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Structural characterisation of the exopolysaccharide produced by *Streptococcus thermophilus* EU20

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Abstract

Streptococcus thermophilus EU20 when grown on skimmed milk secretes a high-molecular-weight exopolysaccharide that is composed of glucose, galactose and rhamnose in a molar ratio of 2:3:2. Using chemical techniques and 1D and 2D-NMR spectroscopy (^{1}H and ^{13}C) the polysaccharide has been shown to possess a heptasaccharide repeating unit having the following structure:

→6)–β-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→3)-β-L -Rha*p*-(1→4)-β-D-Glc*p*-(1→6)-α -D-Gal*f*-(1→6)-β-D-Glc*p*-(1→
2
↑
1
α-L -Rha*p*

Treatment of the polysaccharide with mild acid (0.5 M TFA, 100 °C for 1 h) liberates two oligosaccharides; the components correspond to the repeating unit and a hexasaccharide equivalent to the repeating unit minus the terminal α-L-Rha*p*.

Keywords: *Streptococcus thermophilus*; Exopolysaccharide; Heptasaccharide repeating unit

1. Introduction

A significant number of lactic acid bacteria (LAB) secrete heteropolysaccharides into the surrounding medium during growth.[1,2] The secreted polysaccharides, exopolysaccharides (EPSs), when present in aqueous solution provide thickening and gelling properties.[3] It is the rheological characteristics of aqueous solutions of EPS that gives rise to our interest in EPS production and EPS biosynthesis.[4] EPS production has both positive and negative attributes. EPS formation by LAB during the production of fermented milk products imparts favourable rheological properties.[5–7] The negative aspect of EPS production is connected with the role they play in the formation of biofilms and biofouling,[8] with the EPS providing a matrix on which bacteria can adhere and grow.[9] The functional role of EPS in the life cycle of bacteria is not clear. It is thought that they are produced in order to

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protect the microbial cell rather than their being a potential food reserve.[10]

There is a need to develop an understanding of structure–function relationships, i.e., to relate EPS structure with 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 EPS will help in determining the pathways by which the EPSs are biosynthesised.

The chemical composition and structures of a number of bacterial EPSs have been determined. Structures have been reported for EPSs from *streptococci*,[11–14] *lactobacilli*[15–24] and *lactococci*.[25–27] The structures are very diverse. Whilst the main constituent sugars are frequently galactose, glucose and rhamnose, there is no obvious pattern in the frequency at which they occur and EPSs having different ratios of the three sugars are found. The complexity of the structures is further compounded by the variety of linkages that exist between the monosaccharides. Only when further structures have been reported will it be possible to develop meaningful structure–function relationships. We are currently engaged on a project directed at determining the structure of EPSs generated by a number of LAB that are used widely throughout Europe for the production of fermented milk products. Here we report the structure of the exopolysaccharide produced by *Streptococcus thermophilus* EU20 when grown on skimmed milk.

Table 1
Methylation analysis data of *S. thermophilus* EU20

Derivative	Linkage type
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol	t-Rha*p*
1,2,3,5-Tetra-*O*-acetyl-4-*O*-methyl-rhamnitol	1,2,3-Rha*p*
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol	1,4-Glc*p*
1,4,6-Tri-*O*-acetyl-2,3,5-tri-*O*-methyl-galactitol	1,6-Gal*f*
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol	1,6-Glc*p*
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-galactitol	1,6-Gal*p*

2. Results and discussion

Purity and molecular weight determination.—Analysis of the NMR spectrum of the EPS recovered from a culture of *S. thermophilus* EU20 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, indicating that the EPS isolated is pure and free from low-molecular-weight polysaccharides. When the same protocols are used to isolate polysaccharides from cultures grown in broth supplemented with various nitrogen sources (yeast and peptone) low-molecular-weight poly-mannans are extracted with the EPS.

The molecular weight of the EPS is 140 kDa and is significantly smaller than the value recorded for the EPS isolated from *S. thermophilus* Sts.[14] In a separate series of experiments, where the pH of the fermentation was allowed to fall, the isolated EPS had associated with it a substantial amount of lactic acid, even after exhaustive dialysis. Bubb et al.[11] have described the adhesion of small acids, lactic acid and acetic acid, to EPS samples. The strong adhesion of small molecules may provide evidence for molecular association between polysaccharide chains. These associations would perturb molecular weight measurements.

