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Carbohydrate Research 338 (2003) 61–67
CARBOHYDRATE RESEARCH
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# Structural characterisation of a perdeuteriomethylated exopolysaccharide by NMR spectroscopy: characterisation of the novel exopolysaccharide produced by *Lactobacillus delbrueckii subsp. bulgaricus* EU23

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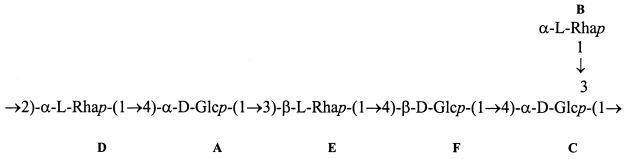
Received 13 May 2002; received in revised form 14 August 2002; accepted 24 September 2002

**Abstract**

The exopolysaccharide (EPS) from *Lactobacillus delbrueckii subsp. bulgaricus* EU23 was perdeuteriomethylated and the perdeuteriomethylated EPS (pdm-EPS) purified by elution from a $C_{18}$ Sep-Pak cartridge. Both 1D and 2D NMR spectra were recorded for the pdm-EPS and these were interpreted to provide assignments for the individual $^{1}H$ and $^{13}C$ resonances of the sugar residues of the repeating unit. Using a combination of the results from monomer analysis and linkage analysis of the native EPS and the ROESY and HMBC NMR spectra of the pdm-EPS the following structure has been determined for the repeating unit:




A process for characterising polysaccharides having low solubility in aqueous solution is reported.


## 1. Introduction

Lactic acid bacteria generate a variety of polysaccharides that are either associated with the cell surface[1] or are excreted into the medium surrounding the cells.[2–7] The polysaccharides that are excreted into the environment surrounding the cells are referred to as exopolysaccharides (EPSs) and are associated with the 'ropy'-character of the medium surrounding many microorganisms. Whilst the nature of the polysaccharides varies, the analytical procedures used to characterise these materials are very similar. The structures of the different classes of polysaccharides are routinely characterised using a combination of NMR spectroscopy[8,9] and chemical methods.[10–12]

The success of the various NMR spectroscopic techniques used to characterise bacterial polysaccharides is highly reliant on the production of significant quantities of a material that is soluble in aqueous solution. In our laboratories EPS samples of very sparing solubility are frequently obtained,[13] and there are reports in the literature of the production of material which, when dissolved in aqueous solution, provides highly viscous solutions giving rise to broad NMR signals.[14] One solution to the problem is to degrade the polysaccharide, either by treatment with acids or chemical degradation to give soluble fragments that can subsequently be used to deduce the structure of the native polysaccharide. We were interested in determining whether it was possible to record NMRs of a perdeuteriomethylated-EPS. Permethylated polysaccharides are routinely

