A New Gene Required for Cellulose Production and a Gene Encoding Cellulolytic Activity in Acetobacter xylinum Are Colocalized with the bcs Operon

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Recently, it was shown that a cellulose-negative mutant (Cel1) of Acetobacter xylinum ATCC 23769 carried an insertion of an indigenous transposable element (IS1031A) about 500 bp upstream of the bcs operon, required for cellulose synthesis. Here we show that Cel1 can be complemented by wild-type DNA covering the insertion point. Nucleotide sequencing of this region revealed the presence of two open reading frames, ORF1 and ORF2. ORF2, which is disrupted by the IS1031A insertion in Cel1, potentially encodes the complementing function. ORF1 encodes a protein (CMCAx) with significant homology to previously described endoglucanases. A cloned DNA fragment containing ORF1 expressed a carboxymethyl cellulose-hydrolyzing activity in Escherichia coli. In A. xylinum, CMCAx is secreted into the culture growth medium. The CMCAx mature protein consists of 322 amino acids and has a molecular mass of 35.6 kDa.

The bacterium Acetobacter xylinum represents the dominating biological model system for studies of the biochemistry and genetics of cellulose biosynthesis (24). The synthesis of cellulose in A. xylinum may be considered a two-step process involving polymerization and then crystallization of the individual glucan chains into native cellulose I. The production of cellulose I probably requires a certain topological organization of the biosynthetic apparatus, and it is believed that in vitro cellulose synthesis in extracts from A. xylinum results in the production of the crystalline cellulose II (7). The complexity of the total process suggests that there exist, in addition to the enzyme activities needed to synthesize the individual glucan chains, several functions required for in vivo cellulose production.

The precursor for cellulose synthesis in A. xylinum is uridine diphosphoglucose (UDPG), which is synthesized from glucose 1-phosphate and UTP by the enzyme UDP pyrophosphorylase. Glucose 1-phosphate is formed by the isomerization of glucose 6-phosphate, a reaction catalyzed by phosphoglucomutase. The glucose unit in UDPG is incorporated into the β(1,4)-glucan polymer by a membrane-bound cellulose synthase, which is dependent on the activator cyclic diguanylic acid (c-di-GMP). c-di-GMP is formed from two molecules of GTP in a reaction catalyzed by diguanylate cyclase (25).

Several genes involved in cellulose biosynthesis have been identified. Two of these have been shown to represent the structural genes for phosphoglucomutase and UDPG pyrophosphorylase (13, 32). The genetics of the steps following precursor synthesis are not yet fully understood, but an operon consisting of four genes (bcsABCD) is known to be essential for the final steps in cellulose biosynthesis (28, 29, 36). The biochemical functions of the gene products encoded by the bcs operon are not clear, but the bcsA and bcsB gene products have been suggested to be involved in the polymerization of glucose residues, whereas the bcsC gene is required for the synthesis of β(1,4)-glucan in vivo but not in vitro. Lack of the bcsD gene product results in a 40% reduction in the rates of cellulose synthesis in vivo (36).

A. xylinum contains several insertion sequences constituting an IS1031 family. Transpositions of IS1031 elements were associated with a loss of the ability to produce cellulose in several spontaneous mutants of A. xylinum ATCC 23769 (8, 9). One of these cellulose-negative (Cel−) mutants, Cel1, was found to carry an IS1031A insertion about 500 bp upstream of a gene equivalent to the first gene (bcsA) of the bcs operon in A. xylinum (8). Here we show that the IS1031A insertion inactivates a function not described previously. In addition, DNA sequence analysis and expression studies with Escherichia coli showed that a gene encoding a carboxymethyl cellulose (CMC)-hydrolyzing activity is localized directly upstream of the gene inactivated by the element. The former gene is also expressed in A. xylinum, and the colocalization of this gene with the bcs operon is intriguing. Moreover, it has not been reported previously that A. xylinum can hydrolyze cellulose or any other β-glucan.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Growth of cells and conjugative matings. A. xylinum and Acetobacter aceti cells were grown in the medium described by Hestrin and Schramm (19), while E. coli cells were grown in LB broth (26). A. xylinum cellulose-producing (Cel+) cells were grown statically at 30°C, while Cel− mutants and A. aceti and E. coli cells were grown with shaking at 30 and 37°C, respectively. Harvesting of A. xylinum cells from cellulose pellets and conjugative matings with E. coli S17-1 as a donor were performed as described previously (32-34). The antibiotic concentrations used for the selection of A. xylinum transcon-
jugants were 400 μg of ampicillin per ml, 500 μg of streptomycin per ml, and 100 μg of kanamycin per ml.

