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Structural characterization of pellicle polysaccharides of *Acetobacter tropicalis* SKU1100 wild type and mutant strains

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ABSTRACT

Mutants of *Acetobacter tropicalis* SKU1100 (R) strain, Δ*polE* and Δ*galE*, defective in pellicle formation, excrete exopolysaccharide (EPS) instead of capsular polysaccharide (CPS) that is produced by the wild-type. We carried out structural analysis of wild type CPS and mutant EPSs using monosaccharide composition analysis, methylation analysis, and ^{1}H and ^{13}C NMR spectroscopy. Wild-type CPS and Δ*polE* mutant EPS had a branched hexasaccharide repeating unit composed of 2 moles of 2,3-α-L-rhamnopyranosyl, and 1 mole each of 6-β-D-galactopyranosyl and 2-α-D-glucopyranosyl residues, of which the rhamnosyl residues were branched by terminal-β-D-galactofuranosyl and terminal-α-D-glucopyranosyl residues. The EPS of Δ*galE* mutant showed a branched tetrasaccharide repeating unit composed of 2,3-α-L-rhamnopyranosyl, 2-α-D-glucopyranosyl, and 3-α-L-rhamnopyranosyl residues and terminal-α-D-glucopyranosyl branch residue. By comparing the three structures, it was suggested that PolE may control the switching of EPS to CPS by adding some residue, e.g. β-D-galactopyranosyl residue, to 2,3-α-L-rhamnopyranosyl residue to make 2,3,4-α-L-rhamnosyl residue which leads to CPS formation.

1. Introduction

Polysaccharide production is common among both Gram-positive and Gram-negative bacteria. These polysaccharides are classified according to their cellular association into capsular polysaccharide (CPS), which is permanently attached to outer surface of the cells, and exopolysaccharide (EPS) which is secreted into the growth medium. Attention has been paid to bacterial polysaccharides due to their importance in bacteria–host interaction, and biofilm formation (Roberts, 1996), stress adaptation (Ferreira et al., 2010), resistance to desiccation (Ophir & Guntnick, 1994), and their applications in food industry (Sutherland, 1994).

Acetic acid bacteria are Gram-negative obligate aerobes belonging to α-proteobacteria subdivision and well known as vinegar producers. In order to keep high aeration state, almost all *Acetobacter* species have ability to grow floating in static culture by producing a pellicle in the surface of culture medium. This pellicle is an aggregation of cells in the liquid–air interface in which cells are tightly associated with each other by polysaccharides and other extracellular matrix on the cell surface. The pellicle polysaccharides occur as homopolysaccharide of cellulose which is produced by *Gluconacetobacter xylinus*, or as heteropolysaccharides such as CPS produced by *Acetobacter tropicalis* SKU1100 consisting of glucose, galactose, and rhamnose (Moonmangmee, Toyama et al., 2002) or CPS of *Acetobacter aceti* IFO3284 (reclassified as *Acetobacter pasteurianus* subsp. Lovaniensis) consisting of glucose and rhamnose (Moonmangmee, Kawabata et al., 2002). *A. pasteurianus* IFO3284 produces two different types of colony on agar surface; rough surfaced colony (R strain) and smooth surfaced colony (S strain). The R strain can produce pellicle which allows it to float on the medium surface in static culture, while S strain cannot produce pellicle in static culture. The R and S strains are interconvertible by spontaneous mutation (Matsushita, Ebisuya, Ameyama, & Adachi, 1992).

The genetic study of polysaccharides in acetic acid bacteria has shown that the *acs* (Saxena, Kudlicka, Okuda, & Brown, 1994) and the *bcs* (Wong et al., 1990) operons, in addition to ORF2 gene (Nakai, Nishiyama, Kuga, Sugano, & Shoda, 2002), are involved in cellulose biosynthesis, and the *aceRQP* operon in acetan biosynthesis in *Ga. xylinus* (Ishida, Sugano, & Shoda, 2002). Moreover, a gene cluster, *polABCDE*, required for pellicle formation in the R strain of *A. trop-*

Abbreviations: CPS, capsular polysaccharide; EPS, exopolysaccharide; Galf, galactofuranose; Galp, galactopyranose; Glcp, glucopyranose; PCP, polysaccharide co-polymerase proteins; PMAA, partially methylated alditol acetates; R, rough surfaced colony; Rhap, rhamnopyranose; S, smooth surfaced colony; TFA, trifluoroacetic acid; TSP, sodium 3-trimethylsilyl-(2,2,3,3,-$^{2}H_4$)-propanoate.

