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Structural analysis of the *Lactobacillus rhamnosus* strain KL37C exopolysaccharide

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Abstract

The exopolysaccharide from the lactic acid bacterium *Lactobacillus rhamnosus* strain KL37C isolated from human intestinal flora was prepared by sonication of bacterial cell mass suspended in water followed by centrifugation and cold ethanol precipitation of the supernatant. The polysaccharide material was purified by gel permeation chromatography on an TSK HW-50 column and characterised using chemical and enzymatic methods. On the basis of sugar and methylation analysis and ^{1}H, ^{13}C, 1D and 2D NMR spectroscopy the exopolysaccharide was shown to be composed of the following pentasaccharide repeating unit:

→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→

Keywords: *Lactobacillus rhamnosus*; Exopolysaccharide; Structural analysis; Probiotic bacteria

1. Introduction

Selected strains of lactic acid bacteria belonging to *Lactobacillus* genus, present in the human gastrointestinal tract as its natural inhabitants, can be beneficial for the host and therefore are regarded as probiotics.[1] The probiotic bacteria possess several activities which are considered to be crucial for their role in maintaining intestinal integrity:[2] adhesion to the intestinal mucosa,[3] an antagonistic activity exerted against enteropathogens,[4] modulation of the immune system,[5] and improved healing of damaged gastric and intestinal mucosa.[6]

Surface components of the bacterial cell e.g., polysaccharides and proteins, play an essential role in this adhesion phenomenon, which is considered important for colonisation of host surfaces by both pathogens and resident microorganisms,[7] whilst exopolysaccharides (EPSs) produced by some intestinal bacteria including *Lactobacillus* are excreted into the growth medium or remain attached to the bacterial cell wall but their role in bacterial interactions with the host remains largely unknown. There is also a growing interest in these polysaccharides as they have rheological properties appropriate for the dairy products industry. Structures of the EPSs from several *Lactobacillus* strains have been reported,[8] and sometimes more than one polysaccha-

Abbreviations: EPS, exopolysaccharide; 1D and 2D, one- and two-dimensional; ROESY, rotating frame 2D NOE spectroscopy; HMBC, ^{1}H-detected heteronuclear multiple-bond correlation spectroscopy; TSP-d_4, sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate; HSQC, ^{1}H-detected heteronuclear single quantum coherence spectroscopy; PFG, pulsed field gradient; HSQC–TOCSY, the two-dimensional HSQC–TOCSY sequence using PFG-assisted heteronuclear sequence of Wider and Wüthrich.

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ride can be secreted by a single strain. Little is known about the surface properties of *Lactobacillus* that mediate attachment to human epithelial cell surfaces, but the presence of EPS has been demonstrated in *Lactobacillus* strains isolated from healthy newborns.[9] Still, the role of the EPS in the adherence of lactobacilli to epithelial surfaces as well as the structures of EPSs of strains adhering to the epithelial cells is not well understood.

Here we report the structure of the EPS from *Lactobacillus rhamnosus* strain KL37C. This strain was isolated from the feces of a newborn in the coaggregating cluster of four strains, namely KL37A–D.[9] The coaggregation is rather common feature of *Lactobacillus* strains.[10] The strain KL37B appeared to be a different entity and is a subject of a separate investigation to be published elsewhere. Strains KL37A, C and D were found to be similar and the representative one, KL37C was selected previously for studies of the surface properties.[9] These three strains KL37A, C and D appeared to have the same capsular polysaccharide.

2. Results and discussion

Lactobacillus rhamnosus strains KL37A, C and D were grown on supplemented MRS broth under anaerobic conditions. The polysaccharides were isolated from each strain by sonication of the bacterial mass suspended in water, followed by centrifugation and precipitation of the supernatant with cold ethanol. Polysaccharide was then isolated by gel filtration on a TSK HW-50 column. The fraction eluting in the void volume was collected and analysed, whereas the fraction containing low molecular mass material was not considered further. The sugar composition analysis performed on each strain revealed, in all cases, the presence of glucose and galactose in the ratio 1.0:1.4, respectively. The structure of the polysaccharide, determined for the *L. rhamnosus* strain KL37C, was deduced by sugar analysis, methylation analysis and NMR spectroscopy. The D series of the glucose and galactose residues was established by quantitative reaction of a hydrolysate with D-glucose oxidase and D-galactose oxidase, respectively. Methylation analysis of the polysaccharide material showed the presence of 3-substituted galactofuranose (1,3,4-tri-*O*-acetyl-2,5,6-*O*-methyl-hexose derivative found in the methylation analysis), 2-substituted galactofuranose (the 1,2,4-tri-*O*-acetyl-3,5,6-tri-*O*-methyl-hexose derivative found), 3-substituted glucopyranose, 6-substituted glucopyranose and 6-substituted galactopyranose residues in a 0.5:0.4:0.5:1.0:0.9 molar ratio. The same results were obtained from methylation analysis of the EPSs from *L. rhamnosus* strains KL37A and D. These results indicate that the polysaccharide is linear. The NMR results proved this supposition, and also show that the polymer is composed of pentasaccharide repeating units.