Repeating unit composition.—The results of the monomer analysis indicate that the polysaccharide is composed of glucose, galactose and rhamnose, in ratios of 2:3:2. Whilst an identical monomer composition has been found for the EPS isolated from *Lactococcus lactis* subsp. *cremoris* B39[27] the NMR spectra of the two are very different. The results of the methylation analysis (Table 1) viewed in combination with the ^{1}H NMR spectra indicate that the EPS has a heptasaccharide repeating unit and that the repeating unit is branched. Of the 14 LAB EPS structures published to date, the largest repeating unit is a heptasaccharide. Including the present structure, a total of five EPSs containing a heptasaccharide repeating unit are known, the other four are the EPSs isolated from *S. thermophilus* 0R901,[11] *S. thermophilus* Rs and Sts

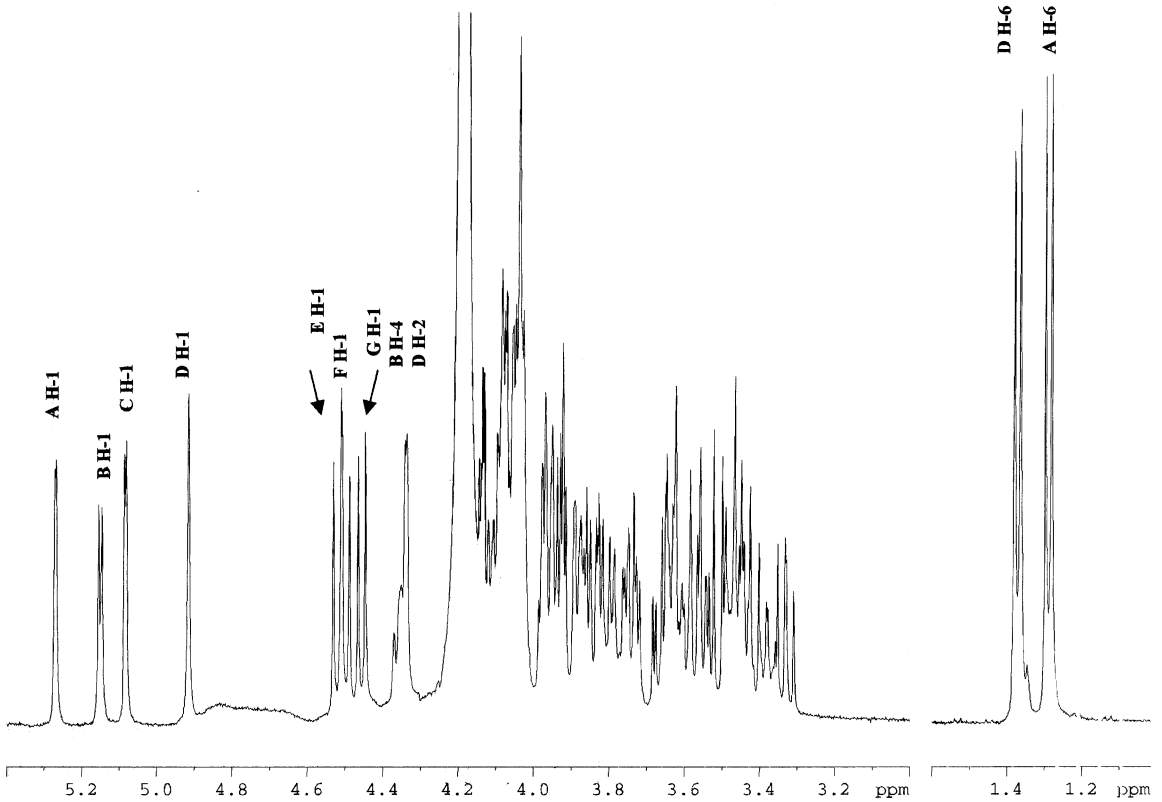
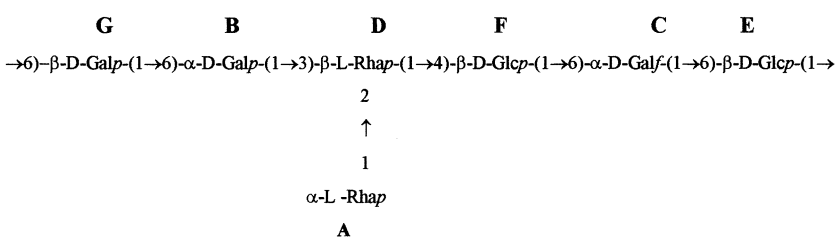


Fig. 1. 400-MHz ^{1}H NMR spectra of EPS from *S. thermophilus* EU20, recorded in D_2O at 70 °C.

(these two have identical structures),[14] *L. lactis* subsp. cremoris B39[27†] and *Lactobacillus helveticus* TY1-2.[21] It would appear that a heptasaccharide unit is the largest building block that LAB can utilise in the synthesis of secreted EPS. The EPS from *S. thermophilus* EU20 contains two rhamnose residues, one of the two rhamnose sugars is a branch terminus whilst the second rhamnose sugar forms the branching point. From inspection of the ^{1}H NMR (Fig. 1) and ^{13}C NMR (Fig. 2) chemical shifts it is clear that, with the exception of a single galactofuranose residue, the monosaccharides are all present in their pyranose ring form.

