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Structural characterisation of a highly branched exopolysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074

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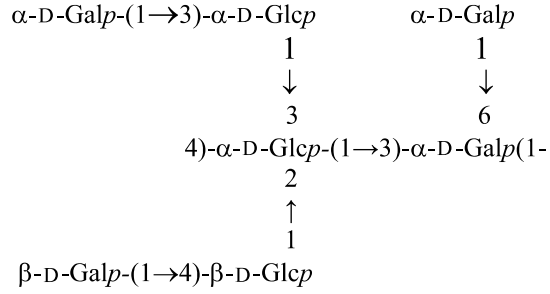
Abstract—*Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074 when grown in skimmed milk secretes a highly branched exopolysaccharide. The exopolysaccharide has a heptasaccharide repeat unit and is composed of glucose and galactose in the molar ratio 3:4. Using chemical techniques and 1D and 2D NMR spectroscopy the polysaccharide has been shown to possess the following repeat unit structure:



Keywords: Exopolysaccharide, EPS; Lactic acid bacteria, LAB; Polysaccharide; Structure

1. Introduction

In common with many lactic acid bacteria (LAB) *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074 secretes a heteropolysaccharide into the surrounding medium during growth.[1–3] The secreted polysaccharides, exopolysaccharides (EPSs), provide a highly viscous local aqueous environment for the bacteria. Whilst the functional role of EPS in the life cycle of the bacteria is not clear, it is thought that they are produced in order to protect the microbial cell rather than they being a potential food reserve.[4] It is the rheological characteristics of aqueous solutions of EPSs that give rise to our interest in EPS production: exopolysaccharides have potential for use as thickening agents.[5] EPS formation by LAB during the production of fermented milk products imparts favourable rheological properties.[6,7] There is a need to develop an understanding of structure–function relationships that is to relate EPS structure to the rheological properties of their aqueous solutions, in order to maximise the advantages of EPS synthesis during the production of fermented products. Knowledge of the structures of EPSs will also help in determining the metabolic pathways by which they are synthesised. The

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chemical composition and structure of a number of bacterial EPSs have been determined. Structures have been reported for EPSs from streptococci,[8–16] lactobacilli[11,17–29] and lactococci.[30–35] The structures are very diverse. Only when further structures have been reported will it be possible to develop meaningful structure–function relationships. Here we report the unique structure of the exopolysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074 when grown in skimmed milk.

2. Results and discussion

Analysis of the NMR spectra of the EPS recovered from a number of repetitive cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074 grown on skimmed milk indicates that a single polysaccharide is secreted into the medium. The EPS elutes as a single early eluting peak from a size-exclusion chromatography column, suggesting that the EPS isolated is free from low molecular weight polysaccharides. The molecular weight of the EPS is greater than 1800 kDa, a value that is similar to those recorded for the EPS isolated from other species of *Lactobacillus*. Monomer analysis and determination of the absolute configuration indicates that the polysaccharide is composed solely of D-glucose and D-galactose in a molar ratio of 3:4. Results of the methylation analysis, viewed in combination with the ^{1}H NMR spectrum, indicate that the EPS has a heptasaccharide repeat unit and that the repeat unit is highly branched. Of the 21 unique LAB EPS structures published to date,[8–16,11,17–35] the largest repeat unit produced is a heptasaccharide. The chemical shift data (Table 1), particularly that for the H-5 and C-5 resonances, indicate that the monosaccharides are all present in their pyranose ring form. The results from the linkage analysis indicate that the repeat unit contains 3 terminal galactoses, a 3-substituted glucose, a 4-substituted glucose, a 3,6-disubsituted galactose and a 2,3,4-trisubstituted glucose.

The anomeric region of the ^{1}H spectrum (δ 5.64–4.49) 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 five low-field signals (**A–E**) have $^3J_{1,2}$ coupling constants of less than 4 Hz and represent sugars having α-anomeric configuration. The remaining two sugars (**F** and **G**) have $^3J_{1,2}$ coupling constants of 7.4 Hz and 7.6 Hz, respectively, and are of β-anomeric configuration. Assignments of resonances, ^{1}H and ^{13}C, to the protons and carbons of the individual monosaccharides (Table 1) are based on interpretations of 2D-spectra: 2D-COSY, 2D-TOCSY, 2D ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC and ^{1}H–^{13}C-HSQC–TOCSY spectra. At 600 MHz the anomeric protons for residues **B** and **C** cannot be resolved; subsequent assignments for protons and carbons in these residues started at C-2/H-2 and made use of the ^{1}H–^{13}C-HSQC–TOCSY spectrum. The resonance positions for ^{1}H and ^{13}C are indicated on the ^{1}H–^{13}C HSQC spectrum (Fig. 1).

