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ARTICLE

Structural analysis of the exopolysaccharide produced by *Streptococcus thermophilus* ST1 solely by NMR spectroscopy

Elin Säwén · Eine Huttunen · Xue Zhang · Zhennai Yang · Göran Widmalm

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Abstract The use of lactic acid bacteria in fermentation of milk results in favorable physical and rheological properties due to in situ exopolysaccharide (EPS) production. The EPS from *S. thermophilus* ST1 produces highly viscous aqueous solutions and its structure has been investigated by NMR spectroscopy. Notably, all aspects of the elucidation of its primary structure including component analysis and absolute configuration of the constituent monosaccharides were carried out by NMR spectroscopy. An array of techniques was utilized including, inter alia, PANSY and NOESY-HSQC TILT experiments. The EPS is composed of hexasaccharide repeating units with the following structure: → 3)[α-D-Glc*p*-(1 → 4)]-*β*-D-Gal*p*-(1 → 4)-*β*-D-Glc*p*-(1 → 4)[*β*-D-Gal*f*-(1 → 6)]-*β*-D-Glc*p*-(1 → 6)-*β*-D-Glc*p*-(1 →, in which the residues in square brackets are terminal groups substituting backbone sugar residues that consequently are branch-points in the repeating unit of the polymer. Thus, the EPS consists of a backbone of four sugar residues with two terminal sugar residues making up two side-chains of the repeating unit. The molecular mass of the polymer was determined using translational diffusion experiments which resulted in M_w = 62 kDa, corresponding to 64 repeating units in the EPS.

Keywords Carbohydrates · Polysaccharide · Biopolymer · NMR spectroscopy · Absolute configuration · Dynamic light scattering

Abbreviations
EPS Exopolysaccharide
TILT Time-domain increments linked together

Introduction

Streptococcus thermophilus is a major dairy starter used mainly in the production of yogurt and some varieties of cheese (Delorme, 2008). Traditional yogurt production includes the fermentation of milk with a mix of thermophilic cultures consisting of strains of *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus.* However, the current yogurt industrial practice prefers the use of *S. thermophilus* alone, since it results in a mild flavor of the final product (Purwandari et al. 2007). In addition to flavor formation and the pH lowering effect, *S. thermophilus* plays a major role in the creation of yogurt and cheese texture through in situ exopolysaccharide (EPS) production. The EPS-producing starter cultures have been shown to improve the texture and sensory characteristics of yogurt (mouthfeel, shininess, clean cut, ropiness and creaminess) (Purwandari et al. 2007; Folkenberg et al. 2006). The use of an EPS-producing *S. thermophilus* strain improved the functional properties of low-fat or part-skim Mozzarella cheese by increasing the moisture content and improving

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E. Säwén · G. Widmalm (✉)
Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, 106 91 Stockholm, Sweden
e-mail: gw@organ.su.se

E. Huttunen · X. Zhang
Department of Food Technology, University of Helsinki, 00014 Helsinki, Finland

Z. Yang
Center of Agro-food Technology, Northeast Agricultural Research Center of China, 130124 Changchun, Jilin, People's Republic of China

the melting properties of the product (Broadbent et al. 2003). However, the final textural characteristics of yogurts are strongly dependent on physical and structural properties of the EPS, such as type (capsular or ropy), degree of ropiness, sugar composition and degree of branching (Purwandari et al. 2007).

Compositional and structural analyses of the EPSs produced by lactic acid bacteria (LAB) revealed a large variation in chemical composition, monomer ratio, and molecular structure of the repeating unit depending on the particular strain considered. In addition, the LAB EPSs differed in the attachment site of the side-chain, as well as the monosaccharides of the side-chain. The EPSs of *S. thermophilus* are neutral or polyanionic heteromers primarily composed of D-galactose, D-glucose, and L-rhamnose, but also *N*-acetyl-D-galactosamine, acetylated D-galactose, D-ribose, and a nononic acid were discovered in these molecules.

The first structural characterization of an EPS was that produced by *S. thermophilus* strains (CNCMI 733, CNCMI 734 and CNCMI 735), and it was found to be composed of tetrasaccharide repeating units of D-galactose, D-glucose, and *N*-acetyl-D-galactosamine (Doco et al. 1990). EPSs with identical repeating unit structure to that of the CNCMI strains have later been shown to be produced by some other *S. thermophilus* strains: Sfi6 (Stingele et al. 1996), IMDO 01, IMDO 02, IMDO 03, NCFB 859 and 21 (Marshall et al. 2001a), SFi20 (Navarini et al. 2001) and LY03 (Degeest et al. 2001). Another EPS with a tetrasaccharide repeating unit of D-galactose and D-glucose was produced by the strains SFi39 (Lemoine et al. 1997), SY89, SY102 (Marshall et al. 2001a), STD and CH101 (De Vuyst et al. 2003). Strains SFi12 (Lemoine et al. 1997) and S3 (Faber et al. 2001) produced EPSs with hexasaccharide repeating units, the former containing D-galactose, D-glucose, and L-rhamnose, and the latter D-galactose, L-rhamnose and acetylated D-galactose. Furthermore, three different kinds of heptasaccharide repeating units of the EPSs were indicated: (1) a heptasaccharide repeating unit of D-glucose, D-galactose and L-rhamnose produced by the strain EU20 (Marshall et al. 2001b), (2) a branched heptasaccharide repeating unit of D-galactose and L-rhamnose with a disaccharide side-chain produced by the strains OR901 (Bubb et al. 1997), Rs and Sts (Faber et al. 1998) and ST 111 (Vaningelgem et al. 2004), and (3) a complex heptasaccharide repeating unit of D-glucose, D-galactose, D-ribose, *N*-acetyl- D-galactosamine, and an open chain nononic acid produced by the strain 8S (Faber et al. 2002a; Faber et al. 2002b). Not only does the repeating unit structure of *S. thermophilus* EPS vary considerably, but also the variability in the molecular mass of structurally identical polymers has been reported (Faber et al. 1998).