The combined results of the NMR analysis and the chemical analysis of the polysaccharide point to the structure of the EPS of *S. thermophilus* EU20 having a repeating unit with the following structure:



1D NMR of the polysaccharide.—The ^{13}C spectrum for the EPS isolated from *S. thermophilus* EU20 (Fig. 2) is consistent with the EPS having a heptasaccharide repeating unit and there are 42 distinct carbon resonances. The two high-field resonances are associated with the rhamnose methyl groups and there are seven resonances in the anomeric region. The anomeric resonance at unusually low field (δ 109.2) is derived from the C-1 of a furanose

† *L. lactis* subsp. *cremoris* B39 and *S. thermophilus* 0R901 have similar structures, the only difference is in the substitution of a branching β-D-Gal*p*-(1→6)-β-D-Glc*p* with a β-D-Gal*p*-(1→4)-β-D-Glc*p*.

sugar. The ^{13}C DEPT(135) spectrum (not shown) has five methylene carbon resonances which appear as negative peaks; this result is consistent with two of the repeating unit sugars being 6-deoxy sugars. As four of the methylene resonances are significantly above 65 ppm, the heptasaccharide must contain a significant proportion of (1→6)-links.

The ^1H NMR spectrum (Fig. 1) of the EPS from *S. thermophilus* EU20 is also consistent with a heptasaccharide repeating unit with seven proton resonances being observed in the anomeric region (δ 5.3–4.4). The sugar residues are designated **A**–**G** according to decreasing chemical shift of the anomeric protons. The configuration of the anomeric protons **E**, **F** and **G** were assigned on a basis of their chemical shifts and the $^3J_{1,2}$ coupling constants. The signal at δ 4.53 (residue **E**, β-D-Glc*p*, $^3J_{1,2}$ 8.1 Hz) δ 4.49 (residue **F**, β-D-Glc*p*, $^3J_{1,2}$ 8.1 Hz) and δ 4.45 (residue **G**, β-D-Gal*p*, $^3J_{1,2}$ 7.8 Hz) are all of β configuration. For the remaining residues, **A**, **B**, **C** and **D**, the configuration of the anomeric protons were assigned by consideration of chemical shifts and $^1J_{H-C}$ coupling constants (determined from a coupled HMQC experiment-spectra not shown). The signals at δ 5.27 (residue **A**, α-L-Rha*p*, $^1J_{H-C}$ 173 Hz) δ 5.15 (residue **B**, α-D-Gal*p*, $^3J_{1,2,}$ 3.4, $^1J_{H-C}$ 170 Hz)

