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Structural features and bioremediation activity of an exopolysaccharide produced by a strain of *Enterobacter ludwigii* isolated in the Chernobyl exclusion zone

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ABSTRACT

The bacterium *Enterobacter ludwigii* Ez-185-17, member of the family *Enterobacteriaceae*, was isolated from the root nodules of plants harvested in the nuclear power region of Chernobyl. Under batch culture conditions, the bacteria produce a high-molecular-mass exopolysaccharide (EPS). After purification, the structure of this EPS was determined using a combinatory approach including monosaccharide composition (GC-FID, HPAEC-PAD) and branching structure determination (GC–MS), as well as 1D/2D NMR (^{1}H, ^{13}C) and ESI-MS (HR, MS/MS) studies of oligosaccharides obtained from mild acid hydrolysis. The EPS was found to be a charged hexasaccharide with a repeating unit composed of D-galactose, D-glucose, L-fucose, D-glucuronic acid (2:1:2:1) and substituted with acyl and pyruvyl groups. The metal-binding properties of the exopolysaccharide were then investigated, and the results seem to indicate that the EPS decreased Cd sequestration in flax seeds.

1. Introduction

Enterobacter ludwigii, is a Gram-negative bacteria from the *Enterobacteriaceae* family, which belongs to the *Enterobacter cloacae* complex (Hoffmann et al., 2005). This species has been described to be a human pathogen, although a commensal character has been suggested (Paauw et al., 2008). Indeed, *E. ludwigii* has been isolated not only from clinical samples but also from fermented vegetables (Kwon et al., 2007). Moreover, *E. ludwigii* was found in plant rhizosphere, where some strains have been reported to be plant-associated bacteria with plant growth-promoting and biocontrol capacities (Shoebitz et al., 2009) or hydrocarbon degradation (Yousaf, Afzal, Reichenauer, Brady, & Sessitsch, 2011). *E. ludwigii* has also been recovered from biofilms associated to corrosion in oil pipelines (Neria-Gonzalez, Tao Wang, Ramirez, Romero, & Hernandez-Rodriguez, 2006), where they are potential producers of exopolysaccharides (EPS). However, no data on the EPS composition is available. Recently, the strain *E. ludwigii* Ez-185-17 was isolated from a root nodule of a plant of *Medicago lupulina* harvested in the Chernobyl exclusion zone (Pawlicki-Jullian et al., 2009) where high levels of radio-elements and heavy metals are present. Under batch culture conditions, this bacterial strain, able to fix nitrogen, produced a high-molecular-mass EPS consisting of fucose, glucose, galactose and uronic acid. Among the genus *Enterobacter*, several species have also been reported to secrete exopolysaccharides with neutral sugar residues and uronic acids. *E. cloacae* produces an acidic polysaccharide composed of fucose, galactose, glucose, glucuronic acid, pyruvate and acetate (Nishikawa, Oi, & Yamamoto, 1979). *E. sakazakii* produces a heteropolysaccharide composed of galactose, fructose, glucose, glucuronic acid and acetate (Sheepe-Leberkuhne & Wagner, 1986). *Enterobacter* sp. CNCM 1-2744 is described to produce an EPS in which fucose, galactose, glucose and glucuronic residues are present (Philbe, 2002) *Enterobacter* sp. SSYL (KCTC 0687BP), isolated from Chinese elm, produces an EPS in which glucuronic acid represents 40–70% of the sugar content (Yang, 2002) *E. amnigenus*, isolated from sugar beets, produces a heteropolymer composed of fucose, galactose, glucose, mannose, glucuronic acid and pyruvate (Cescutti et al., 2005). The accumulation of metals by microbial exopolysaccharides, which contain a large number of negatively charged functional groups, has been recognized for a few decades (Brown & Lester, 1979) but received more attention in recent years. It has been reported that the marine strain of *E. cloaceae* could produce an EPS with excellent chelating properties for heavy metal cations (Iyer, Mody, & Jha, 2004, 2005).

The main aim of this study was to report the complete structural characterization of the EPS produced by *E. ludwigii* Ez-185-17

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using chemical, spectrometric and spectroscopic techniques, and to estimate the metal binding properties of this EPS in order to find potential applications in environmental protection.

2. Materials and methods

2.1. Bacterial strain and EPS production

The strain *E. ludwigii* Ez-185-17 was isolated from a root nodule of a *M. lupulina* plant harvested in the Chernobyl exclusion zone (Pawlicki-Jullian et al., 2009). For the production of EPS, the strain was cultivated in 2 l flasks containing 1 l of Glutamate–Mannitol salt (GMS) medium (pH 7.0), and incubated on a rotary shaker at 250 rpm at 30 °C. A three-day old culture of bacteria was centrifuged (14,000 × *g*, 30 min), and the crude EPS was precipitated from the supernatant by addition of 3 volumes of cold isopropanol (overnight, 4 °C). The precipitate was dissolved in deionized water (1/10 of initial volume), dialyzed against deionized water, and lyophilized. The native EPS was then analyzed for its carbohydrate content using the PhOH–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) as well as the reaction by the metahydroxybiphenyl reagent (Filisetti-Cozzi & Carpita, 1991). The protein content analysis was carried out by the procedure of Bradford (Bradford, 1976) with bovine serum albumin (BSA) as standard. For the methylation analysis, as well as for the partial acid hydrolysis, the crude polysaccharide was de-acetylated (KOH 2 M for 12 h at 50 °C, pH 11.8) as described by Tavernier et al. (2008).

2.2. Structural characterization of the EPS

2.2.1. Molecular weight determination

The average molecular weight (M_w) of EPS was determined according to the method described by Guilloux, Gaillard, Courtois, Courtois, and Petit (2009) by high-performance size-exclusion chromatography (SEC) on Shodex SB-806 HQ, SB-804 HQ and SB-803 HQ columns in series, coupled to a differential refractometer (RI, Shimatzu, Germany) and a multi angle-laser light scattering detector (MiniDAWN, Wyatt Technology, Santa Barbara, CA).

2.2.2. Infrared spectrophotometry (IR)

The IR spectra of the polysaccharide were recorded with a Shimadzu FTIR IRPrestige-21 spectrometer equipped with IR solution software. Hundred interferograms were coadded to obtain good signal to noise ratio. An attenuated total reflectance (ATR) with diamond crystal was used. The purified polysaccharide was pressed into pellets for FTIR–ATR measurement in the frequency range of 4000–750 cm^{-1}.

