JOURNAL OF BACTERIOLOGY, Sept. 1994, p. 5735–5752
0021-9193/94/$04.00+0

Vol. 176, No. 18

Characterization of Genes in the Cellulose-Synthesizing Operon (*acs* Operon) of *Acetobacter xylinum*: Implications for Cellulose Crystallization

INDER M. SAXENA, KRYSTYNA KUDLICKA, KAZUO OKUDA, AND R. MALCOLM BROWN, JR.*

Department of Botany, The University of Texas at Austin, Austin, Texas 78713-7640

Received 7 February 1994/Accepted 11 July 1994

The synthesis of an extracellular ribbon of cellulose in the bacterium *Acetobacter xylinum* takes place from linearly arranged, membrane-localized, cellulose-synthesizing and extrusion complexes that direct the coupled steps of polymerization and crystallization. To identify the different components involved in this process, we isolated an *Acetobacter* cellulose-synthesizing (*acs*) operon from this bacterium. Analysis of DNA sequence shows the presence of three genes in the *acs* operon, in which the first gene (*acsAB*) codes for a polypeptide with a molecular mass of 168 kDa, which was identified as the cellulose synthase. A single base change in the previously reported DNA sequence of this gene, resulting in a frameshift and synthesis of a larger protein, is described in the present paper, along with the sequences of the other two genes (*acsC* and *acsD*). The requirement of the *acs* operon genes for cellulose production was determined using site-determined Tn*phoA*/Kan^r GenBlock insertion mutants. Mutant analysis showed that while the *acsAB* and *acsC* genes were essential for cellulose production in vivo, the *acsD* mutant produced reduced amounts of two cellulose allomorphs (cellulose I and cellulose II), suggesting that the *acsD* gene is involved in cellulose crystallization. The role of the *acs* operon genes in determining the linear array of intramembranous particles, which are believed to be sites of cellulose synthesis, was investigated for the different mutants; however, this arrangement was observed only in cells that actively produced cellulose microfibrils, suggesting that it may be influenced by the crystallization of the nascent glucan chains.

Cellulose is an extracellular polysaccharide, synthesized as long β-1,4 glucan chains that associate to form the microfibrils commonly observed in cellulose-synthesizing organisms. The synthesis of this biopolymer takes place via a single polymerization step, utilizing UDP-glucose as the substrate and catalyzed by the enzyme cellulose synthase (UDP-glucose: 1,4-β-D-glucosyltransferase), without the apparent involvement of any intermediates (40). However, the crystalline structure of cellulose microfibrils and their association with organized intramembranous particles observed in most cellulose-synthesizing organisms suggest a more intricate mechanism of cellulose biogenesis than that deduced from the polymerization reaction alone. Because of their association with cellulose microfibrils, the intramembranous particles are believed to be sites of cellulose synthesis and extrusion. The nature of these particles is incompletely understood at present, and it is not known at the molecular level how they become organized into structures that are referred to as terminal synthesizing complexes (TCs) that are characteristic for most organisms (5). In higher plants, the TC is arranged as a rosette; in a large number of algal species, the TC is arranged as a linear row(s) of particles (7). Even though the cellulose produced by all organisms chemically is the same in its primary composition, the degrees of polymerization and crystallinity and other physical properties are unique to each organism. To a large extent, these differences in the properties of cellulose may be a reflection of the organization of these complexes within the specified organism. We are interested in understanding the composition and organization of the cellulose-synthesizing complexes and how the organization of these complexes influences the assembly of the final cellulose product.

The gram-negative bacterium *Acetobacter xylinum* synthesizes an extracellular ribbon of cellulose microfibrils from membrane-localized cellulose-synthesizing complexes (10) that are organized in a linear row along the long axis of the bacterial cell (8, 56). Among the organisms known to produce cellulose, the mechanism of cellulose synthesis at present is best understood in this bacterium. To determine the various components required for cellulose biosynthesis, cellulose synthase was purified from this bacterium, and two polypeptides with molecular masses of 83 and 93 kDa were identified as major components of this enzyme (27). The substrate-binding site in cellulose synthase was localized to the 83-kDa polypeptide by photoaffinity labeling (28). Using an oligonucleotide probe, designed from the N-terminal amino acid sequence of the 83-kDa polypeptide, a 9.5-kb *Hin*dIII fragment from *A. xylinum* ATCC 53582 was identified by Southern hybridization and cloned in the plasmid pIS532 (44). DNA sequence from this plasmid showed two adjacent open reading frames that coded for polypeptides with molecular masses of 80 and 86 kDa, the deduced amino acid sequences of which matched with the N-terminal amino acid sequences determined for the 83- and 93-kDa polypeptides, respectively. These observations suggested that the 83-kDa (cellulose synthase catalytic subunit) and 93-kDa polypeptides were encoded by two separate genes that were organized in an operon, possibly with other unidentified genes, and in which the cellulose synthase catalytic subunit gene was the first gene (45). The 83- and 93-kDa polypeptide genes were found to be similar to the first two genes (*bcsA* and *bcsB*) of the *bcs* operon that was isolated from another strain of *A. xylinum* and in which the second gene (*bcsB*) was identified as the cellulose synthase catalytic subunit gene (52). Even though the functions of the other three genes

* Corresponding author. Mailing address: Department of Botany, The University of Texas at Austin, Austin, TX 78713-7640. Phone: (512) 471-3364. Fax: (512) 471-3573. Electronic mail address: rmbrown@ccwf.cc.utexas.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
CC118	Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	C. Manoil
CC202	F42 *lacI3 zzf-2*::Tn*phoA*/CC118	C. Manoil
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*)	55
S17-1	*thi pro hsdR recA*; mobilizer strain	47
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	This study
AY201-41-4-1	AY201 *acsAB*::Tn*phoA*; Kan^r	This study
AY201-95-4-1	AY201 *acsAB*::Tn*phoA*; Kan^r	This study
AY201-95-9-1	AY201 *acsAB*::Tn*phoA*; Kan^r	This study
AY201-15-2	AY201 *acsAB*::Tn*phoA*; Kan^r	This study
AY201-15-3	AY201 *acsAB*::Tn*phoA*; Kan^r	This study
AY201-95-1-1	AY201 *acsC*::Tn*phoA*; Kan^r; Kan^r	This study
AY201-*acsD*::Km	AY201 *acsD*::Kan^r GenBlock	This study
Plasmids		
pMAL-c	Amp^r; *lacI*^{q} P_{tac} *malE*Δ2-26-fx-*lacZ*α	New England BioLabs
pRK311	Tet^r; broad-host-range cosmid vector	14
pIS532	Amp^r; approximately 9.5-kb *Hin*dIII fragment of *A. xylinum* ATCC 53582 cloned in pUC18	44
pIS353	Tet^r; approximately 20-kb fragment of *A. xylinum* ATCC 53582 cloned in the cosmid pRK311	43
pMAL-c-4	Amp^r; 4.92-kb *Eco*RI-*Hin*dIII fragment from pIS532 cloned in pMAL-c	This study
pMAL-c-15	Amp^r; 4.92-kb *Eco*RI-*Hin*dIII fragment from pIS353 cloned in pMAL-c	This study
pMAL-c-41	Amp^r; 1.4-kb *Eco*RI fragment from pIS353 cloned in pMAL-c-4	This study
pMAL-c-95	Amp^r; 1.4-kb *Eco*RI fragment from pIS353 cloned in pMAL-c-15	This study
pMAL-*acsD*	Amp^r; 1.64-kb *Eco*RI-*Hin*dIII fragment from pIS353 carrying the 3′ end of the *acsC* gene and the complete *acsD* gene cloned in pMAL-c	This study
pIS-*acsD*	Amp^r; 1.64-kb *Eco*RI-*Hin*dIII fragment from pIS353 carrying the 3′ end of the *acsC* gene and the complete *acsD* gene cloned in pUC18	This study
pIS-*acsD*::Km	Amp^r Kan^r; Kan^r GenBlock cloned in the *Pst*I site of pIS-*acsD* leading to disruption of the *acsD* gene	This study
pIS311-*acsD*	Tet^r; 3.5-kb *Eco*RV-*Hin*dIII fragment from pMAL-*acsD* carrying the P_{tac}-*malE*-′*acsC*-*acsD* region cloned in pRK311	This study
pIS311-95	Tet^r; 8.1-kb *Hpa*I-*Hin*dIII fragment from pMAL-c-95 carrying the P_{tac}-*malE*-′*acsAB*-′*acsC* region cloned in pRK311	This study

(*bcsA*, *bcsC*, and *bcsD*) in the *bcs* operon were not determined, it was suggested that all four genes present in this operon were required for maximal cellulose synthesis in *A. xylinum* (52).

Although a single enzymatic step catalyzed by cellulose synthase is responsible for polymerization of glucose units from UDP-glucose into cellulose, other components are required for the final assembly and export of native cellulose I. Some of these components may be encoded by genes in the cellulose-synthesizing operon, and it is therefore important to identify these genes and determine their functions. In this paper, we show the presence of three genes in the *Acetobacter* cellulose-synthesizing (*acs*) operon and, on the basis of sequence analysis and study of mutants with site-determined insertions, suggest the possible roles for these genes in cellulose biosynthesis.

MATERIALS AND METHODS

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

Reagents and enzymes. Ampicillin, 5-bromo-4-chloro-3-indolyl-phosphate (XP), kanamycin, lysozyme, and tetracycline were purchased from Sigma Chemical Co. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and the Sequenase version 2.0 DNA Sequencing kit were obtained from U.S. Biochemical. Isopropylthio-β-galactoside (IPTG), phenol, restriction endonucleases, and T4 DNA ligase were from Bethesda Research Laboratory. The *Hin*dIII linker and Prime-a-Gene labeling system were obtained from Promega Corporation. Kan^r GenBlock was obtained from Pharmacia. 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 Industries. Oligonucleotides used as primers in DNA sequencing and for PCR were synthesized by Operon Technologies, Inc. Reagents for PCR, including *Taq* DNA polymerase, were obtained as a kit from Perkin-Elmer Cetus.

Media and growth conditions. *A. xylinum* strains were grown in SH medium (46) 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. *Escherichia coli* strains were grown in LB medium (29) at 37°C

on a rotary shaker. For selection of resistance markers, antibiotics were used at the following concentrations, unless otherwise indicated: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml.

DNA techniques. Total genomic DNA from *A. xylinum* and other bacteria was isolated by a procedure (35) modified from Dhaese et al. (13). Plasmid DNA from 0.5- to 1.0-ml bacterial cultures was isolated according to the screening method described by Birnboim (4). Large-scale purified plasmid DNA was prepared according to the procedure whose description was supplied with the Qiagen plasmid kit (37). DNA fragments for cloning and preparation of radioactive probes were isolated from gel slices using the reagents and 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 the GeneScreen hybridization transfer membrane essentially according to the method described by Reed and Mann (38). DNA probes were labeled with [α-^{32}P]dCTP by using the Prime-a-Gene labeling system based on the method described by Feinberg and Vogelstein (16). Prehybridization and hybridization of immobilized DNA were done at 65°C in the buffer system described by Church and Gilbert (11). Membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature, and when required, a high-stringency wash in 0.2× SSC was performed. The sequences of the *acsC* and *acsD* genes were obtained from pIS353 fragments cloned in M13mp18/mp19. Sequencing was done according to the dideoxy chain-termination method (41) using the Sequenase version 2.0 kit and single-stranded DNA from M13mp18/mp19 derivatives or double-stranded plasmid DNA. For verification of DNA sequence in a region of the *acsAB* gene, a 706-bp DNA fragment was obtained from the chromosomal DNA of *A. xylinum* ATCC 53582 and AY201 by PCR using primers E3S2 (5′-GAACGTGCCTACCTGCT) and P23 (5′-GGTCAGAGGCTTCGGGC).

Plasmid constructions. DNA fragments from pIS532 and pIS353 were cloned in the vector pMAL-c for analysis of the *acs* operon genes (Fig. 1). The *malE* gene in this vector has an exact deletion of the signal sequence, leading to cytoplasmic expression of MBP (maltose-binding protein) fusion proteins. For disruption of the *acsD* gene in *A. xylinum*, pIS-*acsD*::Km was constructed by cloning a *Pst*I-digested Kan^r GenBlock in the *Pst*I site of pIS-*acsD*. For complementation of *A. xylinum* mutants in which the *acsD* gene had been disrupted, plasmid pIS311-*acsD* was constructed. The 3′ end of the *acsC* gene and the complete *acsD* gene were obtained as a 1.64-kb *Eco*RI-*Hin*dIII fragment from pIS353. This fragment was cloned in the vector pMAL-c to construct pMAL-*acsD* such that the *malE*Δ2-26 gene on the vector was fused in frame with the 3′ end of the *acsC* gene, leading to the synthesis of a MBP-′AcsC fusion protein and an intact AcsD protein. Since pMAL-*acsD* cannot be maintained in *A. xylinum*, a 3.5-kb *Eco*RV-*Hin*dIII fragment from this plasmid carrying the P_{tac}-*malE*-′*acsC*-*acsD* region was cloned into the broad-host-range vector pRK311. The *Eco*RV site of the 3.5-kb fragment was converted to a *Hin*dIII site using *Hin*dIII linker, following which the fragment was cloned in *Hin*dIII-cleaved pRK311 to form pIS311-*acsD*. An 8.1-kb *Hpa*I-*Hin*dIII fragment of pMAL-c-95 carrying the P_{tac}-*malE*-′*acsAB*-′*acsC* region was cloned in pRK311 in a similar manner to form pIS311-95.