Recombinant DNA techniques, DNA sequencing, and computer analysis of DNA and amino acid sequences. Isolation of plasmids from E. coli, cloning, transformation, and Southern blot hybridization were performed by standard protocols (26). Isolation and purification of total DNA from A. xylinum were performed by the CTAB (cetyltrimethyl-ammonium bromide) extraction procedure (3). Subclones were generated from plasmids pTDU21 and pDCB188 by cloning of overlapping restriction endonuclease fragments into plasmid pUC18. DNA sequencing was performed as described by Sanger et al. (27) with a version 2.0 Sequenase kit (United States Biochemical Corp.) and appropriate commercially available primers. Computer sequence analysis was done with the Genetics Computer Group software package (10). DNA and amino acid sequence homology searches were performed at the National Center for Biotechnology Information with the BLAST network service and the nonredundant DNA and protein sequence data bases (2).

Measurements of enzyme activities, purification of CMCase, and amino acid sequencing. Measurements of β(1,4)-glucan-synthesizing activities in permeabilized cells were performed as described previously (13), and the activity in the polyethylene glycol-precipitated membrane fraction (PEG-MF) was measured as described by Aloni et al. (1).

For measurements of CMCase-hydrolyzing activity in E. coli, cells were harvested and washed twice in 25 mM sodium phosphate buffer (pH 6.0). The washed cells were sonicated in the same buffer and then centrifuged at 12,000 × g for 15 min. The supernatants were used directly as a source of crude enzyme for measurements of CMCase-hydrolyzing activity.

CMCase-hydrolyzing activity in A. xylinum cells was measured in the culture growth medium. Cells were removed from the medium by centrifugation at 12,000 × g for 10 min. The pH was adjusted to 6, and proteins were precipitated by the addition of solid ammonium sulfate to 65% saturation at 0°C. The precipitates were dissolved in 10 mM sodium phosphate (pH 5.5) and desalted. The cellulolytic activity was reprecipitated at between 40 and 55% saturation with ammonium sulfate at 0°C and dissolved in 10 mM sodium phosphate (pH 5.5). The samples were dialyzed overnight against the same buffer and finally concentrated by lyophilization. These preparations were used for zymogram analysis. For amino acid sequencing, the novel CMCase-hydrolyzing activity, CMCase, was further purified by isoelectric separation in a Bio-Trap apparatus (Schleicher & Schuell). Electrophoresis was done with 40 mM boric acid–10 mM imidazole (pH 7.8). After concentration on a Centricon-10 concentrator (Amicon), the proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 0.1% SDS and 10% polyacrylamide (20) and blotted onto a polyvinylidene difluoride membrane (Millipore). A band corresponding to a protein with the expected molecular mass (35 kDa) was cut out of the

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tr>
<td><strong>A. xylinum</strong></td>
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<td>Spontaneous nonreverting Cel− mutant of wild-type ATCC 23769</td>
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<tr>
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<td>pHB18</td>
<td>Ap†; 1.8-kb HindIII-BamHI fragment from pDCB188 subcloned into pUC18</td>
<td>This study</td>
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* MCS, multiple cloning site.
* ATCC, American Type Culture Collection, Rockville, Md.; BRL, Bethesda Research Laboratories, Inc., Gaithersburg, Md.
membrane and used directly for amino acid sequencing with a model 477A protein sequencing apparatus from Applied Biosystems.

CMC-hydrolyzing activities were determined by measuring the generation of reducing sugar ends. Enzyme preparations were incubated at 30°C in 1% CMC (Sigma C 4888)–25 mM sodium phosphate (pH 7.0 or 5.5). Aliquots of 0.5 ml were mixed with an equal volume of the dinitrosalicylic acid reagent described by Sumner (31), except that the concentrations of the components were raised to 14 g of sodium hydroxide per liter, 300 g of sodium potassium tartrate per liter, and 10 g of 3,5-dinitrosalicylic acid per liter. After being heated at 100°C for 5 min, the tubes were cooled on ice, 1 ml of water was added, and the optical density at 540 nm of the solutions was finally determined. Glucose was used as a standard.

