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Structure and properties of the exopolysaccharide produced by *Streptococcus macedonicus* Sc136

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***Streptococcus macedonicus* is a Gram positive lactic acid bacterium that is part of the starter flora present in Greek sheep and goat cheeses. The *S. macedonicus* Sc136 strain produces a high-molecular-mass, highly texturizing exopolysaccharide composed of D-glucose, D-galactose, and *N*-acetyl-D-glucosamine in the molar ratio of 3:2:1. The structure of the exopolysaccharide produced by *S. macedonicus* Sc136 was determined by chemical analysis, mass spectrometry, and nuclear magnetic resonance spectroscopy. The repeating unit was shown to be:**

β-D-Gal*f*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc
1
↓
3
→4)-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→

The polysaccharide sidechain β-D-Gal*f*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc is a key factor in the highly texturizing properties of the *S.macedonicus* Sc136 exopolysaccharide. Finally, the trisaccharide sequence β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p* corresponds to the internal backbone of the lacto-*N*-tetraose and lacto-*N*-neotetraose units, which serve as a structural basis for the large majority of human milk oligosaccharides, an additional property offering an important potential for the development of improved infant nutrition products.

Key words: exopolysaccharide/lactic acid bacteria/nuclear magnetic resonance spectroscopy/*Streptococcus macedonicus*

Introduction

Exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) present texturizing properties combined with a GRAS (Generally Recognized As Safe) status (Sandford and Bard, 1983; Sutherland, 1998). Studies of the structure of bacterial EPSs of the types *Streptococcus* (Doco *et al.*, 1990; Bubb *et al.*, 1997; Lemoine *et al.*, 1997; Faber *et al.*, 1998; Stingele *et al.*, 1999), *Lactococcus* (Gruter *et al.*, 1992; Nakajima *et al.*, 1992; Marshall *et al.*, 1995; van Casteren *et al.*, 2000) and *Lactobacillus* (Kooiman, 1968; Mukai *et al.*, 1990; Gruter *et al.*, 1993; Yamamoto *et al.*, 1994, 1995; Robijn *et al.*, 1995a,b, 1996a,b; Staaf *et al.*, 1996; Stingele *et al.*, 1997; Staaf *et al.*, 1996) have shown a large structural diversity related to differences in texturizing properties. In addition to a large number of glycoconjugates and oligosaccharides, polysaccharides have also been shown to have health-beneficial properties such as the modulation of the immune system (Oda *et al.*, 1982; Nakajima *et al.*, 1995) and anti-ulcer activities (Nagaoka *et al.*, 1994).

Streptococcus macedonicus Sc136 is a Gram positive LAB that is part of the starter flora present in Greek sheep and goat cheeses. Recently, in a phylogenetic study based on 16S rRNA sequence comparison, it has been described as a new species differing from *Streptococcus thermophilus* (Tsakalidou *et al.*, 1998). *S. macedonicus* Sc136 is a strain capable of both mesophilic and thermophilic growth, that is, growth is sustained between 25°C and 42°C. It produces a high-molecular-mass, highly texturizing EPS composed of glucose (Glc), galactose (Gal), and *N*-acetylglucosamine (GlcNAc). Considering the texturizing properties of the EPS, the analysis of the EPS structure will contribute to the understanding of the relationship between rheological properties of EPSs and their structure. We present the production, isolation, and the structural determination of the EPS produced by *S. macedonicus* Sc136. The potential of the EPS, which was shown to contain a milk oligosaccharide related fragment, for the development of improved infant nutrition products will be discussed.

According to recent proposals (Editorial, 1997, 1999; Stubbs, 1997; White, 1997; Zyzik and Goldmann, 1999), authorship contributions are provided: S.J.F.V. collected data, coordinated study, managed data, analyzed data, interpreted data, wrote and edited paper; E.J.F. collected data, analyzed data, interpreted data, edited paper; J.R.N. conceived study, selected bacterial strains, secured funding, edited paper; F.S. investigated bacterial strains, collected part of data, advised on the study, edited paper; J.P.K. designed the study, secured funding, coordinated study, interpreted data, edited paper.

Results

Production, purification, and composition of native exopolysaccharide

The native EPS produced by *S. macedonicus* Sc136 in 10% reconstituted skim milk medium (n-EPS) was isolated by TCA

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precipitation followed by acetone precipitation. A further purification was carried out by size-exclusion chromatography (Superose 6), yielding a similar carbohydrate content for all molecular weights ranging from very large (>2×10^6 Da) to small (<1×10^4 Da). The high-molecular-mass fractions (>1×10^5 Da) were found to have Glc, Gal, and GlcNAc in a constant molar ratio of 3:2:1. These fractions were pooled and lyophilized to yield about 8 mg/l of n-EPS (expressed in milligrams of glucose equivalents per liter of medium). Absolute configuration determination showed for each monosaccharide the D configuration. The low-molecular-mass fractions (< 1×10^5 Da) probably contained contaminating material from the fermentation broth, including lactose.

Composition analysis of degraded exopolysaccharide probes

Mild acid hydrolysis of n-EPS (0.3 M TFA; 20 min, 100°C) and subsequent fractionation on a size-exclusion chromatography column (Bio-Gel P-2) gave a degraded polysaccharide material (hyd-PS) with a monosaccharide composition of D-Glc, D-Gal, and D-GlcNAc in a molar ratio of 3:1:1.