The designation of sugars as either galactose (**B**, **C**, **E** and **G**) or as glucose (**A**, **D** and **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[8–16,11,17–35] show that the H-4 resonances for galactose lie in the range δ 4.30–3.85 whilst those for glucose lie in the range δ 3.45–3.75. Other spectral data are in agreement with these assignments including: the high-field location of the H-2

Table 1. ^{1}H NMR and ^{13}C chemical shifts for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB207, 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							
A	5.64	4.31	4.37	3.75	4.34	3.77	3.73
B	5.30	3.74	3.72	3.96	4.18	3.99	3.77
C	5.30	3.88	3.99	3.91	4.41	3.86	3.79
D	5.08	3.66	3.86	3.71	4.22	3.82	4.00
E	5.00	3.83	3.85	4.04	3.95	3.84	3.84
F	4.72	3.35	3.60	3.63	3.65	3.80	3.79
G	4.49	3.58	3.68	3.96	4.30	3.77	3.73
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							
A	97.44	76.22	78.07	71.18	71.87	62.50	
B	101.02	76.56	71.87	81.49	71.87	67.31	
C	95.85	69.76	70.85	70.98	72.64	61.15	
D	100.52	71.37	82.88	71.18	72.64	61.92	
E	99.61	69.85	70.98	70.67	72.19	62.40	
F	104.88	74.34	75.89	80.34	76.22	61.92	
G	104.21	72.37	74.10	69.96	71.18	62.50	

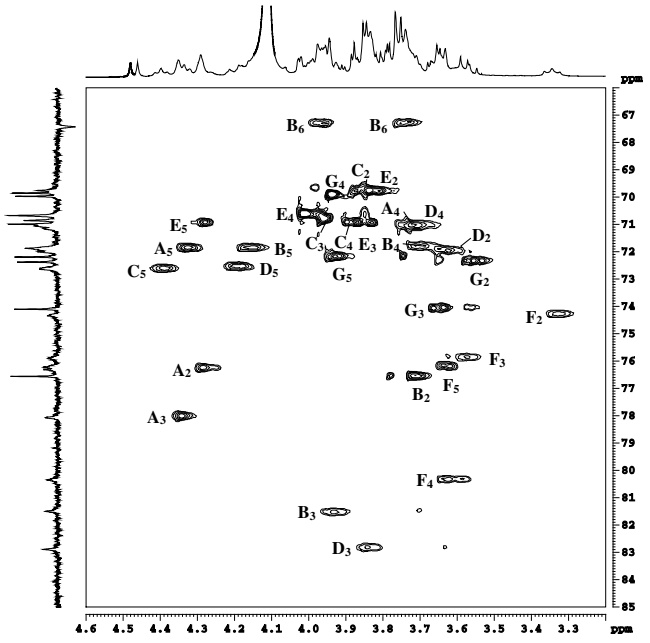


Figure 1. 400-MHz ^{1}H–^{13}C HSQC spectrum of EPS from *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB207, recorded in D_2O at 70 °C. The identity of cross-peaks is noted by the sugar residue, as **A**–**G**, and by indentifying the location of hydrogens/carbons within the ring as **1**–**6**.

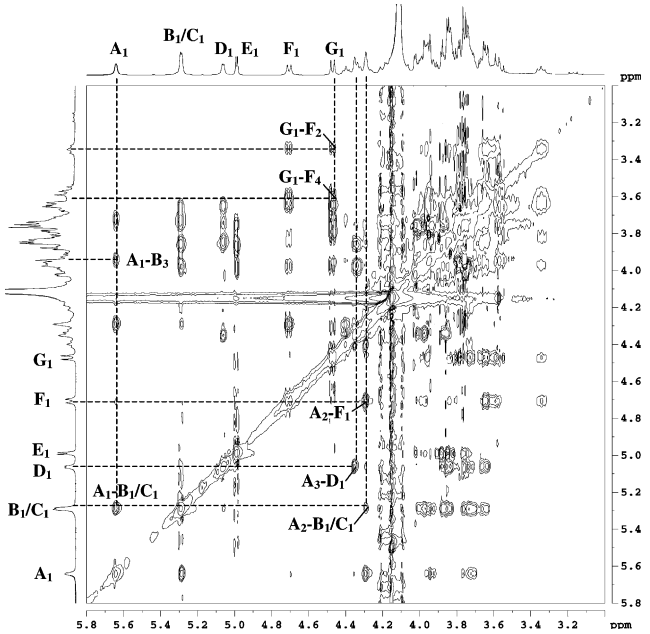
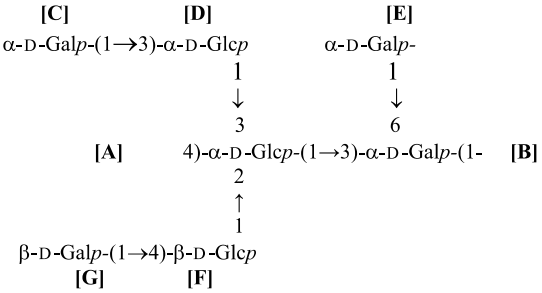


Figure 2. 400-MHz 2D ROESY spectrum of EPS from *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB207, recorded in D_2O at 70 °C. Cross-peaks identifying inter-residue NOEs are identified as intersecting dashed lines. Sugar residues are identified as **A**–**G** and the proton ring position is labelled **1**–**6**.

resonance of the **F** H-2 (β-Glc); the lower field resonance position for **G** H-2 (β-Gal); the inter-residue NOE and the transmission of coupling information from H-4 to H-5 on the TOCSY, which was only visible for the glucose residues. The low-field shift of the **B** C-6 resonance (δ 67.31) immediately identifies this sugar as the 3,6-disubstituted hexose.

