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Determination of the structure and molecular weights of the exopolysaccharide produced by *Lactobacillus acidophilus* 5e2 when grown on different carbon feeds

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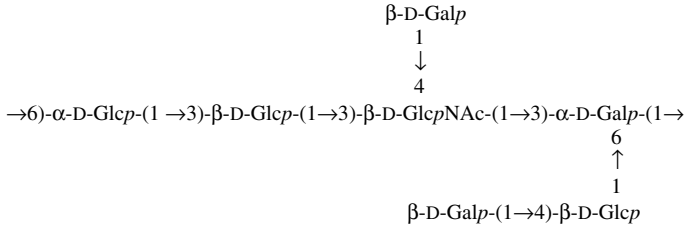
Abstract—*Lactobacillus acidophilus* 5e2 when grown on skimmed milk, skimmed milk supplemented with sodium formate and skimmed milk supplemented with glucose secretes a branched heteropolysaccharide having a weight average molecular weight less than 450 kDa. The exopolysaccharide has a heptasaccharide repeat unit and is composed of D-glucose, D-galactose and *N*-acetyl-D-glucosamine in the molar ratio 3:3:1. Using chemical techniques and 1D and 2D-NMR spectroscopy the polysaccharide has been shown to possess the following repeat unit structure:



Keywords: Exopolysaccharide; NMR; LAB; Molecular weight; *Lactobacillus acidophilus*

1. Introduction

Many bacteria secrete polysaccharides into the surrounding medium during growth, those polysaccharides which are not attached to the cell wall are termed exopolysaccharides (EPS).[1–3] The physical properties of the excreted EPSs provide a highly viscous local environment for the bacteria,[4] trapping water and nutrients, helping bacteria to survive in otherwise hostile environments.[5] It has recently been shown that EPSs from Lactic acid bacteria (LAB) alter the adhesion of pathogenic bacteria to intestinal mucus altering the host's gut microflora[6–8] and as such, there is much interest in understanding how the structure and 3D-shape of EPSs influence their ability to inhabit such environments. Whilst structures of a number of LAB EPSs are known[9–11] only recently has information about the factors that influence the degree of polymerisation (DP) of the repeat unit been investigated.[12,13]

We have initiated a programme of work directed at determining the weight average molecular weight (related to DP) and the repeat-unit structures of LAB EPSs; this information along with knowledge of the 3D-structure of the repeat units will provide a better understanding of how the structure of repeat units in

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EPSs influences their physical properties. Here we report the unique structure of the EPS produced by *Lactobacillus acidophilus* 5e2 when grown on skimmed milk, skimmed milk supplemented with glucose and skimmed milk supplemented with sodium formate.

2. Results and discussion

L. acidophilus 5e2 when cultured on skimmed milk for 24 h produces small quantities, less than 50 mg L^{-1}, of EPS. The EPS is eluted from a size exclusion column as three different weight fractions (Fig. 1a): a small amount of material eluted as an early peak having a molecular weight of 400 kDa (representing less than 10% of the total as determined from the concentration dependent refractometer response—not shown); the second fraction corresponded to approximately 50% of the sample and had a weight average molecular weight of 150 kDa; the final late eluting peak, had a weight average molecular weight of 130 kDa and corresponded to approximately 40% of the total. It had previously been reported[14,15] that low concentrations of sodium formate stimulate the growth of LAB and therefore we attempted to observe if there was a similar effect on EPS production. Addition of sodium formate (500–1000 ppm) led to a reduction in yield. In contrast, supplementation of the carbon feed with glucose (0.166 M) resulted in the production of a significantly higher yield (200 mg L^{-1}): the majority of the EPS eluted from a size exclusion column as a single peak having a weight average molecular weight of 250 kDa (>90%) there was a small amount of high molecular weight material having a weight average molecular mass of 350 kDa (<10%) (Fig. 1b). Similar increases in the yield of EPS on supplementation with glucose have been reported for other LAB.[16–18]

^{1}H NMR spectra were recorded for EPS samples isolated from *L. acidophilus* 5e2 grown under a wide variety of different conditions (different carbon supplements and different times of incubation—see experimental conditions). No matter how the EPS was produced, the resulting spectra were identical indicating that *L. acidophilus* 5e2 is capable of producing a single EPS structure that has a fixed repeat unit. The different fractions eluting from the size exclusion column differ only in their DP.