De-*N*-acetylation and deamination of n-EPS followed by fractionation on a size-exclusion chromatography column (Bio-Gel P-2) resulted in the isolation of a degraded polysaccharide (deNAc-PS) and an oligosaccharide fraction (deNAc-oligo). Monosaccharide analysis of deNAc-PS showed the presence of D-Glc and D-Gal in a molar ratio of 2:1. Reduction of the deNAc-oligo fraction and subsequent MALDI-TOF measurements yielded two $[M+Na]^+$ pseudomolecular ions at *m/z* 512 and 350 compatible with the presence of Hex_2anhydroHex-ol-*1-d* and HexanhydroHex-ol-*1-d*, respectively. Monosaccharide analysis of reduced deNAc-oligo revealed the presence of D-Glc and D-Gal in a molar ratio of 1.7:1 and anhydroHex-ol-*1-d*, which in view of the presence of D-GlcNAc in n-EPS was identified as being 2,5-anhydroMan-ol-*1-d*. Combining these results, reduced deNAc-oligo was constituted of (GalGlc)-2,5-anhydroMan-ol-*1-d* and Glc-2,5-anhydroMan-ol-*1-d* in a molar ratio of 1:0.7. The formation of the disaccharide compound was due to the acid lability of the Gal*f*→Glc linkage (see below).

Methylation analysis

Methylation analyses were performed on n-EPS, hyd-PS, and deNAc-PS (Table I). The methylation analysis data of n-EPS indicated the presence of a branched hexasaccharide repeating unit containing five internal monosaccharides (for a proof of the pyranose rings, see NMR section); the Gal*p* residue forms a branching point, whereas the Gal*f* residue occurs in a terminal position. Comparison of the methylation analysis data of n-EPS and hyd-PS showed the disappearance of terminal Gal*f* and the complete shift of 6-substituted Glc*p* into terminal Glc*p*, demonstrating the occurrence of a terminal Gal*f*-(1→6)-Glc*p* fragment. Methylation analysis of deNAc-PS suggested the presence of a linear trisaccharide repeating unit built up from two 4-substituted Glc*p* residues and one 4-substituted Gal*p* residue (for a proof of the pyranose rings, see NMR section), thereby indicating that the GlcNAc residue is part of the sidechain.

Based on the monosaccharide and linkage analysis of the three polysaccharide probes, and taking into account pyranose ring forms for all internal monosaccharide residues (see NMR section), at this stage a tentative proposal for the structure of n-EPS can be made:

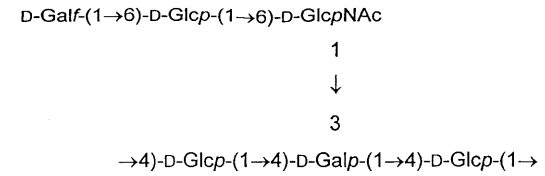


Table I. Methylation analysis data of n-EPS, hyd-PS and deNAc-PS

Derivative[a]	Monosaccharide	Molar ratios		
		n-EPS	hyd-PS	deNAc-PS
2,3,5,6-Gal	Gal*f*-(1→	1	—	—
2,3,6-Glc	→4)-Glc*p*-(1→	2	2	2
2,3,4-Glc	→6)-Glc*p*-(1→	1	—	—
2,6-Gal	→3,4)-Gal*p*-(1→	1	1	—
2,3,4-GlcNAc	→6)-Glc*p*NAc-(1→	1	1	—
2,3,4,6-Glc	Glc*p*-(1→	—	1	—
2,3,6-Gal	→4)-Gal*p*-(1→	—	—	1

[a]Partially methylated alditol acetates have been analyzed by GLC and GLC-EIMS. 2,3,5,6-Gal = 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-D-galactitol-*1-d*, etc. . . .

1D NMR spectroscopy

The 1D ^{1}H NMR spectra of the three polysaccharides n-EPS, hyd-PS and deNAc-PS (Figure 1) showed that even at 67°C, all ^{1}H NMR spectra presented broad lines (Table II) resulting from high viscosity. However, going from n-EPS to hyd-PS to deNAc-PS, a sharpening of the signals is observed, reflecting the trimming of the sidechains.

The 1D ^{1}H NMR spectrum of n-EPS (Figure 1a) has five anomeric proton resonances with relative integrals 1:1:1:2:1 confirming the hexasaccharide repeating unit; two anomeric resonances overlap between 4.58 ppm and 4.57 ppm. The six Hex components of the n-EPS repeating unit were designated **A** to **F** following decreasing anomeric proton chemical shifts. Ring forms including anomeric configurations were deduced from H-1 chemical shifts and one-bond C-1, H-1 scalar couplings measured on the ^{13}C-^{1}H HMBC spectrum. For residue **A** ($\delta_{\mathbf{A}(H\text{-}1)}$ 5.11; $^1J_{C1H1}$ 179 Hz) a furanose ring was identified without it

Table II. ^{1}H linewidths measured in 1D spectra (**Figure 1**) for n-EPS, hyd-PS, and deNAc-PS

Linewidth (Hz)	n-EPS	hyd-PS	deNAc-PS
Aliphatic[a]	15	14	10
Methyl[b]	10	9	—[c]

[a]Aliphatic proton **B**(H-1) signal at 4.97 p.p.m.
[b]*N*-Acetyl methyl group singlet at 2.08 p.p.m.
[c]After chemical de-*N*-acetylation and deamination of n-EPS, GlcNAc is no longer present.

being possible to determine the anomeric configuration of the residue on this basis alone (Bock and Pedersen, 1983), whereas residue **B** ($\delta_{B(H-1)}$ 4.97; $^1J_{C1H1}$ 173 Hz) correlated with an α-Hex*p* residue. For units **C** to **F**, the high-field H-1 chemical shifts ($\delta_{C(H-1)}$ 4.76 to $\delta_{F(H-1)}$ 4.54) and the low $^1J_{C1H1}$ values (all around 163 Hz) corresponded with β-Hex*p* residues. An *N*-acetyl methyl signal observed at 2.08 ppm belonged to the GlcNAc residue.

In the 1D ^{1}H NMR spectrum of deNAc-PS (Figure 1c) three separated H-1 resonances with relative integrals 1:1:1 were found. The three monosaccharide units, denoted **B**, **D**, and **F** by analogy with the n-EPS resonances have δ_{H-1} values of 4.93, 4.53, and 4.48, respectively (Table III). No *N*-acetyl methyl signal was observed.

