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Structure of the high-molecular weight exopolysaccharide isolated from *Lactobacillus pentosus* LPS26

Miguel A. Rodríguez-Carvajal [a,*], J. Ignacio Sánchez [b], Ana B. Campelo [b], Beatriz Martínez [b], Ana Rodríguez [b], Antonio M. Gil-Serrano [a]

[a] *Department of Organic Chemistry, Faculty of Chemistry, University of Seville, 41012 Sevilla, Spain*
[b] *Instituto de Productos Lácteos de Asturias (IPLA-CSIC), 33300 Villaviciosa, Asturias, Spain*

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ABSTRACT

The strain *Lactobacillus pentosus* LPS26 produces a capsular polymer composed of a high- (2.0×10^6 Da) (EPS A) and a low-molecular mass (2.4×10^4 Da) (EPS B) polysaccharide when grown on semi-defined medium containing glucose as the carbon source. The structure of EPS A and its deacetylated form has been determined by monosaccharide and methylation analysis as well as by 1D/2D NMR studies (^{1}H and ^{13}C). We conclude that EPS A is a charged heteropolymer, with a composition of D-glucose, D-glucuronic acid and L-rhamnose in a molar ratio 1:2:2. The repeating unit is a pentasaccharide with two *O*-acetyl groups at O-4 of the 3-substituted α-D-glucuronic acid and at O-2 of the 3-substituted β-L-rhamnose, respectively.

→4)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A4Ac-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*A-(1→3)-β-L-Rha*p*2Ac-(1→

This unbranched structure is not common in EPSs produced by Lactobacilli. Moreover, the presence of acetyl groups in the structure is an unusual feature which has only been reported in *L. sake* 0–1 [Robijn et al. *Carbohydr. Res.*, **1995**, *276*, 117–136].

1. Introduction

Several lactic acid bacteria (LAB) produce exopolysaccharides (EPSs) that are secreted into the growth media or remain tightly attached to the cell surface.[1] These polymers have found a major application in the manufacture of fermented dairy products, specially low-fat yoghurt and cheese, providing thickening and gelling properties at low concentrations.[2] Likewise, certain EPSs produced by LAB are also claimed to have beneficial effects on human health.[3] Among EPSs produced by LAB, heteropolysaccharides (HePS) vary largely according to sugar composition, sugar linkages, chain length, presence of repeated side chains and substitutions. All of these factors determine their technological and biological properties.[4] Therefore, the determination of the chemical composition and structure of novel EPSs is relevant for predicting their potential applications.

Recently, we have reported the production of EPSs by the species *Lactobacillus pentosus*.[5] The strain *L. pentosus* LPS26, isolated from a natural fermentation of green olives, produces a capsular polymer constituted of a high- (EPS A) and a low-molecular mass (EPS B) polysaccharide. According to the preliminary monosaccharide analysis, they are composed of glucose and rhamnose, and glucose and mannose, respectively. It has been observed that culture conditions have a clear influence on the ratio and concentration of each EPS. Here, we report the structural analysis of EPS A when grown in a modified semi-defined medium (mSDM) containing glucose as the carbon source.

2. Results and discussion

The isolation and identification of new bacterial polysaccharides providing novel chemical, rheological, and biological properties are of great interest for the scientific community. In this regard, the production by the strain *L. pentosus* LPS26 of a capsular polymer composed of two HePSs that differed in molecular weight and sugar composition has been recently reported.[5]

2.1. Exopolysaccharide purification

Polysaccharide samples obtained from 1 L culture of *L. pentosus* LPS26 (260 mg of total EPS) were analyzed by size-exclusion chromatography. The analysis revealed the presence of the two

Abbreviations: LAB, lactic acid bacteria; EPS, exopolysaccharide; HePS, heteropolysaccharide; SDM, semi-defined medium; PMAAs, partially O-methylated alditol acetates; DQF, double-quantum filtered; HSQC, ^{1}H-detection mode hetero single-quantum correlation.

