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Effects of Acetan on Production of Bacterial Cellulose by *Acetobacter xylinum*

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Acetan is a water-soluble polysaccharide produced by a bacterial cellulose (BC) producer, *Acetobacter xylinum*. An acetan-nonproducing mutant, EP1, was generated from wild-type *A. xylinum* BPR2001 by the disruption of *aceA*, which may act to catalyze the first step of the acetan biosynthetic pathway in this bacterium. EP1 produced less BC than the wild-type strain. However, when EP1 was cultured in a medium containing acetan, BC production was stimulated and the final yield of BC was equivalent to that of BPR2001. The culture broth containing acetan was more viscous and the free cell number was higher than that of the broth without the polysaccharide, so acetan may hinder the coagulation of BC in the broth. The addition of 1.5 g/l agar also increased BC production; we concluded that acetan and BC syntheses were not directly related on the genetic level.

Key words: *Acetobacter xylinum*; bacterial cellulose; acetan; viscosity

Bacterial cellulose (BC), produced by several bacterial species, has attracted attention as a biodegradable material.[1,2] *Acetobacter xylinum*, which is a producer of BC,[3,4] also accumulates a water-soluble polysaccharide called acetan,[5] the structure of which is similar to that of xanthan.[6,7] BC is an insoluble β-1,4-glucan, and acetan consists of glucose, mannose, glucuronic acid, and rhamnose in the proportions of 4:1:1:1. Genetic analysis of BC showed that the *bcs* operon is associated with cellulose synthesis.[8] In contrast, the genetics of the acetan biosynthetic process has not been clarified[9,10] although the biochemical pathway has been elucidated.[11–14] Because the production patterns during culture of the two extracellular polysaccharides are similar, we have speculated that there is a relationship between BC and acetan in their productivity; however, to our knowledge, there has been no report on a relationship between acetan and BC biosynthesis. If a mutant that lacks only acetan biosynthetic ability can be obtained, the speculation will be clarified. For investigation of any relationship between BC and acetan biosynthesis, the disruption of acetan-synthesizing genes is needed.

In this work, we cloned *aceA*, which encodes a β-glucosyltransferase in acetan biosynthesis,[15] from *A. xylinum* BPR2001, and prepared an acetan-nonproducing mutant by the disruption of *aceA*. By comparing BC production rates between the parent strain and the mutant, we made deductions about the role of acetan in BC production.

Materials and Methods

Bacterial strains and plasmids. A. xylinum subsp. *sucrofermentans* BPR2001, which produces BC and acetan, was used as the wild-type strain.[16] *Escherichia coli* DH5α was used for cloning and sequencing of *aceA* from BPR2001. pAP1 was a cosmid clone harboring *aceA* of the BPR2001 strain. pKF18k and pKF19k were used for DNA sequencing. pUCA-EP, which is pUC18 harboring a 0.7-kb *Eco*RV-*Pst*I fragment of *aceA*, was used for the disruption of *aceA* on the chromosome of BPR2001 with homologous recombination. A genomic library of *A. xylinum* BPR2001 strain[17] was obtained from Dr. T. Hayashi of Kyoto University.

Media. A. xylinum was grown on FPY consisting of 2% fructose, 1% Polypepton (Nihon Seiyaku Co., Ltd., Tokyo, Japan), 0.5% yeast extract (Oriental Yeast Co., Ltd., Tokyo), 0.25% K_2HPO_4 (pH 5.0), or else on a medium of corn steep liquor (Nihon Starch Industry, Kagoshima, Japan) and fructose (CSL-Fru), the composition of which was described elsewhere,[18] at 30°C. *E. coli* DH5α was grown on Luria-Bertani medium consisting of 1% Tryptone peptone (Becton Dickinson and Company, Sparks, Maryland, USA), 0.5% yeast extract (Becton Dickinson) and 1% NaCl at 37°C. Ampicillin (Sigma Chemical Co., St. Louis, Missouri, USA) was added at 50 ppm for *E. coli* and at 100 ppm for *A. xylinum*, and kanamycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added at 100 ppm for *E. coli* when necessary.

