Formation of Highly Twisted Ribbons in a Carboxymethylcellulase Gene-Disrupted Strain of a Cellulose-Producing Bacterium

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Cellulases are enzymes that normally digest cellulose; however, some are known to play essential roles in cellulose biosynthesis. Although some endogenous cellulases of plants and cellulose-producing bacteria are reportedly involved in cellulose production, their functions in cellulose production are unknown. In this study, we demonstrated that disruption of the cellulase (carboxymethylcellulase) gene causes irregular packing of de novo-synthesized fibrils in Gluconacetobacter xylinus, a cellulose-producing bacterium. Cellulose production was remarkably reduced and small amounts of particulate material were accumulated in the culture of a cmcax-disrupted G. xylinus strain (F2-2). The particulate material was shown to contain cellulose by both solid-state 13C nuclear magnetic resonance analysis and Fourier transform infrared spectroscopy analysis. Electron microscopy revealed that the cellulose fibrils produced by the F2-2 cells were highly twisted compared with those produced by control cells. This hyper-twisting of the fibrils may reduce cellulose synthesis in the F2-2 strains.

Cellulose is one of the most abundant biopolymers on earth and is produced by plants, algae, and some types of bacteria (1). It is a linear polymer of β-1,4-linked glucan chains, which are held together by hydrogen bonds to form cellulosic microfibrils (2). In cellulose I, glucan chains are arranged in a parallel fashion. Although cellulose I is the most prominent type of crystalline form in microfibrils, it can be converted to cellulose II by treatment with chemical agents, such as strong alkali (3). Plant and bacterial celluloses are synthesized on the plasma membrane, and several glucan chains assemble to form crystalline microfibrils outside the plasma membrane (4, 5). Plant cellulose molecules produced on the plasma membrane assemble into cellulose networks within cell walls, and the crystallized celluloses are Iα-enriched cellulose. In contrast, bacterial cellulose molecules produced by the cellulose synthase complex on the plasma membrane must pass through the bacterial outer membrane to form cellulose fibrils, and most bacterial cellulose molecules after crystallization become cellulose Iβ (6, 7). Although cellulose synthase complexes located on the plasma membrane play essential roles in cellulose formation, other proteins are also involved.

Plants and cellulose-producing bacteria have several cellulosomes. Endo-β-1,4-glucanases, the Korrigan (KOR) protein of Arabidopsis thaliana (a plant), and carboxymethylcellulase (CMCax) of Gluconacetobacter xylinus (a cellulose-producing bacterium) are endogenous cellulosomes that putatively digest celluloses. Several studies have shown that the KOR protein is involved in cellulose synthesis (8, 9). An insertional mutant and a mutant carrying a point mutation for an endogenous plant cellulase, known as KOR1, show typical phenotypes defined by the prevention of cellulose synthesis in primary and secondary cell walls (9, 10). Several hypotheses regarding the function of KOR have been proposed, including (i) separation of a postulated cellulose primer from the nascent polymer, (ii) separation of completed cellulose molecules from the cellulose synthase complex, and (iii) cleavage of noncrystalline glucan molecules from crystalline cellulose microfibrils to remove the tensional stress that might arise during the assembly of a large number of glucan chains into fibrils (6). However, the role of KOR in cellulose synthesis is unclear.

We used G. xylinus as a model system for investigating the role of endogenous cellulase in cellulose fibril formation. G. xylinus cellulose is produced by a cellulose synthase complex on the plasma membrane with protein components encoded by a single operon (bcsABCD or acsABCD) (11-14). The other operon constructed from two open reading frames (ORFs) is located upstream of the bcs operon (Fig. 1A), which encodes CMCax, which might be responsible for cellulose hydrolysis (15). Several findings support the possible involvement of CMCax in bacterial cellulose synthesis. The addition of endogluccanase to the culture or over-production of cellulase by G. xylinus enhances the yield of cellulose synthesis (16, 17), whereas the addition of antibodies to recombinant glucanase inhibits the formation of the cellulose fibrils (18). In the present study, we describe a novel type of cellulose ribbon in a mutant strain of G. xylinus carrying a disrupted cellulase gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. G. xylinus strain BPR2001 and Escherichia coli strains DHSα and TB1 were used in this study. Strain G. xylinus BPR2001 and its derivative strains (F2-2 and BPR2001Δcmca) were grown in a semisynthetic medium (2). Bacterium were grown aerobically in a fermentor with vigorous mixing at 25°C and 200 rpm for 48 h. The culture medium used for the experiments included 1% corn steep liquor, 1% yeast extract, 0.5% peptone, 0.5% glucose, 1 g of KH2PO4, 0.5 g of K2HPO4, and 1 g of MgSO4·7H2O. The 

