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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

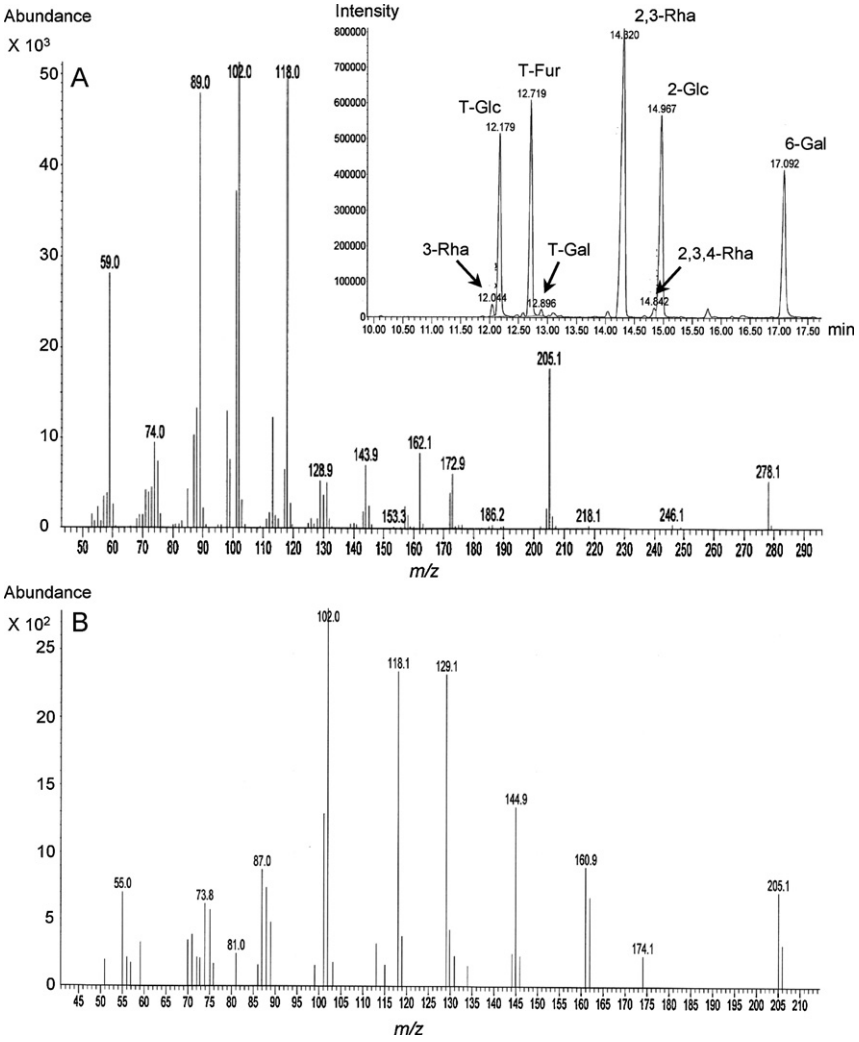

Fig. 1. Electron impact ionization mass spectrum of terminal Galf residue (A) and of terminal Galp residue (B) of *A. tropicalis* SKU1100 (R) wild-type CPS. The analysis was carried out with the two peaks of retention times, 12.719 min of a terminal furanose residue (T-Fur) and 12.896 min of a terminal galactose residue (T-Gal), in GC–MS analysis of partially methylated alditol acetates of *A.tropicalis* SKU1100 wild-type CPS (Inset). As described in the text, the two peaks (T-Fur and T-Gal) are regarded as Terminal-Galf and Terminal-Galp, respectively (Table 1). Another abbreviations are also used in the Inset: T-Glc; terminal glucose, 3-Rha; 3-rhamnose, 2,3-Rha; 2,3-rhamnose, 2-Glc; 2-glucose, 2,3,4-Rha; 2,3,4-rhamnose, and 6-Gal; 6-galactose.

30 °C/min and to 240 °C (20 min) at a rate of 4 °C/min. The resulted peaks were identified by comparing their retention times and mass spectra with those of PMAA of standard sugars, and quantified as mentioned above.