Previously we presented a branched pentasaccharide repeating unit of D-galactose and D-glucose with a trisaccharide side-chain of the EPS produced by *S. thermophilus* THS (Nordmark et al. 2005). The high viscosity of this EPS was possibly explained by the particular side-chain composition of the polymer. In the present study, *S. thermophilus* strain ST1 was found to produce a viscous EPS when grown in skim milk. In order to further understand the viscosity properties of the EPS of strain ST1, the polysaccharide was studied structurally by NMR spectroscopy, showing a new EPS structure with a hexasaccharide repeating unit of D-galactose and D-glucose. The branching pattern present in the repeating unit of the EPS may also in this case contribute to the high viscosity of the skim milk culture. The determination of the primary structure of a polysaccharide includes analysis of sugar components, their absolute configuration, ring-size of monosaccharides, their anomeric configuration, sequence of sugar residues and substituents and their location if present. In particular, all aspects of the structural analysis of the ST1 EPS were carried out by NMR spectroscopy. In addition, the molecular mass of the polymer determined by NMR translational diffusion measurements was complemented with a determination based on dynamic light scattering.

Materials and methods

Growth of the organism

Streptococcus thermophilus ST1, a strain from the Center of Agro-food Technology, Northeast Agricultural Research Center of China (NARCC, Changchun, China), was used as the EPS-producing strain throughout this study. The ST1 strain culture was prepared by inoculating a loopful of freeze-drying culture into 5 mL reconstituted skim milk powder at 11% (w/v, Valio Ltd., Finland) and incubated at 42°C for 16 h. For exopolysaccharide isolation, the strain ST1 was propagated twice, in 100 and 500 mL skim milk, at 42°C for 8 h with a 3% inoculum.

Isolation and purification of the exopolysaccharide

The fermentation broth (490 mL) was stirred in 4% (w/v) trichloroacetic acid (Merck, Darmstadt, Germany) for 2 h at room temperature, and then left at 4°C overnight. Cells and precipitated proteins were removed by centrifugation at 10,000 rpm for 45 min (4°C). The supernatant was filtered through an AcroCap filter (0.45 μm, Pall Gelman, USA) and one volume of cold absolute ethanol was added gradually with a pear-shaped funnel in order to precipitate EPS (4 h, 4°C). The supernatant and the precipitating EPS

were kept at 4°C overnight. After centrifugation (10,000 rpm, 45 min, 4°C), the EPS precipitates were washed with 50% ethanol 3 times and dissolved in distilled water. The aqueous solution of the EPS was again filtered through an AcroCap filter (0.45 μm) and lyohpilized on a DURA-Dry freeze-dryer (FTS Systems Inc., Stone Ridge, NY, USA). Part of the material (10 mg) was dissolved and transferred into a cellulose dialysis membrane with a nominal MWCO of 12,000–14,000 (Cellu Sep T3, Membrane Filtration Products Inc., San Antonio, TX, USA) and dialyzed in 5 L of distilled water for 2 days at 4°C, water being changed twice a day. After dialysis the EPS was used for structural analysis.

The uniformity of the EPS material was further checked by size exclusion chromatography using a column (75 × 1.5 cm) of Bio-Gel P-10 polyacrylamide gel (exlusion limit 20 kDa, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA, USA). A sample (1.5 mg) was loaded onto the column and eluted with 0.05 M NH_4OAc (Riedel-de Haën AG, Seelze, Germany) with UV monitoring at 280 nm (Econo UV monitor, Model EM1, Richmond, CA, USA). The presence of sugar in the fractions was tested qualitatively with a Molish test (Miller and Neuzil 1982).

Absolute configuration determination

The EPS (0.7 mg) was hydrolyzed with 200 μL of 2 M trifluoroacetic acid at 120°C for 90 min and the solvent was evaporated under a stream of dry air. The hydrolyzed EPS and 2 mg each of the reference samples (D-Glc, L-Glc, D-Gal and L-Gal) were treated with (*S*)-(+)-2-methylbutyric anhydride (Sigma-Aldrich, Inc.) (100 μL) and pyridine (100 μL) and were incubated at 120°C for 4 h. The reagents were evaporated under a stream of dry air and toluene was added and evaporated three times. The samples were dissolved in CH_2Cl_2 (1 mL) and were extracted twice with aqueous 2 M Na_2CO_3 (2 mL) followed by water (2 mL). The organic phase was concentrated with a stream of dry air and dried under reduced pressure. The samples were dissolved in acetone-d_6.

^{1}H NMR spectra of the (*S*)-(+)-2-methylbutyryl (SMB) derivatives were recorded at 25°C (York et al. 1997). Chemical shifts were measured relative to internal acetone-d_5 (δ 2.050). 1D spectra were recorded with 64 k data points over a spectral width of 10 ppm at 700 MHz. The spectra were recorded with 64 scans and a repetition time of 6.7 s was used. Zero-filling to 128 k points and an exponential weighting function was applied prior to Fourier transformation for all samples; for the reference samples a line-broadening of 0.3 Hz was used and for the hydrolyzed EPS sample a line-broadening of 0.5 Hz was applied.