2.2.3. Monosaccharides and ionic composition determination

The monosaccharide components were determined after hydrolysis with 4 M CF_3CO_2H (80 °C, 20 h), followed by the addition of pyridine–BSTFA (1:1, v/v), overnight at 4 °C (Banoub, Michon, & Shaw, 1985). The trimethylsilylated derivatives were analyzed on a GC-FID (DSQ II Trace GC, Thermo, France) with a TR-5MS capillary column (5% phenyl polysilphenylene-siloxane) (30 mm × 0.25 mm × 0.25 μm), and equipped with an automated sample injector (TriPlus). The GC temperature program was: 120 °C for 10 min, linear temperature gradient of 2 °C/min to 210 °C, ramp at 10 °C/min to 240 °C, and hold at 240 °C for 10 min. The carrier gas was helium at flow rate 1 ml/min (inlet 230 °C, transfer line 280 °C, source 220 °C, electron impact ionization (EI) 70 eV). Monosaccharides were identified by comparison of their retention times with that of pure standards analyzed under the same conditions. Mesoinositol was used as an internal standard (retention time, 45.22 min). Glycosyl linkage analysis was performed by the preparation of methylated alditol acetates according to Ciucanu and Kerek (1984). Hydrolysis of the permethylated polysaccharide was carried out with 4 M CF_3CO_2H (100 °C, 4 h). The methylated monosaccharides were reduced with $NaBD_4$, and converted to their alditol acetates with Ac_2O in pyridine, and analyzed by GC–MS as described by van Casteren, Dijkema, Schols, Beldman, and Voragen (1998).

Uronic acids were analyzed by high-performance anion exchange chromatography (HPAEC) on a CarboPak PA 1 column (Dionex, France) after hydrolysis with 4 M CF_3CO_2H (100 °C, 4 h) with a pulsed amperometric detector (Dionex ICS 3000 system) as described by Pillon et al. (2010). For the determination of the absolute configuration of the sugar residues, a sample of native EPS was hydrolyzed with 2 M CF_3CO_2H at 125 °C for 1 h, and subsequently subjected to butanolysis with (+)-2-butanol followed by GC-FID analysis of the derived 2-butyl glycoside products (Gerwig, Kamerling, & Vliegenthart, 1979).

2.2.4. Partial acid hydrolyses

The native polysaccharide (10 mg/ml) was submitted to two different mild hydrolyses. The progress of the different partial hydrolyses was followed by TLC. First, the EPS was hydrolyzed with 2 M CF_3CO_2H (100 °C, 2.5 h). The oligosaccharides produced were separated by low-pressure gel-filtration chromatography on a Bio-Gel P-2 column (90 cm × 1.5 cm), eluted with 50 mM NH_4HCO_3 using refractive index monitoring (RID-10A, Shimadzu). Second, the EPS was hydrolyzed with 4 M HCl (100 °C, 4 h), and the oligosaccharides produced were separated by high-performance anion exchange chromatography (HPAEC) on a CarboPak PA 100 with a pulsed amperometric detector (Dionex ICS 3000 system). The different fractions collected, from HCl and TFA hydrolyses, were freeze dried, and only the pure fractions (F_1 and F_2 for the TFA hydrolysis, oligo 1 and oligo 2 for the HCl hydrolysis) were analyzed by GC–EIMS, Q-ToF MS and NMR spectroscopy.

2.2.5. NMR spectroscopy

Before NMR analysis, samples were exchanged twice with 99.9% D_2O, freeze dried, and dissolved in 99.96% D_2O (<5 mg/1 ml). ^1H NMR spectra were recorded, at 80 °C for the native EPS and 25 °C for the oligosaccharides, on a Bruker Avance 500 spectrometer equipped with a 5 mm BBI probe and Topspin 1.3 software. ^1H NMR spectra were accumulated using a 30° pulse angle, a recycle time of 1 s and an acquisition time of 2 s for a spectral width of 3000 Hz for 32 K data points with a presaturation of the HOD signal using a presaturation sequence provided by Bruker. ^{13}C NMR experiments were conducted on the same spectrometer operating at 125.48 MHz with 2 s as relaxation delay.

The 2D $^1H/^1H$ COSY, $^1H/^1H$ TOCSY and $^1H/^{13}C$ HSQC spectra were acquired with standard pulse sequences delivered by Bruker. For the 2D $^1H/^{13}C$ HMBC, spectra were obtained with 512 increments of 2048 real points over a spectral width of 2997.6 Hz in the acquisition domain F_2 and 15,095.1 Hz in the time domain F_1. A total of 64 scans were used per increment with a delay of 50 ms for the evolution of long-range couplings.

All spectra were processed with NMR Notebook 2.6 software provided by NMRtec S.A.S., France. Chemical shifts (δ) are expressed in ppm relative to internal sodium 3-trimethylsilylpropanoate-d_4 (TSP-d_4 = 0 ppm), and to the residual proton of the solvent as a secondary reference. Assignment were carried out with the help of the CASPER simulator (Stenutz, Jansson, & Widmalm, 1998).

2.2.6. ESI-HRMS and MS/MS of oligosaccharides

High-resolution electrospray mass spectra (ESI-HRMS) in the positive ion mode were obtained on a Q-TOF *Ultima Global* hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray)

Table 1
Methylation analyses of native EPS.

Methylated alditol acetate	Average retention time (min)	EI-MS major primary fragments (*m*/*z*)	Linkage positions
1,4,5-Tri-*O*-acetyl-1-deuterio-2,3-Di-*O*-methylfucitol	29.69	118; 143; 203	→4)-Fucp-(1→
1,3,4,5-Tetra-*O*-acetyl-1-deuterio-2-*O*-methylfucitol	33.07	118; 173; 275	→3,4)-Fucp-(1→
1,3,5-Tri-*O*-acetyl-1-deuterio-2,4,6-Tri-*O*-methylglucitol	36.11	118; 161; 234	→3)-Glcp-(1→
1,3,5-Tri-*O*-acetyl-1-deuterio-2,4,6-Tri-*O*-methylgalactitol	37.49	118; 161; 234	→3)-Galp-(1→
1,4,5,6-Tetra-*O*-acetyl-1-deuterio-2,3-Di-*O*-methylgalactitol	44.77	118; 217; 261; 320	→4,6)-Galp-(1→
1,4,5,6-Tetra-*O*-acetyl-1,6,6-trideuterio-2,3-Di-*O*-methyl-glucitol	61.88	118; 162; 263; 307	→4)-GlcpA-(1→

for the reference compound. The oligosaccharide fractions were dissolved in water (0.01 mg/ml) and the solutions were directly introduced (5 μl/min) through an integrated syringe pump into the electrospray source. The source and desolvation temperatures were 80 °C and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 l/h, respectively. The capillary voltage was 3 kV, the cone voltage 100 V and the RF lens1 energy 50 V. Lock mass correction, using appropriate cluster ions of an *ortho*-phosphoric acid solution (0.1% in H_2O/CH_3CN, 50/50, v/v), was applied for accurate mass measurements. The mass range was typically *m*/*z* 50–2500 and spectra were recorded at 2.5 s/scan in the profile mode at a resolution of 10,000 (FWHM). For collision-induced dissociation (CID) experiments, argon was used as collision gas at an indicated analyzer pressure of 5×10^{-5} Torr and the collision energy was optimized for each precursor ion (20–70 eV). Data acquisition and processing were performed with MassLynx 4.0 software.

