Structure determination of the exopolysaccharide produced by *Lactobacillus rhamnosus* strains RW-9595M and R

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Exopolysaccharides (EPSs) were isolated and purified from *Lactobacillus rhamnosus* strains RW-9595M, which has been shown to possess cytokine-stimulating activity, and R grown under various fermentation conditions (carbon source, incubation temperature and duration). Identical ¹H NMR spectra were obtained in all cases. Molecular masses were determined by gel permeation chromatography. The primary structure was elucidated using chemical and spectroscopic techniques. Organic acid, monosaccharide and absolute configuration analyses gave the following composition: pyruvate, 1; D-glucose, 2; D-galactose, 1; and L-rhamnose, 4. Methylation analysis indicated the presence of three residues of 3-linked rhamnose, and one residue each of 2,3-linked rhamnose, 2-linked glucose, 3-linked glucose and 4,6-linked galactose. The EPS was submitted to periodate oxidation followed by borohydride reduction. Monosaccharide

analysis of the resulting polysaccharide gave the new composition: rhamnose, 4; and glucose, 1. Methylation analysis confirmed the loss of the 2-linked glucose and 4,6-linked galactose residues. On the basis of one- and two-dimensional ¹H and ¹³C NMR data, the structure of the native EPS was consistent with the following heptasaccharide repeating unit: {3Rhaα-3Glcβ-3[Gal4,6(R)Pyα-2]Rhaα-3Rhaα-3Rhaα-2Glcα-}_n where Rha corresponds to rhamnose (6-deoxymannose) and Py corresponds to pyruvate acetal. Complete ¹H and ¹³C assignments are reported for the native and the corresponding pyruvate-hydrolysed polysaccharide. Electrospray MS and MS/MS data are given for the oligosaccharide produced by Smith degradation.

Key words: lactic acid bacteria, sequence, heptasaccharide repeating unit, pyruvate substituent, NMR.

INTRODUCTION

A variety of polysaccharides produced by plants (cellulose, pectin and starch), algae (agar, alginate and carrageenan) and bacteria (alginate, dextran, gellan, pullulan and xanthan gum) are commonly used as food additives for their gelling, stabilizing or thickening properties [1]. However, the use of polysaccharides excreted during the manufacture of food, such as yoghurt, might be attractive for the food industry and should constitute a new generation of food thickeners. To date, exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) have received increasing interest mainly because of their GRAS (generally regarded as safe) status [1] and their rheological properties in food to improve the texture of fermented products [2]. Some EPSs produced by LAB present potential health-beneficial properties, such as immune stimulation [3,4], and anti-ulcer [5] and cholesterol-lowering activities [6].

Microbial polysaccharides are characterized by a considerable diversity in their composition and structure. Most polysaccharides from microbial origin are heteropolysaccharides; this means there is a wide range of possible structures and differences in the properties of EPSs due to the many possible linkages and configurations. Microbial polysaccharides which are capable of interacting with the immune system to up-regulate or down-regulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers [7]. The use

of polysaccharide immunomodulators as an alternative to classical antibiotic treatment for enhancing host defence responses is very attractive. However, few polysaccharides of microbial origin, with specific activity for both T-cells and antigen-presenting cells, such as monocytes and macrophages, have been examined in detail for both structure–function and mechanism of action [7–12]. The knowledge of structure–function relationships between polysaccharides and the regulation of cytokine networks should provide a foundation for the development of compounds with novel immunomodulating activities [7], in particular, stimulation of the specific cellular component of the immune system, the ‘Th1 response’. Th1 cytokines, interleukin-2 and gamma interferon (IFN-γ), for example, appear to have prominent roles in cellular immunity resulting in resistance against most infectious agents and reducing the manifestations of allergy [13]. Hence, in order to understand the relationship between rheological and immunomodulating properties and the three-dimensional (3-D) structure of a polysaccharide, knowledge of its primary structure is a prerequisite.

The primary structure of the EPS produced by *Lactobacillus rhamnosus* strain C83 was the only one previously reported for this species of LAB [14]. In the present paper we report the structure determination of the EPS from two new strains of *L. rhamnosus*, which are of interest because they are among the highest EPS-producing strains of LAB [15]. Moreover their EPS possesses interesting rheological properties as a viscosifying agent

Abbreviations used: 1-, 2- or 3-D, one-, two- or three-dimensional; APT, attached proton test; BMM, basal minimum medium; CHORTLE, carbon–hydrogen correlations from one-dimensional polarization-transfer spectra by least-squares analysis; CI, chemical ionization; CID, collision-induced dissociation; EI, electron impact ionization; EPS, exopolysaccharide; ES-MS, electrospray MS; GPC, gel permeation chromatography; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; IFN-γ, gamma interferon; LAB, lactic acid bacteria; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid.

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[15] and health-promoting properties in the stimulation of the pro-inflammatory cytokines [4].

EXPERIMENTAL

Bacterial strains and culture conditions

L. rhamnosus RW-9595M and R were obtained from the culture collection of Dr D. Roy (Centre de recherche et de développement sur les aliments, Saint-Hyacinthe, Québec, Canada). All strains were subcultured in 20 ml of Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) [16] and incubated anaerobically at 37 °C for 48 h. Stock cultures were stored at -40 °C in brain-heart infusion broth (Difco Laboratories) with 15% (v/v) glycerol. Before experimental use, the cultures were propagated twice in Lactobacilli MRS broth at 37 °C for 16 h.