The results of monomer analysis and determination of the absolute configuration indicate that the polysaccharide is composed of D-glucose, D-galactose and either *N*-acetyl-D-glucosamine or D-glucosamine in a molar ratio of 3.1:2.9:0.7. Inspection of the NMR spectra clearly indicates that the amino sugar is acetylated and a single C-2 resonance is observed at δ 56.08 ppm. The ratio of the acetyl protons to the anomeric proton is 3.1 and this confirms that there is complete acetylation of the monomer.

Full characterisation of the structure of the EPS was achieved through the inspection of a series of 2D-NMR spectra including 2D-COSY, 2D-TOCSY, 2D-^{1}H–^{13}C HMBC and ^{1}H–^{13}C–HSQC–TOCSY spec-

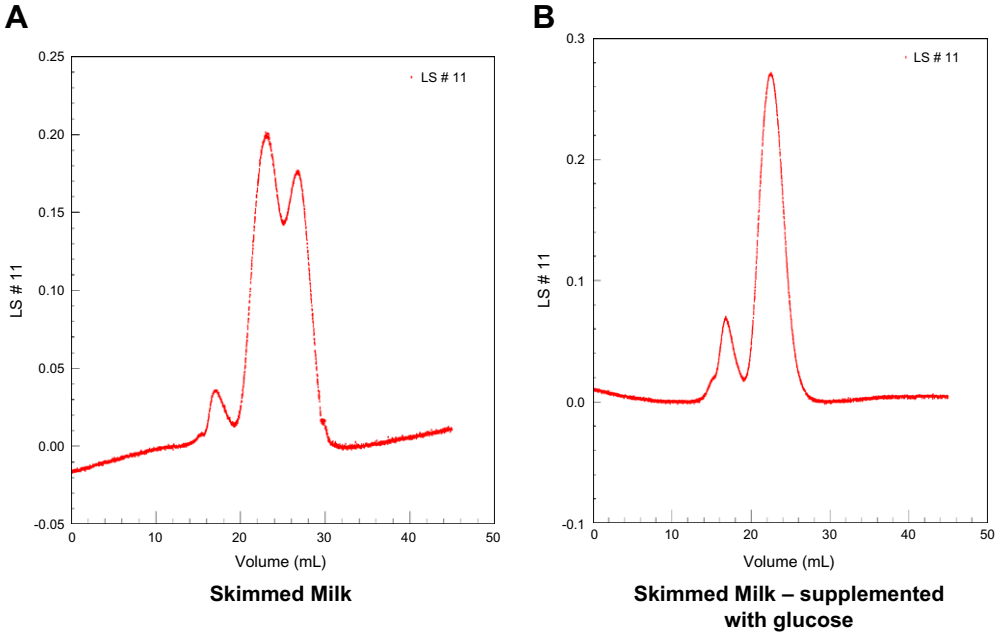

Figure 1. HPSEC–MALLS mass sensitive light scattering traces for EPS solutions (1 mg/mL): (A) EPS isolated from skimmed milk; (B) EPS isolated from skimmed milk supplemented with glucose.