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Abbreviations: BC, bacterial cellulose; CSL, corn steep liquor

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Primers. The sequences of aceA1, aceA2, aceE2, and aceF1 were 5′-ATGGCAAGGGGAGCTGTATC-3′, 5′-ACGCATTCCGTGAGAAAATTTC-3′, 5′-CATTTGATCAGAAGCCACAGC-3′, and 5′-TGACAGCAACGGATGTAACC-3′, respectively. The sequences of M13F and M13R were 5′-TTTCCCAGTCACGACGTTG-3′ and 5′-GGATAACAATTTCACACAGG-3′, respectively. The sequence of aceA214c for primer extension was 5′-AAATGCGCTGCCAGGCGATG-3′, and this was labeled with infrared dye-800 (IRD-800, Nisshinbo Industries Inc., Tokyo).

Genetic manipulation. Total DNA from *A. xylinum* was isolated as described elsewhere.[19] All PCR except for sequencing was done in a volume of 50 μl with denaturation at 94°C for 5 min and then 30 cycles of 1 min at 94°C, 1 min at 55–60°C, and 1–3 min at 72°C with r*Taq* polymerase (Takara Shuzo Co., Ltd., Shiga, Japan). Restriction digestion, DNA modification, ligation, and primer extension were done as the supplier suggested. Minipreparation of plasmids from *E. coli* was done by alkaline extraction with a FlexiPrep kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). Total RNA from *A. xylinum* BPR2001 was extracted with an RNeasy mini kit (Qiagen GmbH, Hilden, Germany). A digoxygenin (DIG) DNA labeling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for labeling the probe DNA, detection of colonies, and Southern analysis. DNA was sequenced by dideoxy chain termination, with a ThermoSequenase cycle sequencing kit (Amersham Pharmacia), IRD-800-labeled M13F and M13R primers, and a DNA sequencer (Model 4000L, Li-Cor Inc., Lincoln, Nebraska, USA). Vector NTI Suite (InforMax Inc., North Bethesda, Maryland) was used for sequence analysis. DNA and deduced amino acid sequence similarity searches were done with FASTA and BLAST network services. Transformation of *E. coli* and agarose gel electrophoresis were done by standard protocols.[20] Transformation of *A. xylinum* was done by electroporation[20] with a Cell-Porator (Life Technologies Inc., Gaitherburg, Maryland).

Culture conditions and analytical methods. A. xylinum BPR2001 and the acetan-nonproducing mutant EP1 were first cultured in 120 ml CSL-Fru medium in 500-ml Erlenmeyer flasks with three slant baffles[21] (Altair Co., Ltd., Yokohama, Japan) and shaken at 30°C at 180 rpm for one day. A 5-ml portion of this culture was used to inoculate fresh medium in a flask, with acetan or agar added (Shimizu Shokuhin, Shizuoka, Japan), and the flask was shaken at 30°C at 180 rpm. The culture broth was homogenized with an Excel Auto Homogenizer (Nihonseiki Kaisha Ltd., Tokyo) at 10,000 rpm for 1 min and diluted with 0.1 M potassium acetate buffer (pH 5.0) before being examined for the number of viable cells and the concentrations of BC, acetan, and residual fructose. After treatment of the culture broth containing BC with cellulase (Celluclast, Novo Nordisk A/S, Bagsværd, Denmark) at 30°C for 1 h, cells were counted by the dilution plate method and expressed as colony-forming units (cfu). The value found was defined as the total cell number because the number was the sum of cells entrapped in BC and cells suspended in the culture broth. The cell number found by the plate dilution method without homogenization or cellulase treatment was defined as the free cell number, that is, the number of cells suspended in the culture broth. After the homogenized culture broth was centrifuged, the precipitate was treated with 0.1 M NaOH for 20 min at 80°C, rinsed three times with deionized water, vacuum-dried, and assayed for BC. The supernatant obtained by centrifugation of the homogenized culture broth was then treated with two volumes of ethanol, and the precipitate was rinsed with the potassium acetate buffer and assayed for acetan by the phenol-sulfate method.[18,22] Residual fructose was assayed by HPLC as previously described.[18]

Preparation of acetan. The supernatant of the culture broth of *A. xylinum* BPR2001 after cultivation for 5 days in CSL-Fru medium was obtained by centrifugation, and was treated with two volumes of ethanol. The acetan that precipitated was air-dried and the residue was dissolved in 0.1 M NaOH. An equal volume of chloroform was added to the acetan solution and the mixture was gently mixed in a 50-ml conical tube (Becton Dickinson). After the tube was centrifuged, the aqueous phase was precipitated with ethanol and air-dried. The residue was dissolved in deionized water. The final concentration of acetan was about 30 g/l.