2.6. NMR spectroscopy

NMR analysis was done using a Bruker 400 MHz spectrometer with a 5 mm sample tube, which was kept at 60 °C. Polysaccharide samples were first exchanged with D_2O by dissolving in D_2O and lyophilizing 3 times to minimize the residual HDO. Standard pulse sequences from Bruker were used to obtain 1D proton, ^{13}C, 2D HSQC, COSY, TOCSY, and NOESY spectra. Chemical shifts were reported in ppm downfield from internal sodium 3-trimethylsilyl-(2,2,3,3,-$^2H_4$)-propanoate (TSP).

3. Results

3.1. Glycosyl composition and molecular size analysis

Glycosyl compositional analysis using trimethylsilyl methylglycosides revealed that CPS of *A. tropicslis* SKU1100 (R) wild type strain was composed of glucose, galactose, and rhamnose in approximately equimolar ratio. Δ*polE* mutant EPS showed the same glycosyl composition as wild type (R) CPS, while Δ*galE* mutant EPS composition was glucose and rhamnose in approximately equimolar ratio. In gel filtration chromatography, EPSs of both Δ*polE* and Δ*galE* mutants were eluted monodispersely at almost the same elution volume, which is earlier than that of wild type CPS (120 kDa) (Moonmangmee, Toyama et al., 2002), and the molecular size of both EPSs were estimated to be around 620 kDa.

3.2. Glycosyl linkage analysis

In order to know the sugar linkages of these polymers, methylation analysis of purified polysaccharides was carried out. As summarized in Table 1, wild type CPS contains 2,3-linked rhamnose residue, terminal and 2-linked glucose residues, terminal furanose residue, and 6-linked galactopyranose (Galp) residue as major components, and some minor components, double branched 2,3,4-rhamnose, terminal Galp, and 3-linked rhamnose (see inset of Fig. 1). EPS of Δ*polE* mutant had the same major components as those of the CPS and increased number of minor components such as glucose, galactose, and rhamnose residues. For Δ*galE* mutant

Table 1
Methylation analysis of *A. tropicalis* SKU1100 wild type CPS, Δ*polE* mutant EPS, and Δ*galE* mutant EPS.

Sugar residue	Moles%		
	SKU100 (R)	ΔpolE mutant	ΔgalE mutant
2,3-Rhamnose	33.5	28.5	23.9
2-Glucose	18.6	18.1	28.0
Terminal-Galf	17.3	18.1	NF
6-Galactose	14.1	12.8	NF
Terminal-glucose	14.8	17.0	21.5
2,3,4-Rhamnose	0.69	NF	NF
3-Rhamnose	0.34	1.70	24.2
Terminal-galactose	0.34	3.10	NF
6-Glucose	NF	0.34	0.34
3-Glucose	NF	0.34	0.30
3,4-Glucose	NF	0.34	NF
2,6-Glucose	NF	0.34	0.34
2-Rhamnose	NF	0.20	0.34
4,6-Galactose	NF	0.10	NF
4-Glucose	NF	NF	0.68
2,3-Glucose	NF	NF	0.23

NF: not found.

EPS, 2,3-linked rhamnose, 3-linked rhamnose, terminal and 2-linked glucose residues were the major peaks and some minor peaks of glucose, and rhamnose. The absence of the furanose residue was observed in Δ*galE* mutant EPS suggesting that the terminal furanose residue observed in CPS and Δ*polE* EPS may be a galactofuranose (Galf) as Δ*galE* mutant cannot produce galactose. To further check this possibility, we examined the electron impact ionization mass spectrum of the terminal furanose residue (Fig. 1A) compared with the terminal Galp residue (Fig. 1B). The mass spectrum of the former peak was different from that of the latter peak, but rather the same spectrum as terminal Galf electron impact ionization spectrum (Sassaki, Iacomini, & Gorin, 2005). Thus, these results confirmed that the terminal furanose residue was a Galf.