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icalis SKU1100, has been identified (Deeraksa et al., 2005). In this operon, *polABCD* showed high similarity to *rfbBACD* genes which are involved in dTDP-L-rhamnose biosynthesis, while *polE*, a novel gene downstream of *polABCD,* had a relatively low similarity to glycosyltransferases. The disruption of this gene caused the bacteria not to form pellicle in static culture because of no capsular polysaccharide production. Interestingly instead, the mutant cells secreted EPS in the culture medium (an S strain like phenotype). Moreover, the mutation sites in S strain of *A. tropicalis* SKU1100 are found to be in a 7 C repeated residues in the coding region of *polE* gene. Hence, it was hypothesized that the *polE* gene may be involved in the switching of CPS to EPS. In addition, *galE*, a gene that encodes UDP-galactose 4-epimerase involved in UDP-galactose biosynthesis, has also been identified in *A. tropicalis* SKU1100. The disruption of this gene also resulted in no pellicle formation in static culture but secretion of EPS composed of glucose and rhamnose in culture medium (Deeraksa, Moonmangmee, Toyama, Adachi, & Matsushita, 2006).

In this study, we elucidated the structures of the wild type CPS as well as EPSs of Δ*polE* and Δ*galE* mutants of *A. tropicalis* SKU1100 (R). Based on the structural information, the function of *polE* gene was also discussed here.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. tropicalis SKU1100 (R) strain and its mutants Δ*polE* and Δ*galE* (Deeraksa et al., 2005, 2006) were used in this study. In Δ*polE* mutant, the *polE* gene was disrupted by insertion of Tn10 transposon, while, in Δ*galE,* the *galE* gene (UDP-galactose 4-epimerase) was disrupted by insertion of non-polar kanamycin cassette. All strains were grown at 30 °C in potato medium (Deeraksa et al., 2005). Antibiotics, kanamycin or tetracycline, were added at the concentration of 50 μg/ml, or 12.5 μg/ml, respectively.

2.2. Purification of A. tropicalis SKU1100 CPS

The CPS was purified according to the method of Moonmangmee, Toyama et al. (2002). Briefly, 10% of the seed culture was inoculated to 1 L of potato medium and incubated at 30 °C with shaking for 30 h. The cells were then collected, washed 2 times with 50 mM phosphate buffer (pH 6.5), and resuspended in the same buffer (1 g cells/10 ml buffer). The suspension was ultrasonicated using sonicating microprobe (TP-040, 3 mm diameter, TOMY TECH, INC) for 20 min, centrifuged, and ultra-centrifuged to remove cell debris. DNase (50 μg/ml) was added to the supernatant and it was incubated at 37 °C overnight, followed by an additional overnight incubation with 100 μg/ml proteinase K at 37 °C. The suspension was then subjected to dialysis against 25 mM Tris–HCl buffer (pH 8.5) overnight. After centrifugation to remove precipitate, the supernatant was applied to a DEAE–cellulose column and eluted with 25 mM Tris–HCl (pH 8.5). Polysaccharide fractions were determined by phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Robers, & Smith, 1956), pooled, ultra-centrifuged, and precipitated with 2 volumes of cold ethanol. The precipitated polysaccharide was then dissolved in 0.1 M NaCl and applied to a Superdex S-200 column. The polysaccharide fractions were pooled and precipitated with 2 volumes of cold ethanol.