Zymograms were prepared by a modification of the procedure described by Morag et al. (21). Samples containing cellulytic activity were heated for 3 min at 50°C, and the proteins were separated on an SDS-10% polyacrylamide gel containing 0.1% CMC. The proteins were renatured by washing the gel twice in 25% isopropanol–25 mM sodium phosphate (pH 5.5) for 30 min each time and twice in the same buffer without isopropanol for 30 min each time. The gel was incubated overnight at 30°C in the same buffer and stained with 0.1% Congo red as described by Morag et al. (21).

**Nucleotide sequence accession number.** The nucleotide sequence data reported here have been deposited in the EMBL, GenBank, and DDBJ data bases under accession number M96060.

**RESULTS**

**Biochemical analysis and complementation of the spontaneous Cel− mutant, Cell, of A. xylinum ATCC 23769.** The synthesis of β(1,4)-glucan was measured in permeabilized cells and in a fraction obtained from sonicated cells (designated PEG-MF) of Cell and the wild-type parent strain A. xylinum ATCC 23769. As can be seen from Table 2, permeabilized cells of Cell1 were strongly reduced in their capacity to synthesize β(1,4)-glucan in comparison with the corresponding wild-type strain. However, PEG-MF prepared from Cell1 and the wild-type strain had similar β(1,4)-glucan-synthesizing capacities. These results indicated that the bcs operon was not inactivated in Cell1. Assuming that the IS1031A insertion caused the Cel− phenotype in Cell1, it consequently follows that there probably exists upstream of this operon a new function required for cellulose synthesis in vivo.

We tested the hypothesis described above in a complementation analysis by using a previously cloned 2.5-kb PstI wild-type DNA fragment which contains a DNA sequence upstream of the equivalent of the bcs operon. This PstI fragment was cut out of plasmid pTDU21 and subcloned into the polylinker site of plasmid pTDK21.
of broad-host-range mobilizable plasmid pKH3. The resulting recombinant plasmid, pTDK21 (Fig. 1), was then transferred to a spontaneous streptomycin-resistant derivative of Cell (CellS). The transconjugant colonies obtained in this mating experiment had a Cell− colony morphology, and growth experiments with liquid medium also showed that the cells formed the characteristic irregular aggregates in shake cultures or floating pellicles in static cultures. Cells plated from liquid shake cultures grown under nonselective conditions gave rise to colonies of both the Cell− and the Cell+ appearances. Retesting of these colonies on agar medium containing ampicillin showed that there was a 100% correlation between Cell+ colony morphology and the presence of pTDK21. These results indicated that CellS was complemented by pTDK21, implying that the sequence upstream of the bcs operon encodes a gene product acting in trans. To confirm this hypothesis, we also performed a hybridization analysis of PstI-digested total DNA from the transconjugant. The resulting PstI fragments, separated by agarose gel electrophoresis, were Southern blotted and hybridized with the 32P-labelled 2.5-kb PstI fragment. Figure 2 shows that the probe hybridized, as expected, to the corresponding fragment from wild-type DNA and to a 3.4-kb fragment in CellS (because of the insertion of IS031A in this strain). In the case of the transconjugant, the probe hybridized to both of the above-mentioned fragments. This result shows that pTDK21 is present in the cells as an autonomous replicon and that the chromosomal IS031A insertion is unaffected. Thus, it could be concluded that the region upstream of the bcs operon in pTDK21 contains a gene which complements the deficiency in CellS.

DNA sequence analysis of the complementing region. Approximately 700 bp of the wild-type DNA sequences flanking the IS031A insertion point in Cell was determined previously (8), and we have now extended the sequence analysis in both directions. Figure 4 shows the restriction endonuclease map of approximately 3.5 kb of the sequence in this region, including the localization of the start site for the bcs operon. The nucleotide sequence of the region upstream of the bcs operon is shown in Fig. 3. For simplicity of presentation, these data can be divided into two parts. The first part (region 1) covers nucleotides (nt) 1 through 1426, while the second part (region II) covers nt 1423 through 2731. Region I is described in more detail below, while region II seems to encode the function inactivated by the IS031A insertion between nt 2194 and 2195. The ATG corresponding to the start codon of the previously described bcsA gene is localized at nt 2709 through 2711.

