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Lactobacillus helveticus Lh59 secretes an exopolysaccharide that is identical to the one produced by *Lactobacillus helveticus* TN-4, a presumed spontaneous mutant of *Lactobacillus helveticus* TY1–2 [1]

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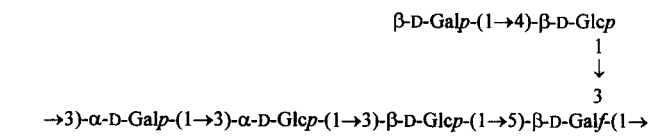
Abstract

Lactobacillus helveticus Lh59 produces a high-molecular-mass exopolysaccharide ($\geq 2 \times 10^6$ Da) when cultured in skimmed milk. Compositional analysis, methylation analysis and NMR experiments (^{1}H and ^{13}C) recorded from the native polysaccharide as well as from oligosaccharides released by partial acid hydrolysis, allowed the complete structural determination of this polysaccharide, which consists of the following hexasaccharide repeating unit:



This structure is identical to the one of an EPS produced by *L. helveticus* TN-4, which was claimed to be a spontaneous mutant of strain TY1–2. © 1997 Elsevier Science Ltd.

Keywords: *Lactobacillus helveticus* Lh59; Exopolysaccharide; Texture

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[1] Preliminary patent and report: [1,2].

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1. Introduction

Interest in exopolysaccharides (EPS) from lactic acid bacteria (LAB) has increased over the last decade in the food industry. Because of their rheological properties they represent an attractive alternative to the currently used thickeners [3,4]. In 1994, we began screening for EPS-producing LAB, including the species *Lactobacillus helveticus*, from our industrial collection containing more than 3500 strains. From 170 *L. helveticus* strains we identified only two EPS-producing microorganisms, both secreting an EPS composed of D-glucose and D-galactose in the molar ratio of 1:1. The structural analysis of the EPS produced by our representative strain *L. helveticus* Lh59, revealed the following repeating unit [1,2]:

→ 3)-α-D-Gal*p*-(1 → 3)-α-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 5)-[β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 3)]-β-D-Gal*f*-(1 → .

It should be noted that in the Lh59 EPS repeating unit, the lactosyl side chain is the only branched sequence attached to the main polysaccharide backbone, a structural property which makes this molecule a 'cluster' of lactose sequences and which may confer this polymer specific biological properties. This potential biological activity prompted us to apply for a patent [1]. At the same time, a report of Yamamoto et al. [5] appeared in this Journal describing the structure of the EPS of *L. helveticus* TY1–2 which contained 2-acetamido-2-deoxy-D-glucose as an additional constitutive residue, and which consisted of the following repeating unit:

→ 6)-β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 6)-[α-D-Gal*p*-(1 → 4)]$_{0.8}$-α-D-Glc*p*NAc-(1 → 3)-[β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 6)]-β-D-Gal*p*-(1 → .

The only similar feature in the two structures was the lactosyl-side chain, which in TY1–2 was linked β-(1 → 6) to a galactopyranose residue, whereas in Lh59 it was linked β-(1 → 3) to a galactofuranose residue. Furthermore, it is important to note that these two EPSs do not share a common backbone and, according to today's understanding of polysaccharide biosynthesis, are therefore produced by a different set of glycosyltransferases. In 1995, Yamamoto et al. [6] published the structure of a new *L. helveticus* EPS, which was identical to our *L. helveticus* Lh59 EPS. Surprisingly though, they claimed that it was produced by a spontaneous mutant of strain TY1–2 named TN-4.

From our studies on the genetics of EPS production in *Streptococcus thermophilus* we know that each monosaccharide of the EPS-repeating unit is transferred by a distinct glycosyltransferase and each glycosyltransferase is encoded by a distinct gene [7]. Hence the mutation from the TY1–2 EPS to the TN-4 EPS would entail a simultaneous mutation in at least five to six genes coding for the glycosyltransferases and probably one to three genes involved in the polymerization and export of the EPS. TN-4 was obtained from a vial stock of strain TY1–2 stored for 1 year at −19 °C in 1.5% yeast extract, 1% lactalbumin hydrolysate, and 2% glucose. Since to our knowledge there is no report of spontaneous mutations arising under these types of conditions, such drastic multi-locus mutations seem highly improbable, if not impossible. Given the fact that Yamamoto et al. provided no evidence that TN-4 is actually a mutant derived from TY1–2, it seems more reasonable to assume that *L. helveticus* TY1–2 and TN-4 coexisted as two independent strains in a common vial-stock and TN-4 was only detected later. We report the structural characterization of the *L. helveticus* Lh59 EPS to highlight the fact that this type of EPS is actually strain specific and not a mutated TY1–2-type EPS. This consideration is of particular relevance for a multidisciplinary field such as carbohydrate engineering where the competences from microbiologists, geneticists and chemists have to converge.