Genetic manipulations. Preparation and transformation of *E. coli*-competent cells were according to the procedure described by Miller (34). Nonconjugative plasmids were introduced into *A. xylinum* cells by electroporation using the Bio-Rad Gene Pulser apparatus attached to a pulse controller. *A. xylinum* cells were prepared essentially as described elsewhere (3) and were transformed with plasmid DNA purified by the Qiagen plasmid purification procedure (37). Electroporation was performed in 0.1-cm cuvettes at a field strength of 15 kV/cm, with the capacitor and the resistor set at 25 μF and 200 Ω, respectively. Transformed cells were grown in SH medium for 3 h before plating on selective plates. Mobilizable plasmids were transferred to *A. xylinum* by conjugation with *E. coli* S17-1. Matings between plasmid-bearing *E. coli* S17-1 and *A. xylinum* cells were performed on 0.22-μm-pore-size filters placed on SH agar plates at 28°C for 4 to 5 h.

Isolation of Tn*phoA* insertions in plasmids carrying *acs* operon genes. Tn*phoA* was transferred from F42 *lacI3 zzf-2*::Tn*phoA* present in *E. coli* CC202 into plasmids carrying the *acs* operon genes by the method described by Manoil and Beckwith (31). *E. coli* CC202 cells were transformed with pMAL-c-derived plasmids carrying DNA fragments from the *acs* operon region fused to the *malE*Δ2-26 sequence of pMAL-c. Single colonies of ampicillin-resistant transformants were suspended in LB medium and plated on LB agar containing ampicillin (50 μg/ml), kanamycin (300 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (40 μg/ml) to select for Tn*phoA* transpositions into the multicopy plasmids. The plates were incubated for 2 days at 37°C, after which colonies that had active alkaline phosphatase fusions appeared blue. Plasmid DNA was isolated from a number of blue colonies and introduced into *E. coli* CC118. Cells from the kanamycin-resistant blue colonies of *E. coli* CC118 carried plasmids with Tn*phoA* insertions at sites coding for extracytoplasmic regions of membrane proteins. To screen for fusions in which kanamycin-resistant blue colonies arose as a result of Tn*phoA* insertion in the β-lactamase coding region of the vector pMAL-c, colonies were tested for resistance to ampicillin, and sensitive colonies were discarded. Plasmid DNAs, which were isolated from colonies resistant to both kanamycin and ampicillin, were analyzed by restriction analysis. The locations of the Tn*phoA* transposition site on the plasmids were determined by DNA sequencing using Qiagen-purified plasmid DNA and a synthetic primer, 5′-AATATCGCCCTGAGC, complementary to the sequence of the 5′ region of the alkaline phosphatase gene present in Tn*phoA* (48).

Transfer of plasmid-borne insertions into the *A. xylinum* chromosome. Plasmids carrying Tn*phoA* insertions in the *acsAB* and *acsC* genes and a Kan^r GenBlock insertion in the *acsD* gene were introduced into *A. xylinum* AY201 by electroporation, and transformants were selected on SH agar plates containing kanamycin (50 μg/ml). Since the pMAL-c-derived and pUC18-derived plasmids that carried these insertions cannot be maintained in *A. xylinum*, insertions from these plasmids were transferred into the *A. xylinum* chromosome by recombination. Southern analysis was performed using the wild-type DNA fragment, the vector DNA, and the insert DNA (Tn*phoA* DNA fragment or Kan^r GenBlock) as probes in separate hybridizations to determine if insertions in the mutants resulted from a single recombination or from double-recombination events. The absence of vector sequences and the wild-type DNA fragment and the presence of DNA fragment(s) corresponding to those obtained upon insertion of Tn*phoA* or the Kan^r GenBlock confirmed that the mutants analyzed in this study resulted from double recombination. Cells in which cellulose synthesis was disrupted because of insertions in the *acs* operon genes appeared as smooth colonies in comparison with the rough colonies formed by wild-type cells.

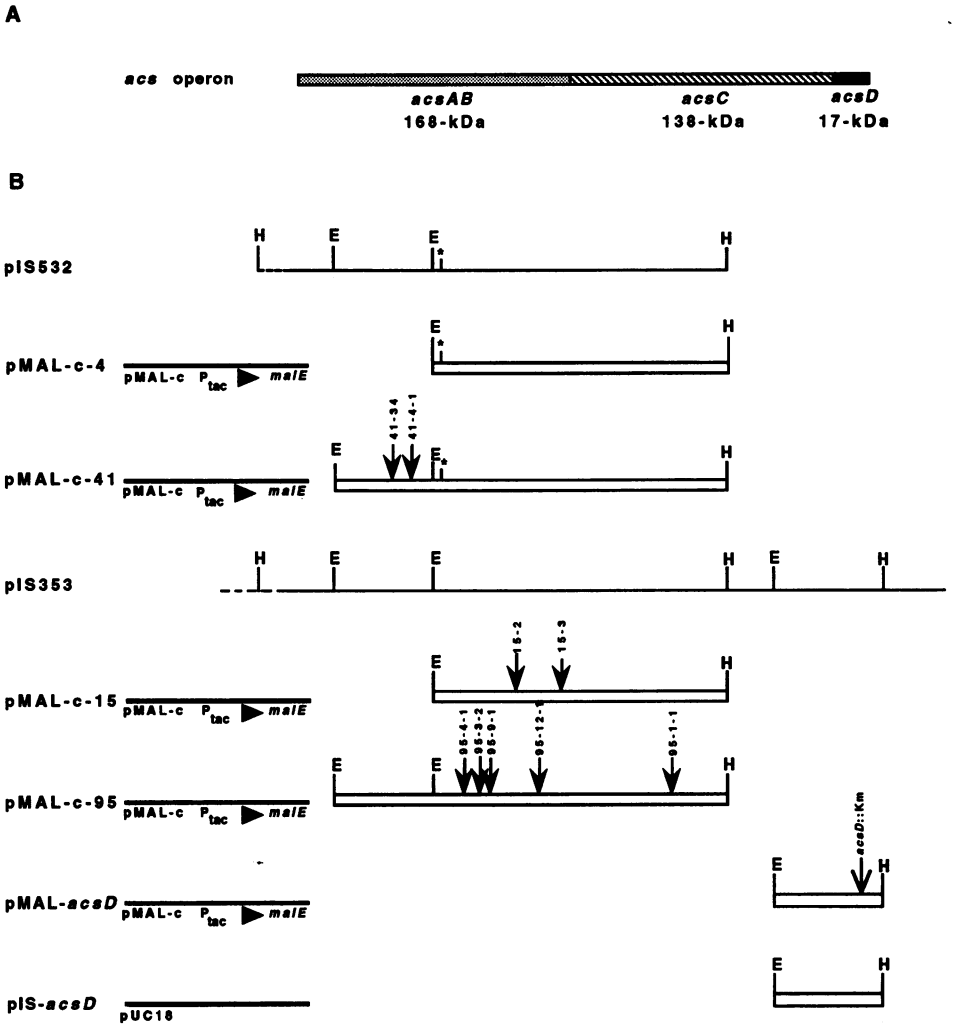

FIG. 1. (A) The *acs* operon of *A. xylinum* ATCC 53582 showing organization of the *acsAB*, *acsC*, and *acsD* genes and the predicted sizes of polypeptides encoded by these genes. (B) Map of DNA fragments from *A. xylinum* ATCC 53582 cloned in plasmids pIS532 and pIS353 that were used for determining the sequence of the *acs* operon. The position of an extra T residue observed in the sequence from pIS532 is marked with an asterisk. DNA fragments cloned in the vector pMAL-c for expression of the *acs* operon genes in *E. coli* and for isolation of Tn*phoA* insertions are shown under the map of pIS532 and pIS353. The 1.4-kb *Eco*RI fragment cloned in pMAL-c-41 and pMAL-c-95 was obtained from pIS353. Tn*phoA* insertions, which were isolated in plasmids pMAL-c-41, pMAL-c-15, and pMAL-c-95 (Table 2), are shown at locations determined by DNA sequencing and are indicated by arrows with filled arrowheads. The Kan^r GenBlock insertion in the *acsD* gene, which was isolated in the plasmid pIS-*acsD*, is indicated by an arrow with an open arrowhead and shown at a similar position in plasmid pMAL-*acsD*. The restriction endonuclease sites shown are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII.

Enzyme assays. Cellulose synthase activity was determined in membrane preparations of *A. xylinum* essentially as described by Lin and Brown (27). Approximately 150 μg of membrane proteins 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 of c-di-GMP (a 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. Alkaline phosphatase activity was determined by measuring the rate of *p*-nitrophenyl phosphate hydrolysis in permeabilized cells (30). Protein concentrations were determined by Peterson's modification of the micro-Lowry method (36) using a protein assay kit (Sigma).

Electron microscopy. *A. xylinum* cells were attached to Formvar-coated grids by touching the grids to the surface of cell suspensions. The grids, with adhering cells, were washed with a few drops of water and 0.25% Triton X-114, and the samples were negatively stained with 1.5% aqueous uranyl acetate containing bacitracin (0.1 mg/ml) as a spreading agent. For freeze-etching, *A. xylinum* cells from colonies, cell suspensions, and active cellulose pellicles were mounted on gold double-replica holders and immediately frozen in liquid nitrogen slush at −208°C. The samples were fractured at −106°C and etched for 15 s with a Balzers BA 360M apparatus at a vacuum of 2×10^{-6} Torr. Specimens were rotary shadowed for 6 s with platinum-carbon and stabilized with carbon for 20 s. Replicas were released from the holders into water, cleared overnight in a solution of 5% $Na(CrO_3)_2$–50% H_2SO_4, washed by floating three times on water, and picked on Formvar-coated copper grids. Negatively stained specimens and replicas

were examined with a Philips EM 420 electron microscope at 100 kV.

X-ray diffraction. Cellulose samples were packed in 0.5-mm-diameter glass capillaries, and powder diffraction patterns were recorded with Ni-filtered Cu Kα radiation (35 kV, 25 mA, 45-min exposure time) using a Philips PW 1729 X-ray generator and a Debye Scherrer camera.

Sequence analysis. Nucleotide and derived protein sequences were analyzed using version 7.0 of the GCG (Genetics Computer Group) sequence analysis software package (12). Percent identity and similarity for predicted amino acid sequences were determined by the BESTFIT program of the GCG package. Transmembrane segments were predicted in the deduced amino acid sequences by the method described by Klein et al. (22) using the PC/GENE program (IntelliGenetics, Inc.). A search of data bases for sequences homologous to the *acs* operon genes and gene products was also performed with the BLAST program (1).

Nucleotide sequence accession number. The accession number for the complete sequence is X54676.

RESULTS

Complete DNA sequence of the *acs* operon from *A. xylinum* ATCC 53582. Following different approaches, genes for cellulose biosynthesis were identified initially for two strains of *A. xylinum*. While the identification and sequencing of the cellulose synthase catalytic subunit (83-kDa polypeptide) gene (44) and a 93-kDa polypeptide gene as part of an operon (45) were reported for *A. xylinum* ATCC 53582, the complete DNA sequence of a cellulose synthase operon (*bcs* operon) carrying four genes was described for *A. xylinum* 1306-3 (52). The genes coding for the 83- and 93-kDa polypeptides were found to be similar to the first two genes (*bcsA* and *bcsB*) of the *bcs* operon, suggesting a similar organization of cellulose-synthesizing genes in these two strains of *A. xylinum*. The DNA sequences of the 83-kDa polypeptide and the 93-kDa polypeptide genes were determined from a 9.5-kb *Hin*dIII fragment of *A. xylinum* ATCC 53582 cloned in pIS532 (44). To obtain the complete sequence of genes present downstream of the previously sequenced region, plasmid pIS353 that carried the 9.5-kb *Hin*dIII fragment present in pIS532 along with additional DNA (Fig. 1B) was identified from a library of *A. xylinum* ATCC 53582, constructed in the broad-host-range cosmid vector pRK311 (43). However, the DNA sequence from pIS353 differed from that determined for pIS532 by the absence of a single T residue at position 2668 in the previously published sequence (44). The change resulted in a frameshift after codon 677 of the original 83-kDa polypeptide gene to fuse this open reading frame with that of the originally defined 93-kDa polypeptide gene so as to give a gene (*acsAB*) encoding a polypeptide with a molecular mass of 168 kDa. To determine which of the two sequences (that from pIS532, encoding two smaller proteins, or that from pIS353, encoding a single larger protein) was correct, a 706-bp fragment was synthesized by PCR from chromosomal DNA of *A. xylinum* strains ATCC 53582 and AY201, using primers E3S2 and P23. The sequences of the PCR fragments (Fig. 2) confirmed that the sequence obtained from pIS353 in this region was the actual sequence in the chromosome of *A. xylinum* strains ATCC 53582 and AY201. It is not clear at this time whether the extra T residue observed at position 2668 in pIS532 was due to a mutation present in the *A. xylinum* cell from which the DNA was isolated or was introduced during the cloning steps. Since the 83-kDa polypeptide, which was shown to bind the substrate (28), and the 93-kDa polypeptide are both derived from the 168-kDa