3.3. NMR analysis

Proton and ^{13}C NMR spectroscopies were used to determine the number of residues in the repeating units in *A. tropicalis* SKU1100 polysaccharides. The 1D proton spectrum of *A. tropicalis* SKU1100 wild type CPS showed five signals (A/B, C, D, E, and G in Fig. 2A; A/B is overlapped as shown in Fig. 3) in the anomeric region (4.4–5.5 ppm), crowded ring region, and two signals corresponding to methyl group of 6-deoxysugars (CH_3 in Fig. 2A). EPS of Δ*polE* mutant exhibited a typical 1D proton spectrum as much the same as wild type CPS (Fig. 2B). While, Δ*galE* mutant EPS showed four signals (A, C, D, and F in Fig. 2C) in the anomeric region, and the characteristic methyl group signal of 6-deoxysugars. Furthermore, ^{13}C NMR analysis exhibited six anomeric signals (A, B, C, D, E, and G) in the case of wild type CPS and Δ*polE* mutant EPS (Fig. 3A and B). Since anomeric signal A in ^{1}H spectrum showed correlation to two signals of ^{13}C in HSQC spectrum (A and B, data not shown), A and B signals were shown to be overlapped in ^{1}H spectrum (Fig. 2A and B). Whereas, Δ*galE* mutant EPS showed the same four anomeric signals (A, C, D, and F in Fig. 3C) as in ^{1}H spectrum (Fig. 2C). Together with the methylation analysis results, these findings implied that SKU1100 wild type CPS and Δ*polE* mutant EPS had the same hexasaccharide repeating unit, while Δ*galE* mutant EPS had a tetrasaccharide repeating unit.

The 2D ^{1}H–^{1}H COSY, TOCSY, and ^{1}H, ^{13}C HSQC experiments, alongside with 2D NOESY, 1D ^{1}H, and ^{13}C analysis, were used to assign the proton and carbon chemical shifts, absolute configurations, and anomeric configurations of the polysaccharides. Proton chemical shifts assignment was hindered by signal overlapping in the ring region of SKU1100 wild type CPS and Δ*polE* EPS. However, together with the analysis of ^{13}C NMR and ^{1}H–^{13}C HSQC,

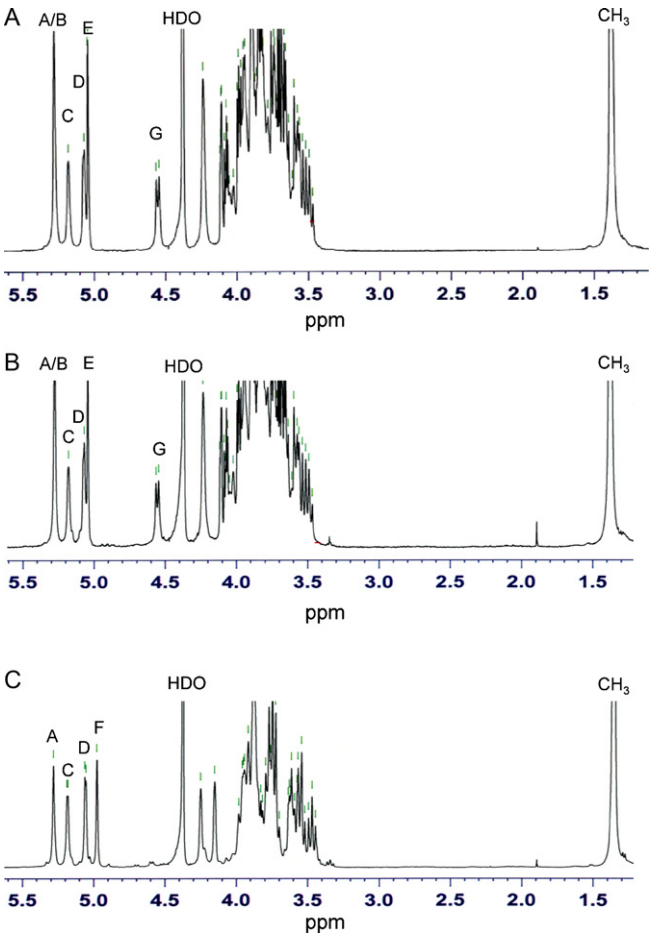

Fig. 2. Proton NMR spectra of *A. tropicalis* SKU1100 wild-type (R) CPS (A), Δ*polE* mutant EPS (B), and Δ*galE* mutant EPS (C). The spectra were recorded in D_2O at 60 °C with TSP as internal standard. The peaks named as A to G as described in the text (section 3.3).