Fermentation

Most fermentations were performed at 32 or 37 °C in 7 litre Chemap fermenters (Chemap, Woodbury, NY, U.S.A.) containing 6 litres of basal minimum medium (BMM) as described previously [17], with glucose or lactose (20 g/l) as the carbon source. The pH was maintained at pH 6 with 7 M NH_4OH . The fermenters were agitated by constant stirring at 100 rev./min and no air was added. The culture medium was inoculated with 1% (v/v) of a 16 h active culture and fermentation was allowed to proceed for 24 or 72 h. Some fermentations with *L. rhamnosus* R were performed at 37 °C in 19 litre fermenters (NLF-19, Bioengineering AG, Wald, Switzerland) containing 15 litres of BMM with glucose or lactose (20 g/l) as the carbon source for 24, 30, 48, 72 or 96 h. When the carbon source was lactoserum, fermentations were conducted at 32 °C for 48–56 h in 4 litres of medium composed of 5% (w/v) whey permeate and 1% (w/v) yeast nitrogen base with agitation at 100 rev./min under aerobic conditions. The pH was maintained at pH 6 with 3 M NH_4OH . Samples were cooled on ice immediately after removing them from the fermenters.

EPS isolation and purification

EPSs were isolated and purified according to Cerning et al. [18]. The cultures were heated at 100 °C for 15 min to inactivate enzymes potentially capable of polymer degradation and the cells were removed by centrifugation at 12785 g for 30 min at 4 °C. The EPS was precipitated with 3 vols. of chilled 95% ethanol. After standing overnight at 4 °C, the resultant precipitate was collected by centrifugation (11325 g for 20 min). The EPS was dissolved in deionized water, dialysed against deionized water at 4 °C for 24 h and freeze-dried. The freeze-dried powder was dissolved in 10% (w/v) trichloroacetic acid to remove proteins. The supernatant was dialysed at 4 °C against deionized water for 5 days and freeze-dried. These preparations were referred to as purified EPSs and were stored at 4 °C.

Molecular mass determination

Molecular masses were determined by gel permeation chromatography (GPC) on a Waters (Milford, MA, U.S.A.) HPLC system at 26 °C. Three 8 mm $\times$ 300 mm Shodex OHPak columns (Waters) were connected in series: KB-802.5, exclusion limit 8.5×10^3 Da; KB-805, exclusion limit 2×10^6 Da; and KB-806M, exclusion limit 1×10^7 Da. The mobile phase was 0.1 M NaCl and the flow rate was 0.4 ml/min. A volume of 50 μl was injected, the total amount, depending on the molecular mass, ranging from 5–83 μg for standards and 3–4 μg for purified

EPSs. Detection was performed with both a refractometer and a fluorescence detector used as a right-angle light-scattering detector at a wavelength of 300 nm for excitation and emission. A calibration curve was constructed with dextran standards (Pharmacia, Uppsala, Sweden) having molecular masses of 1×10^3 , 5×10^3 , 1×10^4 , 4×10^4 , 7×10^4 , 5×10^5 and 2×10^6 Da.

Periodate oxidation, Smith degradation and electrospray MS (ES-MS)

To the purified EPS from *L. rhamnosus* RW-9595M grown on glucose at 37 °C for 24 h (48–60 mg) dissolved in 10 ml of 0.2 M sodium acetate buffer (pH 3.9) was added sodium metaperiodate to a final concentration of 0.05 M, and the solution was kept in the dark for 7 days at 4 °C. Ethylene glycol (2 ml) was added and the solution was agitated at 20–25 °C for 2 h to destroy any excess reagent. The solution was dialysed against deionized water for 48 h and freeze dried. The oxidized polysaccharide was reduced with 5 ml of 0.5 M NaBH_4 in 2 M NH_4OH at 60 °C for 4 h. Acetone (2.5 ml) was added and the solution was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in water, neutralized with Amberlite IR-120 (H^+ form) ion-exchange resin, and boric acid was eliminated as methyl borate by co-evaporating several times with methanol under reduced pressure. The resulting polysaccharide was submitted to sugar and linkage analyses as described below.

The oxidized-reduced polysaccharide was further submitted to Smith degradation. The mild acid hydrolysis was performed at 80 °C for 1 h in 0.1 M trifluoroacetic acid (TFA). After evaporation of the TFA under a stream of nitrogen, reduction was repeated as above with NaBH_4 or NaB^2H_4 . The resulting product was analysed by ES-MS.

Positive-ion ES-MS was carried out on a ThermoQuest LQC Classic spectrometer with a spray voltage of 4.5 kV, a capillary voltage of 46 V, a capillary temperature of 200 °C and a tube lens offset of 35 V. Samples dissolved in methanol were infused at 5 $\mu\text{l}/\text{min}$ and single scans were acquired by scanning between m/z 500 and 2000. Collision-induced dissociation (CID)-MS/MS (30 V) spectra were obtained with a mass window of 2 atomic mass units (a.m.u.).

Monosaccharide analysis

Samples were hydrolysed in 2 M TFA at 121 °C for 1 h [19]. The sugar and organic acid compositions were determined by HPLC. A Waters chromatography system was used with a 30 cm $\times$ 7.8 mm ION300 column (InterAction Chromatography, San Jose, CA, U.S.A.), the mobile phase was 2.5 mM H_2SO_4 at a flow rate of 0.4 ml/min and a temperature of 70 °C, with refractive-index detection for sugars and UV detection at 210 nm for organic acids. Sugars were also analysed after reduction with NaBH_4 and acetylation [19] by GLC on a Hewlett-Packard model 5890 gas chromatograph equipped with a 30 m $\times$ 0.32 mm, 1.0 μm film DB-5 capillary column (J&W Scientific, Folsom, CA, U.S.A.) using the temperature program: 150 °C for 5 min, linear temperature gradient of 15 °C/min to 190 °C, hold for 20 min, ramp at 15 °C/min to 230 °C and hold for 20 min. The temperatures of the injector and the flame ionization detector were 225 and 250 °C respectively. Effective carbon responses were used for quantification [20].