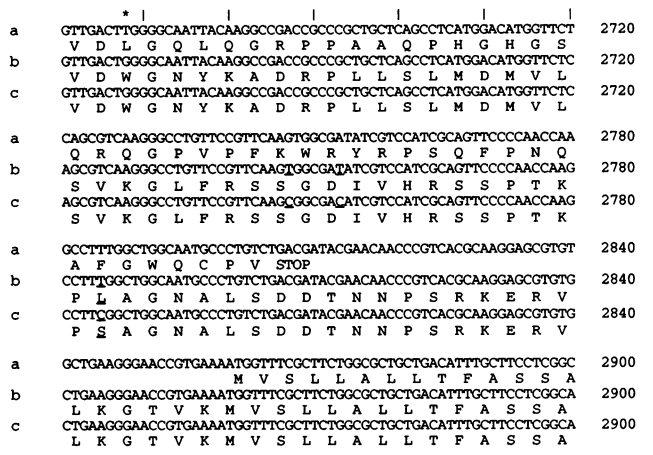

FIG. 2. DNA sequence of a region of the *acs* operon from positions 2661 to 2900 and the deduced amino acid sequence of the open reading frames (ORFs) determined for the DNA fragments from plasmid pIS532 (a) and DNA fragments synthesized by PCR using primers E3S2 and P23 (b and c) and chromosomal DNAs from *A. xylinum* strains ATCC 53582 (b) and AY201 (c). The extra T residue at position 2668 (shown with an asterisk), which was observed in the sequence from pIS532, results in termination of an ORF at position 2804 and the start of the next ORF at position 2860. DNA sequences determined from pIS353 and the PCR fragments (b and c) show an ORF (*acsAB*) extending from positions 636 to 5285 and coding for a polypeptide of 1550 amino acids. Nonidentical residues in the sequences of PCR fragments from strains ATCC 53582 (b) and AY201 (c) are underlined.

polypeptide, this large polypeptide (AcsAB) will be referred to as cellulose synthase. Analysis of the derived amino acid sequence of the AcsAB polypeptide predicts the presence of 11 transmembrane segments, as determined by the method described by Klein et al. (22), and on the basis of this prediction the polypeptide is classified as an integral membrane protein.

DNA sequence from pIS353 led to the identification of two more genes downstream of *acsAB*, with sequence similarity to the *bcsC* and *bcsD* genes. These two genes were named *acsC* and *acsD* (Fig. 1A). The *acsC* gene starts at nucleotide position 5285 and terminates at position 9190 (Fig. 3), coding for a polypeptide with a molecular mass of 138 kDa. The start codon of the *acsC* gene was identified as GTG, and this codon overlaps with the termination codon of the *acsAB* gene. The *acsD* gene begins at position 9193 and terminates at position 9660, coding for a polypeptide with a molecular mass of 17 kDa (Fig. 3). The start codon of the *acsD* gene overlaps with the termination codon of the *acsC* gene. Analysis of the derived amino acid sequences of the AcsC and AcsD polypeptides by the method described by Klein et al. (22) predicted the presence of a single transmembrane segment in both of these polypeptides, suggesting that they were also membrane localized.

Relationship of genes for cellulose biosynthesis in strains of *A. xylinum*. The organization of genes in the *acs* operon of *A. xylinum* ATCC 53582 (Fig. 1A) is similar to that of the *bcs* operon described for *A. xylinum* 1306-3 (52), except that in place of the *bcsA* and *bcsB* genes, a single gene (*acsAB*) is shown to be present in the *acs* operon. In addition, when the gene products were compared using the BESTFIT program, the BcsA polypeptide showed 81% similarity and 69.3% identity with the N-terminal half of AcsAB, while the BcsB polypeptide showed 80.6% similarity and 63% identity with the

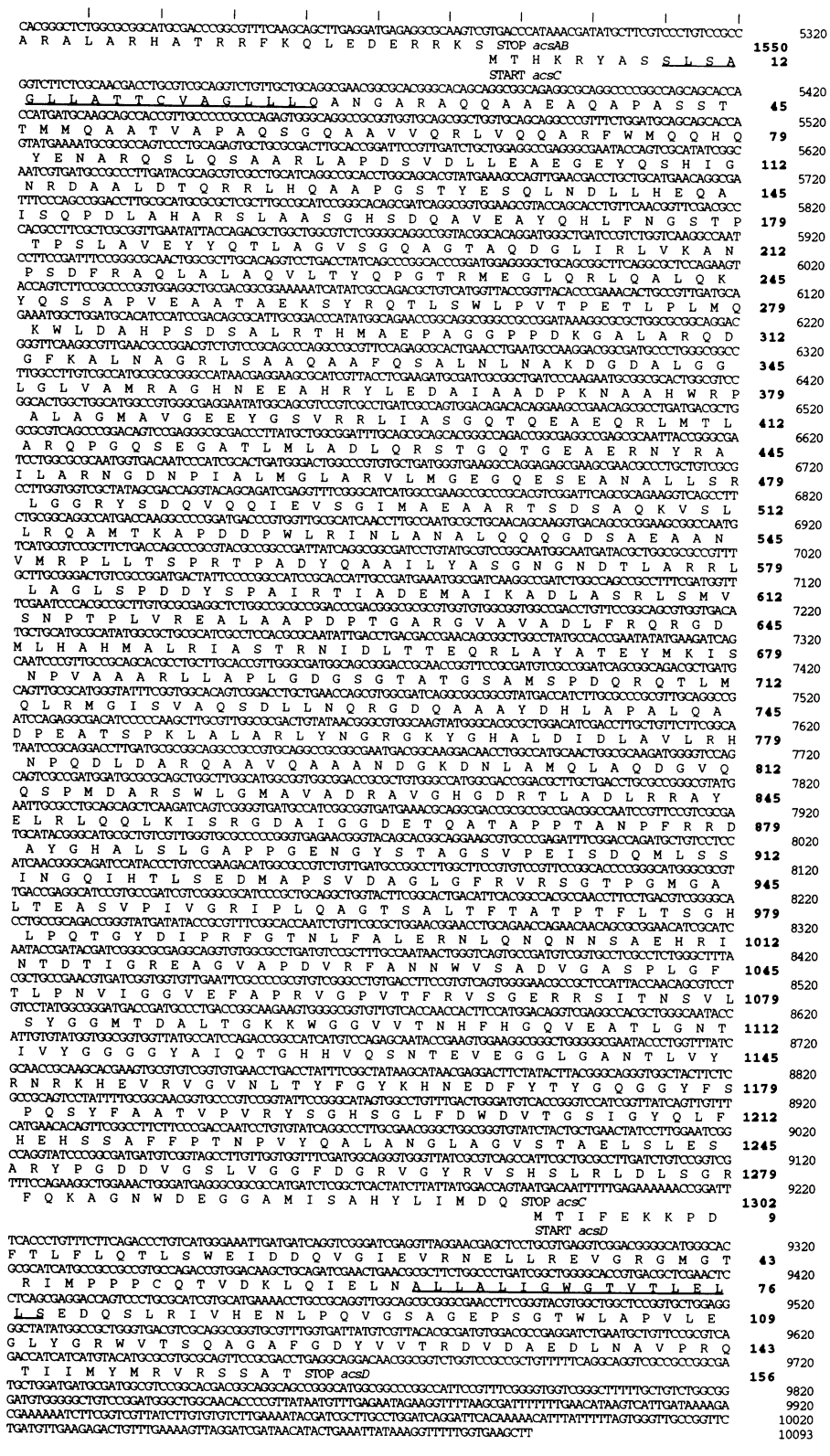

FIG. 3. DNA sequence of a region of the *acs* operon showing the *acsC* and *acsD* open reading frames along with their deduced amino acid sequence. Potential membrane-spanning segments in the AcsC and AcsD polypeptides, which were determined by the method described by Klein et al. (22), are underlined. The DNA sequence is numbered in continuation of the sequence reported earlier (44, 45) and takes into account the deletion of a T residue at position 2668, as mentioned in the present paper.

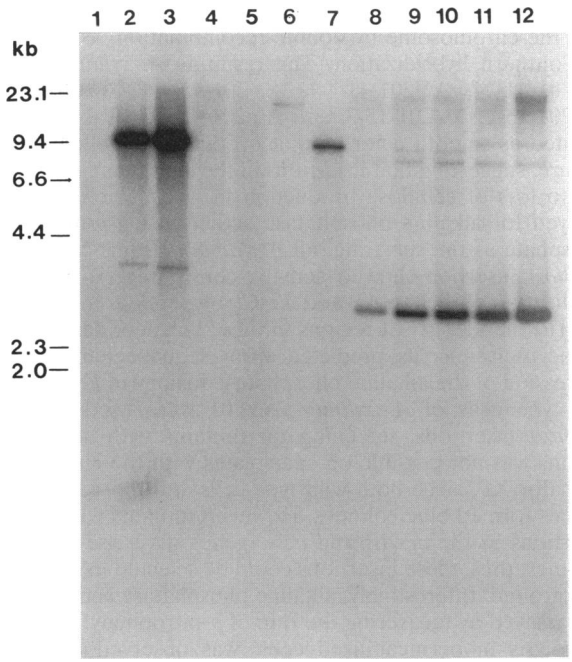

FIG. 4. Southern hybridization of *Hin*dIII-cleaved DNA from strains of *A. xylinum* (lanes 2, 3, and 6 to 12), *A. aceti* (lane 4), and *A. tumefaciens* (lane 5) with ^{32}P-labeled *acsAB* gene probe (pIS532 *Eco*RI fragment, nucleotide positions 240 to 1215). Lanes denoting *A. xylinum* strains are as follows: 2, ATCC 53582; 3, AY201; 6, NIST; 7, LG-1; 8, Hum-1; 9 and 10, different colony types of TG24; 11 and 12, different colony types of TG75. Lane 1 shows *Hin*dIII fragments of bacteriophage lambda. The numbers on the left are the sizes (in kilobases) of the markers.

C-terminal half of the AcsAB polypeptide. Deletion of a single nucleotide in the 3′ region of the *bcsA* gene would lead to a frameshift and continuation of the open reading frame into the *bcsB* gene, resulting in the synthesis of a single large protein that would be similar to the AcsAB polypeptide. However, there is no further information available on the *bcs* operon at present. Alignment of the AcsC polypeptide with the BcsC polypeptide (79.2% similarity, 67.8% identity) and the AcsD polypeptide with the BcsD polypeptide (86.5% similarity, 76.9% identity) showed that overall, a 63 to 77% identity was present in gene products from the *acs* and *bcs* operons. Since no other gene sequences have been reported from *A. xylinum* strains ATCC 53582 and 1306-3, in which the *acs* and *bcs* operons are described, respectively, it was not possible to determine the level of identity expected for the cellulose-synthesizing genes from these two strains.

Since polypeptides encoded by genes for cellulose biosynthesis isolated from two strains of *A. xylinum* were found to be similar but nonidentical, we decided to study the homology of the cellulose synthase gene from *A. xylinum* ATCC 53582 with DNA from other strains of *A. xylinum*, as well as to investigate if sequences homologous to this gene were present in the related bacterium *Acetobacter aceti* and a cellulose-producing bacterium, *Agrobacterium tumefaciens*. A DNA probe carrying the 5′ end of the *acsAB* gene from *A. xylinum* ATCC 53582 (976-bp *Eco*RI fragment, nucleotide positions 240 to 1215) was used in Southern hybridization with *Hin*dIII-cleaved DNA from *A. xylinum* AY201, NIST, LG-1, Hum-1, TG24, and TG75 and *Hin*dIII-cleaved DNA from *A. aceti* and *A. tumefaciens* (Fig. 4). While DNA fragments homologous to the *acsAB* gene probe were observed in all of the *A. xylinum* strains with various degrees of homology (Fig. 4, lanes 2, 3, and 6 to 12), hybridization of this probe was not detected with DNA from *A. aceti* (Fig. 4, lane 4) and *A. tumefaciens* (Fig. 4, lane 5) under the conditions used for DNA-DNA hybridization (35°C below T_m). Thus far, DNA fragments homologous to the *A. xylinum* cellulose-synthesizing genes have not been identified in any other cellulose-producing organism.

The results from a number of hybridizations showed that DNA from *A. xylinum* ATCC 53582 was highly homologous to DNA from *A. xylinum* AY201, a derivative of ATCC 23769. Both strains showed hybridization of similar-sized DNA fragments to probes from the *acs* operon region, suggesting a relationship between these strains. An almost identical sequence was also obtained for a region of the *acsAB* gene in PCR fragments synthesized using chromosomal DNAs from these two strains (Fig. 2). Although the *acs* operon was isolated from *A. xylinum* ATCC 53582, this strain was found to be unsuitable for genetic analysis, since it was not possible to obtain efficient DNA transfer in this strain either by conjugation or by electroporation. On the other hand, both of these methods were found to be effective for DNA transfer in *A. xylinum* AY201. Consequently, the sequence identity between these two strains was exploited to generate site-determined *A. xylinum* AY201 mutants for genetic analysis using cloned DNA fragments isolated from *A. xylinum* ATCC 53582.

