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Carbohydrate Research 337 (2002) 2469–2480

CARBOHYDRATE RESEARCH

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Extracellular polysaccharides of *Erwinia futululu*, a bacterium associated with a fungal canker disease of *Eucalyptus* spp.

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Received 9 July 2002; accepted 5 September 2002

Abstract

Extracellular polysaccharides (EPSs) produced by an *Erwinia* spp. associated with a fungal canker disease of *Eucalyptus* were fractionated into two polysaccharides, one that was identified with that produced by *Erwinia stewartii*. The other has a similar structure, but with one terminal Glc residue replaced by pyruvic acid to give 4,6-*O*-[(*R*)-1-carboxyethylidene)-Gal*p*. Their structures were determined using a combination of chemical and physical techniques including methylation analysis, periodate oxidation, low-pressure gel filtration and anion-exchange chromatographies, high-pH anion-exchange chromatography, mass spectrometry and 1D and 2D ^{1}H NMR spectroscopy. The new polysaccharides, identified as EPS Futululu FF-1 and FF-2, have the following structures:

β-D-Glc*p*
1
↓
6
→ 3)-β-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1→ 6)-β-D-Glc*p*-(1 →
4
↑
1
β-D-Glc*p*-(1 → 6)-α-D-Gal*p*-(1 → 4)-β-D-GlcA*p*

1

β-D-Glc*p*
1
↓
6
→ 3)-β-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1→ 6)-β-D-Glc*p*-(1 →
4
↑
1
4,6-*O*-[(*R*)-1-carboxyethylidene]-α-D-Gal*p*-(1 → 4)-β-D-GlcA*p*

2

Abbreviations: EPS, extracellular polysaccharide; EPS6, EPS from Ech6, etc.; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; TFA, trifluoroacetic acid; MALDITOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; EI MS, electron impact mass spectrometry at 70 eV; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; LS, light scattering; [η], intrinsic viscosity; M_w, weight average molecular weight; R_w, weight average root mean square radius.

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PII: S0008-6215(02)00349-X

The molecular weights of the polysaccharides range from $1.3–2.1 \times 10^6$ and their hydrodynamic properties are those of polydisperse, polyanionic biopolymers with pseudoplastic, non-thixotropic flow characteristics in aqueous solutions.

Keywords: *Erwinia* spp.; *Eucalyptus* spp.; extracellular polysaccharide; structure; ribotyping

1. Introduction

Eucalyptus trees in South Africa are being endangered by a fungal disease that produces cankerous stem lesions that go into the wood, thus damaging it for timber, pulp and paper uses. The canker that is threatening the tree is caused by *Coniothyrium zuluense*,[1–3] which is associated with a bacterium that has many properties similar to an *Erwinia* spp. Another bacterium, *Erwinia teranera*, was isolated from infected *Eucalyptus* in the Teranera region of KwaZulu-Natal and shown to produce different extracellular polysaccharide (EPS) from those reported here.[4] The present paper reports on the chemotaxonomy and ribotyping of the bacterium, which for the present is identified as *Erwinia futululu*.

2. Methods

Preparation of extracellular polysaccharides.—The EPS from *E. futululu* (EPS Futululu) was prepared exactly as described for EPS Teranera.[4] Yield was 2.3 g L^{-1}.

Purification of polysaccharides by anion-exchange chromatography.—Crude EPS (80 mg) as isolated from a Fernbach culture broth by EtOH precipitation was dissolved in water (40 mL) and loaded on a low-pressure anion-exchange column (Toyopearl DEAE-650M, 2.5×10 cm), which had been washed with 100 mL of 1.5 M pyridinium acetate (pH 5.5) and equilibrated with water. The column was washed with water (100 mL), and then irrigated with a step gradient of pyridinium acetate (100 mL each of 0.2, 0.4, 0.6 and 0.9 M; pH 5.5) (Fig. 1). The elution was followed by the phenol–H_2SO_4 method.[5] Fractions containing carbohydrate were appropriately pooled and concentrated to dryness in vacuo at 43 °C to give two polysaccharides identified FF-1 and FF-2 (Fig. 1).

Analytical and general methods.—The methods used for methylation analysis, gas–liquid chromatography with flame ionization (GLC) or mass selective (GC–MS) detector, monosaccharide analysis by high-pH anion-exchange chromatography pulsed amperometric detection (HPAEC-PAD), matrix-assisted laser desorption/ionization time of flight mass spectrometric analysis (MALDI-TOF MS) of per-*O*-methylated derivatives, and 600 MHz ^{1}H NMR spectroscopy, have been described previously.[6–13] The determination of pyruvic acid released by mild acid hydrolysis, the analysis of purified EPSs by light scattering (LS), and the determination of viscosity have been described elsewhere.[4]

Aldobiouronic acid.—Native EPS (2 mg) was hydrolyzed by 2 M TFA (4 mL, 120 °C, 1 h), a condition for monosaccharide composition analysis, and freeze dried. In such hydrolyzates aldobiouronic acid is always

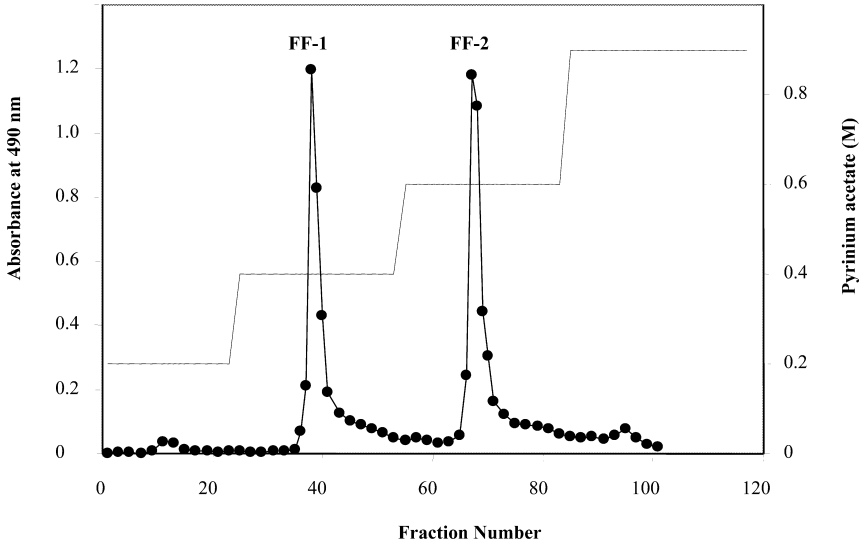


Fig. 1. Anion-exchange chromatographic fractionation of EPS Futululu on a DEAE650 column (2.5×12 cm). The column was eluted with a step gradient of 0.2, 0.4, 0.6, and 0.9 M pyridinium acetate (pH 5.5) and the elution was monitored by the phenol H_2SO_4 method.

present, together with monosaccharides. The resulting products, reconstituted in water (0.5 mL), were loaded on a weak anion-exchange column (2 mL, AG3-X4, OH-form, Bio-Rad, Hercules, CA, USA) and neutral monosaccharides were eluted with water (2 × 2 mL). The bound acids were eluted with 1.5 M pyridinium acetate (2 × 2 mL, pH 5.5) and reduced with $NaBH_4$ (10 mg mL^{-1}) after excess of pyridinium acetate had been removed in vacuo. The borate was evaporated away as methyl borate (co-evaporation with 10% AcOH in MeOH) and the resulting NaOAc was removed by chromatography on a spin column (2 mL, AG50W-X8, H-form, Bio-Rad, Hercules, CA, USA).