2.3. Cadmium uptake analyses

2.3.1. Cadmium metal binding to EPS

After 72 h of culture, the bacterial batch was supplemented with different metallic solutions of $CdCl_2$ (concentrations ranged from 0.1 to 1 mM). The batch cultures were shaken at room temperature in an orbital shaker at 200 rpm for 5 h, the EPS was recovered as described previously. After dissolution of EPS in MilliQ water (1 g/l), the solution was sampled through a 30,000 Da porosity filter and acidified with 1% nitric acid solution for analysis. The metal concentration in the EPS was determined by atomic absorption spectrophotometry (Varian).

2.3.2. Plant germination assays

Flax seeds (*Linum usitatissimum* L.) were surface-sterilized and transferred aseptically to a 500 ml culture flask containing 0.8% (w/v) agar Murashige and Skoog medium (1962) supplemented with $CdCl_2$ from 0.05 to 1 mM. EPS (0.1 or 1%) was added to the culture medium after autoclaving. After ten days, at 25 °C, under darkness, the root length of seedlings was measured. The assay was repeated three times with 24 seeds for each treatment.

3. Results and discussion

3.1. EPS purification, monosaccharide identification and linkage analysis

The EPS of *E. ludwigii* Ez-185-17 was purified from a three-day-old batch culture, and the yield was approximately 0.7 g/l. The protein amount was estimated to be less than 1%, and the HPSEC-MALLS elution profile of the native EPS showed a 2.9×10^6 Da molecular weight. Quantification by colorimetric methods gave 83% of neutrals sugars and 17% of acidic sugars. This high-molecular-mass EPS was subjected to total acid hydrolysis with TFA and the neutral sugars were converted to their alditol acetates. GC-FID analysis showed the presence of fucose, glucose and galactose in the molar ratio 2:1:2. The analysis of the acid hydrolysate by HPAEC-PAD showed, in addition to the neutral monosaccharides listed above, the presence of one glucuronic acid. The absolute configuration was shown to be L for the fucose residues and D for all the other sugars by GC-FID analysis of their trimethylsilylated (+)-2-butyl glycosides.

GC–MS analyses of the methylated EPS of *E. ludwigii* Ez-185-17 showed the presence of 4-substituted fucopyranose, 3,4-disubstituted fucopyranose, 3-substituted glucopyranose, 3-substituted galactopyranose, 4-substituted glucuronic acid and 4,6-disubstituted galactopyranose (Table 1). FTIR spectra were obtained both on the acetylated and de-*O*-acetylated EPS (supplementary data), and absorption bands of the FTIR analysis were assigned based on literature (Pau-Roblot et al., 2002; Tavallaie, Talebpour, Azad, & Soudi, 2011). In the region 1800–1200 cm^{-1}, we noted two bands at 1598 and 1414 cm^{-1} assigned to the asymmetric and symmetric stretching modes of the planar carboxylate groups from glucuronic acid, respectively. However, we observed, for the acetylated polymer, the presence of two specific bands at 1727 and 1247 cm^{-1}. These bands correspond to C=O stretching of ester groups and C—O—C antisymmetric stretching, respectively. Moreover, the deconvolution of the peak at 1598 cm^{-1} indicated the presence of two bands (1596 and 1604 cm^{-1}) attributed to carboxylate of glucuronic acid and pyruvate group, respectively. The deconvolution of peaks was also used to estimate the average degree of acetylation (DA) of the EPS. Two methods were used for the estimation of DA: the ratio of the area of the band at 1247 cm^{-1} to the area of the band at 1596 cm^{-1} or the ratio of the area of the band at 1727 cm^{-1} to the area of the band at 1596 cm^{-1}. The DA of the EPS was 41%.

3.2. NMR studies of the native EPS

In order to elucidate the structure of the polysaccharide, NMR investigation of the native core EPS was carried out in tandem with the de-*O*-acetylated EPS in order to facilitate peak assignments (Fig. 1). The ^1H NMR spectrum of the native EPS (Fig. 1a) exhibited in the low-field region (δ 5.5–4.4) the presence of overlapping anomeric proton signals. In the high-field region of the spectrum, we observed 3 signals at δ 2.1–2.3 attributed to *O*-acetyl methyl groups. A signal at δ 1.44 was attributed to the pyruvate methyl group, and several overlapping signals characteristic of 6-deoxy-sugar methyl groups were observed between δ 1.1 and 1.3. The proportion of the substituted groups (acetyl and pyruvyl groups) was determined with the integration of signals of ^1H NMR spectra from downfield, upfield and substituted regions. Therefore, we noted the presence of 2.3 *O*-acetyl groups and 1 pyruvyl group per repeating unit. The degree of *O*-acetyl substitution calculated from NMR (DA = 38%) was in accordance with the FTIR data (DA = 41%).

The ^1H NMR spectrum of the de-*O*-acetylated EPS (Fig. 1b) shows six defined resonances labeled A–F according to their decreasing chemical shift values that support the presence of the hexasaccharide repeating unit, previously suggested by GC. Chemical shifts and coupling constants indicate that the four resonances at δ 5.46 (residue A), δ 5.33 (residue B), δ 5.30 (residue C), δ 4.99 (residue D) are characteristic of the α configuration. The two resonances at δ