Analysis of the *acs* operon genes expressed in *E. coli* and isolation of *acsAB*::*phoA* and *acsC*::*phoA* fusions. DNA fragments from the *acs* operon region of *A. xylinum* ATCC 53582 were cloned in the expression vector pMAL-c, and the gene present at the 5′ end of the fragment was fused to the *malE* gene of the vector. The gene(s) present on the cloned fragment was expressed in *E. coli* under the control of the *tac* promoter to synthesize MBP fusion protein, along with either truncated or intact polypeptides, depending on the coding region present in the 3′ end of the DNA fragment. The *malE* region coded for a 42-kDa MBP portion in the fusion proteins. Fusions of *malE* to DNA fragments from pIS353 carrying the 3′ half of the *acsAB* gene (pMAL-c-15) and an almost complete *acsAB* gene (pMAL-c-95) were isolated (Fig. 1B). Upon IPTG induction of *E. coli* JM109 containing these two plasmids, synthesis of polypeptides with molecular masses of 137 kDa for pMAL-c-15 and 184 kDa for pMAL-c-95 were observed, consistent with the expected size of polypeptides predicted for a single AcsAB protein. When similar DNA fragments from pIS532, the plasmid containing an additional T residue and therefore two separate reading frames, were cloned into the pMAL-c vector, the resulting proteins were the sizes expected if translation terminates at the position of the asterisk shown in Fig. 1B (pMAL-c-4 and pMAL-c-41). *E. coli* extract containing the MBP-AcsAB fusion synthesized from pMAL-c-95 was assayed for cellulose synthase activity; however, no activity was detected, suggesting that the N-terminal region (residues 1 to 193) of the AcsAB polypeptide that was not present in this fusion may be essential for enzyme activity.

Transposon Tn*phoA* (31) was used in the present study to generate translational fusions of the *acsAB* and *acsC* genes with the *E. coli phoA* gene to understand the membrane topology of polypeptides encoded by these genes. The *phoA* gene codes for alkaline phosphatase which is active only upon export from the cytoplasm; thus, alkaline phosphatase fusions have been used in the analysis of a number of membrane proteins (32). Expression of regions of the *acsAB* and *acsC* genes in *E. coli* using pMAL-c-derived plasmids made it possible to isolate Tn*phoA* insertions with active fusions in these genes that could be analyzed in *E. coli* and later on

TABLE 2. Position of Tn*phoA* insertions isolated on pMAL-c derived plasmids carrying DNA fragments from the *acs* operon of *A. xylinum*

Insertion	Plasmid[a]	Gene	Nucleotide position[b]	Amino acid position[c]
41-34	pMAL-c-41	*acsAB*	1870	411 (AcsAB)
41-4-1	pMAL-c-41	*acsAB*	2104	489 (AcsAB)
95-4-1	pMAL-c-95	*acsAB*	3196	853 (AcsAB)
95-3-2	pMAL-c-95	*acsAB*	3232	865 (AcsAB)
95-9-1	pMAL-c-95	*acsAB*	3316	893 (AcsAB)
15-2	pMAL-c-15	*acsAB*	3856	1073 (AcsAB)
95-12-1	pMAL-c-95	*acsAB*	4177	1180 (AcsAB)
15-3	pMAL-c-15	*acsAB*	4615	1326 (AcsAB)
95-1-1	pMAL-c-95	*acsC*	6327	347 (AcsC)

[a] Plasmid on which the insertion was isolated.

[b] Position of Tn*phoA* insertion in the nucleotide sequence of the *acs* operon published earlier (44, 45) and continued with modification (deletion of T residue at position 2668) in the present paper.

[c] Position of the C-terminal-most amino acid of the polypeptide (indicated in parentheses) before Tn*phoA*-encoded residues.

transferred to the *A. xylinum* chromosome. Plasmids carrying these fusions were analyzed by restriction analysis, and the Tn*phoA* insertion site was determined by DNA sequencing of the fusion site. Figure 1B shows the locations of Tn*phoA* insertions with active fusions in the *acsAB* and *acsC* genes isolated on the different plasmids. The nucleotide position at the site of insertion and the protein fusion site determined from the predicted amino acid sequence of the corresponding genes are indicated in Table 2. Permeabilized cells of *E. coli* CC118 containing plasmids with these fusions were assayed for alkaline phosphatase activity. All fusions showed high levels of activity, confirming the presence of an extracytoplasmic region(s) as predicted from analysis of the derived amino acid sequences of the AcsAB and AcsC polypeptides. The presence of multiple transmembrane segments in the AcsAB polypeptide was confirmed from identification of a large number of sites in this polypeptide that were exposed to the periplasm. A single transmembrane segment was predicted in the derived amino acid sequence of the AcsC polypeptide, and although only a single periplasmic site was identified, it provides evidence to support the prediction that this polypeptide may be completely exposed to the periplasm, since it is anchored in the inner membrane at its N-terminal end.

Isolation of *A. xylinum* mutants with insertions in genes of the *acs* operon. Thus far, genetic analysis of cellulose biosynthesis in *A. xylinum* has been performed using mutants that were isolated either by chemical mutagenesis or those that originated spontaneously. A number of nitrosoguanidine-induced mutants that formed no cellulose pellicle were found to produce highly reduced amounts of cellulose that was characterized as a cellulose II allomorph (39). In all of these mutants, cellulose synthase activity was observed, suggesting that the mutations affecting cellulose production were not in the gene for cellulose synthase but at some other loci. The identification of the cellulose synthase gene and its organization into an operon provided information on other genes in the operon that may be involved in cellulose production. To determine if all of the genes in the *acs* operon were involved in cellulose production and to analyze the role of the various genes in this process, mutants of *A. xylinum* with insertions at known sites in the *acs* operon were isolated for analysis. Mutants with insertions in the *acsAB* and *acsC* genes were obtained by introducing pMAL-c-derived plasmids carrying the Tn*phoA* insertions 41-34, 41-4-1, 95-4-1, 95-9-1, 15-2, 15-3, and 95-1-1 (Table 2) into *A. xylinum* AY201. Plasmid-borne insertions were transferred into the chromosome by double recombination, as confirmed by Southern hybridization. The resulting *A. xylinum* strains, AY201-41-34, AY201-41-4-1, AY201-95-4-1, AY201-95-9-1, AY201-15-2, AY201-15-3, and AY201-95-1-1 (Table 1), were isolated as smooth colonies that produced no cellulose upon culture, suggesting that insertions in the *acsAB* and *acsC* genes led to loss of cellulose production in vivo. When they were assayed for alkaline phosphatase activity using *p*-nitrophenyl phosphate as the substrate, all *A. xylinum* strains carrying the Tn*phoA* insertion showed activity, confirming (i) membrane localization of the AcsAB and AcsC polypeptides in *A. xylinum* and (ii) the identity of regions in these polypeptides that were extracytoplasmic, as predicted from sequence analysis and expression of the alkaline phosphatase fusions in *E. coli*. Since wild-type cells of *A. xylinum* AY201 were used to isolate Tn*phoA* insertions, screening for mutants with active *phoA* fusions was not possible on agar plates with the chromogenic indicator XP, since both wild-type cells and those with active fusions formed blue colonies. However, mutants with Tn*phoA* insertions in the *acsAB* and *acsC* genes gave rise to smooth colonies; thus, these insertions could be selected in a wild-type background. Interestingly, alkaline phosphatase activity, which was assayed by measuring the rate of *p*-nitrophenyl phosphate hydrolysis in permeabilized cells, was observed only in *A. xylinum* mutants that carried active *phoA* fusions, thereby making it possible to analyze Tn*phoA* fusions in wild-type *A. xylinum* AY201.

For isolation of an *A. xylinum acsD* mutant, plasmid pIS-*acsD*::Km that carries a Kan^r GenBlock in the *acsD* gene was introduced into *A. xylinum* AY201 by electroporation, and kanamycin-resistant colonies were selected. The transfer of the Kan^r GenBlock from the plasmid to the chromosome by a double recombination event was confirmed by Southern hybridization. The kanamycin-resistant colonies were smooth and produced highly reduced amounts of cellulose in culture, suggesting that the *acsD* gene product was required for normal cellulose production, even though some cellulose was synthesized in the absence of this gene product.

Insertions in the *acsAB* and *acsC* genes resulted in polar mutations in which expression of the downstream gene(s) was affected; however, analysis of the mutants generated by these insertions provided evidence that polypeptides encoded by these two genes were essential for cellulose synthesis in vivo. No cellulose production was observed in mutants AY201-95-4-1, AY201-95-9-1, AY201-15-2, and AY201-15-3, in which truncated AcsAB polypeptides were synthesized as AcsAB-PhoA fusions, and in mutant AY201-95-1-1, in which the complete AcsAB polypeptide and an AcsC′-PhoA fusion protein were synthesized. Synthesis of these proteins was inferred from the Tn*phoA*-encoded alkaline phosphatase activity detected in all of the mutants. The presence of the AcsAB and AcsC polypeptides in AY201-*acsD*::Km was established by the fact that this mutant could be recovered to wild type by transfer of the plasmid pIS311-*acsD* that carries the *acsD* gene (discussed below). However, transfer of pIS311-*acsD* into AY201-41-34, AY201-95-4-1, AY201-15-3, and AY201-95-1-1 did not lead to recovery of cellulose production in any of the mutants, suggesting that the AcsAB and AcsC polypeptides were required along with the AcsD polypeptide for normal cellulose I synthesis. Since mutants with insertions in the *acsAB* gene also led to a loss of *acsC* function, the possibility remained that the AcsC polypeptide could support cellulose production in the absence of the *acsAB* gene product. To verify this probability, plasmid pIS311-95 which carries a DNA fragment containing the P_{tac}-*malE*-′*acsAB-acsC* region from pMAL-c-95 was

TABLE 3. Cellulose synthase activity in the membrane fraction from mutant strains of *A. xylinum* AY201

Strain	Location of Tn*phoA* insertion	Cellulose synthase sp act[a]
AY201-41-34	*acsAB*	387
AY201-41-4-1	*acsAB*	306
AY201-95-4-1	*acsAB*	473
AY201-95-9-1	*acsAB*	206
AY201-15-2	*acsAB*	324
AY201-15-3	*acsAB*	294
AY201-95-1-1	*acsC*	—[b]

[a] Specific activity is defined as picomoles of UDP-glucose incorporated into alkali-insoluble product per minute per milligram of protein. Cellulose synthase specific activity for wild-type AY201 was 728.
[b] —, not shown.

cloned in the vector pRK311 and mobilized into mutants AY201-41-34 and AY201-15-3. A thin cellulose pellicle was produced only in the AY201-15-3(pIS311-95) transconjugant, in which the AcsAB′-PhoA fusion protein (containing residues 1 to 1326 of the AcsAB polypeptide) encoded by the chromosome and the MBP-′AcsAB fusion (containing residues 194 to 1550 of the AcsAB polypeptide) and AcsC′ (residues 1 to 754) polypeptides encoded by the plasmid, were synthesized. Although a truncated AcsC polypeptide (residues 1 to 754) was present in both AY201-41-34(pIS311-95) and AY201-15-3 (pIS311-95) transconjugants, cellulose production was observed only in AY201-15-3(pIS311-95), suggesting that by itself this region of the AcsC polypeptide was not sufficient and that a relatively large region of the AcsAB polypeptide was required as well for cellulose synthesis. No cellulose production was observed in the AY201-41-34(pIS311-95) transconjugant in which the AcsAB′-PhoA fusion protein (containing residues 1 to 411 of the AcsAB polypeptide) was synthesized along with the MBP-′AcsAB fusion protein and the AcsC′ polypeptide. From these studies, it was possible to determine the upper limit for regions in the AcsAB (residues 1 to 1326) and the AcsC (residues 1 to 754) polypeptides that were found to be essential for cellulose synthesis.

Cellulose synthase activity in *A. xylinum* AY201 mutants with Tn*phoA* insertions in the *acsAB* and *acsC* genes. Analysis of AY201 insertion mutants suggested that products of the *acsAB* and *acsC* genes were essential for in vivo cellulose production because of their possible role in the formation of the cellulose-synthesizing and extrusion complex. Since the 83- and 93-kDa polypeptides, derived from the 168-kDa AcsAB polypeptide, were found to be major components of purified cellulose synthase (27), we decided to determine if the AcsAB polypeptide by itself was sufficient for in vitro enzyme activity using membrane preparations from the different mutants. While mutants AY201-41-34, AY201-41-4-1, AY201-95-4-1, AY201-95-9-1, AY201-15-2, and AY201-15-3 were expected to synthesize AcsAB-PhoA fusion proteins containing various lengths of the AcsAB polypeptide, but no AcsC and AcsD polypeptides, synthesis of the complete AcsAB polypeptide and a truncated AcsC polypeptide was expected in AY201-95-1-1. Surprisingly, when assays for in vitro cellulose synthesis were performed, cellulose synthase activity was detected in membrane preparations from all of the mutants (Table 3). The activity observed in mutants was less than that observed for wild-type AY201; however, it was activated by the cellulose synthase activator c-di-GMP to the same extent as that observed in wild-type cells. Even though the levels of activity varied among the mutants, no correlation could be found between the observed activity and the site of Tn*phoA* insertion. The presence of cellulose synthase activity in membranes from these mutants suggested that this activity was encoded by a gene(s) other than those present in the *acs* operon; this was much more evident in mutants AY201-41-34 and AY201-41-4-1, in which only the N-terminal 411 and 489 residues of the AcsAB polypeptide were synthesized as part of the AcsAB-PhoA fusion protein, respectively. As suggested, a second cellulose synthase gene (*acsAII*) was identified in *A. xylinum* AY201, and using mutants with insertions in both the *acsAB* and the *acsAII* genes, it was shown that the in vitro cellulose synthase activity observed in the *acsAB* and *acsC* mutants was determined by the *acsAII* gene (42). The *acsAII* gene codes for a polypeptide with a molecular mass of 175 kDa that shows similarity to the 168-kDa AcsAB polypeptide, providing further evidence that cellulose synthase is synthesized as a large protein.