the analysis of Δ*galE* mutant which has fewer number of residues than wild type CPS and Δ*polE* mutant EPS enabled us to complete the signal assignment. Anomeric proton and carbon signals labeled as A to E and G in wild type CPS (Figs. 2 and 3A), and Δ*polE* mutant EPS (Figs. 2 and 3B), and A, C, D, and F in Δ*galE* mutant EPS (Figs. 2 and 3C) from downfield upwards were identified by comparing their chemical shifts (ppm) with those reported in the literature (Gargiulo et al., 2008; Grimmecke et al., 1991; Hounsell, 1995; Landersjö, Yang, Huttunen, & Widmalm, 2002; Perepelov et al., 2010; Roslund, Tähtinen, Niemitz, & Sjöholm, 2008). As shown in Table 2, SKU1100 wild type CPS constituents were identified as 2-linked-α-D-glucopyranose (Glcp), terminal-α-D-Glcp, 2,3-linked-α-L-rhamnopyranose (Rhap) (2 residues), terminal-β-D-Galf, and 6-linked-β-D-Galp residues. EPS of Δ*polE* mutant showed the same sugar residues and configuration as wild type CPS, while Δ*galE* mutant EPS contained 2-linked-α Glcp, terminal-α-D-Glcp, 2,3-linked-α-L-Rhap, and 3-linked-α-L-Rhap. This identification was confirmed by ^{13}C assignment (Table 3) which showed the same constituents as mentioned above. Moreover, the characteristic down field shift of glycosylated carbon peaks confirmed the linkage analysis results. The small $J_{H1,H2}$ coupling constants (J = 2.2, 2.8 Hz) suggested that A and D residues were in α configuration. The anomeric carbon E resonated at 110.41 ppm (Table 3) indicating furanosidic ring form, and the small coupling (J = 1.6) confirmed its β-furanose configuration. Residue G showed large coupling constant (J = 7.5) of β-pyranose configuration (Table 2).