2D NMR spectroscopy of n-EPS

A set of standard polysaccharide NMR experiments were recorded on n-EPS at 67°C: DQF-COSY, TOCSY with different mixing times (Figure 2), NOESY with different mixing times, HSQC (Figure 3a), and HMBC (Figure 4). The HSQC spectrum (Figure 3a) contained six cross-peaks in the anomeric region, confirming the size of the proposed hexasaccharide repeating unit. The ^{1}H and ^{13}C NMR assignments for n-EPS at 67°C are collected in Table III.

The ^{1}H assignment of n-EPS started from the H-1 resonances of each residue **A** to **F** in the TOCSY spectra recorded with increasing mixing times (10 to 80 ms; Figure 2). Connectivities from H-1 to H-2,3,4 were traced for all residues, but due to overlap of the three resonances **D**(H-1), **E**(H-1), and **F**(H-1) (4.58–4.54 ppm) and their linewidths on the order of the chemical shifts difference (LW ~ 20 Hz = 0.03 ppm for Δδ 0.04 ppm), some uncertainties could not be resolved on the basis of the TOCSY data alone. Additional assignments and confirmation of assignments were obtained from NOESY cross-peaks and by correlating the ^{1}H resonances to the corresponding ^{13}C resonances in the HSQC spectrum, while simultaneously assigning the ^{13}C chemical shifts. The carbon position(s) involved in a glycosidic bond were inferred from the ^{13}C chemical shifts by identifying significant shifts (>5 ppm) towards low-field compared to standard monosaccharide methyl glycoside references (Bock and Thøgersen, 1982; Bock and Pedersen, 1983; Bock *et al.*, 1983).

For residue **A** (Hex*f*), the assignments of H-2,3,4 followed from TOCSY experiments; H-5,6a,6b were identified following **A**(H-2,3,4) traces in the TOCSY spectra and from the HSQC. The ^{13}C chemical shifts of residue **A** were found to be virtually identical to those of β-D-Gal*f*1Me (average chemical shift difference $\langle\Delta\delta\rangle$ = 0.5 ± 0.2 ppm) (Bock *et al.*, 1983). Furthermore, comparison of the complete set of ^{13}C chemical shifts with the chemical shifts from α-D-Gal*f*1Me ($\langle\Delta\delta\rangle$ = 1.2 ± 1.5 ppm) unambiguously proved the β anomeric configuration.

Table III. ^{1}H and ^{13}C NMR chemical shifts of two polysaccharides, n-EPS and deNAc-PS

		Monosaccharide	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b	Nac CH_3
n-EPS	**A**	β-D-Gal*f*-(1→	5.11	4.17	4.12	4.05	3.89	3.76	3.72	
			109.1	82.1	78.0	84.2	72.0	63.9		
	B	→4)-α-D-Glc*p*-(1→	4.97	3.59	3.88	**3.73**	4.32	4.03	3,95	
			99.7	72.7	72.3	**79.6**	71.2	61.1		
	C	→6)-β-D-Glc*p*NAc-(1→	4.76	**3.71**	3.59	3.47	3.64	**3.90**	**4.25**	2.08
			103.9	**56.7**	75.3	71.5	76.0	**70.2**		83.4
	D	→4)-β-D-Glc*p*-(1→	4.58	3.47	3.73	**3.72**	3.68	3.86	3.93	
			104.2	74.1	75.1	**80.0**	75.8	61.2		
	E	→6)-β-D-Glc*p*-(1→	4.57	3.37	3.52	3.46	3.63	**3.77**	**4.09**	
			103.5	74.2	77.0	71.0	76.0	**68.1**		
	F	→3,4)-β-D-Gal*p*-(1→	4.54	3.72	**3.84**	**4.28**	3.81	3.93	3.86	
			104.3	71.8	**81.6**	**76.3**	76.8	62.6		
deNAc-PS	**B**	→4)-α-D-Glc*p*-(1→	4.93	3.59	3.86	**3.66**	4.19	3.84	3.84	
			100.7	72.6	72.3	**79.8**	71.7	60.9		
	D	→4)-β-D-Glc*p*-(1→	4.53	3.37	3.66	**3.65**	3.61	3.89	3.97	
			103.2	74.0	75.3	**79.7**	75.8	61.2		
	F	→4)-β-D-Gal*p*-(1→	4.48	3.57	3.71	**4.03**	3.77	3.82	3.89	
			104.2	72.0	73.3	**78.4**	76.3	61.3		

Values were determined in ^2H_2O at 67°C and are given in ppm relative to external [^{13}C-1]glucose ($\delta_{H-1(\alpha)}$ 5.15 and $\delta_{C-1(\alpha)}$ 92.90). Top, *Streptococcus macedonicus* Sc136 native EPS (n-EPS) and, bottom, de-*N*-acetylated and deaminated *S. macedonicus* polysaccharide (deNAc-PS). Chemical shifts highlighted in bold typeface indicate positions at which a glycosidic linkage was identified based on significant differences (Δδ > 5 p.p.m.) with corresponding reference chemical shifts (Bock and Thøgersen, 1982; Bock and Pedersen, 1983; Bock *et al.*, 1983).