* Corresponding author. Tel.: +34 954 559 997; fax: +34 954 624 960.
E-mail address: rcarvaj@us.es (M. A. Rodríguez-Carvajal).

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peaks corresponding to EPS A and EPS B as previously described.[5] However, the modification of the method improved the separation of both EPSs and provided a more accurate determination of molecular mass, with EPS A and EPS B being of 2.0×10^6 Da and 2.4×10^4 Da, respectively (Fig. S1, in Supplementary data). These two peaks were not detected in non-inoculated medium processed in the same way as the cultures. By contrast, the third peak (retention time of 40.117 min) corresponds to a component of the culture medium as it was also detected in non-inoculated medium (data not shown). Fractions of EPS A were collected, dialyzed against water, and freeze-dried for further chemical and structural analyses.

2.2. Monosaccharide composition analysis

GLC–MS analysis of the trimethylsilylated methyl glycosides of EPS A showed that it contains glucose (Glc) and rhamnose (Rha) (in a 3:1 ratio, respectively), and traces of glucuronic acid (GlcA); besides, two peaks whose mass spectra and retention times are characteristic of aldobiuronic acids were observed. The presence of GlcA had not been reported in the previous publication[5] because it could not be detected by the sugar analysis method employed there.

When the methanolysis was performed in higher concentrated HCl/MeOH (1.5 M, 80 °C, 24 h, instead of 0.625 M, 80 °C, 16 h), the intensity of the aldobiuronic acid peaks was considerably reduced. This treatment changed the results of the monosaccharide composition analysis to a 3:2:1 ratio (Glc:Rha:GlcA). The equivalent Rha and GlcA increasing agrees with an aldobiuronic acid composed of rhamnose and glucuronic acid. However, it was not possible to achieve a good agreement between quantification by GLC–MS and NMR results (see below) because of the resistance of the glucuronic acid–rhamnose linkage to acid conditions.

The monosaccharide composition analysis of the carboxyl-reduced polysaccharide (EPSA_R) obtained according to a modification of the method of Taylor and Conrad[6] indicated that it contains glucose, and rhamnose in about a 3:1.3 ratio, respectively. This analysis also indicated that the EPS A reduction was not completed and rhamnose was partially present as aldobiuronic acid, which could explain the disagreement with the expected (see NMR analysis) 3:2 ratio (Glc:Rha). Thus, GLC–MS results were considered in a semiquantitative way and consequently, the relative ^{1}H NMR peak areas were used to accurately quantify the ratio of the different residues in this polysaccharide.

The absolute configuration of the monosaccharides in the polysaccharide was achieved by the formation of their trimethylsilylated (*S*)- and (*R,S*)-2-butyl glycosides and by their GLC–MS analysis. Both glucose and glucuronic acid residues showed the D configuration, whereas the L configuration was found in the rhamnose residues.

2.3. NMR analysis

Figure 1a shows the 500 MHz ^{1}H NMR spectrum of EPS A (303 K). Although signals corresponding to anomeric protons (5.7–4.5 ppm), acetyl groups (ca. 2.1 ppm), and methyl groups of rhamnose (1.4–1.2 ppm) can be assigned, the peaks are broad and poorly defined. In order to improve the solubility of the polysaccharide in water and to avoid any potential broadening of signals by a random distribution of acetyl groups, an aliquot of EPS A was deacetylated, yielding the polysaccharide EPSA_D. This polysaccharide was thoroughly studied by NMR at 323 K (Fig. 1b) using the experiments DQF-COSY (Fig. S2, in Supplementary data), TOCSY (Fig. 2a and Fig. S3, in Supplementary data, NOESY (Fig. 2b), and HSQC (Fig. 3a).

