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Structural characterisation of the exocellular polysaccharide produced by *Streptococcus thermophilus* OR 901

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Abstract

The exocellular polysaccharide of *Streptococcus thermophilus* OR 901, isolated from partially deproteinised whey, is a heteropolymer of D-galactopyranose and L-rhamnopyranose residues in the molar ratio 5:2. The structure was established by methylation analysis and 1D and 2D NMR spectroscopy of the native polysaccharide, in combination with characterisation of oligosaccharide fragments, obtained by partial acid hydrolysis, using methylation analysis and 1D ^{1}H NMR spectroscopy. The polysaccharide has a branched heptasaccharide repeating unit with the following structure:

$$\begin{array}{l} \beta\text{-D-Gal}_p\text{-}(1\rightarrow6)\text{-}\beta\text{-D-Gal}_p\text{-}(1\rightarrow4) \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ \rightarrow2)\text{-}\alpha\text{-D-Gal}_p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Gal}_p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Gal}_p\text{-}(1\rightarrow3)\text{-}\alpha\text{-L-Rha}_p\text{-}(1\rightarrow2)\text{-}\alpha\text{-L-Rha}_p\text{-}(1\rightarrow \end{array}$$

Keywords: Lactic acid bacteria; *Streptococcus thermophilus*; Exopolysaccharide structure; NMR data

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1. Introduction

It has been reported that many microorganisms produce exopolysaccharides, i.e. polysaccharides found outside the cell wall, either attached to it in the form of capsules or secreted into the extracellular environment in the form of slime. Interest in the improvement of the body and texture of dairy products has led to the characterisation of slimy polysaccharides elaborated by several Lactobacillus and Lactococcus species, including *Lactobacillus delbrückii* subsp. *bulgaricus* [1,2], *Lactobacillus kefiranofaciens* K_1 [3], *Lactobacillus helveticus* [4–6], *Lactococcus lactis* subsp. *cremoris* [7,8], *Lactobacillus acidophilus* [9], *Lactobacillus paracasei* [10] and *Lactobacillus sake* 0–1 [11].

Since *Streptococcus thermophilus*, together with *L. bulgaricus*, are constituents of yogurt starter, polysaccharides produced by the Streptococci are of commercial interest in dairy technology. For *S. thermophilus*, Doco et al. [12] reported the chemical structure of a polysaccharide which contained D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-galactose in the molar ratio of 1:2:1, while Cerning et al. [13] reported a polysaccharide which was composed mainly of D-glucose and D-galactose, with small amounts of D-xylose, L-arabinose, L-rhamnose and D-mannose. During the course of our investigations of *S. thermophilus* exopolysaccharides we found that *Streptococcus thermophilus* OR 901, isolated from market yogurt, produced an exocellular polysaccharide containing D-galactose and L-rhamnose when cultured in a partially deproteinised whey medium [14]. Here we report the chemical structure of this polysaccharide.

2. Experimental

Organisms.—*Streptococcus thermophilus* OR 901 was isolated from a commercial yogurt of the Snow Brand Milk Products Co., Ltd. (Tokyo, Japan) as previously described [14].

Preparation of the polysaccharide from culture.—The culture was inoculated into partially deproteinised whey containing 1% peptone and incubated as previously described [14]. After incubation, the exocellular polysaccharide was collected from the culture by precipitation with cold EtOH and purified by passage through DEAE-Sephadex A-50 ion exchange resin [14].

Methylation analysis of the polysaccharide.—The polysaccharide was methylated by the method of Hakomori [15] and the methylate, in $CHCl_3$ (1 mL), was subjected to silica gel column chromatography at 37 °C. Elution with $CHCl_3$ (5 mL) removed incompletely methylated polysaccharide, while the permethylated saccharide was obtained by subsequent elution with MeOH (3 mL). Alditol acetate derivatives of partially methylated sugars were prepared from the permethylated polysaccharide by the method of Stellner et al. [16]. The partially methylated alditol acetates were analysed on a Hitachi 163 gas chromatograph equipped with a flame ionisation detector. The instrument was fitted with a glass column (0.2 × 200 cm) packed with 2% OV-17 on chromosorb W and programmed from 150 to 250 °C at 3 °C/min. Gas chromatography-mass spectrometry was performed on a JEOL HX-105 mass spectrometer (ionisation current, 100 μA; voltage 70 eV) equipped with a GC column (OV-1, 0.25 mm × 60 m) operated at 150–250 °C, 3 °C/min.