2. Results and discussion

Fast protein liquid chromatography (FPLC) and compositional analysis of the EPS.—After 24 h of a one liter fermentation the EPS was extracted and yielded 272 mg of polysaccharide (83.3% of dry weight). FPLC analysis on Superose 6 showed that the polysaccharide was eluted at the exclusion limit of approximately 2×10^6 Da (data not shown). After acid hydrolysis of the EPS, the monosaccharides were subjected to derivatization as *O*-methyloxime acetates and analyzed by GLC. The presence of D-galactose and D-glucose in a molar ratio 1:1 were detected.

NMR spectroscopy.—The 400 MHz ^{1}H NMR spectrum of the native polysaccharide recorded in Me_2SO-d_6 at 80 °C showed 4 signals at 5.201, 5.158, 4.568, and 4.350 ppm, which are characteristic of anomeric protons in a ratio of 1:2:2:1 and thus suggest a hexasaccharide repeating unit (Table 1). This was confirmed by the 100 MHz ^{13}C spectrum that

Table 1
NMR chemical shifts for the EPS from *L. helveticus* Lh59 in Me_2SO-d_6 at 80 °C (internal standard: acetone)

Assignment	Residue					
	β-Gal*f* **A**	β-Glc*p* **B**	α-Glc*p* **C**	α-Gal*p* **D**	β-Glc*p* **E**	β-Gal*p* **F**
^1H (ppm)						
H-1	5.158	4.568	5.201	5.158	4.568	4.350
H-2	4.240	3.289	3.542	3.888	3.206	3.470
H-3	4.419	3.532	3.752	3.776	3.488	3.470
H-4	4.125	3.533	3.512	3.999	3.533	3.796
H-5	4.118	3.354	3.920	4.108	3.455	3.582
H-6a	3.723	3.84	3.61	3.62	3.90	3.67
H-6b	3.62	3.66	3.61	3.62	3.70	3.67
^{13}C (ppm)						
C-1	108.95	103.08	99.87	100.26	102.52	103.74
C-2	80.52	72.72	71.19	68.33	73.47	71.19
C-3	85.08	85.15	82.90	77.31	74.90	75.32
C-4	81.64	70.14	70.20	69.19	80.19	68.69
C-5	78.84	76.43	72.48	71.27	73.52	75.80
C-6	61.68	61.38	61.07	61.26	61.12	60.99

exhibited six anomeric carbon signals (108.95, 103.74, 103.80, 102.52, 100.26, and 99.87 ppm) (Table 1). Differences between the ^1H assignments carried out here and those previously performed by Yamamoto et al. [6] arise from the use of different solvents, namely Me_2SO-d_6 in the present study and D_2O in Ref. [6].

According to the ^1H spin system, on individual monosaccharide units depicted in the two-steps relayed COSY spectrum, nearly all the monosaccharides were identified: β-D-Gal*f* (residue **A**), β-D-Glc*p* (residues **B** and **E**), α-D-Glc*p* (residue **C**), and α-D-Gal*p* (residue **D**). Further examination of the relayed COSY spectra of oligosaccharide alditols IVB and IIA obtained by partial acid hydrolysis (see below) led to the obvious identification of β-D-Gal*p* (residue **F**). As indicated by the COSY spectrum of the native polysaccharide, H-2 and H-3 of this β-D-Gal*p* **F** residue exhibited a strong coupling constant which did not allow to analyze their multiple patterns. Correlation peaks observed in the ^1H–^{13}C heteronuclear COSY spectrum showed that one of the proton resonances (5.158 ppm) was connected to the carbon resonance deshielded at 108.95 ppm, which proved a β-anomeric configuration for the Gal*f* **A** residue. Most of the proton resonances were assigned in the homonuclear COSY spectrum except for the H-5 and H-6 spin systems of the β-D-Gal*p* **F** residue. The carbon resonances were assigned by direct correlation to their attached protons. The two remaining unassigned carbons at 73.52 and 61.12 ppm were deduced to correspond to the C-5 and C-6 resonances of the β-D-Gal*p* **F** residue.

In summary, these assignments provided the substitution pattern of each monosaccharide unit according to their downfield shifted carbon resonances: C-3 and C-5 for β-D-Gal*f* **A**, C-3 for α-D-Glc*p* **C**, C-3 for α-D-Gal*p* **D**, C-3 for β-D-Glc*p* **B**, and C-4 for β-D-Glc*p* **E**.

