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Genetics

Genetic organization of the cellulose synthase operon in *Acetobacter xylinum*

(cellulose biogenesis/cloning/sequencing)

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ABSTRACT **An operon encoding four proteins required for bacterial cellulose biosynthesis (*bcs*) in *Acetobacter xylinum* was isolated via genetic complementation with strains lacking cellulose synthase activity. Nucleotide sequence analysis indicated that the cellulose synthase operon is 9217 base pairs long and consists of four genes. The four genes—*bcsA*, *bcsB*, *bcsC*, and *bcsD*—appear to be translationally coupled and transcribed as a polycistronic mRNA with an initiation site 97 bases upstream of the coding region of the first gene (*bcsA*) in the operon. Results from genetic complementation tests and gene disruption analyses demonstrate that all four genes in the operon are required for maximal bacterial cellulose synthesis in *A. xylinum*. The calculated molecular masses of the proteins encoded by *bcsA*, *bcsB*, *bcsC*, and *bcsD* are 84.4, 85.3, 141.0, and 17.3 kDa, respectively. The second gene in the operon (*bcsB*) encodes the catalytic subunit of cellulose synthase. The functions of the bcsA, bcsC, and bcsD gene products are unknown. Bacterial strains mutated in the *bcsA* locus were found to be deficient in cellulose synthesis due to the lack of cellulose synthase and diguanylate cyclase activities. Mutants in the *bcsC* and *bcsD* genes were impaired in cellulose production *in vivo*, even though they had the capacity to make all the necessary metabolic precursors and cyclic diguanylic acid, the activator of cellulose synthase, and exhibit cellulose synthase activity *in vitro*. When the entire operon was present on a multicopy plasmid in the bacterial cell, both cellulose synthase activity and cellulose biosynthesis increased. When the promoter of the cellulose synthase operon was replaced on the chromosome by *E. coli tac* or *lac* promoters, cellulose production was reduced in parallel with decreased cellulose synthase activity. These observations suggest that the expression of the *bcs* operon is rate-limiting for cellulose synthesis in *A. xylinum*.**

Cellulose is the most abundant biopolymer in nature and is an indispensable raw material for many industries. In spite of the importance of cellulose, its mechanism of biosynthesis is still poorly understood (1). Since the bacterium *Acetobacter xylinum* synthesizes a cellulose that is structurally similar to the cellulose produced by plants, it has been a model system of choice for the study of cellulose biogenesis.

The enzymatic pathway for cellulose synthesis in *A. xylinum* has been extensively investigated and four essential enzymatic steps have been identified (2). Glucose is transported through the bacterial membrane and phosphorylated to glucose 6-phosphate by glucose kinase. Glucose 6-phosphate is isomerized by phosphoglucomutase to glucose 1-phosphate. Glucose 1-phosphate is converted to uridine 5′-diphosphate glucose (UDPG) by UDPG pyrophosphorylase. Finally, UDPG is polymerized to cellulose by cellulose synthase.

Cellulose synthesis is an energy-consuming and nonreversible process. Cellulose synthase is the only enzyme known to be unique to the cellulose synthetic pathway. Therefore, one would expect that cellulose synthase activity is under strict regulatory control. It is known that cellulose synthase is specifically activated by the unique nucleotide cyclic diguanylic acid (3). This nucleotide is synthesized from GTP by diguanylate cyclase and is degraded by phosphodiesterases A and B (3). It is likely that cellulose synthase is further regulated at the genetic level.

In this paper, we describe the cloning, sequencing, and characterization of the cellulose synthase operon as a necessary step toward a better understanding of the regulation of cellulose production in *A. xylinum*.‖

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains, Plasmids, and Media. *A. xylinum* 1306-3 is an isolate from *A. xylinum* B42 (North Regional Research Laboratories, Peoria, IL). *A. xylinum* 824 was obtained from the Cetus Master Culture Collection. *A. xylinum* 1306-21 is a glucose dehydrogenase mutant of 1306-3 obtained through ethyl methanesulfonate mutagenesis (4).