NMR spectroscopy

^{1}H and ^{13}C chemical shift assignments of the EPS from ST1 were performed in D_2O (4 mg in 0.55 mL) at 25°C on three different spectrometers: a Varian INOVA 600 MHz spectrometer equipped with a 5 mm PFG triple resonance probe, a Varian INOVA 900 MHz spectrometer equipped with a 5 mm Cold Probe and a Bruker AVANCE III 700 MHz spectrometer with dual receivers equipped with a 5 mm TCI Z-Gradient CryoProbe. ^{1}H chemical shifts were referenced to internal TSP (δ 0.00) and ^{13}C chemical shifts were referenced to external dioxane in D_2O (δ 67.40). The chemical shifts were compared to those of the corresponding monosaccharides (Jansson et al. 1989; Baumann et al. 1992).

^{1}H NMR assignments were performed using ^{1}H,^{1}H-TOCSY experiments recorded at 700 MHz over 5 ppm with 2,048 × 256 points in F2 × F1, respectively, and 16 scans per t_1-increment using the States-TPPI method. An MLEV-17 spin-lock ($\gamma B_1/2\pi = 9.6$ kHz) was used with five different mixing times, viz., 10, 30, 60, 90 and 110 ms. Zero-filling was performed to 4,096 × 4,096 points. Prior to Fourier transformation a 90° shifted squared sine-bell function was applied in both dimensions.

A PANSY experiment (Kupče et al. 2006) in which a ^{1}H,^{1}H-TOCSY experiment with 1.5 s presaturation was performed with a 120 ms DIPSI spin-lock ($\gamma B_1/2\pi =$ 10 kHz) during which a ^{13}C experiment was recorded on a parallel receiver. The ^{1}H,^{1}H-TOCSY experiment was recorded with the States-TPPI method over 4 ppm with 2,048 × 64 points in F2 × F1, respectively; eight scans per t_1-increment were used. Prior to Fourier transformation zero-filling was carried out to 8,192 × 4,096 points and a shifted squared sine-bell function was applied in both dimensions. The ^{13}C experiment was recorded during the 120 ms mixing time over a region of 200 ppm with 8,192 points and was zero-filled to 131,072 points; forward linear prediction using all points was applied. An exponential window function with a line broadening factor of 9 Hz was applied.

A ^{1}H,^{13}C-H2BC experiment (Petersen et al. 2006) was recorded at 700 MHz with a spectral width of 8 ppm in the direct dimension and 80 ppm in the indirect dimension with 1,024 × 512 points and 128 scans per t_1-increment using the echo/antiecho method. A constant time delay of 22 ms was employed for $^3J_{H,H}$ evolution. The third-order low-pass J-filter was set for $140 < {}^1J_{C,H} < 170$ Hz. Zero-filling to 2,048 × 2,048 points was performed. A 90° shifted squared sine-bell function was applied in F2 and a 60° shifted squared sine-bell function was applied in F1 prior to Fourier transformation. The ^{1}H,^{13}C-H2BC spectrum was processed in magnitude mode.

^{13}C NMR chemical shifts were assigned using a ^{1}H,^{13}C-HSQC experiment recorded with the ^{1}H,^{1}H NOESY-^{1}H,^{13}C HSQC-TILT (Time-domain increments linked together) pulse sequence at 900 MHz. A spectral width of 4.4 ppm was used for ^{1}H and a 70 ppm spectral width was used for ^{13}C. The experiment was recorded with 650 × 128 points and 8 scans per t_1-increment. Zero-filling to 2,048 × 2,048 was performed and a Gaussian function was applied prior to Fourier transformation.

To assign multiplicities for the signals a multiplicity-edited ^{1}H,^{13}C-HSQC experiment was recorded at 700 MHz with 1,024 × 256 points and 128 scans per t_1 increment over a spectral region of 10 ppm for ^{1}H and 80 ppm for ^{13}C employing the echo/antiecho method. Adiabatic pulses were used for inversion in F2. Prior to Fourier transformation zero-filling to 2,048 × 2,048 points was performed and 90° shifted squared sine-bell functions were applied in both dimensions.

For inter-residue correlations ^{1}H,^{13}C-HMBC and ^{1}H,^{1}H-NOESY experiments were used. The gradient selected ^{1}H,^{13}C-HMBC experiment was recorded at 700 MHz, and processed in magnitude mode. A spectral region of 10 ppm in the direct dimension and 80 ppm in the indirect dimension with 4,096 × 256 points and 128 scans per t_1-increment was used. A 65 ms evolution time was used. Zero-filling was performed to 16,384 × 4,096 points and sine-bell functions were applied prior to Fourier transformation in both dimensions. The ^{1}H,^{1}H-NOESY experiment was recorded at 600 MHz with a spectral region of 2.5 ppm and 1,746 × 128 points and 32 scans per t_1-increment. A mixing time of 100 ms was used; during the mixing time a zero-quantum filter was applied with a 50 ms simultaneous 180° adiabatic WURST-pulse swept over 14 kHz and a gradient pulse of 1.8 G s^{-1}. Prior to Fourier transformation zero-filling was performed to 2,048 × 2,048 points and a 90° shifted squared sine-bell function was applied in both dimensions.

To resolve ambiguities due to spectral overlap in the 2D ^{1}H,^{1}H-NOESY spectrum, tilted projections of a three-dimensional gradient-enhanced ^{1}H,^{1}H-NOESY-^{1}H,^{13}C-HSQC spectrum (Kupče and Freeman 2005; Kupče et al. 2005) were recorded at 900 MHz with tilt angles $\alpha = +15$ and $\alpha = -15°$. Spectral regions of 4.4 ppm in F2, 70 ppm in F1 and 16,000 Hz in the mixed plane F* were used and the experiment was recorded with 650 × 128 points in F2 × F1, respectively; 54 scans per increment was recorded. A 55 ms mixing time was used in the NOESY part. Prior to Fourier transformation zero-filling was performed to 2,048 × 2,048 points and a 90° shifted squared sine-bell function was applied in both dimensions.