The DNA sequences proximal to and upstream of the bcs operon were reported previously for two different A. xylinum strains (28, 36). In addition, we have independently cloned and partially characterized the equivalent of the bcs operon and its upstream region for strain ATCC 23768 by complementation of Cell− mutants. The open reading frames (ORFs) present in all available bcs upstream region sequences were analyzed and compared with each other. This comparison showed that the downstream part of one of the ORFs was very similar in all four strains (Fig. 4). In strain ATCC 23769, this ORF, designated ORF2, spans nt 1324 through 2484, and the first putative translational start site is a GTG codon located at nt 1423 through 1425. ORF2 contains a series of potential GTG translational initiation start sites, while the first ATG in this ORF is located at nt 1984 through 1986. The GTG codons at nt 1423 through 1425 and 1807 through 1809 have upstream sequences similar to the consensus sequence for E. coli ribosome binding sites (RBSs) (16), and in both cases, these sequences are located at appropriate distances from the corresponding GTG codons (Fig. 3). This result indicates that ORF2 encodes a putative protein containing 353 or 245 amino acids. The amino acid sequence deduced from ORF2 starting at nt 1423 is very rich (17%) in proline residues, and some of these are clustered (residues 81 to 85 and 99 to 102). A computer search of protein data bases did not lead to an identification of sequences with a high degree of similarity to the deduced amino acid sequence of the ORF2 protein.

Evidence indicating that the Cell1-complementing function in pTDK21 is expressed from an A. xylinum promoter. The orientation of the complementing 2.5-kb PstI fragment in pTDK21 (Fig. 1) is such that transcription from the lac promoter would proceed in the same direction as that from ORF2. Since it has also been demonstrated that the lac promoter is active in Acetobacter spp. (14), it seemed possible that the complementation of Cell1 was dependent on transcription from this promoter. To analyze whether this was the case, we inserted the Km-Km interposon from plasmid pJF3550 as a 3.4-kb HindIII fragment into the polylinker site of pTDK21 (between the lac promoter and the insert), generating plasmid pTDK21::Km-Km (Fig. 1). The insertion of Km was expected to block the transcription of A. xylinum DNA from the lac promoter because of strong transcriptional terminators at both ends of the interposon. Conjugation of pTDK21::Km-Km into Cell1 resulted in transconjugants that displayed a Cell+ phenotype. DNA hybridization analysis also confirmed that pTDK21::Km-Km, like pTDK21, is present as an autonomous replicon in the transconjugants (Fig. 2). The complementing function is therefore probably expressed from a native promoter localized downstream of the PstI site at nt 953. It should be noted, however, that the close coupling between ORF1 and ORF2 in the chromosome suggests that they also could be cotranscribed.

A DNA sequence upstream of ORF2 encodes a CMCDerivating activity. An analysis of the DNA sequence in region I (Fig. 3) demonstrated that the longest ORF starts at nt 326 but that the first translational start codons with putative upstream RBSs are the ATG at nt 398 through 400 and the GTG at nt 431 through 433. The ORFs terminate with a TGA stop codon at nt 1424 through 1426. The putative RBS sequence upstream of the ATG shares some similarity with consensus RBS sequences from several Acetobacter strains (36), while the corresponding sequence upstream of the GTG
FIG. 3. Nucleotide sequence of the region upstream of the bcs operon in \emph{A. xylinum} ATCC 23769. Putative RBSs and translational start sites are underlined. Translational termination sites are marked by asterisks. Deduced amino acid sequences for ORF1 (nt 398 to 1423), ORF2 (nt 1423 to 2481), and the 5' end of the equivalent of the \emph{bcsA} gene (nt 2709 to 2729) are shown above the DNA sequence. The amino acid residues identified by N-terminal sequencing of CMCase are shown in boldface type, and the arrow indicates the site of proteolytic cleavage of CMCase.

is more similar to consensus RBS sequences from \emph{E. coli} genes (16). A comparison of the amino acid sequence deduced from the ORF starting at the ATG codon (ORF1) with published sequences in protein sequence data bases revealed that the \emph{A. xylinum} sequence shares significant homology with two previously reported endoglucanase sequences, those from \emph{Erwinia chrysanthemi} (CelY) and \emph{Cellulomonas uda} (CMCase) (Fig. 5A). The region of homology starts from amino acid 1 in the

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FIG. 4. Alignment of deduced amino acid sequences for the region upstream of the bcs operon in four different \emph{A. xylinum} strains: 1306-3 (36), ATCC 23768 and ATCC 23769 (this study), and ATCC 53582 (28). Positions with identical amino acids are marked with asterisks. The first amino acid in the ATCC 23769 sequence corresponds to amino acid 288 in ORF2.
CeY and CMCase mature peptides and from amino acid 28 in the *A. xylinum* sequence.