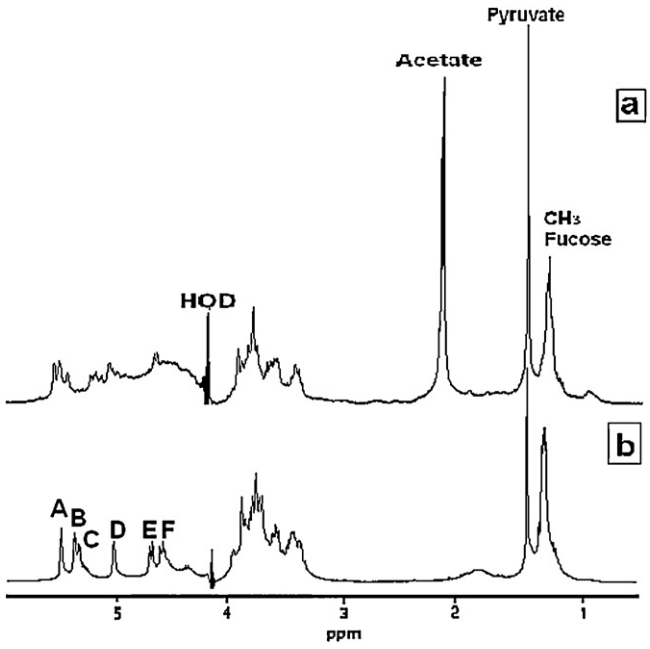


Fig. 1. ^{1}H NMR spectra (500 MHz, 80 °C) of (a) native EPS and (b) de-*O*-acetylated EPS.

4.67 (residue E) and δ 4.58 (residue F) are characteristic of the β configuration.

The ^{13}C NMR spectrum of the native EPS shows inter alia six anomeric carbon signals at δ 98.0, 99.8, 100.4, 100.6, 103.6 and 104.5. In addition, we noted the characteristic signals at low field δ 176.4, 175.2 and 174.9 corresponding to the presence of three CO groups within the repeating unit. The carbon resonance at δ 176.4 was assigned to the CO group of pyruvic acid, and signals at δ 175.2 and 174.9 to CO group of glucuronic acid and CH_3 of acetyl group, respectively. Partial assignments for the ^{1}H and ^{13}C resonances of the EPS were determined by 2D ^{1}H/^{1}H COSY and TOCSY experiments and 2D ^{1}H/^{13}C HSQC.

Due to the high viscosity of the polymer, the EPS was submitted to a partial hydrolysis with TFA or HCl.

3.3. Structural analysis of oligosaccharides

3.3.1. Oligosaccharides from TFA hydrolysis

After the partial hydrolysis of the EPS with TFA and the separation by gel chromatography, two pure fractions (F_1 and F_2) were recovered and analyzed. The GC-FID analysis of fraction F_1 revealed the presence of galactose and glucuronic acid in a molar ratio of 1:1. The ESI-HRMS spectrum of fraction F_1 showed a sodiated $[M+Na]^+$ ion at *m*/*z* 379.1, which indicated the presence of one hexose unit linked to one hexuronic acid unit. The elemental composition $[C_{12}H_{20}O_{12}Na]^+$ was confirmed by accurate mass measurement (found: 379.0848, calculated: 379.0852). To confirm the structure, NMR analyses were performed.

Three anomeric signals were observed in the ^{1}H NMR of fraction F_1 (Fig. 2a). The signal at δ 4.70 was attributed to H-1 of β-D-Glc*p*A and resonances at δ 4.65 and δ 5.30 to H-1 of β-D-galactose and H-1 of α-D-galactose, respectively. The assignment of the proton and carbon chemical shifts was completed by a combination of 2D COSY, TOCSY and HSQC (Table 2). The HMBC spectrum allowed us to confirm the linkage between H-1 of β-D-Glc*p*A (δ 4.70) and C-3 of galactose (δ 79.8 and δ 82.8) determined by the shift of chemical shift due to the substitution. From these results we propose the following structure for fraction F_1:

β-D-Glc*p*A-(1 → 3)-D-Gal*p*

For fraction F_2, GC-FID analysis indicated one fucose residue in addition to the precedent monosaccharide residues. The ESI-HRMS of this fraction produced a sodiated molecule $[M+Na]^+$ at *m*/*z* 525.1 confirming the presence of a trisaccharide. The elemental composition $[C_{18}H_{30}O_{16}Na]^+$ was established by accurate mass measurement (found: 525.1428; calculated: 525.1432). The MS/MS spectrum confirmed the successive losses of one glucuronic acid (−176 u, *m*/*z* 349.1), and one fucose unit (−146 u, *m*/*z* 203.0).

This fraction F_2 was subjected to 1D- and 2D NMR analysis. The ^{1}H NMR spectrum (Fig. 2b) showed four signals in the anomeric region. We observed two α- and two β-anomeric protons at δ 5.30, 5.22, 4.68 and 4.62, respectively. A partial overlapping of resonance at a δ 4.68 and HOD signal was observed in Fig. 2b. In the high field region, two signals at δ 1.20 and δ 1.26 were assigned to C-6 of fucose. The assignment of the proton and carbon chemical shifts was completed as previously (Table 2). On the basis of chemical shift values, the resonance at δ 5.30 was attributed to H-1 of α-galactose, at δ 5.22 to H-1 of α-fucose and resonances at δ 4.68 and δ 4.62 to H-1 of β-glucuronic acid and H-1 of β-fucose, respectively. The HMBC spectrum showed interesting inter-residue connectivities: H-1 of glucuronic acid (δ 4.68) was connected to a signal at δ 78.9 belonging to C-3 of galactose. H-1 of the galactose residue (δ 5.30) was connected to C-3 of both α- and β-fucose residue. From these results, we proposed the following structures of fraction F_2:

β-D-Glc*p*A-(1 → 3)-α-D-Gal*p*-(1 → 3)-L-Fuc*p*

3.3.2. Oligosaccharides from HCl hydrolysis

When the native EPS was hydrolyzed with 4 M HCl for 4 h at 100 °C, we obtained longer oligosaccharides. Ten fractions were recovered by HPAEC-PAD. The evaluation of these different fractions by ESI-HRMS showed mainly $[M+Na]^+$ ions corresponding to penta-, hexa- and hepta-oligosaccharides (*m*/*z* 919.3, 1065.3 and 1211.4, respectively). We will describe the structural investigations of the major fraction containing a mixture of one pentasaccharide (*m*/*z* 919.3) and one hexasaccharide (*m*/*z* 1065.3), labeled oligo 1 and oligo 2, respectively. The spectrum (Fig. 3) clearly indicates that the hexasaccharide (oligo 2) is the major component.