Isolation and characterisation of oligosaccharide hydrolysis products.—Freeze-dried precipitate (160 mg) from the culture was dissolved in 0.2 M trifluoroacetic acid (16 mL) and the solution heated at 100 °C for 1 h and then evaporated to dryness on a rotary evaporator. The partial hydrolysate was dissolved in distilled water (4 mL) and subjected to preparative paper chromatography in an ascending system of 6:4:3 butan-1-ol–pyridine–water, using five developments. The zones containing the oligosaccharides were located with alkaline $AgNO_3$ reagent and extracted with distilled water and the extracts freeze-dried. Each extract was dissolved in distilled water (1 mL) and further separated by passage through a column (2.5 × 100 cm) of Bio Gel P-2 (−400 mesh, extra fine). The oligosaccharide fractions, identified by the phenol–H_2SO_4 method [17], were collected and freeze-dried.

Each oligosaccharide was treated with ^2H_2O (99.75 atom % ^2H), freeze-dried and finally dissolved in ^2H_2O (99.96 atom % ^2H) and examined by ^1H NMR using a JEOL JNM-GSX-400 spectrometer at 400.13 MHz with the probe temperature set to 27 °C. Chemical shifts are expressed relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS), but were actually measured by reference to internal acetone (δ 2.225). Partially methylated alditol acetates were prepared from each oligosaccharide and analysed by GC as described above and GC–MS on a mass spectrometer equipped with a GC column (SPE-1, 0.25 mm × 60 m) operated at 140–250 °C, 3

°C/min. Each oligosaccharide was reduced with NaB^2H_4 at room temperature for one day prior to the methylation.

NMR studies of the polysaccharide.—All spectra of the polysaccharide were recorded on a Bruker AMX-600 spectrometer using a 5 mm broadband inverse-detection probe with standard Bruker pulse sequences (UXNMR 930601) modified for off-resonance water suppression (1.4–1.8 s) as previously described [18]. The data was processed directly on the spectrometer or on a Silicon Graphics R4600PC Workstation running XWIN-NMR version 1.2.

Samples were prepared as described above for the oligosaccharides but concentrated and transferred to a 5 mm o.d. susceptibility-matched microcell (Shigemi) for the two-dimensional studies. This resulted in a gradual increase in the intensity of the residual HO^2H signal as it was not possible to seal the microcell as effectively as a conventional NMR tube. Spectra were obtained over a probe temperature range of 30–80 °C but, with the exception of the $^1J_{^{13}C-^1H}$ coupling constants which were obtained from a spectrum recorded at 57 °C, all data are reported for spectra recorded at 70 °C, with chemical shifts referenced to internal DSS which was added to the sample after it had been fully characterised. Chemical shifts of clearly resolved resonances of anomeric and methyl protons were determined from a 1-dimensional spectrum but all other ^1H and all ^{13}C chemical shifts were obtained from a heteronuclear multiple quantum coherence (HMQC) [19] spectrum.

Spectral widths were 2958 Hz for ^1H and 15091 Hz for ^{13}C, except for the spectra used for determination of chemical shifts for which the ^1H spectral width was increased to 4033 Hz for the 1D experiment and to 3521 Hz and 20374 Hz, respectively, for the ^1H and ^{13}C dimensions of the HMQC experiment. Apart from the heteronuclear multiple bond correlation (HMBC) [20] experiments, all spectra were recorded in phase-sensitive mode and routinely processed with one level of zero-filling in F1 and a $\pi/2$ shifted sine-bell in each dimension and independently with $\pi/2-\pi/4$ shifted sine-squared functions for resolution enhancement.

2D Total correlation spectra (TOCSY) [21] were recorded for mixing times of 51, 70, 102 and 203 ms, with the spin-lock field strength adjusted for a 90° pulse-length of 25–28 μs. 2D Rotating-frame nuclear Overhauser enhancement spectra (ROESY) [22] were recorded for mixing times of 80 and 140 ms, with the spin-lock field strength adjusted for a 90° pulse-length of approximately 100 μs. 2D nuclear Overhauser enhancement spectra (NOESY) [23] were recorded with mixing times of 50 and 100 ms. HMQC spectra were recorded at 57 °C with and without ^{13}C decoupling and at 70 °C, for determination of chemical shifts, over 600 t_1 increments of 2048 data points with 88 scans per increment. HMBC spectra were recorded with delays of 40, 50, 60 and 70 ms for evolution of long-range $^{13}C-^1H$ couplings. The spectra were processed with one level of zero-filling in F1 and Gaussian broadening of -10 Hz in each dimension and independently with a $\pi/2$ shifted sine bell in each dimension.

3. Results and discussion

Identification of constituent monosaccharides.—Some chemical characteristics of the exocellular polysaccharide produced by *Streptococcus thermophilus* OR 901 have been described previously [14]. It contains L-rhamnopyranose and D-galactopyranose as the monosaccharide constituents and its ^1H NMR spectrum has signals for 7 anomeric protons, designated **A–G** (Fig. 1). In addition, doublets (J 6.1 Hz) at δ 1.294 and δ 1.329 were consistent with their being two rhamnose residues. From this information and the chemical shifts of the anomeric protons [24,25], the polysaccharide repeating unit comprises two rhamnosyl and three α-galactosyl linkages (**A–E**), with two β-galactosyl linkages (**F**, **G**) displaying characteristic *trans* couplings. The ratio of rhamnose to galactose residues is lower than the 1:1.47 estimated from GC peak areas of an acid hydrolysate of the polysaccharide and reported in our previous paper [14]. As there is no reason to suspect inaccuracies in the NMR data obtained from the native polysaccharide, it appears that the chemical manipulations involved in the GC study lead to underestimation of the relative amount of galactose.