To examine whether the cloned fragment encoded an enzyme with cellulolytic activity, cell extracts prepared from *E. coli* cells containing plasmid pH81 were tested experimentally for such activity. pH81 was constructed by cloning the 1.8-kb HindIII-BamHI fragment (Fig. 1) containing ORF1 into the corresponding site of the pUC18 polylinker. An analysis of the enzyme extracts (Fig. 6) clearly demonstrated that a CMC-hydrolyzing activity (CMCase) was expressed in cells containing pH81, while no such activity was detected in the corresponding extracts prepared from cells containing pUC18 without the insert.

**CMCase belongs to family D of cellulosomes.** The amino acid sequence homology studies indicated that CMCase is evolutionarily strongly related to the endoglucanases produced by *E. chrysanthemi* (CeY) and *C. uda* (CMCase). CeY and CMCase are members of family D of cellulosomes (15, 18), and the results reported here therefore indicate that CMCase should be classified as a new member of this enzyme family.

The family D cellulosomes contain a conserved motif with the consensus sequence DYYSSXXXLVLXXLXXV localizes close to the carboxy-terminal end of the proteins (Fig. 5B). This motif could also be deduced from the DNA sequence of the corresponding region from *A. xylinum* ATCC 23768 (CMCase68 in Fig. 5B), indicating that the gene organization is the same in the *A. xylinum* ATCC 23768 and ATCC 23769. However, the motif could not be found in the CMCase sequence. Interestingly, we discovered that the motif could be deduced from one of the other two reading frames in the *C. uda* DNA sequence, and the corresponding amino acid sequence (CMCase) is shown in Fig. 5B. In this frame, there is a stop codon that would make CMCase about the same size as CMCase and CeY. The explanation for these observations is not known, but they may indicate that a frameshift mutation has taken place near the 3' end of the gene encoding CMCase or simply that there is a mistake in this region of the previously reported *C. uda* DNA sequence.

**A. xylinum expresses the gene encoding CMCase, and similar enzymes are also produced by other cellulose-synthesizing Acetobacter strains.** To our knowledge, it has not yet been reported that *A. xylinum* produces enzymes involved in polysaccharide hydrolysis. The observed expression of CMCase in *E. coli* therefore raised the question of whether similar activities are expressed in *A. xylinum*. As shown in Fig. 7, the culture growth medium of *A. xylinum* contains a CMCase-hydrolyzing activity.

It was shown previously that cellulolytic activities can be visualized directly after SDS-PAGE of crude protein mixtures (21). By performing such an analysis on the proteins present in the culture growth medium of *A. xylinum*, we found that one such activity band could be clearly visualized (both from the wild type and from Ce1) (Fig. 8). The molecular masses of the corresponding proteins appeared to be about 35 kDa. In addition, weak activities corresponding to proteins of higher molecular masses were also detected (not easily visible in Fig. 8). Since the protein solution was not heated to 100°C prior to
application to the gel, it is possible that these activities were caused by aggregate forms rather than by separate polypeptides.

The zymogram studies described above showed that a CMC-hydrolyzing activity is expressed in *A. xylinum*. To analyze whether this activity originated from the cloned gene, we partially purified the protein corresponding to the activity band from the wild type. This protein was subjected to N-terminal amino acid sequencing, and the amino acids identified in this analysis matched the CMCase sequence (Fig. 3). The first amino acid in the mature protein is the aspartate corresponding to residue 21 in the deduced amino acid sequence of the ORF1 protein. The mature protein therefore consists of 322 amino acids, corresponding to a molecular mass of 35.6 kDa. This probably means that CMCase is first translated as a pre-protein and then proteolytically cleaved at the amino-terminal end. A comparison of the N-terminal sequence of CMCase with known signal peptide sequences by use of the weight matrix proposed by von Heijne (35) revealed some similarity to signal peptides. The predicted cleavage site is located between amino acids 20 and 21, in agreement with the N-terminal sequencing results.