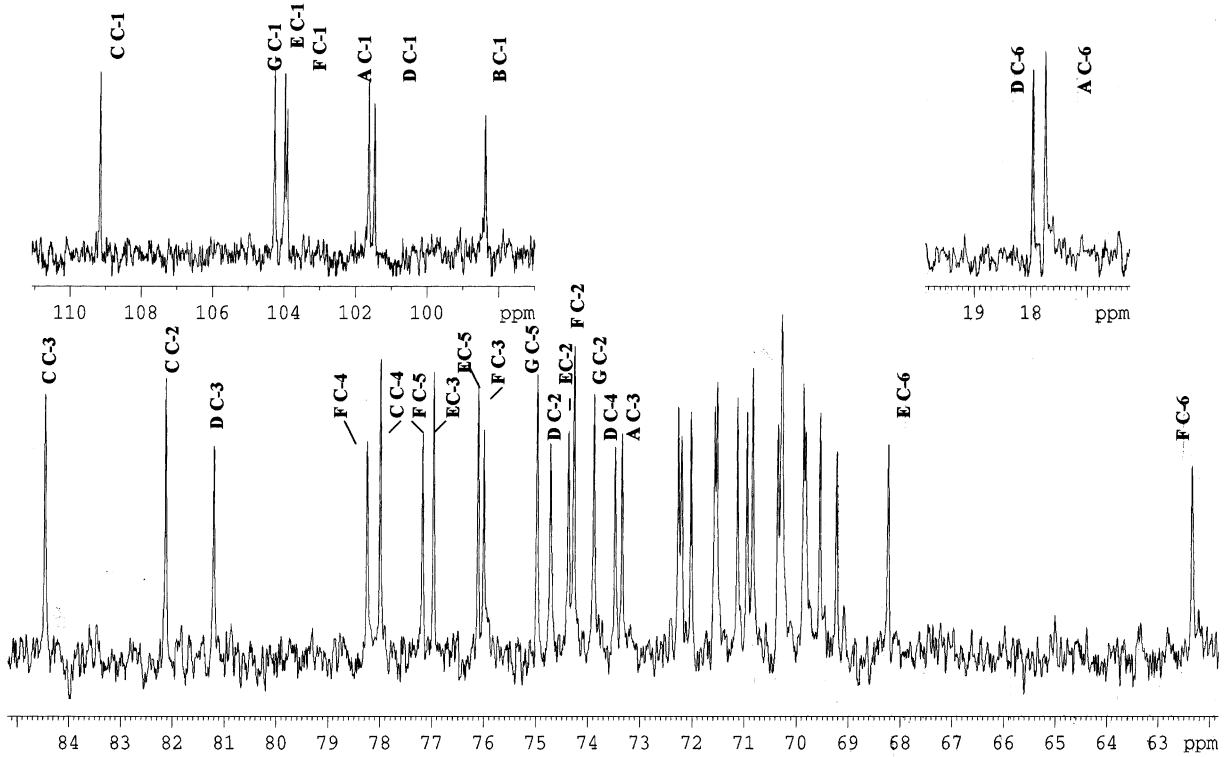


Fig. 2. 100-MHz ^{13}C NMR spectra of EPS from *S. thermophilus* EU20, recorded in D_2O at 70 °C.

Table 2
^1H NMR chemical shifts of EPS (1) recorded in D_2O at 70 °C

Residue	H-1	H-2	H-3	H-4	H-5	H-6a, H6b	CH_3
A	5.27	4.07	3.85	3.47	4.07		1.29
B	5.15	3.92	4.05	4.36	3.89	3.94, 4.08	
C	5.08	4.13	4.14	4.03	3.78	3.51, 4.04	
D	4.91	4.33	3.75	3.44	3.54		1.36
E	4.53	3.33	3.51	3.43	3.60	3.73, 4.07	
F	4.49	3.36	3.63	3.48	3.80	3.80, 3.98	
G	4.45	3.56	3.66	3.97	3.88	3.97, 4.06	

Table 3
^{13}C NMR chemical shifts of EPS (1) recorded in D_2O at 80 °C [a]

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	101.62	~71.55	73.33	~70.28	69.54	17.74
B	98.33	~70.28	69.21	70.92	~71.55	69.85
C	109.13	82.10	77.98	84.43	~70.28	72.24
D	101.46	74.69	81.14	73.47	72.24	17.96
E	103.96	74.36	76.95	71.11	76.09	68.21
F	103.89	74.25	75.98	78.23	77.17	62.33
G	104.24	73.86	72.01	~69.85	74.97	70.28

[a] ~, symbol used to identify partially overlapping carbon resonances.

and δ 5.08 (residue **C**, α-D-Gal*f*, $^3J_{1,2,}$ 1.8, $^1J_{H-C}$ 173 Hz) have the α configuration. The remaining residue, which will subsequently be shown to be the bridging rhamnose, δ 4.91 (residue **D**, β-L-Rha*p*) has a $^1J_{H-C}$ of 161 Hz which is consistent with it being a β linked sugar. The assignment of specific residues to **A**–**G** is based on the analysis of the 2D-spectra that follows.

2D NMR of the polysaccharide.—Assignments of 1D-resonances, ^1H and ^{13}C, to the protons and carbons of the individual monosaccharides (Tables 2 and 3, respectively) are based on interpretations of 2D-spectra. A large number of 2D-spectra were recorded including: 2D-COSY, 2D-TOCSY (400 and 600 MHz), 2D $^{13}C-^1H$ HMQC, $^{13}C-^1H$ HMBC and $^{13}C-^1H$ HMBC-TOCSY spectra.

For the rhamnose sugars, residues **A** and **D**, the assignment of shifts was achieved using the tracks on the TOCSY spectrum and starting from either end of the molecule, i.e., using a combination of cross peaks derived from the methyl resonances (δ 1.29 **A** H-6 and δ 1.36 **D** H-6) and the anomeric protons (δ 5.27 **A** H-1 and δ 4.91 **D** H-1). From the TOCSY spectrum (mixing time of 60 ms) the **A** H-6 track (δ 1.29) shows cross peaks to **A** H-5, 4 and 3 whilst on the **A** H-1 track (δ 5.27) there are cross peaks to **A** H-2, 3 and 4. Similarly, the **D** H-6 track (δ 1.36) shows cross peaks to **D** H-5, 4, 3 and 2 whilst on the **D** H-1 track (δ 4.91) there are cross peaks to **D** H-2 and 3. For the remaining sugars assignments start from the tracks derived from the anomeric protons. On the TOCSY (mixing time 210 ms) the **B** H-1 track (δ 5.15) has cross peaks from **B** H-1 to B H-2, and 3 and a very small cross peak is observed for **B** H-4. The 210 ms TOCSY spectrum recorded at 600 MHz has a modest cross peak, clearly visible on either side of the diagonal, at δ 4.36 confirming the location of **B** H-4. The low field resonance of **B** H-4 (δ 4.36) allows the connection from **B** H-4 to H-3 and 5 to be made from the **B** H-4 track. The location of the two remaining protons cannot be identified using the TOCSY spectra and were established by identifying the position of the methylene carbons using a ^{13}C DEPT(135) spectrum and then locating the corresponding hydrogens on a $^{13}C-^1H$ HMQC spectrum. The chemical shifts for the methylene carbon C-6 of **B** and **G** are known (δ 70.3 and 69.9). These were identified as methylene carbons from a DEPT spectrum and were assigned to **B** and **G** after all the other signals had been identified. Both resonances are at low field positions and the positions suggest that they are both involved in (1→6)-links.