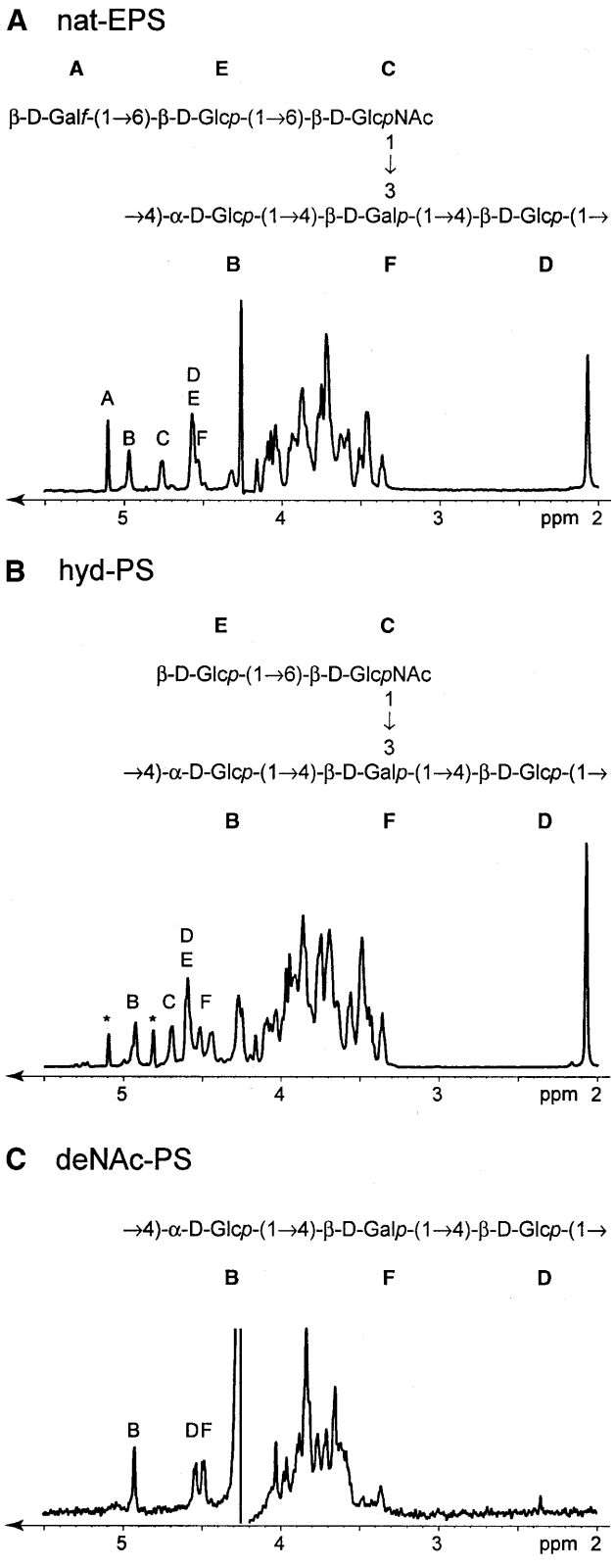


Fig. 1. Structures and 1D ^{1}H NMR spectra of (**a**) n-EPS, (**b**) hyd-PS, and (**c**) deNAc-PS. All spectra were recorded in ^2H_2O at 600 MHz and 67°C. Anomeric (H-1) resonances are identified by a residue letter code (**A** to **F**) as shown on the corresponding structure. Peaks marked with an asterisk derived from contaminants.

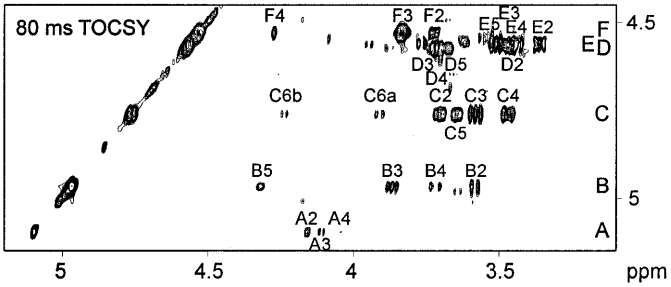


Fig. 2. Anomeric region from TOCSY of n-EPS recorded with a mixing time of 80 ms in ^2H_2O at 600 MHz and 67°C. Cross-peaks in the six anomeric protons traces are indicated with the residue letter code (**A** to **F**) and the corresponding position number (**1** to **6**).

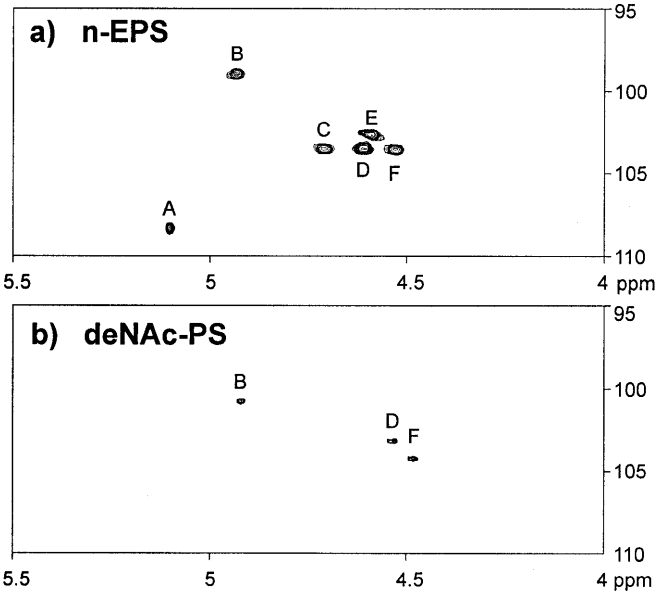


Fig. 3. Anomeric HSQC region of (**a**) n-EPS and (**b**) deNAc-PS, recorded in ^2H_2O at 600 MHz and 67°C. (C-1, H-1) cross-peaks are marked with the residue letter code (**A** to **F**).

Therefore, residue **A** was identified as a terminal β-D-Gal*f*-(1→ unit.

TOCSY measurements yield an assignment for H-2,3,4,5 of residue **B** (α-Hex*p*), while the assignments of **B**(H-6a,6b) resulted from **B**(H-2,3,4) TOCSY traces and HSQC. The ^{13}C chemical shifts for residue **B** were similar to those of α-D-Glc*p*1Me ($<\Delta\delta> = 0.9 \pm 0.6$ ppm) (Bock and Pedersen, 1983), at the exception of the C-4 position ($\delta_{B(C\text{-}4)}$ 79.6, $\Delta\delta$ = 9.0 ppm), indicating that residue **B** was a →4)-α-D-Glc*p*-(1→ unit.