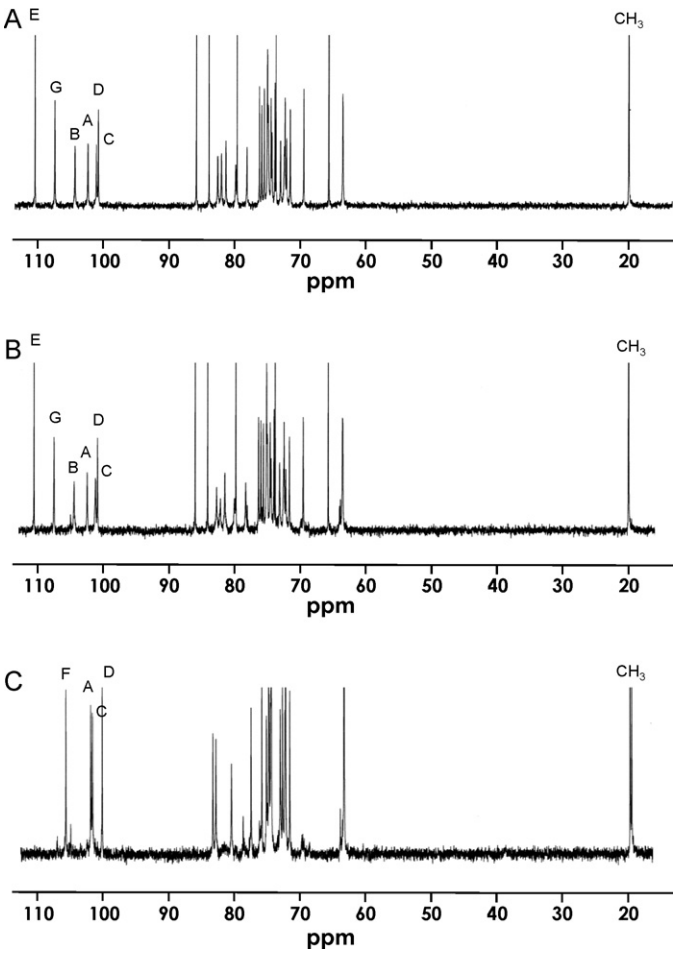

Fig. 3. ^{13}C NMR spectra of *A. tropicalis* SKU1100 wild-type (R) CPS (A), Δ*polE* mutant EPS (B), and Δ*galE* mutant EPS (C). The peaks named as in Fig. 2.

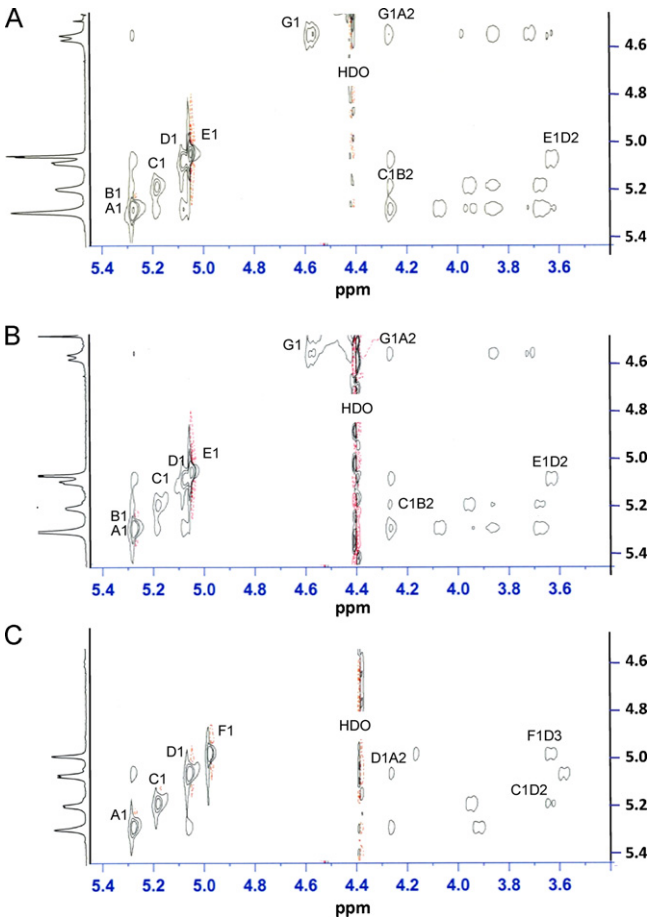

Fig. 4. Part of NOESY spectra of *A. tropicalis* SKU1100 wild-type (R) CPS (A), Δ*polE* mutant EPS (B), and Δ*galE* mutant EPS (C). The signal terminology is the same as described in Fig. 2. For example, D1A2 means the signal assigned as the interaction between H1-D and H2-A.

Fig. 4 shows the inter-residual correlations between the anomeric protons and nearby protons giving information about saccharides sequence in repeating unit. SKU1100 wild type CPS exhibited cross peaks between anomeric proton G and H-2 of A, indicating the link between A and G, and further did that anomeric protons of C and E show a correlation to H-2 of B and D respectively, indicating that C and E are branches. For Δ*polE* mutant EPS, similar cross peaks were observed (Fig. 4A and B). Whereas, in Δ*galE* mutant EPS, inter-residual correlations were observed between

Table 2
Proton chemical shifts of *A. tropicalis* SKU1100 CPS, Δ*polE* mutant EPS, and Δ*galE* mutant EPS. $J_{H1,H2}$ coupling constants (Hz) included in braces.