GC–MS and estimation of GC peak areas of the partially methylated alditol acetates prepared from the polysaccharide showed 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,3,4,5-tetra-*O*-acetyl-2-*O*-methylrhamnitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol, and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol with 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol in the molar ratio 1:1:1:1:3. Although four peaks were reported for the chromatogram in our previous paper [14], five alditol acetate peaks were obtained from the polysaccharide in this study. The

use of a 2% OV-17 column separated the second peak in the previously reported gas chromatogram into two peaks in the present work. Moreover, the mass spectrum of the fourth peak in the OV-17 chromatogram indicates the presence of 2- as well as 3-substituted galactose. The data showed that the heptasaccharide repeating unit consists of *O*-2 substituted rhamnose, non-reducing galactose, *O*-3,4 disubstituted rhamnose, *O*-6 substituted galactose, and three moles of *O*-2/*O*-3 substituted galactose, respectively. The *O*-3,4 disubstituted rhamnose residue is a branching point in the heptasaccharide unit.

Characterisation of hydrolysis products.—Two oligosaccharide spots, with R_{Gal} 0.61 and R_{Gal} 0.44, were detected on the paper chromatogram of the partial hydrolysate of the polysaccharide. The spot with R_{Gal} 0.61 corresponded to a single oligosaccharide, designated PO-1, but that with R_{Gal} 0.44 was further separated by Bio Gel P-2 chromatography (void volume 129 mL) into three oligosaccharide fractions, with elution volumes 216 mL (PO-2), 193 mL (PO-3) and 157 mL (PO-4).

PO-1 was isolated by preparative PC, purified by gel filtration on Bio Gel P-2 and characterised by ^{1}H NMR together with GC–MS of its partially methylated alditol acetates. Identification of the acetates, 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, together with NMR data for the anomeric protons ((δ 5.153 (J 4.0 Hz), δ 5.296 (J 3.5 Hz), δ 4.645 (J 8.1 Hz)) consistent with α-substitution by the non-reducing galactose, confirmed α-D-Gal*p*-(1 → 3)-D-Gal as the structure of PO-1.

PO-2 was shown to have the sequence Gal-(1 → 6)-Gal by characterisation of the alditol acetate derivatives 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylgalactitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol. Signals at δ 5.256 (J 3.7 Hz) and δ 4.594 (J 7.7 Hz) in the ^{1}H NMR spectrum were assigned to the α- and β-anomers of reducing galactose and further signals at δ 4.454 (J 7.7 Hz) and δ 4.445 (J 7.7 Hz), with the same ratio of intensities as for the α- and β-anomers of the reducing residue, to the anomeric proton of the non-reducing galactose residue, confirming β-D-Gal*p*-(1 → 6)-D-Gal as the structure of PO-2.

A ^{1}H NMR spectrum of the fraction identified as PO-3 indicated that it contained two oligosaccharides, a major saccharide (designated PO-3A) and minor saccharide (designated PO-3B). The partially methylated alditol acetates prepared from the PO-3 mixture contained 4-*O*-acetyl-1,2,3,5-tetra-*O*-methylrhamnitol, 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol. From comparison of their relative intensities with those of the low-frequency NMR signals (δ 1.342 (J 6.2 Hz), δ 1.357 (J 5.9 Hz)) attributed to the associated methyl groups, resonances at δ 5.102 and δ 4.896 could be assigned to the anomeric protons of reducing rhamnose residues (α and β, respectively) in the major saccharide. Similarly, resonances at δ 4.691 (J 8.1 Hz) and δ 4.465 (J 7.7 Hz) could be assigned to two β-Gal residues. The anomeric protons of the minor saccharide could in turn be assigned to a reducing galactose (δ 5.296 (J 4.0 Hz) and δ 4.646 (J 7.7 Hz) for the α and β protons, respectively) and two α-Gal residues (δ 5.199 (J 4.0 Hz) and δ 5.171 (J 3.7 Hz)). The