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nibenzoic acid, 0.002 mg/liter folic acid, and 0.002 mg/liter biotin (pH 5.0). The cell suspension was inoculated into fresh medium in a baffled shake flask and cultured at 30°C on a rotary shaker (180 rpm) for 5 days. To select transformants with resistance markers, the following antibiotics were used: ampicillin (50 µg/ml), chloramphenicol (50 µg/ml), and kanamycin (100 µg/ml). The *E. coli* plasmid vectors used in this study were pUC119, pHSG399, and pHSG299 (Takara Bio Inc., Otsu, Japan). The plasmid vector pSA19 (22) was used as a shuttle vector between *G. xylinus* and *E. coli*.

**Electroporation.** For preparing *E. coli*-competent cells, *E. coli* was grown in LB medium and washed with ice-cold 10% glycerol. Plasmid DNA (1 µg) was introduced into the competent *E. coli* cells by electroporation using the Cell-Porator apparatus (Gibco BRL, Carlsbad, CA). Electroporation was performed in 0.15-cm cuvettes at a field strength of 1.9 kV/cm, with the capacitor and resistor set at 330 µF and 4 kΩ, respectively. After electroporation, the cells were incubated in 1 ml of LB medium at 37°C for 1 h and then plated onto LB agar plates containing appropriate antibiotics. The plates were incubated at 37°C until colonies were formed.

For preparing *G. xylinus*-competent cells, *G. xylinus* was grown in FPY medium containing 1% (vol/vol) cellulase (Celluclast; Novo Nordisk A/S, Bagsard, Denmark) and washed with ice-cold 10% glycerol. Plasmid DNA (5 µg) was introduced into *G. xylinus* by electroporation using the Cell-Porator apparatus. Electroporation was performed in 0.15-cm cuvettes at a field strength of 2.1 kV/cm, with the capacitor and resistor set at 330 µF and 8 kΩ, respectively. After electroporation, the cells were incubated in 1 ml of FPY medium containing 1% (vol/vol) cellulase at 30°C for 3 h and then plated onto FPY agar plates containing appropriate antibiotics. The plates were incubated at 30°C until colonies were formed.

**Gene disruption and generation of recombinant strains.** The β-lactamase gene, which provides ampicillin resistance (Amp<sup>+</sup>), was amplified as a 1.1-kb PCR product from pUC119 using oligonucleotide primers Amp1 (5′-GTACAGTGTCGACTTTGGG-3′) and Amp2 (5′-GTCGAC TGGAAACGAAAACTCAGG-3′). To disrupt the gene encoding CMCax, a 0.8-kb Clai-HincII fragment from cosmID pAM9, isolated as a plasmid containing the entire region of the bcs operon from a wild-type genomic library (Fig. 1A) (23), was subcloned into pHSG399. The nucleotide sequence data of *cmcax* appears in the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank nucleotide sequence databases under the accession number AB010645. The *cmcax* gene corresponds to nucleotides (nt) 869 to 1994 in this nucleotide sequence. The Amp<sup>+</sup> gene was inserted at the Stul site on the 0.8-kb Clai-HincII fragment, and the resultant plasmid was linearized and transformed into strain BPR2001 (wild type), yielding the mutant strain F2-2. To confirm the insertion of the Amp<sup>+</sup> gene into the F2-2 genomic DNA, genmic Southern hybridization was performed by the capillary transfer method (24) (see Fig. S1 in the supplemental material). The probe DNA was prepared using the digoxigenin (DIG) labeling and detection system (Roche Diagnostics, Tokyo, Japan). The 0.9-kb BamHI fragment (Fig. 1A) from pAM9 (23) was digested with SalI and XhoI. The probe DNA was prehybridized and hybridized into 68°C according to the instructions for the DIG labeling and detection kit (Roche Diagnostics). The blotting membranes were washed twice with 2× salt-sodium citrate (SSC) buffer (20× SSC contains 3 M NaCl and 0.3 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min and then washed twice with 0.1× SSC-0.1% SDS at 68°C for 15 min. The 1.2-kb BamHI-HindIII fragment containing the full-length *cmcax* operon was inserted into a shuttle vector plasmid vector pSA19 (22) and then self-ligated. The resultant plasmid was transformed into *E. coli* BPR2001 (wild type), yielding the mutant strain F2-2. To confirm the insertion of the *cmcax* gene into the F2-2 genomic DNA, genmic Southern hybridization was performed by the capillary transfer method (24) (see Fig. S1 in the supplemental material). The probe DNA was prepared using the digoxigenin (DIG) labeling and detection system (Roche Diagnostics, Tokyo, Japan). The 0.8-kb BamHI fragment (Fig. 1A) from pAM9 (23) was digested with SalI and XhoI. The probe DNA was prehybridized and hybridized into 68°C according to the instructions for the DIG labeling and detection kit (Roche Diagnostics). The blotting membranes were washed twice with 2× salt-sodium citrate (SSC) buffer (20× SSC contains 3 M NaCl and 0.3 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min and then washed twice with 0.1× SSC-0.1% SDS at 68°C for 15 min.
DNA by PCR using the primers Km-1 (5'-GTGAAAGGTGTGCTGAC-3') and Km-R1 (5'-CTGGCGTAATAGGAGGG-3'). The resultant plasmid was designated pSA-CMCase/k.