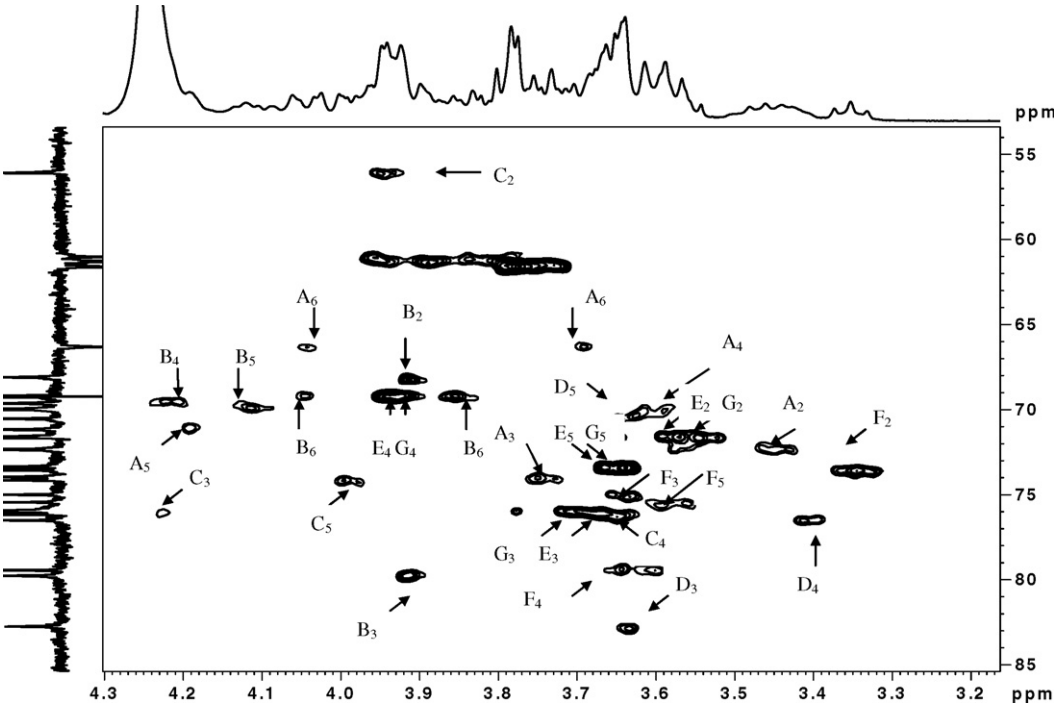

Figure 2. 400-MHz ^{1}H–^{13}C HSQC of a selected region of the spectrum of EPS from *Lactobacillus acidophilus* 5e2 recorded in D_2O at 70 °C. The identity of cross peaks is noted by the sugar residue, as **A**–**G**, and by identifying the location of hydrogens/carbons within the ring as **2**–**6**.

tra. The resonance positions for the non-anomeric ^{1}H signals and ^{13}C signals are indicated on the ^{1}H–^{13}C HSQC spectrum (Fig. 2), the anomeric ^{1}H signals are indicated on the HMBC spectrum (Fig. 3) and the combined data are presented in Table 1.

The anomeric region of the ^{1}H spectrum (δ 5.35–4.46, Fig. 3) is consistent with the EPS having a heptasaccharide repeat unit; the sugar residues are designated **A**–**G** according to the decreasing chemical shift of the anomeric protons. The two low field signals (**A** & **B**) have $^{3}J_{1,2}$ coupling constants of less than 4 Hz and represent sugars having α-anomeric configuration; all the high field signals (**C**–**G**) have coupling constants greater than 7.5 Hz and are of β-anomeric configuration. The chemical shift data for the H-5 and the C-5 resonances indicate that the monosaccharides are all present in their pyranose ring form.[19] The designation of monomers as either galactose (**B**, **E** & **G**) or as glucose (**A**, **D** & **F**) is based primarily on the location of the H-4 resonance. The H-4 resonance for a galactose is shifted substantially to a lower field than that of a glucose, regardless of the anomeric configuration and linkage: data collected from the assignments for LAB EPS structures show that the H-4 resonances for a galactose lie in the range δ 4.30–3.85 whilst those for glucose lie in the range δ 3.45–3.75.[20]