***A. xylinum* AY201-*acsD*::Km produces both cellulose I and II in vivo.** In contrast to mutants that had insertions in the *acsAB* and *acsC* genes and in which no cellulose production was observed, cellulose synthesis was observed in cells of the *acsD* mutant of *A. xylinum*. The mutant AY201-*acsD*::Km exhibited smooth colonies of two sizes—small and large (Fig. 5A), and while small colonies gave rise to large colonies, the large colonies remained stable. However, cellulose production was observed only in cells of the small-colony type (Fig. 5B). AY201-*acsD*::Km cells produced a thin cellulose pellicle during static growth (Fig. 5E), in contrast to a thick pellicle produced by wild-type AY201 cells (Fig. 5D). In agitated cultures of AY201-*acsD*::Km, flocs consisting of cells and attached material were observed in place of the cellulose pellicle. A quantitative estimate of the amounts of cellulose produced in static cultures showed that AY201-*acsD*::Km produced very little cellulose in comparison with wild-type AY201 (Table 4). In spite of the difference in cellulose yield, the cellulose synthase activities in membrane preparations from wild-type AY201 and AY201-*acsD*::Km were found to be similar (Table 4), suggesting that the *acsD* gene product is not required for cellulose synthase activity in vitro but may regulate it in the intact cell. Although no cellulose was produced by cells of the AY201-*acsD*::Km large-colony type, cellulose synthase activity was detected in membranes from these cells as well.

When cells of AY201-*acsD*::Km were grown under static and agitated conditions, differences were observed not only in the amounts of cellulose produced but also in the morphology of the cellulose products. To determine if this change was related to cellulose crystalline structure, X-ray diffraction patterns were obtained for cellulose produced by AY201-*acsD*::Km under static and agitated growth conditions. Reflections, typical of the cellulose I crystalline allomorph, were observed in cellulose produced under static conditions by cells of AY201-*acsD*::Km (Fig. 6C). However, when these cells were grown under agitation, reflections of both cellulose I and cellulose II allomorphs were observed in the cellulose product (Fig. 6B). These observations suggested that growth conditions influenced the crystalline structure of the final cellulose product in AY201-*acsD*::Km. Mostly, this influence was related to the amount of cellulose produced, with higher yields reported for static cultures of wild-type *A. xylinum* cells (54) and observed in cultures of AY201-*acsD*::Km as well (Table 4). The low yield of cellulose, obtained in agitated cultures of AY201-*acsD*::Km, could possibly be due to the production of cellulose II, which by its inherent structure may block cellulose synthesis (discussed below). A similar situation was also observed in mutants of *A. xylinum* ATCC 53582 that produced

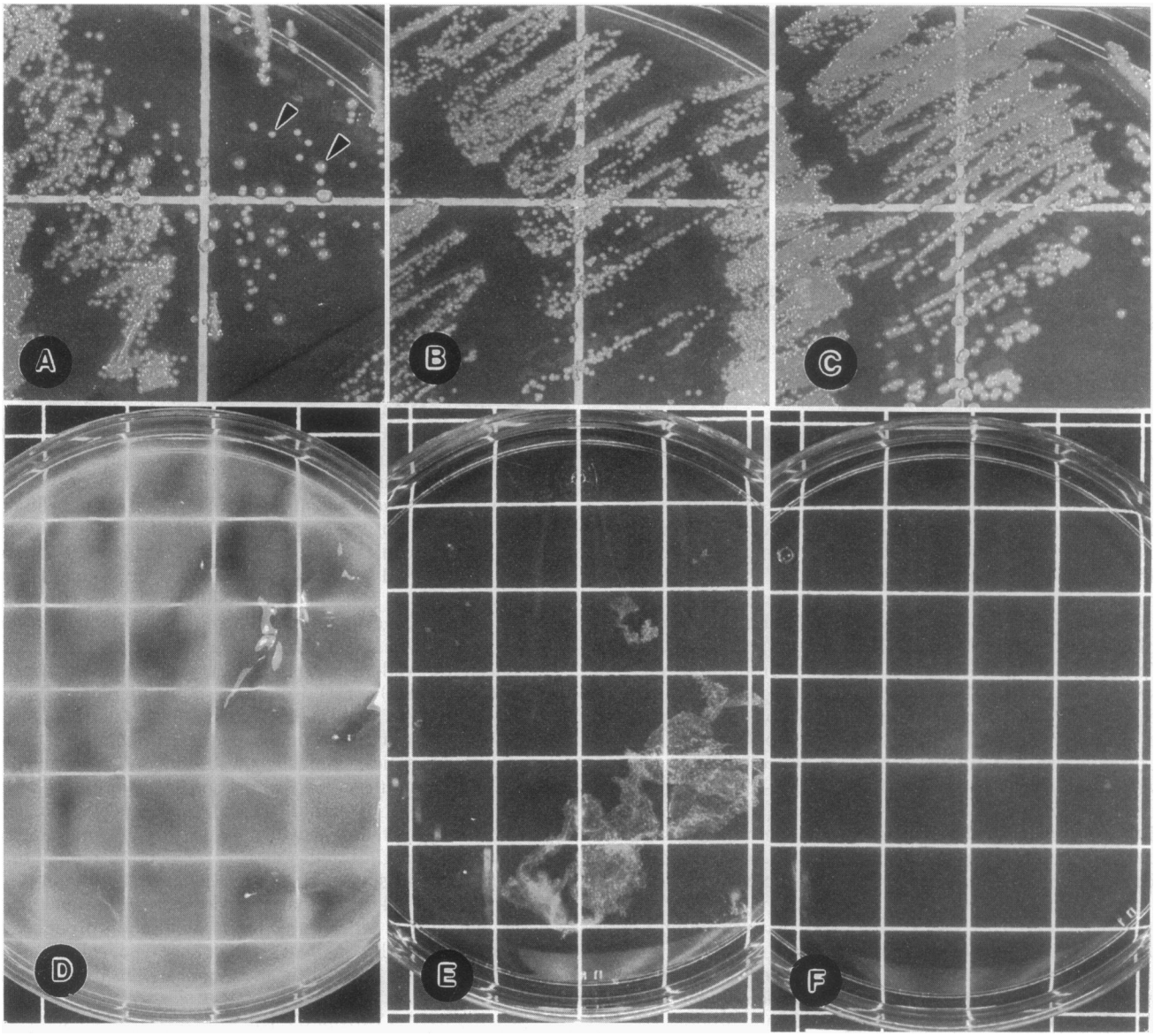

FIG. 5. Morphology of *A. xylinum* AY201-*acsD*::Km colonies and the cellulose produced in static cultures. Two sizes of colonies (A) obtained from cultures of AY201-*acsD*::Km were isolated to show small (B)- and large (C)-colony types. In static culture, the small-colony type produces a thin cellulose pellicle (E), whereas the large-colony type produces a thin surface film but no cellulose pellicle (F). Under similar culture conditions, wild-type AY201 cells produce a thick cellulose pellicle (D).

very small amounts of cellulose and in which the cellulose product was characterized as cellulose II (39).

Recovery of normal cellulose production in AY201-*acsD*::Km upon introduction of pIS311-*acsD*. The defect in cellulose production in the mutant AY201-*acsD*::Km was due to disruption of the *acsD* gene, and this was confirmed by the ability of the wild-type *acsD* gene to complement this mutation. The plasmid pIS311-*acsD*, which carries a DNA fragment (P_{tac}-*malE*-'*acsC*-*acsD*) from the plasmid pMAL-*acsD* cloned in the broad-host-range vector pRK311, was mobilized into *A. xylinum* AY201-*acsD*::Km, and transconjugants were selected on SH agar plates containing kanamycin and tetracycline. Cells of AY201-*acsD*::Km(pIS311-*acsD*) produced cellulose under static as well as agitated conditions, and the yields and properties of cellulose were similar to those obtained from

TABLE 4. Cellulose yield and cellulose synthase activity in AY201-*acsD*::Km

Strain	Amt of cellulose produced in vivo (mg)[a]	Cellulose synthase sp act[b]
AY201 wild type	37.07	500.28
AY201-*acsD*::Km (small-colony type)	1.25	507.81
AY201-*acsD*::Km (large-colony type)	ND[c]	330.54

[a] Dry weight of cellulose from 20-ml culture harvested after 4 days of static growth. AY201-*acsD*::Km (small-colony type) grown for 4 days with agitation produced 0.43 mg of cellulose.

[b] In picomoles of UDP-glucose incorporated into alkali-insoluble product per minute per milligram of protein.

[c] ND, not detected.

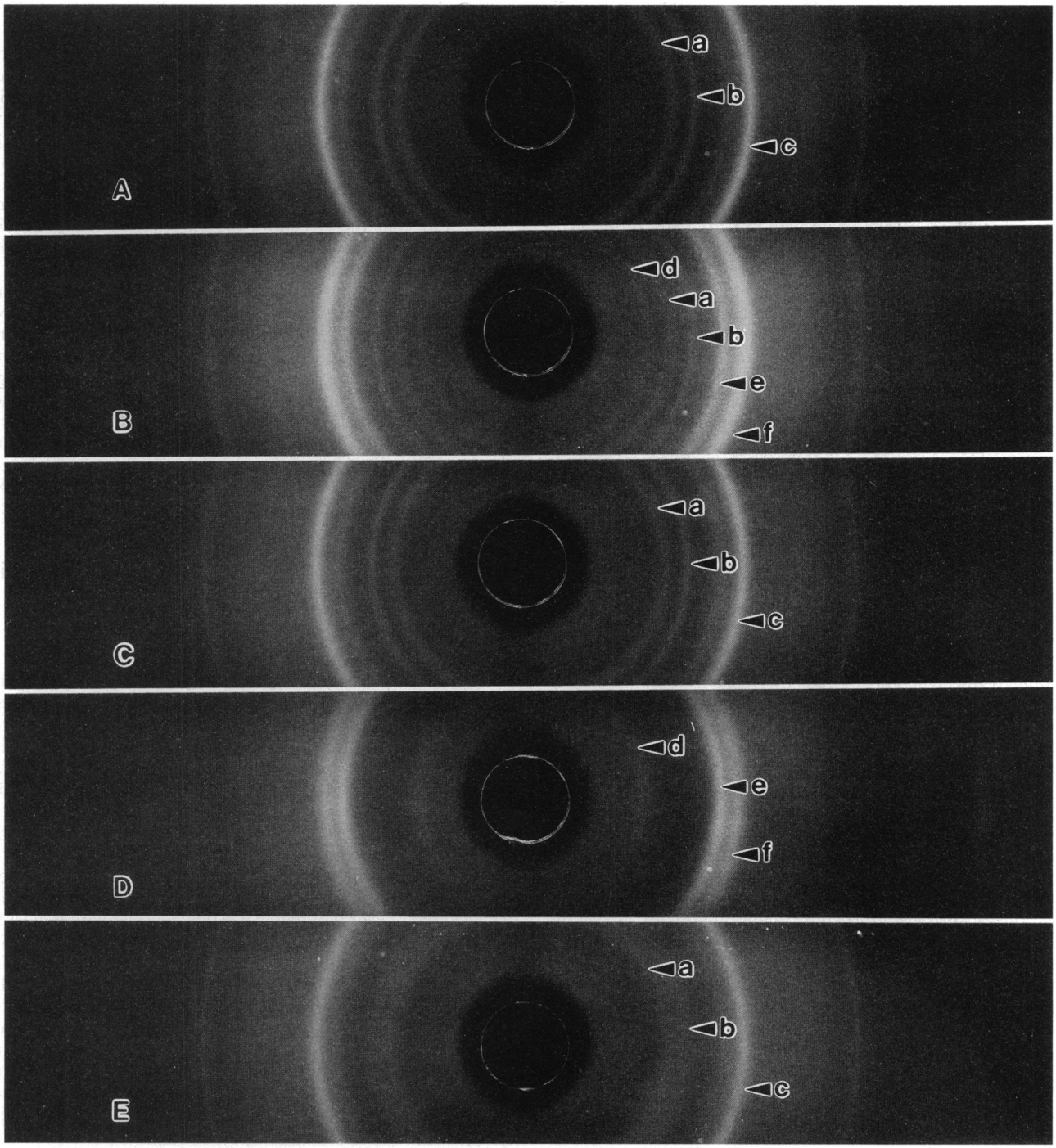

FIG. 6. X-ray diffraction patterns of cellulose products. (A) *A. xylinum* AY201 wild-type (static culture) showing cellulose I (see reflections [a, 6.08 Å, where 1 Å is 0.1 nm; b, 5.26 Å; c, 3.89 Å]); (B) AY201-*acsD*::Km (agitated culture) showing cellulose II (see reflections [d, 7.22 Å; e, 4.40 Å; f, 3.97 Å]) and cellulose I (see reflections [a, 6.11 Å; b, 5.21 Å]); (C) AY201-*acsD*::Km (static culture) showing cellulose I (see reflections [a, 6.10 Å; b, 5.25 Å; c, 4.02 Å]); (D) rayon (cellulose II control; see reflections [d, 7.50 Å; e, 4.41 Å; f, 4.07 Å]); (E) Sigmacell (cellulose I control; see reflections [a, 5.89 Å; b, 5.36 Å; c, 3.91 Å]).

wild-type AY201. To demonstrate that normal cellulose production in the transconjugants was due to the *acsD* gene expressed from pIS311-*acsD* and was not due to a recombination event, plasmid DNA was isolated from AY201-*acsD*::Km(pIS311-*acsD*), and the presence of the *acsD* gene in the recovered plasmid was confirmed by Southern hybridization. However, the complementation of the *acsD* mutation by pIS311-*acsD* was observed only in the small-colony-type cells of AY201-*acsD*::Km and not in cells of the large-colony type, suggesting the presence of secondary mutations that affect cellulose production in the large-colony type cells.