Degradation of polysaccharide by Lithium in ethylenediamine.—The EPS FF-1 and the de-pyruvated EPS FF-2 by mild acid hydrolysis[4] were treated with Li in ethylenediamine[14,15] and the Li-degradation products were fractionated by chromatography on a Bio-Gel P-2 column, as described previously.[7]

β-Elimination.—The freeze-dried EPS FF-2 (50 μg) (**2**) was methylated by the $NaOH/Me_2SO/MeI$ procedure as described previously.[16] The resulting product was re-methylated to induce β-elimination using the same reagent and the subsequent degradation product was fractionated by chromatography on a Sephadex LH-20 column (1 × 23 cm; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The column was eluted with 50% $CHCl_3$ in MeOH. The low molecular weight products, eluted at the total volume of the column, were pooled, analyzed directly by GLC and identified by GLC–MS as an α- and β-mixture referenced to the per-*O*-methyl derivative of proven (4,6)-pyruvated Gal from the EPS6[7] (α anomer: *m*/*z*, 43 (70), 44 (62), 45 (19), 75 (100), 85 (26), 88 (63), 127 (14), 155 (7), 247 (5); β anomer: *m*/*z*, 43 (95), 44 (11), 45 (39), 75 (100), 85 (29), 88 (75), 127 (70), 145 (13), 155 (11), 247 (24)). The high molecular weight component that eluted at the exclusion volume of the column was pooled, reduced with Superdeuteride (100 μL, $Li(Et)_3BD$) and hydrolyzed (2 M TFA, 120 °C, 1 h) for analysis by GLC–MS as the alditol acetate derivatives.

Smith degradation.—A Smith degradation of native EPSs (8 mg) or the modified polysaccharide (3–5 mg) derived from the native EPS by Li degradation was performed as described previously.[12] The product was purified by gel permeation chromatography on a BioGel P-2 column (2.5 cm × 16, −400), eluting with water, and finally by HPAEC-PAD on a CarboPac MA1 column (4 × 250 mm), using isocratic elution (0.4 mL min^{-1}) of 15 mM NaOAC in 100 mM NaOH.

3. Results and discussion

The bacterium in these studies is a Gram-negative, motile rod, showing a yellowish growth on nutrient agar containing glucose. The organism shows a good selectivity (81%) for *Pantoea agglomerans* by Vitek and API 20E evaluations (which offer identification systems for *Enterobacteriaceae* and other Gram-negative rods, BioMerieux, St. Louis, MO, USA). Many of the *Erwinia* strains in the clinical laboratories are classified most often as *P. agglomerans* (*Enterobacter agglomerans*). The organism does not ferment lactose and does not produce pectate lyase. It does, however, show a relationship to *Erwinia* spp. in that the EPSs include one identical in primary structure to that produced by *Erwinia stewartii*,[17,18] and the second EPS has structural similarities to the first but with the modification of its side chain. Tentatively, the bacterium has been designated *E. futululu*.

Purification and composition analyses of EPS.—The crude EPS, as isolated by fractional precipitation with ethanol, was composed of Glc, Gal, and GlcA, determined by HPAEC-PAD analysis of acid hydrolyzates. GLC analysis of the methanolyzates as trimethyl silyl derivatives[19] showed additionally the presence of a pyruvated Gal residue.

Thirteen anomeric proton signals each of unit stoichiometric ratio were observed in the 600 MHz 1D ^1H NMR spectrum of the crude EPS, together with methyl proton signals of pyruvic acid in a half mole ratio. Anion-exchange chromatography of the crude EPS resulted in two different acidic EPSs (FF-1 and FF-2). The EPS FF-1 was eluted at 0.4 M pyridinium acetate and the other EPS FF-2 at 0.6 M (Fig. 1). Under such conditions a significant portion of one polysaccharide was co-eluted with the other. Following repetitive rechromatographic purification, the EPS FF-1 of 95% purity was isolated, based on the peak area of anomeric proton signal pertinent to the respective polysaccharide in the 1D ^1H NMR spectrum, but the EPS FF-2 still contained 27% of the EPS FF-1 for unknown reasons (Fig. 2). No neutral oligo- or other polysaccharides were present in the EPS preparations.

HPAEC-PAD analysis of the acid hydrolyzate (2 M TFA, 120 °C, 1 h) revealed that the EPS FF-1 has a composition of D-Glc, D-Gal, and D-GlcA in the ratio, 3:3:1 and the EPS FF-2 that of D-Glc, D-Gal, D-GlcA, and pyruvic acid in the approximate ratio, 2:3:1:1. The pyruvic acid released by mild acid hydrolysis (pH 2.2, 100 °C, 4 h) was analyzed by HPLC[4] and the absolute configurations of the sugar residues were determined as the trimethyl silyl derivatives of the *R*-(−)-butane-2-ol glycosides by GLC analysis.[20]

EPS FF-1.—Glycosyl linkage analysis of the EPS FF-1 after reduction of the per-*O*-methylated EPS with Super-Deuteride, $Li(Et)_3BD$, (to convert the GlcA to Glc), showed the presence of two residues of terminal Glc, one residue each of 6-linked Glc, 3,4,6-linked Gal, 3-linked Gal, 6-linked Gal and 4-linked GlcA (Table 1). It is clear from the glycosidic linkage analysis and other

