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The structure of the O-antigen of *Cronobacter sakazakii* HPB 2855 isolate involved in a neonatal infection

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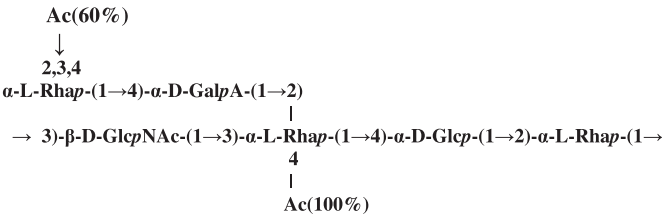
ABSTRACT

Strains of *Cronobacter sakazakii* (previously known as *Enterobacter sakazakii*) are medically recognized important Gram-negative bacterial pathogens that cause enterocolitis, septicemia, and meningitis, with a high mortality rate in neonates. The structure of their O-antigens, that form part of their somatic lipopolysaccharide (LPS) components, is of interest for their chemical and serological identification and their relationship to virulence. The O-polysaccharide (O-PS) of *C. sakazakii* HPB 2855 (SK 81), a strain isolated from an infant at the Hospital for Sick Children in Toronto in 1981, was shown to be a polymer of a partially O-acetylated-repeating hexasaccharide unit composed of D-glucose, D-galacturonic acid, 2-acetamido-2-deoxy-D-galactose, and L-rhamnose (1:1:1:3). From composition and methylation analysis, and the application of 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, the O-PS was determined to be a polymer of a repeating oligosaccharide unit having the structure:



1. Introduction

The taxonomy of *Enterobacter sakazakii* initially described as a motile peritrichous Gram-negative bacteria has recently, on the basis of phenotypic and genotypic characterization and other observations, been assigned a novel genus *Cronobacter*[1] with a division of species based on previously described biogroups.[2] As such, there are now six distinct genomospecies comprising *Cronobacter sakazakii*, (formerly *E. sakazakii*), *Cronobacter malonaticus, Cronobacter turicensis*, *Cronobacter* genomospecies 1, *Cronobacter muytjensii*, and *Cronobacter dublinensis.* While a rare infection, *C. sakazakii* can cause life-threatening neonatal meningitis, sepsis, and necrotizing enterocolitis and is thus an emerging pathogen of medical interest.

Currently no agreed-upon typing protocol has been developed to trace *C. sakazakii*, however, biochemical profiling, real-time polymerase chain reaction, and differential selective agars have proven useful for its identification. In view of the demonstrated usefulness of LPS O-antigen typing systems for other Gram-negative bacterial species we have undertaken the structural analysis of the O-PS of selected *Cronobacter* spp. with a view to identifying particular O-serotypes with pathogenicity. With the limited number of *Cronobacter* O-PS antigen structures reported to date, the variety in composition and structure suggests that many O-serotypes will be discovered. The current investigation of the O-PS structure of the *C. sakazakii* strain 2855, the causal agent of invasive infection in an infant in Toronto, determined it to be a polymer of a repeating hexasaccharide unit. Interestingly, this O-PS is structurally similar to the repeating heptasaccharide unit found in the O-PS *C. sakazakii* strain 767,[3] an isolate from a neonatal meningitis death in Paris.[4] This is the first reported finding of a *Cronobacter* spp. O-PS having strikingly similar structures. Unlike the close structural relationship between the O-PS antigens of *C. sakazakii strains* 767 and HPB 2855, previously determined O-PS structures of *C. sakazakii* HPB 3290,[5] *C. muytjensii* HPB 3270,[6] and *C. malonaticus* HPB 3267[7] are composed of structurally unrelated repeating oligosaccharide units.

