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Overproduction of Cellulose in *Acetobacter xylinum* KCCM 10100 Defective in GDP-Mannosyltransferase

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Abstract GDP-mannosyltransferase (GMT) is an enzyme responsible for the addition of a mannose to glucose (α[1→3]) during biosynthesis of the water-soluble branched polysaccharide acetan in *Acetobacter* species. In an effort to obtain a cellulose-overproducing bacterium, a mutant defective in GMT of *Acetobacter xylinum* KCCM 10100 was constructed by single crossover homologous recombination using part of the *aceA* gene encoding GMT amplified by polymerase chain reaction. The GMT-disrupted mutant produced 23% more cellulose, but 16% less water-soluble polysaccharide than those of the wild-type strain. Analysis of the sugar composition by gel permeation chromatography revealed that water-soluble polysaccharides produced by the GMT-defective mutant contained no mannose molecule.

Key words: *Acetobacter xylinum*, cellulose, *aceA*, GDP-mannosyltransferase, water-soluble polysaccharide

Bacterial cellulose is a useful and attractive material, mainly because of its valuable properties including biodegradability, low toxicity, excellent water absorption, and high crystal and mechanical strengths [1, 16, 20]. The cost of cellulose production by bacteria, however, is very high, because bacteria produce a very small amount of cellulose from expensive carbon sources such as glucose and fructose. Therefore, it would be useful to develop a cellulose-overproducing mutant for commercial purposes.

Acetobacter xylinum produces water-insoluble cellulose as well as water-soluble polysaccharides, including acetan [5, 6, 15]. Cellulose is a β-1,4-glucose polymer, whereas acetan is a polymer consisting of glucose, mannose, glucuronic acid, and rhamnose in proportions of 4:1:1:1 [6, 12] (Fig. 1). Cellulose and acetan share a linear glucose polymer structure;

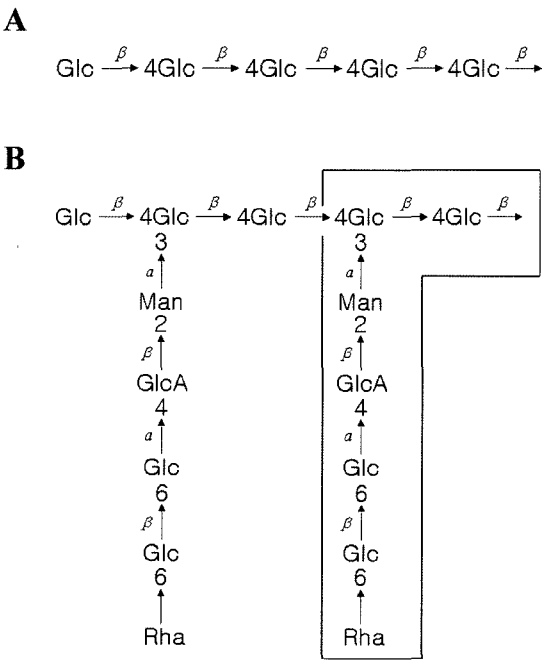

Fig. 1. Proposed structures of cellulose and acetan.
A. Cellulose structure [20, 23]. **B.** Proposed structure of acetan [6, 23]. The solid frame indicates the repeating unit. Abbreviations: Glc, glucose; Man, mannose; GlcA, glucuronic acid; Rha, rhamnose.

however, acetan has side chains of an oligomer (Man←GlcA←Glc←Glc←Rha) attached to the linear glucose polymer in a "Man(α1→3)Glc" manner (Fig. 1) by GDP-mannosyltransferase (GMT). It has been reported that certain strains of *A. xylinum* produce more amounts of acetan than of cellulose, whereas others produce more cellulose than acetan [6, 7, 9, 12, 19, 22]. This result together with the structural characteristics of cellulose and acetan suggests that it may be possible to control the productivity of cellulose and acetan through genetic manipulation of

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A. xylinum, resulting in the construction of a cellulose-overproducing mutant that produces no acetan.

We previously isolated and characterized *Acetobacter xylinum* KCCM 10100 from cellulose pellicles, formed in conventional vinegar plants in Korea, for the ability to produce cellulose [24]. In this study, as an initial effort to develop an *A. xylinum*-based system for cellulose production, we generated and characterized a mutant of *A. xylinum* KCCM 10100 defective in GMT activity.