The reference in the tilted projections was set from calculated frequencies according to Eqs. 1 and 2, where f_H is the ^{1}H frequency in Hertz from a 1D ^{1}H-spectrum and f_C is the ^{13}C frequency in Hertz from the pure HSQC experiment.

$$f(+) = f_H \cos\alpha + f_C \sin\alpha \quad (1)$$

$$f(-) = f_H \cos\alpha - f_C \sin\alpha \quad (2)$$

The pure frequencies f_H and f_C were derived from measurements made in the positively tilted plane $f(+)$ and the negatively tilted plane $f(-)$ according to Eqs. 3 and 4.

$$f_H = [f(+) + f(-)]/2\cos\alpha \quad (3)$$

$$f_C = [f(+) - f(-)]/2\sin|\alpha| \quad (4)$$

Translational diffusion measurements were made at 25°C with a sample concentration of 0.6 mg/mL. The experiment was repeated 5 times. A pulsed field gradient spin-echo experiment (PFG-LED) was used with a fixed diffusion time and PFGs increasing linearly over 32 steps (Damberg et al. 2001). The pulsed field gradients were calibrated using a doped water sample (1% H_2O in D_2O + 1 mg/mL $GdCl_3$) and a literature value of $D_t = 1.90\times10^{-9}$ m^2 s^{-1} for the HDO diffusion coefficient in D_2O at 25°C (Mills 1973). The molecular mass was calculated from the following relationship (Viel et al. 2003).

$$D_t = 8.2 \times 10^{-9} M_W^{-0.49} \quad (5)$$

The measured diffusion coefficients were corrected with a factor of 1.053 for using 27°C in the calculations.

Dynamic light scattering

Dynamic light scattering measurements of the hydrodynamic radius were performed at 25°C on an ALV/CGS-3 instrument with a detection angle of 150° using a filtered sample of the ST1 EPS in D_2O with a concentration of 0.6 mg/mL. Ten measurements were performed for 30 s each and presented as autocorrelation functions. The data were processed with a distribution function fit from the manufacturer's software. The translational diffusion was calculated with the Stokes–Einstein relationship and the molecular mass was calculated as described above for NMR translational diffusion measurements.

Results and discussion

The exopolysaccharide produced by *Streptococcus thermophilus* ST1 in reconstituted skimmed milk was isolated and purified by 50% ethanol precipitation followed by dialysis in water. The uniformity of the EPS sample ($M_w > 20$ kDa) was confirmed by size exclusion chromatography. The preparation of the EPS in D_2O for NMR

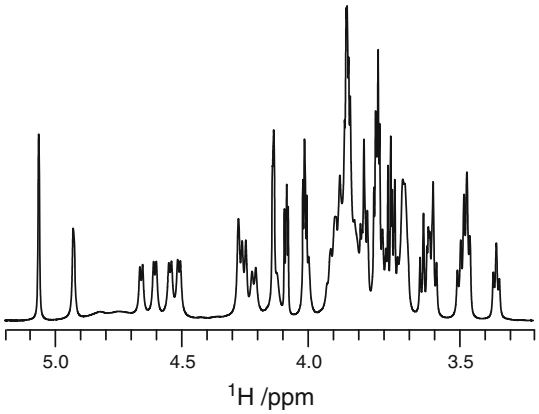

Fig. 1 ^{1}H NMR spectrum at 700 MHz of the EPS from ST1 in D_2O with presaturation of the HDO resonance

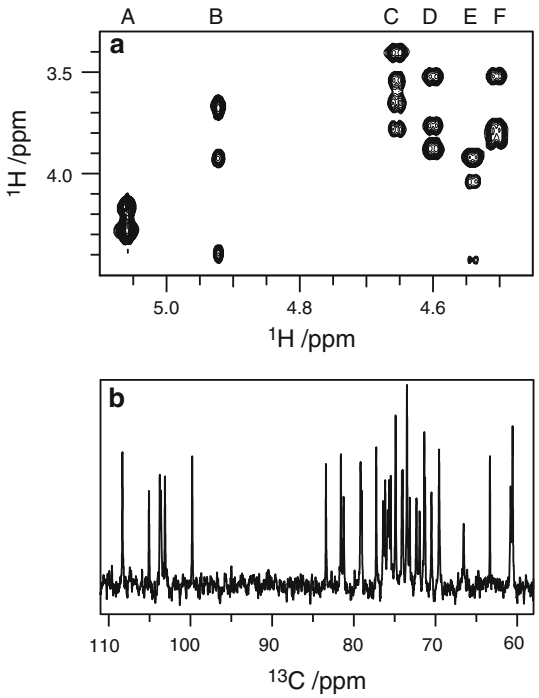

Fig. 2 Spectra resulting from a PANSY NMR experiment that utilizes dual receivers. A ^{1}H,^{1}H-TOCSY experiment carried out with a 120 ms isotropic mixing time (**a**). During the spin-lock a separate ^{13}C experiment with proton decoupling was acquired (**b**)

analysis gave a highly viscous sample. However, the quality of the NMR spectra at 25°C was still very high which facilitated further analysis and structural studies of the EPS. The ^{1}H NMR spectrum of the EPS (Fig. 1) showed resonances between 3.3 and 5.1 ppm. There are six resonances in the region for anomeric protons, in the present case between 4.5 and 5.1 ppm; the remaining resonances are all present within <1 ppm emphasizing the well-known fact that carbohydrates are notorious for their limited spectral dispersion.