In the case of residue **C** (β-Hex*p*), TOCSY cross-peaks were observed between **C**(H-1) and **C**(H-2,3,4,5,6a,6b). The carbon chemical shifts of **C**(C-2) ($\delta_{C(C\text{-}2)}$ 56.7) and **C**(C-3) ($\delta_{C(C\text{-}3)}$ 75.3) and the cross-peak between **C**(H-2) and **C**(NAc-CH_3) in the TOCSY spectra are characteristic for a Glc*p*NAc residue. The **C**(C-6) signal ($\delta_{C(C\text{-}6)}$ 70.2) shifted towards low-field by 9.1 ppm when compared to C-6 of β-D-Glc*p*NAc1Me (Bock *et al.*, 1983), indicated that residue **C** was a →6)-β-D-Glc*p*NAc-(1→ unit.

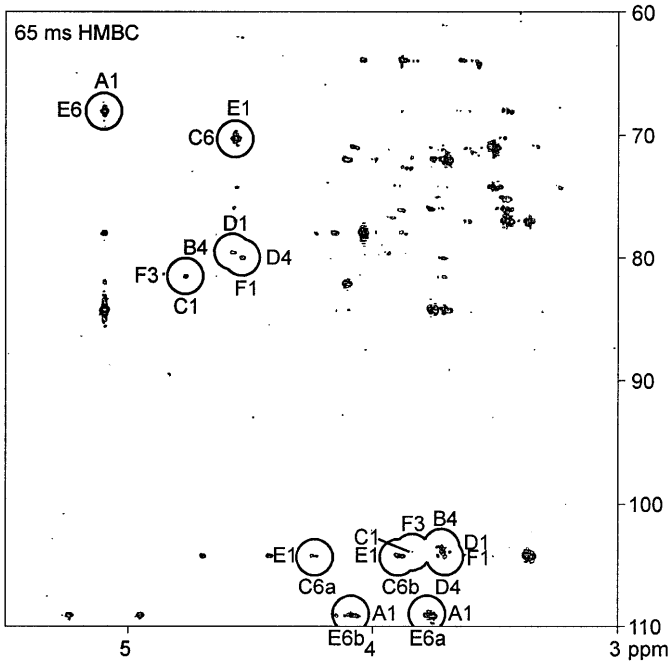


Fig. 4. 65 ms ^{1}H-^{13}C HMBC of n-EPS. The circled cross-peaks (upper part: H-1→C; lower part: C-1→H) identify interglycosidic linkages listed in Table IV. The resonances are identified by the residue letter code (**A** to **F**) and the corresponding atom (either ^{1}H or ^{13}C) number (**1** to **6**).

For residue **D** (β-Hex*p*), TOCSY transfers were found from **D**(H-1) towards **D**(H-2,3,4,5,6a,6b) but the assignment of **D**(H-5) needed to be confirmed by the presence of a NOESY cross-peak between **D**(H-1) and **D**(H-5) as well as HMBC peaks between **D**(H-4) – **D**(C-5) and **D**(C-4) – **D**(H-5). The carbon chemical shifts of residue **D** as compared to those of relevant reference compounds (Bock and Thøgersen, 1982; Bock and Pedersen, 1983; Bock *et al.*, 1983) and the NOESY cross-peak pattern (weak **D**(H-1) – **D**(H-2), strong **D**(H-1) – **D**(H-3,5)) identified residue **D** as a β-Glc*p* residue. The **D**(C-4) resonance ($\delta_{\mathbf{D}(C-4)}$ 80.0) shifted by 9.4 ppm towards low-field compared to C-4 of β-D-Glc*p*1Me (Bock and Thøgersen, 1982) defined residue **D** as a →4)-β-D-Glc*p*-(1→ unit.

The assignment of the proton signals of residue **E** (β-Hex*p*) followed from TOCSY cross-peaks between **E**(H-1) and **E**(H-2,3,4,5,6a,6b). The four non-anomeric ring protons **E**(H-2) to **E**(H-5) covered a small ^{1}H chemical shifts range ($\langle\delta\rangle$ = 3.50 ± 0.13 ppm). The carbon chemical shifts of residue **E** were almost identical to those of β-D-Glc*p*1Me ($\langle\Delta\delta\rangle$ = 0.2 ± 0.2 ppm) (Bock and Pedersen, 1983), at the exception of that of **E**(C-6) ($\delta_{\mathbf{E}(C-6)}$ 68.1, Δδ = 6.3 ppm), defining residue **E** as a →6)-β-D-Glc*p*-(1→ unit.

For residue **F** (β-Hex*p*), no TOCSY cross-peaks were found from **F**(H-1) towards **F**(H-5,6a,6b). However, the assignments of **F**(H-5) and **F**(H-6a,6b) were obtained from NOESY data (cross-peaks between **F**(H-1) and **F**(H-5,6a,6b)) and HMBC data (cross-peaks **F**(H-4) – **F**(C-5) and **F**(C-4) – **F**(H-5) as well as **F**(H-5) – **F**(C-6) and **F**(C-5) – **F**(H-6a,6b)), and by correlating the ^{1}H resonances to the corresponding ^{13}C resonances in the HSQC spectrum. The low-field **F**(H-4) ^{1}H chemical shift ($\delta_{\mathbf{F}(H-4)}$ 4.28) together with the NOESY cross-peak pattern (weak **F**(H-1) – **F**(H-4,6a,6b), strong **F**(H-1) – **F**(H-3,5)) indicated a β-D-Gal*p* rather than a β-D-Glc*p* residue (Bock and Thøgersen, 1982; Bock and Pedersen, 1983; Bock *et al.*, 1983). Both the **F**(C-3) and **F**(C-4) ^{13}C resonances ($\delta_{\mathbf{F}(C-3)}$ 81.6, Δδ = 6.3 ppm; $\delta_{\mathbf{F}(C-4)}$ 76.3, Δδ = 6.3 ppm) shifted towards low-field with respect to the corresponding signals in β-D-Gal*p*1Me (Bock and Thøgersen, 1982), indicated a 3,4-disubstituted residue. Residue **F** was therefore identified as a →3,4)-β-D-Gal*p*-(1→ branching unit.