We considered it puzzling that the gene encoding CMCase is localized close to the *bcs* operon, required for cellulose synthesis. To examine whether cellulolytic activities were produced by other *Acetobacter* strains, we measured the activities of CMC-hydrolyzing enzymes in the culture growth medium of seven cellulose-producing *A. xylinum* strains and two non-producing *A. aceti* strains. The results of these analyses showed that all tested cellulose-producing *A. xylinum* strains (ATCC 10245, ATCC 10821, ATCC 11142, ATCC 14851, ATCC 23767, ATCC 23768, and ATCC 23770) produced such an enzyme activity, while no activity was detected in the corresponding protein fractions from the two tested non-cellulose-producing strains, *A. aceti* ATCC 15973 and ATCC 23747 (data not shown). These results therefore indicate that the production of a CMC-hydrolyzing activity may be specifically associated with cellulose-producing *Acetobacter* strains.

**DISCUSSION**

The results presented in this paper show that the insertion of IS1031A upstream of the *bcs* operon in the spontaneous Cel− mutant Cel1 has identified a new gene required for cellulose synthesis in vivo. The protein encoded by this gene is probably not directly involved in polymerization, since PEG-MF prepared from Cel1 synthesized normal quantities of β(1,4)-glucan. The stimulation of PEG-MF by GTP (Table 2) suggests that Cel1 is not deficient in diguanylate cyclase activity, and this suggestion has been confirmed by direct measurements (data not shown). It also seems clear that the deficiency in vivo cellulose formation in Cel1 is not due to an inactivation of some step prior to the synthesis of the precursor UDPG. This conclusion is based on the observation that permeabilized Cel1 cells supplemented with UDPG were impaired in β(1,4)-glucan synthesis, compared with the synthesis in permeabilized wild-type cells. The biochemical and DNA sequence data therefore strongly indicate that the gene complementing the deficiency in Cel1 represents a new function essential for in vivo cellulose biosynthesis. The nature of this function is not known, but it seems obvious that cellulose biosynthesis may require many functions in addition to those directly involved in polymerization. Such functions may be related to transport from the sites of polymerization in the cell membrane and into the extracellular environment, the regulation of gene expression, and the organization of the crystallization process.

An interesting result reported here is that *A. xylinum* has at least one gene encoding a CMC-hydrolyzing activity, and this gene is also localized close to the *bcs* operon. This finding was quite unexpected, since no activity associated with cellulose degradation in *A. xylinum* has yet been described. In addition, it has not been reported that the organism can utilize the cellulose produced as a carbon source. Our results clearly
demonstrate that the enzyme (CMCax) encoded by the cloned gene is produced and secreted by A. xylinum under the growth conditions used in the laboratory. Apparently CMCax undergoes prokaryotic processing, and it seems reasonable to believe that this process is related to the secretion of CMCax.

The biological function of CMCax in A. xylinum is not known. Mutants in which the corresponding gene is interrupted could certainly add some additional information, but such mutants are currently not available. In a recent report (4), it was shown that the protein encoded by a gene (exok) located on a 22-kb gene cluster involved in the biosynthesis of exopolysaccharide I in Rhizobium meliloti displays homology to secreted endo-β-1,3-1,4-glucanases. Interestingly, an exok mutant strain produced reduced quantities of exopolysaccharide I. On the basis of these observations, one might speculate that CMCax is somehow involved in the biosynthesis of cellulose.

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T-G. Iversen and R. Standal contributed equally to this work. We thank Knut Sletten for help with the amino acid sequencing.

REFERENCES

AUTHOR’S CORRECTION

A New Gene Required for Cellulose Production and a Gene Encoding Cellulolytic Activity in Acetobacter xylinum Are Colocalized with the bcs Operon

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Volume 176, no. 3, p. 665-672: A citation of the earlier work of Husemann and Werner (E. Husemann and R. Werner, Makromol. Chem. 59:43-60, 1963), who reported that Acetobacter xylinum culture supernatants contained cellulolytic activity, was inadvertently omitted.