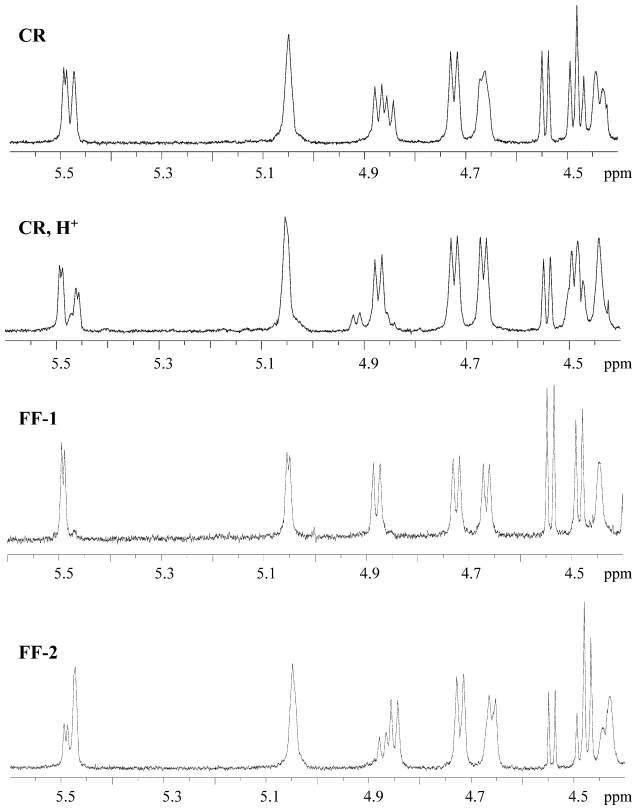


Fig. 2. The 600 MHz 1D ^{1}H NMR spectra of anomeric proton region of EPS Futululu before (CR) and after (FF-1 and FF-2) purification on an anion-exchange column (DEAE650, 2.5 × 16 cm). The spectra were recorded for samples in D_2O containing 1% NaCl at 65 °C. The spectrum of crude EPS after mild acid hydrolysis (pH 2.2, 98 °C, 4 h) is also included for comparison (CR, H^+).

information that all of the residues in the EPS are in the pyranose form and that the EPS has a branched heptasaccharide repeating unit containing two side chains.

The evidence of a heptasaccharide repeat unit in the native EPS FF-1 was supported by the presence of equal proportions of seven anomeric proton signals in the 1D ^{1}H NMR spectrum (Fig. 2 and Table 2). The chemical shifts and coupling constants indicated that the resonances at δ 5.492 ppm ($J_{1,2}$ 3.7 Hz) and at δ 5.052 ppm ($J_{1,2}$ 3.1) are characteristic of an α-*gluco* or *galacto* configuration. The five signals at δ 4.880 ppm ($J_{1,2}$ 7.9 Hz), δ 4.725 ppm ($J_{1,2}$ 7.7 Hz), δ 4.666 ppm ($J_{1,2}$ 7.4 Hz), δ 4.541 ppm ($J_{1,2}$ 8.0 Hz), and δ 4.485 ppm ($J_{1,2}$ 7.9 Hz) are characteristic of β-*gluco* or *galacto* configuration.

Aldobiouronic acid, fractionated from acid hydrolyzates (2 M TFA, 120 °C, 1 h) on an anion-exchange spin column, was identified to be glucuronosyl galactose by HPAEC-PAD analysis of the monosaccharide composition. Galactose was present as a 4-linked residue by glycosyl linkage analysis of the reduced aldobiouronic acid (with $NaBH_4$), which detected 1,5,6-tri-*O*-acetyl-1,6,6-trideuterio-2,3,4-tri-*O*-methylglucitol (derived from the reduction of the methyl ester of GlcA with Super-Deuteride) and 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylgalactitol. One signal of anomeric proton in the 1D ^{1}H NMR spectrum of the reduced aldobiouronic acid was seen at δ 4.495 ppm (7.8 Hz), which was attributed to the β configuration of the GlcA residue. These data provided the following structure of the aldobiouronic acid (**3**).

β-D-GlcA*p*-(1 → 4)-D-Gal*p*

3

Degradation of the EPS FF-1 by lithium, where lithium in ethylenediamine causes cleavage of the uronosyl residue and partial reduction of the released saccharide,[14,15] generated a modified polysaccharide and low molecular weight components, separated by fractionation on a BioGel P-2 column. The low molecular weight components, which eluted like disaccharides on the column, were further reduced with $NaBH_4$ to convert completely the released saccharide to its alditol sugar. The principal resulting product was a glucosyl galactitol, as determined by GLC–MS analysis of alditol acetate derived by acetylating the acid hydrolyzate (2 M TFA, 120 °C, 1 h). The galactitol in this disaccharide was present as a 6-linked residue by methylation analysis with the detection of one residue of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and one residue of 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylgalactitol. The 1D ^{1}H NMR spectrum of the glucosyl galactitol contained

Table 1
Glycosyl linkage analyses of EPS FF-1 and FF-2 before (I) and after (II) Li degradation. Modified polysaccharide (III) derived from EPS FF-2 by β-elimination using NaOH/DMSO/CH_3I system also included

Me sugar [a]	EPS FF-1		EPS FF-2		
	I	II	I	II	III
2,3,4,6-Me_4Glc	1.6	1.0	1.2	0.9	1.0
2,3,4,6-Me_4Gal					
2,4,6-Me_3Gal	1.0	1.0	1.0	1.0	1.0
2,3,4-Me_3Glc	0.9	1.0	1.1	0.8	1.0
2,3,4-Me_3Gal	0.7		0.2		<0.1
2,3-Me_2Gal [b]			0.7		0.2
2,4-Me_2Gal		1.0		1.0	
2,3-Me_2GlcA [c]	0.8		0.8		0.3
2-MeGal	1.1		1.2		1.0

[a] 2,3,4,6-Me_4Glc = 1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methylglucitol.

[b] Derived from pyruvated (4,6)Gal.

[c] Observed as 1,4,5,6-tetra-*O*-acetyl-1,6,6-trideuterio-2,3-di-*O*-methylglucitol derived from the reduction of the methyl ester of 2,3-Me_2GlcA with Superdeuteride and etc.

Table 2
NMR data of anomeric protons for crude EPS (CR), purified EPS FF-1 and FF-2 before (I) and after (II) Li-modification. The spectra of EPSs were obtained in D_2O containing 1% NaCl, 65 °C

Residue of inferred linkage in the native EPS	δ_H (ppm) [a] and coupling constant ($J_{1,2}$)				
	CR	FF-1		FF-2	
		I	II	I	II
-6)-α-D-Gal*p*-(1-	5.491 (1 H) 3.5	5.492 (1 H) 3.7		5.494 (0.3 H) 3.6	
Py(4,6)-α-D-Gal*p*-(1-	5.473 (1 H) broad			5.472 (1.0 H) 3.7	
-3,4,6)-α-D-Gal*p*-(1-	5.049 (2 H) broad	5.052 (1 H) 3.1	5.035 (1 H) 3.0	5.049 (1.3 H) ~3	5.035 (1 H) 3.3
-4)-β-D-GlcA*p*-(1-	4.872 (1 H) 7.8	4.880 (1 H) 7.9		4.873 (0.3 H) 8.5	
-4)-β-D-GlcA*p*-(1-	4.850 (1 H) 7.8			4.850 (1.0 H) 7.7	
-6)-β-D-Glc*p*-(1-	4.724 (2 H) 7.8	4.725 (1 H) 7.7	4.722 (1 H) 7.8	4.722 (1.3 H) 8.0	4.722 (1 H) 7.7
-3)-β-D-Gal*p*-(1-	4.662 (2 H) broad	4.666 (1 H) 7.4	4.678 (1 H) 7.8	4.659 (1.3 H) 7.4	4.677 (1 H) 7.3
β-D-Glc*p* [1]-(1-	4.543 (1 H) 7.8	4.541 (1 H) 8.0		4.543 (0.3 H) 7.9	
β-D-Glc*p* [2]-(1-	4.488 (1 H) 7.8	4.485 (1 H) 7.9	4.501 (1 H) 7.8	4.487 (0.3 H) 8.3	
β-D-Glc*p* [2]-(1-	4.474 (1 H) 7.8			4.473 (1.0 H) 8.1	4.501 (1 H) 7.7
CH_3–pyruvate	1.444 (3 H) singlet			1.443 (3.1 H) singlet	