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PII: S0008-6215(02)00354-3

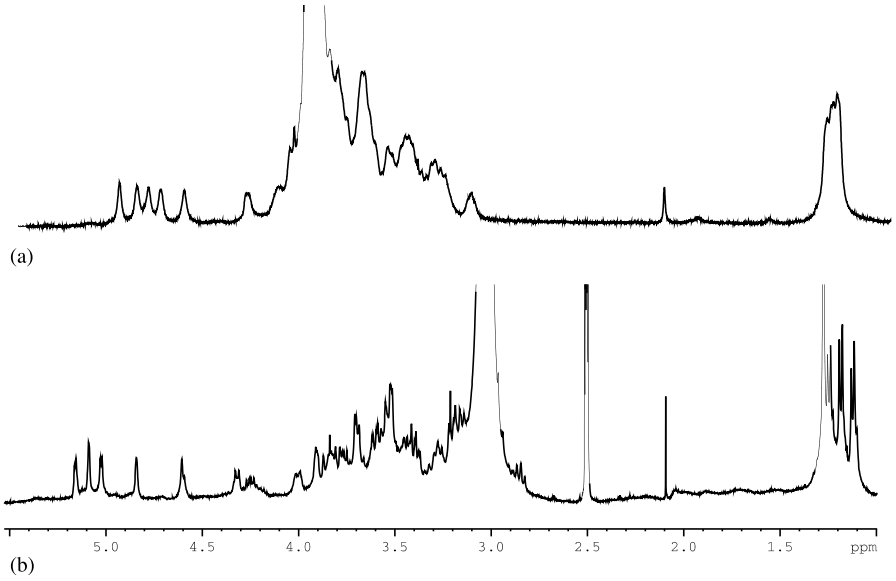



Fig. 1. 400-MHz $^1$H NMR spectra of: (a) the native EPS from *Lactobacillus delbrueckii subsp. bulgaricus* EU23, recorded in $D_2O$ at 70 °C; (b) the pdm-EPS from *Lactobacillus delbrueckii subsp. bulgaricus* EU23, recorded in DMSO-$d_6$ at 70 °C.

prepared for use in linkage analysis and, as such, the literature contains a variety of procedures for their preparation.[11,15–17] We report here the characterisation of a novel EPS produced by *Lb. delbrueckii subsp. bulgaricus* EU23 using chemical methods and from inspection of the NMRs of the perdeuteriomethylated derivative (pdm-EPS).

## 2. Results and discussion

### 2.1. Isolation and attempted characterisation of the native EPS

The EPS isolated *from Lb. delbrueckii subsp. bulgaricus* EU23 was isolated and purified by the procedures reported in Section 3. The purified EPS elutes as a single high molecular mass peak on size exclusion chromatography and the average molecular mass was determined to be in excess of 1800 kDa. The molecular mass of the EPS is similar to that recorded for EPS isolated from other strains of *Lb. delbrueckii subsp. bulgaricus*.[18] A $^1$H NMR spectrum of the native EPS (Fig. 1a) contained 6 resonances in the anomeric region. Unfortunately, the EPS is only sparingly soluble in water and attempts to determine further information about the structure of the repeating unit, using 2D NMR, failed. Existing methods for improving the sample's solubility e.g., adding salt and subjecting the sample to an extended period of ultrasonication, failed to improve the spectra of the native-EPS.

Sugar composition analysis of the purified native EPS, and determination of the absolute configuration,[10] identified D-glucose and L-rhamnose as present in equal proportions. The results of sugar linkage analysis (Table 1) showed the presence of terminally-linked rhamnose, 2-substituted rhamnose, 3-substituted rhamnose, two 4-substituted glucoses and a 3,4-disubstituted glucose. The results of the chemical analysis of the native EPS and inspection of the 1D NMR indicate that the EPS has a repeating unit of six pyranose residues. None of the twenty-six EPS structures reported to date[2] have the same sugar composition and $^1$H spectrum as those reported here. The EPS isolated from *Lb. delbrueckii subsp. bulgaricus* EU23 is thus a novel EPS.

### 2.2. Perdeuteriomethylation

In an attempt to evaluate if perdeuteriomethylation would provide fully permethylated samples suitable for

Table 1
Sugar linkage composition of EPS from *Lactobacillus delbrueckii subsp. bulgaricus* EU23

| Derivative | Molar ratios | Linkage type |
|---|---|---|
| 1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol | 1 | *t*-Rha*p* |
| 1,2,5-Tri-*O*-acetyl-3,4-*O*-dimethyl-rhamnitol | 1 | 1,2-Rha*p* |
| 1,3,5-Tri-*O*-acetyl-2,4-*O*-dimethyl-rhamnitol | 1 | 1,3-Rha*p* |
| 2 × 1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol | 2 | 1,4-Glc*p* |
| 1,3,4,5-Tetra-*O*-acetyl-6-*O*-methyl-glucitol | 1 | 1,3,4-Glc*p* |