The sequence of the monosaccharide residues was deduced from the presence of cross-peaks in the ^{1}H-^{13}C HMBC and in the NOESY spectra. Relevant cross-peaks are summarized in Table IV. In case of ambiguities with the type of glycosidic linkage, the methylation analysis data and the ^{13}C NMR assignments were also used.

The sequence β-D-Gal*f*-(1→6)-β-D-Glc*p* (**A**-(1→6)-**E**) is reflected by HMBC cross-peaks between **A**(H-1) and **E**(C-6) and between **A**(C-1) and **E**(H-6a,6b). The sequence α-D-Glc*p*-(1→4)-β-D-Gal*p* (**B**-(1→4)-**F**) was deduced from the NOESY cross-peak **B**(H-1) – **F**(H-4), no HMBC signal being visible, while the sequence β-D-Glc*p*NAc-(1→3)-β-D-Gal*p* (**C**-(1→3)-**F**) resulted from both HMBC (**C**(H-1) – **F**(C-3) and **C**(C-1 – **F**(H-3))

Table IV. HMBC and NOESY information available for the determination of n-EPS interresidue correlations

HMBC		NOESY		Linkages
ω_1[a]	ω_2[a]	ω_1[a]	ω_2[a]	
A(H-1)	**E**(C-6)			
A(C-1)	**E**(H-6a)			**A**-(1→6)-**E**
A(C-1)	**E**(H-6b)			β-D-Gal*f*-(1→6)-β-D-Glc*p*
		B(H-1)	**F**(H-4)	
		B(H-1)	**F**(H-5)	**B**-(1→4)-**F**
		B(H-1)	**F**(H-6a)	α-D-Glc*p*-(1→4)-β-D-Gal*p*
		B(H-1)	**F**(H-6b)	
C(H-1)	**F**(C-3)	**C**(H-1)	**F**(H-3)	
C(C-1)	**F**(H-3)	**C**(H-1)	**F**(H-5)	**C**-(1→3)-**F**
		C(H-1)	**F**(H-6a)	β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*
		C(H-1)	**F**(H-6b)	
D(H-1)	**B**(C-4)	**D**(H-1)[b]	**B**(H-2)[b]	
D(C-1)	**B**(H-4)	**D**(H-1)[b]	**B**(H-5)[b]	**D**-(1→4)-**B**
		D(H-1)[b]	**B**(H-6a)[b]	β-D-Glc*p*-(1→4)-α-D-Glc*p*
E(H-1)	**C**(C-6)			
E(C-1)	**C**(H-6a)			**E**-(1→6)-**C**
E(C-1)	**C**(H-6b)			β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc
F(H-1)	**D**(C-4)	**F**(H-1)	**D**(H-3)	
F(C-1)	**D**(H-4)	**F**(H-1)	**D**(H-4)	**F**-(1→4)-**D**
		F(H-1)	**D**(H-5)	β-D-Gal*p*-(1→4)-β-D-Glc*p*
		F(H-1)	**D**(H-6b)	

[a]ω_1 refers to the first (indirect) frequency dimension, while ω_2 refers to the second (direct) frequency dimension.
[b]The overlap between **B**(H-4), **D**(H-3), **D**(H-4), and **D**(H-5) made the use of NOESYs for this linkage unreliable, although not incompatible with the assignment.

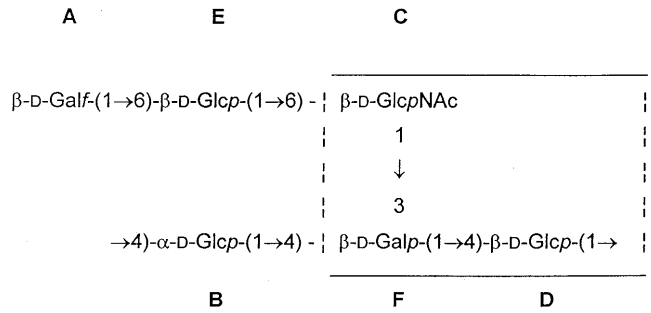
and NOESY (**C**(H-1) – **F**(H-3)) cross-peaks. For the sequence β-D-Glc*p*-(1→4)-α-D-Glc*p* (**D**-(1→4)-**B**), two HMBC cross-peaks were indicative: **D**(H-1) – **B**(C-4) and **D**(C-1) – **B**(H-4). It should be noted that the overlap between **B**(H-4), **D**(H-3), **D**(H-4), and **D**(H-5) (Table III) made the use of NOESY for this linkage unreliable although not incompatible with the assignment. For the sequence β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc (**E**-(1→6)-**C**), no NOESY cross-peaks were visible but the HMBC cross-peaks were unambiguous (**E**(H-1) – **C**(C-6) and **E**(C-1) – **C**(H-6a,6b)). Finally, the sequence β-D-Gal*p*-(1→4)-β-D-Glc*p* (**F**-(1→4)-**D**) was reflected both by HMBC (**F**(H-1) – **D**(C-4) and **F**(C-1) – **D**(H-4)) and by NOESY (**F**(H-1) – **D**(H-4)) cross-peaks.