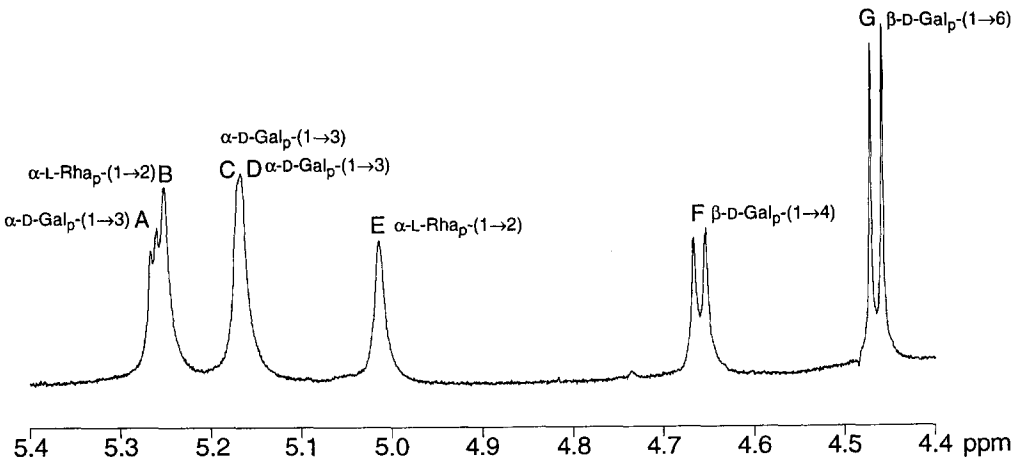

Fig. 1. Assignment of anomeric protons in the 600 MHz ^{1}H NMR spectrum of the polysaccharide (**1**) produced by *S. thermophilus* OR 901, recorded in ^2H_2O at 70 °C.

Table 1
NMR data for the exocellular polysaccharide produced by *S. thermophilus* OR 901

	Residue and chemical shifts (δ)						
	A	**B**	**C**	**D**	**E**	**F**	**G**
^{1}H Proton							
H-1	5.262	5.251	5.16(7)	5.16(5)	5.013	4.660	4.463
H-2	4.02	4.05	3.96	4.04	4.35	3.50	3.53
H-3	4.15	3.86	4.10	4.04	4.12	3.69	3.64
H-4	4.17	3.47	4.03	4.21	3.98	3.95	3.93
H-5		3.70	4.21	4.23	3.84	3.82	3.67
H-6,6′	3.70–3.77		3.70–3.77	3.70–3.77		3.90, 4.04	3.75–3.81
CH_3		1.294			1.329		
^{13}C Carbon							
C-1	95.7	102.6	97.8	98.4	104.4	105.4	105.8
C-2	69.3	81.3	76.5	69.3	67.8	73.4	73.4
C-3	77.5	72.6	71.9	77.6	75.6	75.4	75.4
C-4	68.8	74.7	72.4	68.8	78.1	71.6	71.3
C-5		71.8	73.5	72.9	70.7	76.3	77.7
C-6	63.5	19.4	63.5	63.5	19.8	71.3	63.5