Determination of absolute configuration

The absolute configuration of the sugar residues was determined after hydrolysis by polarimetry at 589 nm on an Autopol III polarimeter (Rudolph Research, Flanders, NJ, U.S.A.). Alterna-

tively it was determined on the hydrolysate after glycoside formation with (+)-2-octanol followed by peracetylation [21] including an additional washing step [19], and analysed by GLC as described above but with the temperature program: 150 °C for 5 min followed by a temperature gradient of 15 °C/min to 230 °C and maintained for 30 min.

Linkage analysis

Permethylation of the polysaccharides was performed according to Harris et al. [19] modified as follows: an ultrasonic bath was used for dissolution, 100 µl of DMSO and 100 µl of 1,1,3,3-tetramethylurea [22] were used instead of 150 µl of DMSO to improve solubility, and three preliminary methylations were necessary for complete methylation. Hydrolysis, reduction, acetylation and GLC analysis were performed as described above for sugar analysis. GLC–MS analyses were performed on a 30 m × 0.25 mm, 0.25 µm film J&W Scientific DB-5 capillary column on a Finnigan Incos 50 mass spectrometer equipped with a Varian 3400 gas chromatograph [inlet, 250 °C; transfer line, 225 °C; source, 180 °C; electron impact ionization (EI), 70 eV] or a 30 m × 0.25 mm, 0.1 µm film J&W Scientific DB-5 column on a Finnigan GCQ [inlet, 250 °C; transfer line, 275 °C; source, 110 °C; EI, 15 eV; isobutane chemical ionization (CI), 50 millitorr]. In the first case, GLC analyses were obtained using the temperature program: 50 °C for 2 min, linear gradient at 10 °C/min to 125 °C, then at 5 °C/min to 250 °C, hold for 5 min, followed by a linear gradient at 20 °C/min to 320 °C and hold for 5 min. In the second case, the temperature program was: 40 °C for 5 min, ramp at 15 °C/min to 275 °C and hold for 10 min.

Mild acid hydrolysis

The purified EPS from *L. rhamnosus* RW-9595M grown on glucose at 37 °C for 24 h (60 mg) was submitted to mild acid hydrolysis in 4 ml of 0.1 M acetic acid for 4 days at 80 °C, and the reaction mixture was neutralized with aqueous ammonia.

NMR spectroscopy

The polysaccharides were exchanged twice in ²H₂O (99.9 atom % ²H) with intermediate freeze-drying and dissolved at 1% (w/v) in ²H₂O (99.96 atom % ²H) for NMR experiments. A Chemagetics (Fort Collins, CO, U.S.A.) CMX Infinity 300 spectrometer was used for all experiments using a Nalorac (Martinez, CA, U.S.A.) 5 mm dual ¹³C/¹H probe for ¹H (300 MHz) and homonuclear spectra, and a Nalorac 10 mm broad-band probe for ¹³C (75.5 MHz), ³¹P (121.5 MHz) and heteronuclear spectra. The temperature was maintained at 80 °C in order to obtain a reasonable line width in proton spectra. ¹H and ¹³C chemical shifts (δ in p.p.m.) are both referenced with internal 2,2-dimethyl-2-silapentane-5-sulphonate at δ 0 as recommended by Wishart et al. [23]. The two-dimensional (2-D) COSY spectra [24] were acquired in magnitude mode. The 2-D magnitude COSY with relay coherence transfer (42 ms transfer time) experiment was performed according to Bax and Drobny [25]. The 2-D phase-sensitive TOCSY spectra were recorded with an effective spin lock time of 90–170 ms [26]. The 2-D phase-sensitive NOESY [27] and rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) [28] experiments were performed with a mixing time of 300 ms. The one-dimensional (1-D) ¹³C NMR spectra were recorded with wideband alternating-phase low-power technique for zero residue splitting ('WALTZ') proton decoupling [29], and the 1-D attached proton test (APT) [30] with a 7 ms evolution time. The 2-D phase-sensitive heteronuclear

single-quantum coherence (HSQC) [31] spectra were acquired with the frequency switched decoupling pulse sequence MPF9 [32] or without carbon decoupling during acquisition. A bilinear rotation decoupling ('BIRD') sandwich [33] was included at the beginning of the sequence to suppress the signal from protons bonded to ¹³C. The 2-D magnitude heteronuclear multiple-bond correlation (HMBC) [34] was run with an evolution time of 72 ms without carbon decoupling. Most of the reported proton chemical shifts were obtained from the carbon–hydrogen correlations from one-dimensional polarization-transfer spectra by least-squares analysis (CHORTLE) experiment [35] run with four increments for cosine and sine subspectra: 0.4, 1.0, 2.4 and 3.2 ms.

RESULTS

Culture, isolation and purification

Under fermentation conditions at pH 6, *L. rhamnosus* RW-9595M yielded between 931–1275 mg of purified EPS when grown on glucose or lactose at 32 or 37 °C, whereas *L. rhamnosus* R produced between 438–601 mg of purified EPS/l.

Molecular mass determination

The average molecular masses extrapolated from the dextran calibration curves for the EPSs of strains RW-9595M and R grown on glucose at 37 °C for 24 h were 5.0 × 10⁷ and 2.1 × 10⁷ Da respectively.

EPS composition

Elemental analysis (C, H, N and S) revealed the absence of nitrogen and sulphur. GLC data of monosaccharide analysis of EPSs are summarized in Table 1. Sugar and organic acid analysis was also performed on the EPS from strain RW-9595M grown on glucose at 37 °C for 24 h by HPLC, giving the following relative composition (mean ± S.D. for 15 determinations): pyruvate, 0.70 ± 0.16; glucose, 2.10 ± 0.14; galactose, 1; and rhamnose, 3.87 ± 0.13. For the EPS of both strains, the data are consistent with a repeating unit of the following composition: rhamnose, 4; glucose, 2; galactose, 1; and pyruvate, 1. Oxidation of the EPS destroys one residue of glucose and one residue of galactose, while new peaks for peracetylated threitol and glycerol appear (Table 1). Quantification was not exact for the latter (and for ethylene glycol which was not observed) due to the high volatility of their peracetylated derivatives. Glucose and galactose were found to be of the D configuration, whereas rhamnose was of the L configuration.