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2. Experimental

2.1. Bacterial growth and LPS and O-PS production

C. sakazakii HPB 2855 (NRCC 6464) was grown in 3.7% brain–heart infusion broth (Difco) at 3% oxygen concentration and 37 °C in a New Brunswick 25 L fermenter. The bacteria were killed with phenol (1% final concentration at 4 °C, 2 h) and collected by centrifugation as a wet paste (295 g). Saline-washed cells (250 g) were extracted by vigorous stirring in 50% aqueous phenol (1.2 L) at 65 °C for 15 min. The water phase of the cooled (4 °C) extract was dialyzed against running tap water until free from phenol, and then lyophilized. The lyophilizate dissolved in 0.02 M sodium phosphate buffer (pH 7.0, 160 mL) was treated sequentially with deoxyribonuclease, ribonuclease, and proteinase K (2 h each at 37 °C) and the solution was then subjected to ultracentrifugation (150,000 *g*, 10 h, 4 °C) and the precipitated LPS was taken up in water (80 mL) and lyophilized (yield 1.70 g).

In a parallel study, an identical growth and extraction from a cell paste (533 g) of *C. sakazakii* 767 (HPB 5723)[3] afforded LPS (5.6 g), a portion of which was used to produce an O-PS under the same conditions as those described below for *C. sakazakii* HPB 2855.

LPS from *C. sakazakii* HPB 2855 (1.0 g) was hydrolyzed with 2% acetic acid for 2 h at 100 °C and insoluble lipid A (145 mg) was removed from the cooled hydrolyzate by low speed centrifugation. The lyophilized water-soluble products dissolved in 0.05 M pyridinium acetate (pH 4.5, 8 mL) were subjected to Sephadex G-50 column (2.5 × 95 cm) chromatography using the same buffer. The eluate was monitored by refractive index and colorimetrically for neutral aldose,[8] hexuronic acid,[9] and 2-amino-2-deoxyhexose[10] and collected fractions were lyophilized to yield O-PS (K_{av} 0.02, 289 mg) and core oligosaccharide (K_{av} 0.42, 32 mg).

2.2. Gel electrophoresis

Deoxycholate–polyacrylamide gel-electrophoresis (DOC–PAGE) was performed on separating gels of 14% acrylamide and detection was made by silver staining after periodate oxidation.[11]

2.3. Monosaccharide analysis

O-PS samples (2–5 mg) were hydrolyzed with 4 M trifluoroacetic acid (115 °C, 2 h) and the concentrated residues were reduced ($NaBH_4$) and acetylated as previously described. The acetylated alditol derivatives were analyzed by GLC using a HP5 capillary column (30 m × 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) in a temperature gradient from 170 to 260 °C at 4 °C/min, and by GLC–MS using a Varian 200 ion-trap instrument with the same column conditions. Mobilities are quoted relative to hexa-*O*-acetylglucitol (T_{GA} = 1.0).

Chromatographically pure samples of component aldose hydrolyzates of O-PS (100 mg) were obtained by preparative paper chromatography on water-washed Whatman 3MM filter paper using 1-butanol–pyridine–water (10:3:3 v/v) mixture as the eluant. Detection of glycoses on excised guide strips was made by sprays of 2% *p*-anisidine HCl (in EtOH) and 2% ninhydrin (in acetone). Chromatographic mobilities are quoted relative to D-galactose (R_{Gal} = 1.0).

2.4. Methylation analysis

Samples of O-PS (3 mg) and carboxylic acid-reduced ($NaBD_4$) O-PS (3 mg) dissolved in dimethylsulfoxide (1 mL) were methylated by the addition of NaOH following the Ciucanu–Kerek procedure,[12] washed through Sep-Pak C18 cartridges, and hydrolyzed with 2 M trifluoroacetic acid (120 °C, 2 h), reduced ($NaBD_4$), acetylated, and analyzed by GLC–MS (as described above) using a temperature gradient 170 °C (4 min delay) to 260 °C at 4 °C/min. Retention times of derivatives are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (T_{GM} = 1.0).

2.5. NMR Spectroscopy and mass spectrometry

^1H and ^{13}C spectra were recorded at 42 °C using a Varian Inova 500 MHz with samples dissolved in 99% D_2O and internal acetone standard (2.225 ppm for ^1H and 31.45 ppm for ^{13}C) employing standard parameters for COSY, TOCSY (mixing time 100 ms), ROESY (mixing time 400 ms), ^1H,^{13}C-HSQC, and heteronuclear correlation gHMBC, for 6 Hz long-range coupling constants.