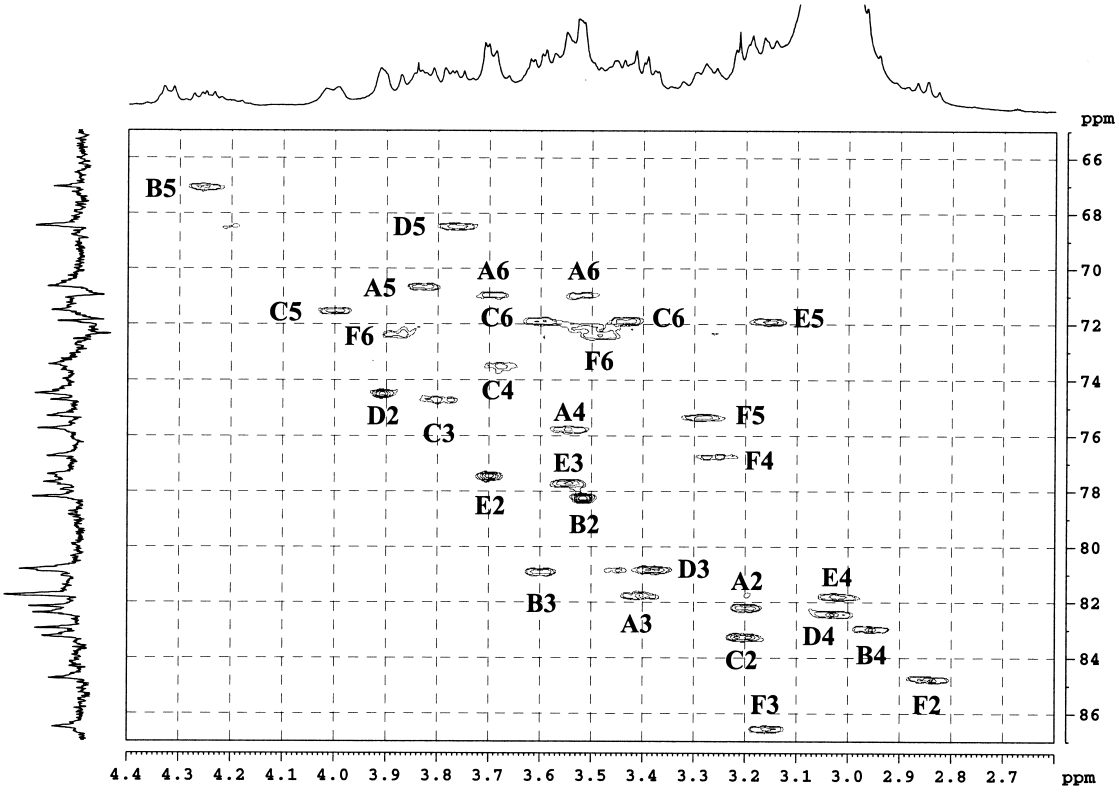



Fig. 2. 400-MHz $^{13}C-^{1}H$ HSQC spectrum of the pdm-EPS from *Lactobacillus delbrueckii subsp. bulgaricus* EU23, recorded in DMSO-$d_6$ at 70 °C. The location of cross peaks is identified by noting the sugar residue as **A**–**F** and by identifying the location of carbons/hydrogens within the ring as **1**–**6**.

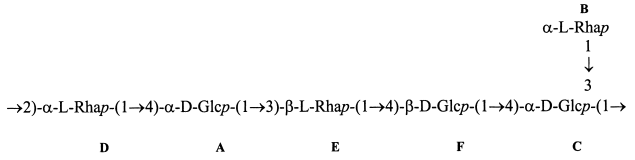
characterisation by NMR, the native EPS was permethylated using a modified version of the Stellner and Hakomori procedure[11] (see Section 3). For ease of analysis the reactions were performed with deuteriodomethane-$d_3$ in place of the protio-reagent. The crude perdeuteriomethylated EPS (pdm-EPS) sample was purified by elution through a $C_{18}$ Sep-Pak cartridge using the procedures described by Mort and co-workers.[19] The resulting sample was soluble in organic solvents (methanol and dimethylsulfoxide) allowing high quality 1D and 2D NMR to be recorded. The $^{1}H$ NMR spectrum of the pdm-EPS is presented in Fig. 1b. From comparison of the spectra of the native EPS and the pdm-EPS it is clear that the repeating unit has not been modified or destroyed during the permethylation procedure. More importantly, there is greater dispersion of signals for both the anomeric and the methyl proton resonances. The dispersion of the rhamnose methyl resonances significantly simplifies the interpretation of the 2D-spectra by providing well-resolved H-6/C-6 tracks on the TOCSY and the HSQC–TOCSY spectra.