The sixth sugar unit β-D-Gal*p* **F**, which did not possess any downfield shifted ^{13}C resonance, occurred in a non-reducing position.

Methylation analysis.—The NMR results were supported and confirmed by GLC–MS analysis of the partially methylated alditol acetates and methyl glycosides obtained from the permethylated EPS (Table 2). In fact, the GLC–MS analysis demonstrated the presence of a terminal galactopyranosyl residue, a 3-linked glucopyranosyl residue, a 4-linked glucopyranosyl residue, a 3-linked galactopyranosyl residue, and a 3,5-linked galactofuranosyl residue in a ratio of 1:2:1:1:1.

Table 2
Methylation analysis of the native EPS from *L. helveticus* Lh59

Derivative	Molar ratio	
	Partially methylated alditol acetates	Partially methylated methyl glycosides
2,3,4,6-Me_4Gal	1	1
2,4,6-Me_3Glc	2.2	1.9
2,3,6-Me_3Glc	0.9	1.3
2,4,6-Me_3Gal	0.7	1.6
2,6-Me_2Gal	1	0.9

Table 3
NMR chemical shifts of the oligosaccharide-alditols obtained by partial acid hydrolysis of the EPS from *L. helveticus* Lh59 in D_2O at 353 K

Assignment ^1H (ppm)	Residues in IVA			Residues in IVB			Residues in IIA				
	Gal-ol **A**	β-Glc*p* **E**	β-Gal*p* **F**	Gal-ol **A**	β-Glc*p* **B**	β-Glc*p* **E**	Gal-ol **A**	β-Glc*p* **B**	α-Glc*p* **C**	α-Glc*p* **D**	β-Gal*p* **E**
H-1a	3.77	4.686	4.557	3.77	4.539	4.428	3.77	4.707	5.340	4.590	4.460
H-1b	3.77	–	–	3.77	–	–	3.77	–	–	–	–
H-2	4.118	3.328	3.320	4.056	3.421	3.541	4.120	3.423	3.559	3.379	3.542
H-3	4.141	3.500	3.484	3.915	3.661	3.661	4.148	3.647	3.748	3.647	3.665
H-4	3.907	3.424	3.406	3.797	3.674	3.927	3.911	3.748	3.463	3.678	3.928
H-5	4.301	3.543	3.453	4.149	3.62	3.732	4.299	3.47	4.025	3.590	3.73
H-6a	3.77	3.899	3.899	3.68	4.02	3.79	3.77	3.97	3.80	3.90	3.79
H-6b	3.77	3.758	3.758	3.68	3.80	3.79	3.77	3.85	3.80	3.75	3.79

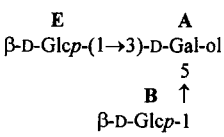
Table 4
Methylation analysis of oligosaccharide-alditols (partially methylated and acetylated methyl glycosides) IVA, IVB and IIA obtained by partial acid hydrolysis of the native EPS

Derivative	Molar ratio		
	IVA	IVB	IIA
1,2,4,5,6-Me_5Gal-ol	–	1	–
1,2,4,6-Me_4Gal-ol	1	–	1
2,3,4,6-Me_4Glc	1.2	–	1.0
2,3,4,6-Me_4Gal [a]	–	1.4	0.9
2,4,6-Me_3Glc	–	–	1.3
2,3,6-Me_3Glc	–	1.0	1.2

[a] Due to its high volatility the value is lower than expected.

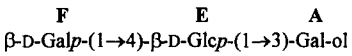
Partial acid hydrolysis.—In order to elucidate the position of the branched terminal galactosyl residue, oligosaccharides were produced by partial acid hydrolysis of the native polysaccharide. Six major fractions were separated by gel filtration chromatography on Fractogel HW40 F. Two of them (fractions II and IV) were subjected to HPAEC-PAD (data not shown), and the structures of the subfractions, denoted IIA, IVA and IVB were investigated both by NMR (Table 3) and methylation analysis (Table 4).

Oligosaccharide-alditol IVA contained two Glc residues and one hexitol residue, as shown by the ^1H spin system depicted in the two-steps relayed COSY spectrum. The two Glc residues occurred at the non-reducing terminal position, as confirmed by the methylation analysis which identified 2,3,4,6-tetra-*O*-methylglucitol. The last derivative showed a pattern corresponding to a O-3 and O-5 substitution characteristic of a furanose ring form. From the previous NMR data, this hexitol originated from the β-D-Gal*f* **A** unit. These findings led to the following structure:



Due to the symmetry displayed by the ^1H spin system of galactitol, the ^1H NMR assignment of the compound was achieved after the elucidation of the structure of compound IVB.