The linkages of the different monomers in the oligosaccharide repeat unit were determined using a combination of the carbon chemical shift data and linkage analysis. Linkage analysis identified the presence of two terminal hexoses; a 1,3-linked hexose; a 1,4-linked hexose; a 1,6-linked hexose; a 1,3,6-linked hexose and a 1,3,4-linked hexosamine. In the ^{13}C spectrum, the glycosidic links for the simple hexoses (**A**, **B**, **D**–**G**) are signalled by a shift to lower field (approximately 4 ppm) of the bridging carbons. The combined results identify **A** as a 1,6-linked α-D-glucose; **B** as a 1,3,6-linked α-D-galactose; **C** as the 1,3,4-linked β-*N*-acetyl-D-glucosamine; **D** as a 1,3-linked β-D-glucose; **E** & **G** as terminal β-D-galactoses and **F** as a 1,4-linked β-D-glucose.

Finally, the sequence of the monomers in the oligosaccharide repeat unit was determined by the examination of the 2D ^{1}H–^{13}C HMBC and 2D-NOESY spectra. Cross peaks linking different sugars are highlighted on the corresponding spectra (Figs. 3 and 4). On the NOESY spectrum (Fig. 4) there are inter-residue cross peaks between: **A** H-1 and **D** H-4/2 which is consistent with a **A(1→3)D** linkage (see HMBC results for confirmation of the presence of this linkage); **B** H-1 and **A** H-6 confirming a **B(1→6)A** linkage; a strong cross peak between **D** H-1 and **C** H-3 confirming a **D(1→3)C** linkage; **E** H-1 and **C** H-4 confirming a **E(1→4)C** linkage and finally, there is a weak cross peak between **F** H-1 and **B** H-6 suggesting a **F(1→6)B** linkage.

On the HMBC spectra inter-residue cross peaks are observed between: **A** H-1 and **D** C-3 confirming a **A(1→3)D** linkage; **C** H-1 and **B** C-3 confirming a