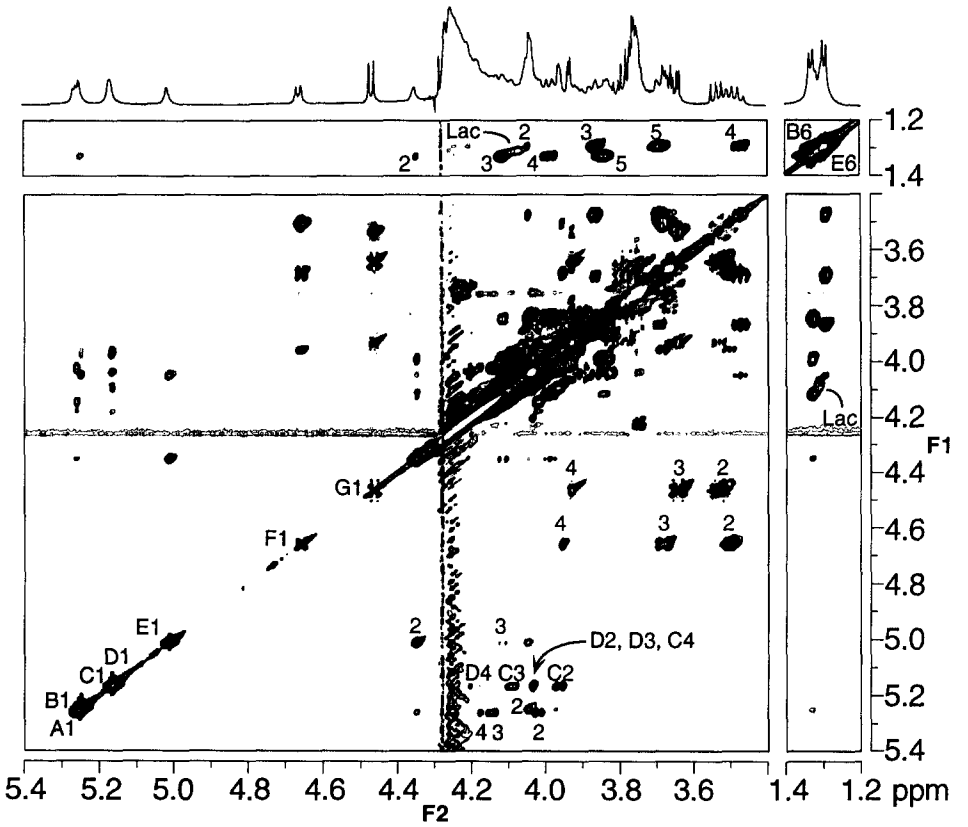

Fig. 2. 600 MHz ^{1}H TOCSY spectrum (mixing time 102 ms) of the polysaccharide (**1**) recorded in ^2H_2O at 70 °C. The spectrum is shown in phase-sensitive mode after processing with a $\pi/2$ shifted sine-bell in each dimension. Negative contours, identifying ROESY transfer effects, are shown in lighter shading. The projection is of the 1-dimensional spectrum of which part is shown in Fig. 1. Diagonal peaks and correlated protons are indicated for the anomeric protons, rhamnose methyl groups and lactate (Lac) using the assignments defined in Fig. 1.

analysis of partially methylated alditol acetates obtained from PO-3 had shown that the GC peak area of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol was larger than that of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, indicating that the 6-substituted galactose was associated with the major saccharide and the 3-substituted galactose with the minor saccharide. The major product (PO-3A) must therefore have the structure β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-L-Rha and the minor product (PO-3B) must be α-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-D-Gal.

The fraction PO-4 contained several tetrasaccharides for which no specific structures could be determined. However, it is apparent from the ^{1}H NMR spectrum and methylation analysis of the polysaccharide that the oligosaccharides PO-3A and PO-3B, together with an *O*-2 substituted rhamnose, constitute the elements of the polysaccharide repeating unit.

NMR studies of the polysaccharide.—NMR chemical shifts for the polysaccharide are summarised in Table 1. The resonances at δ 5.251 and δ 5.013 were assigned to the anomeric protons of rhamnose residues **B** and **E**, respectively, by correlation with their associated methyl groups through TOCSY ex-