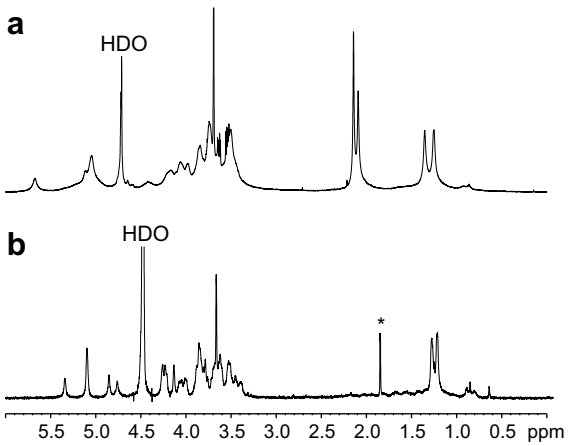

Figure 1. 500 MHz ^{1}H NMR spectra of the high-molecular weight exopolysaccharide isolated from *L. pentosus* LPS26 (a) and the resulting polysaccharide after a deacetylation treatment (b). Impurities are indicated by asterisks.

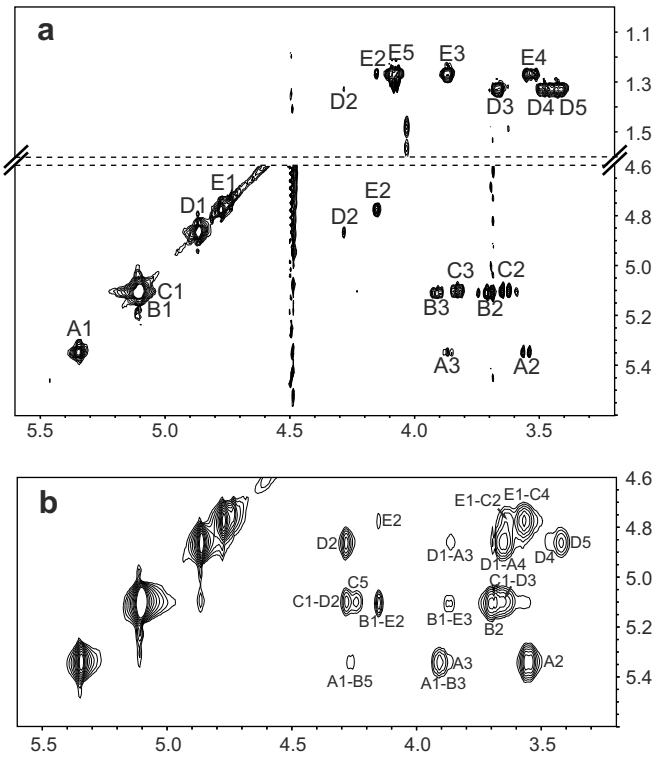

Figure 2. 500 MHz TOCSY (a) and NOESY (b) spectra of deacetylated exopolysaccharide A isolated from *L. pentosus* LPS26.

The anomeric region in ^{1}H NMR of EPSA_D shows four signals whose area ratio (1:2:1:1) indicates the existence of five sugar residues, which were labeled A–E according to their chemical shifts.

Residue A: 500 MHz TOCSY spectrum (Fig. 2a and Fig. S3, in Supplementary data shows cross-signals between H-1 (δ_H 5.35 ppm) and a set of signals at δ_H 3.55 (H-2), 3.87 (H-3), 3.65 (H-4), and 4.02 (H-5) ppm. The assignment of the latter was based on the DQF-COSY spectrum (Fig. S2, in Supplementary data). On the other hand, the HSQC spectrum (Fig. 3a) shows the hydroxymethyl group signals of the glucopyranose residue at δ_H 3.89 and 3.79 ppm (δ_C 60.8 ppm), which correlate with H-5 in the DQF-COSY spectrum. The set of coupling constants (Table 1) agrees with the *gluco* configuration for this residue, whereas the small $^3J_{1,2}$ (<2 Hz) and the