Methylation analysis

GLC retention times [36], EI–MS fragmentation patterns [36,37] and CI–MS molecular ion mass determination were used to confirm linkage or substitution positions. Molecular pseudo-ions [M + H]⁺ were observed on CI spectra, but in all cases the base peak was [M + H – CH₃COOH]⁺. The results (Table 2) indicate the presence of three residues of 3-linked rhamnose, and one residue each of 2,3-linked rhamnose, 2-linked glucose, 3-linked glucose and 4,6-linked galactose for the EPS of both strains. In the oxidized–reduced polysaccharide, the 2-linked glucose and the 4,6-linked galactose were lost as expected. In addition, 2,3,4-tri-*O*-acetyl-1-*O*-methylthreitol, resulting from the oxidation of the galactose residue between C-2 and C-3, was observed. The other decomposition products, i.e. glycerol and ethylene glycol derivatives, were not detected due to their high volatility. A small amount of 1,3,4,5-tetra-*O*-acetyl-2-*O*-methylrhamnitol was al-

Table 1 GLC monosaccharide analysis data for the native and oxidized–reduced polysaccharides

The EPSs from *L. rhamnosus* strains RW-9595M and R and the oxidized–reduced polysaccharide were hydrolysed, reduced and acetylated, and the resulting alditol acetates were analysed by GLC as described in the Experimental section. Relative molar composition results are reported as means $\pm$ S.D. (*n* values are given in parentheses). Rha, rhamnose.

Alditol acetate	Average retention time (min)	Relative molar composition			
		EPS from RW-9595M grown on glucose at 37 °C for 24 h (8)	EPS from R grown on glucose at 37 °C (4)	EPS from R grown on lactose at 37 °C (5)	Oxidized–reduced polysaccharide (2)
Glycerol	1.88	—	—	—	0.84 $\pm$ 0.06*
Threitol	6.44	—	—	—	0.57 $\pm$ 0.03*
Rha	9.65	3.66 $\pm$ 0.14	3.62 $\pm$ 0.13	3.63 $\pm$ 0.06	3.85 $\pm$ 0.11
Glc	16.99	2.00 $\pm$ 0.09	1.92 $\pm$ 0.07	2.01 $\pm$ 0.10	1
Gal	17.34	1	1	1	0.04 $\pm$ 0.05

* Due to their high volatility, these residues gave values lower than expected.

ways detected in the GLC chromatograms (Table 2) and was much higher when tetramethylurea was not used and less than three premethylations were performed, suggesting hindrance to the alkylation of position 4 of the corresponding residue in the EPS.

NMR spectroscopy

NMR experiments were performed on a 1 % solution of EPS in $^2\text{H}_2\text{O}$. When higher concentrations were used, precipitation occurred with time at 80 °C.

Native EPS

Identical 1-D ^1H NMR spectra were obtained for the native EPSs, irrespective of the culture conditions: carbon source, incubation temperature and duration.

The 1-D proton spectrum (Figure 1) can be divided into three regions: anomeric protons (4.6–5.2 p.p.m.), other sugar protons (3.2–4.4 p.p.m.) and methyls (1.2–1.5 p.p.m.). The anomeric region integrates for seven signals, which correspond to seven sugar residues in the repeating unit. One signal is at higher field (δ 4.636) with a large coupling constant (7.5 Hz), therefore corresponding to a β configuration of glucose or galactose. A broad signal (1.2–1.4 p.p.m.) corresponding to the H-6's of the rhamnose residues, which integrates for four methyls, is found in the methyl region. Finally there is another unknown methyl signal (δ 1.445), which integrates for one methyl per repeating unit. To help in the analysis, the residues were labelled with A through G with increasing chemical shift of their anomeric proton.

From the 2-D COSY spectrum (Figure 1) correlations are observed between the H-6's (1.2–1.4 p.p.m.) of the rhamnose residues and the corresponding H-5's (3.8–4.1 p.p.m.). In all cases the chemical shift is characteristic of an α anomeric configuration, H-5 in a β -rhamnose residue would resonate at approx. 0.5 p.p.m. to lower frequency [38]. Analysis of the sugar region (Figure 1) allows the identification of some residues. The β anomeric proton A1 (δ 4.636) is coupled to A2 (δ 3.257), which is coupled to A3 (δ 3.582), in turn coupled to A4 (δ 3.406). All cross-peaks show large couplings, meaning that residue A corresponds to β -glucose. All other anomeric protons have a small coupling and therefore have the α anomeric configuration, since the rhamnose residues were already determined to be α . Identification of four rhamnose residues (B, D, E and G) is possible from their small $J_{2,3}$ value (rhamnose has the *manno* configuration, i.e. H-2 is equatorial) as observed from the cross-

peak. The other two residues (C and F) could only be galactose or glucose because they have large $J_{2,3}$ values. The spin system for all rhamnose residues can be followed up to H-3 (residues B, D and G) and H-4 (residue E). Table 3 reports chemical shift data.

Adding a relay coherence transfer step to the 2-D COSY experiment allows the assignment of new resonances: B4 (δ 3.612), D4 (δ 3.55), G4 (δ 3.601) and F3 (δ 3.933). The latter shows a large $J_{3,4}$ value, which indicates that the residue is α -glucose. Residue C therefore corresponds to the galactose detected by sugar analysis.