[a] Chemical shifts relative to acetone (δ_H 2.225 ppm) and the number of proton include in bracket.
[1] Glc in the side chain of trisaccharide.
[2] Glc attached to the tri-substituted branching point.

one doublet (7.8 Hz) at δ 4.508, which was attributed to the anomeric proton of β-Glc*p*. These data provide evidence that one side chain of the EPS FF-1 has a unit of the trisaccharide the glucosyl galactosyl glucuronate.

The Li-modified EPS was composed of equimolar amounts of Glc and Gal. Methylation analysis showed the presence of one mole each of terminal Glc, 3-linked Gal, 6-linked Glc and 3,6-linked Gal (Table 1). These analyses demonstrate that the Li-modified polysaccharide consists of a branched tetrasaccharide repeating subunit. Moreover, the quantitative appearance of 3,6-linked Gal at the expense of 3,4,6-linked Gal illustrates that the GlcA residue, and consequently the side chain of glucosyl galactosyl glucuronate, is linked to O-4 of a tri-substituted branching Gal residue in the native EPS. This is also consistent with the 4-linked Gal residue being present as a constituent of the aldobiouronic acid (**2**).

The four resonances of anomeric protons, in equimolar ratio, at δ 5.035 ppm ($J_{1,2}$ 3.0, 1 H), δ 4.722 ppm ($J_{1,2}$ 7.8, 1 H), δ 4.678 ppm ($J_{1,2}$ 7.8, 1 H) and δ 4.501 ppm ($J_{1,2}$ 7.8, 1 H) in the 1D ^{1}H NMR spectrum of the Li-modified polysaccharide (Fig. 3) is consistent with a tetrasaccharide repeating subunit consisting of two residues each of Glc and Gal. The analyses of anomeric resonances before and after the degradation of the EPS FF-1 by Li, i.e., the losses of three anomeric protons (one α and two β) in the Li-modified polysaccharide, two of which are attributed to one residue each of β-Glc and β-GlcA, indicates the 6-linked Gal residue attached to the 4-*O* position of β-GlcA is present in α-configuration, thus providing the complete structure of the side chain trisaccharide and the Gal residue in the main chain to which it is attached (**4**).

β-D-Glc*p*-(1 → 6)-α-D-Gal*p*-(1 → 4)-β-D-GlcA*p*-(1-4)-Gal*p*

4

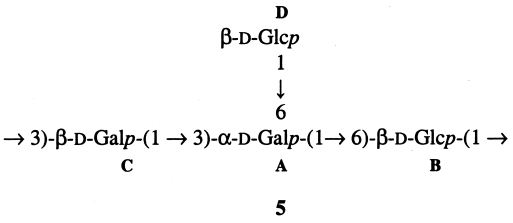
In the 2D ^{1}H NMR spectrum of the Li-modified polysaccharide (Fig. 4), the spin system of the residues, A and C (see **5**), is characteristic of a Gal residue, as assigned by a COSY experiment (Table 3). The chemical shifts of equatorial H-4 in both A and C residues

were observed far downfield over H-2 and H-3 signals. The cross-signals of H-5 and H-6 in the TOCSY spectrum were not seen due to the small coupling constant of the H-4 to H-5. All of these data provided the evidence of both residues present in the *galacto* configuration.

The chemical shift and coupling constant showed that the residue A was present in α-configuration (δ 5.035 ppm, $J_{1,2}$ 3.1 Hz) and the residue C in β configuration (δ 4.678 ppm, $J_{1,2}$ 7.5 Hz). This assignment was supported by the absence of the intra-residue NOE contacts from H-1 to either H-3 or H-5 for the residue A and by the appearance of the intra-residue NOE contacts from H-1 to H-3 and H-5 for the residue C.

Both B and D residues, as assigned again by the COSY experiment, were β-Glc, characterized by the upfield chemical shifts of H-1 and the large coupling constants attributed to the diaxial configuration of vicinal protons. In the NOESY spectrum there were also intra-residue connective signals of H-1 to H-3 and H-5, supporting the Glc residues present in β-configuration. The signals from H-1 to H-6 of the Glc residue detected in the TOCSY spectrum allowed the complete assignments of proton resonances.

The inter-residue contacts in the NOESY spectrum supported the sequence of the glycosyl residues in the backbone, as seen by clear NOE contacts of A1 to B6, B1 to C3, and C1 to A3, consequently establishing the sequence of A-B-C. The NOE signal of D1 to A6 was weak and close to noise signals, but clearly visible. These overall inter-residue contacts provided the sequence of monosaccharide residues, -A(D)-B-C-, in the Li-modified EPS (**5**) (Table 4).



5

The Smith degradation product derived from **5** was a disaccharide–glycerol (m/z 565.8 as sodium adduct; calcd 565.3, as determined by MALDI-TOF analysis of the per-*O*-methylated derivative), which was composed of two residues of Gal and one residue of glycerol by GLC analysis of the alditol acetate derivative. Methylation analysis revealed that the two Gal residues were present as one residue each of a non-reducing terminal Gal and a 3-substituted Gal, as detected by 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol. The 1-*O*-acetyl-2,3-di-*O*-methylglycerol, derived from the 6-substituted Glc residue in **5**, was not detected due to its volatility under the experimental conditions. Thus, these data provided the sequence of the Smith product, **6**, confirming that two Gal residues were linked to each other, the branching Gal residue in the main chain being present in