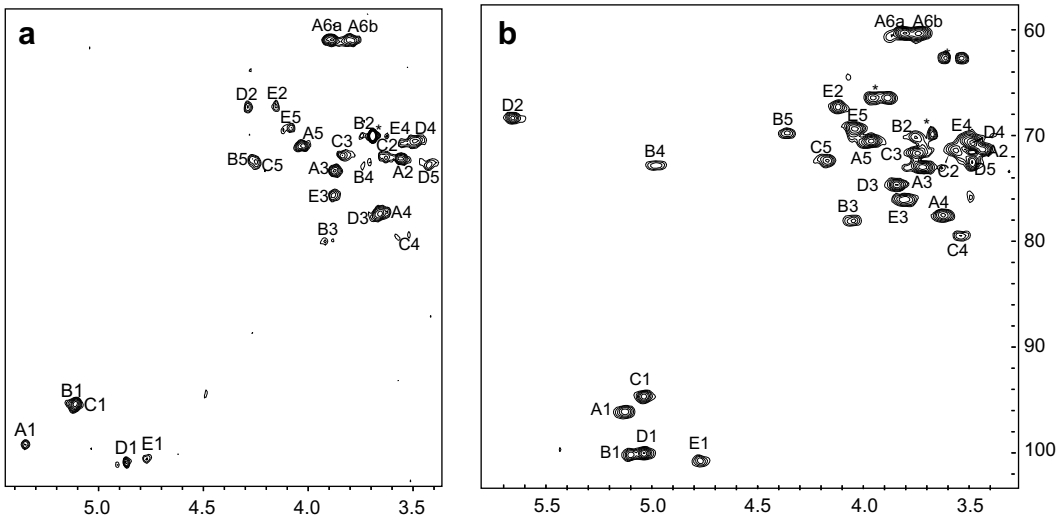

Figure 3. ^{1}H–^{13}C HSQC spectra obtained for the exopolysaccharide A of *L. pentosus* LPS26. (a) Deacetylated. (b) Native polysaccharide.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm), and coupling constants ($^{3}J_{H,H}$, Hz) for the deacetylated exopolysaccharide A of *L. pentosus* LPS26

Unit		1	2	3	4	5	6
A	^{1}H	5.35	3.55	3.87	3.65	4.02	3.89; 3.79
	$^{3}J_{H,H}$	$J_{1,2}$ <2	$J_{2,3} \sim 10$	$J_{3,4} \sim 8$	$J_{4,5} \sim 8$	[b]	$J_{6a,6b} \sim 12$
	^{13}C	99.4	72.1	73.3	77.2	71.0	60.8
B	^{1}H	5.11	3.70	3.91	3.72	4.26	—
	$^{3}J_{H,H}$	$J_{1,2}$ <5	$J_{2,3} \sim 10$	$J_{3,4} \sim 8$	$J_{4,5} \sim 8$	—	—
	^{13}C	95.5[a]	~70	80.0	72.6	72.3	[b]
C	^{1}H	5.10	3.63	3.83	3.57	4.24	—
	$^{3}J_{H,H}$	$J_{1,2}$ <5	$J_{2,3} \sim 10$	$J_{3,4} \sim 8$	$J_{4,5} \sim 8$	—	—
	^{13}C	95.6[a]	72.0	71.8	79.7	72.6	[b]
D	^{1}H	4.86	4.28	3.67	3.48	3.42	1.33
	$^{3}J_{H,H}$	$J_{1,2}$ <2	$J_{2,3}$ <4	$J_{3,4} \sim 8$	$J_{4,5} \sim 8$	$J_{5,6} \sim 6$	—
	^{13}C	101.1	67.3	77.4	70.5	72.8	17.0
E	^{1}H	4.77	4.15	3.87	3.53	4.08	1.27
	$^{3}J_{H,H}$	$J_{1,2}$ <2	$J_{2,3}$ <4	$J_{3,4} \sim 8$	$J_{4,5} \sim 8$	$J_{5,6} \sim 6$	—
	^{13}C	100.8	67.1	75.6	70.7	69.2	16.8

[a] Exchangeable assignment.
[b] Not determined.

chemical shift of H-1 (>5 ppm) demonstrate that the anomeric configuration is α. The downfield shift of C-4 (77.2 ppm, Table 1) indicates that this position is substituted. Thus, this residue corresponds to →4)-α-D-Glc*p*.