For residue **C**, the relay of coupling from the track for the anomeric proton of residue **C** H-1 (δ 5.08) identified the locations of **C** H-2, 3, 4 and 5. The resonances for H-2 and H-4 partially overlap, determination of the individual chemical shifts was only possible with reference to the 2D $^{13}C-^1H$-HMQC spectra and the HSQC-TOCSY spectra. On the HSQC-TOCSY spectra the **C** H-1 has very strong cross peaks to all six carbons of the galactose furanose residue. The only carbon resonance that has a chemical shift that is coincident with that of a methylene carbon is the signal at δ 72.24. This is the methylene resonance at the lowest field and therefore its position is consistent with it being derived

from a furanose sugar. The **C** carbon tracks on the HSQC-TOCSY spectrum **C** C-1 (δ 109.1), **C** C-2 (δ 82.1), **C** C-3 (δ 84.4), and **C** C-4 (δ 78.0) show progressive development of cross peaks locating the positions of **C** H-1, 2, 3, 4 and H-5 (C-1 to H-1 and H-2; C-2 to H-2, 3 and 4; C-3 to H-2, 3 and 4; C-4 to H-3, 4 and H-5). Finally, the assignments for the proton residues for **C** H-6a and H-6b were obtained from the $^{1}H-^{13}C$ HMQC spectrum. The H-1 track for residue **E** δ 4.53 shows cross peaks for **E** H-2, 3, 4, 5 and H-6a, H-6b, the ease with which the coupling is transmitted from H-1 through to H-6 is indicative of residue **E** being a glucopyranose sugar. For residue **F** the TOCSY spectrum (60 ms mixing time) has a H-1 track δ 4.49 with cross peaks to **F** H-2, 3 and 4. On the **F** H-4 track, δ 3.48, only two additional cross peaks are visible (the same is true for the spectrum recorded with a mixing time of 210 ms) suggesting that at least two of the proton resonances overlap. The location of the resonances were determined by establishing the location of **F** C-6 using the **F** H-1 track of the HSQC-TOCSY spectrum which has a cross peak that is coincident with one of the methylene carbon signals. The latter cross peak must be derived from **F** C-6 as this is a clean area of the spectrum. The location of **F** H-6a and H-6b can be read directly from the $^{13}C-^{1}H$-HMQC spectrum. The ease with which the coupling information is transmitted around the ring is indicative of residue **F** being a second glucopyranose sugar. For residue **G**, the **G** H-1 track (δ 4.45) on the TOCSY spectrum (mixing time of 60 ms) shows cross peaks to **G** H-2, 3 and 4. With a much longer mixing time (210 ms) a small cross peak from **G** H-1 to **G** H-5 is visible. Identification of the exact location of the **G** H-6a and **G** H-6B using only the TOCSY spectrum is not possible. The position of **G** C-6 (δ 70.28) was identified from a ^{13}C DEPT(135) spectrum once the position of the other methylene resonances had been determined. The locations of the **G** H-6 proton resonances were then extracted as cross peaks on the $^{13}C-^{1}H$-HMQC spectrum.

For each of the carbons, the position of the ^{13}C resonances (Table 3) were determined on inspection of the $^{13}C-^{1}H$ HMQC and the $^{13}C-^{1}H$ HSQC-TOCSY spectra.