2.3. Purification of ΔpolE and ΔgalE mutants EPSs

The EPSs of Δ*polE* and Δ*galE* mutants were purified from culture media by basically the same method as described (Deeraksa et al., 2006). The cultivations were performed in 1 L of YPG medium (5% glycerol, 0.5% peptone, and 0.5% yeast extract) for 2 days. The cells were removed by centrifugation (9000 × *g* for 10 min at 4 °C). The culture media was then collected and concentrated to one third using ultra filtration (20 kDa cutoff, Advantec), followed by DEAE–cellulose column chromatography as described above. Polysaccharide fractions were then pooled, and treated as described above, and applied to a Sephacryl S-400 column equilibrated with 0.1 M NaCl. Polysaccharide fractions were combined and precipitated with 2 volumes of cold ethanol.

2.4. Monosaccharide composition and molecular size analysis

The monosaccharide composition was analyzed using trimethylsilyl (TMS) methylglycoside method as described by Wozniak et al. (2003). Aliquots of 300-μg of each polysaccharide were lyophilized, and then, mixed with 500 μl of 1 M methanolic HCl and heated for 16 h at 80 °C. The methanolysis product was dried, followed by the addition of 20 drops of methanol and drying 2 times. The samples were then, acetylated with pyridine and acetic anhydride in methanol in a 1:1:2 ratios at room temperature for 30 min, followed by per-O-trimethylsilylation with 200 μl of Tri-Sil (Pierce) and heating for 20 min. Then it was dried, dissolved in 2 ml of hexane, centrifuged, filtered through glass wool, and dried down to 100 μl. Aliquots of 1 μl were analyzed by GC–MS (Agilent 6890N), using a HP-5 m capillary column (Agilent 30 m × 0.25 mm) and mass selective detector (electron impact ionization mode). The oven temperature was programmed to increase from 80 °C (2 min), to 140 °C (2 min) at a rate of 20 °C/min, to 200 °C, at a rate of 2 °C/min, and to 250 °C, (5 min) at a rate of 30 °C/min. The resulting peaks were identified by comparing their retention times with those of standard sugars using inositol as internal standard. For quantification, detector response factors (RF) were calculated for each standard sugar from peak area and weight of standard sugars and internal standard. RF values were used to calculate the weight of each component.

Molecular size was determined by gel filtration chromatography on Sephacryl S-400 column (1.6 cm × 90 cm) equilibrated and eluted with 0.1 M NaCl at a flow rate of 1 ml/min. Polysaccharide fractions were monitored by phenol-sulfuric acid assay. Pullulan P-100 (100 kDa), P-400 (376 kDa), and P-800 (758 kDa) were used as molecular size standards (Showa Denko K.K., Tokyo, Japan).

2.5. Glycosyl linkage analysis

The purified polysaccharides were methylated, hydrolyzed, reduced, and acetylated. The partially methylated alditol acetates (PMAAs) thus obtained were analyzed by GC–MS according to York, Darvill, McNeil, Stevenson, and Albersheim (1985). Aliquots of each polysaccharide (1.0 mg) were permethylated using the method of Ciucanu and Kerek (1984). The samples were suspended in 1 ml of DMSO, mixed with 0.7 ml of 1 M NaOH in DMSO, and incubated for 10 min. Then, 0.1 ml of methyl iodide was added to the suspension and it was incubated for 10 min. The permethylation was repeated twice using 0.2 ml of methyl iodide for 40 min to facilitate complete methylation of the polysaccharides in the second methylation. After that, the permethylated polysaccharides were extracted in organic phase of 1:1 dichloromethane–water, then hydrolyzed with 2 M trifluoroacetic acid (TFA) for 2 h at 121 °C, and reduced with sodium borodeuteride (10 mg/ml in 1 M ammonia) overnight. The samples were neutralized with methanolic acetic acid, dried in methanol, and acetylated with 0.25 ml of acetic anhydride and 0.23 ml of TFA at 50 °C for 10 min. After extraction into the organic phase of dichloromethane–Na_2CO_3, the PMAAs obtained were analyzed by GC–MS (Hewlett–Packard) using a Sp2330 capillary column (Supelco, 30 m × 0.25 mm) and mass selective detector (electron impact ionization mode). The oven temperature was programmed to increase from 80 °C (2 min) to 170 °C at a rate of