Residue B: In the TOCSY spectrum, H-1 (δ_H 5.11 ppm) appears correlated to signals at 3.70 ppm (H-2, according to DQF-COSY) and 3.91 ppm (H-3). Besides, signals at 3.72 ppm (H-4) and 4.26 ppm (H-5) are identified. The coupling constants in signals H-1 to H-5 (Table 1) are in agreement with an alpha *gluco* configuration. The relatively high value of H-5 indicates that this residue corresponds to a glucopyranuronic acid unit. The low-field shift of C-3 (80.0 ppm) indicates the linkage point, this residue being a unit of →3)-α-D-Glc*p*A.

Residue C: DQF-COSY and TOCSY allow the identification of H-2 (3.63 ppm), H-3 (3.83 ppm), H-4 (3.57 ppm), and H-5 (4.24 ppm) from H-1 signal (5.10 ppm). In this case, the linkage position is *O*-4 according to the low-field shift of C-4 (79.7 ppm). The coupling constants indicate an α-*gluco* configuration, this residue being a unit of →4)-α-D-Glc*p*A.

Residue D: TOCSY spectrum shows only a correlation between H-1 (4.86 ppm) and H-2 (4.28 ppm). The identification of the rest of the spin system arises from the correlation peaks with a methyl group at 1.33 ppm (H-6) (Fig. 2a), and their assignments are based on DQF-COSY. The set of coupling constants, $^{3}J_{1,2}$ very small (< 2 Hz), $^{3}J_{2,3}$ small (< 4 Hz), $^{3}J_{3,4}$ large (~8 Hz), and $^{3}J_{4,5}$ large (~8 Hz), are characteristic of a *manno* configuration, this residue being a unit of rhamnopyranose. The linkage position in this residue is indicated by the chemical shift of C-3 (77.4 ppm) (Table 1). A coupled HSQC experiment allowed the determination of $^{1}J_{C,H}$ in all residues. The obtained value (165 Hz) indicates that it is the β-anomer, this unit being a →3)-β-L-Rha*p* residue.

Residue E: Likewise the preceding residue, TOCSY spectrum from H-1 (4.77 ppm) shows only a correlation peak with H-2 (4.15 ppm), whereas the rest of the spin system can be obtained from the methyl signal at 1.27 ppm (H-6). The HSQC spectrum indicates that C-3 (75.6 ppm) is the linkage position in this unit. Finally, $^{1}J_{C,H}$ (173 Hz) is characteristic of an α anomer, this residue being a unit of →3)-α-L-Rha*p*.

Coupling constants and ^{13}C chemical shifts indicate that all the residues are in pyranosidic forms.

Determination of the sequence: The sequence of the units in the repeating unit can be obtained from NOESY experiment (Fig. 2b). Relatively intense cross-peaks between H-1 of residue A and H-3 of residue B indicate the proximity of these residues. In the same way, H-1(B) correlates with H-2 and H-3 of residue E, H-1(E) generates intense NOE cross-peaks with signals of residue C, and H-1(C) correlates with H-2 and H-3 (intense) of residue D. Finally, H-1(D) presents an intense cross-peak with H-4 of residue A. Thus, the sequence of residues in the polysaccharide is A-B-E-C-D.