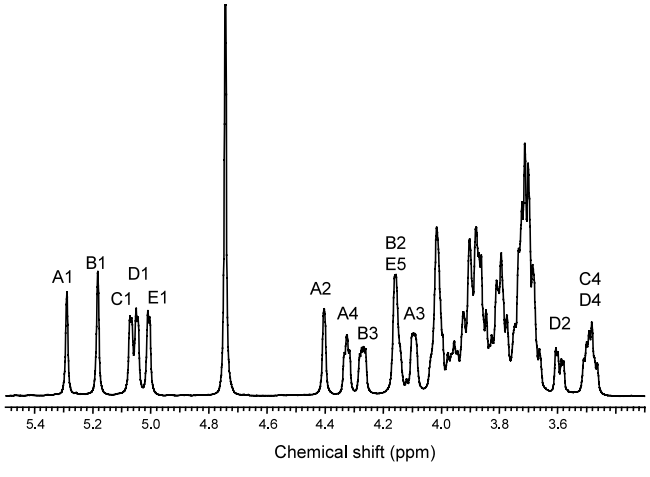

Fig. 1. Partial 500 MHz one-dimensional ^{1}H NMR spectrum of the EPS from *L. rhamnosus* strain KL37C, obtained at 30 °C. The assignments of some resolved peaks are indicated, using the abbreviations for the residues used elsewhere in the text.

The one-dimensional ^{1}H NMR spectrum at 500 MHz (Fig. 1) of the EPS from *L. rhamnosus* strain KL37C contains five proton resonances in the anomeric region (δ 5.00–5.29), designated A–E according to decreasing values of chemical shifts. No highfield anomeric resonance, characteristic of β-linked gluco- or galactopyranosyl residues was observed. Full ^{1}H and ^{13}C assignments (Table 1), and information on the sequence and linkages between the sugars, were obtained from TOCSY, double quantum filtered COSY, HSQC, HSQC–TOCSY, and ROESY spectra. Correlations in the TOCSY and COSY spectra were consistent with anomeric resonances C and D arising from α-Glc*p* residues, with $^{3}J_{H4,H5}$ estimated as 10 Hz. Anomeric E, on the other hand, arises from an α-Gal*p* residue, as suggested by the estimated value of $^{3}J_{H3,H4}$ and the failure to observe a TOCSY or COSY correlation between H-4 and H-5 ($^{3}J_{H4,H5}$ small). Anomeric resonances A and B exhibited small values of $^{3}J_{H1,H2}$ and correlated to anomeric carbon resonances at low field (108.53 and 106.01 ppm, respectively), consistent with their assignment as arising from →3- or →2-substituted β-Gal*f* residues, respectively.[11,12] Assignment of the ^{13}C spectrum was achieved from HSQC spectra optimised for $^{1}J_{C,H}$ of 150 Hz, an HSQC–TOCSY spectrum obtained with a mixing time of 50 ms, and an HSQC spectrum optimised for $^{n}J_{C,H}$ of 20 Hz by increasing the fixed delay to 25 ms.[13] The latter is particularly valuable as it unambiguously identifies the C-5 resonance in pyranose residues (and the C-4 resonance in furanose residues). Use of this approach was described previously[14] and the observed ^{13}C chemical shifts are in agreement with published data.[15]

Table 1
^{1}H and ^{13}C assignments for the EPS from *L. rhamnosus* strain KL37C, at 30 °C

	Residue A β-Gal*f*	Residue B β-Gal*f*	Residue C α-Glc*p*	Residue D α-Glc*p*	Residue E α-Gal*p*
H-1	5.29	5.19	5.07	5.05	5.01
C-1	108.53	106.01	98.07	99.16	98.30
H-2	4.40	4.16	3.72	3.60	3.86
C-2	79.66	86.63	71.29	71.27	68.44
H-3	4.10	4.27	3.85	3.72	3.92
C-3	84.39	75.52	79.23	73.03	69.46
H-4	4.33	4.01	3.50	3.49	4.02
C-4	82.01	82.33	68.02	69.89	69.47
H-5	3.88	3.88	3.81	4.03	4.16
C-5	70.93	70.51	72.44	71.06	69.77
H-6	3.71	3.73	3.90	3.96	3.92
H-6′		3.69	3.81	3.79	3.74
C-6	62.98	62.97	60.70	66.46	67.03

The anomeric configurations of the residues were determined from the ^{1}H and ^{13}C chemical shifts and the values of $^{3}J_{H1,H2}$. For the three pyranose residues, $^{3}J_{H1,H2}$ was about 3.5 Hz, and chemical shift data are consistent with the α anomeric configuration. The coupling to the anomeric resonances of the two galactofuranose residues was unresolved (typically <2 Hz in the β anomeric form and 4 Hz in the α anomeric form[16,17]), and the lowfield shifts of the two C-1 are also indicative of the β anomeric configuration.