2.6. Reduction of O-PS by the carbodiimide-borodeuteride method

Following the method of Taylor and Conrad[13] a solution of O-PS (70 mg) in water (10 mL) was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (250 mg) and the stirred mixture was maintained over 4 h at pH 4.5 by adjustment with 0.05 M HCl. The reaction mixture was reduced by the slow addition of $NaBD_4$ (100 mg) solution with a maintained pH ~7.0 by M HCl addition. After 16 h the mixture was acidified (AcOH), dialyzed against water, and the reduced O-PS (55 mg) was recovered by lyophilization.

2.7. CE-MS analysis

CE-MS was accomplished using a Prince CE system coupled to a 4000 Q-trap mass spectrometer employing a sheath solution of 2:1 isopropanol–MeOH and a 5-kV electrospray-ionizing voltage for the positive ion detection mode.

2.8. Specific optical rotations

Specific optical rotations were determined at 20 °C using a 10-cm. microtube and a Perkin–Elmer 343 Polarimeter.

3. Results and discussion

Fermenter-grown cells of *C. sakazakii* HPB 2855 were extracted by a modified hot-aqueous phenol method[14] and ultracentrifugation of the concentrated aqueous phase afforded LPS in 6% based on dry cell mass. DOC–PAGE analysis of the LPS showed a typical pattern for a smooth endotoxin product (Fig. 1) in which the bands in the silver-stained gels were consistent for a LPS composed of a high molecular mass O-PS chain composed of a repeating hexasaccharide unit.

Mild acid hydrolysis of the LPS from *C. sakazakii* HPB 2855 afforded precipitated lipid A (14% yield) and Sephadex G-50 column chromatography of the water-soluble products afforded a high molecular mass O-PS (56% yield) having $[\alpha]_D$ +36 (*c* 0.5, water) and a core oligosaccharide (7% yield).

Preparative paper chromatography of the acid (M H_2SO_4)-hydrolyzed O-PS afforded chromatographically pure L-rhamnose (R_{Gal} 3.54), D-glucose (R_{Gal} 1.26), and 2-amino-2-deoxy-D-glucose (R_{Gal} 0.56) that were identified from their proton NMR spectra, specific optical rotations, and by GLC of their acetylated alditol derivatives. A hydrolyzate of the reduced ($NaBD_4$) O-PS prepared by the carbodiimide method[13] afforded, in addition to the above-identified glycoses, the new derivative D-galactose-6-d_2 that was characterized by proton NMR and by GLC–MS of its hexa-*O*-acetyl-D-galactitol-6-d_2 derivative (T_{GA} 1.02), thus identifying the uronic acid component of the O-PS as D-galacturonic acid.

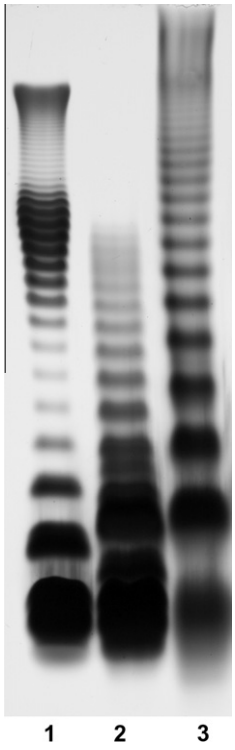


Figure 1. DOC–PAGE analysis of the lipopolysaccharide of [**1**] *C. sakazakii* 767, [**2**] *C. sakazakii* HPB 2855, and [**3**], *S. milwaukee* (standard reference).[22]