The sequence of the sugar residues in the heptasaccharide repeat unit was determined with reference to the ^{1}H–^{13}C HMBC spectrum and the ROESY spectrum (Fig. 2). The ROESY spectrum contains cross-peaks identifying a number of inter-residue NOEs and these are highlighted on the spectrum. A substantial number of NOEs are observed for residue **A**. On the **A** H-1 track (δ 5.64) there is a strong NOE to **B** H-3 (δ 3.72) and to either **B** H-1 or **C** H-1 (δ 5.30). On the **A** H-2 track (δ 4.31) there is a strong NOE to **F** H-1 (δ 4.72) and a medium NOE to either **B** H-1 or **C** H-1 (δ 5.30). On the **A** H-3 track (δ 4.37) there is a very strong NOE to **D** H-1 (δ 5.08). The combination of NOEs observed for **A** are consistent with there being a **A**(1→3)**B** linkage, a **F**(1→2)**A** linkage and a **D**(1→3)**A** linkage. The existence of the latter linkages identifies **A** as the 2,3,4-trisubstituted hexose though it not clear why we should see an NOE from **A** H-1 to either **B** H-1 or **C** H-1. On the ROESY spectrum there is also a strong NOE on the **G** H-1 track (δ 4.49) to **F** H-4 (δ 3.63) and a moderate NOE to **F** H-2 (δ 3.35), which are consistent with there being a **G**(1→4)**F** linkage and **F** being the 4-substituted hexose. A number of inter-residue scalar couplings are visible on the HMBC spectrum, the most significant of which are between: **E** H-1 to **B** C-6 indicating a **E**(1→6)**B** linkage; **G** H-1 to **F** C-4 confirming a **G**(1→ 4)-**F** linkage; **C** H-1 to **D** C-3 indicating a **C**(1→3)**D** linkage.

The combined results of the NMR analysis and the chemical analysis of the polysaccharide point to the structure of the EPS of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074 having a repeat unit with the following structure:



The structure of the EPS is different to that reported for other LAB polysaccharides.[8–16,11,17–35] It is notable that the terminal sugars are galactoses, which is a common feature of a large proportion of the EPS structures reported to date. An unusual feature of the EPS structure however, is the degree of branching: the EPS has a very highly branched heptasaccharide repeating unit.

3. Experimental

The bacterial culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074 was purchased from the National Collection of Food Bacteria (Norwich, UK) and was maintained in M17 broth (Oxoid). From a pure working culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2074, 1% (v/v) was inoculated into 10 mL of reconstituted skim milk powder (10% w/v supplied by St. Ivel Ltd, UK) to provide a milk master culture by incubation for between 18 and 24 h at 37 °C. This culture (1%) was used to inoculate a larger working volume (1 L) and was incubated at 37 °C for between 18 and 24 h. The procedure used for EPS extraction was developed in our laboratories. To the working cultures, an 80% (w/v) trichloroacetic acid 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 the 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 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 (sample heated as above) and then small neutral sugars 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 (Sephacryl® S-500 high resolution) and NMR analysis.

The average molecular mass of the polysaccharide was determined by size-exclusion chromatography; analyses were performed on a Sephacryl® S-500 high resolution (Amersham Pharmacia Biotech, Uppsala, Sweden) column (70 cm × 1 cm) eluting with 50 mM $NaHCO_3$ at a flow rate of (1 mL min^{-1}). The molecular mass range and retention characteristics of the column were determined using dextran standards. Product sugars were detected using a RI detector (ERC-7510, Erma optical works Ltd).

For analysis of the sugar composition the 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 consideration of the total ion count for the different alditol acetates and by comparison with the ion count determined for a mixture of standard alditol acetates. The standard alditol acetates were generated by subjecting an intimate mixture of equal proportions of glucose and galactose to the same experimental conditions that were applied to the polysaccharide. The absolute configuration of the sugars (D or L) was determined by the method of Gerwig et al.[36] The linkages of the different sugars were determined by methylation analysis. The isolated EPS was per-methylated using the procedures described by Stellner and coworkers.[37] The methylated polysaccharide was hydrolysed by treatment with 2 M TFA (120 °C for 2 h) and the monosaccharides converted to their corresponding methylated alditol acetates. The structures of the constituent methylated alditol acetates were determined by GC–MS analysis.[37,38]

For NMR spectroscopy, samples were dissolved directly in D_2O (99.9% D) (Goss Scientific Instruments Ltd., Essex). NMR spectra were recorded at probe temperatures of either 70 °C or 80 °C unless otherwise stated; the elevated temperature 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. The majority 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. The 600 MHz ^1H spectrum was recorded on a JEOL Spectrometer. 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 inverse 2D-heteronuclear ^1H–^{13}C HSQC, HMQC and phase sensitive HSQC–TOCSY were recorded using Bruker pulse sequences and 512 experiments of 1024 data points. For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions.

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