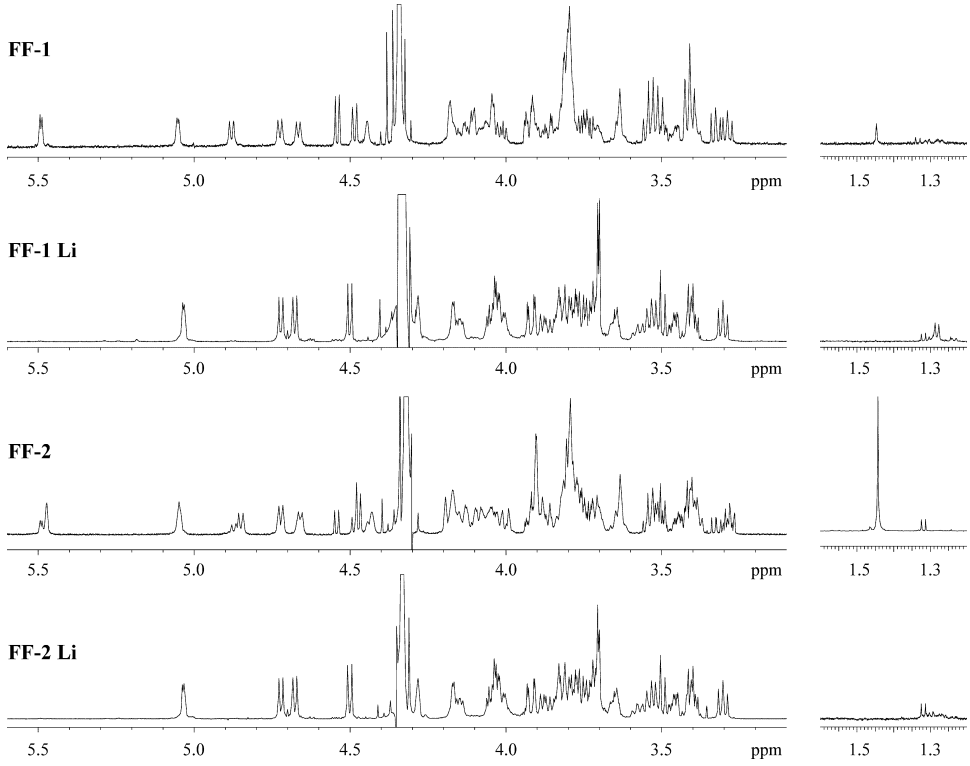


Fig. 3. The 600 MHz 1D ^{1}H NMR spectra of EPS FF-1 and FF-2 before and after Li-degradation. The spectra were obtained of the EPS in D_2O containing 1% NaCl at 65 °C.

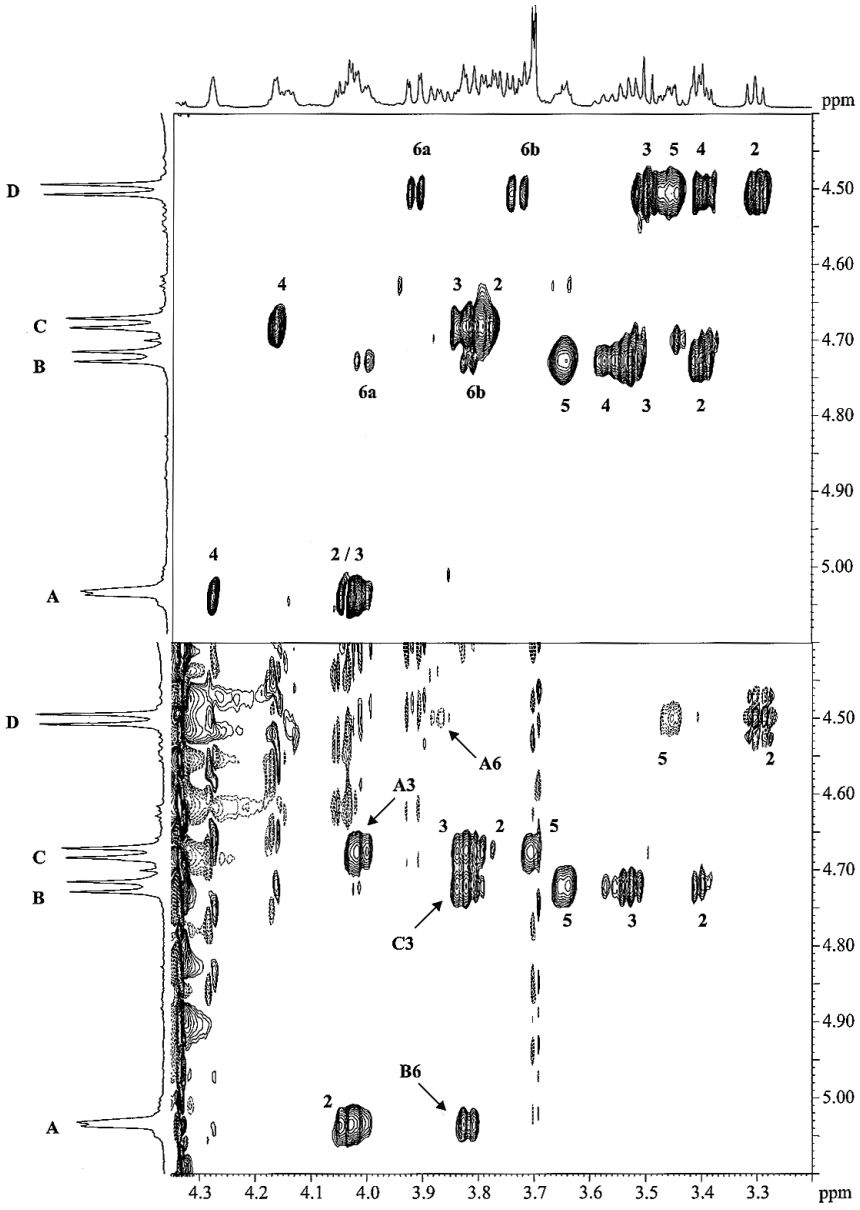


Fig. 4. A part of the 600 MHz 2D ^{1}H NMR spectra of Li-modified EPS FF-1 recorded in D_2O at 65 °C. TOCSY (top, 119 ms mixing time) and NOESY (bottom, 0.5 s mixing time).

α-configuration, which is consistent with the methylation analysis of the Li-modified EPS.

β-D-Gal-(1 → 3)-α-D-Gal-(1 → 1)-glycerol
C' A'
6

Two resonances of anomeric protons in 1D ^{1}H NMR spectrum of the Smith product (**6**) were present in an equimolar ratio at δ 4.980 ppm ($J_{1,2}$ 3.6 Hz) and at δ 4.600 ppm ($J_{1,2}$ 7.7), attributed to one residue each of α- and β-Gal residues.

The β-configuration of the residue C′ was clear from the chemical shift and coupling constant of H-1 (δ 4.600 ppm, $J_{1,2}$ 7.7 Hz). Two NOE contacts of intra-residue, H-1 to H-2 at δ 3.612 and H-1 to H-3 at δ 3.666 ppm, confirm the β-configuration of Gal residue.

The residue A′ was present in α configuration, as demonstrated by the downfield resonance of H-1 and its coupling constant (δ 4.980, $J_{1,2}$ 3.6 Hz) and by the appearance of only one intra-residue NOE contact between H-1 to H-2 at δ 4.001 ppm.