The GC-FID analysis of oligo 1 revealed the presence of fucose, glucose, galactose and glucuronic acid in a molar ration of 1:1:2:1. Fig. 2c shows the ^{1}H NMR spectrum of oligo 1 and oligo 2. The smallest signals were attributed to oligo 1 in accordance with mass spectrometry results. In the anomeric region between δ 5.5 and 4.4, five resonances were observed with overlapping of two signals with those from oligo 2. The anomeric configuration of each monosaccharide unit was assigned on the basis of the $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constants. The chemical shifts of each spin system were attributed based on COSY, TOCSY, HSQC and HMBC experiments (Table 2 and supplementary data). In comparison to fraction F_2, we noted for oligo 1 the presence of two more resonances in the anomeric region. The chemical shift observed for the first of the new residues allowed us to assign these resonances to galactose substituted by pyruvate with a 4,6 linkage. The second residue presents the characteristic of a reducing end with two resonances at δ 5.20 attributed to α-D-Glc*p* and δ 4.50 to β-D-Glc*p*. Moreover, the HMBC spectrum shows an interesting inter-residue connectivity between H-1 at δ 5.38 of Pyr-4,6)-α-D-Gal*p*-(1→ and C-4 at δ 78.2 of the glucuronic acid residue. Another inter-residue connectivity was observed between H-1 of the fucose residue at δ 5.22 and C-4 of the glucose unit at δ 69.2.

The oligo 1 $[M+Na]^+$ ion elemental composition was found to be $[C_{33}H_{52}O_{28}Na]^+$. Fig. 4a shows the CID-MS/MS spectrum of the

Table 2
^{1}H and ^{13}C NMR chemical shifts of F_1 to F_4 fraction, de-*O*-acetylated EPS and native EPS.

Residues	Nucleus	Chemical shifts (ppm)					
		1	2	3	4	5	6
Fraction F_1							
β-D-GlcA-(1→	^{1}H	4.70	3.44	3.54	3.55	3.77	
	^{1}C	103.6	73.2	74.9	71.8	76.1	175.2
→3)-β-D-Gal	^{1}H	4.65	3.66	3.80	4.19	3.72	3.77
	^{13}C	96.1	70.9	82.8	68.1	74.9	61.2
→3)-α-D-Gal	^{1}H	5.30	3.98	3.98	4.28	4.10	3.77
	^{13}C	92.2	67.3	79.8	68.7	70.3	61.0
Fraction F_2							
β-D-GlcA-(1→	^{1}H	4.68	3.43	3.53	3.60	3.74	
	^{13}C	103.6	73.1	75.1	71.8	76.1	175.2
→3)-α-D-Gal-(1→	^{1}H	5.30	3.86	3.90	4.14	3.90	3.75
	^{13}C	100.5	67.7	78.9	68.9	70.8	61.0
→3)-α-L-Fuc	^{1}H	5.22	3.92	3.84	3.86	4.03	1.20
	^{13}C	92.2	67.2	77.4	70.6	66.1	16.1
→3)-β-L-Fuc	^{1}H	4.62	3.62	3.67	3.87	3.79	1.26
	^{13}C	96.1	70.9	81.0	71.4	70.4	16.2
Oligo 1							
Pyr-4,6)-α-D-Gal-(1→	^{1}H	5.38	3.84	3.84	4.11	3.72	3.91
	^{13}C	100.4	69.4	69.4	72.3	63.7	65.7
→4)-β-D-GlcA-(1→	^{1}H	4.69	3.41	3.72	3.77	3.83	
	^{13}C	104.5	74.2	76.9	78.2	77.5	175.2
→3)-α-D-Gal-(1→	^{1}H	5.31	3.91	3.92	4.12	4.15	3.73
	^{13}C	101.4	68.8	80.8	70.1	71.7	62.3
→3)-α-L-Fuc-(1→	^{1}H	5.22	4.03	3.87	3.85	4.30	1.21
	^{13}C	100.4	69.3	78.8	72.9	67.5	16.3
→3)-β-D-Glc	^{1}H	4.50	3.28	3.51	3.40	3.47	3.88
	^{13}C	97.4	74.2	83.4	70.5	76.9	61.8
→3)-α-D-Glc	^{1}H	5.20	3.71	3.77	3.50	3.86	3.76
	^{13}C	93.2	72.4	81.3	69.2	72.3	61.8
Pyr	^{1}H			1.44			
	^{13}C	176.4	101.4	26.0			
Oligo 2							
Pyr-4,6)-α-D-Gal-(1→	^{1}H	5.38	3.84	3.84	4.11	3.72	3.91
	^{13}C	100.4	69.4	69.4	72.3	63.7	65.7
→4)-β-D-GlcA-(1→	^{1}H	4.71	3.41	3.72	3.77	3.83	
	^{13}C	104.5	74.2	76.9	78.2	77.5	175.2
→3)-α-D-Gal-(1→	^{1}H	5.27	3.91	3.92	4.12	4.15	3.73
	^{13}C	101.4	68.8	80.8	70.1	71.7	62.3
α-L-Fuc-(1→	^{1}H	5.19	3.81	3.87	3.85	4.30	1.21
	^{13}C	100.4	69.3	70.8	72.9	67.5	16.3
→3,4)-α-L-Fuc-(1→	^{1}H	4.92	4.03	3.87	4.10	4.51	1.21
	^{13}C	100.6	69.9	78.2	80.6	68.5	16.3
→3)-β-D-Glc	^{1}H	4.50	3.28	3.51	3.40	3.47	3.88
	^{13}C	97.4	74.2	83.4	70.5	76.9	61.8
→3)-α-D-Glc	^{1}H	5.20	3.71	3.77	3.50	3.86	3.76
	^{13}C	93.2	72.4	81.3	69.2	72.3	61.8
Pyr	^{1}H			1.44			
	^{13}C	176.4	101.4	26.0			
De-*O*-acetylated EPS							
Pyr-4,6)-α-D-Gal-(1→	^{1}H	5.46	3.92	3.90	4.17	3.80	3.99
	^{13}C	100.4	69.3	69.3	72.3	63.7	65.7
→4)-β-D-GlcA-(1→	^{1}H	4.67	3.47	3.74	3.78	3.89	
	^{13}C	104.5	74.3	76.8	78.2	77.4	175.3
→3)-α-D-Gal-(1→	^{1}H	5.33	3.98	3.98	4.19	4.23	3.74
	^{13}C	101.3	68.8	80.7	70.2	71.8	62.3
→3)-β-D-Glc-(1→	^{1}H	4.58	3.60	3.66	3.45	3.40	3.88
	^{13}C	103.6	74.4	83.2	70.6	77.1	61.6
→3,4)-α-L-Fuc-(1→	^{1}H	4.99	4.05	4.11	4.18	4.55	1.29
	^{13}C	100.6	69.9	78.2	80.6	68.5	16.3
→4)-α-L-Fuc-(1→	^{1}H	5.30	3.89	3.98	3.89	4.41	1.27
	^{13}C	100.2	69.5	70.1	80.9	67.5	16.3
Pyr	^{1}H			1.44			
	^{13}C	176.4	101.4	26.0			
Native EPS							
Pyr-4,6)-α-D-Gal-(1→	^{1}H	5.48	3.94	3.94	4.19	3.81	4.00
	^{13}C	100.4	69.4	69.4	72.3	63.7	65.7
→4)-β-D-GlcA-(1→	^{1}H	4.83	3.40	3.75	3.78	3.93	
	^{13}C	104.5	74.3	76.9	78.2	77.4	175.2
→3)-α-D-Gal-(1→	^{1}H	5.37	*5.15*	4.33	4.24	4.23	3.74
	^{13}C	99.8	*70.5*	77.6	67.2		
→3)-β-D-Glc-(1→	^{1}H	4.60	3.62	3.99	3.48	3.45	3.90
	^{13}C	103.6	74.4	83.2	70.6	77.2	61.7
→3,4)-α-L-Fuc-(1→	^{1}H	5.07	4.05	4.11	4.18	4.55	1.33
	^{13}C	100.6	69.9	78.2	80.7	68.5	16.3