2D NMR spectroscopy of deNAc-PS

For the ^{1}H and ^{13}C NMR elucidation of deNAc-PS, which was found to be composed of trisaccharide repeating units, a similar strategy as described for n-EPS was applied. A speedy assignment was possible as a result of the greatly decreased viscosity, of the disappearance of the overlap between anomeric resonances and of the existing assignment available for n-EPS. The HSQC spectrum (Figure 3b) showed three cross-peaks from the clearly separated anomeric resonances. The ^{1}H and ^{13}C data are given in Table III, demonstrating the occurrence of the →4)-α-D-Glc*p*-(1→ unit **B**, the →4)-β-D-Glc*p*-(1→ unit **D**, and the →4)-β-D-Gal*p*-(1→ unit **F**. For residue **B** a virtual equivalence of all chemical shifts in n-EPS and deNAc-PS was observed ($<\Delta\delta(^{1}H)> = 0.08$ ppm and $<\Delta\delta(^{13}C)> = 0.3$ ppm). A similar finding occurred in the case of residue **D** ($<\Delta\delta(^{1}H)> = 0.06$ ppm and $<\Delta\delta(^{13}C)> = 0.2$ ppm). For residue **F**, small deviations in the ^{1}H and ^{13}C chemical shifts from n-EPS versus deNAc-PS were found ($<\Delta\delta(^{1}H)> = 0.11$ ppm and $<\Delta\delta(^{13}C)> = 0.8$ ppm). These deviations were induced by the absence of a substituent at *O*-3 of residue **F**, reflected by the shift towards high-field of **F**(C-3) (deNAc-PS $\delta_{F(C-3)}$ 73.3; n-EPS $\delta_{F(C-3)}$ 81.6). The NMR data of deNAc-PS entirely supported the conclusions made for the structure of n-EPS. It was interesting to note that the anomeric proton chemical shifts of **B** and **D** were identical to the corresponding values in synthetic β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Gal (Koeman *et al.*, 1993).

Discussion

Based on monosaccharide analysis, methylation analysis, mass spectrometry, and NMR spectroscopy of n-EPS, hyd-PS, and deNAc-PS, the structure of the repeating unit of *S. macedonicus* Sc136 EPS can be formulated as follows:



The partially hydrolyzed polysaccharide hyd-PS had the terminal β-D-Gal*f*-(1→ residue missing and the sidechain terminated by a β-D-Glc*p*-(1→ residue, a structure directly resulting from the original n-EPS. The de-*N*-acetylated and deaminated Sc136 polysaccharide deNAc-PS was found to have a linear backbone composed of (1→4)-linked α-D-Glc*p*, β-D-Gal*p*, and β-D-Glc*p*. By complete cleavage of the sidechain as obtained by selective chemical de-*N*-acetylation and deamination, the viscosity of the EPS was greatly decreased; this change in viscosity was supported by large changes in the NMR linewidths of deNAc-PS as compared to n-EPS (Table II).

Interestingly, the outlined trisaccharide sequence β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p* is the minimal trisaccharide sequence common to both lacto-*N*-tetraose and lacto-*N*-neotetraose units found in human milk oligosaccharides. Human milk oligosaccharides are a complex mixture in which lactose is the main component (around 70 g/l), while larger structures (total around 20 g/l) are based on lactose and tetraoses backbones substituted by fucosyl and sialic acid residues bound by several types of linkages (Kobata, 1977; Kobata *et al.*, 1978; Strecker *et al.*, 1989; Finke *et al.*, 1999). Many biological functions have been postulated for human milk oligosaccharides, including bifidogenicity and the ability to inhibit the adhesion of pathogenic microbes to the infant gut mucosa. Moreover, the use of lactic acid bacteria to acidify infant formulas in order to improve their nutritional value has been applied for a long time. Thus, the use of a bacterial strain like *S. macedonicus* Sc136 for infant formula preparation could present the additional advantage to bring a non-digestible complex polysaccharide available for endogenous fermentations in the infant's gut, finally releasing carbohydrates similar to the main molecular structures found in human milk oligosaccharides.

Materials and methods

Production and isolation of S. macedonicus Sc136 EPS

Streptococcus macedonicus Sc136 from the Nestlé Culture Collection (NCC 2419) was grown in 10% reconstituted skim milk with addition of a mixture of amino acids in quantities corresponding to those found in 1% Proteose Peptone No. 3. Fermentations were carried out in a 1 l scale fermentor with a magnetic stirrer (60 rpm) at 30°C at pH 5.5 under pH regulation (2 M NaOH) for 24 h. Then, proteins were removed from the fermented culture broth by precipitation with 25% TCA (w/v). After centrifugation (1 h at 10,000 × *g*), the EPS was precipitated by addition of 1 volume of acetone (20 h, 4°C), recovered by centrifugation (1 h at 10,000 × *g*), and redissolved in 0.1 M NH_4HCO_3. The solution was adjusted to pH 7.0, dialyzed against water (24 h, 4°C), centrifuged (1 h at 10,000 × *g*), and lyophilized to give purified EPS. The molecular weight distribution of the EPS was estimated by size-exclusion chromatography (Superose-6 column) on a fast protein liquid chromatography (FPLC) system (Pharmacia) calibrated for molecular weight estimation with commercially available dextrans (Sigma). The total neutral carbohydrate content was determined for all FPLC fractions by the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Monosaccharide analysis

Quantitative monosaccharide analyses of poly- and oligosaccharides were performed by GLC after methanolysis followed by trimethylsilylation (Chaplin, 1982; Kamerling and Vliegenthart, 1989). Absolute configurations were determined by GLC analysis of the trimethylsilylated (-)-2-butyl glycosides as described (Gerwig *et al.*, 1978, 1979).