Escherichia coli MM294 (*thi-1*, *hsdR17 endA1 supE44*), DG98 [*thi-1*, *hsdR17 endA1 supE44 lacIq lacZ* M15 *proC*::Tn*10*(F′ *lacIq lacZ* M15 *proC*$^+$)], and K802 *recA* (*lacY1 sup E44 galK galT22 rtbD1 metB1 hsdR2*) (5) were kindly provided by D. Gelfand (Cetus). Bacteriophage M13mp10 and M13mp11 and plasmids pUC18, pBR322, pACYC184, pKT230, and pRK2013 have been described (6, 7). The cosmid pKT230cos5 was constructed by cloning the 1.85-kilobase (kb) *Bgl* II fragment carrying the λ cos site from plasmid pVK100 (8) into the *Bam*HI site of pUC19. The cos site was then reisolated as an *Sma* I/*Hin*dIII fragment and subcloned into the *Sma* I/*Hin*dIII sites of pKT230. Plasmids from *E. coli* and double-stranded DNA of M13 phages were prepared according to Katz *et al.* (9). Plasmids from *A. xylinum* were purified as described (10).

The R-70-2 medium contains 7.3 mM KH_2PO_4 (pH 5.0), 25 mM $(NH_4)_2SO_4$, 4.0 mM sodium citrate, 1.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_3$, 0.001 mM Na_2MoO_4, 0.005 mM $ZnSO_4$, 0.005 mM $MnSO_4$, 0.001 mM $CuSO_4$, 0.001 mM $CoCl_2$, 0.001 mM $NiCl_2$, and 2% or 4% (wt/vol) glucose. The R-20 medium contains 5 g of Na_2HPO_4 (pH 5.0) per liter, 5 g of Bacto-peptone per liter, 5 g of yeast extract per liter, 1.15 g of citric acid per liter, and 2% (wt/vol) glucose.

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Abbreviation: UDPG, uridine 5′-diphosphate glucose.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37202).

Chromosomal DNA Isolation, Gene Bank Construction, and Conjugation. High molecular weight chromosomal DNA from *A. xylinum* 1306-3 was partially digested with *Sau*3A and fragments of ≈30 kb were isolated from a sucrose gradient. Approximately 1 μg of 27- to 38-kb DNA was ligated into *Bam*HI-cleaved and dephosphorylated pKT230cos5 vector, and packaged into λ phage particles as described (11). The phage particles were used to infect *E. coli* K802 *recA*. Cosmids from the individual transformants were transferred into *A. xylinum* via conjugation. Conjugation was performed on an agar surface essentially as described (12) except that mobilization of the cosmid was provided by an *E. coli* strain host carrying the helper plasmid pRK2013.

Primer Extension and Nucleotide Sequence Analysis. Total cellular RNA from *A. xylinum* cells was extracted and purified as described (13). Primer extension was carried out by the procedure of Jones *et al.* (14) with the oligonucleotide-containing sequence 5′-TGCGCGATAAGTGCACA-3′. Sequences of both strands of an 11-kb region containing the *bcs* operon were determined by the chain-termination method (15) with specifically synthesized primers.

Promoter Replacement and Gene Disruption in *A. xylinum*. A 2.5-kb *Hin*dIII restriction fragment containing the *bcs* operon promoter and its flanking regions was cloned into M13mp10. An *Sst* I/*Sma* I/*Bam*HI linker was then introduced into the operon promoter 59 base pairs (bp) 3′ of the transcriptional initiation site by site-specific mutagenesis (6). This mutated fragment was subcloned into the *Hin*dIII sites of pACYC184 to construct the plasmid pACYC184:Pcs-10.