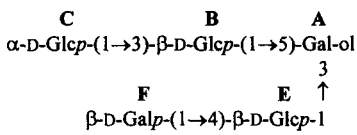
Oligosaccharide-alditol IVB contained β-D-Gal*p* and β-D-Glc*p* in the molar ratio 1:1, as shown by the pattern of the vicinal coupling constants. The methylation analysis (Table 4) indicated the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose and 1,2,4,5,6-penta-*O*-methylgalactitol. Therefore, oligosaccharide-alditol IVB possesses the following structure:



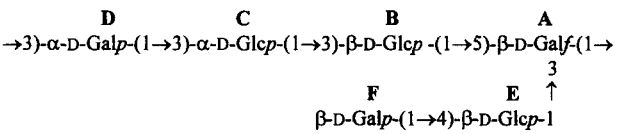
The attachment of β-D-Glc*p* at O-3 of Gal-ol was not susceptible to modify dramatically the chemical shift of the H-1 and H-2 resonances of the hexitol, when compared to the NMR data from compound IVA. Therefore, the two signals at 3.77 and 4.056 ppm could be assigned to H-1 and H-2 of Gal-ol, whereas the H-5 and H-6 resonances were upfield shifted at 4.149 and 3.68 ppm by comparison with compound IVA (Table 3). In the NMR spectra of IVA and IVB, the H-5 resonance of Gal-ol was the most upfield shifted resonance of the molecule.

Oligosaccharide-alditol IIA contained two β-D-Glc*p*, one β-D-Gal*p*, and one α-D-Glc*p* residues (Table 3), whereas the presence of O-3- and O-5-substituted Gal-ol could easily be deduced by the similarity in chemical shifts of H-1 to H-6 of Gal-ol in

compound IVA. Since the two β-D-Glc*p* units and the non-reducing terminal β-D-Gal*p* residue have already been located in IVA and IVB, and given the fact that the β-D-Glc*p* **B** residue was substituted at O-3, the oligosaccharide-alditol IIA has the following structure:



The sixth monosaccharide unit, an α-D-Gal*p* residue, was not observed in the products of the partial hydrolysis. However, the facts that the α-D-Glc*p* unit **C** was substituted at O-3, and that the only β-D-Gal*p* unit of the polysaccharide occupied the non-reducing position (see NMR data) permitted the conclusion that α-D-Gal*p* is attached to the α-D-Glc*p* residue. Therefore, the structural unit of the polysaccharide was defined as follows:



3. Experimental

Fermentation conditions.—*Lactobacillus helveticus* Lh59 was a ropy strain from the Nestlé strain collection. From 168 *L. helveticus* strains of the Nestlé collection, only 2 strains produced exopolysaccharides (EPS). The growth medium, which consisted of 10% reconstituted skimmed milk sterilized at 115 °C for 35 min, was inoculated with 1% of a preculture. Fermentations were carried out in a one liter-scale fermenter with a magnetic stirrer (60 rpm) for 24 h at 40 °C, and regulated at pH 5.5 with 2 M NaOH.

Extraction of the polysaccharide.—An equal volume of CCl_3CO_2H (40%) was added to the spent culture to remove proteins by precipitation. After centrifugation (17,000 g, 20 min), the same volume of acetone was added to the supernatant. The precipitated polysaccharides were recovered by centrifugation (17,000 g, 20 min) and dissolved in distilled water. Then, the pH of the EPS solution was adjusted to 7.0 with 1 M NaOH. After dialysis against distilled water for 24 h, insoluble substances were removed by ultracentrifugation (110,000 g, 1 h) and the EPS solution was lyophilized to obtain the crude dehydrated EPS. The total neutral sugar content expressed as glucose equivalents was determined by the phenol–H_2SO_4 method [8].

Molecular mass of the exopolysaccharide.—Gel-permeation chromatography, using a Superose 6 column (1.0 cm × 30 cm) connected to a FPLC system (Pharmacia), was conducted to confirm the purity and estimate the molecular mass of the EPS. Samples (200 μL) containing 200–400 μg glucose equivalents were loaded onto the column and eluted with 50 mM phosphate buffer at pH 7.2 at the flow rate of 0.5 mL/min. 1 mL fractions were collected and the glucose equivalents in each tube were determined [8].

Monosaccharide composition.—The monosaccharide composition was analyzed using GLC from *O*-methyloxime acetate derivatives obtained after acid hydrolysis of the EPS in 4 M CF_3CO_2H [9].