A full assignment of the $^{1}H$ and $^{13}C$ NMR spectra of the pdm-EPS required analysis of the 1D $^{1}H$ and $^{13}C$ NMR spectra along with that of 2D $^{1}H-^{1}H$ gs-DQF COSY, $^{1}H-^{13}C$ HSQC (Fig. 2), the $^{1}H-^{1}H$ TOCSY (variable mixing times) and the $^{1}H-^{13}C$ HSQC–TOCSY spectra. Both the assignments of the $^{1}H$ resonances, recorded in dimethyl-$d_6$ sulfoxide, and the assignments of the $^{13}C$ resonances are given in Table 2. The sugar residues are designated **A**–**F** according to decreasing chemical shift of the anomeric protons in the pdm-EPS. Relay of coupling from the anomeric signals through to the methyl resonances on the $^{1}H-^{1}H$ TOCSY spectra (mixing time 120 and 150 ms) identified residues **B**, **D** and **E** as the rhamnose sugars and, by default, **A**, **C** and **F** as glucose. The configuration of the anomeric protons of **A**, **C** and **F** were assigned on the basis of their chemical shift and $^{3}J_{1,2}$ coupling constants. Residues **A** ($\delta$ 5.14, $^{3}J_{1,2}$ 3.0 Hz) and **C** ($\delta$ 5.02, $^{3}J_{1,2}$ 3.0 Hz) are both α-D-Glc*p*. In contrast, residue **F** ($\delta$ 4.32, $^{3}J_{1,2}$ 7.5 Hz) is a β-D-Glc*p*. For the rhamnose sugars the position of the H5 proton resonance and the $^{1}J_{H1-C1}$ coupling constant (determined from a coupled HMQC experiment—spectrum not shown) were used to assign anomeric configurations; residue **E** ($\delta$ H-5 3.15, $^{1}J_{H-C}$ 162 Hz) is a β-L-Rha*p* and residues **B** and **D** are α-L-Rha*p* ($\delta$ H-5 4.24 and 3.76, $^{1}J_{H-C}$ 170 and 172 Hz).

### 2.3. Residue sequence

The structure of the repeating unit of the EPS was determined using a combination of the NMR spectra

and the chemical analyses; the EPS has the following structure:




The residue sequence was determined almost entirely by inspection of the ROESY spectrum (see Fig. 3) which contains a considerable number of both intra-residue and inter-residue NOE. A HMBC spectrum was recorded for the pdm-EPS, however, with one exception, only weak HMBC couplings were visible.

For residue **A** strong NOEs were observed from **A** H-1 ($\delta$ 5.14) to **A** H-2 ($\delta$ 3.20) and to either **A** H-6 ($\delta$ 3.69) or to **E** H-2 ($\delta$ 3.69). Medium NOEs were observed to **A** H-3 ($\delta$ 3.41) and to either **A** H-4 ($\delta$ 3.55) or **E** H-3 ($\delta$ 3.54). In addition to the NOEs there is also a strong inter-residue contour on the HMBC spectrum linking **A** H-1 ($\delta$ 5.14) and **E** C-3 ($\delta$ 77.7). The NOE and HMBC data are consistent with the presence of a **A**($1\rightarrow3$)**E** linkage. For residue **B** there is strong intra-residue NOE from **B** H-1 ($\delta$ 5.07) to **B** H-2 ($\delta$ 3.51) and a weak intra-residue NOE from **B** H-1 ($\delta$ 5.07) to **B** H-4 ($\delta$ 2.96). On the **B** H-1 ($\delta$ 5.07) track a strong inter-residue NOE is observed from **B** H-1 ($\delta$ 5.07) to **C** H-3 ($\delta$ 3.78), a medium NOE is observed to **C** H-4 ($\delta$ 3.68), and a weak NOE is observed to **C** H-2 ($\delta$ 3.20). The inter-residue NOE are consistent with a **B**($1\rightarrow3$)**C** linkage.