The substituted positions within each sugar residue was suggested by the downfield chemical shifts of the ^{13}C resonances, and sequence and linkage deduced from the HSQC experiment optimized for long-range $^{1}H-^{13}C$ correlations (Fig. 2, Table 2). Correlations across the glycosidic linkage were observed between **A** H-1 and **C** C-3, between **B** H-1 and **E** C-6, between **C** H-1 and **B** C-2, between **D** H-1 and **A** C-3, and between **E** H-1 and **D** C-6. Confirmatory correlations between **C** C-1 and **B** H-2 and between **D** H-1 and **A** H-3 were also observed. This implies that the structure of the pentasaccharide repeat unit is

C **B**
→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)

E **D**
-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→3)

A
-β-D-Gal*f*-(1→

The sequence and linkages between the sugars were confirmed with a ROESY spectrum obtained with a mixing time of 150 ms (Table 3). Strong correlations were observed between **A** H-1 and **C** H-3 (and weak correlations to **C** H-2 and **C** H-4), between **B** H-1 and **E** H-6 and H-6′, between **C** H-1 and **B** H-1, **B** H-2, **B** H-3 and **B** H-4, between **D** H-1 and **A** H-1, **A** H-2, **A** H-3 and **A** H-4, and between **E** H-1 and **D** H-6 and **D** H-6′.

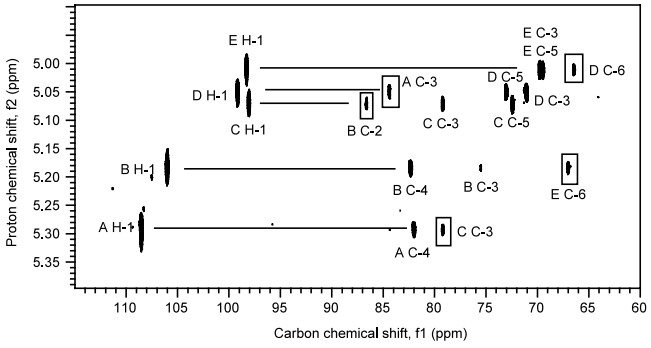

Fig. 2. Partial 500 MHz HSQC spectrum of the EPS from *L. rhamnosus* strain KL37C, optimised for $^{n}J_{C,H}$ of 20 Hz, and obtained at 30 °C. Horizontal traces show correlations to the anomeric protons, and the transglycosidic correlations are highlighted.

Table 2
Correlations involving anomeric atoms observed in the long-range $^{1}H-^{13}C$ HSQC spectra of the EPS from *L. rhamnosus* strain KL37C

Residue	Intra-residue	Inter-residue
A H-1	C-4, C-3 (v. weak)	**C** C-3
B H-1	C-4, C-3 (v. weak)	**E** C-6
C H-1	C-5, C-3	**B** C-2
D H-1	C-5, C-3	**A** C-3
E H-1	C-5, C-3, C-2 (v. weak)	**D** C-6
C C-1		**B** H-2
D C-1		**A** H-3

Table 3
Correlations from anomeric protons atoms observed in the ROESY spectrum of the EPS from *L. rhamnosus* strain KL37C

Residue	Intra-residue	Inter-residue
A H-1	H-2 (TOCSY), H-4 (TOCSY, weak), H-3 (TOCSY)	**C** H-4 (weak), **C** H-2, **C** H-3, **C** H-6, **C** H-6′
B H-1	H-3 (TOCSY), H-2 (TOCSY), H-4 (TOCSY, v. weak)	**E** H-6, **E** H-6′
C H-1	H-3, H-2, H-5 (weak), H-4 (weak)	**B** H-2, **B** H-3, **B** H-4
D H-1	H-3, H-2, H-4	**A** H-3, **A** H-2, **A** H-4, **A** H-5 (weak)
E H-1	H-2, H-3, H-5 (TOCSY, weak), H-4 or **D** H-5?	**D** H-6, **D** H-6′ (weak), **D** H-5

The EPS from *L. rhamnosus* strain KL37C is a neutral heteropolysaccharide containing galactofuranose residues, which are often found as components of *Lactobacillus* EPSs.[8] Strains *L. rhamnosus* KL37A and D have the same EPS structures, as indicated not only by sugar composition and methylation analysis, but also by the proton and the $^{1}H-^{13}C$ heteronuclear correlation spectra of the exopolysaccharide material from *L. rhamnosus* KL37A and D. These are identical to the spectrum of the KL37C EPS. However, composition analysis of the EPS from the KL37B strain indicates that it contains additional rhamnose and glucosamine components: determination of the structure of this polysaccharide is in progress. The studies of surface properties of several human *Lacobacillus* strains revealed that the KL37C strain possesses a distinct surface activity expressed as autoaggregation in physiological saline solution and evident adherence to Caco2 human intestinal cell line derived from enterocytes.[9] As the surface carbohydrate structures of these micro-organisms may participate in the interactions with host tissue as probiotic factors, determination of their structures, and of those from other lactobacilli, may help in understanding the complexity of the probiotic activity of these bacteria.