Petroni and Ielpi [18] previously reported that that all bacterial α-mannosyltransferases have a short C-terminal amino acid sequence in common. Accordingly, two degenerate primers, desiganated as GM1 (5'-WSNACNCAYGGNG-GNTTYTTYC-3') and GM2 (5'-CNGCNGCNARNCCR-AANCCYTC-3'), were designed based on the amino acid sequences STHGGFF (amino acid residues 125–131) and FGLAAVE (amino acid residues 289–295) in GMT from *A. xylinum* NRRL B42 (GenBank U37258). Total DNA from *A. xylinum* KCCM 10100 was prepared according to the method of Ausubel *et al.* [2]. Indeed, application of these primers in a PCR reaction with total genomic DNA from KCCM 10100 amplified a ~500-bp fragment, as expected from a potential GMT gene target. PCR was carried out in a 25-μl reaction volume as follows: after incubation for 5 min at 94°C, 30 cycles of incubation for 1 min at 94°C, 1.5 min at 60°C, and 1.5 min at 72°C, followed by post-elongation for 7 min at 72°C. The PCR fragment was then cloned into pGEM T-easy vector to make pST200. Subsequent sequencing and analysis of the 525-bp PCR product revealed 95% and 91% identity to the GMTs from *Acetobacter xylinum* BPR2001 [14] and *A. xylinum* NRRL B42 [18], respectively.

Since the above sequence analysis data indicate that the PCR-cloned fragment is a part of GMT from *A. xylinum* KCCM 10100, attempts were made to disrupt the putative GMT gene in KCCM 10100. The insert in pST200 was recovered by digestion with EcoRI and transferred into pSHG398 (Takara Shuzo, Japan). The resultant recombinant plasmid, designated as pST210, was transformed into *E. coli* DH5α by using the $CaCl_2$ method [10]. Subsequently, the pST210 plasmid DNA was purified by a plasmid minipreparation kit (Qiagen, Hilden, Germany), and then introduced into the cells of KCCM 10100 by electrotransformation using a Gene Pulser (Biorad) under the conditions of 2.5 kV, 800 ohms, and 25 μF. Competent cells were prepared by the general electroporation-competent cell preparation method [21]. After transformation, chloramphenicol-resistant colonies were picked as recombinant candidates harboring the pTS210 in the chromosome, because pST210 was unable to replicate in KCCM 10100. The insertion of pTS210 by homologous recombination in chloramphenicol-resistant cells was confirmed by PCR and nucleotides sequence analysis. A 2.76-kb DNA fragment that was identical with

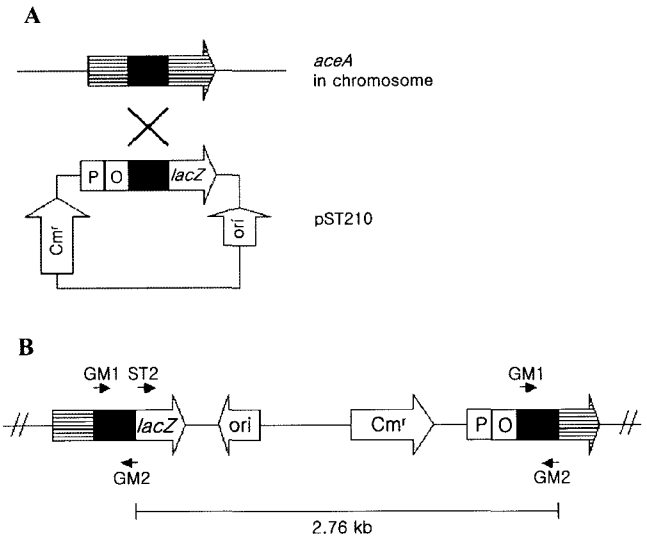

Fig. 2. Diagrams for homologous recombination of the *aceA* gene.

A. A partial *aceA* gene in pST210 and the *aceA* on the chromosome. Black squares in arrow shape diagrams on the pTS210 and the *aceA* in chromosome indicate the amplified PCR product and the corresponding region of the PCR product on the chromosome, respectively. **B.** Diagram of chromosomal *aceA* gene disrupted by pTS210 after single crossover homologous recombination. The small arrows indicate the PCR primers used to amplify the partial *aceA* gene (GM1 and GM2) and to confirm the insertional mutation of the chromosomal *aceA* gene by the 2.76-kb pST210 plasmid in chloramphenicol-resistant transformants (ST2 and GM2).

the size of pTS210 was amplified (data not shown) by PCR, using total DNA from the recombinant candidate as a template, and the GM2 and ST2, an internal sequence of pHSG398 (5'-GATCCTCTAGAGTCGACCTGC-3'), as primers (Fig. 2). This means that the recombinant resulted from a single crossover homologous recombination between the cloned DNA fragment in the pST210 and the corresponding region of the putative GMT gene on the chromosome. This was further confirmed after analysis of the nucleotide sequence of the 2.76-kb insert in the recombinant (data not shown).