On the **C** H-1 track there is a strong intra-residue NOE to **C** H-2 ($\delta$ 3.20). There is a strong inter-residue NOE on the **C** H-1 ($\delta$ 5.02) track to **D** H-2 ($\delta$ 3.91) and a moderate NOE to **D** H-1 ($\delta$ 4.83). The inter-residue NOE are consistent with a **C**($1\rightarrow2$)**D** linkage. For the second L-rhamnose residue there is a strong intra-residue NOE from **D** H-1 ($\delta$ 4.83) to **D** H-3 ($\delta$ 3.38). There is a moderate intra-residue NOE signal observed from **D** H-1 to **D** H-2 ($\delta$ 3.91). The two remaining NOE signals observed on the **D** H-1 ($\delta$ 4.83) track are a very strong inter-residue NOE to **A** H-4 ($\delta$ 3.55) and medium NOEs to **A** H-5 ($\delta$ 3.83) and to **C** H-1 ($\delta$ 5.02). The inter-residue NOE are consistent with there being a **D**($1\rightarrow4$)**A** linkage being present in the repeating unit. For the final L-rhamnose residue, a strong intra-residue NOE is observed from **E** H-1 ($\delta$ 4.60) to **E** H-3 ($\delta$ 3.54) a moderate NOE is observed to **E** H-2 ($\delta$ 3.69) and a weak NOE is observed to **E** H-4 ($\delta$ 3.03). The **E** H-1 track also shows a strong NOE to $\delta$ 3.16; this could be either an inter-residue NOE to **F** H-3 or an expected intra-residue NOE to **E** H-5. The H-1 track also contains a moderate NOE to either **F** H-4 ($\delta$ 3.26) or **F** H-5 ($\delta$ 3.28). As the linkage analysis suggests that the remaining glucose residue must adopt a ($1\rightarrow4$) link it is clear that the inter-residue NOE are indicative of a **E**(1–4)**F** linkage in the repeating unit. For residue **F** there is strong intra-residue NOE from **F** H-1 ($\delta$ 4.32) to **F** H-3 ($\delta$ 3.16), one to either **F** H-4 ($\delta$ 3.26) or to **F** H-5 ($\delta$ 3.28) and a weak NOE to **F** H-2 ($\delta$ 2.84). The remaining NOE is an inter-residue NOE from **F** H-1 to **C** H-4 ($\delta$ 3.68) which indicates the presence of a **F**($1\rightarrow4$)**C** linkage in the repeat unit.

Table 2
$^{1}$H and $^{13}$C NMR chemical shifts of the pdm-EPS of *Lactobacillus delbrueckii subsp. bulgaricus* EU23, recorded in DMSO-$d_6$ at 70 °C

Proton

| Residue | Residue | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b |
|---|---|---|---|---|---|---|---|---|
| **A** | 1,4-Glc*p* | 5.14 | 3.20 | 3.41 | 3.55 | 3.83 | 3.52 | 3.69 |
| **B** | *t*-Rha*p* | 5.07 | 3.51 | 3.59 | 2.96 | 4.24 | 1.11 | |
| **C** | 1,3,4-Glc*p* | 5.02 | 3.20 | 3.78 | 3.68 | 4.00 | 3.43 | 3.60 |
| **D** | 1,2-Rha*p* | 4.83 | 3.91 | 3.38 | 3.04 | 3.76 | 1.18 | |
| **E** | 1,3-Rha*p* | 4.60 | 3.69 | 3.54 | 3.03 | 3.15 | 1.24 | |
| **F** | 1,4-Glc*p* | 4.32 | 2.84 | 3.16 | 3.26 | 3.28 | 3.48 | 3.88 |