The *E. coli tac* and *lac* promoter fragments were synthesized with an *Eco*RI site at the 5′ end and *Bam*HI/*Hin*dIII at the 3′ end. Both synthetic fragments carry sequence from nucleotide −45 to nucleotide +3 of its corresponding promoter. These fragments were cloned into the *Eco*RI/*Hin*dIII site of pALF20, a derivative of pBR322 with the *Alw*NI site changed to an *Sst* I site. The *Sst* I/*Bam*HI fragments containing the promoter and the β-lactamase genes from the resultant plasmids were subcloned into the *Bam*HI/*Sst* I site of pACYC184:Pcs-10. The resultant plasmids were digested with *Xba* I and the linearized fragments were used to transform *A. xylinum* 1306-21. Ampicillin-resistant transformants were isolated and their chromosomal DNA was used in Southern blot analysis to confirm the identity of the transformants. The ampicillin-resistant colonies that were obtained reflect a recombination between the plasmid's cellulose synthase regions, which flanked the marker–promoter sequence, and homologous regions in the chromosome. Since the heterologous promoter precedes the ribosome binding site of the *bcsA* gene, the transcription of the *bcs* operon was under the control of the inserted promoter.

To construct a *bcsD* mutant, a 3.7-kb *Sma* I/*Bam*HI fragment carrying the 3′ portion of *bcsC* and the entire coding region of *bcsD* was cloned into the *Eco*RV/*Bam*HI site of pACYC184. This plasmid was designated pALF23. The Tn*3* β-lactamase gene was then isolated as an *Eco*RI/*Alw*NI fragment from pBR322. The ends of the fragment were repaired and it was subcloned into the *Eco*RV site of pALF23. The resultant plasmid was digested with *Xba* I and the linearized plasmid was used to transform *A. xylinum* 1306-21.

Other methods used for DNA manipulations were described by Sambrook *et al.* (11).

Transformation of *A. xylinum*. Cells were grown in R20-2 at 30°C with shaking in the presence of 0.1% (vol/vol) cellulase. When the cultures reached midlogarithmic phase growth (OD_{660} = 0.5), cells were harvested, extensively washed with 10% (vol/vol) glycerol, concentrated 300-fold in 10% (vol/vol) glycerol, and stored frozen at −70°C until use. They were thawed on ice and mixed with DNA in a 2-mm Bio-Rad electroporation cuvette. Electroporation was carried out at a field force of 9.0 kV and a pulse duration of 25 μF/750 Ω. The transformed cells were grown in R20-2 medium for 3 hr before they were plated on selective plates. Plasmid DNA for electroporation was purified by CsCl ultracentrifugation and extensively dialyzed against distilled water before use.

Assay for Cellulose Production and Cell Growth. Cells were grown in R70-2 medium containing 10 μM $FeCl_3$, 25 mM 3,3-dimethylglutaric acid (pH 5.0), 2% (wt/vol) corn steep liquor, and 1% (wt/vol) fructose (for strain 1306-3) or 1% (wt/vol) glucose (for strain 1306-21). Cell growth and cellulose production were analyzed after 3 days of incubation when all the carbon source was consumed. The cultures (25 ml) were harvested by centrifugation, washed with saline twice, and resuspended in 15 ml of 0.1 M NaOH and incubated at 60°C with mild agitation for 60 min. The heat-treated samples were centrifuged. The concentration of protein in the supernatants was determined and used to calculate the cell concentration in the culture assuming a cell with 65% protein content. The dry weight of the precipitate represents the amounts of cellulose in the sample.

Purification and N-Terminal Amino Acid Sequencing of Cellulose Synthase. Exponentially growing cells were broken in a French press and the membrane fraction was isolated as described (16). The membrane preparation [10 mg of cells (dry wt) per ml] was then treated with trypsin (80 μg/ml) in buffer containing 100 mM Tris·HCl (pH 8.0), 20% (wt/vol) sucrose at 4°C for 1 hr. The digestion was terminated by addition of 80 μg of trypsin inhibitor per ml. The trypsin-treated membranes were then solubilized in 2% (wt/vol) digitonin by sonication at 4°C for 2 min. Cellulose synthase was further purified from the soluble proteins by entrapment in cellulose as adapted from the procedure developed for chitin synthase (17). The entrapped enzyme was subjected to SDS/PAGE analysis (18). After Coomassie staining, the major bands were cut out and eluted from the gel. N-terminal amino acid sequences were determined by the Edman degradation method (19).