In the NOESY spectrum of the Smith product **6**, one each of inter-residue NOE contacts was seen from the respective anomeric protons, namely, C′1 to A′3 at δ 4.034 and A′1 to H-1 of glycerol at δ 3.580, thus supporting the sequence of **6**, consistent with that established by methylation analysis above. Moreover, the NOE contact of A′1 to H-1 of glycerol permitted the assignment of the glycerol residue, together with the COSY experiment.

The periodate oxidation on the native EPS FF-1 generated the same Smith product as the Li-modified EPS. However, the oxidation was not complete. MALDI-TOF MS analysis of the fractions eluted at the beginning of the column chromatography of the Smith products detected a sodiated mass m/z 784.0 (calcd 783.4), which is that for the per-*O*-methylated GlcAGal$_2$Glycerol. Thus, a portion of the GlcA residue was not oxidized.

Table 3
^{1}H NMR chemical shifts and coupling constants for the Li-modified EPS FF-1 (**5**) in D_2O at 65 °C and Smith product (**6**) in D_2O at 25 °C

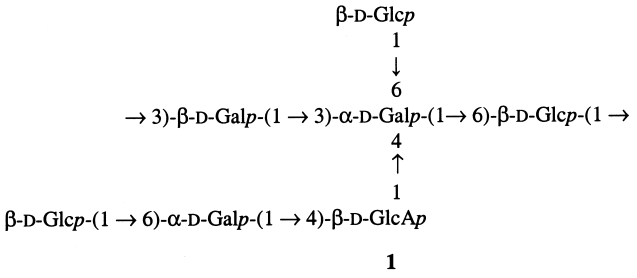
	Residue of inferred linkage	δ [a] (ppm) ($^3J_{H/H}$ (Hz))						
		H-1	H-2	H-3	H-4	H-5	H-6	H-6′
A	-3,6)-α-D-Gal*p*-(1-	5.035 (3.1)	4.040 (7.0)	4.028 (3.3)	4.282 (<1.0)	4.154 (4.8/2.4)	4.046 (11.4)	3.898
B	-6)-β-D-Glc*p*-(1-	4.722 (7.8)	3.396 (9.5)	3.533 (9.0)	3.577 (9.4)	~3.65 (3.0/3.0)	4.199 (13.2)	3.818
C	-3)- β-D-Gal*p*-(1-	4.678 (7.5)	3.798 (9.6)	3.836 (3.7)	4.172 (2.3)	3.709 (4.2/)	3.785 (11.5)	3.77
D	β-D-Glc*p*-(1-	4.501 (7.7)	3.304 (8.6)	3.505 (9.2)	3.410 (9.3)	3.463 (2.4/6.0)	3.919 (12.4)	3.736
A′	-3)-α-D-Gal*p*-(1-	4.980 (3.6)	4.001 (10.2)	4.034 (3.0)	4.260 (0.8)	3.966 (2.4/2.4)	3.765 (12.6)	3.732
C′	β-D-Gal*p*-(1-	4.600 (7.7)	3.612 (9.6)	3.666 (3.4)	3.922 (<1.0)	~3.67		
	Glycerol	3.776 (3.6) 3.580 (3.6) $J_{1,1'}$ 10.8	3.96 (5.4/)	3.640 3.69 $J_{3,3'}$11.7				

[a] Relative to the internal signal of acetone (δ 2.225 ppm).

Table 4
NOE data for the Li-modified EPS FF-1 (**5**) and Smith product (**6**). The spectra of **5** were recorded in D_2O at 65 °C with 0.5 s mixing time but the spectra of **6** in D_2O at 25 °C

	Residue	δ (ppm) of H-1	Connectivity residue and chemical shifts (ppm)	
A	-3,6)-α-D-Gal*p*-(1-	5.035	A2	4.040
			B6	3.818
B	-6)-β-D-Glc*p*-(1-	4.722	B2	3.396
			B3	3.533
			B5	3.65
			C3	3.836
C	-3)-β-D-Gal*p*-(1-	4.678	C2	3.798
			C3	3.836
			C5	3.709
			A3	4.028
D	β-D-Glc*p*-(1-	4.501	D2	3.304
			D5	3.463
			A6b	3.898
A′	-3)-α-D-Gal*p*-(1-	4.980	A′2	4.001
			Glycerol H-1	3.580
C′	β-D-Gal*p*-(1-	4.600	C′2	3.612
			C′3	3.666
			A′3	4.034

All of the data support the presence of the branched heptasaccharide repeating unit for EPS FF-1 (**1**).



1

EPS FF-2.—The 600 MHz 1D ^{1}H NMR spectrum of the EPS FF-2 contained the methyl protons of a pyruvic acid constituent and thirteen anomeric protons that were in a major set of six equal signals and a minor set of seven equal signals. The ratio of the areas of the major to the minor sets was 1.00:0.27 (Fig. 2). The resonances of the minor set had identical chemical shifts and coupling constants with those of the EPS FF-1, as summarized in Table 2. These analyses suggested that the EPS of the principal component has a hexasaccharide repeating unit in the EPS FF-2, one residue of which is quantitatively capped by pyruvic acid, based on the signal area of its methyl protons.

The chemical shifts and coupling constants of the EPS FF-2 indicate that two resonances at δ 5.472 ppm ($J_{1,2}$ 3 Hz) and at δ 5.049 ppm ($J_{1,2}$ 3 Hz) are present in an α-*gluco* or *galacto* configuration and the four signals at δ 4.850 ppm ($J_{1,2}$ 7.7 Hz), δ 4.722 ppm ($J_{1,2}$ 8.0 Hz), δ 4.659 ppm ($J_{1,2}$ 7.4 Hz), and δ 4.473 ppm ($J_{1,2}$ 8.1 Hz) are characteristic of an β-*gluco* or *galacto* configuration.

Glycosyl linkage analysis of the EPS FF-2, as described above for the EPS FF-1, showed the presence of one residue each of a terminal Glc, 6-linked Glc, 4-linked GlcA, terminal Gal, 3-linked Gal, and 3,4,6-linked Gal (Table 1), of which the terminal Gal was substituted by pyruvic acid, 4,6-*O*-[(*R*)-1-carboxyethylidene]-Gal*p*, as detected by 1,4,5,6-tera-*O*-acetyl-2,3-di-*O*-methylgalactitol. This analysis indicated the presence of a branched hexasaccharide repeating unit in the EPS FF-2.

There was an additional residue of 6-linked Gal, present in approximately 0.2 molar ratio. This minor residue was derived from the EPS FF-1 present in the EPS FF-2 preparation, as described earlier, and the extent of which was also reflected by the elevated amounts of other residues, such as a terminal Glc and by the reduced amount of pyruvated Gal residue. This is consistent with what was seen by analyses of the anomeric proton signals in the 1D ^{1}H NMR spectrum of EPS FF-2, consequently supporting the presence of a branched hexasaccharide repeating unit in the EPS FF-2.