2.4. Methylation analysis

The position of the glycosidic linkages in the polysaccharide EPS A was confirmed by methylation analysis. The polysaccharide was methylated, carboxyl-reduced using $NaB^{2}H_4$, hydrolyzed, reduced with $NaB^{2}H_4$, and acetylated. GLC–MS analysis of the resulting partially methylated alditol acetates showed the presence of 1,3,5-tri-*O*-acetyl-1-deuterio-2,4-di-*O*-methylrhamnitol, arising from a 3-linked rhamnopyranose residue, 1,4,5-tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methylglucitol, arising from 4-linked glucopyranose, 1,3,5,6-tetra-*O*-acetyl-1,6,6-trideuterio-2,4-di-*O*-methylglucitol, derived from a 3-linked glucopyranuronic acid residue, and 1,4,5,6-tetra-*O*-acetyl-1,6,6-trideuterio-2,3-di-*O*-methylglucitol, arising from 4-linked glucopyranuronic acid residue. The degradation of uronic acids in alkali conditions must be responsible for the very low ratio (about 0.1) of the PMAAs arising from the 3- and 4-linked

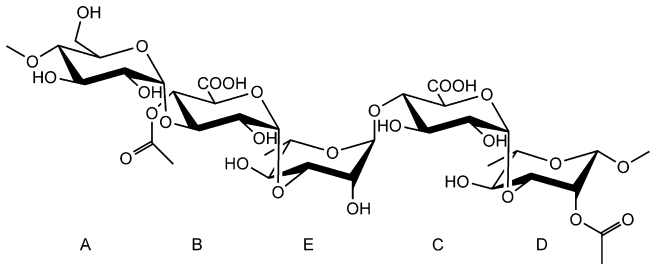

Figure 4. Repeating unit of the native exopolysaccharide A of *L. pentosus* LPS26.

glucuronic acid residues in the methylation analysis; nevertheless, the ratio of 3-linked rhamnopyranose to 4-linked glucopyranose (2.5:1.0) is close to that expected from the proposed structure (2:1).

To avoid this alkali degradation, carboxyl-reduced polysaccharide (EPSA_R) was also studied by methylation analysis, which gave the PMAAs corresponding to 3-linked rhamnopyranose, 3-linked glucopyranose, and 4-linked glucopyranose residues in a ratio 2.3:0.5:2.0, respectively. The remaining glucuronic acid in EPSA_R could be responsible for the differences with the expected ratio 2:1:2.

From the above results, we propose the structure of the deacetylated polysaccharide as

→4)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*A-(1→3)-β-L-Rha*p*-(1→

Location of acetyl groups: The assignment of the NMR signals and the structure of the deacetylated polysaccharide make the assignment of the NMR spectra of the native exopolysaccharide isolated from *L. pentosus* LPS26 easier. NMR studies included experiments DQF-COSY, TOCSY (Fig. S4, in Supplementary data), and HSQC (Fig. 3b), and the results are summarized in Table S1 (in Supplementary data).

The relative area in ^{1}H NMR (Fig. 1a) of signals at ~2 ppm (CH_3CO–) indicates that the number of acetyl groups in the repeating unit is two. The large variations in chemical shifts of H-4 of residue B and H-2 of residue D observed when compared with its deacetylated derivative[7] ($\Delta\delta_H$ 1.26 and 1.39 ppm, respectively) indicate that acetyl groups are located at *O*-4 in residue B and at *O*-2 in residue D. Besides, vicinal protons appear slightly shifted (~0.15 ppm). On the other hand, the presence of acetyl groups increases the value of the chemical shift of the substituted carbon (0.4–1.2 ppm), and generates an upfield shift of the vicinal carbons (1.3–2.6 ppm).

From the above results, we propose the following structure as the repeating unit of the high-molecular weight exopolysaccharide isolated from *L. pentosus* LPS26 (Fig. 4):

→4)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A4Ac-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*A-(1→3)-β-L-Rha*p*2Ac-(1→

3. Concluding remarks

Monosaccharide analysis and determination of the absolute configuration of the high-molecular weight exopolysaccharide (EPS A) produced by *L. pentosus* LPS26 revealed the presence of D-glucose, D-glucuronic acid, and L-rhamnose, and NMR results indicate a molar ratio of 1:2:2. D-Glucose and L-rhamnose are quite common components among the HePSs produced by *Lactobacillus* strains, but D-glucuronic acid has been only detected in *L. acidophilus* LMG 9433.[8]