Morphology of cells and cellulose products in mutant

strains of *A. xylinum* AY201. Synthesis of the thermodynamically less-favorable crystalline cellulose I allomorph is suggested to be the result of a precise arrangement of cellulose-synthesizing sites in *A. xylinum*. The arrangement of these sites has been inferred from examination of cells along with the attached cellulose product and by freeze-fracture analysis of the cell surface. To determine if genes in the *acs* operon were involved in the arrangement of these sites, we examined the morphology of the cellulose products and the organization of the intramembranous particles in mutants of *A. xylinum*. Like other *A. xylinum* strains, wild-type AY201 cells produced a typical, twisted ribbon of cellulose (Fig. 7A and B). Among mutants with insertion in genes of the *acs* operon, cellulose production was seen only in AY201-*acsD*::Km. This strain produced a cellulose pellicle during static growth conditions, and an examination of cells from the pellicle showed that they produced ribbons of cellulose (Fig. 7C and D) that did not differ in morphology from those produced by wild-type cells. When AY201-*acsD*::Km was grown with agitation and the cells were examined at a high magnification, rodlets of the cellulose II allomorph attached to the cells could be clearly observed (Fig. 8B). However, these cells produced both cellulose I and cellulose II, as shown by X-ray diffraction analysis of the cellulose product (Fig. 6B). Although production of both microfibrils of cellulose I and rodlets of cellulose II was observed from the same cell of AY201-*acsD*::Km (Fig. 8A), it is not clear at present if both of these allomorphs were synthesized from the same site or from different sites in the cell.

To determine if the linear arrangement of cellulose-synthesizing sites was present at all times in cells of *A. xylinum*, we examined wild-type AY201 cells by freeze-fracture. A distinct linear row of intramembranous particles was observed only in cells producing cellulose microfibrils during pellicle formation (Fig. 9A), and a very diffuse organization was observed in cells frozen from colonies (Fig. 9B). No arrangement was observed in wild-type cells when they were collected by centrifugation or when they were treated with cellulase to dissolve the cellulose product. These results suggested that the cellulose-synthesizing sites in *A. xylinum* assumed an ordered arrangement during the synthesis of cellulose microfibrils and that this organization was not preserved in conditions of stress or when the cellulose microfibrils were even digested. When mutants of *A. xylinum* AY201 were examined by freeze-fracture, the linear arrangement of particles correlated strictly with production of a cellulose pellicle. Even though AY201-*acsD*::Km produced a thin pellicle during static growth, cells from this pellicle showed an ordered array of particles (Fig. 10A). A similar organization was observed in cells of AY201-*acsD*::Km (pIS311-*acsD*), which produced a thick pellicle and in which cellulose production was indistinguishable from that of wild-type cells (Fig. 10B). *A. xylinum* AY201-41-34, AY201-95-4-1, AY201-15-3 (Fig. 8C), and AY201-95-1-1 were found to produce no cellulose product, and an examination of cells from these strains by freeze-fracture revealed no organization of intramembranous particles (Fig. 8D).

Homology of the AcsC polypeptide with bacterial pore-forming proteins. From analysis of *A. xylinum* mutants, a requirement of the AcsC polypeptide was established for in vivo cellulose production, and on the basis of its predicted hydrophobicity and membrane localization, it was suggested that this polypeptide is involved in formation of pore-like structures for extrusion of the cellulose product. A data base search for identification of related proteins revealed that the AcsC polypeptide exhibited sequence relationship with a few proteins that were involved in formation of membrane complexes. Alignment of the AcsC sequence with the sequences of the *E. coli* TrbI protein (49.1% similarity, 25.3% identity), *A. tumefaciens* VirB10 protein (47.0% similarity, 23.8% identity), and *orfG* gene product of the *ptl* operon of *Bordetella pertussis* (45.7% similarity, 25.3% identity) was done using the BESTFIT program of the GCG package to determine the relationship of these proteins with the AcsC polypeptide. The VirB10, TrbI, and OrfG proteins were predicted to be anchored in the cytoplasmic membrane by a single hydrophobic segment in much the same manner as that of the AcsC polypeptide, suggesting that the structural organization of these proteins may be related to a common function. The VirB10 protein (49, 50) has been suggested to form an aggregate with itself or other membrane proteins to form a complex pore structure that spans both membranes and through which the T-DNA from *A. tumefaciens* is passed into the plant cell. The TrbI protein is encoded in the Tra2 region of the plasmid RP4 that is involved in mating-pair formation during bacterial conjugation in *E. coli* (26), and the *ptl* operon is involved in secretion of the pertussis toxin in *B. pertussis* (51). Sequence similarity has been observed between protein products encoded by genes in the *virB* region of *A. tumefaciens*, the Tra2 region of *E. coli*, and the *ptl* operon of *B. pertussis*, suggesting that these regions are involved in the formation of membrane complexes for the secretion of macromolecules from bacteria. The similarity of the AcsC polypeptide with these polypeptides emphasizes its role in the formation of a membrane complex in *A. xylinum* for extrusion of the cellulose product.

DISCUSSION

Genes involved in biosynthetic pathways are commonly organized in an operon or in a cluster, as seen for the synthesis of a number of bacterial exopolysaccharides (24). Compared with some of the more complex polysaccharides that require a number of steps for their synthesis (18, 20), the polymerization of cellulose is catalyzed in a single step by the enzyme cellulose synthase. In *A. xylinum*, cellulose synthase activity is regulated by the activator c-di-GMP, and thus in its simplest form the cellulose synthase from this bacterium is visualized as a membrane protein with a substrate-binding and a regulatory site. Purification of this enzyme from *A. xylinum* ATCC 53582 showed the presence of two major polypeptides with molecular masses of 83 and 93 kDa (27), and the 83-kDa polypeptide was identified as the substrate-binding subunit by photolabeling with [^{32}P]azido-UDP-glucose (28). The function of the 93-kDa polypeptide could not be established from these studies, except that it was observed in purified preparations in approximately the same amount as the 83-kDa polypeptide, suggesting that both of these polypeptides were present in an equimolar ratio in cellulose synthase. However, cellulose synthase purified from *A. xylinum* 1306-21 was shown to have three major polypeptides with molecular masses of 90, 67, and 54 kDa and in which the activator and substrate-binding sites were associated with the 67- and 54-kDa polypeptides, respectively (33). N-terminal sequencing of these polypeptides showed that the 90-kDa polypeptide was encoded by the second gene (*bcsB*) in the *bcs* operon (52) and that the 67-kDa polypeptide was a cleavage product of the 90-kDa polypeptide. In these studies, the *bcsB* gene was identified as the cellulose synthase catalytic subunit gene (33, 52). Moreover, the 83-kDa polypeptide, encoded by the first gene (*bcsA*) in the *bcs* operon, was not observed in purified cellulose synthase that was treated with sample buffer at 95°C for 2 min and analyzed by SDS-polyacrylamide gel electrophoresis. Only in later experiments, when purified cellulose synthase was treated at 37°C for 15

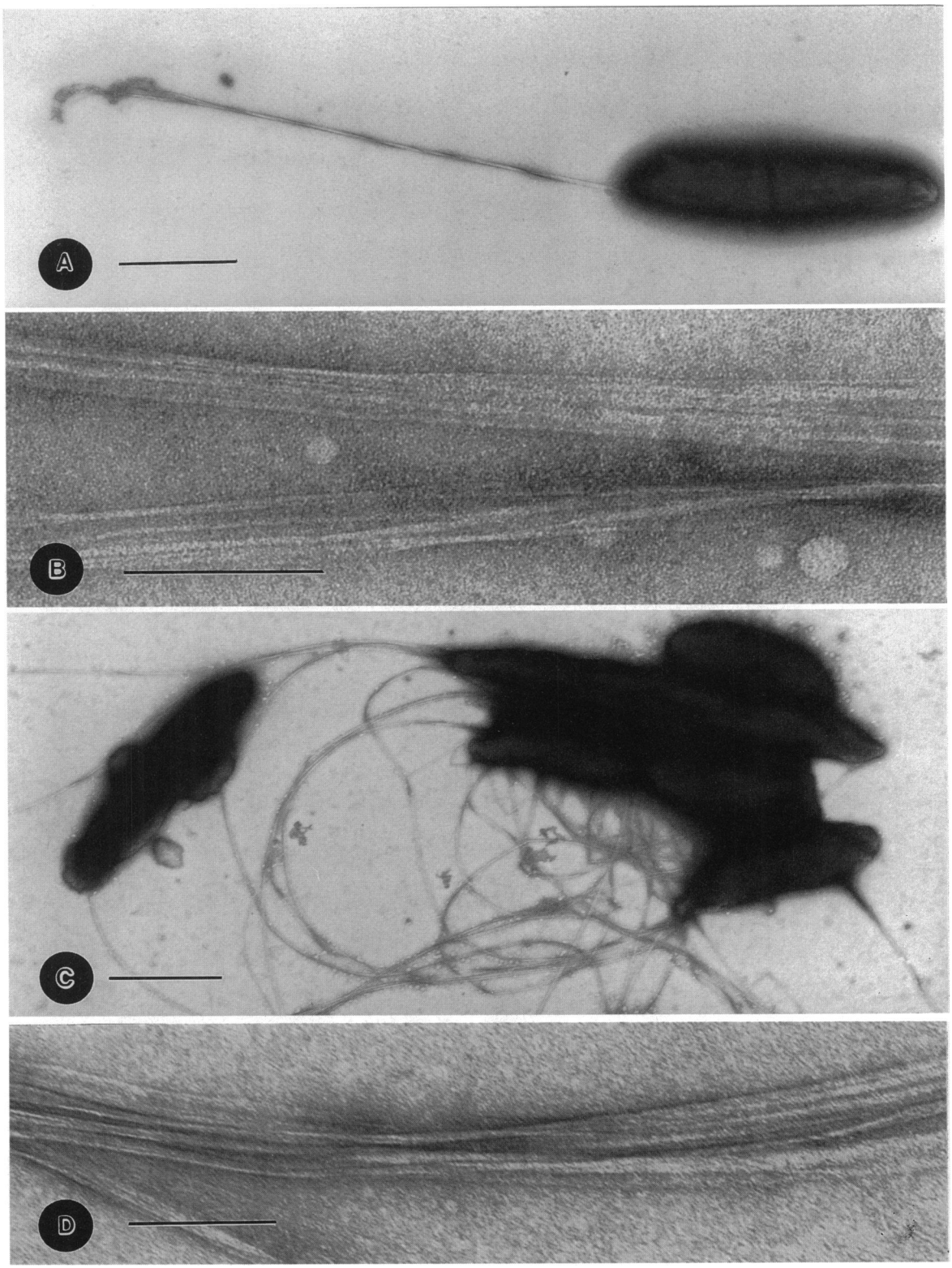

FIG. 7. Production of a typical cellulose ribbon by *A. xylinum* wild-type AY201 (A) and ribbons from AY201-*acsD*::Km cells (C) in static culture. No differences were observed in the cellulose ribbon structure from AY201 wild type (B) and AY201-*acsD*::Km (D) when they were examined by high-resolution negative staining. Scale bars: 1.0 (A), 0.1 (B), 1.0 (C), and 0.1 (D) μm.