**Cellulose analyses.** The amount of cellulosomes in the culture was measured according to the method described by Chao et al. (26).

For the analysis of solid-state $^{13}$C nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy, specimens were prepared according to the following method. Using forceps, we removed sheets of cellulose film from cellulose pellicles produced by the control cells. In the mutant F2-2 culture, particulate material was collected by centrifugation. The pellet was resuspended in 10 mM K-acetate buffer (pH 5.0) and recentrifuged at 5,000 rpm for 20 min. In the F2-2 culture, cells grew rapidly to a large concentration. In order to avoid contamination of the cell fragments, we washed the resultant pellet with a sodium hydroxide solution. The cellulose product (pellicle or particulate material of the mutant) was stored in 0.1 M NaOH at 80°C overnight and then washed several times with hot water. The purified cellulose and alkali-insoluble materials were dried at 60°C overnight. Avicel (microcrystalline cellulose) (Merck, Darmstadt, Germany) was treated with 4 M NaOH at 80°C and washed several times with hot water. These products were analyzed by solid-state $^{13}$C NMR and FTIR spectrometry.

High-resolution solid-state $^{13}$C NMR spectra were recorded on a Chemagnetics Infinity 400 spectrometer ($^{13}$C: 100.6 MHz) (Varian, Inc., Santa Clara, CA) using cross-polarization–magic-angle sample spinning (CP-MAS). The spectral width, acquisition time, and repetition time for Santa Clara, CA) using cross-polarization–magic-angle sample spinning and then analyzed.

**RESULTS**

**Cellulose production in the culture of the cmcax-disrupted strain.** To investigate the role of CMCax in bacterial cellulose synthesis, we prepared an artificial mutant strain, F2-2, with a disrupted cmcax sequence accompanied by insertion of the Amp' gene (Fig. 1A; see also Fig. S1 in the supplemental material). We also constructed a cmcax complement strain, F2-2(pSA-CMCase/k), in which the complementation plasmid pSA-CMCase/k (an approximately 1.9-kb fragment of *G. xylinus* DNA with cmcax and a Km' gene included in the shuttle vector PSA19) was inserted into F2-2. A 5-mm-thick pellicle (Fig. 1B, arrow) was formed after 1 week of static culture of the wild-type strain. Pellicles were not formed in the 1-week static F2-2 culture, but small amounts of particulate material (Fig. 1C, arrowheads) were observed at the bottom of the flask or attached to the inner surface of the flask at the border between the medium and the air. As expected, a thick pellicle (Fig. 1D, arrow) was also formed in the static F2-2(pSA-CMCase/k) culture.

Changes in the amounts of cellulose were compared among these strains in agitation cultures prepared over 5 days on a rotary shaker. The amount of cellulose produced by F2-2(pSA-CMCase/k) cultured for 5 days was 77% of that produced by the wild-type strain; however, the amount of cellulose produced by F2-2 was very small (Fig. 1E). These results indicate that disruption of *cmcax* causes a remarkable reduction in cellulose production by *G. xylinus*.