On the 2-D TOCSY spectrum with 90 ms mixing time correlations are observed for E2/E5 (δ 4.30/ δ 3.876) and F1/F4 (δ 5.141/ δ 3.490), as well as two new signals for residue A (δ 4.636/ δ 3.92 and δ 4.636/ δ 3.70) and one for residue C (δ 5.054/ δ 4.243), detected by magnetization transfer from the corresponding anomeric protons, left to be assigned. With 110 ms mixing time one more correlation in the spin system of residue F (δ 5.141/ δ 3.80) is observed. With 140 ms mixing time there is one more correlation in the spin system of residue C (δ 5.054/ δ 4.04). When the mixing time is increased to 170 ms correlations are observed between the anomeric and methyl protons of the rhamnose residues. It is then possible to assign the H-5's of the rhamnose residues (Table 3).

So far the number of residues, their identity and their anomeric configuration are known, and most of the resonances have been assigned. All rhamnose resonances are known. There is one resonance missing in the spin system of residue A, probably due to overlap, and two resonances (δ 3.92 and 3.70) are unassigned. Only four resonances in the spin system of residue C have been detected, of which two (δ 4.243 and 4.04) are unassigned. For residue F five resonances have been observed, one of which is unassigned (δ 3.80).

The 1-D carbon spectrum (Figure 2) shows peaks in four different regions: carbonyl (weak), anomeric, other sugar carbons and methyls. Integration of all but the carbonyl region gives 44 carbons, i.e. two more than the 42 expected for seven hexoses in the repeating unit. One is in the anomeric region, the other one is a methyl, probably corresponding to the unknown methyl seen in the proton spectrum. The other methyl signals correspond to the four rhamnose residues. One signal (δ 103.30), probably a quaternary carbon, in the anomeric region was not present on the APT spectrum. Three signals (δ 67.54, 63.97 and 63.32), which are inverted in the APT spectrum, correspond to carbons having two attached protons. These are C-6's of the glucose and galactose residues. Two of these signals are in the normal

Table 2 GLC and GLC-MS linkage analysis data for the native and oxidized-reduced polysaccharides

The EPSs from *L. rhamnosus* strains RW-9595M and R and the oxidized-reduced polysaccharide were methylated, hydrolysed, reduced and acetylated, and the resulting partially methylated alditol acetates (1-Me-Threitol, 2,3,4-tri-O-acetyl-1-O-methylthreitol; 2,4-Me₂-Rha, 1,3,5-tri-O-acetyl-2,4-di-O-methyl-6-deoxymannitol; 2-Me-Rha, 1,3,4,5-tetra-O-acetyl-2-O-methyl-6-deoxymannitol; 4-Me-Rha, 1,2,3,5-tetra-O-acetyl-4-O-methyl-6-deoxymannitol; 3,4,6-Me₃-Glc, 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylglucitol; 2,4,6-Me₃-Glc, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol; 2,3-Me₂-Gal, 1,4,5,6-tetra-O-acetyl-2,3-di-O-methylgalactitol) were analysed by GLC and GLC-MS as described in the Experimental section. Relative molar composition results are reported as means $\pm$ S.D. (*n* values are given in parentheses).

Methylated alditol acetate	Average retention time (min)	EI-MS major fragments (<i>m/z</i>)	CI-MS [<i>M</i> +H] ⁺ (<i>m/z</i>)	Relative molar composition			
				EPS from RW-9595M grown on glucose at 37 °C for 24 h (3)	EPS from R grown on glucose at 37 °C (4)	EPS from R grown on lactose at 37 °C (5)	Oxidized-reduced polysaccharide (2)
1-Me-Threitol	4.07	45	263	—	—	—	0.59 $\pm$ 0.10*
2,4-Me ₂ -Rha	7.88	117, 131, 233, 247	321	2.57 $\pm$ 0.07	2.42 $\pm$ 0.13	2.56 $\pm$ 0.12	3.04 $\pm$ 0.11
2-Me-Rha	8.66	117, 275	349	0.15 $\pm$ 0.08	0.07 $\pm$ 0.07	0.03 $\pm$ 0.01	0.09 $\pm$ 0.12
4-Me-Rha	8.88	131, 261	349	1	1	1	1
3,4,6-Me ₃ -Glc	9.63	161, 189, 205	351	0.89 $\pm$ 0.14	1.01 $\pm$ 0.10	0.87 $\pm$ 0.14	0.13 $\pm$ 0.18
2,4,6-Me ₃ -Glc	9.72	117, 161, 233, 277	351	0.99 $\pm$ 0.05	0.98 $\pm$ 0.07	0.99 $\pm$ 0.16	1.06 $\pm$ 0.12
2,3-Me ₂ -Gal	12.61	117, 261, 305	379	0.89 $\pm$ 0.10	0.78 $\pm$ 0.15	0.73 $\pm$ 0.06	0.04 $\pm$ 0.06

* Due to its high volatility, this residue gave a value lower than expected.

position but the other one is somewhat unshielded (δ 67.54). This suggests substitution on C-6 of this residue. There is no signal in the region near 90 p.p.m. suggesting that there is no furanose ring, i.e. all sugars have the pyranose ring configuration.