Sugar residue	Chemical shifts (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
CPS from SKU1100 wild type						
A → 2)-α-D-Glcp-(1 →	5.28{2.2}	4.23	4.15	3.82	3.63	3.7/3.72
B → 2,3)-α-L-Rhap-(1 →	5.28	4.23	4.02	3.60	3.8	1.38
C → α-D-Glcp-(1 →	5.18	3.61	3.91	3.44	3.57	3.79/3.77
D → 2,3)-α-L-Rhap-(1 →	5.07{2.8}	3.65	3.97	3.61	3.93	1.37
E → β-D-Galf-(1 →	5.04{1.6}	4.11	4.08	4.06	3.98	3.73
G → 6)-β-D-Galp-(1 →	4.56{7.5}	3.71	3.63	3.98	3.81	3.59
EPS from Δ*polE* mutant						
A → 2)-α-D-Glcp-(1 →	5.28{2.2}	4.23	4.15	3.82	3.61	3.65/3.72
B → 2,3)-α-L-Rhap-(1 →	5.28	4.23	4.02	3.61	3.8	1.38
C → α-D-Glcp-(1 →	5.18	3.60	3.94	3.47	3.57	3.79/3.75
D → 2,3)-α-L-Rhap-(1 →	5.07{2.8}	3.65	3.97	3.63	3.95	1.38
E → β-D-Galf-(1 →	5.04{1.6}	4.11	4.06	3.98	3.73	3.56
G → 6)-β-D-Galp-(1 →	4.56{7.5}	3.71	3.63	3.98	3.81	3.59
EPS from Δ*galE* mutant						
A → 2)-α-D-Glcp-(1 →	5.28{2.2}	4.25	3.91	3.59	3.63	3.88/3.76
C → α-D-Glcp-(1 →	5.19	3.61	3.91	3.44	3.57	3.79/3.77
D → 2,3)-α-L-Rhap-(1 →	5.06{2.8}	3.72	3.62	3.96	3.94	1.36
F → 3)-α-L-Rhap-(1 →	4.98	4.15	3.95	3.57	3.7	1.35

Chemical shift values were rounded to two digits.

Table 3
^{13}C chemical shifts of *A. tropicalis* SKU1100 CPS, Δ*polE* mutant EPS, and Δ*galE* mutant EPS.

Sugar residue	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
CPS from SKU1100 wild type						
A → 2)-α-D-Glcp-(1 →	102.35	78.15	74.47	75.89	74.89	63.46
B → 2,3)-α-L-Rhap-(1 →	104.31	81.35	82.61	72.08	74.31	19.91
C → α-D-Glcp-(1 →	101.07	72.33	71.52	73.02	74.89	63.52
D → 2,3)-α-L-Rhap-(1 →	100.76	83.94	79.89	75.51	74.31	19.88
E → β-D-Galf-(1 →	110.41	82.03	85.86	72.42	76.25	65.63
G → 6)-β-D-Galp-(1 →	107.41	75.01	79.67	73.70	73.88	69.46
EPS from Δ*polE* mutant						
A → 2)-α-D-Glcp-(1 →	102.36	78.16	74.47	75.90	74.89	63.46
B → –2,3)-α-L-Rhap-(1 →	104.32	81.37	82.62	72.08	74.32	19.92
C-α-D-Glcp-(1 →	101.08	72.33	71.53	73.03	74.89	63.52
D → 2,3)-α-L-Rhap-(1 →	100.77	83.95	79.90	75.52	74.32	19.88
E → β-D-Galf-(1 →	110.42	82.03	85.87	72.34	76.26	65.64
G → 6)-β-D-Galp-(1 →	107.42	75.02	79.68	73.71	73.89	69.47
EPS from Δ*galE* mutant						
A → 2)-α-D-Glcp-(1 →	101.77	77.50	74.44	74.73	74.80	63.41
C → α-D-Glcp-(1 →	101.53	72.33	72.22	75.85	74.80	63.37
D → 2,3)-α-L-Rhap-(1 →	100.06	83.27	82.82	73.04	74.38	19.95
F → 3)-α-L-Rhap-(1 →	105.60	71.64	80.44	75.15	72.70	19.70