Carbon

| Residue | Residue | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|---|
| **A** | 1,4-Glc*p* | 93.0 | 82.2 | 81.8 | 75.8 | 70.7 | 70.9 |
| **B** | *t*-Rha*p* | 97.8 | 78.2 | 80.8 | 83.0 | 67.1 | 18.7 |
| **C** | 1,3,4-Glc*p* | 95.6 | 83.2 | 74.7 | 73.5 | 71.5 | 72.0 |
| **D** | 1,2-Rha*p* | 98.3 | 74.5 | 80.8 | 82.4 | 68.5 | 18.3 |
| **E** | 1,3-Rha*p* | 102.2 | 77.4 | 77.7 | 81.8 | 71.9 | 18.5 |
| **F** | 1,4-Glc*p* | 101.9 | 84.8 | 86.5 | 76.8 | 75.3 | 72.4 |

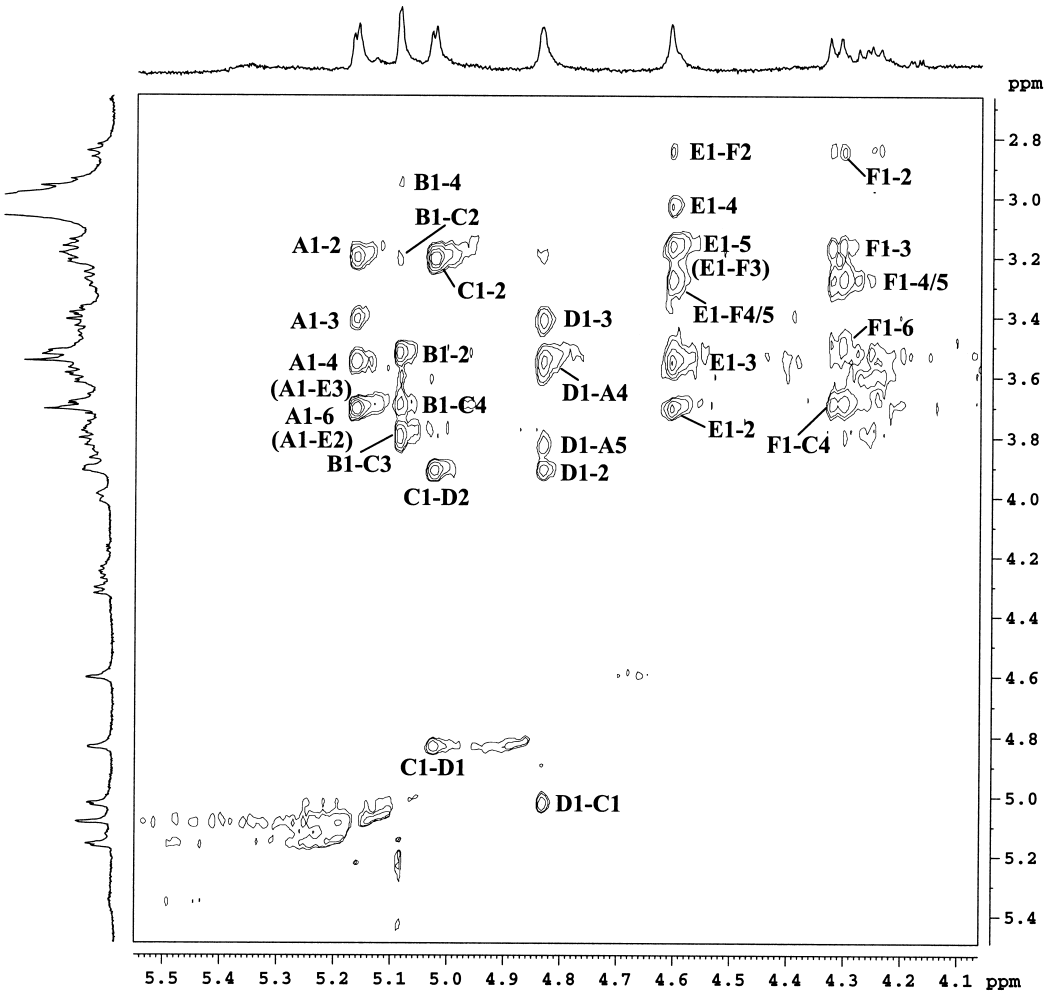



Fig. 3. 400-MHz 2D ROESY spectrum of the pdm-EPS from *Lactobacillus delbrueckii subsp. bulgaricus* EU23, recorded in DMSO-$d_6$ at 70 °C. The sugar residues are identified as **A**–F and the proton ring position is labelled **1**–**6**.