The next step in the structural analysis employed a PANSY NMR experiment (Kupče et al. 2006) in which a ^{1}H,^{1}H-TOCSY experiment was carried out in parallel with the acquisition of the FID from a 1D ^{13}C NMR experiment. The isotropic mixing time in the TOCSY experiment was 120 ms, sufficiently long to identify most ^{1}H,^{1}H correlations for a monosaccharide. Six spin-systems originating from the anomeric protons were present in the EPS (Fig. 2a). Resonances from the different sugar residues are denoted by **A**–**F** starting from the anomeric proton having the highest chemical shift. The acquisition of a 1D ^{13}C NMR spectrum is essential, albeit not obligatory, in structural analysis of polysaccharides since non proton-bearing carbons may be present at quite different ^{13}C chemical shifts. In the present case the ^{13}C resonances were only observed in the spectral region 60–110 ppm (Fig. 2b). These results indicate that the EPS is built of repeating units consisting of six sugar residues, i.e., hexasaccharide repeating units.

A ^{1}H,^{13}C-HSQC NMR experiment in conjunction with its multiplicity-edited variant confirmed that six cross-peaks were present in the anomeric region (Fig. 3a) and that the number of cross-peaks in the region for methene and methylene groups (Fig. 3b) indicated that the sugars were six-carbon residues, i.e., hexoses. The four resonances between 4.5 and 4.7 ppm from anomeric protons all had large $^3J_{H1,H2}$ coupling constants of 7.5 Hz indicative an anti-periplanar arrangement between the H1 and H2 protons in these sugar residues, as e.g. in β-linked *gluco*- or *galacto*-pyranosides but not in α- or β-linked *manno*-pyranosides. The residues having their anomeric protons at δ_H 4.92 and 5.06 were both unresolved but the width at half peak-height indicated that their values were smaller and much smaller, respectively.

In structural analysis of glycans the determination of constituent sugar components is usually carried out by chromatographic methods. In addition, the absolute configuration of the sugar residues, i.e. the D- or L-configuration, is subsequently determined in a separate procedure, also employing chromatographic methods, by separation of diastereomers. In the present study we determine the sugar components, their absolute configuration and their relative proportions of the ST1 EPS by ^{1}H NMR spectroscopy using SMB, (*S*)-(+)-2-methylbutyryl, derivatives of the constituent sugars as devised by York et al. (1997). The ST1 EPS was hydrolyzed and peracylated using SMB anhydride. After workup the derivatized material was dissolved in acetone-d_6 for ^{1}H NMR analysis. Derivatized D- and L-sugars of monosaccharides which have different ^{1}H NMR chemical shifts (since they are diastereomers) were prepared as reference compounds. ^{1}H resonances in the spectral region 6.0–6.5 ppm were indicative of the sugars present and their absolute configurations. The ^{1}H NMR spectrum of the material from the ST1 EPS (Fig. 4a)

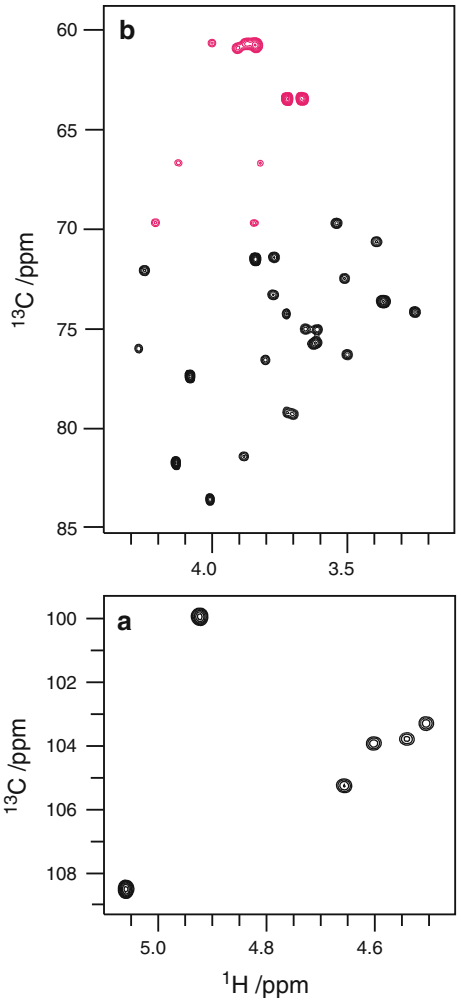

Fig. 3 ^{1}H,^{13}C-HSQC NMR spectrum of the EPS from ST1 showing (**a**) the anomeric region and (**b**) the region for ring and hydroxymethyl atoms. Correlations for methine groups ($\delta_C > 69.7$) are shown in *black* and for methylene groups ($\delta_C < 69.7$) in *red* (spectrum edited by information from a multiplicity-edited experiment)

showed a mixture of compounds that were identified by comparison to the reference ^{1}H spectra of derivatized D-galactose (Fig. 4b) and of D-glucose (Fig. 4c). ^{1}H spectra of the L-galactose and of L-glucose derivatives showed chemical shift differences to that of the ST1 EPS derivatized material. Integration of ^{1}H resonances from D-glucose vs. D-galactose between 6.0 and 6.5 ppm showed a relative ratio of 3.7:2. Thus, this NMR analysis demonstrates that D-glucose and D-galactose are constituents of the ST1 EPS and indicate that they are present in a relative ratio ~4:2.