The 2-D HSQC spectrum shows correlations in the methyl region for the rhamnose 6 positions (19.3–19.5 p.p.m./1.2–1.4 p.p.m.) and the unknown methyl (δ_{C} 27.97/ δ_{H} 1.445). The assignments are straightforward for the anomeric signals. In the sugar region (Figure 2), all resonances for the rhamnose residues can easily be found. These are H-2, H-3, H-4 and H-5 for residues B, D, E and G, with some overlap for: B2 and D2; B4, D4, E4 and G4; and B5, D5 and E5. Resonances for the spin system of residue A can easily be identified from their known proton shifts: A2, A3, A4 and the two previously unassigned resonances (δ_{H} 3.92 and 3.70), which correspond to the CH₂ in position 6 (δ_{C} 63.97). For the spin system of residue F, F2, F3 and F4 are found. The signal at δ_{H} 3.80, previously unassigned, actually corresponds to the two protons of F6 (δ_{C} 63.32). For the spin system of residue C, C2 (δ_{C} 70.86) is observed. The third CH₂ (δ_{C} 67.54) must correspond to C6, meaning that the galactose is the residue that is substituted on position 6. Five signals (δ_{C} 78.61/ δ_{H} 3.42, δ_{C} 74.57/ δ_{H} 3.957, δ_{C} 74.07/ δ_{H} 4.243, δ_{C} 70.75/ δ_{H} 3.952 and δ_{C} 65.27/ δ_{H} 4.129), which correspond to A5, C3, C4, C5 and F5, are left unassigned. Since there is considerable overlap on the 2-D HSQC for some of the signals (see above) the CHORTLE experiment was performed. Complete assignments are found in Table 3. Due to overlap of their carbon signals, some proton chemical shifts are reported with less precision since they are taken from the 2-D HSQC spectrum and not from the CHORTLE experiment.

When the HSQC experiment is performed without carbon decoupling, the anomeric region shows two signals for each residue separated in the proton dimension by $^1J_{\text{C,H}}$. This value is characteristic of the anomeric configuration [38]. It provides further evidence for the β configuration of the upfield-proton glucose and the α configuration for all other residues (Table 3).

In the methyl region of the 2-D HMBC spectrum, apart from the direct correlation observed to the methyl carbon of the unknown signal, the methyl proton shows correlations to the quaternary carbon in the anomeric region and to a carbonyl carbon. This corresponds to a pyruvate substituent in the ketal form, usually linked to a residue to form either a six-membered ring, when substituting positions 4 and 6, or a five-membered ring when substituting positions 2 and 3, or 3 and 4 [39].

Careful examination of the carbon chemical shifts in Table 3 gives indication as to the glycosidation or substitution position of the sugar residues. An α glycosidation shift of 5–9 p.p.m. is observed for carbons A3, B3, D3, E2, E3, F2 and G3 when compared with the corresponding methyl glycosides [40]. In the case of residue C, carbons C4, C5 and C6 are shifted noticeably from their respective positions in methyl α -D-galactopyranoside, suggesting that the pyruvate is substituted at positions 4 and 6 of the galactose residue. In particular C5 has a large negative β shift due to the presence of two neighbouring substituted carbons. These results confirm the linkage analysis data, i.e. three 3-substituted rhamnose residues (B, D and G), one 2,3-disubstituted rhamnose (residue E), one 2-substituted glucose (residue F), one 3-substituted glucose (residue A) and one 4,6-substituted galactose (residue C). In the latter case the pyruvate substituent is not lost during the methylation step as opposed, for example, to an acetate substituent which is labile under basic conditions [41].

By forming a ketal with the pyruvate a chiral centre is introduced. From the chemical shift of the methyl group, C-2 of the pyruvate substituent must have the *R* configuration,

Figure 1 2-D ^1H COSY spectrum

The 2-D spectrum of the EPS from *L. rhamnosus* RW-9595M grown on lactoserum at 32 °C for 48–56 h was obtained in $^2\text{H}_2\text{O}$ at 80 °C. Increments (512) of 1024 complex data points were acquired in magnitude mode with a digital resolution of 1.9 Hz/point in the horizontal dimension and 3.8 Hz/point in the vertical dimension. The horizontal dimension was processed by multiplication with an unshifted sinebell squared window function and complex Fourier transform, and the vertical dimension by linear prediction of 512 points, multiplication with an unshifted sinebell squared window function, complex Fourier transform and magnitude calculation. The projection corresponds to the 1-D ^1H spectrum of the EPS from *L. rhamnosus* R grown on lactose at 37 °C for 48 h obtained in $^2\text{H}_2\text{O}$ at 80 °C. Complex data points (8192) were acquired with a digital resolution of 0.24 Hz/point and processed by exponential multiplication, complex Fourier transform, phase correction and polynomial baseline correction. Rha, rhamnose.

because the corresponding carbon having the *S* configuration, being axial, would resonate at approx. 9 p.p.m. to lower frequency [39].

No signal was detected by ^{31}P NMR spectroscopy, suggesting the absence of a phosphate substituent. The latter, or any other substituent, can also be ruled out since no carbon has an unexplained substitution shift.

Sequence information is given from the 2-D NOESY spectrum (Figure 3). The following interresidue correlations are observed: A1/E3, B1/F2, C1/E2, D1/B3, E1/D3, F1/G3 and G1/A3. In addition, a correlation between C1 and E1 is present. The data are consistent with the following sequence:

The cross-peaks observed on the 2-D ^1H ROESY and $^1\text{H}/^{13}\text{C}$ HMBC are consistent with this sequence (Table 4). In addition to interresidue correlations, characteristic intraresidue connectivities are present, in agreement with the type of sugar and its anomeric configuration [42].

Modified polysaccharide

The NMR experiments were repeated with the polysaccharide submitted to mild acid hydrolysis and the data were analysed in a similar way. Table 3 reports both ^1H and ^{13}C chemical shifts

Table 3 ^1H and ^{13}C NMR chemical shift assignments of the native and pyruvate-hydrolysed polysaccharides

Chemical shifts of the EPS from *L. rhamnosus* RW-9595M grown on glucose at 37 °C for 24 h and the pyruvate-hydrolysed polysaccharide obtained in $^2\text{H}_2\text{O}$ at 80 °C are reported. ^1H chemical shifts were obtained from the CHORTLE experiment or, with a lower precision, from the 2-D HSQC spectrum. ^{13}C chemical shifts were obtained from the CHORTLE experiment in the case of protonated carbons and from the 1-D ^{13}C spectrum or, with a lower precision, from the 2-D HMBG spectrum in the case of quaternary carbons. n.d., not determined.