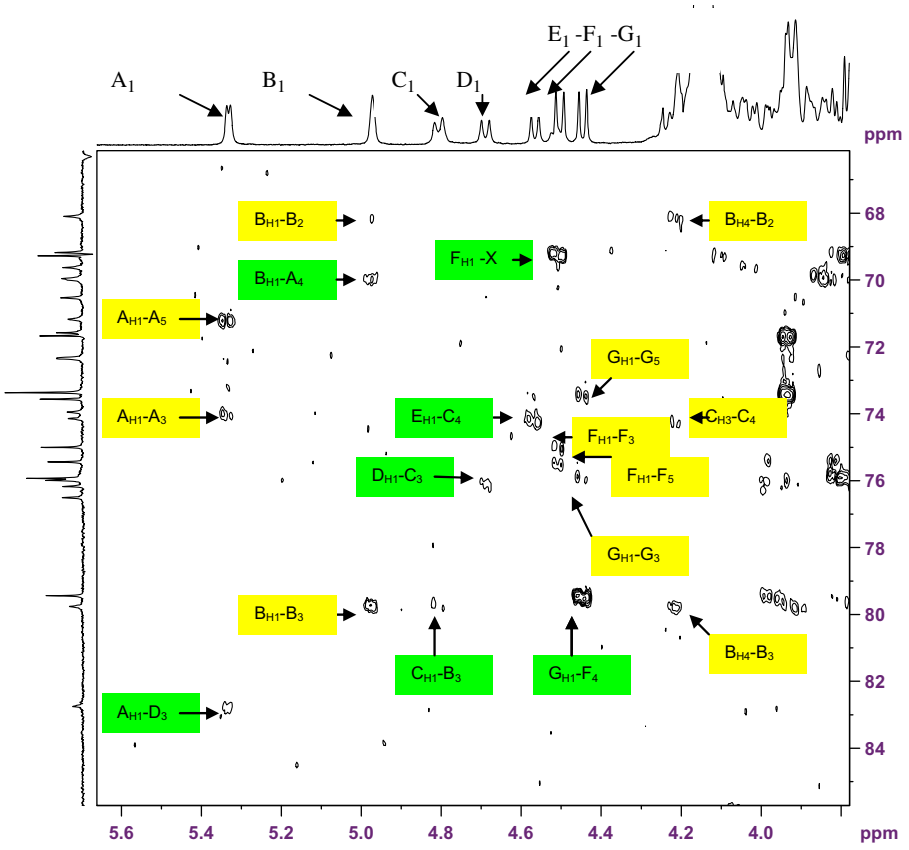

Figure 3. 400-MHz ^{1}H–^{13}C HMBC spectrum of EPS from *Lactobacillus acidophilus* 5e2 recorded in D_2O at 70 °C. The identity of cross peaks is noted by the sugar residue, as **A**–**G**, and by identifying the location of coupled hydrogens/carbons within the ring as **1**–**6**. (Intra-residue couplings are highlighted in yellow, inter-residue couplings are highlighted in green.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1. ^{1}H and ^{13}C NMR chemical shifts of EPS (1) recorded in D_2O at 70 °C

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
^{1}H NMR chemical shifts of EPS (1) recorded in D_2O at 70 °C							
Glc-A	5.35	3.57	3.61	3.75	4.21	4.04	3.68
Gal-B	4.97	3.93	3.91	4.21	4.11	4.04	3.85
NAcGlc-C	4.80	3.96	4.22	3.99	3.67	4.04	3.86
Glc-D	4.71	3.46	3.63	3.42	3.66	3.87	3.74
Gal-E	4.58	3.59	3.67	3.95	3.67	3.80	3.72
Glc-F	4.52	3.36	3.67	3.62	3.60	3.97	3.83
Gal-G	4.46	3.57	3.66	3.95	3.66	3.80	3.80

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
^{13}C NMR chemical shifts of EPS (1) recorded in D_2O at 70 °C						
Glc-A	99.44	72.35	69.96	73.94	71.17	66.32
Gal-B	99.08	68.10	79.76	69.63	69.96	69.23
NAcGlc-C	102.83	56.08	75.99	74.15	76.17	61.29
Glc-D	101.30	72.35	82.76	76.51	70.53	61.29
Gal-E	102.21	71.58	75.99	69.18	73.37	61.59
Glc-F	102.98	73.57	75.00	79.44	75.48	61.03
Gal-G	103.57	71.67	75.93	69.28	73.37	61.62

C(1→3)B linkage; **D** H-1 and **C** C-3 confirming a **D(1→3)C** linkage; **E** H-1 and **C** C-4 confirming a **E(1→4)C** linkage and **G** H-1 and **F** C-4 confirming a **G(1→4)F** linkage.