The presence of a pyruvated residue in the EPS was shown by HPLC analysis of pyruvic acid released from the native EPS by mild acid hydrolysis. Under such conditions, 85% of pyruvic acid, together with some monosaccharides was cleaved from the EPS, based on the signals of the methyl protons and anomeric protons in the 1D ^{1}H NMR spectrum of the resulting EPS. Broad signals of anomeric protons, at δ 5.473 ppm, δ 5.049 ppm and δ 4.662 ppm in the EPS FF-2 were sharpened and the shift of the signal from δ 5.472 ppm to δ 5.459 ppm due to the de-pyruvation was also observed.

In preparation for lithium degradation of the EPS FF-2 it was found that the native EPS FF-2 was not soluble in ethylenediamine until the pyruvate was removed. The de-pyruvated EPS FF-2 was degraded by lithium in ethylenediamine to generate two fragments, one of high- and another of low-molecular weight, that were isolated on a BioGel P-2 column (data not shown). The high molecular weight fraction was composed of two residues each of Glc and Gal, which were present as a terminal Glc, 3-linked Gal, 6-linked Glc and 3,6-linked Gal in equimolar ratio. These data showed the presence of a branched tetrasaccharide in the Li-modified EPS FF-2. Furthermore, the quantitative conversion of 3,6-linked from 3,4,6-linked Gal by Li illustrated the substitution of GlcA at *O*-4 of a branch point Gal residue in the native EPS, as seen for the EPS FF-1.

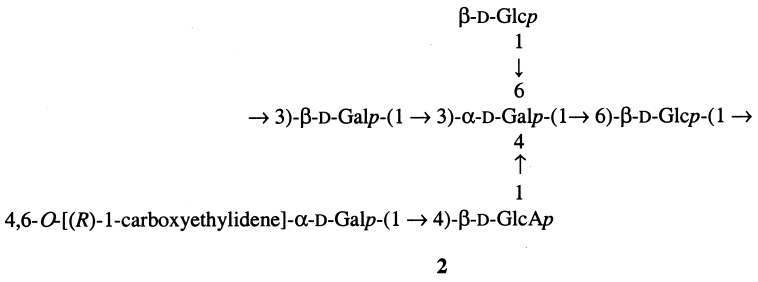
Evidence of the branched tetrasaccharide repeating subunit in the Li-modified EPS FF-2 was supported by the appearance of four anomeric proton signals in the 1D ^{1}H NMR spectrum, one resonance of α-configuration at δ 5.035 ppm ($J_{1,2}$ 3.3, 1 H) and three resonances of β-configuration at δ 4.722 ($J_{1,2}$ 7.7, 1 H), δ 4.677 ($J_{1,2}$ 7.3, 1 H) and δ 4.501 ($J_{1,2}$ 7.7, 1 H). The analysis of the 1D ^{1}H NMR spectrum revealed that the Li-modified EPS FF-2 has the structure of a branched tetrasaccharide repeating subunit, identical to that derived from the EPS FF-1 by lithium degradation (Fig. 3). Moreover, the losses of two signals (one each of α and β-anomeric protons) in the modified EPS FF-2, one of which was due to the loss of β-GlcA, show that the Gal residue in the side chain has the α-configuration of anomeric proton.

These overall data, together with identical spectra of both Li-modified EPSs in the 1D ^{1}H NMR spectra, provided the sequence of the EPS FF-2 (**2**).

The new terminal residues generated by mild acid hydrolysis of the EPS FF-2 complicated the conclusive assignment of the side chain from the analysis of the low molecular weight fractions derived from the resulting EPS by lithium. Thus the penultimate residue, attached to O-4 of GlcA residue, was released by β-eliminative degradation from re-methylation of the per-*O*-methylated EPS FF-2. The resulting product was

separated from high-molecular weight materials on a Sephadex LH-20 column (1x 21 cm). The fractions eluted at the total volume of the column were directly analyzed by GLC and identified by GLC–MS as the per-*O*-methyl pyruvated galactosides of the α- and β-anomeric mixtures. There was an additional minor component, co-eluted with per-*O*-methyl pyruvated Gal derivative on the column, identified as per-*O*-methylated glucosyl galactoside (obsd *m*/*z* 477.8 as sodium adduct; calcd *m*/*z* 477.2) by MALDI-TOF MS. This compound again was due to the cross contamination of the EPS FF-1.

PAD analysis, MALDI-TOF analysis of the per-*O*-methylated derivative (*m*/*z*, 565.9) and 1D ^{1}H NMR spectroscopy (δ 4.980, 1 H, J_{12} 3.6 Hz; δ 4.600, 1 H, J_{12} 7.7 Hz). These data added confirmation to the structural similarity of EPS FF-2 and EPS FF-1, particularly the identical structural unit of the backbone. Thus, the native EPS FF-2 (**2**) is similar to the EPS FF-1 (**1**) but with the terminal glucose replaced by pyruvic acid. The chemical shift (δ 1.443 ppm) of the methyl group of the pyruvic acid indicates an equatorial conformation[22,23] of the methyl group and accordingly the *R* configuration to the pyruvic acid substituent.



2

The high-molecular weight fraction of β-eliminative products, by subsequent glycosidic linkage analysis was composed of 1 mol each of a terminal Glc, 3-linked Gal, 6-linked Glc and 3,4,6-linked Gal (Table 1). The unsaturated glucuronate (4-deoxy-hex-4-enoglucuronate) derived from glucuronic acid was not detected due to the losses of volatile furan derivatives that result from its degradation during acid hydrolysis.[21] Approximately 70% of glucuronosyl residue by this reaction was eliminated, as determined by the extent of the remaining 4-linked GlcA and terminal pyruvated Gal residues in the analysis. These data demonstrated the side chain to be the pyruvated galactosyl glucuronate and the high molecular weight component was present as a tetrasaccharide repeating unit. This is also in good agreement with the data from Li-degradation.

The same Smith degradation product **6** was derived from the native EPS FF-2, as determined by HPAEC-

Table 5
The weight-average molecular weight (M_w), root mean square radius (RMS), and intrinsic viscosity ([η]) of EPS FF-1 and FF-2 in 0.15 M Na_2SO_4 containing 0.015 M EDTA at 25 °C

	M_w[a] ($\times 10^6$)	RMS[a] (nm)	[η][b] (mL g^{-1})
EPS FF-1	2.08 ± 0.01	82.8 ± 0.6	253
EPS FF-2	1.25 ± 0.01	54.6 ± 0.3	148

[a] EPS solution of 0.1–0.5 (mg mL^{-1}) determined by light scattering (batch mode) spectroscopy.

[b] EPS solution of 1.0–5.0 (mg mL^{-1}) determined at the lowest reliable shear rate by Brookfield viscometry.