H/C	Native EPS			Pyruvate-hydrolysed polysaccharide		
	^1H		^{13}C δ (p.p.m.)*	^1H		^{13}C δ (p.p.m.)*
	δ (p.p.m.)*	Standard error (p.p.m.)†		δ (p.p.m.)*	Standard error (p.p.m.)†	
A1	4.636	0.007	106.82 (162)‡	4.710	0.006	106.59 (163)‡
A2	3.257	0.006	76.86	3.483	0.005	76.81
A3	3.582	0.005	85.22	3.620	0.003	85.36
A4	3.406	0.010	71.38	3.478	0.004	71.28
A5	3.42	n.d.	78.61	3.467	0.010	78.55
A6	3.92	n.d.	63.97	3.93	n.d.	63.82
	3.70	n.d.		3.74	n.d.	
B1	5.050	0.006	104.62 (172)‡	5.03	n.d.	104.58 (173)‡
B2	4.156	0.029	72.67	4.162	0.010	72.50
B3	3.853	0.008	80.58	3.87	n.d.	80.79
B4	3.612	0.013	73.95	3.612	0.004	73.94
B5	3.814	0.009	72.23	3.802	0.004	72.21
B6	1.318	0.004	19.47	1.324§	0.003§	19.45§
C1	5.054	0.007	100.76 (170)‡	5.051	0.003	100.24 (173)‡
C2	3.917	0.007	70.86	3.825	0.003	71.09
C3	3.952	0.007	70.75	3.93	n.d.	72.12
C4	4.243	0.054	74.07	4.022	0.003	71.63
C5	4.129	0.005	65.27	4.281	0.020	73.12
C6	4.04	n.d.	67.54	3.769	0.002	63.34
	3.85	n.d.		n.d.		
D1	5.059	0.004	104.52 (172)‡	5.07	n.d.	104.58 (173)‡
D2	4.16	n.d.	72.52	4.153	0.004	72.74
D3	3.925	0.007	81.12	3.93	n.d.	81.12
D4	3.55	n.d.	74.13	3.582	0.002	74.12
D5	3.847	0.009	71.93	3.88	n.d.	72.12
D6	1.30	n.d.	19.33	1.314	0.001	19.36
E1	5.139	0.006	102.24 (170)‡	5.163	0.004	102.14 (172)‡
E2	4.30	n.d.	78.61	4.275	0.010	78.43
E3	4.056	0.005	81.69	4.120	0.006	81.20
E4	3.62	n.d.	74.13	3.692	0.004	74.41
E5	3.876	0.006	72.07	3.883	0.004	71.97
E6	1.30	n.d.	19.33	1.32§	n.d.§	19.38§
F1	5.141	0.007	97.74 (170)‡	5.127	0.009	98.11 (174)‡
F2	3.66	n.d.	80.97	3.67	n.d.	80.73
F3	3.933	0.008	75.10	3.934	0.003	75.17
F4	3.490	0.040	72.59	3.497	0.010	72.62
F5	3.957	0.006	74.57	3.982	0.003	74.61
F6	3.85	n.d.	63.32	3.85	n.d.	63.39
	3.75	n.d.		3.77	n.d.	
G1	5.174	0.007	103.18 (172)‡	5.207	0.006	103.16 (174)‡
G2	4.193	0.006	70.10	4.205	0.008	70.35
G3	3.88	n.d.	78.54	3.893	0.008	79.04
G4	3.601	0.007	73.19	3.617	0.006	73.18
G5	4.016	0.003	71.58	4.010	0.009	71.92
G6	1.289	0.003	19.39	1.31	n.d.	19.38
Pyruvate C-1			178.4			
Pyruvate C-2			103.30			
Pyruvate C-3	1.445	0.003	27.97			

* For both ^1H and ^{13}C , downfield from internal 2,2-dimethyl-2-silapentane-5-sulphonate at δ 0.

† Obtained from the CHORTLE experiment.

‡ $^1J_{\text{C,H}}$ in Hz as measured on the HSQC spectrum without ^{13}C decoupling in parentheses.

§ Values in the same column could be interchanged as an ensemble.

Figure 2 Sugar region of the 2-D $^1\text{H}/^{13}\text{C}$ HSQC spectrum

The 2-D spectrum of the EPS from *L. rhamnosus* RW-9595M grown on glucose at 37 °C for 24 h was obtained in $^2\text{H}_2\text{O}$ at 80 °C. Increments (256) of 1024 complex data points were acquired in the hypercomplex mode with a digital resolution of 2.2 Hz/point in the proton dimension and 42 Hz/point in the carbon dimension. The proton dimension was processed by multiplication with an exponential window function, complex Fourier transform and phase correction, and the carbon dimension by linear prediction of 256 points, multiplication with a Hanning window function, complex Fourier transform and phase correction. The projection corresponds to the 1-D ^{13}C spectrum of the EPS from *L. rhamnosus* RW-9595M grown on lactoserum at 32 °C for 48–56 h obtained in $^2\text{H}_2\text{O}$ at 80 °C. Complex data points (8192) were acquired with a digital resolution of 2.0 Hz/point and processed by exponential multiplication, complex Fourier transform, phase correction and polynomial baseline correction.