The ^{1}H and ^{13}C NMR chemical shifts of the six sugar residues denoted **A**–**F** (vide supra) were assigned using a number of different 2D NMR experiments. A series of ^{1}H,^{1}H-TOCSY experiments were utilized with increasing mixing times from 10 to 110 ms giving information on the spin-systems of sugar residues. All of the anticipated one-bond ^{1}H,^{13}C-correlations were identified in the proton-

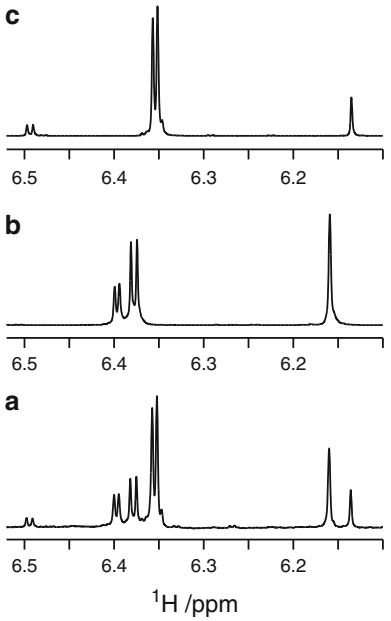

Fig. 4 Component analysis of the EPS from ST1 by ^{1}H NMR showing **a** EPS-SMB hydrolysate, **b** D-galactose-SMB, and **c** D-glucose-SMB

decoupled ^{1}H,^{13}C-HSQC-spectrum (Fig. 3), i.e., 42 from the six hexoses. Methylene and methine groups were differentiated by a multiplicity-edited HSQC-experiment revealing that the methylene groups had ^{13}C chemical shifts <69.7 ppm whereas for the methine groups $\delta_C > 69.7$ (cf. Fig. 3). The assignment process was aided by a ^{1}H,^{13}C-H2BC experiment in which two-bond ^{1}H,^{13}C-correlations are observed and by a ^{1}H,^{13}C-HMBC experiment where $^{2}J_{C,H}$ and $^{3}J_{C,H}$ mediated correlations may be observed (Fig. 5). Resonance overlap and small $^{3}J_{H,H}$ coupling constants, e.g. in *galacto*-pyranose $J_{H4,H5} < 1$ Hz, hamper the resonance assignments. Therefore, a 2D NOESY-HSQC TILT NMR experiment (Kupče et al. 2005) was performed in which ^{1}H,^{1}H-correlations are observed via through-space interactions but ^{13}C frequency-labeled using a tilt angle $\alpha = \pm 15°$ (Fig. 6). The 3D NOESY-HSQC spectrum where the F_1 and F_3 dimensions correspond to ^{1}H frequencies and the F_2 dimension that corresponds to ^{13}C is projected onto a tilted plane F*/F_3 which allows overlapping cross-peaks to be resolved. Subsequently, the pure proton and carbon frequencies can be obtained by trigonometric functions (vide supra) and consequently the corresponding chemical shifts of the cross-peaks can be derived. For example, the cross-peak from H1 in residue **B** to H4 in residue **E** (cf. Fig. 6) has the same carbon-13 frequency as C1 in **B**. In an analogous way the cross-peak from H1 in **C** to H3 in **E** is ^{13}C-labeled by C1 in **C**. The TILT experiment, which is an order of magnitude faster than the 3D NOESY-HSQC experiment, resolved the remaining ^{1}H and ^{13}C chemical shift assignments for the

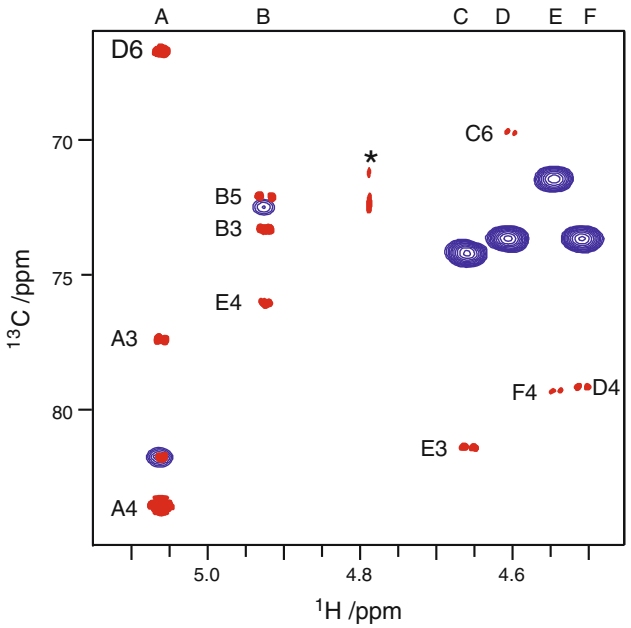

Fig. 5 Overlay of a ^{1}H,^{13}C-H2BC spectrum (*blue*) and a ^{1}H,^{13}C-HMBC spectrum (*red*) of the EPS from ST1. *Cross-peaks* from the latter experiment are annotated showing, *inter alia*, interresidue $^{3}J_{C,H}$ correlations. The asterisk denotes artifacts from the HDO resonance

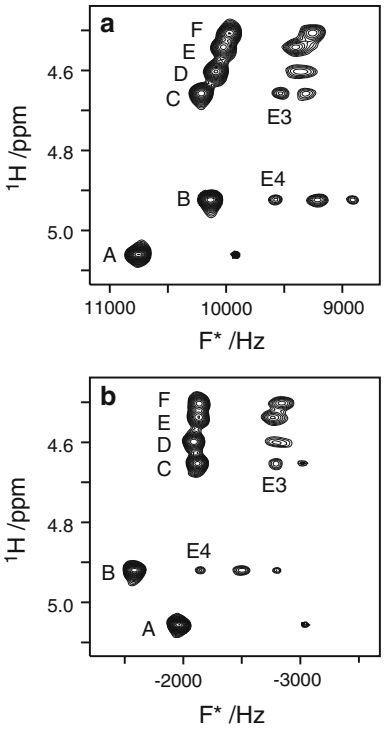

Fig. 6 A 2D NOESY-HSQC TILT NMR experiment of the EPS from ST1 with a tilt angle of $\alpha = +15°$ (*top*) and $\alpha = -15°$ (*bottom*)

ST1 EPS. The ^{1}H and ^{13}C chemical shifts of the six sugar residues are given in Table 1.