Viscosity measurement of media. The viscosity of the CSL-Fru medium containing acetan or agar was measured with an Ostwald viscometer (capillary inner diameter, 1.0 mm; Sibata Scientific Technology Ltd., Tokyo). Viscosity was calculated relative to the viscosity of H_2O. Measurements were done at 30°C.

Results

Cloning and sequencing of aceA *from* A. xylinum *BPR2001*

An 8.9-kb DNA fragment was amplified by PCR with total DNA of *A. xylinum* BPR2001 as the template and primers aceF1 and aceE2, and three fragments were obtained when the fragmet was digested with *Eco*RI. BLAST and FASTA analyses of the DNA sequence of one of the three fragments (2.7-kb) showed 77.5% identity with that of *aceA* of *A. xyli-*

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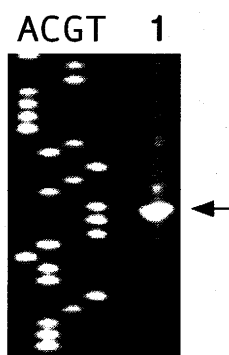

Fig. 1. Transcriptional Initiation Site of *aceA* (lane 1).
Total RNA was extracted from BPR2001 and extended with primer aceA214c. The length of the extension product was measured against the terminated products produced in DNA sequencing reactions with the same primer. A, C, G, and T indicate the individual sequencing reactions. The arrow indicates the transcriptional initiation site.

num C1.[15] Primers aceA1 and aceA2 were designed from the sequence data of the 2.7-kb *Eco*RI fragment. PCR was done with BPR2001 total DNA as the template, and a 1.5-kb fragment was generated. This PCR product was labeled with DIG and used to screen a genomic library of *A. xylinum* BPR2001. Nine clones gave positive signals among about 3,000 clones. These clones were digested with *Eco*RI, and a 2.7 kb-DNA fragment in each clone was hybridized with the *aceA* probe by Southern blotting. One clone, pAP1, was selected.

The 2.7-kb *Eco*RI fragment of pAP1 was cloned into pKF18k and pKF19k, and its DNA was sequenced. Computer analysis of the sequence showed the presence of an open reading frame (ORF). The transcriptional initiation site of this ORF was identified by the primer extension method (Fig. 1). We assumed that the gene product of this ORF was 523 amino acid residues, from results of computer analysis of its DNA sequence, but the transcriptional initiation site was downstream from the putative translational initiation codon, suggesting that the number of amino acid residues was 469. The amino acid sequence deduced from this ORF, assigned to *aceA*, was very similar to that of AceA (84.2% identity, 90.0% similarity) from *A. xylinum* C1 and GumD (29.9% identity, 45.2% similarity) from *Xanthomonas campestris*. The accession number of this sequence in the DNA Data Bank of Japan is AB059427.

Disruption of aceA *gene in BPR2001*

To obtain an acetan-nonproducing mutant, we used a truncated gene strategy[9] to disrupt *aceA*. *A. xylinum* BPR2001 was transformed by electroporation with pUCA-EP (described in Materials and Methods). Ampicillin-resistant colonies appearing on FPY agar plates containing ampicillin were homologous-recombinant on the chromosome because pUCA-EP was unable to replicate in *A. xylinum*. One of these transformants, EP1, was selected, and the recombination was confirmed by PCR. Using the total DNA of each strain as the template with primers aceA1 and aceA2, we obtained 1.5-kb and 4.9-kb DNA fragments were obtained from *A. xylinum* BPR2001 and EP1, respectively. This result indicated that a 3.4-kb DNA fragment had been taken into *aceA*. In combinations of primers aceA1 and M13F, or aceA2 and M13R, 0.9- or 1.2-kb DNA fragments were amplified from EP1, but no product was obtained from *A. xylinum* BPR2001. By arrangement of these data, pUCA-EP was seen to be integrated into the chromosome of the EP1 strain, and *aceA* was seen to be disrupted.