**Presence of cellulose II in the particulate material produced by the F2-2 culture.** To examine whether the F2-2 culture produced cellulose, the chemical nature of the particulate material in the static culture was investigated. The sample was prepared from the static culture because the amount of particulate material was greater than that found in the agitated culture. Solid-state $^{13}$C NMR spectra were obtained with a powder sample of the particulate material from the F2-2 culture. The $^{13}$C CP-MAS NMR spectra of alkali-insoluble materials from wild-type (Fig. 2Aa) and F2-2(pSA-CMCase/k) (Fig. 2Ab) pellicles were found to be very similar to previously published spectra of *G. xylinus* (7). In contrast, the C6 signal in the spectrum of the alkali-insoluble material obtained from the F2-2 culture was shifted upfield (Fig. 2Ac). Furthermore, the C4 signal (Fig. 2Ac, dagger) was also shifted slightly upfield, and the C1 signal became a doublet at 104.5 and 105.6 ppm. The spectrum of the particulate material obtained from the F2-2 culture was similar to that of NaOH-treated Avicel (see Materials and Methods), which represents typical cellulose II (Fig. 2Ad). These observations (Fig. 2A) suggest that the particulate material from the F2-2 culture contained cellulose with a crystalline structure similar to that of cellulose II.

FTIR spectrometry also revealed a significant difference in the spectra of the cellulose of the wild-type and F2-2 cultures (Fig. 2B). In the OH-stretching frequency region from 3,600 to 3,000 cm$^{-1}$, the spectrum of the cellulose specimen from the F2-2 culture contained two bands near 3,440 and 3,480 cm$^{-1}$ (Fig. 2Bc, dagger). These bands were not present in the spectrum of the wild-type strain (Fig. 2Ba) and were assigned to the intramolecular hydrogen bonds of O3 H···O5 in cellulose II (27). Similar bands were also detected in the NaOH-treated cellulose (Fig. 2Bd, dagger), suggesting that the main component of the alkali-insoluble fraction from F2-2 is cellulose II. In contrast, these bands were absent in the spectra of F2-2(pSA-CMCase/k). Instead, a band was present near 3,240 cm$^{-1}$ (Fig. 2Bb, asterisk). This absorption band was observed only for cellulose I$_{a}$ (28).

**Highly twisted cellulose fibrils associated with F2-2.** To investigate the morphology of the cellulose fibrils produced by strain F2-2, negatively stained bacteria were examined by transmission electron microscopy. Approximately 10% of the examined cells had cellulose fibrils in the wild-type and F2-2(pSA-CMCase/k)
cultures (Fig. 3A and C). Although the frequencies were very low (approximately 1% of all cells), cellulose fibrils associated with the F2-2 cells were observed (Fig. 3B). The existence of cellulose fibrils in F2-2 cells was also confirmed by fluorescence microscopy of cells stained with 0.005% calcofluor white M2R (see Fig. S2 in the supplemental material). The cellulose fibrils produced by F2-2 cells (Fig. 3E to G) were more twisted than those produced by the wild-type (Fig. 3D) or F2-2(pSA-CMCax/k) (Fig. 3H) cells.

To characterize the morphology of the fibrils, we further quantified their half-pitches and widths (Fig. 3I). The half-pitches of the fibrils produced by F2-2 cells were 103.0 ± 3.5 nm (mean ± standard error of the mean, n = 50), which was significantly different from those of the fibrils produced by the wild-type (906.1 ± 37.4 nm, n = 34, P < 0.0001 by two-tailed t test) and F2-2(pSA-CMCax/k) (825.4 ± 30.2 nm, n = 47, P < 0.0001) strains. The widths of the fibrils produced by the F2-2 strain (25.0 ± 1.2 nm, n = 50) were approximately half of those of the fibrils produced by the wild-type strain (45.8 ± 2.0 nm, n = 34, P < 0.0001) and two-thirds of those of the fibrils produced by the F2-2(pSA-CMCax/k) strain (38.6 ± 1.5, n = 47, P < 0.0001) (Fig. 3G).
contrast, the widths of the fibrils produced by the complement strain were slightly less than those of the fibrils produced by the wild-type strain ($P = 0.005$), although no significant differences were observed between the half-pitches of the two fibrils ($P = 0.094$). These results indicate that fibrils produced by the F2-2 strain were highly twisted compared with those produced by the control cells.