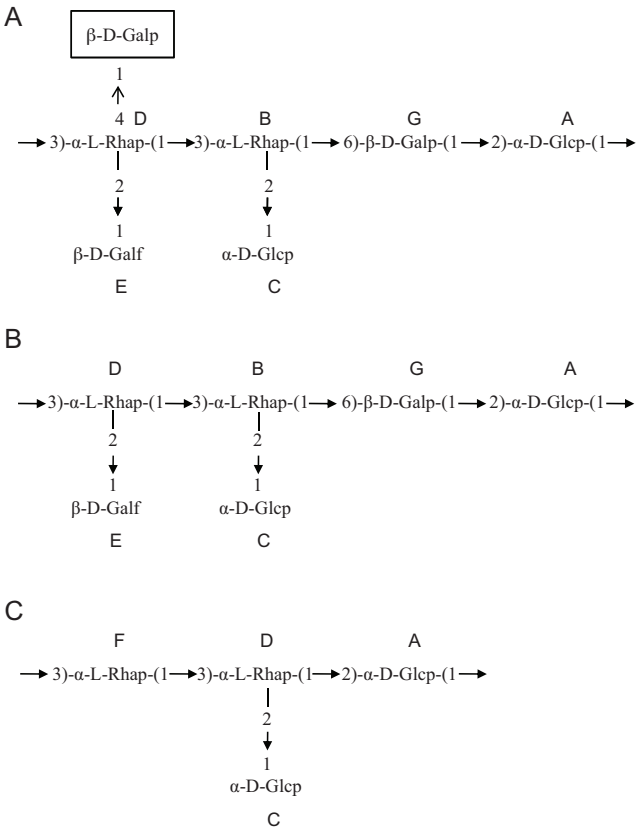

Fig. 5. Proposed repeating unit structures of *A. tropicalis* SKU1100 wild-type (R) CPS (A), Δ*polE* mutant EPS (B), and Δ*galE* mutant EPS (C). A to G on each residue in the repeating units refer to the residues named in Fig. 2. The β-D-Galp residue in box is not a part of the repeating unit of wild type CPS, it rather substitutes 2,3-L-rhamnosyl residue to make 2,3,4-L-rahamnosyl double branch which occurred only once or few times in the whole structure of the CPS.

anomeric proton D and H-2 of A, between anomeric proton C and H-2 of D, and between anomeric proton F and H-3 of D. These correlations, together with methylation analysis results and comparison between wild type CPS and Δ*galE* mutant EPS, allowed us to estimate the monosaccharide sequences of the polysaccharides (the minimum repeating units), as shown in Fig. 5.

In wild type CPS structure, there was an additional 2,3,4-α-L-Rhap double branched residue albeit with small amount (Table 1), which could not be seen in any mutants EPSs. Thus, as discussed later, it could be possible that this residue occurs in only part of many repeating units, together with another minor residues such as β-D-Galp branch residue (in box in Fig. 5A), which could not be detected in NMR analysis because of its low abundancy (Table 1).

4. Discussion

We identified the structure of *A. trpoicalis* SKU1100 wild type CPS, as well as Δ*polE* and Δ*galE* mutants EPSs. Wild type CPS and Δ*polE* mutant EPS appears to have the same hexasaccharide repeating unit, while Δ*galE* mutant EPS appears to have a tetrasaccharide repeating unit. Monosaccharide composition analysis of wild type CPS, and Δ*polE* EPS show similar results as the previous studies (Deeraksa et al., 2005; Moonmangmee, Toyama et al., 2002), while Δ*galE* mutant ESP had glucose and rhamnose in approximately equimolar ratio, which is different from the previous study (Deeraksa et al., 2006), (1:1.7 rhamnose:glucose). This discrepancy may resulted from loses in rhamnose during acid hydrolysis and derivatization. The results obtained here showed no acidic residue in these CPS and EPSs, by using different method (TMS) which could detect acidic residues if any, indicating the neutral nature of these polysaccharides. Methylation linkage analysis showed increased number of minor glucose, galactose, and rhamnose residues in Δ*polE* mutant EPS, while Δ*galE* mutant EPS has glucose and rhamnose minor residues. These minor residues, detected only by GC–MS analysis, may be present in the edges of the CPS or EPS polymer complex. Especially, one minor residue 2,3,4-α-L-rhamnose, detected only in wild type CPS but not in the mutant EPSs, would be important to consider the difference in the localization between CPS and EPS. Since this double branched residue represents only about 0.69% of the CPS composition (Table 1), it may occurs only once or few times in large numbers of the repeating units of the CPS structure. Thus, it can be speculated that this double branched residue may play a role in CPS formation, in which the modification of the 2, 3-α-L-rhamnose inside the repeating units may terminate the polymerization reaction, and lead the polysaccharide binding to the cell surface. Since actually Δ*polE* mutant EPS have much larger size than wild type CPS, the continuous polymerization reaction would occur to make much larger polymer in the absence of PolE which may cause such the termination reaction with the minor residues.