Table 2 (*Continued*)

Residues	Nucleus	Chemical shifts (ppm)					
		1	2	3	4	5	6
→4)-α-L-Fuc-(1→	^{1}H	5.30	*4.80*	4.14	3.89	4.41	1.27
	^{13}C	98.0	*71.8*	67.7	81.3	68.5	16.3
Pyr	^{1}H			1.44			
	^{13}C	176.4	101.4	26.0			
O-Ac	^{1}H		2.16				
	^{13}C	174.9	21.5				
O-Ac	^{1}H		2.18				
	^{13}C	174.9	21.5				
O-Ac	^{1}H		2.20				
	^{13}C	174.9	21.5				

The chemical shifts are given relative to internal TSP-d_4. Italicized values indicate *O*-acetyl substitution.

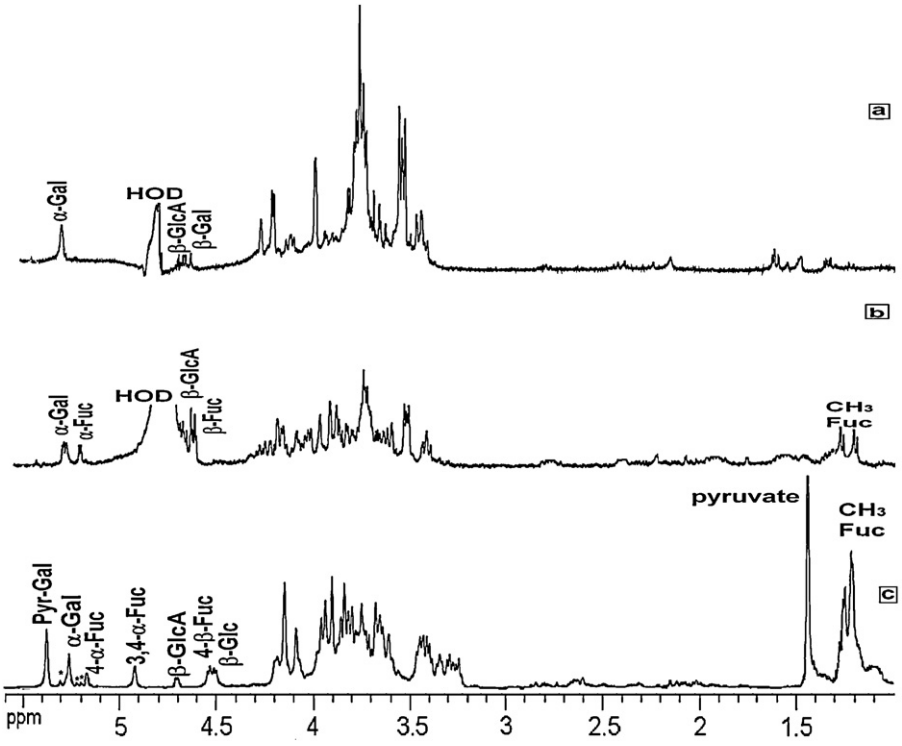


Fig. 2. ^{1}H NMR spectra (500 MHz, 25 °C) of (a) fraction F_1, (b) fraction F_2, (c) oligo 1 + 2. *Small signals from oligo 1.

$[M+Na]^+$ ion at *m*/*z* 919.3, annotated according to the systematic fragmentation nomenclature of Domon and Costello (1988) and reported in Table 3. We noted the presence of abundant interglycosidic product ions belonging to the Y and Z series, as expected for a linear oligosaccharide with a high degree of polymerization (DP) (Lesur et al., 2006). In addition, weaker ions at *m*/*z* 593.1, 431.1 and 255.1, corresponding to the B series, were identified. These fragments, in accordance with NMR data, suggested the following sequence for oligo 1:

Pyr-4, 6-α-D-Gal*p*-(1 → 4)-*β*-D-Glc*p*A-(1 → 3)-*α*-D-Gal*p*-(1 → 3)-*α*-L-*Fuc*p-(1 → 3)-D-Glc*p*

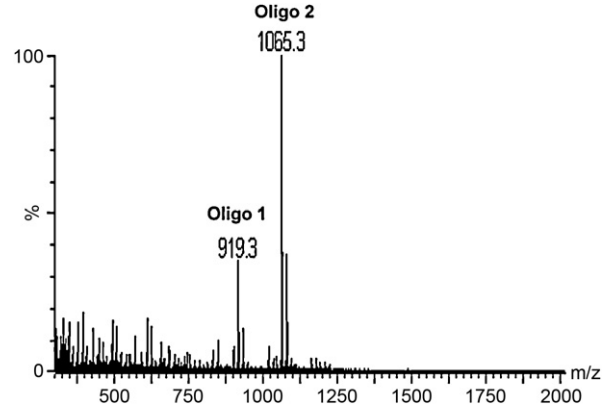


Fig. 3. ESI-HRMS spectrum of the major fraction obtained after HCl hydrolysis.

In addition, negative electrospray ionization investigations (data not shown) confirm the deprotonated molecule at *m*/*z* 1041.3, the product ions corresponding to the C series (*m*/*z* 249.1, 425.1 and 587.3) and the pyruvate moiety at *m*/*z* 87.0 (CH_3—CO—COO^-; found: 87.0089, calculated: 87.0082).