Enzymatic Assay. Exponentially growing cultures were harvested and passed through cheesecloth to remove the cellulose from the cells. The cells were washed twice in 50 mM KH_2PO_4 buffer (pH 6.0), resuspended to 20 mg (dry wt) per ml and sonicated in the appropriate assay buffers. The cellulose synthase, diguanylate cyclase, phosphoglucomutase, and UDPG pyrophosphorylase were assayed as described (3, 16, 20). Cyclic diguanylic acid was prepared as described by Ross *et al.* (3).

RESULTS

Isolation and Characterization of *A. xylinum* Strains Deficient in Cellulose Synthesis. Cellulose-producing (Cel^+) *A. xylinum* strains form small rough and dry colonies, whereas cellulose nonproducers (Cel^-) form large flat and shiny colonies on agar plates. This phenotypic difference permits isolation of Cel^- mutants from *A. xylinum* strain 1306-3 after mutagenesis with ethyl methanesulfonate. These Cel^- mutants can be biochemically classified into five groups. Group I mutants are deficient in one of the enzymes involved in the conversion of glucose to UDPG. The group II and group III mutants lack the cellulose synthase or diguanylate cyclase activities, respectively. Group IV mutants are defective in both diguanylate cyclase and cellulose synthase activities. The group V mutants have normal levels of all the known enzymes involved in the conversion of glucose to cellulose. Thus, the mutants in this group are deficient in the gene(s) that is essential for cellulose synthesis *in vivo* but that remains undefined. One of the group II mutants, 1306-24, was host in the genetic complementation experiments used to isolate the cellulose synthase operon.

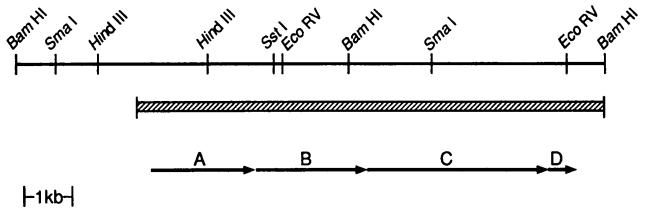

FIG. 1. Physical map of the *bcs* locus. The extent of the coding regions of the *bcs* genes and their transcription direction are indicated by the arrows. Hatched bar indicates the sequenced region.

Cloning of the Cellulose Synthase Operon. The broad host range plasmid pKT230 (7) was found to be stably maintained in *A. xylinum* (data not shown). We constructed an *A. xylinum* gene library in a cosmid vector derived from the plasmid pKT230 in *E. coli*. A total of 2010 individual streptomycin-resistant transformants were picked and their recombinant cosmids were mobilized with the helper plasmid pRK2013 and transferred into the cellulose synthase-deficient mutant 1306-24 via the tripartite mating method. Cosmid T19G9 was found to restore the Cel^+ phenotype of strain 1306-24. A physical map of the region containing the *bcs* operon on the *A. xylinum* chromosome was constructed (Fig. 1) based on restriction analysis of this cosmid. The molecular organization of the *bcs* locus was also confirmed by genomic Southern analysis (data not shown).