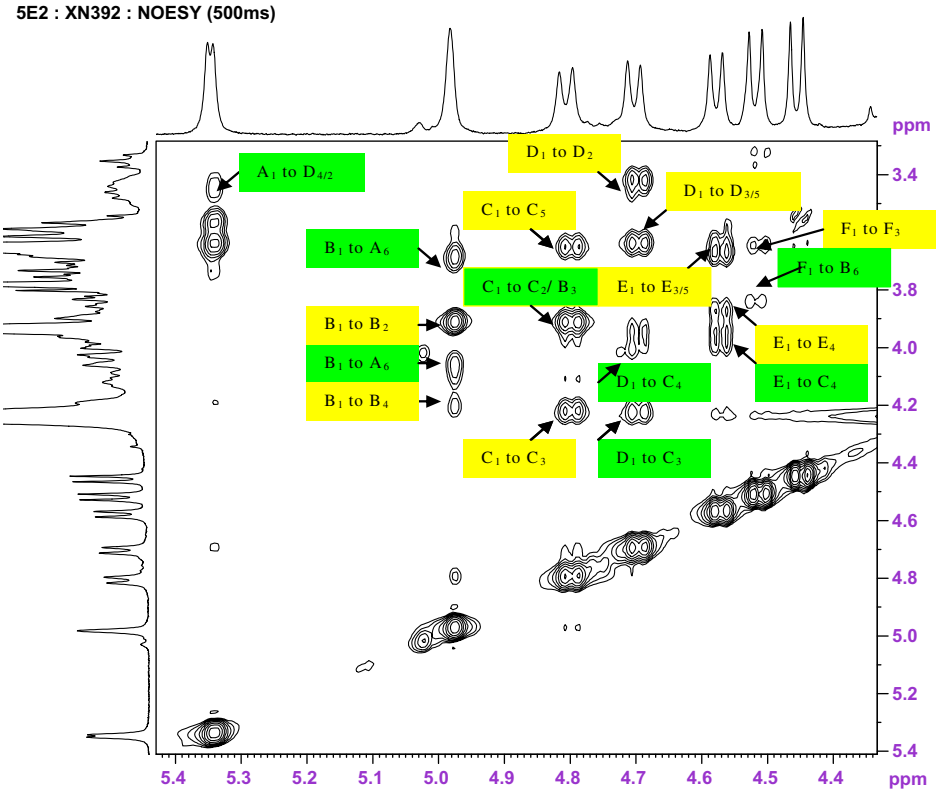

Figure 4. 400-MHz ^{1}H–^{1}H NOESY spectrum of EPS from *Lactobacillus acidophilus* 5e2 recorded in D_2O at 70 °C. The identity of cross peaks is noted by the sugar residue, as **A**–**G**, and by identifying the location of coupled hydrogens/carbons within the ring as **1**–**6**. (Intra-residue NOEs are highlighted in yellow, inter-residue NOEs are highlighted in green.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The combined results indicate that the EPS from *L. acidophilus* 5e2 has the following repeat unit structure:

E

β-D-Gal*p*

1

↓

4 **B**

→6)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→

A **D** **C** 6

↑

1

β-D-Gal*p*-(1→4)-β-D-Glc*p*

G **F**

In conclusion, *L. acidophilus* 5e2 produces a low molecular weight EPS, the EPS is smaller than that of other LAB EPSs.[21] The repeat unit structure of the EPS is independent of the fermentation conditions and a single repeat unit structure is synthesised by the bacterium. In a future report we will indicate how the molecular weight of the EPS varies as a function of the incubation period.

3. Experimental

The bacterial culture of *L. acidophilus* 5e2 was from Rhodia food Biolactica, Poland, and was maintained in MRS broth (Oxoid). From a pure working culture of *L. acidophilus* 5e2, 1% was inoculated into 10 mL of

reconstituted skimmed milk powder (10% w/v supplied by St. Ivel Ltd, UK) to provide a milk master culture by incubation for between 18 and 72 h at either 37 °C or 42 °C. This culture (1%) was used to inoculate a larger working volume (1.5 L for static fermentations. For formate supplementation, fermentations were performed with added sodium formate (500–1000 ppm). For glucose supplementation, fermentations were performed with skimmed milk containing 0.166 M glucose. The procedure used for EPS extraction was developed in our laboratories.[22] To the working cultures, an 80% (w/v) trichloroacetic acid (TCA) solution was added to provide a final concentration of 14% TCA. The resulting mixture was centrifuged at 25,000*g* (using a Beckman J2-MC centrifuge) for 30 min at 4 °C to remove cells and protein. Crude EPS was precipitated by the addition of an equal volume of chilled absolute ethanol to the supernatant fluid. After overnight precipitation at 4 °C the sample was centrifuged, as above, and the pellet retained. The sample was redissolved in distilled water (100 mL) with gentle heating (less than 50 °C) and the EPS was recovered by precipitation on the addition of an equal volume of chilled absolute ethanol. The sample was centrifuged at 25,000*g* for 25 min at 4 °C. The resulting EPS pellet was redissolved in not more than 20 mL of distilled water with gentle heating (less than 50 °C) and then small neutral sugars were removed by dialysis, for 72 h at 4 °C, against three changes of distilled water per day. The contents of the dialysis bag were freeze-dried to provide EPS. The purity of the EPS was determined by size exclusion chromatography (details below) and NMR analysis (details below).