Methylation analysis

For methylation analyses, poly- and oligosaccharides were permethylated using CH_3I and NaOH in DMSO as described (Chaplin, 1982; Ciucanu and Kerek, 1984). After treatment with $Na_2S_2O_3$ (100 mg/ml) and extraction with $CHCl_3$, the permethylated samples were hydrolyzed with 2 M TFA (2 h, 120°C), then reduced with NaB^2H_4. After neutralization and removal of boric acid by co-evaporation with MeOH, samples were acetylated with acetic anhydride (3 h, 120°C). The resulting partially methylated alditol acetates were extracted with CH_2Cl_2 and analyzed by GLC-EIMS (Jansson *et al.*, 1976).

Mild acid hydrolysis

Native *S. macedonicus* Sc136 EPS (n-EPS, 4 mg) was hydrolyzed in 0.3 M TFA (20 ml; 20 min, 100°C). After lyophilization, the partially hydrolyzed material was fractionated on a size-exclusion chromatography column (Bio-Gel P-2, 200–400 mesh, 50 × 2.5 cm; Bio-Rad), eluted with 5 mM NH_4HCO_3 monitoring the effluent with a differential refractive index detector (LKB Bromma). The degraded polysaccharide (hyd-PS) was collected and lyophilized.

De-N-acetylation and deamination

A solution of n-EPS (3 mg) in anhydrous hydrazine (0.3 ml), containing hydrazine sulfate (15 mg), was stirred under argon for 20 h at 100°C. The solution was concentrated *in vacuo* and co-evaporated repeatedly with toluene. Then, the residue was dissolved in water and the solution was desalted on a HighTrap Desalting column (Sephadex G-25 Superfine, Pharmacia) eluted with 5 mM NH_4HCO_3 at a flow rate of 2 ml/min. The resulting preparation was dissolved in a solution containing 33% acetic acid (1 ml), 5% sodium nitrite (1 ml), and water (0.5 ml), and the solution was stirred for 2 h at room temperature, then neutralized with 2 M NH_4OH. The resulting mixture was desalted on a cation-exchange column (Dowex AG 50W-X12,100–200 mesh, H^+-form; Bio-Rad), reduced using NaB^2H_4, and fractionated on a size-exclusion chromatography column (Bio-Gel P-2) as described above. The degraded polysaccharide (deNAc-PS) and an oligosaccharide fraction (deNAc-oligo) were collected and lyophilized.

Gas-liquid chromatography and mass spectrometry

Gas-liquid chromatography (GLC) analyses were performed on a CP-Sil 5CB fused silica capillary column (Chrompack CP9002, 25 m × 0.32 mm) using a temperature program from 130°C to 230°C at 4°C/min.

Gas-liquid chromatography–electron ionization mass spectrometry (GLC-EIMS) analyses were carried out on a Fisons MD800/8060 system (electron energy, 70 eV) equipped with a DB-1 fused silica capillary column (J&W Scientific, 30 m × 0.32 mm) using a temperature program from 150°C to 250°C at 4°C/min.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) experiments were performed using a Voyager-DE mass spectrometer equipped with a nitrogen laser. Samples were prepared by mixing directly on the target 1 µl oligosaccharide solution with 2 µl aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

NMR spectroscopy

Samples were exchanged two times in 99.9 atom% ^2H_2O (Euriso-Top) by successive lyophilization before being dissolved in 99.96 atom% ^2H_2O (Euriso-Top). All experiments were recorded on a three-channel Bruker DRX 600 MHz spectrometer equipped with an actively shielded pulsed-field z-gradient inverse triple-resonance probe. Chemical shifts are expressed in ppm by reference to the α-anomeric signal of external [^{13}C-1]-glucose ($\delta_{H\text{-}1}$ 5.15 and $\delta_{C\text{-}1}$ 92.90).

The following phase-sensitive two-dimensional experiments were recorded using time proportional phase increments (TPPI) (Marion and Wüthrich, 1983): double-quantum filtered correlation spectroscopy (DQF-COSY) (Piantini *et al.*, 1982; Rance *et al.*, 1983), total correlation spectroscopy (TOCSY) (Braunschweiler and Ernst, 1983) with mixing times between 10 ms and 80 ms, nuclear Overhauser effect spectroscopy (NOESY) (Jeener *et al.*, 1979; Kumar *et al.*, 1980) with mixing times between 50 ms and 250 ms, and gradient sensitivity-enhanced ^{1}H-^{13}C heteronuclear single-quantum coherence (HSQC) (Kay *et al.*, 1992). A magnitude mode gradient-filtered ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) (Bax and Summers, 1986) was recorded with a *J*-evolution time of 65 ms. The following number of complex points were acquired (F_1, F_2): 128×512 (TOCSY and HSQC), 256×512 (NOESY), and 128×1024 (HMBC), with averaging over 32 scans (TOCSY and NOESY) or 256 scans (HSQC and HMBC). Spectral widths (ω_1, ω_2) of 4000 Hz × 4000 Hz (TOCSY and NOESY), 16350 Hz × 4000 Hz (HSQC), and 12577 Hz × 4000 Hz (HMBC) were used. A 60° shifted square sine-bell was used in all cases, with zero-filling once. All data were processed using Bruker XWINNMR 2.x software.

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Abbreviations

deNAc-PS, de-*N*-acetylated deaminated polysaccharide; DQF-COSY, double-quantum filtered correlation spectroscopy; EPS, exopolysaccharide; FPLC, fast protein liquid chromatography; Gal*p*, galactopyranose; Gal*f*, galactofuranose; GLC, gas-liquid chromatography; GLC-EIMS, gas-liquid chromatography–electron ionization mass spectrometry; Glc*p*, glucopyranose; Glc*p*NAc, *N*-acetylglucopyranosamine; GRAS, generally recognized as safe; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; hyd-PS, partially hydrolyzed polysaccharide; LAB, lactic acid bacteria; MALDI-TOF-MS, matrix-assisted laser desorption time-of-flight mass spectrometry; n-EPS, native *S.macedonicus* Sc136 EPS; NMR, nuclear magnetic resonance; NOESY,

nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; TPPI, time proportional phase increments.

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