Nucleotide Sequence of Cellulose Synthase Operon. Cosmid DNA recovered from the Cel^+ transconjugate of strain 1306-24 was significantly smaller than the parental cosmid. This cosmid, designated T19G9 106, still retained its ability to restore the Cel^+ phenotype of strain 1306-24. This indicated that the cosmid T19G9 106 contained the *bcs* gene(s) even though a significant amount of *Acetobacter* DNA was deleted from the parental plasmid. Southern analysis (data not shown) indicated that the insert in cosmid T19G9 106 spanned the 7.2- and 5.5-kb *Bam*HI fragments shown in Fig. 1. Therefore, we believed that the 7.2- and 5.5-kb *Bam*HI restriction fragments contained the *bcs* genes. Approximately 11 kb of nucleotide sequence in this region was determined. (A copy of the sequence will be provided upon request.) A schematic representation of the sequence data for the *bcs* operon is shown in Fig. 2.

There are four open reading frames in the sequenced region. They are 2262, 2406, 3957, and 468 bp long and they encode four proteins of 84.4, 85.3, 141, and 17.3 kDa. To examine whether these genes are transcribed as a polycistronic mRNA, we used the primer-extension method to identify the transcriptional initiation site of the *bcs* genes. Total RNA was isolated from exponentially growing cultures of *A. xylinum* 1306-3 and used as a template for the primer-extension reactions. The 17-mer oligonucleotide, used as the primer in these reactions, contained the sequence complementary to the *bcsA* coding strand from nucleotides 453–436

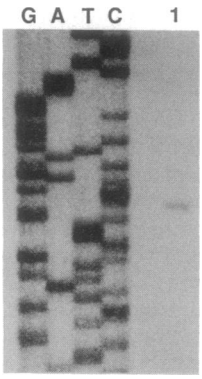

FIG. 3. Transcription initiation site of the *bcs* operon. RNA was extracted from *A. xylinum* 1306-3 and hybridized with the ^{32}P-labeled extension primer. cDNA was then synthesized with the avian myeloblastosis virus reverse transcriptase and the four deoxyribonucleotide triphosphates. The length of the extension product was measured against the terminated products produced in DNA sequencing reactions using the same primer. G, A, T, and C indicate the individual sequencing reactions.

shown in Fig. 2. The analysis indicated that transcription of the operon started at the nucleotide 97 bp 5′ of the translation initiation codon of the *bcsA* gene (Figs. 2 and 3). The absence of an intergenic region is consistent with a single initiation site.

The *bcs* Operon Genes Are Essential for Cellulose Synthesis *in Vivo*. We used genetic complementation and gene disruption to examine whether these genes were required for *in vivo* cellulose synthesis.

Genetic complementation in *Acetobacter* was facilitated by our discovery that *A. xylinum* could be transformed by DNA via electroporation and by the isolation of a small *Acetobacter* plasmid. A 3.6-kb cryptic plasmid with a unique *Sst* I site was isolated from *A. xylinum* strain 824. Two *Acetobacter–E. coli* shuttle plasmids, designated as pUC18-824 and pUC19-824, were constructed by ligating this cryptic plasmid with the *E. coli* plasmids pUC18 and pUC19 at the *Sst* I site. These shuttle plasmids confer *A. xylinum* ampicillin resistance and also serve as expression vectors due to the presence of the *E. coli lac* promoter. The *E. coli lac* promoter was previously shown to be functional in *Acetobacter* (21). A gene cloned into the linker region of pUC18-824 or pUC19-824 downstream from the *lac* promoter will be actively transcribed in *A. xylinum*.

For the genetic complementation tests, we subcloned the *bcs* genes individually into the linker region of the shuttle plasmid pUC18-824 or pUC19-824 in *E. coli*. These plasmids were then introduced into the five groups of Cel^- mutants of *A. xylinum*. The Cel^+ phenotype of the group II, group IV, and group V mutants was restored only by plasmids carrying the *bcsB*, *bcsA*, and *bcsC* fragments, respectively. Since the group II mutants were deficient in cellulose synthase activity and were complemented by the *bcsB* gene, we believe that *bcsB* encodes the catalytic subunit of the cellulose synthase. All of our group V mutants could be complemented by the *bcsC* but not by the *bcsD* gene. Thus, the group V mutants are deficient in *bcsC*. Interestingly, the group IV mutants, which were deficient in both the cellulose synthase and diguanylate cyclase activities and originally classified as