The assignments of sugars (D-glucose and D-galactose) to **A**–**F** were performed based in particular on ^{1}H and ^{13}C NMR chemical shifts. Of the β-linked hexopyranosides, having $^{1}J_{C1,H1}$ values of 162 or 163 Hz, residues **C**, **D** and **F** correspond to D-glucose whereas residue **E** stems from D-galactose. Residue **B** corresponds to a D-glucose residue that is α-linked since $^{1}J_{C1,H1} = 172$ Hz (Bundle and Lemieux 1976). That residue **A** has a furanose ring-form is evident from its ^{13}C chemical shifts of the anomeric carbon C1 as well as C4 (Gorin and Mazurek 1975; Ritchie et al. 1975; Linnerborg et al. 1997). This conclusion was corroborated by a three-bond correlation observed in the ^{1}H,^{13}C-HMBC spectrum between H1 and C4 in residue **A** (Fig. 5). The $^{1}J_{C1,H1}$ coupling constant does not readily distinguish the α- from the β-anomeric configuration. Additionally, the $^{3}J_{H1,H2}$ coupling constant is not resolved but the width at half peak-height indicates a small value. β-Linked D-galactose in a furanoid ring form, i.e. β-D-Gal*f*, is present in many bacterial polysaccharides, whereas β-D-Glc*f* has only been reported once, to the best of our knowledge, as a constituent in a lipopolysaccharide from *Erwinia amylovora* T which also contained an α-D-Gal*p* residue in the repeating unit (Ray et al. 1987). A comparison of the ^{13}C chemical shifts of residue **A** to those of the methyl glycosides of β-D-Glc*f* and β-D-Gal*f* (Beier et al. 1980) indicates that the deviation, based on the chemical shifts from C2 to C6, is larger for the former, $\approx$1.2 ppm/resonance, than for the latter, $\approx$0.6 ppm/resonance. Therefore, residue **A** is assigned to β-D-Gal*f*, resulting in two galactose and four glucose residues in the repeating unit of the EPS, consistent with results from the component analysis (vide supra).

^{13}C NMR glycosylation shifts, i.e., the difference in chemical shifts to those of the corresponding monosaccharide, are highly indicative of the substitution position(s) in an oligo- or polysaccharide (Söderman et al. 1998); they have a magnitude of 5–10 ppm and are shifted to a higher chemical shift. The resonances from the anomeric carbons are shifted by 6.2–8.5 ppm in the ST1 EPS. The substituted carbons show glycosylation shifts in the range 4.8–8.6 ppm. Small positive and negative glycosylation shifts will also be present at non-substituted positions. Thus, residues **C** and **F** are 6- and 4-substituted, respectively, residues **D** and **E** are 4,6- and 3,4-disubstituted, respectively, which necessitates branching at these residues and consequently residues **A** and **B** are terminal groups. The sugar residues, ring forms, substitution patterns and their ^{1}H and ^{13}C chemical shifts of the ST1 EPS are summarized in Table 1.

The sequence of sugar residues making up the repeating unit of the EPS were determined by ^{1}H,^{13}C-HMBC and ^{1}H,^{1}H-NOESY experiments and the correlations are compiled in Table 2 (cf. also Figs. 5 and 6). From this information the primary structure of the hexasaccharide

Table 1 ^{1}H and ^{13}C chemical shifts (ppm) at 25°C of the EPS from ST1[a]

Sugar residue		1	2	3	4	5	6
β-D-Gal*f*-(1→	**A**	5.06 [4.6][b]	4.13	4.08	4.01	3.84	3.67, 3.72
		(−0.16)[c]	(0.11)	(−0.02)	(−0.04)	(0.03)	
		108.45 {175}	81.75	77.40	83.60	71.53	63.49
		(6.25)	(−0.61)	(0.46)	(0.42)	(−0.16)	(−0.16)
α-D-Glc*p*-(1→	**B**	4.92 [8.3][b]	3.51	3.77	3.54	4.25	3.85, 3.85
		(−0.31)	(−0.03)	(0.05)	(0.12)	(0.41)	
		99.96 {172}	72.49	73.32	69.71	72.10	60.69
		(6.97)	(0.02)	(−0.46)	(−1.00)	(−0.27)	(−1.15)
→6)-β-D-Glc*p*-(1→	**C**	4.66 [7.5]	3.25	3.50	3.39	3.62	3.84, 4.21
		(−0.01)	(0.00)	(0.00)	(−0.03)	(0.16)	
		105.28 {163}	74.17	76.30	70.65	75.83	69.66
		(8.44)	(−1.03)	(−0.46)	(−0.06)	(−0.93)	(7.82)
→4,6)-β-D-Glc*p*-(1→	**D**	4.60 [7.5]	3.37	3.61	3.72	3.73	3.82, 4.12
		(−0.07)	(0.12)	(0.11)	(0.30)	(0.27)	
		103.94 {163}	73.65	75.06	79.15	74.27	66.68
		(7.10)	(−1.55)	(−1.70)	(8.44)	(−2.49)	(4.84)
→3,4)-β-D-Gal*p*-(1→	**E**	4.54 [7.5]	3.77	3.88	4.27	3.80	3.84, 3.91
		(0.01)	(0.32)	(0.29)	(0.38)	(0.15)	
		103.79 {162}	71.44	81.40	76.01	76.60	60.98
		(6.42)	(−1.52)	(7.62)	(6.32)	(0.67)	(−0.86)
→4)-β-D-Glc*p*-(1→	**F**	4.51 [7.5]	3.37	3.65	3.69	3.61	3.85, 4.00
		(−0.16)	(0.12)	(0.15)	(0.27)	(0.15)	
		103.31 {162}	73.65	75.03	79.29	75.66	60.69
		(6.47)	(−1.55)	(−1.73)	(8.58)	(−1.10)	(−1.15)