**DISCUSSION**

Cellulose ribbons produced by wild-type *G. xylinus* strains are gently twisted, with a pitch of approximately 900 nm (Fig. 3D). In contrast, *de novo*-synthesized ribbons became entangled and highly twisted with a pitch of approximately 103 nm in the F2-2 strain (Fig. 3E to G). Morphological alterations of cellulose ribbons by the addition of cellulose-binding molecules have been reported. Calcofluor white (29) is a fluorescent agent that binds to cellulose and induces fibrils with broad bands that do not condense laterally in the ribbon. The cellulose-binding domain (CBD) (30) is a polypeptide that binds to cellulose and interferes with its synthesis. Electron microscopic observations have shown that the fibrils produced in the presence of the CBD are splayed ribbons composed of separate fibrillar subunits. Similar splayed ribbons have also been reported in wild-type bacteria cultured in the presence of CMCax (16). The highly twisted ribbon in the *cmca*AX-disrupted strain represents a morphology that is markedly different from that of the cellulose ribbons altered by these cellulose-binding molecules; thus, the ribbons presented in this study may represent a novel type of alternation of cellulose ribbons. The addition of CMCax (16) and loss of function of CMCax (in this study) affected the morphologies of the cellulose ribbons; the addition produced a splayed ribbon, and the loss of function formed a highly twisted ribbon. These observations suggest an essential role of CMCax in cellulose assembly.

Cellulose production decreased remarkably in the F2-2 strain (Fig. 1); however, highly twisted ribbons associated with a bacterium were observed by electron microscopy (Fig. 3). Solid-state $^{13}$C NMR and FTIR spectroscopy experiments on particulate materials from the F2-2 culture (Fig. 2) showed the existence of cellulose in the F2-2 strains. These results suggest that the highly twisted ribbons reported in this study were cellulose ribbons. The low productivity of cellulose in the mutant can be explained if the formation of highly twisted ribbons causes reduction of cellulose synthesis or if the synthesis stops prematurely in the mutant when highly twisted ribbons are formed.

*G. xylinus* strain AY201 has two genes coding for cellulose synthetase: *acsAB* of the *acs* operon and *acsAII* (31, 32). In mutants with disrupted *acsAB*, in *vivo* cellulose production was not detected, but in *vivo* cellulose synthase activity was observed (11). The disruption of both *acsAB* and *acsAII* was necessary to inhibit in *vivo* cellulose synthase activity (31). A comparative genomic analysis of *G. xylinus* NBRC 3288, a cellulose-nonproducing strain, with other cellulose-producing strains showed that a nonsense mutation in the catalytic subunit of cellulose synthase (BcsB1) caused the cellulose-nonproducing phenotype (33). This evidence indicates that the loss of function of the cellulose synthase genes completely inhibited cellulose synthesis. In this study, residual cellulose was detected in the culture of the *cmca*AX-disrupted mutant, suggesting the functional differences between cellulose synthase and CMCax. Complementation experiments using the point-mutated CMCax based on available information about its crystal structure (34) and hydrolysis activity (15) should be helpful for elucidating the function of CMCax in cellulose production.

It is well established that cellulose polymerization and crystallization are coupled reactions during cellulose synthesis in *G. xylinus* and that crystallization limits the rate of polymerization (2, 11, 35). CMCax digests amorphous cellulose; however, it cannot digest the crystalline form (15). One possible explanation for the hypertwisted ribbons is that disruption of *cmca*AX causes irregular packing of the *de novo*-synthesized fibrils (Fig. 4). The cellulose produced by the wild-type *G. xylinus* strain is rich in cellulose I*α* (7). The present results show that the cellulose produced by the F2-2 cells was cellulose I*β* (Fig. 2). Because cellulose II is thermodynamically more stable than cellulose I (36), inhibition of cellulose I crystallization in the mutant strain may cause accumulation of cellulose II in the particulate cellulose of the mutant culture. In this study, sodium hydrate solution was used to prepare samples for solid-state $^{13}$C NMR and FTIR analysis from the F2-2 culture. A high concentration of sodium hydrate solution (>4 M) digested the hydrogen bonds between β-1,4-d-glucan chains and caused changes in the cellulose allomorph from cellulose I to the more stable form, cellulose II. However, the concentration of sodium hydrate solution used in this study was very low (0.1 M [0.4%]), and cellulose I may not have been converted to cellulose II (3). Of course, there still remains the possibility that cellulose I can be converted to cellulose II even in low concentrations of sodium hydrate solution under conditions where the cellulose fibrils do not crystallize completely.