The structure of the EPS is very different to those reported for other *Lb. delbrueckii subsp. bulgaricus* strains and is different to the polysaccharides secreted by other lactic acid bacteria.

## 3. Experimental

### 3.1. Production, isolation and purification of EPS

The bacterial culture of *Lb. delbrueckii subsp. bulgaricus* EU23 was a kind gift from Rhodia foods (Dange St Romain, France) and was maintained in MRS broth (Oxoid). From a pure working culture of *Lb. delbrueckii subsp. bulgaricus* EU23 1% 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 42 °C. This culture (1%) was used to inoculate a larger working volume (1 L for static fermentations) and was incubated as above 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 (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 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 re-dissolved in not more than 20 mL of distilled water with gentle heating (less than 50 °C) 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, see below for conditions) and NMR analysis.

### 3.2. Molecular mass determination

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 × 1 cm) eluting with 50 mM $NH_4HCO_3$ 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.).

### 3.3. Sugar composition

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 alditol acetates. The standard alditol acetates were generated by subjecting an intimate mixture of equal proportions of rhamnose and glucose to the same experimental conditions that were applied to the polysaccharide. GLC–MS analyses were performed on a Varian GC (3400) coupled to a Finnigan Mat ion trap detector (ITD 800). The samples were eluted from a SGE column (BPX5, 25 m × 0.32 mm-id, 0.5 μm film) 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 $min^{-1}$). Absolute configurations of monosaccharides were determined according to the method reported by Gerwig and co-workers.[10]

### 3.4. Sugar linkage analysis

The isolated EPS was permethylated using the procedures described by Stellner and co-workers.[11] 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.[20]

### 3.5. Perdeuteriomethylation of the EPS

Methylation was performed using a modified version of the procedure described by Stellner and Hakomori.[11] Samples of EPS (100 mg) were suspended in DMSO-$d_6$ (1.5 mL) and left stirring under a nitrogen atmosphere for 24 h. Separately, sodium hydride (102.0 mg) was dissolved in DMSO-$d_6$ (1.5 mL) at 65 °C under a stream of dry nitrogen. The two solutions were combined and the mixture stirred under an atmosphere of nitrogen at room temperature for 24 h. Iodomethane-$d_3$ (250 μL) was added and the solution was stirred for a further 75 h. The reaction was quenched by the addition of an equal volume of water and the sample purified directly.

### 3.6. Purification of the deuteriomethylated exopolysaccharide (pdm-EPS)

The crude pdm-EPS was purified using the procedures described by Mort and co-workers.[19] $C_{18}$ Sep-Pak reverse phase cartridges were obtained from Waters Inc., Massachusetts, USA. The cartridges were pre-conditioned by washing with MeOH (5 mL), EtOAc (5 mL), MeOH (5 mL) and water (10 mL). The aqueous DMSO solution of pdm-EPS was applied to the cartridge and the cartridge eluted with water (10 mL), a solution containing MeOH and water (6 mL, 1:1 v/v) and finally with MeOH (4 mL). Fractions were collected as the eluent was applied to the cartridge. The pdm-EPS eluted in the methanol fractions which were evaporated to dryness at 45 °C under a stream of nitrogen.

### 3.7. NMR spectroscopy

Samples were dissolved directly in either $D_2O$ for the native-EPS or in DMSO-$d_6$ for pdm-EPS. The deuteriosolvents used in the experiments and in recording NMR spectra were purchased from 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, when recording the spectra of native-EPS. 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; $\delta$ 2.225 for $^1H$ and $\delta$ 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 with a mixing time of 80 ms. The ROESY spectrum was recorded using a Bruker pulse sequence and 256 experiments of 1024 data points and 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.

## Acknowledgements

This work was funded by the European Commission Framework IV fair (FAIR CT-98-4267) and by the University of Huddersfield (L. Harding). We also wish to thank Dr Neil McLay for recording NMR spectra of the native EPS.

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