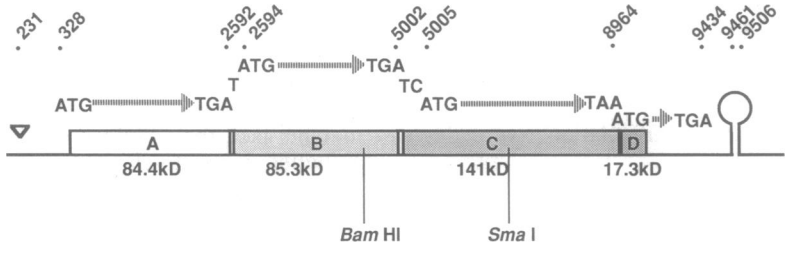

FIG. 2. Summary of the nucleotide sequence analysis of the *bcs* operon and its flanking regions. Boxes A–D represent the coding regions of the *bcs* genes. Arrowhead and stem–loop structure indicate the transcription initiation site and the putative transcription terminator, respectively. ATG, TGA, and TAA represent the translation initiation or termination codons of the respective genes. The size of each gene product is indicated under its coding region. The numbers above the figure represent the nucleotide position(s) of the transcriptional initiation site, the translational initiation and termination codons, and the transcriptional terminator within the sequenced region.

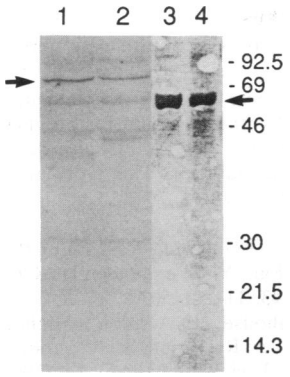

FIG. 4. Immunoblotting analysis of the bcsB and diguanylate cyclase proteins *in vivo*. Cellular extracts prepared from *A. xylinum* 1306-42 (a *bcsA* mutant; lanes 1 and 3) and 1306-21 (the wild-type strain; lanes 2 and 4) were fractionated by SDS/PAGE, transferred to nitrocellulose paper, and probed with the antiserum raised against the purified bcsB peptide (lanes 1 and 2) or the diguanylate cyclase (lanes 3 and 4). The immunoreactions were visualized by the horseradish peroxidase/ TMB/dextran sulfate method (22). Arrows indicate the position of the bcsB protein or diguanylate cyclase in the gel. Protein marker sizes (kDa) are shown on the right.

double mutants, were complemented by the *bcsA* gene alone. Immunoblotting indicated that the *bcsA* mutants produced normal levels of diguanylate cyclase and bcsB protein (Fig. 4). This implies that these two proteins were in an inactive form in the *bcsA* mutants.

To demonstrate whether the *bcsD* is important for cellulose synthesis, we disrupted the *bcsD* on the bacterial chromosome. The *bcsD* mutant was found to have a partial Cel^+ phenotype on agar plates. This mutant produced ≈40% less cellulose than its parental strain in agitated cultures. This result demonstrated that *bcsD* is required for maximal production of cellulose.

The *bcsB* Gene Encodes the Catalytic Subunit of the Cellulose Synthase. To confirm that the *bcsB* gene indeed encodes the catalytic subunit of cellulose synthase, we purified sufficient amounts of this enzyme for amino acid sequence analysis. Purification of the catalytic subunit of cellulose synthase was achieved by trypsin treatment of cell membrane followed by enzyme–product entrapment. Two major bands of ≈91 and ≈67 kDa were obtained. The sequence of the first 18 amino acids of the 91-kDa protein was determined. It is identical to the deduced amino acid sequence of bcsB starting from the 25th codon. The sequence obtained for the 67-kDa protein also showed a good match with the deduced amino acid sequence of bcsB from codons 196–206. Thus, the 91-kDa protein is the catalytic subunit of cellulose synthase encoded by the *bcsB* gene, and the 67-kDa protein appears to be a proteolytic fragment of the 91-kDa protein.