Preliminary 1D ^{1}H NMR of the O-PS indicated it to be significantly O-acetylated and subsequent-described analyses were made on a column chromatographically (Sephadex G-50) purified de-O-acetylated O-PS prepared by treatment with anhydrous hydrazine under previously described conditions.[7] The de-O-acetylated O-PS had $[\alpha]_D$ +38 (*c* 0.8, water) and its ^{1}H NMR spectrum (Fig. 2, Table 1) showed six anomeric proton signals in the region 5.29–4.68 ppm indicative of five residues having α-configurations and one β-configuration. The spectrum showed inter alia three methyl signals (1.25–1.27, 9H) attributed to the presence of three L-Rha residues, and a single methyl signal (2.03 ppm, 3H) from an acetyl function of the D-GlcNAc component residue. Consistent with the proton NMR spectrum the related ^{13}C spectrum of the O-PS (Fig. 3) also showed six anomeric carbon signals (104.0–97.2 ppm) inter alia with three methyl resonances (17.9, 17,6, and 17.3 ppm) from the L-Rha residues, and a C-2 ring carbon resonance (57.2 ppm) of the acetamido-substituted function in the D-GlcNAc residue that was also associated with a methyl resonance (23.5 ppm) and carbonyl resonance (175.2 ppm). A second carbonyl resonance at 173.8 ppm (pD 3.2) shifted upfield to 174.4 ppm at pD 8.5 was assigned to the carboxyl group of the D-GalA residue. The combined experimental evidence leads to the O-PS composition as D-Glc (1 part), L-Rha (3 parts), D-GlcNAc (1 part), and D-GalA (1 part).

For a more detailed NMR analyses (Fig. 4) the component sugars were assigned the letters **A** to **F** in order of their decreasing anomeric proton shifts. The small $J_{H\text{-}1,H\text{-}2}$ (<1.5 Hz) coupling constants of residues **A**, **C**, and **D** identified the three *manno*-configured residues whose spin systems terminated with methyl groups revealed in TOCSY and COSY spectra. Residue **B** had a *galacto*-configuration determined from its $^3J_{H,H}$ coupling pattern and contained a carboxylic group at the C-6 position (173.8 ppm), further confirming **B** as a D-Gal*p*A residue. Residue **F** was confirmed as a GlcpNAc residue with an amino function at C-2 (57.2 ppm). Residue **E** was identified by its $^3J_{H,H}$ coupling constant pattern, as the α-D-Glc*p* residue.

Inter-residue correlations were made from ^{1}H–^{1}H NOESY and ^{1}H–^{13}C HMBC spectra obtained for the de-O-acetylated O-PS. The observed anomeric NOE proton connectivities were H-1**A** to H-4**B** (4.5 ppm), H-1**B** to H-2**C** (4.30 ppm), H-1**C** to H-4**E** (3.64 ppm), H-1**D** to H-3**F** (3.67 ppm), and H-1**F** to H-3**C** (3.85 ppm, weak signal). HMBC spectra showed long-range connectivities of H-1**A** to C-4**B** (77.1 ppm), H-1**B** to C-2 **C** (75.1 ppm), H-1**C** to C-4**E** (78.5 ppm), C-1**D** to H-3**F** (3.67 ppm), and H-1**F** to C-3**C**. The spectral data were consistent with the proposed sequence shown below [**I**].

The anomeric configurations of the glycose residues were established by the coupling constants $^1J_{H\text{-}1,C\text{-}1}$ determined in a HMQC experiment recorded without decoupling. Residues **A** to **E** having $^1J_{H\text{-}1,C\text{-}1}$ values >170 Hz (Table 1) were assigned to α-configuration and the D-Glc*p*NAc residue **F** ($^1J_{H\text{-}1,C\text{-}1}$ 165 Hz) was assigned the β-configuration. A consideration of the above composition and