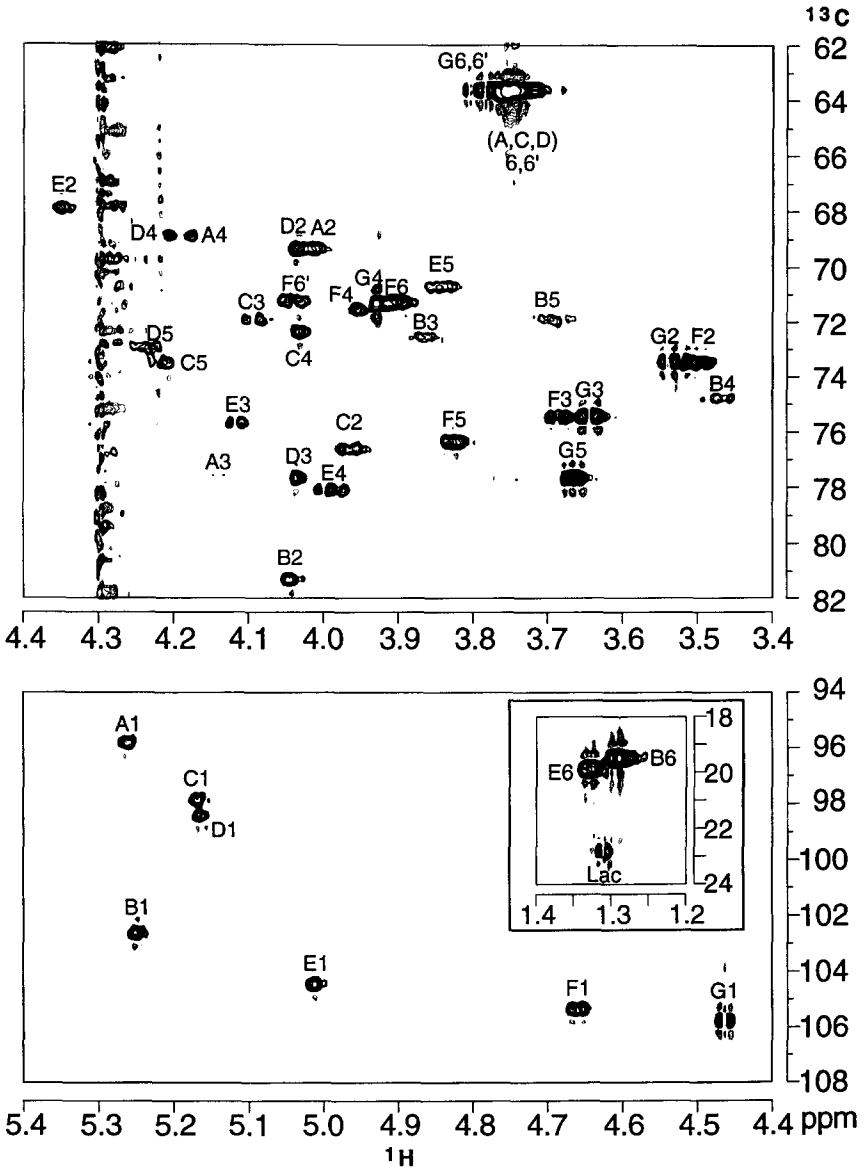

Fig. 3. 600 MHz HMQC spectrum of the polysaccharide (**1**) recorded in ^2H_2O at 70 °C. The spectrum is shown in phase-sensitive mode after processing with a $\pi/4$ shifted sine-squared function in each dimension, with negative contours due to negative sidelobes on relatively sharp resonances shown in lighter shading. Assignments, following the definitions of Fig. 1, plus lactate (Lac), are indicated next to each cross-peak. Note that relatively weak correlations such as A3 and the lactate methine (partially overlapped with F6′) were well-defined in less severely resolution-enhanced spectra.

periments (Fig. 2). The remaining high-frequency resonances, at δ 5.262 and δ 5.17, are therefore attributable to the anomeric protons of α-Gal residues, designated **A** and **C/D**, respectively. An HMQC experiment (Fig. 3) showed that each of the anomeric carbons had chemical shifts consistent with these assignments and also distinguished the α-Gal residues **C** and **D**. A further HMQC experiment, obtained without ^{13}C decoupling, revealed $^{1}J_{CH}$ values of 173–176 Hz for residues **A–E** and 161–163 Hz for **F** and **G**, confirming α and β substitution for the respective groups of monosaccharides [26].

Non-anomeric proton resonances for the rhamnose residues **B** and **E** and H-1–H-4 of the galactose residues **A**, **F** and **G** were sufficiently dispersed to be assigned directly from phase-sensitive COSY [27] and TOCSY spectra. The COSY experiment also provided the locations of H-2 and H-3 of the α-Gal residue **C** but the comparable two protons were apparently coincident in **D** as suggested by strongly distorted cross-peaks at the frequency of H-2 in both COSY and TOCSY spectra and the lack of an additional cross-peak in the latter. Assignments for H-4 of **C** and **D** were established from a TOCSY experiment obtained with a relatively short (51 ms) mixing time and processed with resolution enhancement in order to reduce the complexity of the spectrum near the diagonal. With the exception of a region at δ 4.03–4.04 in the ^{1}H spectrum, the ^{13}C assignments corresponding to the above identified protons were established directly from HMQC spectra in which characteristic multiplet structures observed in the TOCSY spectra permitted differentiation amongst partially overlapped resonances.

An HMBC experiment (Table 2) with a 70 ms delay for evolution of long-range couplings confirmed the above assignments for **G** and identified C-5 and C-6 of **G**, together with C-6 of the other β-Gal residue **F** to which it was known, from the characterised hydrolysis products, to be linked. The assignments for **F** and **G** were completed by reference to the COSY and short mixing time TOCSY spectra. Apart from correlations to the relatively mobile rhamnose methyl groups and H-6,6′ of some α-Gal residues, cross-peaks involving residues other than **G** in the HMBC experiment with a 70 ms delay were very weak. HMBC experiments with progressively shorter delays for evolution of long-range couplings revealed several additional cross-peaks and eliminated most of the remaining ambiguities in assignments. Thus, cross-peaks from H-1 of **C** and **D** enabled the assignment of their C-5 and H-5 resonances from the recognised [28] distinct coupling pathway between H-1 and C-5 in α-Gal residues, although the differentiation of assignments for **C** and **D** was tentative because of marginal resolution in the line-broadened HMBC spectra. However from the joint assignments, cross-peaks observed in the 70 ms HMBC experiment to the envelope of α-Gal H-6,6′ protons could be assigned to H-6,6′/C-5 for **C** and **D**. At the distinct ^{1}H frequency of one of these cross-peaks, a second cross-peak coincided with the ^{13}C frequency of one of the unassigned HMQC correlations at $\delta(^{1}H)$ 4.03. As the well-resolved C-4 of residue **D** had already been assigned, these correlations could be attributed to coupling with C-4 of residue **C**, i.e. H-6,6′/C-4 and H-4/C-4 in the HMBC and HMQC experiments, respectively. This, in turn, confirmed the differentiation of the C-5 resonances of residues **C** and **D**.