Table 1. Relative Viscosity of Media

Medium	Relative viscosity*
CSL-Fru	1.10
CSL-Fru + 1.5 g/l acetan	2.43
CSL-Fru + 2.9 g/l acetan	5.38
CSL-Fru + 1.5 g/l agar	5.38

Each medium was autoclaved, and the measurement was at 30°C.
* The viscosity of H_2O was taken to be 1.00.

Viscosity of media

The relative viscosity increased when the acetan concentration increased (Table 1). The CSL-Fru medium with 1.5 g/l agar had the same relative viscosity as with 2.9 g/l acetan.

Cultivation of EP1 strain

A. xylinum BPR2001 and the acetan-nonproducing mutant EP1 produced BC at the same rate in the first two days, but production by EP1 became lower than that of BPR2001 after day 5. When 1.5 g/l acetan or agar was added to the EP1 culture medium at the start of the experiment, the production rate of BC for the first two days was increased, being higher than that of BPR2001, and the final yield of BC was almost the same as that of BPR2001 on day 5. With 2.9 g/l acetan, with the same relative viscosity as 1.5 g/l agar, the production rate further increased, and the final concentration of BC was higher than that of BPR2001 (Fig. 2).

The culture broth of EP1 containing 1.5 g/l acetan was more dispersed than that of BPR2001 or EP1 without acetan, in which large flocks of BC formed (Fig. 3). The free cell numbers of EP1 increased with the addition of acetan or agar although the total cell numbers were similar (Fig. 4). Perhaps the increase in free cell numbers, which corresponded to cells that actively produced BC, was associated with the high relative viscosity of the media.

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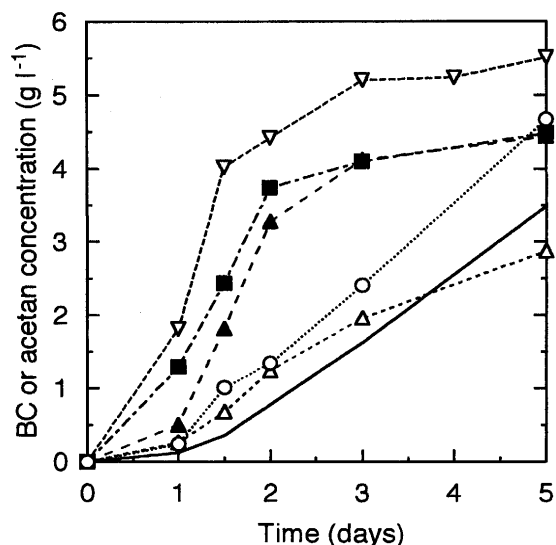


Fig. 2. Bacterial Cellulose (BC) Concentration When *A. xylinum* BPR2001 and Acetan-Nonproducing Mutant EP1 Are Cultivated in CSL-Fru Medium.

Symbols: ○, BPR2001; △, EP1; ▲, EP1 with 1.5 g/l acetan; ▽, EP1 with 2.9 g/l acetan; ■, EP1 with 1.5 g/l agar. — shows acetan production by BPR2001.

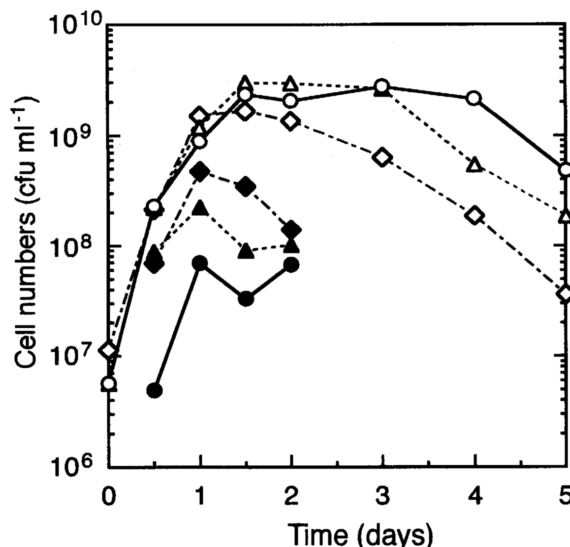
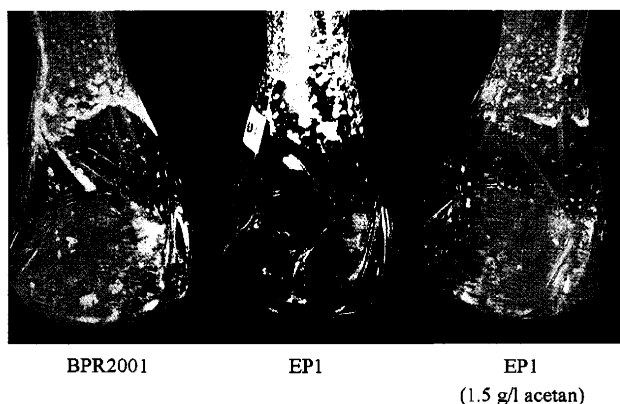