4. Physical properties of EPS FF-1 and FF-2

The molecular weights, root mean square radii (RMS) and the intrinsic viscosities of these EPSs were determined by light scattering spectrometer (Dawn DSP Laser Photometer, Wyatt Technology Co. Santa Barbara, CA, USA) using a batch mode technique and viscometer (DV-III, Brookfield Engineering Lab. Inc., Middleboro, MA, USA). It is recognized that EPS FF-2 contains approximately 30% of EPS FF-1 so that the values of the EPS FF-2 are not accurate, but these properties are similar for both EPSs, as summarized in Table 5 and Fig. 5.

The rheological properties of the EPSs include pseudoplastic, non-thixotropic flow as characterized by shear rate dependence of viscosity (Fig. 5). Addition of NaCl significantly decreases the viscosity of the aqueous polysaccharide solution, in particular at the range of 0.5 M NaCl ($I^{-0.5} = 1.4$). All these represent the typical viscometric behavior of polyelectrolytes.[24–26]

The effect of Ca ion on the intrinsic viscosity [η] was different for the two polysaccharides. The viscosity of EPS FF-1 proportionally increased with the concentration of calcium chloride added, as was seen for other similar families of *Erwinia's* EPS, such as Ech9.[27] This increase of the viscosity is considered to be due to the resulting complex formation of the calcium ion with two molecules of polysaccharide, consequently increasing the molecular weight. However, the viscosity of the EPS FF-2 with the first addition of calcium chloride was not significantly changed until the calcium ion reached one molar concentration. In such a pyruvated polysaccharide of FF-2, two proximal carboxyl groups

in the same repeating unit form a complex, preferentially with calcium ion in its dilute concentration, thus delaying the formation of an intermolecular complex until excess of calcium ion is present. The implication of pyruvate on this behavior was also seen in other pyruvated EPSs such as EPS 6[27] and EPS Teranera PK-2.[4]

5. Summary

The bacterium *E. futululu* produces two EPSs in equal amounts with similar monosaccharide compositions. The crude EPS product, as isolated from the fermentation broth by ethanol precipitation, contained half a mole residue of pyruvate. Fractional amounts of organic constituents, such as acetyl, pyruvyl, or methyl groups, in polysaccharides is not unusual and frequently considered to be partial substitution of such a molecule. In the case of EPS Futululu such an assumption would have resulted in an erroneous structural proposal. Because of the similarities of the two polysaccharides as demonstrated here, it was not possible by the many chromatographic procedures tried to completely remove the EPS FF-1 from the EPS FF-2. However, with the isolation of pure EPS FF-1, its 30% contamination of the EPS FF-2 could be interpreted for structural studies. The separation of one EPS demonstrated the pyruvic acid was present as one stoichiometric constituent in one polysaccharide, not randomly substituted in the EPS Futululu.

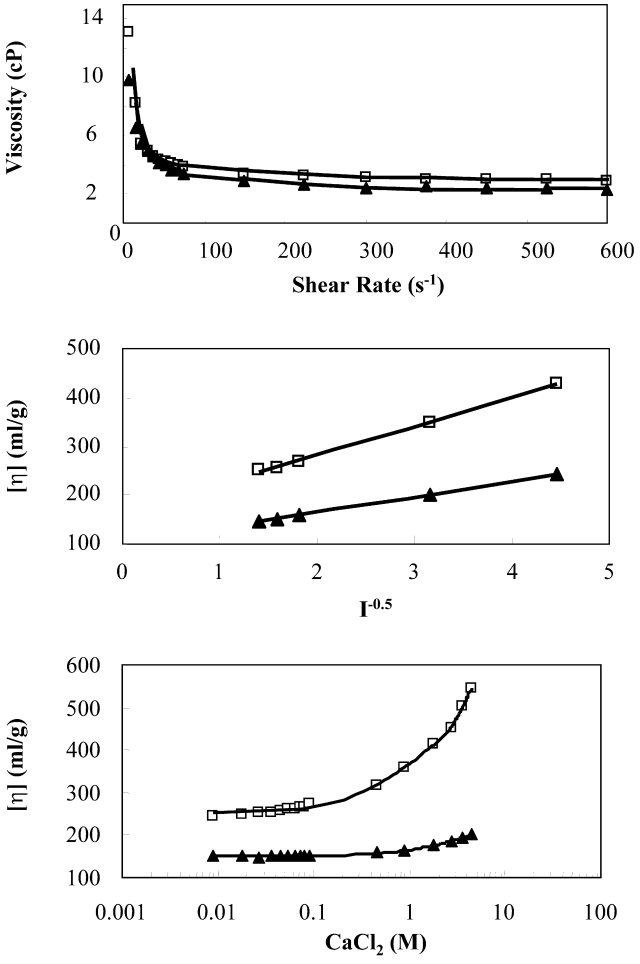


Fig. 5. Viscometric properties of EPS Futululu. The effect of shear rates on the viscosity of EPS FF-1 (open square) and FF-2 (filled triangle) in water at 25 °C (I), as determined by Brookfield viscometer. The Effect of Na(II) and Ca (III) concentration on the reduced viscosity of EPSs at 25 °C, as determined by capillary viscometer. NaCl was added to the aqueous solution of polysaccharide and plotted against ionic strength ($I^{-0.5}$) and $CaCl_2$ added to the polysaccharide solution dissolved in 0.5 M NaCl.

The persistent occurrence of some EPS FF-1 in the FF-2 fraction, even after repeated chromatography, suggests some binding or linkage between the two polysaccharides. Were there to exist a covalent linkage in 30% of EPS FF-1 in EPS FF-2, then a high molecular weight dimer of approximately 3.3×10^6 Da would have been clearly seen in LS studies. This was not the case and the behavior of the EPS FF-2 is without explanation.

Both *E. futululu* and *E. teranera* are similar in that they produce two polysaccharides, one of which is pyruvated EPS. Moreover, they are both associated with the fungal canker disease of *Eucalyptus* spp. However, they distinctly differ in their ribotypes[6] and the structures of the EPSs produced by these bacteria. Also, the EPS FF-1 has the same primary structure as Stewartan from *E. stewartii* strain DC283,[17,18] the causative agent of Stewart's wilt of maize.[28–30] The structure of the EPS FF-2 is similar to that of amylovoran from *Erwinia amylovora*,[31] but with the Glc of the backbone replaced by a Gal. It is again clear that ribotyping is valuable in differentiating bacteria but does not relate to the nature of the EPSs.

Acknowledgements

The authors thank the Biotechnology Byproducts Consortium (USDA Grant No. 98-34188-5902) and the Carbohydrate Structure Facility for the use of its equipment. We also wish to thank Dr. T. A. Coutinho and Dr. L. M. VanZyl, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa for the bacterial cultures, Dr. James S. S. Gray for many discussions, John Snyder for recording the ^{1}H NMR spectra, Richard J Hollis for the ribotyping and Carol Yang for the clinical microbiological characterizations.

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