Concerning the second oligosaccharide (oligo 2), the monosaccharide contents was similar to oligo 1 but in a different molar ratio 2:2:1:1. The NMR data confirmed the precedent structure with the presence of a supplementary fucose residue as shown by two signals of CH_3 of fucose in the δ 1.1–1.3 region. Chemical shifts of each spin system were attributed based on COSY, TOCSY, HSQC and HMBC experiments (Table 2, supplementary data). We

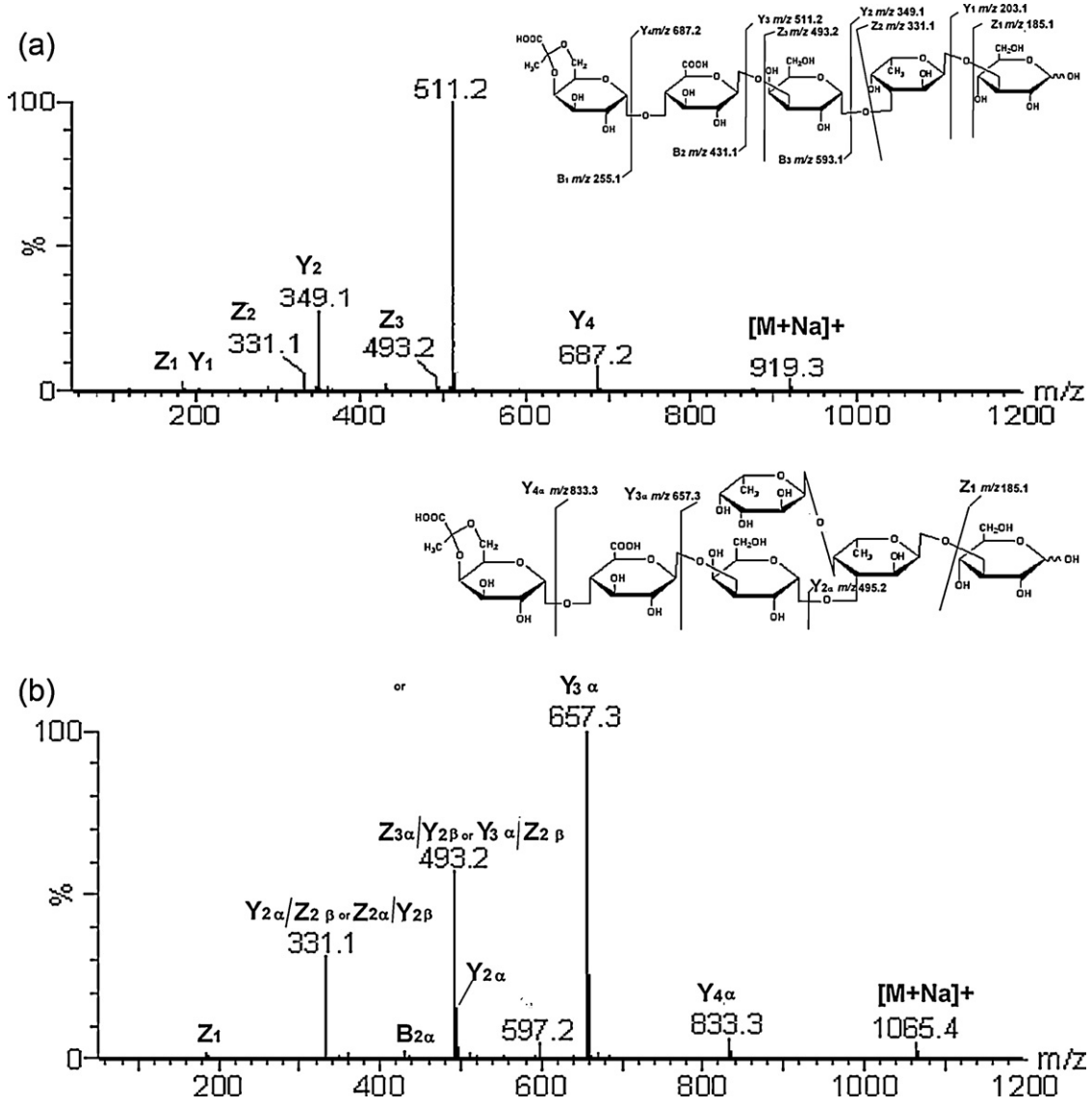


Fig. 4. CID-MS/MS spectra (40 eV) of (a) oligo 1 and (b) oligo 2. Fragment ions corresponding to inter-glycosidic cleavages, allowing the oligosaccharide sequence determination, are indicated on the structure.

observed a shift from oligo 1 to oligo 2 of chemical shift for H-4 from δ 3.85 to δ 4.10 and for C-4 from δ 72.9 to δ 80.6 of →3)-α-L-Fuc-(1→, pointing out a substitution in position 4 in oligo 2.

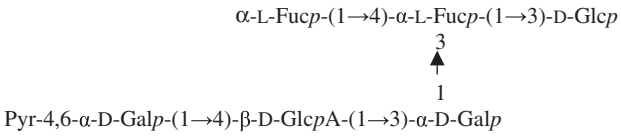
As presented in Fig. 3, oligo 2 is characterized by its sodiated ion $[M+Na]^+$ at m/z 1065.3. The exact mass determination lead to the formula $[C_{39}H_{62}O_{32}Na]^+$ confirming the additional fucose unit. The CID-MS/MS spectrum is presented in Fig. 4b, and the main fragments are reported in Table 3. The presence of the Y_α series ($Y_{2\alpha}$ m/z 495.2, $Y_{3\alpha}$ m/z 657.3 and $Y_{4\alpha}$ m/z 833.3) seems to confirm the branched structure (Kilz, Waffenschmidt, & Budzikiewicz, 2000) with a supplementary fucose residue probably linked to the fucose located on oligo 1. Additionally, the release of fragment $Y_{2\alpha}/Z_{2\beta}$ or $Z_{2\alpha}/Y_{2\beta}$ (m/z 331.1) and $Z_{3\alpha}/Y_{2\beta}$ or $Y_{3\alpha}/Z_{2\beta}$ (m/z 493.2), was observed, corresponding to a spontaneous loss of the additional fucose residue. The negative electrospray MS/MS spectrum of the deprotonated molecule at m/z 1041.3 (data not shown) revealed a more complex fragmentation pattern. However, the

Table 3
ESI-HRMS and MS/MS data of oligosaccharide fractions obtained after HCl hydrolysis of the native EPS.