Cellulose Synthase Activity Is Rate-Limiting for Cellulose Synthesis in *A. xylinum*. To examine whether the expression of the *bcs* operon is the rate-limiting step in cellulose production, we altered the activities of the *bcs* operon genes by replacing the *bcs* promoter on the chromosome with the heterologous *lac* or *tac* promoters by a gene replacement technique. The cellulose synthase activities of cells with the *bcs* operon under control of the *lac* and *tac* promoter were ≈60% and ≈85% of the level of the wild-type strain, respectively (Table 1). The amount of cellulose accumulated in the strain containing the *lac* and *tac* promoter was also ≈30% and ≈80% of the level of the parental strain (Table 1). Such a correlation between cellulose production and cellulose synthase activity suggested that the expression of the *bcs* operon in *A. xylinum* was rate-limiting for cellulose synthesis.

The *bcs* operon was cloned into the shuttle vector pUC18-824, and the plasmid was designated pABCD. As shown in Table 1, strain 1306-3 containing the pABCD plasmid, accumulated significantly greater amounts of cellulose than did the strain carrying the parental plasmid pUC18-824. This observation further supports our conclusion that expression of the cellulose synthase operon was rate-limiting for cellulose synthesis in *A. xylinum*.

DISCUSSION

Our data on the DNA sequence analysis of the *bcs* locus indicates that the *bcs* genes are organized as an operon. Comparison of the N-terminal amino acid sequence of purified cellulose synthase with the deduced amino acid sequence of the *bcsB* gene provides conclusive evidence that the *bcsB* gene encodes the catalytic subunit of cellulose synthase. This conclusion is also supported by the results of genetic complementation experiments. The cellulose synthase activity of our *A. xylinum* group II mutants was restored by introducing a plasmid carrying the *bcsB* gene into the mutant strains.

The first 24 amino acid residues of the sequence deduced from the gene *bcs* are not found in the N-terminal amino acid sequence of purified cellulose synthase. The structure of this 24-amino acid sequence is remarkably similar to that of signal peptides of secreted proteins in other bacteria (23). This suggests that the catalytic subunit of cellulose synthase is synthesized as a precursor, processed, and deployed in the membrane. This is consistent with the fact that cellulose synthase activity is associated with the cytoplasmic membrane (24). Analysis of the predicted amino acid sequence of bcsB protein for hydrophobicity and secondary structure (25) suggests the presence of transmembrane helices at the N- and C-terminal regions. As mentioned above, the N-terminal region appears to be a leader sequence. A region of high charge density at the C terminus following a hydrophobic region may indicate the anchoring of a transmembrane helix on the cytoplasmic side. In addition to the two regions discussed above, there are a number of hydrophobic regions that may indicate transmembrane β-sheet, as are frequently seen for various porins.

Although the *in vitro* conversion of UDPG to cellulose can be carried out solely by the bcsB gene product *in vitro*, our data showed that cellulose synthesis *in vivo* required the functions of bcsA, bcsC, and bcsD proteins. The exact functions of these proteins in cellulose synthesis are unclear. However, they probably play a role either in the complex processes of transport and crystallization of the cellulose or in the regula-

Table 1. Cell growth, cellulose production, and cellulose synthase activity in recombinant strains

Strain	Plasmid/ promoter	Cellulose synthase, specific activity	Cellulose production, g/liter	Cell concentration, g/liter	Cellulose/ cell
1306-21	*bcs*	3.15	2.99	1.45	2.06
	lac	1.83	0.78	1.28	0.61
	tac	2.56	2.39	1.72	1.39
1306-3	pUC18-824	2.23	2.55	1.51	1.70
	pABCD	3.47	4.04	0.69	5.53