Figure 3 Part of the sugar region of the 2-D ^1H NOESY spectrum

The spectrum of the EPS from *L. rhamnosus* RW-9595M grown on glucose at 32 °C for 72 h was obtained in $^2\text{H}_2\text{O}$ at 80 °C. Increments (512) of 1024 complex data points were acquired in the hypercomplex mode with a mixing time of 300 ms and a digital resolution of 1.8 Hz/point in the horizontal dimension and 3.7 Hz/point in the vertical dimension. The horizontal dimension was processed by multiplication with an exponential window function, complex Fourier transform and phase correction, and the vertical dimension by linear prediction of 512 points, multiplication with a Hanning window function and complex Fourier transform.

and one-bond CH coupling constants for the new compound. The major changes in the carbon spectrum are noticed for C3, C4, C5 and C6, which are shifted by +1.37, −2.44, +7.85 and −4.20 p.p.m. respectively. In the proton spectrum, important shifts are observed for C4, C5 and one proton on C6 (−0.221, +0.152 and −0.27 p.p.m. respectively), as well as for A2 (+0.226 p.p.m.). Noticeable shifts are present for other protons on residues C (C2 and the second proton on C6), A (A1 and A4) and E (E3, E4 and E5), i.e. residues in close proximity to the location of the missing substituent. The data are consistent with the structure of the native EPS lacking the pyruvate substituent.

ES–MS of the Smith degradation product

The following compound is expected after Smith degradation of the EPS:

where Rha corresponds to rhamnose and Gro corresponds to glycerol. The corresponding sodium-cationized species was observed at m/z 861.7 on the ES–MS spectrum of the reaction product, but the base peak was found at m/z 933.7. ES–CID–MS/MS spectra of both compounds showed peaks at m/z 787.2, 769.2, 641.2, 623.2, 495.1 and 477.1, corresponding to sodium-cationized C_5 , B_5 , C_4 , B_4 , C_3 and B_3 fragments respectively, according to the nomenclature proposed by Domon and Costello [43]. In addition, a fragment at m/z 715.2, corresponding to the sodium-cationized Y_4 fragment was present on the spectrum of the m/z 861.7 species. C_1 fragments were much less intense than B_1 and Y_1 fragments. When NaB^2H_4 was used instead of NaBH_4 , the reaction products had m/z values of 862.5 and 933.5, indicating that the minor product underwent reduction but not the major one. It is proposed that the major product:

is formed during mild acid hydrolysis by acetal formation between the glyceraldehyde and glycerol moieties derived from the oxidation of residue F, by analogy to the formation of cyclic *O*-2'-hydroxyethylidene acetals from polyalcohols of 4-*O*-linked polysaccharides [44].

DISCUSSION

The primary sequence of the EPS produced by both strains of *L. rhamnosus* RW-9595M and R was found to be identical by all methods and is always the same, independent of the culture conditions: carbon source, temperature and fermentation time. The structure determined in the present work is complete, including the number and type of residues, their absolute and anomeric configuration, linkage type, sequence and substituent information.

To our knowledge the present structure is unique among EPSs produced by LAB in general (for a review of structures published prior to 1998 refer to De Vuyst and Degeest [45] and Ricciardi and Clementi [46]) and *L. rhamnosus* in particular. Glucose and galactose are present, as commonly found. Novel features include the high number of rhamnose residues, four as opposed to a maximum of two in the literature [47–50], and the presence of

Table 4 Through-space (ROESY) and long-range (HMBC) correlations

The 2-D ROESY (300 ms mixing time) and HMBC spectra of the EPS from *L. rhamnosus* R grown on glucose at 37 °C for 24 h were obtained in $^2\text{H}_2\text{O}$ at 80 °C. Correlations from or to the anomeric protons observed on the ROESY spectrum and from the anomeric or substituent carbons or to the anomeric protons observed on the HMBC spectrum are reported.

Proton	ROESY		HMBC	
	Intraresidue	Interresidue	Intraresidue	Interresidue
A1	A3*, A5	E2, E3, E4*		E3
A2			A1	
A3				G1
B1	B2	F2, F3*, F4	B3*, B5	F2*
B3				D1
C1	C2*	E1, E2	C3, C5	E2*
C6				Pyruvate C-2
D1	D2	B3, B4	D3*, D5	B3*
D3				E1
E1	E2	D2, D3*, D4	E3*, E5	D3*
E3				A1
F1	F2	G3*, G4*	F3*, F5*	G3*
F2				B1
G1	G2	A2, A3*, A4	G3*, G5	A3
G3				F1

* Cross-peak superposition.

a pyruvate substituent but no acetate [49,51], phosphodiester [52,53] or furanose ring configuration [14,49,50]. In addition, no acetamido [54] or uronic acid sugar residues are found. The previously known structure of an EPS from another strain of *L. rhamnosus* is totally different from the one reported in the present paper. The former had no rhamnose residue, possessed two

galactose residues with a furanose ring configuration and bore no substituent [14].

Due to its acidic nature, the EPS of *L. rhamnosus* RW-9595M or R could find applications where high-methoxyl pectin is used in pasteurized and sterilized acidified milk products, such as drinkable yoghurts, fruit juice-containing milks and fruit-flavoured, protein-fortified drinks. In such products the free carboxylic acid groups electrostatically interact with the net positively charged casein micelles, whereas the neutral sugars, which do not interact with the charged flocculating ions, form a stabilizing layer in solution [55]. To explain the superior stabilizing effect of high- over low-methoxyl pectin, it has been proposed [56] that high-methoxyl pectin, with fewer interactive sites, has a larger portion of the chain free to interact with the solvent. Therefore, because of its low charge density (one charge per seven residues), the EPS of *L. rhamnosus* from strains RW-9595M or R presents interesting properties to stabilize acidified milk products.

The EPS from *L. rhamnosus* RW-9595M appears to elicit bioactive IFN- γ in both C57Bl/6 and BALB/c splenocytes on the murine RAW 264.7 macrophage-like cell line [4]. The immunomodulating properties of microbial polysaccharides may

depend on branching pattern, molecular and higher-order structure. The structural basis for the activity of immunomodulator polysaccharides may also lie in their 3-D conformation and overall spatial charge organization [57]. Different polysaccharides may assume similar 3-D structures, e.g. helices, which would define a common scaffold for the presentation of charges. In this respect, molecular modelling calculations of the 3-D structure of the EPS are in progress.

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