Comparing the primary structure of EPS A with the so far published structures of the EPSs produced by other *Lactobacillus*, it is worth noting that most of them show a main chain branched by one or two side chains, and only four contain a pentasaccharide as repeating unit. On the other hand, the presence of acetyl groups in the structure is an unusual feature which has only been reported in *L. sake* 0–1.[9]

According to previous studies,[4] EPS should have high molar mass and stiff chains to provide high viscosity. EPS A fulfils only the first requirement. The presence of a unique β-(1→4) linkage in the repeating unit in contrast to four α-linkages along with the absence of side chains in EPS A suggests certain chain flexibility. In addition, the presence of negatively charged residues in EPS A could also contribute to elasticity instead to viscosity.[10] The two acetyl groups could have marked effects on the polymer properties as observed in other polysaccharides such as bacterial alginates to which acetyl groups provide a reduced ion binding ability and increased enzyme resistance, and prevent gelation.[11]

4. Experimental

4.1. Bacterial culture conditions and polysaccharide isolation

Bacterial cultures and polysaccharide isolation were performed according to Sánchez et al.[5] The EPS-producing strain *L. pentosus* LPS26 was cultured at 20 °C for 72 h without pH control, on mSDM (1 L) containing 30 g/L glucose. Inoculation was at 2% (vol/vol) with an overnight culture. The capsular polymer was detached from cells by sonication (200 mL samples) at 30 W for 10 min with pulses of 1 s using a Vibra Cell CV17 (Sonics & Materials Inc., Danbury, CO) equipped with a 3 mm diameter probe. For cell and protein removal purposes, samples were then treated with 10 g/L trichloroacetic acid (TCA) for 30 min and centrifuged (10,000 *g* for 10 min at 4 °C). Crude EPS was precipitated from the supernatant by addition of 2 volumes of cold ethanol (overnight, at 4 °C), dissolved in distilled water (1/10 of initial volumen), dialyzed (Mw cut-off 12,000–14,000 Da) against distilled water for 48 h at 4 °C with water replacement twice a day, and lyophilized for quantification and structural analysis.

4.2. Purification and quantification of polysaccharides

To separate and quantify the polysaccharides EPS A and EPS B, lyophilized EPS samples were dissolved in milli-Q water (10 μg/μL final concentration) and subjected to size-exclusion chromatography (SEC) in a HPLC system. Two TSK-Gel columns ($G3000PW_{XL}$ + $G5000PW_{XL}$) connected in series and protected by a TSK-Gel pre-column (Tosoh Corporation, Tokyo, Japan) were used. As mobile phase, 0.1 M $NaNO_3$ was used with an operating temperature of 40 °C, a flow rate of 0.45 mL/min, and an injection volume of 50 μL. The polymers were detected by refractive index (RI) using a 410 differential refractometer (Waters Corporation, Milford, MA). A UV 996 detector (Waters) was used to detect the potential presence of proteins. Different concentrations of standard dextrans (Fluka-Chemie, Buchs, Switzerland) with molecular mass ranging from 5×10^3 to 4.9×10^6 Da were run separately and used to obtain calibration curves in order to determine both the concentration (μg/μL) ($R^2 = 0.994$) and the molecular mass (Da) ($R^2 = 0.995$) of the eluting peaks.

4.3. Monosaccharide analysis

Monosaccharides were identified on GLC–MS separation of their trimethylsilylated methyl glycosides obtained as described.[12] The absolute configuration of monosaccharides was assigned following GLC–MS analysis of their trimethylsilylated (*S*)- and (*R,S*)-2-butyl glycosides, prepared as described,[13] and analyzed by GLC–MS. Derivatives from standard glucose, rhamnose, and glucuronic acid were prepared for comparison.