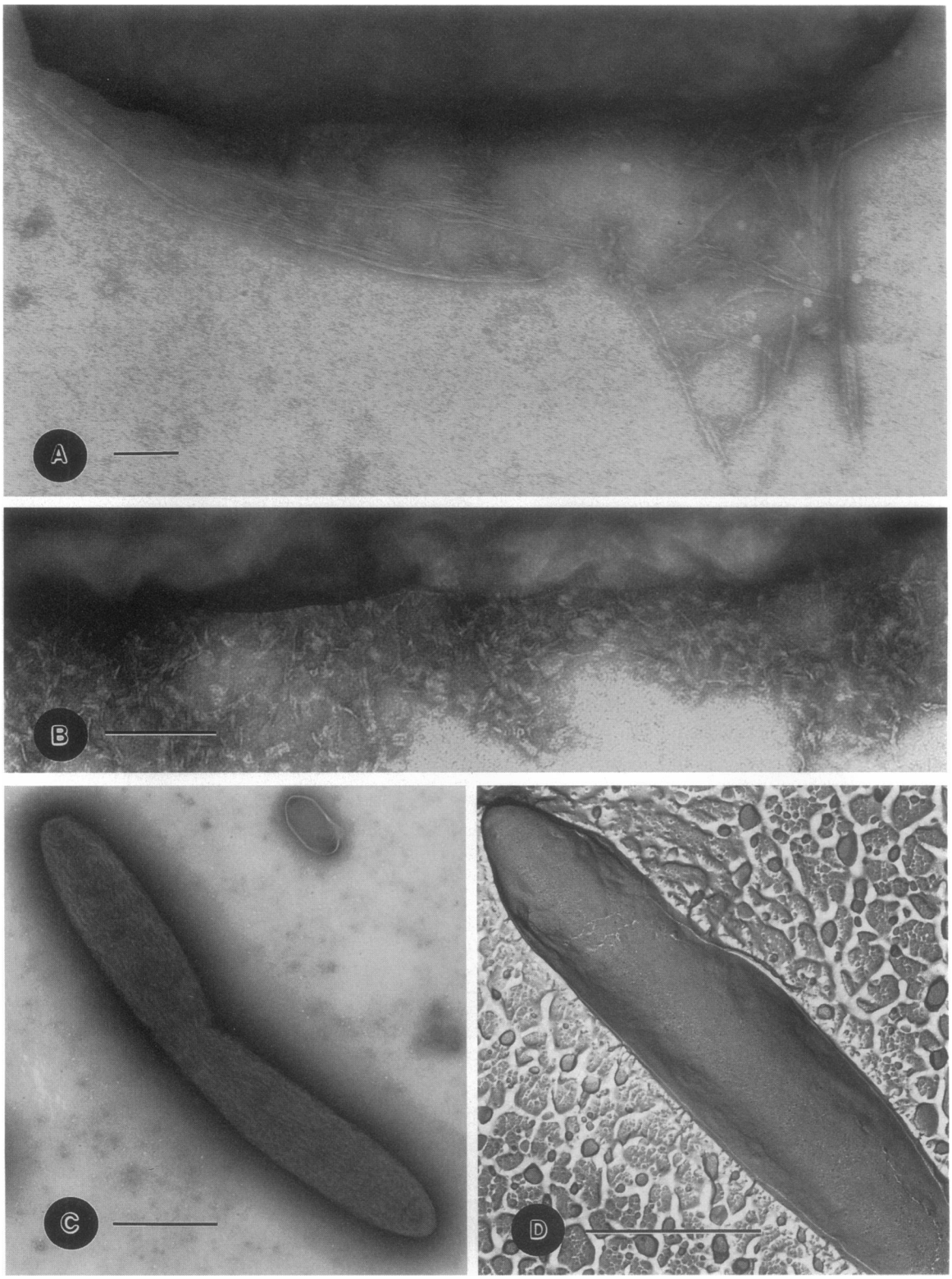

FIG. 8. Production of both allomorphs, corresponding to microfibrils of cellulose I, and rodlets of cellulose II in a single cell of AY201-*acsD*::Km incubated on a grid (A) and of cellulose II in a cell of AY201-*acsD*::Km cultured under agitated conditions (B). A typical mutant (AY201-15-3), in which no cellulose product was observed by negative staining (C), shows no arrangement of intramembranous particles in a freeze-etch replica (D). Scale bars: 0.1 (A), 0.1 (B), 1.0 (C), and 1.0 (D) μm.

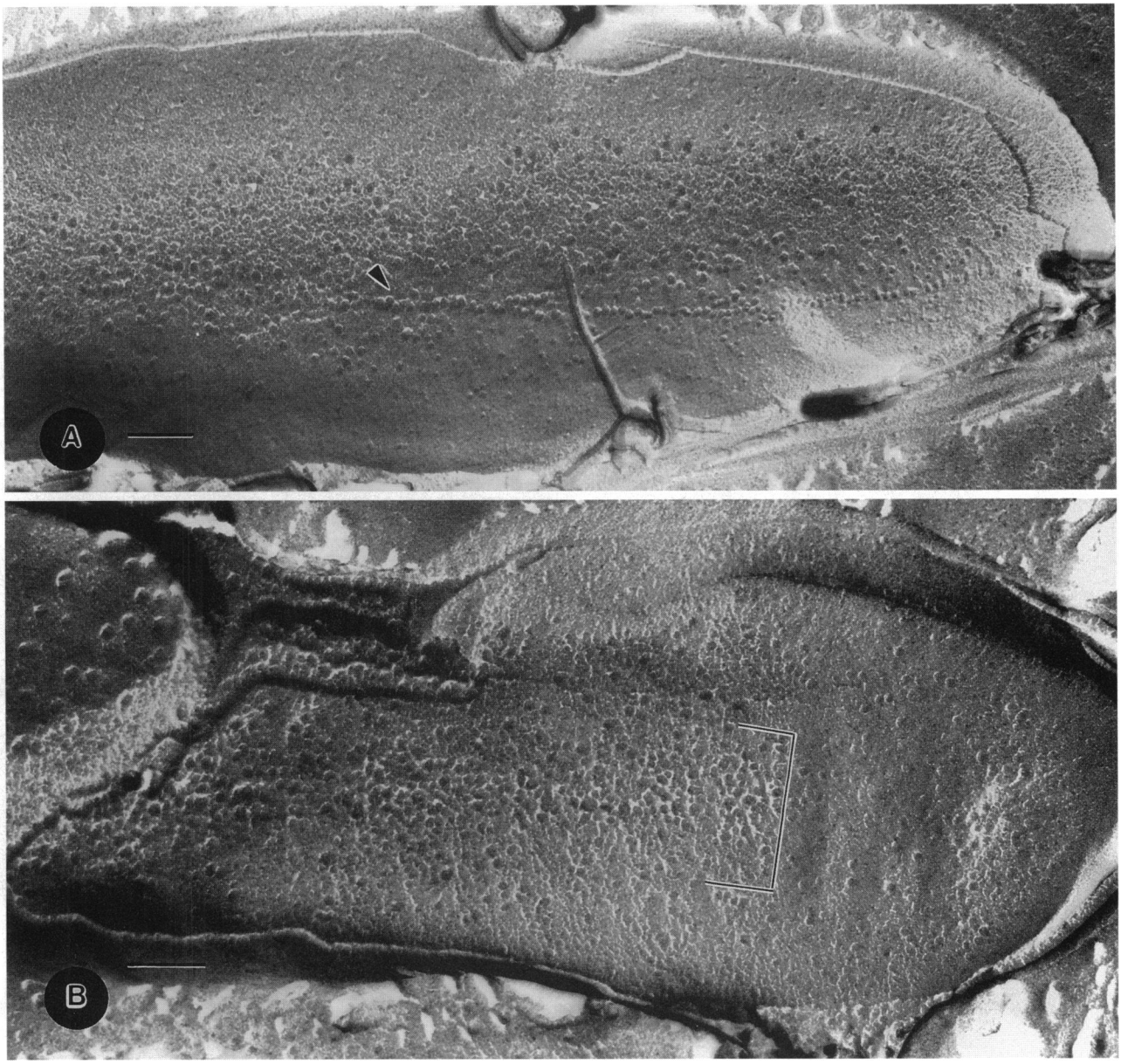

FIG. 9. Freeze-etch replicas of a *A. xylinum* AY201 wild-type cell from a cellulose pellicle showing a linear row of particles (arrowhead) (A) and cell from a colony showing diffuse organization of particles (bracket) (B). Scale bars: 0.1 (A) and 0.1 (B) μm.

min, were both the 83- and the 90-kDa polypeptides observed, and [^{32}P]UDP-glucose was found to bind to the 83-kDa polypeptide (53), confirming our initial results leading to the conclusion that this was the substrate-binding polypeptide (28). In the present study, we have shown that the *acs* operon consists of three genes and that the first gene (*acsAB*) in this operon codes for cellulose synthase. This enzyme is synthesized as a polypeptide with a molecular mass of 168 kDa that carries both the substrate-binding and activator-binding sites. The mechanism by which the 83- and 93-kDa polypeptides are obtained from this large protein is not clear at present.

A search for related proteins showed that the AcsAB polypeptide shared sequence similarities with, among others, the NodC protein (51.8% similarity, 26.5% identity) and the ExoO protein (45.4% similarity, 20.9% identity) from *Rhizobium meliloti* and the HasA protein from group A streptococci (46.1% similarity, 21.8% identity). The *nodC* gene (21) is required for nodulation in all *Rhizobium* species and for synthesis of a nodulation factor such as NodRm-1, a sulfated *N*-acyl-tri-*N*-acetyl-β-1,4-D-glucosamine tetrasaccharide (25) that bears a structural resemblance to chitin (poly-*N*-acetyl-β-1,4-D-glucosamine). The NodC protein showed a 32% identity to chitin synthase III from *Saccharomyces cerevisiae* and on the basis of this homology was suggested to be a glycosyl transferase involved in the synthesis of the NodRm-1 backbone (9). However, no significant homology was observed between the AcsAB polypeptide and the chitin synthases. The ExoO protein from *R. meliloti* (17) is involved in the synthesis of the exopolysaccharide succinoglycan and catalyzes the formation of a β-1,6 glycosidic linkage. HasA has been identified as a hyaluronate synthase in the group A streptococci and is predicted to function in the β-1,4 transfer of *N*-acetylglu-

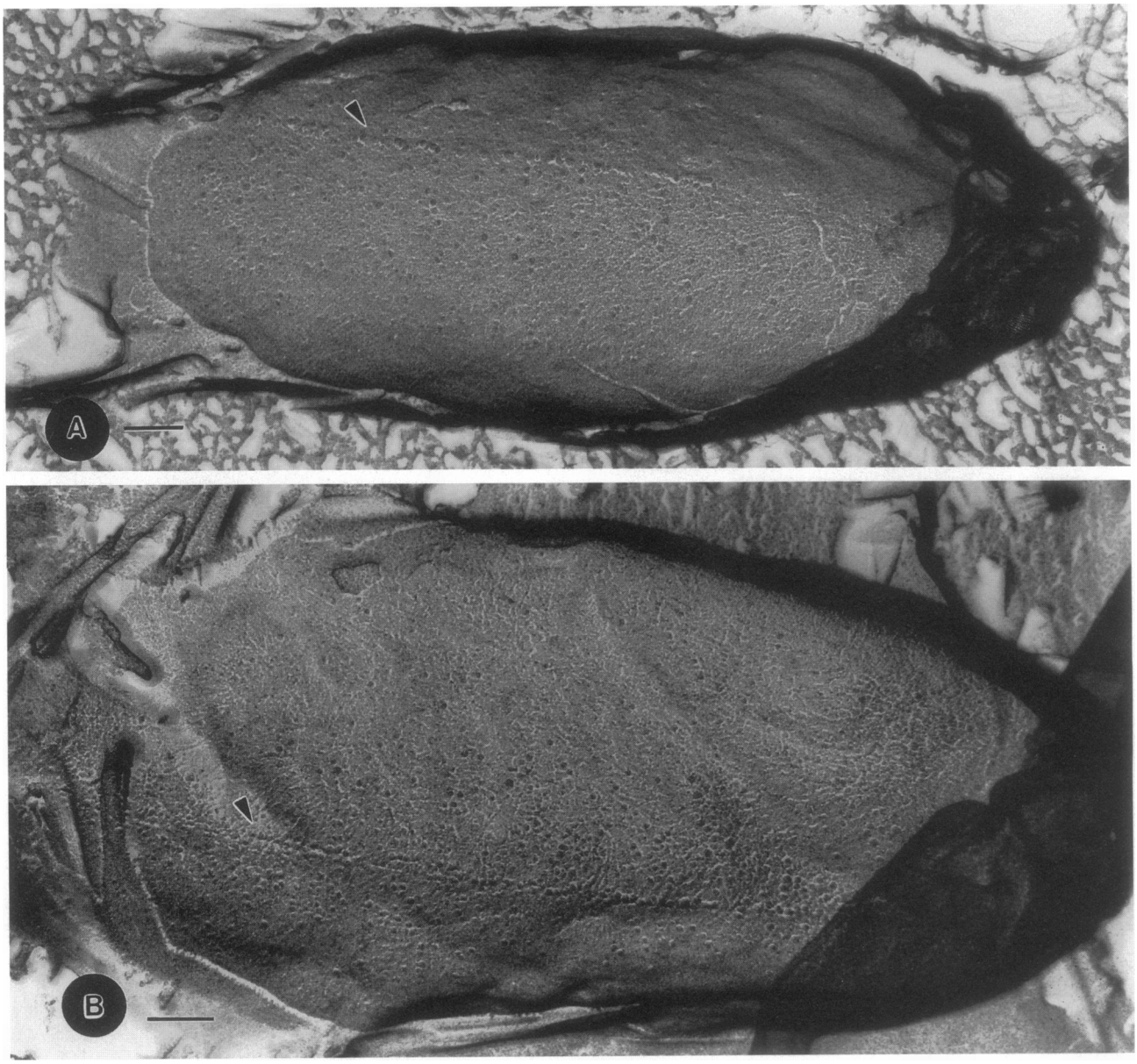

FIG. 10. Freeze-etch replicas of a AY201-*acsD*::Km cell from a cellulose pellicle (A) and a cell of AY201-*acsD*::Km(pIS311-*acsD*) from a cellulose pellicle (B) showing a linear row of particles (arrowheads). Scale bars: 0.1 (A) and 0.1 (B) μm.

cosamine to glucuronic acid during hyaluronate biosynthesis (15). Multiple alignments of these polypeptides with the AcsAB and BcsA polypeptides identify regions of conserved residues present among these various glycosyltransferases that may be involved in formation of β-glycosidic linkages (Fig. 11).