Fig. 4. Total and Free Cell Numbers of EP1 in the Culture with and without Addition of Polysaccharides.

Symbols: ○ and ●, total and free cell numbers without polysaccharides, respectively; ◇ and ◆, total and free cell numbers with addition of 2.9 g/l acetan, respectively; △ and ▲, total and free cell numbers with addition of 1.5 g/l agar, respectively.



BPR2001 EP1 EP1 (1.5 g/l acetan)

Fig. 3. Culture Broth after Cultivation for 2 Days in CSL-Fru Medium.

Left flask, *A. xylinum* BPR2001; Center flask, the acetan-nonproducing mutant EP1; Right flask, EP1 when acetan was added to 1.5 g/l (initial concentration).

Discussion

Acetan-nonproducing mutants without other mutations have not been available, mainly because chemical mutagenesis can cause mutation of other genes such as BC-synthesizing genes. In this study, we obtained an acetan nonproducer by the disruption of only *aceA* by the gene-truncation method.

Because the 2.7-kb *Eco*RI fragment from PCR with *A. xylinum* BPR2001 total DNA as the template contained a region with sequence similarity to *aceA* from *A. xylinum* C1, we sequenced this complete region from pAP1 screened from a BPR2001 genomic library. The deduced amino acid sequence corresponding to *aceA* from *A. xylinum* BPR2001 was homologous to that of AceA from *A. xylinum* C1, that is predicted to be a β-glucosyltransferase that catalyzes the first step of the acetan biosynthetic pathway to construct glucose-diphosphopolyprenol as an intermediate of acetan.[15] We have not elucidated the function of AceA yet, but the disruption of *aceA* led to the derivation of the acetan-nonproducing mutant EP1, suggesting that *aceA* is critical for acetan biosynthesis.

EP1 produced less BC than the parent strain BPR2001. However, with 1.5 g/l acetan in the culture medium of EP1, the production of BC was stimulated during the first two days, and the final concentration of BC was almost identical to that of the control culture of BPR2001. These results suggested that acetan has a physicochemical effect on the culture conditions and that there is no genetic relationship between BC and acetan biosynthesis. When 1.5 g/l agar was present in EP1 culture, BC production was stimulated by acetan as before. When 2.9 g/l acetan, with relative viscosity identical to that of 1.5 g/l agar was present, the production rate and final yield of BC with EP1 were larger than those with BPR2001.

Culture medium containing acetan or agar became viscous. The viscosity of the culture medium increased with the addition of polysaccharides. The culture broth of EP1 after 2 days became a heterogeneous suspension containing large flocks formed by the aggregation of cells and BC. However, the addition of water-soluble polysaccharides such as ace-

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tan or agar led to a more dispersed culture broth where the free cell number was increased. Therefore, we speculate that the addition of acetan increased the viscosity of the culture broth, by hindering coagulation. Probably, increase in viscosity does not influence cell growth because only the free cell number was raised; the total cell number was not affected in EP1 culture until after 2 days. Increased viscosity will lead to more dispersed BC or cells and a more efficient uptake of oxygen or nutrients by the cells.

A. xylinum BPR2001 accumulates BC and acetan in a similar pattern, so the effect of acetan on BC productivity is not as great as when the acetan is added at the start. However, the difference in BC productivity between BPR2001 and EP1 after 5 days reflected the effect of acetan produced by BPR2001 on its BC production rate. If acetan is produced much earlier than BC in *A. xylinum* BPR2001, the cultivation time taken to reach maximum BC production will be shortened.

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