Specific activity is defined as nmol of glucose from UDPG incorporated into β-1,4-glucan per min per mg of protein.

tion of cellulose synthase activity. In *A. xylinum*, the biosynthesis of high molecular weight β-1,4-glucan sub-elementary fibrils by cellulose synthase is believed to occur at the cytoplasmic membrane. These subelementary fibrils are extruded from pores located in the outer membrane and then crystallized (by hydrogen bonding) into microfibrils. The microfibrils then assemble into bundles. Interestingly, polymerization and crystallization processes have been shown to be coupled in *A. xylinum* (26). It is possible that these processes are actually catalyzed by a single enzyme complex, and that the bcs gene products are part of this complex. If this is the case, one would expect overexpression of an individual gene product to have little impact on the production of cellulose in *A. xylinum*. In support of this, we found that the simultaneous and individual increase in the level of the bcsA and bcsB gene products did not improve the cellulose production of *A. xylinum* (data not shown). Furthermore, our nucleotide sequence analysis of the *bcs* operon indicated that the *bcs* genes were translationally coupled. Translational coupling leads to a coordination of gene expression and may be used by *A. xylinum* to maintain the correct balance or appropriate architecture of the multiprotein cellulose synthase complex. The deduced amino acid sequence of *bcsC* at its N terminus is similar to signal sequences of secreted proteins in other bacteria. This may indicate that the bcsC gene product is a membrane protein and a component of the membrane-bound cellulose synthase complex. On the other hand, the bcs gene products may be involved in regulating the activity of cellulose synthase in *A. xylinum*. Mutants deficient in *bcsA* were found to lack cellulose synthase and diguanylate cyclase activities. Immunoblotting of these mutants indicated that they actually contained normal levels of bcsB and diguanylate cyclase proteins (Fig. 4). This implies that the bcsB gene product and diguanylate cyclase require posttranslational modification/activation and that the bcsA protein is involved in such a modification process.

Among *E. coli* promoters, two highly conserved stretches of sequences centered at the −35 and −10 regions are easily identified (27). These regions have proven to be the contact points between the promoter and RNA polymerase. The aldehyde dehydrogenase (*ald*) and the alcohol dehydrogenase (*adh*) genes from *Acetobacter acetii* have been isolated and their nucleotide sequences determined (28, 29). Comparison of the 5′ untranslated regions of these genes with the promoter sequence of the *bcs* operon indicated that they contain scattered regions of homology among them. The homology between the *ald* and *bcs* promoter sequence is particularly striking. For instance, the sequence CAT CGCTG located between nucleotides −11 and −4 in the *bcs* promoter is quite similar to the sequence CATGGCTG positioned 84 bp 5′ of the initiation codon of *ald*. Since the transcriptional initiation sites of *ald* and *adh* promoters have not been identified, the positional significance of such homologous regions between the three promoters is unclear. However, comparison of the sequences adjacent to the initiation codons of the *bcs*, *ald*, and *adh* genes indicated that the sequence GGACGNG is highly conserved approximately 2–6 bases 5′ of the ATG codon. This sequence may represent the ribosome binding site of *Acetobacter* mRNAs.

Computer analysis of the DNA sequence 3′ of the *bcsD* gene reveals a region with an inverted repeat sequence. This sequence has the potential of forming a stable stem–loop structure and $\Delta G = -29.1$ kcal (1 cal = 4.184 J) at the end of a mRNA, which is often used as a transcription termination signal in bacteria (26). This region is positioned 26 bp 3′ of the termination codon of the *bcsD* gene and could be the transcriptional termination of the *bcs* operon.

We do not know of another case in which the genetic organization of a cellulose synthase locus has been disclosed. Immunochemical and enzymatic evidence suggests that similar systems operate in other cellulose-producing bacteria (30) and plants (31). Thus, this work should greatly facilitate the further understanding of cellulose biogenesis.

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