII*	*acsAB*	8.90
AY15-3-895	*acsAB acsAII*	*acsAB*	92.43
AY95-1-1-895	*acsAB*$^+$ *acsAII*	*acsC*	625.66

[a] Specific activity is defined as picomoles of UDP-glucose incorporated into alkali-insoluble product per minute per milligram of protein.

SDS-polyacrylamide gel electrophoresis to identify synthesis of plasmid-encoded polypeptides. Synthesis of a high-molecular-weight polypeptide was observed in cells induced with IPTG, and this polypeptide corresponded with the molecular mass of the maltose-binding protein-AcsAII fusion protein (196 kDa) as predicted from the nucleotide sequence. However, when permeabilized cells and a membrane fraction from *E. coli* DH5αMCR (pIS119) were assayed for cellulose synthase activity, no activity could be detected in vitro. This result suggests that the N-terminal region (residues 1 to 191) of the AcsAII polypeptide, which was not present in the fusion polypeptide, is essential for enzyme activity. Sequence analysis showed that this region contained a part of domain A (Fig. 4) that is presumably involved in the catalytic activity.

DISCUSSION

A. xylinum has at least two similar but nonidentical genes for cellulose synthase, *acsAB* and *acsAII*. The *acsAII* gene encodes a polypeptide, much like the large 168-kDa polypeptide encoded by the *acsAB* gene (28), with a molecular mass of 175 kDa. Although *acsAB* and *acsAII* share sufficient DNA homology, the *acsAII* gene was not identified during cloning of the *acsAB* gene. The *acsAB* gene was isolated with an oligonucleotide probe that hybridized only with the *A. xylinum* ATCC 53582 9.5-kb *Hin*dIII fragment and showed no hybridization with any other DNA fragment (29). In subsequent studies, when a region of the *acsAB* gene was used as a probe, a weak signal was also observed with an 8.2-kb *Hin*dIII fragment from *A. xylinum* ATCC 53582 and AY201, but the identification of the sequence for this fragment was not pursued at the time.

Incubation of purified cellulose synthase from wild-type *A. xylinum* ATCC 53582 with [^{32}P]azido-UDP-glucose always showed labeling of an 83-kDa polypeptide (14), suggesting the presence of a single species of cellulose synthase in *A. xylinum*. To understand the changes in the nitrosoguanidine-induced mutant ATCC 53582-19, which produced highly reduced amounts of cellulose II in vivo yet exhibited normal levels of in vitro cellulose synthase activity (22, 26), cellulose synthase was purified and analyzed by labeling with [^{32}P]azido-UDP-glucose (12). Interestingly, a 75-kDa polypeptide was found to be labeled in the cellulose synthase from this mutant, and the N-terminal amino acid sequence determined for the 75-kDa polypeptide showed that it was not derived from the 83-kDa polypeptide (12) and that instead it was encoded by another gene. The N-terminal amino acid sequence determined for the 75-kDa polypeptide matches with the predicted N-terminal sequence of the AcsAII polypeptide, confirming that the cellulose synthase activity observed in the mutant was encoded by the *acsAII* gene.

It is unusual that the polypeptides encoded by the *acsAB* and *acsAII* genes have not been found together in purified cellulose synthase from wild-type *A. xylinum* ATCC 53582 when such preparations are analyzed by gel electrophoresis and [^{32}P]azido-UDP-glucose labeling (14). This could happen if the synthesis of these two gene products is regulated such that only one of them is present at any one time. Alternatively, both the AcsAB and AcsAII polypeptides are present in the cell at the same time, yet only the AcsAB polypeptide is preferentially isolated during purification of the cellulose synthase from wild-type cells.

On the basis of the available data, the *acsAII* gene is clearly not required for cellulose production in vivo. At the same time, this gene is conserved in a number of *A. xylinum* strains, suggesting that some possible role for *acsAII* exists. Perhaps the *acsAII* gene is the ancestral cellulose synthase gene that duplicated and associated with other genes in an operon during the evolution of cellulose biosynthesis in *A. xylinum*. Although *E. coli* ORFs homologous to the *A. xylinum* cellulose biosynthesis genes recently have been identified (32), the roles of these ORFs in *E. coli* remain to be determined. However, as more sequences showing homology with the *A. xylinum* cellulose synthase gene are identified, it will be possible to determine their functions and the roles they play in the biology of diverse organisms.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bio-Rad Laboratories.** 1993. Gene Pulser electroprotocols. Bio-Rad Laboratories, Hercules, Calif.
3. **Birnboim, H. C.** 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100:**243–255.
4. **Brautaset, T., R. Standal, E. Fjærvik, and S. Valla.** 1994. Nucleotide sequence and expression analysis of the *Acetobacter xylinum* phosphoglucomutase gene. Microbiology **140:**1183–1188.
5. **Brede, G., E. Fjærvik, and S. Valla.** 1991. Nucleotide sequence and expression analysis of the *Acetobacter xylinum* uridine diphosphoglucose pyrophosphorylase gene. J. Bacteriol. **173:**7042–7045.
6. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **Dhaese, P., H. De Greve, H. Decraemer, J. Schell, and M. Van Montagu.** 1979. Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of *Agrobacterium tumefaciens*. Nucleic Acids Res. **7:**1837–1849.
9. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. **179:**125–142.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
11. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815:**468–476.
12. **Lin, F. C., and R. M. Brown, Jr.** Unpublished data.
13. **Lin, F. C., and R. M. Brown, Jr.** 1989. Purification of cellulose synthase from *Acetobacter xylinum*, p. 473–492. *In* C. Schuerch (ed.), Cellulose and wood—chemistry and technology. John Wiley and Sons, New York.
14. **Lin, F. C., R. M. Brown, Jr., R. R. Drake, Jr., and B. E. Haley.** 1990. Identification of the uridine 5′-diphosphoglucose (UDP-glc) binding subunit of cellulose synthase in *Acetobacter xylinum* using the photoaffinity probe 5-azido-UDP-glc. J. Biol. Chem. **265:**4782–4784.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Mayer, R., P. Ross, H. Weinhouse, D. Amikam, G. Volman, P. Ohana, R. D. Calhoon, H. C. Wong, A. W. Emerick, and M. Benziman.** 1991. Polypeptide