For molecular weight determination, solutions of EPS (1 mg/mL) in deionised water were prepared and left for 24 h to completely dissolve. Samples (100 μL) were injected onto an analytical size exclusion system comprising three columns connected in series (aquagel-OH 40, 50 and 60, 15 μm particle size, 25 cm × 4 mm, Polymer Labs. UK). The neutral analytes were eluted with deionised water flowing at 1 mL/min; these conditions are suited to the elution of neutral polysaccharides. The concentration of the EPS fractions eluting from the column were determined by a differential refractometer (Optilab rEX, Wyatt technology, Santa Barbara, USA) and the weight average molecular weights of slices of the chromatogram were measured using a Dawn EOS multi-angle light scattering photometer operating with a 690 nm laser (Wyatt technology, Santa Barbara, USA). The refractive index increment (dn/dc) was measured as 0.198 mL/g by monitoring the response of the differential refractometer to a series of different concentrations of the EPS samples (0.025–1.0 mg L^{-1}). The second viral coefficient (A_2) was taken as zero for individual slices of the chromatogram. For sugar composition determinations, polysaccharides were hydrolysed by treatment with 2 M TFA (120 °C for 2 h); the released sugars were converted to their alditol acetates and analysed by GC–MS. The relative proportions of the different sugars were determined by the consideration of the total ion count for the different alditol acetates and by comparison with the ion count determined for a mixture of alditol acetates. GC–MS analyses were performed on a Thermo (Finnigan) Polaris Q-Trace 2000 GC–MS, (Hemel Hempstead, UK). The samples were eluted from a SGE column (BPX5, 25 m × 0.32 mm-id, 0.5 μm film; HP5, 15 m × 0.32 mm-id) eluting with helium (9 psi, flow rate 1 mL min^{-1}) and using a temperature programme (start temperature 150 °C, hold time 4 min and a final column temperature of 250 °C reached via a rising gradient of 4 °C/min). Under the standard conditions for the analysis of monomers, the integral area for the amino sugar was very small. This is a consequence of on column thermal decomposition, problems with the analysis of amino sugars have been well documented.[23] When a shorter capillary column (HP5, 15 m × 0.32 mm-id) was used the degree of thermal decomposition was reduced. Absolute configurations of monosaccharides were determined according to Gerwig et al.[24]

For sugar linkage analysis, the isolated EPS was permethylated using the procedures described by Stellner et al.[25] The methylated polysaccharide was hydrolysed by treatment with 2 M TFA (120 °C for 2 h) and the monosaccharides were converted to their corresponding methylated alditol acetates. The structures of the constituent methylated alditol acetates were determined by GC–MS analysis.[26]

NMR spectra were recorded for samples that were dissolved (10 mg/mL) directly in D_2O (Goss Scientific Instruments Ltd, Essex). NMR spectra were recorded at a probe temperature of 70 °C unless otherwise stated. The elevated temperature was initially chosen as it shifted the HOD signal to higher field, into a clear region of the spectrum. The higher temperature also increased spectral resolution by reducing the sample viscosity. All of the NMR spectra were recorded on a Bruker Avance DPX400.13 MHz ^1H (100.61 MHz ^{13}C) spectrometer (located at Huddersfield) operating with Z-field gradients where appropriate and using Bruker's pulse programmes. Chemical shifts are expressed in ppm relative to either internal or external acetone; δ 2.225 for ^1H and δ 31.55 for ^{13}C. The 1D ^1H and ^{13}C spectra were processed with 32,768 data points. The 2D gs-DQF-COSY spectrum was recorded in magnitude mode at 70 °C. TOCSY experiments were recorded with variable mixing times (30, 60, 90, 120, 150, 210 ms). The 2D-heteronuclear ^1H–^{13}C HSQC and phase sensitive HSQC–TOCSY were recorded using Bruker pulse sequences and 256 experiments of 1024 data points. The ROESY spectrum was recorded using Bruker pulse sequence and 256 experiments of 1024 data points were recorded using a mixing time of 250 ms.

For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions. After applying zero-filling and Fourier transformation, data sets of 1024–1024 points were obtained.

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