Residue sequence.—The sequence of the sugar residues in the heptasaccharide repeating unit was determined with reference to the $^{13}C-^{1}H$ HMBC (Fig. 3) and ROESY (Fig. 4) spectra of the native EPS. For residue **A** a strong inter-residue NOE was detected from **A** H-1 (δ 5.27) to **D** H-2 (δ 4.33) suggesting a **A**($1\rightarrow2$)**D** linkage, i.e., the terminal α-L-Rha*p* is connected to the 2-position of the branching β-L-Rha*p*. The latter result is evidence that the branch is composed of a single residue and the main chain is a hexasaccharide. The presence of the **A**($1\rightarrow2$)**D**-link was confirmed by the observation of a cross peak on the **A** C-1 track (δ 101.6) at **D** H-2 (δ 4.33) on the $^{13}C-^{1}H$ HMBC spectrum. The **B** H-1 track (δ 5.15) of the ROESY spectrum shows a strong inter-residue cross peak with **D** H-3 (δ 3.75), there is also a weak cross peak between **B** C-1 (δ 98.33) and **D** H-3 (δ 3.75) on the $^{13}C-^{1}H$ HMBC spectrum indicating the presence of a **B**($1\rightarrow3$)**D** linkage. For the H-1 track of residue **C** (δ 5.08) only one clearly identifiable inter-reside NOE is visible and this is to one of the **E** H-6s (δ 3.73). More convincing evidence for a **C**($1\rightarrow6$)**E** linkage can be found on the $^{13}C-^{1}H$ HMBC spectrum which has a strong cross peak from **E** C-6 (δ 68.21) to **C** H-1 (5.08). For residue **D**, the **D** H-1 track (δ 4.91) on the ROESY spectrum shows an inter-residue NOE to **F** H-4 (δ3.48) and a weak inter-residue NOE to **F** H-3 (δ 3.63). Further evidence for the **D**($1\rightarrow4$)**F**-link is available on the $^{13}C-^{1}H$ HMBC spectrum where there is a cross peak between **D** H-1 (δ 4.91) and **F** C-4 (δ 78.23). The H-1 track for residue **E** (δ 4.53) has two strong inter-residue NOE at δ 3.88 and δ 3.94 each of these areas is occupied by a number of resonances including **G** H-5 and **B** H-5 (δ 3.88/3.89) and **G** H-6 and **B** H-6 (3.97/3.94). The **G** C-6 track of the $^{13}C-^{1}H$ HMBC spectrum shows a strong cross peak to **E** H-1. The ROESY and HMBC provides evidence for a **E**($1\rightarrow6$)**G** linkage. For residue **F** the H-1 track (δ 4.49) of the ROESY spectrum shows a strong inter-residue NOE at δ 4.04, this is a crowded region of the spectrum containing a number of proton resonances including **C** H-6. On the $^{13}C-^{1}H$ HMBC spectrum there is a very clear cross peak between **C** C-6 (δ 72.2) and **F** H-1 (δ 4.49) identifying an **F**($1\rightarrow6$)**C**-link between the two residues.

The **G** H-1 track (δ 4.45) of the ROESY spectrum has a weak inter-residue NOE at δ 3.93 and the **G** H-1 track (δ 4.45) on the ^{13}C–^{1}H HMBC spectrum shows a strong cross peak with **B** C-6 (δ 69.85). The latter two sets of results are consistent with there being a **G**(1→6)**B** linkage, a link between **B** and **G** is also required to bring both ends of the hexasaccharide together.

The structure of the EPS is different to that reported for other *S. thermophilus* polysaccharides and is different to the polysaccharides secreted by other LAB. There are, however, a few common elements to several structures, for example many are composed of galactose, glucose and rhamnose. For those containing rhamnose in the backbone chain, it is frequently rhamnose that is the branching point. The published EPS structures nevertheless are diverse suggesting that the substrate specificity of the transferase enzymes which are responsible for EPS assembly are unique to each organism.

Mild acid hydrolysis of the EPS from S. thermophilus *EU20.*—With a view to confirming the structure of the EPS, a sample of polysaccharide was subjected to mild acid hydrolysis. The glycosidic linkage joining the