In order to confirm the functional disruption of the putative GMT gene in the recombinant KCCM 10100, water-soluble polysaccharides in both the wild-type and the mutant strains were analyzed for mannose sugar by chromatographic analysis. Both the wild-type and mutant cells were grown for 5 days at 30°C under agitation (150 rpm [17, 24]) in 250-ml flasks containing 50 ml of Hestrin and Shramm (HS) medium [11]. Water-soluble polysaccharides were isolated from culture supernatants, hydrolyzed by acid, and dried following the method of Couso *et al.* [6]. The sugars in the samples were analyzed at 75°C with water as a carrier (0.6 ml/min) by using a Youngin 9200 gel permeation chromatograph (Young Lin Instrument Co., Ltd., Anyang, Kyonggido, Korea), equipped with a MEI gel CK08EC column, M930 solvent delivery pump, 7725i rheodyne injector, RI750F detector, column heating chamber,

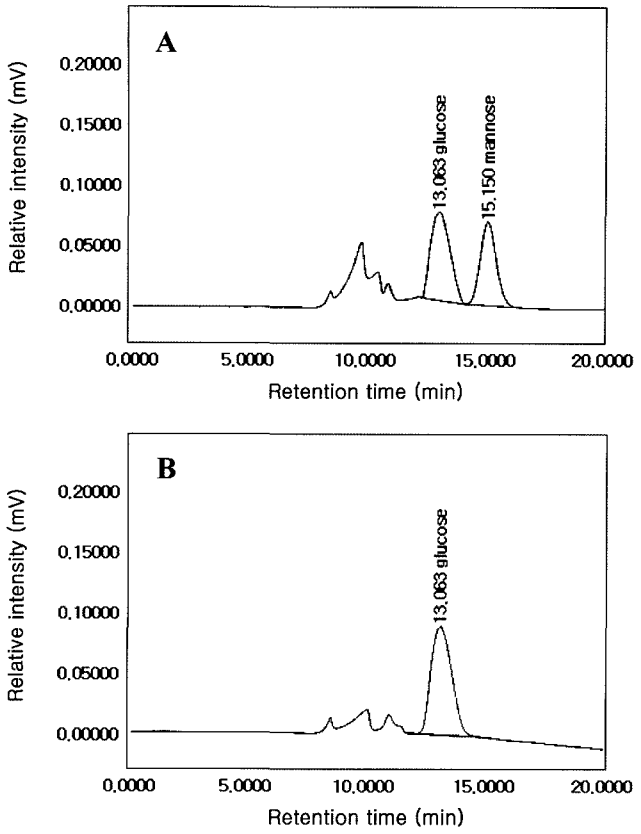

Fig. 3. Chromatograms of representative sugars in water-soluble polysaccharides prepared from wild-type (**A**) and chloramphenicol-resistant recombinant (**B**).

Numbers in X and Y axes are retention time (min) and relative intensities (mV), respectively. The numbers on peaks also represent retention time for each sugar.

and autochro-GPC software. Glucose and mannose were used as standards. The chromatography revealed that mannose sugar was present only in water-soluble polysaccharides produced by wild-type cells, whereas glucose was present in samples from both wild-type and mutant cells (Fig. 3). These results clearly indicate that the mutant did not produce mannose-containing water-soluble polysaccharides like acetan, but produced other modified water-soluble polysaccharides containing no mannose sugar. Thus, coupled with sequence analysis, one can reasonably expect that the disrupted gene encodes GMT, which is responsible for addition of mannose to the glucose molecule in a "α1→3" manner during biosynthesis of acetan in *A. xylinum* (Fig. 1).

The amount of cellulose produced by KCCM 10100 was determined after treatment of washed cells with NaOH, followed by neutralization with acetic acid, as described by Hestrin and Schramm [11]. The total amount of water-soluble polysaccharides was estimated by the phenol-sulfuric acid method [8]. The wild-type and GMT-defective mutant produced 2.03 g dry wt/l and 2.49 g dry wt/l of cellulose after 5 days of cultivation, respectively, indicating that the productivity of cellulose in the GMT-defective mutant is 23% more than that in wild-type cells. The amount of water-soluble polysaccharides produced in the GMT-defective mutant was 0.87 g dry wt/l, which was 16% less than that of the wild-type (1.04 g dry wt/l) containing the water-soluble branched polysaccharide acetan. The increase in the production of cellulose, but decrease in the production of water-soluble polysaccharides, in the GMT-defective mutant may be due to the inability to synthesize the mannose-containing acetan, suggesting that part of the sugar molecules, especially glucose, usually used up for the formation of side chains of acetan in wild-type cells may be supplied as extra sugars for overproduction of cellulose in the *aceA*-disrupted mutant defective in GMT.

It has been recently reported that an acetan-nonproducing mutant of *A. xylinum* BPR2001 defective in a gene for β-glucosyltransterase, growing in a jar fermentor, produced less cellulose than the wild strain because of decrease in the viscosity of the culture broth, which is contradictory to the present results [3].

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