A search of protein databases for identification of sequences homologous to the AcsC polypeptide has provided insight into the role that this polypeptide may have in cellulose assembly. The AcsC polypeptide was found to be homologous to bacterial proteins that are components of membrane channels or pores. Even though pore-like structures have been proposed for the extrusion of the cellulose microfibrils (56) and intramembranous particles have been visualized by freeze-fracture (8), this is the first time that a protein (AcsC polypeptide) has been identified that may have a role in the formation of these structures. The similarity of the AcsC polypeptide with

```
                *   - ---   -       -   -     -  **-
AcsAB   VDIFVPTYNE ELSIVRLTVL GSLGIDWPPE KVRVHILDDG .........R
BcsA    VDIFIPTYDE QLSIVRLTVL GALGIDWPPD KVNVYILDDG .........V
NodC    VDVIVPSFNE DPGILSACLA SIADQDYPGE .LRVYVVDDG SRNREAIVRV
HasA    VAAVIPSYNE DAESLLEILK SVLAQTYPLS ..EIYIVDDG SSNIDAIQLI
ExoO    VTFVVAAYNS ADTIVRA.IE SALAQE..GV TVEVVVVDDC SADAT.....

                                    -       --    --    -  * *
AcsAB   RPEFAAFAAE CGANYIARPT NEHAKAGNLN YAIGHTDGDY ILIFDCDHVP
BcsA    RPEFEQFAKD CGALYIGRVD SSHAKAGNLN HAIKRTSGDY ILILDCDHIP
NodC    RAFYSRDPRF SFILLPE... .NVGKRKAQI AAIGQSSGDL VLNVDSDSTI
HasA    EEYVNREVDI CRNVIVHRSL VNKGKRHAQA WAFERSDADV FLTVDSDTYI
ExoO    .PALVAAIPD PRVRLIA.LD RNRGPGGARN AGIGAARGRW IAVLDSDDIV
```

FIG. 11. Deduced amino acid sequences of the AcsAB, BcsA (52), NodC (21), HasA (15), and ExoO (17) polypeptides were aligned using the GCG program PILEUP, and a region of conserved residues is shown. The amino acids displayed correspond to residues 151 to 241 of AcsAB, 151 to 241 of BcsA, 51 to 145 of NodC, 66 to 163 of HasA, and 12 to 101 of ExoO. Positions with four identical amino acids are denoted by dashes, while those with five identical amino acids are denoted by asterisks.

proteins involved in the secretion of macromolecules from bacteria suggests that the cellulose-synthesizing and extrusion complex has functions other than the polymerization of glucans.

The concept that polymerization and crystallization are separate but tightly coupled reactions has been used to suggest that cell-directed assembly may mediate the production of cellulose ribbons (19). The rate-limiting step has been proposed to be the crystallization step (2). In spite of these interesting observations, no mention has been made of the relationship between the cellulose product and how it could directly or indirectly control its rate of synthesis. Is it produced by the nature of the crystalline allomorph? When the AcsD polypeptide is not present, as in AY201-*acsD*::Km, cells produce much smaller quantities of cellulose, and the crystalline structure of the product is influenced by the conditions during synthesis. Although an explanation for such a behavior is not readily available, a common mechanism for production of the two cellulose allomorphs in AY201-*acsD*::Km under different conditions may be related to the direct effects of the product on the cellulose-synthesizing machinery. Such conditions would arise in which a random crystallization and/or distribution of the exiting polymer product could change the conformation of the synthesizing complex. In this scenario, the *acsD* gene product may have a major role in the perfection of crystallization. From these observations, we believe that the AcsD polypeptide is involved either in the pore structure itself or in the organization of the pores within the linear array of TCs. In the absence of the AcsD polypeptide, the pore structure is present and capable of normal ribbon synthesis; however, the pore may be much more sensitive to the exiting cellulose product. Here, the random crystallization of nascent glucan chains into cellulose II could effectively clog the pore so that further polymerization becomes restricted. This influence could be exerted by regulating the activity of cellulose synthase inside the cell. In *A. xylinum*, cellulose synthase activity is regulated by the activator c-di-GMP, the presence of which inside the cell must be under a strict control. The conditions that regulate the level of c-di-GMP in *A. xylinum* have not been fully ascertained (40). However, under certain conditions, the cellulose product, which has not been extruded during synthesis and which blocks or occludes the cellulose-extrusion pores, may regulate the c-di-GMP level by altering the activities of the membrane-associated diguanylate cyclase and regulatory phosphodiesterases that constitute the c-di-GMP regulatory system. Alternatively, the cellulose product could directly affect the polymerization reaction in a similar manner by influencing the activity of the membrane-localized cellulose synthase.

The present study provides evidence that the organization of the cellulose-synthesizing complexes governs the morphology of the cellulose product and that the product directly or indirectly influences its own synthesis at the polymerization step. As the cellulose microfibrils integrate into a ribbon, the entire structure becomes more rigid and itself can more effectively maintain the alignment of the TCs. This frequently is observed in the organized row of TC structure in cells of wild-type *A. xylinum* AY201 (Fig. 9A) and AY201-*acsD*::Km (Fig. 10A) from pellicles obtained during static culture conditions; however, this organization is significantly altered in wild-type cells from colonies in which, in general, rows are apparent, but they are much more diffuse (Fig. 9B). This suggests that at the time of freezing for analysis, cells in colonies of the wild-type strain that are fully capable of synthesizing cellulose ribbons have a diffuse organization of the cellulose-synthesizing complexes and that this organization could become pronounced as a result of ribbon formation. A diffuse TC structure is also observed in a strain of *A. xylinum* which produces cellulose II (23).

Implications for cellulose crystallization may give insight into the evolutionary history of cellulose biogenesis among primitive organisms, as reflected in the distribution of this process among extant living organisms. For example, a more primitive bacterium of the genus *Sarcina* synthesizes only cellulose II in vivo, whereas representatives of the gram-negative purple bacteria belonging to the genera *Rhizobium*, *Alcaligenes*, *Agrobacterium*, and *Acetobacter* normally synthesize the cellulose I allomorph (6). This implies that perhaps during cellular evolution, these latter organisms acquired additional mechanisms, among which was the facilitation of cellulose I assembly. Since this allomorph is a metastable product, one which is less thermodynamically favorable, the natural selection process may have directed the organization of the synthetic machinery to favor extended glucan chain crystallization. The selective advantage of producing microfibrils or ribbons composed of cellulose I may have included a rapid cellular translocation to provide more nutrients or to secure a protective environment for growth and division. Such a selective advantage may not be provided by the cellulose II allomorph. This form of cellulose is produced only in rare instances and in highly reduced amounts, especially under conditions of stress. Thus, cellulose production may be seen as a mechanism whereby *A. xylinum* cells sense the environment through a directed crystallization of their major metabolic product, cellulose.

ACKNOWLEDGMENTS

We thank Richard Santos for computer assistance and for preparing the plates.

This work was supported by USDA grant 93-37301-9303 to R.M.B. and I.M.S.

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. **Benziman, M., C. H. Haigler, R. M. Brown, Jr., A. R. White, and K. M. Cooper.** 1980. Cellulose biogenesis: polymerization and crystallization are coupled processes in *Acetobacter xylinum*. Proc. Natl. Acad. Sci. USA **77**:6678–6682.
3. **Bio-Rad Laboratories.** 1993. Gene pulser electroprotocols. Bio-Rad Laboratories, Hercules, Calif.
4. **Birnboim, H. C.** 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100**:243–255.
5. **Brown, R. M., Jr.** 1985. Cellulose microfibril assembly and orientation: recent developments. J. Cell Sci. Suppl. **2**:13–32.
6. **Brown, R. M., Jr.** 1990. Algae as tools in studying the biosynthesis of cellulose, nature's most abundant macromolecule, p. 20–39. *In* W. Wiessner, D. G. Robinson, and R. C. Starr (ed.), Cell walls and surfaces, reproduction, and photosynthesis. Springer-Verlag, Berlin.
7. **Brown, R. M., Jr., C. H. Haigler, J. Suttie, A. R. White, E. Roberts, C. Smith, T. Itoh, and K. Cooper.** 1983. The biosynthesis and degradation of cellulose. J. Appl. Poly. Sci. **37**:33–78.
8. **Brown, R. M., Jr., J. H. M. Willison, and C. L. Richardson.** 1976. Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the *in vivo* process. Proc. Natl. Acad. Sci. USA **73**:4565–4569.
9. **Bulawa, C. E., and W. Wasco.** 1991. Chitin and nodulation. Nature (London) **353**:710.
10. **Bureau, T. E., and R. M. Brown, Jr.** 1987. *In vitro* synthesis of cellulose II from a cytoplasmic membrane fraction of *Acetobacter xylinum*. Proc. Natl. Acad. Sci. USA **84**:6985–6989.
11. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
13. **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.
14. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
15. **Dougherty, B. A., and I. van de Rijn.** 1994. Molecular characterization of *hasA* from an operon required for hyaluronic acid synthesis in group A streptococci. J. Biol. Chem. **269:**169–175.
16. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
17. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*. J. Bacteriol. **175:**7033–7044.
18. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J. Bacteriol. **175:**7045–7055.
19. **Haigler, C. H., R. M. Brown, Jr., and M. Benziman.** 1980. Calcofluor White ST alters *in vivo* assembly of cellulose microfibrils. Science **210:**903–906.
20. **Harding, N. E., J. M. Cleary, D. K. Cabanas, R. G. Rosen, and K. S. Kang.** 1987. Genetic and physical analyses of a cluster of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. J. Bacteriol. **169:**2854–2861.
21. **Jacobs, T. W., T. T. Egelhoff, and S. R. Long.** 1985. Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. J. Bacteriol. **162:**469–476.
22. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815:**468–476.
23. **Kuga, S., S. Takagi, and R. M. Brown, Jr.** 1993. Native folded-chain cellulose II. Polymer **34:**3293–3297.
24. **Leigh, J. A., and D. L. Coplin.** 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. **46:**307–346.
25. **Lerouge, P., P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Prome, and J. Denarie.** 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature (London) **344:**781–784.
26. **Lessl, M., D. Balzer, W. Pansegrau, and E. Lanka.** 1992. Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. J. Biol. Chem. **267:**20471–20480.
27. **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.
28. **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.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Manoil, C.** 1991. Analysis of membrane protein topology using alkaline phosphatase and β-galactosidase gene fusions. Methods Cell Biol. **34:**61–75.
31. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
32. **Manoil, C., J. J. Mekalanos, and J. Beckwith.** 1990. Alkaline phosphatase fusions: sensors of subcellular location. J. Bacteriol. **172:**515–518.
33. **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.
34. **Miller, H.** 1987. Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. Methods Enzymol. **152:**145–170.
35. **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.
36. **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.
37. **Qiagen, Inc.** 1992. Qiagen plasmid handbook. Qiagen Inc., Chatsworth, Calif.
38. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:**7207–7221.
39. **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.
40. **Ross, P., R. Mayer, and M. Benziman.** 1991. Cellulose biosynthesis and function in bacteria. Microbiol. Rev. **55:**35–58.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
42. **Saxena, I. M., and R. M. Brown, Jr.** Unpublished results.
43. **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.
44. **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.
45. **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.
46. **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.
47. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
48. **Taylor, R. K., C. Manoil, and J. J. Mekalanos.** 1989. Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171:**1870–1878.
49. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1988. Characterization of the virB operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **263:**5804–5814.
50. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1990. Characterization of the virB operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **265:**4768.
51. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA **90:**2970–2974.
52. **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.
53. **Wong, H. C., A. L. Fear, R. D. Calhoon, G. H. Eichinger, R. Mayer, P. Ross, M. Benziman, A. Ben-Bassat, and R. Tal.** 1991. Genetic organization and regulation of the cellulose synthase operon in *Acetobacter xylinum*, abstr. 12. Abstr. Cellulose '91. American Chemical Society, Washington, D.C.
54. **Yamanaka, S.** 1989. Production and application of bacterial cellulose, p. 175–181. *In* H. Inagaki and G. O. Phillips (ed.), Cellulosics utilization—research and rewards in cellulosics. Elsevier Applied Science, London.
55. **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.
56. **Zaar, K.** 1979. Visualization of pores (export sites) correlated with cellulose production in the envelope of the gram negative bacterium *Acetobacter xylinum*. J. Cell Biol. **80:**773–777.