[a] $J_{H1,H2}$ are given in Hz in square brackets. [b]Signal width at half-height. [c]Chemical shift differences compared to the corresponding monosaccharides are given in parenthesis

Table 2 Interresidue correlations observed in the ^{1}H,^{13}C-HMBC and ^{1}H,^{1}H-NOESY spectra of the EPS from ST1

Residue	Anomeric atom	Residue	HMBC	NOE
A	H1	**D**	C6	H6a
B	H1	**E**	C4	H4, H6a (w), H6b (w)
B	C1	**E**	H4	
C	H1	**E**	C3	H3
C	C1	**E**	H3	
D	H1	**C**	C6	H6a
E	H1	**F**	C4	H4, H6a (w), H6b (w)
E	C1	**F**	H4	
F	H1	**D**	C4	H4
F	C1	**D**	H4	

(w) = weak NOE

repeating unit is determined having two terminal sugar residues, two branching sugar residues being part of the backbone of the polymer, and two sugar residues in the polymer backbone. The repeating unit structure is shown in Fig. 7. The 4,6-disubstituted residue **D** is substituted in an identical way as in the EPS from LGG (Landersjö et al. 2002). The ^{1}H and ^{13}C NMR chemical (glycosylation) shifts of residue **A** in the ST1 EPS are indistinguishable (within differences in experimental conditions) from those of the terminal β-D-Gal*f* residue in the EPS from LGG providing further credence to the assignment of this sugar as D-galactose. In addition, the glycosylation shifts of residues **A** and **F** in the two polysaccharides are highly similar confirming the structure at this branch point even though the disubstituted sugar is D-glucose in the present case but *N*-acetyl-D-glucosamine the EPS form LGG.

The preparation of the EPS for NMR spectroscopy studies resulted in a highly viscous sample as mentioned above. In order to determine its molecular mass by NMR translational diffusion experiments the sample was diluted about one order of magnitude to a concentration of ~0.6 mg/mL, i.e., until no further change was obtained in the translational diffusion constant. It was determined from five NMR experiments giving $D_t = 3.50 \times 10^{-11}$ m^2 s^{-1} at 25°C in D_2O with a standard deviation of 0.38×10^{-11} m^2 s^{-1}. The molecular weight was calculated using a relationship developed for polysaccharides (Viel et al. 2003) which resulted in M_w= 61.9 kDa. Since there are six

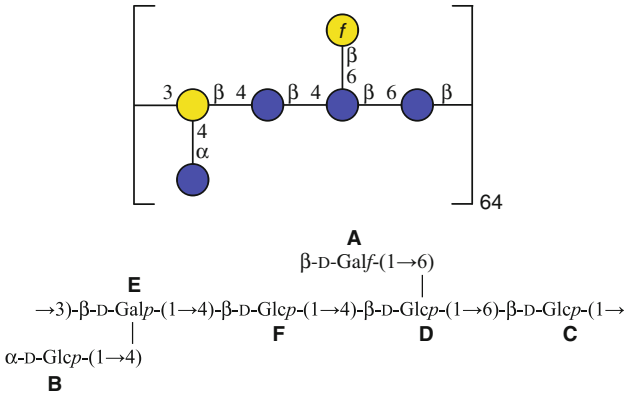

Fig. 7 Structure of the EPS from ST1 in CFG-notation (*top*) and standard nomenclature (*bottom*)

hexoses in the repeating unit of the EPS the M_w corresponds to an EPS having 64 repeating units. The M_w determination from NMR was compared to that measured by dynamic light scattering (DLS) on the same sample. From ten DLS experiments $D_t = 3.81 \times 10^{-11}$ m^2 s^{-1} at 25°C in D_2O with a standard deviation of 0.34×10^{-11} m^2 s^{-1}. This result corresponds to M_w= 51.9 kDa. Thus, the M_w determinations agree within one standard deviation and underscore that NMR spectroscopy can be used with confidence and in conjunction with DLS if available.

In conclusion, all aspects of the primary structure determination of the EPS of *S. thermophilus* ST1 were possible to address by NMR spectroscopy. The highly viscous NMR sample still resulted in NMR spectra of high quality that permitted the structural elucidation of the hexasaccharide repeating unit consisting of glucose and galactose residues. Furthermore, use of efficient NMR techniques based on parallel acquisition and TILT experiments was valuable in the structural analysis. In addition, the molecular mass determination of the EPS was complemented with dynamic light scattering experiments, revealing good agreement with the result from NMR spectroscopy. Knowledge of the primary EPS structure now facilitates further investigations relating polysaccharide structure and dynamics to rheological properties.

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