Table 2
Long-range $^{13}C-^{1}H$ correlations observed for the polysaccharide

Proton	Residue and identified ^{13}C correlations [a]						
	A α-Gal	**B** α-Rha	**C** α-Gal	**D** α-Gal	**E** α-Rha	**F** β-Gal	**G** β-Gal
H-1		B3, B5	C3, C5	D3/A3 [b], D5	B2, E3, E5	E4	G2, G3, F6
H-2			B1			F1, F3	G1, G3
H-3						F2	G2
H-4		B3, B5, B6	C2, C3		E6, F1	F2, F3	G2, G3
H-5						F4	G1, G4, G6
H-6,6′			C4, C5	D5		F5 [c]	G4, G5
CH_3		B4, B5			E4, E5		

[a] Each column corresponds to the proton resonances of specific residues; correlated carbon atoms are identified by a letter and a number.
[b] In principle alternative assignments but see text.
[c] To H-6 only.

G F 1

β-D-Gal$_p$-(1→6)-β-D-Gal$_p$-(1→4)

|

→2)-α-D-Gal$_p$-(1→3)-α-D-Gal$_p$-(1→3)-α-D-Gal$_p$-(1→3)-α-L-Rha$_p$-(1→2)-α-L-Rha$_p$-(1→

C D A E B

As the hydrolysis products had revealed two *O*-3 glycosylated α-Gal residues and only one had been identified, the remaining two peaks in the HMQC spectrum at $\delta(^1H)$ 4.04 could be attributed to C-3 (δ 77.6) and C-2 (δ 69.3) of residue **D** on the basis of the required glycosylation shift for C-3. Consistent with these assignments, the HMBC cross-peak from the anomeric proton of residue **D** to C-3 of either residue **D** or **A** is almost certainly due to intramolecular coupling because no other HMBC cross-peaks involving residue **A** were observed. This appears to be due to rapid relaxation [29] as even the HMQC cross-peak to C-3 (Fig. 3) was very weak unless acquisition times in both t_1 and t_2 were particularly short, while no HMQC cross-peak could be definitively assigned to C-5 due to limited digital resolution in spectra obtained with short acquisition times. Similarly, COSY and TOCSY spectra could not be adequately resolved to distinguish three α-Gal H-5 resonances. As there were no other cross-peaks to the H-6,6′ envelope in the homonuclear experiments, it seems reasonable to assume that the H-5 resonance of **A** is part of the envelope associated with the H-5 resonances of **C** and **D** but that assumption could not be independently verified.

From the HMBC experiments, interresidual cross-peaks between **G** (H-1) and **F** (C-6) and between **E** (H-4) and **F** (C-1) linked the constituent monosaccharides of the trisaccharide unit β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-L-Rha which had been identified as an hydrolysis product. Otherwise, HMBC cross-peaks (**E** (H-1) to **B** (C-2)) showed that the rhamnose residues were linked and (**C** (H-2) to **B** (C-1)) that the non-branched rhamnose **B** was attached to the α-Gal residue **C**. Taken together, these correlations showed that, since α-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-D-Gal had also been characterised as an hydrolysis product, the polysaccharide must contain the structural entity β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)- α-L-Rha*p*(**E**)-(1 → 2)-α-L-Rha*p*(**B**)-(1 → 2)-α-D-Gal*p*-(1 → 3)(**C**)-α-D-Gal*p*-(1 → 3)-D-Gal.

NOESY and ROESY experiments (Table 3) provided further support for each of the interglycosidic linkages characterised by HMBC cross-peaks. In particular, the strong NOE between the anomeric proton of **B** and H-2 of **C** confirmed the identified link to the α-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-D-Gal subunit, while a strong and clearly resolved NOE between the anomeric proton of **A** and H-2 of **E** located the other point of attachment. The polysaccharide therefore has the structure **1** in which the residue **G** forms the end of a more mobile branch.

The location of residue **D** follows from the identification of the linkages between the α-D-Gal*p*-(1 →

Table 3
Observed nuclear Overhauser enhancements for the polysaccharide

		Residue with observed enhancements [a]						
		A α-Gal	**B** α-Rha	**C** α-Gal	**D** α-Gal	**E** α-Rha	**F** β-Gal	**G** β-Gal
NOESY	Proton							
	H-1	A2, E2	B2, C2, E5 (E1, E3, E6)	C2 (D4)	(A3, B5)	B2 (B1, B3, E2)	E4, F3, F5 (A3, F2)	
	C/D, H-1 [b]			A4, D2/D3/C4 [c]				
	CH_3		B4, B5			E4, E5		
ROESY								
	H-1	A2, E2	B2, C2, E5 (C3)	C2 (D4)	(A3, B5)	B2 (E2)	E4, F3, F5 (A3, E3) (F2, F6)	G3, G5 (E6, F5, F6) (G2, G4)
	C/D, H-1 [b]			A4, D2/D3/C4 [c]				
	CH_3		B4, B5			E4, E5, G1		

[a] Relatively weak correlations are enclosed in parenthesis.
[b] Correlations not resolved between anomeric protons.
[c] Correlation to one or more of these protons.