Gas-liquid chromatography–mass spectrometry (GLC–MS) was performed on a Micromass AutoSpec-Q instrument fitted with an OV-1 column (25 m × 0.25 mm). The temperature program for separating the trimethylsilylated methyl and 2-butyl glycosides was isothermal at 150 °C for 2 min followed by a 10 °C/min gradient up to 250 °C. The ionization potential was 70 eV, and spectra were recorded in low-resolution mode.

4.4. Methylation analysis

The vacuum-desiccated sample of polysaccharide was methylated by the method of Ciucanu and Costello.[14] When appropriate, the permethylated polysaccharide was carboxyl-reduced by treatment with 2 mg of NaB^2H_4 dissolved in 500 μL of ethanol/water (3:1, v/v) at room temperature.[15] Finally, the sample was hydrolyzed, reduced with NaB^2H_4, and acetylated as described.[16] Gas-liquid chromatography–mass spectrometry was performed on a Micromass AutoSpec-Q instrument fitted with an OV-1 column (25 m × 0.25 mm). The temperature program for separating the partially methylated alditol acetates was isothermal at 120 °C for 1 min followed by an 8 °C/min gradient up to 250 °C. The ionization potential was 70 eV, and spectra were recorded in low-resolution mode.

4.5. Modifications of polysaccharide

Carboxylic groups were reduced to hydroxymethyl groups by the method of Wustman et al.,[6] slightly modified. Briefly, the polysaccharide (5 mg) was dissolved in 2 mL of sodium acetate buffer (0.01 M, pH 4.75), and *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate (CMC) (0.5 mL of 250 mg CMC/mL of 0.01 M sodium acetate buffer, pH 4.75) was added. The mixture was stirred on ice for 2.5 h. Imidazol-HCl (4 M, pH 7.0, 0.5 mL) was added followed by 300 mg of $NaBH_4$ (dissolved in 1 mL of water) and stirred overnight. The reaction mixture was quenched by addition of a few drops of acetic acid. The reduced polysaccharide was dialyzed using a JumboSep 10 K (Pall Corporation, Ann Arbor, MI, USA) and freeze-dried.

Acetyl groups were removed by treatment with 12.5% NH_4OH (rt, overnight),[17] and further desalted by size-exclusion chromatography.

4.6. NMR spectroscopy

Samples were deuterium-exchanged several times by freeze drying from ^2H_2O, and then examined in solution (~5 mg/750 μL) in 99.98% ^2H_2O. Spectra were recorded at 303 K and 323 K on a Bruker AV500 spectrometer operating at 500.13 MHz (^1H) and 125.75 MHz (^{13}C). Chemical shifts are given in ppm, using the H^2HO signal[18] (4.75 ppm at 303 K and 4.50 ppm at 323 K) (^1H) and external dimethylsulfoxide (39.5 ppm) (^{13}C) as references. The 2D homonuclear DQF-COSY was performed using the Bruker standard pulse sequence. A data matrix of 256 × 1024 points was used to digitize a spectral width of 3788 Hz; 24 scans were used per increment. The TOCSY was acquired using a data matrix of 256 × 2048 points to digitize a spectral width of 3788 Hz; 24 scans per increment and an isotropic mixing time of 80 ms were used. The pure absorption NOESY experiment was performed with a mixing time of 50 ms. A data matrix of 256 × 2048 points was used to digitize a spectral width of 5000 Hz; 32 scans were used per increment. The 2D heteronuclear one-bond proton-carbon correlation experiment was registered in the ^1H-detection mode via single-quantum coherence (HSQC). A data matrix of 256 × 1024 points was used to digitize a spectral width of 3788 and 22523 Hz in F_2 and F_1; 48 scans were used per increment. ^{13}C decoupling was achieved by the GARP scheme. Squared-cosine-bell functions were applied in both dimensions, and zero-filling was used to expand the data to 1024 × 1024 points. A coupled 2D heteronuclear one-bond proton-carbon correlation experiment was also registered in the ^1H-detection mode via single-quantum coherence (coupled HSQC).

NMR spectra have been assigned using the program Sparky.[19]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.08.028.

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