Methylation analysis.—Samples (native polysaccharide and oligosaccharide-alditols) were permethylated as previously described [10]. The methylated products were subjected either to methanolysis (methanolic 0.5 M HCl, 24 h, 80 °C) or acid hydrolysis (4 M CF_3CO_2H, 4 h, 100 °C) followed by reduction with $NaBD_4$. The partially methylated and acetylated (1:1 pyridine–Ac_2O) methyl glycosides and alditols were identified by GLC (BP1 column) and GLC–MS in EI mode on a Nermag R10-10 S mass spectrometer using an electron energy of 70 eV and an ionizing current of 0.2 mA.

Partial acid hydrolysis.—EPS (40 mg) was hydrolyzed in 4 mL 0.5 M CF_3CO_2H for 90 min at 100 °C. Complete hydrolysis and formation of low-molecular-mass oligosaccharides were monitored by TLC on Silica Gel 60 F254 aluminum sheets (Merck) using 2:1:1.5 BuOH–H_2O–HOAc and detection with orcinol–H_2SO_4.

HPAE - PAD Chromatography.—Fractionation of HW40 peaks was performed on a HPAE-PAD Dionex LC system consisting of a Dionex Bio-LC quaternary gradient module, a model PAD 2 detector, and a CarboPac PA-1 pellicular anion-exchange column (250 × 9 mm). The two elution programs had the following ratios of eluent A (0.1 M NaOH) and B (0.1 M NaOH containing 1 M NaOAc) at room temperature and a flow rate of 3 mL/min: 1, 99:1 for 0.2 min then to 65:35 in 60 min; 2, 98:2 then to 70:30 in 60 min. The eluted fractions were immediately neutralized with 1 M HOAc and lyophilized. The fractions were successively desalted on a column

(6 × 1 cm) of Dowex 50 × 8 (H^+) resin and on a column of Fractogel (55 × 2 cm) using deionised water as eluent.

^{1}H-Nuclear magnetic resonance spectroscopy.— For ^{1}H NMR measurements, the deuterium-exchanged polysaccharide was dissolved in Me_2SO-d_6 (10 mg/mL, Aldrich) while deuterium-exchanged oligosaccharides were dissolved in 0.5 mL D_2O (99.96% atom ^{2}H, Aldrich). The 400 MHz ^{1}H NMR experiments were performed with a Bruker AM-400WB spectrometer, equipped with a 5 mm ^{1}H–/^{13}C mixed probe head, operating in the pulsed Fourier transform mode and controlled by an Aspect 3000 computer (Centre Commun de Mesures, Université de Lille Flandres Artois). All the spectra were obtained at a probe temperature of 353 K. One dimensional spectra were obtained with a spectral width of 3000 Hz for a 16 K frequency-domain points and time-domain data points giving a final digital resolution of 0.365 Hz/point.

The 100 MHz ^{13}C NMR experiments were obtained with the standard Bruker pulse program Powgate with ^{1}H composite pulse decoupling. The spectral width was 22.727 Hz for a 32 K frequency-domain data points and time-domain data giving a final digital resolution of 1.387 Hz/point; a ninety-degree pulse (6 μs) and 1 s recycle delay were used. The chemical shifts are given relative to the signal of the methyl group of acetone (2.225 for ^{1}H and 31.55 for ^{13}C).

The 2D-homonuclear COSY 45, COSY with simple, double and triple relay transfers were performed by use of the standard Bruker pulse program library or the programs given by B. Perly (C.E.A., Saclay). For all RCT experiments, refocusing delays of 35 ms were chosen and the relaxation delay was 2 s. In all these experiments, the spectral width was 1840 Hz, the ^{1}H ninety-degree pulse was 10.6 μs; 256 W × 2K data matrices were acquired, which were zero-filled prior to Fourier transform, to obtain a 1 K × 2 K spectral data matrix and a sine-bell squared function was used in both dimensions.

The 2D-^{13}C/^{1}H COSY experiments were performed with simultaneous suppression of ^{1}H homonuclear couplings by use of the standard Bruker pulse program XHCORRD. Refocusing delays were adjusted to an average $^1J_{C,H}$ coupling constant of 150 KHz. ^{1}H and ^{13}C ninety-degree pulse width were 10.6 and 6 μs. The relaxation delay was 0.8 s. 128 W × 4 K data matrix was acquired, which was zero-filled prior to Fourier transform, to obtain a 512 W × 4 K spectral data matrix. An exponential function (LB = 1 Hz) for ^{13}C subspectra and a sine-bell function for ^{1}H spectra were applied to enhance the signal to noise ratio.

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