It is now well established that the polysaccharide co-polymerase proteins (PCP) regulate the chain length and surface assembly of capsular polysaccharides (Collins et al., 2007). Although the PCP homologue could be found in the genome of *Gluconacetobacter diazotrophicus* (GDI_2493) but not of *A. pasteurianus*, however, *polE* gene showed no similarity to PCP protein family. Whereas, such the homology search of *polE* gene showed that there are homologous *polE* genes in many bacteria including acetic acid bacteria, e.g. putative rhamnosyltransferases from *A. pasteurianus, Granulobacter bethesdensis*, and putative glycosyltransferases from *Gluconobacter oxydans* 621H, *Rhodobacter sphaeroides*, and *Nitrosomonas eutropha* C71 (data not shown). Moreover, PolE has clear consensus motifs (catalytic domain), DDGSxD and DQDDxW, near the N-terminus, and additional consensus motifs (estimated as the binding site), HDWxx and xYRQH, near the central part. The former two motifs are similar to domain A of ExoU and HasA families of β-glycosyltransferases, but the latter two are different from the putative substrate binding domain of this family (Deeraksa et al., 2005). Thus, taking together with the structural findings, we propose that PolE may work as β-galactosyltransferase that adds terminal-β-Galp residue to 2,3-α-L-Rhap residue to make 2,3,4-α-Rhap residue (Fig. 5A). This notion could be supported by the following observation. Like Δ*polE* mutant, despite of keeping native *polE* gene, Δ*galE* mutant also produces EPS but not CPS. Since the mutant could not produce UDP-galactose as the precursor of the terminal-β-Galp residue, active PolE could not make the double branched rhamnosyl residue and thus not able to switch the EPS repeating units to the CPS. Thus, it is conceivable that this double branched residue contribute to CPS attachment to the cell surface. However, biochemical characterization of PolE remains to be addressed.

Since the conversion of CPS to EPS occurs spontaneously in the case of S strain besides the artificial mutations described, this indicates the ability of *A. tropicalis* to produce different types of polysaccharides in different niches. For example, in shaking submerge culture, membrane stresses would be generated more than in static culture by the higher acid production due to rapid growth, and thus EPS production may help in stress adaptation (Mao, Doyle, & Chen, 2001). Recently, CPS, and probably EPS, production has been shown to add acetic acid resistance to *A. tropicalis* (Deeraksa et al., 2005) and *A. pasteurianus* (Kanchanarach et al., 2010). Additionally, *A. tropicalis* was recently reported as a major symbiont in the olive fruit fly (Kounatidis et al., 2009), reasonably suggesting that *A. tropicalis* produce EPS when switching from free-living mode to symbiosis mode to aid its colonization of the host insect.

5. Conclusion

We have determined the structure of *A. tropicalis* CPS, based on the structural comparison with mutant EPSs, to be a polymer consisting of a branched hexasaccharide repeating unit composed of two 2,3-α-L-Rhap, and 1 mole each of 6-β-D-Galp and 2-α-D-Glcp, of which the two rhamnosyl residues are branched by β-D-Galf and α-D-Glcp. Furthermore, the study could confer information physiologically important for *Acetobacter* sp., which is directly related to the pellicle formation. EPS could be attached to cell surface by the function of PolE, which may cross-link one 2,3-α-L-Rhap with β-D-Galp to produce CPS.

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