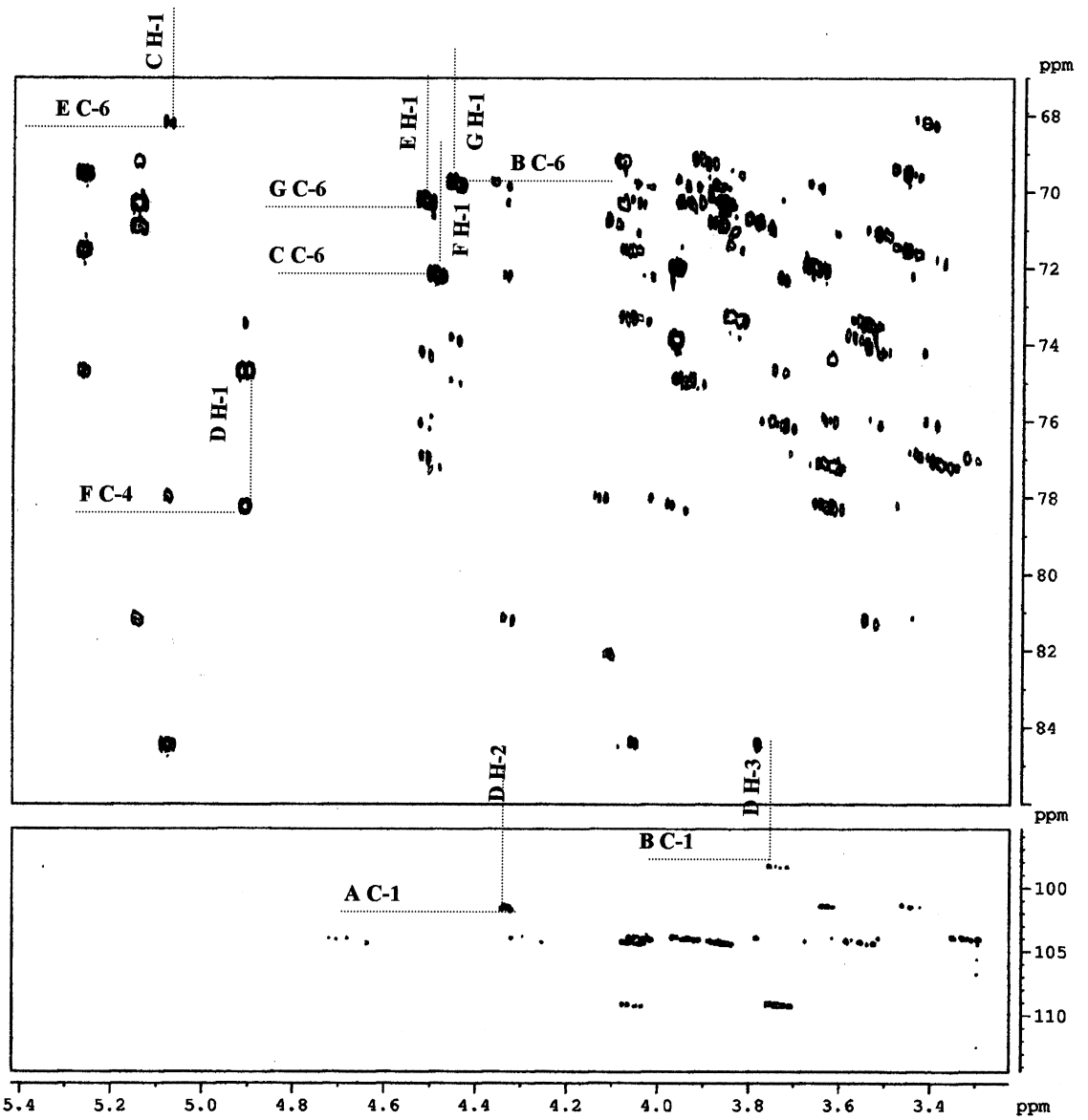


Fig. 3. 400-MHz ^{13}C–^{1}H HMBC spectrum of EPS from *S. thermophilus* EU20, recorded in D_2O at 70 °C. Cross peaks identifying scalar coupling across a glycosidic link are identified as intersecting dashed lines. The sugar residues are identified as A–G and the carbon position is labelled on the horizontal axis and the coupled proton on the vertical axis.