3)-α-D-Gal *p*-(1 → 3)-D-Gal sub-unit at residues **A** and **C** with residues **E** and **B**, respectively, and was supported by NOEs which could be uniquely assigned by strong resolution enhancement of the time-domain data and by rational possible assignments for NOE cross-peaks which remained unresolved. Thus the confirmed NOE between **D** (H-1) and **A** (H-3) allows the unresolved cross-peak to **A** (H-4) to be assigned to NOE enhancement from **D** (H-1) since 3-glycosylated galactose residues commonly have NOEs to both H-3 and H-4 [30]. Similarly, a strong NOE from **C** (H-1) to **D** (H-3) should accompany the confirmed weak NOE to **D** (H-4) and overlap the expected strong intramolecular NOE (H-1 to H-2) for residue **D**. A model showed that the relatively weak 'long-range' NOEs [11,31] between the β-Gal residue **F** (H-1) and α-Gal **A** (H-3) and between **D** (H-1) and **B** (H-5) are consistent with conformations required to produce the strong direct enhancements. The relatively weak cross-peak at the H-1/H-2 frequencies of residue **F** is evidently due to spin diffusion as it was not observed in the NOESY experiment with the shorter mixing time.

NMR spectra of the isolated polysaccharide contained peaks assigned to acetate ($\delta(^1H)$ 1.889; $\delta(^{13}C)$ 25.9) and lactate ($\delta(^1H)$ 1.309, 4.06; $\delta(^{13}C)$ 22.8, 71.1); resonances corresponding to each carboxyl carbon were folded into HMBC spectra. The presence of these anions in the sample was confirmed by comparison of the chemical shifts noted above and reported in Table 1 with published data for acetate and lactate [32] and that for polysaccharides containing *O*-acetyl [33] or 1-carboxyethyl groups [34,35]. Furthermore, Hakomori methylation of a polysaccharide containing 4-*O*-(1-carboxyethyl)-D-glucose yielded an alditol acetate containing an acetoxypropyl group [36] but no comparable product was observed in the current work, while the relative intensity of the peak assigned here to acetate was very small (approximately one CH_3 group for every seven polysaccharide repeating units) but there was no evidence of partial acetylation [11,37] in the spectra. The apparent concentration of lactate (approximately one CH_3 group per polysaccharide repeating unit) was higher than that of acetate but the methyl resonance was relatively broad. The NMR visibility of small organic acids, especially lactate, is known to be significantly diminished by complexation with macromolecules in human blood plasma [38] and serum [39]; similar weak interactions with the polysaccharide may inhibit the release of lactate and acetate during dialysis and ion-exchange chromatography.

Discussion of the structure.—When compared with other *S. thermophilus* exopolysaccharides, the most distinctive feature of the polysaccharide characterised in this work is the presence of rhamnose as a component monosaccharide. The structure is significantly different to the exopolysaccharides produced by *S. thermophilus* and reported by Doco et al. [12] as → 3)-β-D-Gal *p*-(1 → 3)-[α-D-Gal *p*-(1 → 6)]-β-D-Glc *p*-(1 → 3)-α-D-Gal *p*NAc-(1 → and by Cerning et al. [13] as composed mainly of glucose and galactose. The observed structural differences can be attributed to differences in strains used by each group. Similar variations in exopolysaccharides produced by the same species have previously been noted for *Lactococcus lactis* subsp. *cremoris* [7,8] and *Lactobacillus helveticus* [4–6].

As with recent observations for several polysaccharides [11], the NMR studies, particularly the HMBC experiments, provided evidence of extensive differences in internal mobility within the polysaccharide repeating unit. Thus, few long-range ^{13}C–^1H couplings in carbohydrates are larger than 7.1 Hz [26], for which an HMBC experiment with a 70 ms delay for coupling evolution provides optimal transfer of magnetisation. However, as noted, only the most mobile β-Gal residue **G**, located at the terminus of the side-chain, and some internally mobile functional groups provided correlations at this long delay time. As the delay for coupling evolution was progressively reduced (and the efficiency of magnetisation transfer for couplings of 7.1 Hz or less correspondingly diminished), correlations were observed involving first the other side-chain β-Gal residue **F** and each rhamnose residue, and finally the α-Gal residues **C** and **D**. Mobility of the α-Gal residue **A** is evidently sufficiently constrained by its vicinal substitution [40] at the rhamnose linkage that no observable correlations were obtained in HMBC experiments. On the other hand, while all other residues showed strong correlations in conventional NOESY experiments, the segmental motion [29] of residue **G** was clearly unfavourable to NOESY transfer under the conditions used in this study. Given the considerable difference in mobility of various residues in the polysaccharide repeating unit, the acquisition and processing of spectra using a variety of experimental conditions, optimised for resonances of different line-widths, was very important to the ultimate elucidation of the structure.

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