3. Experimental

3.1. Bacterial strains and isolation of exopolysaccharide

Lactobacillus rhamnosus strains KL37A, C and D were isolated from the feces of newborn. These microorganisms were identified by cell morphology, absence of catalase production, arginine hydrolysis, glucose fermentation and growth at 15 °C and by hybridization with DNA probes specific for bacterial DNA coding for 23S rRNA of *Lactobacillus* species occurring in human flora.[9] The strains were stored at −70 °C in MRS broth supplemented with 10% glycerol. Bacteria were cultivated in MRS (Rogosa) liquid broth (Oxoid) (2.4 dm^3) under anaerobic conditions in the chamber with a BBL Anaero-Pac System (Becton Dickinson), at 37 °C for 48 h. Cells were harvested by centrifugation at 8000 rpm (4 °C, 30 min) and washed twice with PBS. The bacterial mass was suspended in water (10 mL) and sonicated three times for 5 min, in an ice bath. After the centrifugation at 6000 rpm (30 min, 4 °C), the supernatant was centrifuged twice at 12,000 rpm at 4 °C for 1 h and then precipitated with 5 vol of cold EtOH (−20 °C, overnight). The precipitated material was recovered by centrifugation at 12,000 rpm, 4 °C for 20 min and freeze-dried. The resuspended polysaccharide was purified by gel filtration on a column of TSK HW-50 (1.6 × 100 cm) in 0.05 M aq pyridine acetate buffer (pH 5.6). The column eluate was monitored with a Knauer differential refractometer. The first fraction, which eluted in the void volume, contained polysaccharide and was the subject of the present investigation.

3.2. Sugar and methylation analysis

The polysaccharide sample (1 mg) was hydrolysed for sugar analysis with 10 M HCl at 80 °C for 30 min followed by evaporation under a stream of N_2, as previously described.[18] The resulting monosaccharides were converted into alditol acetates[18] and analysed by gas–liquid chromatography–mass spectrometry (GLC–MS), using a Hewlett-Packard 5971A system, equipped with an HP-1 capillary column (0.2 mm × 12 m), and the temperature programmed from 150 to 270 °C at 8 °C/min. The ionisation potential was 70 eV. For methylation analysis, the polysaccharide samples were permethylated according to the method of Hakomori[19] and the product was purified by water–$CHCl_3$ extraction. The methylated polysaccharide was hydrolysed with 0.6 M HCl in 80% AcOH at 80 °C for 18 h as previously described,[20] then reduced with $NaBD_4$ and acetylated for GLC–MS analysis using the conditions described in Ref. 18.

3.3. Determination of the absolute configuration of the monosaccharides

Polysaccharide (1 mg) was hydrolysed with 10 M HCl at 80 °C for 20 min and dried by evaporation under a stream of N_2. The material was treated, respectively with D-glucose oxidase or D-galactose oxidase in the presence of peroxidase, according to procedures described.[21,22]

3.4. NMR spectroscopy

Samples (5 mg) were dissolved in 300 μL of deuterated water (> 99.9% D, Aldrich), lyophilised, redissolved in the same volume of deuterated water, and introduced into a 5 mm susceptibility-matched Shigemi tube. Spectra were recorded on a Varian Unity 500 NMR spectrometer equipped with a 5 mm triple-resonance PFG probe as previously described.[23] Spectra were collected at a nominal probe temperature of 30 °C using standard Varian pulse sequences except for: (i) the use of the HSQC experiment developed by Wider and Wüthrich[13] using pulsed field gradients to suppress unwanted signals; and (ii) the HSQC–TOCSY sequence, an adaptation of the HMQC–TOCSY experiment of Crouch and co-workers[24] to incorporate the HSQC sequence of Wider and Wüthrich. Chemical shifts are referenced against internal TSP-d_4 at 0 ppm (^{1}H) and −1.8 ppm (^{13}C).[25] The ROESY spectrum used a 150 ms 2.5 kHz spin lock generated by continuous low power irradiation, whilst the TOCSY spectrum used a 80 ms 10 kHz spin lock generated using an MLEV17 sequence. The spin lock period in the HSQC–TOCSY spectrum was 50 ms, using a 10 kHz spinlock field generated as before.

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