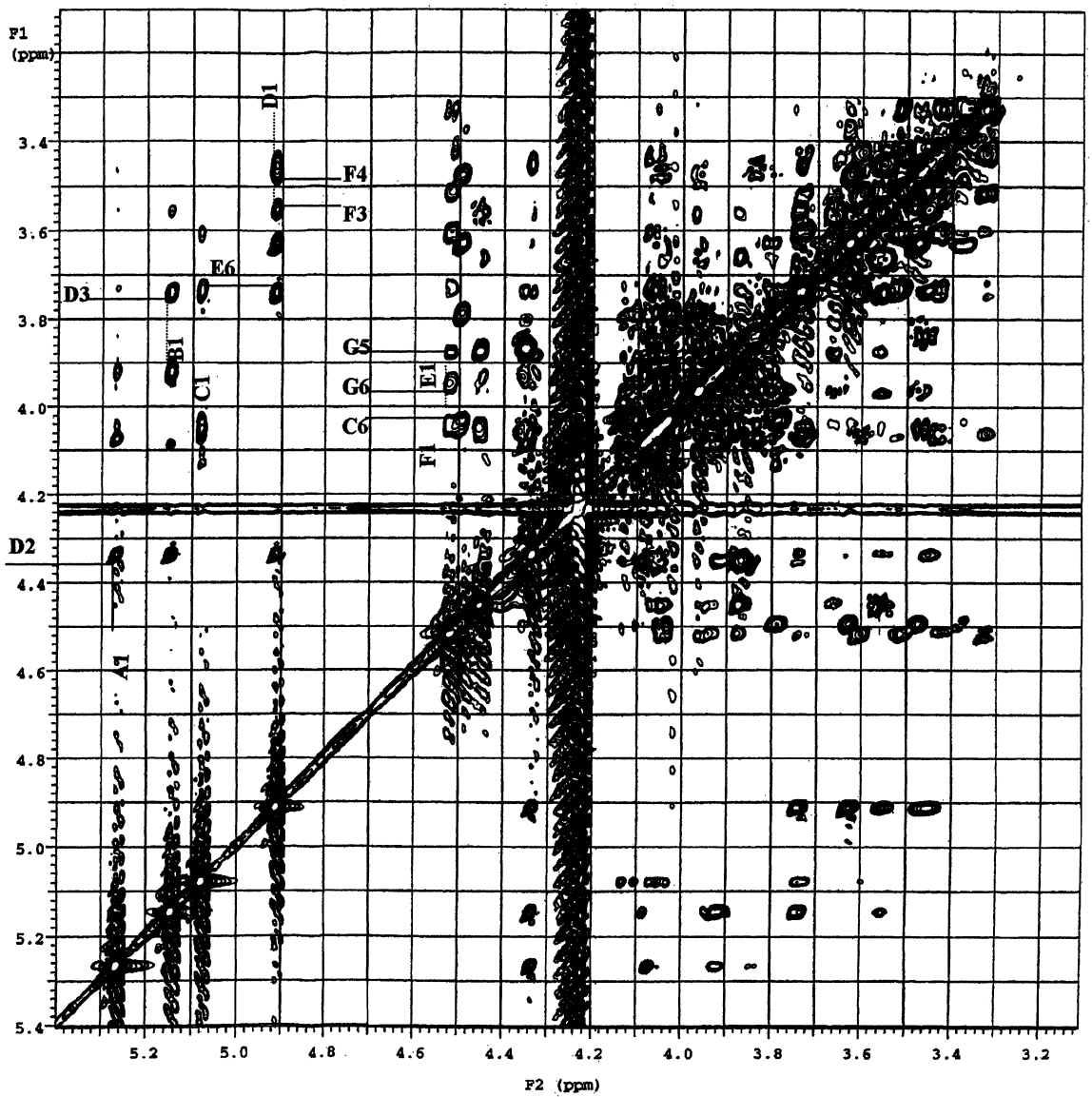


Fig. 4. 600-MHz 2D ROESY spectrum of EPS from *S. thermophilus* EU20, recorded in D_2O at 80 °C. Cross peaks corresponding to interresidue NOEs are identified as intersecting dashed lines. The sugar residues are identified as A–G and the proton ring position is labelled on the axes.

C-1 of a furanose ring to a pyranose sugar is usually acid labile.[28] Comparison of the ^1H NMR spectra for the native EPS shows loss of the H-1 resonance (δ 5.08) for the galactofuranose residue, residue **C**. The latter result would be expected if cleavage of the polysaccharide had occurred between residues **C** and **E**.

3. Experimental

Production, isolation and purification of EPS.—The bacterial culture of *S. thermophilus* EU20 was a kind gift from Rhodia foods (Dange St Romain, France) and was maintained in M17 broth (Oxoid). From a pure working culture of *S. thermophilus* EU20 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 24 h at 37 °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) 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 EtOH 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 EtOH. The sample was centrifuged at 25,000*g* for 25 min at 4 °C. The resulting EPS pellet was redissolved in a maximum of 20 mL of distilled water (sample heated as above) 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 (Sephacryl® S-500 high resolution) and NMR analysis.

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). 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).

Release of oligosaccharide fragments.—EPS (236 mg) isolated as described above, was suspended in an aq trifluoroacetic acid solution (20 mL, 0.5 M) and the solution was heated at 100 °C for 1 h. The resulting oligosaccharides were fractionated by size-exclusion chromatography on a Bio-Gel® P2 (Bio RAD) column (70×1.6 cm).

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, glucose and galactose 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 $\times$ 0.32 mm-id, 0.5 μm film) eluting with helium (9 psi, flow rate 1 mL/min) 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). Absolute configurations of monosaccharides were determined according to Ref. 29.

Sugar linkage analysis.—The isolated EPS was per-methylated using the procedures described by Stellner et al.[30] 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.[31]

NMR spectroscopy.—Samples were dissolved directly in D_2O (99.9% D) (Goss Scientific Instruments Ltd., Essex, UK). NMR spectra were recorded at probe temperatures of either 70 or 80 °C unless otherwise stated. The elevated temperature shifted the HOD signal to a 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 ^{1}H (100.61 MHz ^{13}C) spectrometer (located at Huddersfield) operating with Z-field gradients where appropriate and using Bruker's pulse programmes. The 2D-TOCSY and ROESY spectra were recorded on a Varian 600 MHz spectrometer (located at Astra–Zeneca's Research centre, Loughborough, UK). Chemical shifts are expressed in ppm relative to either internal or external acetone; δ 2.225 for ^{1}H and δ 31.55 for ^{13}C. The 1D ^{1}H 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 at 400 MHz and 60, 210 ms at 600 MHz). The 2D-heteronuclear $^{1}H-^{13}C$ HMBC, HMQC (coupled and decoupled) 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. After either applying zero-filling or linear prediction and Fourier transformation, data sets of 2048×1024 (400 MHz HMQC, HSQC-TOCSY) 1024–1024 points (400 MHz DFQ-COSY, TOCSY) or 4096×4096 points (600 MHz TOCSY, ROESY) were obtained.

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