composition of bacterial cyclic diguanylic acid-dependent cellulose synthase and the occurrence of immunologically crossreacting proteins in higher plants. Proc. Natl. Acad. Sci. USA **88:**5472–5476.
17. **Miller, H.** 1987. Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. Methods Enzymol. **152:**145–170.
18. **Noti, J. D., M. N. Jagadish, and A. A. Szalay.** 1987. Site-directed Tn5 and transplacement mutagenesis: methods to identify symbiotic nitrogen fixation genes in slow-growing *Rhizobium*. Methods Enzymol. **154:**197–217.
19. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83:**346–356.
20. **Qiagen Inc.** 1992. Qiagen plasmid handbook. Qiagen Inc., Chatsworth, Calif.
21. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:**7207–7221.
22. **Roberts, E. M., I. M. Saxena, and R. M. Brown, Jr.** 1989. Biosynthesis of cellulose II in *Acetobacter xylinum*, p. 689–704. *In* C. Schuerch (ed.), Cellulose and wood—chemistry and technology. John Wiley and Sons, New York.
23. **Ross, P., R. Mayer, and M. Benziman.** 1991. Cellulose biosynthesis and function in bacteria. Microbiol. Rev. **55:**35–58.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
25. **Saxena, I. M., and R. M. Brown, Jr.** Unpublished data.
26. **Saxena, I. M., and R. M. Brown, Jr.** 1989. Cellulose biosynthesis in *Acetobacter xylinum*: a genetic approach, p. 537–557. *In* C. Schuerch (ed.), Cellulose and wood—chemistry and technology. John Wiley and Sons, New York.
27. **Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia, and B. Henrissat.** 1995. Multidomain architecture of β-glycosyl transferases: implications for mechanism of action. J. Bacteriol. **177:**1419–1424.
28. **Saxena, I. M., K. Kudlicka, K. Okuda, and R. M. Brown, Jr.** 1994. Characterization of genes in the cellulose-synthesizing operon (*acs* operon) of *Acetobacter xylinum*: implications for cellulose crystallization. J. Bacteriol. **176:**5735–5752.
29. **Saxena, I. M., F. C. Lin, and R. M. Brown, Jr.** 1990. Cloning and sequencing of the cellulose synthase catalytic subunit gene of *Acetobacter xylinum*. Plant Mol. Biol. **15:**673–683.
30. **Saxena, I. M., F. C. Lin, and R. M. Brown, Jr.** 1991. Identification of a new gene in an operon for cellulose biosynthesis in *Acetobacter xylinum*. Plant Mol. Biol. **16:**947–954.
31. **Schramm, M., and S. Hestrin.** 1954. Factors affecting production of cellulose at the air/liquid interface of a culture of *Acetobacter xylinum*. J. Gen. Microbiol. **11:**123–129.
32. **Sofia, H. J., V. Burland, D. L. Daniels, G. Plunkett III, and F. R. Blattner.** 1994. Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 to 81.5 minutes. Nucleic Acids Res. **22:**2576–2586.
33. **Standal, R., T.-G. Iversen, D. H. Coucheron, E. Fjærvik, J. M. Blatny, and S. Valla.** 1994. A new gene required for cellulose production and a gene encoding cellulolytic activity in *Acetobacter xylinum* are colocalized with the *bcs* operon. J. Bacteriol. **176:**665–672.
34. **Wong, H. C., A. L. Fear, R. D. Calhoon, G. H. Eichinger, R. Mayer, D. Amikam, M. Benziman, D. H. Gelfand, J. H. Meade, A. W. Emerick, R. Bruner, A. Ben-Bassat, and R. Tal.** 1990. Genetic organization of the cellulose synthase operon in *Acetobacter xylinum*. Proc. Natl. Acad. Sci. USA **87:**8130–8134.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.