In our previous study, we showed that an orf2-disrupted strain
produced cellulose I, cellulose II, and amorphous cellulose (25). Together, orf2 (located downstream of cmcax) and cmcax comprise an operon (Fig. 1A) (15, 25). In an orf2-disrupted strain, cmcax may be normally expressed because it locates upstream of orf2. Therefore, cellulose I is produced by the function of CMCAx in orf2-disrupted strains. The facts that cellulose production in the F2-2 culture was lower than that in the orf2-disrupted culture (Fig. 1E) (25) and that the F2-2 strain mainly produces cellulose II (Fig. 2) suggest that CMCAx and ORF2 polypeptides collaborate in cellulose crystallization and that cellulose I crystallization is severely interrupted without the function of CMCAx.

The G. xylinus cellulose synthase complex is composed of transmembrane proteins that span the plasma and outer membranes and are arranged in a row on the membrane along the longitudinal axis of the cell (35, 37). A row of pores (Fig. 4, EP) with 3.5-nm diameters has been found on the outer surface of the outer membrane (38). These pores are considered to be extrusion pores for cellulose fibrils and are somehow involved in the correct crystallization of cellulose fibrils. There is a possibility that CMCAx and/or ORF2 near the pore may assist or function as components of the “extrusion machine” (Fig. 4, upper row). CMCAx can cleave the tangled celluloses to crystallize correctly when de novo-synthesized cellulose molecules fail to arrange themselves correctly before or during extrusion, or the CMCAx sorts out tangles in the cellulose fibrils near the pore in case tensional stress arises during the assembly of cellulose fibrils. In the F2-2 strain, without inhibited CMCAx cellulose activity, the cellulose molecules produced by the cellulose synthase complex tend to tangle, and the cellulose fibrils that accidentally crystallize are composed of cellulose II molecules (Fig. 4, lower row).

The AcsD subunit, a product of acsD comprising the cellulose synthase operon of G. xylinus ATCC 53582, is reportedly involved in cellulose crystallization, as the acsD mutant reduced the amounts of two cellulose allomorphs (cellulose I and cellulose II) (11). Wild-type bcsD is a homolog of acsD of G. xylinus ATCC 53582 (11, 23). Cellulose productivity in the N-terminal deletion mutant of bcsD also decreased, although cellulose crystallization produced by the mutant was not investigated (39). These observations suggested that CMCAx worked together with BcsD in cellulose crystallization in the wild-type strain.

Individual β-1,4-D-glucan chains are synthesized by cellulose synthase complexes, which are arranged in a row on the cell membrane and associate to form crystalline fibrils. The structure of BcsD (AxCeSD), a component of the cellulose synthase complex, was found to be a homo-octamer comprised of four dimer subunits with a central pore and cylindrical structure (39). The interfaces between the dimers form four spiral interstices on the wall of the molecular cylinder at a 50° angle from the cylinder (vertical) axis (39). There is a possibility that the spiral interstices may affect the twist formation in cellulose microfibrils without cell rotation. It is also possible that when microfibrils are secreted by bacterial cells, the synthesized cellulose fibrils may become distorted without the action of CMCAx, thereby producing highly twisted ribbons. CMCAx helps to remove the tensional stress of the glucan molecules within the crystalline cellulose microfibrils that may arise during the assembly of a large number of glucan chains into fibrils. Further studies will be necessary to elucidate the twisting mechanism of bacterial cellulose fibrils.

Data obtained from the present study on bacteria show the possibility of hypertwisting of cellulose fibrils upon disruption of the cellulase gene. This may also apply to plant-based cellulases such as KOR, although there are structural differences, such as the lack of the plasma membrane-binding domain of KOR in CMCAx (40). In future studies, in order to further elucidate the functions of cellulases, we will examine whether KOR or other cellulases, such as the celC product in Agrobacterium, contribute to their own cellulose synthesis (41) and whether celC can compensate for CMCAx in G. xylinus cellulose production.

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T.N., D.Y., and Y.M. designed the experiments; T.N., Y.S., and M.S. produced and characterized the mutants; S.T. performed the NMR experiments; T.I. and J.S. performed the FTIR experiments; and T.N., H.S., K.O., M.T., and Y.M. made the EM observations and performed analysis. Each author has discussed the results and commented on the manuscript. We declare no conflicts of interest.

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