	Oligomer	$[M+Na]^+$ found	$[M+Na]^+$ calculated	Elemental composition	Fragment MS/MS ions: m/z (relative intensity %) and identification following Domon nomenclature
Oligo 1	[Pyr]-[Gal]-[GlcA]-[Gal]-[Fuc]-[Glc]-OH	919.2505	919.2543	$C_{33}H_{52}O_{28}Na$	919.3 (4) $[M+Na]^+$, 687.2 (8) Y_4, 593.1 (<1) B_3, 511.2 (100) Y_3, 493.2 (4) Z_3, 431.1 (2) B_2, 349.1 (27) Y_2, 331.1 (6) Z_2, 255.1 (<1) B_1, 203.1 (<1) Y_1, 185.1 (3) Z_1
Oligo 2	[Fuc]-[Fuc]-[Glc]-OH [Pyr]-[Gal]-[GlcA]-[Gal]	1065.3086	1065.3121	$C_{39}H_{62}O_{32}Na$	1065.4 (5) $[M+Na]^+$, 833.3 (6) $Y_{4\alpha}$, 657.3 (100) $Y_{3\alpha}$, 597.2 (4) $Y_{3\alpha}$ —$C_2H_4O_2$, 593.2 (<1) $B_{3\alpha}$, 495.2 (15) $Y_{2\alpha}$, 493.2 (56) $Z_{3\alpha}/Y_{2\beta}$ or $Y_{3\alpha}/Z_{2\beta}$, 431.1 (2) $B_{2\alpha}$, 349.1 (<1) $Y_{2\alpha}/Y_{2\beta}$, 331.1 (31) $Y_{2\alpha}/Z_{2\beta}$ or $Z_{2\alpha}/Y_{2\beta}$, 185.1 (2) Z_1

pyruvatemoiety is clearly identified at *m*/*z* 87.0 (found: 87.0079, calculated: 87.0082).

The combination of NMR and MS data allowed us to propose for oligo 2 the following structure:



The number of anomeric protons present in oligo 2 was the same as in the de-acetylated EPS except for the reducing end. We deduced that this oligosaccharide corresponds to the repeating unit of the de-acetylated EPS and this allowed us to assign all of the signals of the de-acetylated EPS (Table 2).

Comparison of the ^{1}H NMR spectra of the de-acetylated and acetylated polysaccharide (Fig. 1) showed a variation in the chemical shift of H-2 at the →3)-α-D-Gal-(1→ residue, and of H-2 of the →4)-α-L-Fuc-(1→ residue. From these results, we could presume that acetyl groups were attached to these residues and we deduced the total assignment of signals of the native polysaccharide as summarized in Table 2. The proportion of the acetyl groups calculated from ^{1}H NMR spectrum of native EPS was 2.3, and three acetyl signals were present in the spectrum. Another *O*-acetyl group was present in the repeat unit, but its position was not determined in this study.

3.4. Cadmium experiments

The effect of EPS on root elongation of flax germinations in presence of cadmium in the culture medium is shown in Table 4. In the presence of $CdCl_2$, the flax seeds were very sensitive. We did not observe any germination of the seeds when $CdCl_2$ concentrations above 0.1 mM were added. Addition of EPS (0.1 and 1%) in the culture medium enhanced root elongation, even when high concentrations of $CdCl_2$ are present. The analysis by atomic absorption spectrophotometry of cadmium indicated that the EPS, produced by the *E. ludwigii* Ez-185-17 after a three-day-old culture, was able to trap more than 35% of initial cadmium concentration.

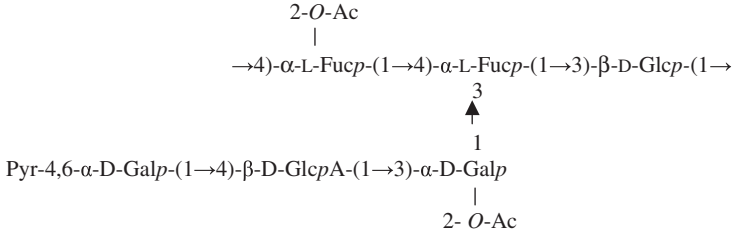
It has been described that the conformational characteristics of EPS produced by bacteria affect the binding of different molecules available to its cells (Costernon, Geesy, & Cheng, 1987). The acidic composition of the EPS produced by the strain *E. ludwigii* Ez-185-17, increased by the presence of a pyruvated galactose, is certainly responsible for this metal binding capacity. These results seem to indicate that the EPS decreased Cd sequestration in flax seeds conferring a protective effect in the presence of heavy metals.

Table 4
Root length (mm) of flax seedlings grown on a culture medium supplemented with different concentrations of $CdCl_2$ in presence or not of EPS (the data are means of three experiments of 24 seedlings each).

Cadmium concentration (mM)	EPS 0	EPS 0.1%	EPS 1%
0.05	24 ± 1.5	58 ± 1.5	64 ± 0.2
0.1	21 ± 1.2	56 ± 0.8	63 ± 1.4
0.25	0	47 ± 1.1	72 ± 1.1
0.5	0	32 ± 1.5	58 ± 2.4
0.75	0	24 ± 1.8	51 ± 2.3
1	0	17 ± 1.5	49 ± 1.9

4. Conclusions

The structural composition of the exopolysaccharide produced by a strain of *E. ludwigii*, *E. ludwigii* Ez-185-17, isolated from a root nodule of a *Medicago* plant was described for the first time. Based on the NMR and spectrometry data presented in this report, the structure of the exopolysaccharide is as follows:



This high-molecular-weight heteropolysaccharide from *E. ludwigii* Ez-185-17 share several structural features with the EPS described by Cescutti, Toffanin, Pollesello, and Sutherland (1999), but also with the EPS produced by *Erwinia chrysanthemi* Ech6 (Yang, Gray, & Montgomery, 1994), and with colanic acid, the EPS of many genera in the *Enterobacteriaceae* (Garegg, Lindberg, Onn, & Sutherland, 1971; Grant, Sutherland, & Wilkinson, 1969). Recently, the structure of colanic acid was again described (Rätto et al., 2006; Verhoef et al., 2005). Compared to the EPS produced by *E. ludwigii* Ez-185-17, the major differences are the anomeric configuration of the galactose residues and of the branched fucose residue.

In the soil of the Chernobyl exclusion zone, the level of radioelements as well as the level of heavy metals is quite high, the bacteria must be well adapted (Giller, McGrath, & Hirsch, 1989) and the produced EPS must protect the bacteria from environmental factors. Future studies will focus on the mechanism of biodisponibility of cadmium by the EPS for plants in order to remediate Cd-contaminated soils.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.09.025.

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