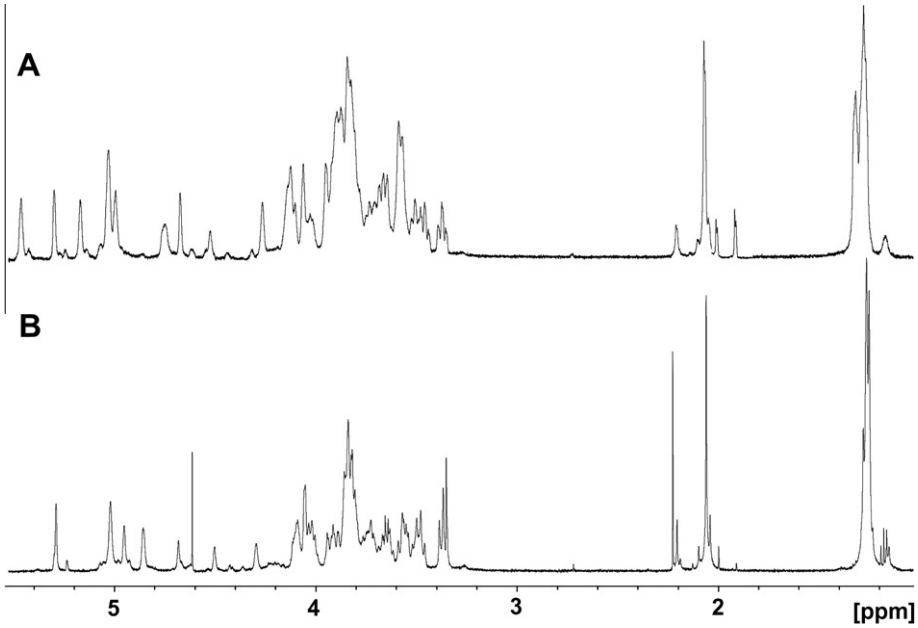


Figure 2. 1D Proton NMR spectra of the de-O-acetylated O-PS from [**A**] *C. sakazakii* 767 and [**B**] *C. sakazakii* HPB 2855.

Table 1
NMR Chemical shifts and coupling constants for the de-O-acetylated O-polysaccharide of *Cronobacter sakazakii* HPB 2855

	Glycose residue	Chemical shifts					
		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
[**A**]	α-L-Rha*p*-(1→	5.29 (<1.5)	4.05	3.83	3.36	3.82	1.26
		101.5 (173)	71.7	71.3	73.5	69.8	17.1
[**B**]	→4)-α-D-Gal*p*A-(1→	5.04 (s)	3.93	4.10	4.50	4.68	
		97.2 (173)	69.2	71.8	77.1	73.1	175.7
[**C**]	→2,3)-α-L-Rha*p*-(1→	5.04 (s)	4.30	3.85	3.57	4.02	1.26
		98.7 (173)	75.1	80.3	72.2	70.4	17.1
[**D**]	→2)-α-L-Rha*p*-(1→	4.95 (<1.5)	3.85	3.90	3.48	4.03	1.26
		99.7 (170)	78.2	70.6	73.1	70.7	17.1
[**E**]	→4)-α-D-Glc*p*-(1→	4.86 (~3)	3.55	3.84	3.64	4.08	3.80/3.8
		98.9 (173)	72.8	72.7	78.5	71.8	61.2
[**F**]	→3)-β-D-Glc*p*NAc-(1→	4.68 (7.4)	3.72	3.67	3.50	3.37	3.74/3.85
		104.0 (161)	57.2	82.4	69.7	77.0	62.4

Chemical shifts are recorded in ppm and coupling constants in Hz are shown in parentheses.

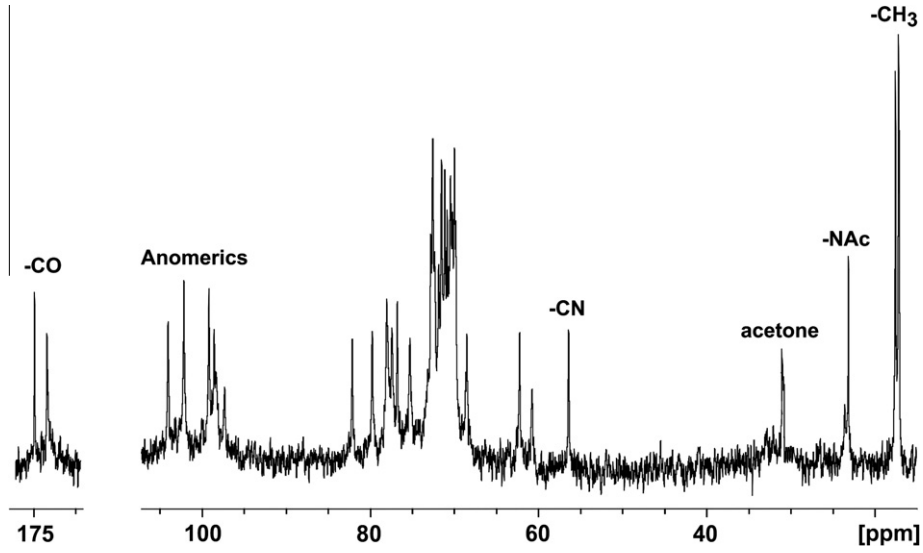
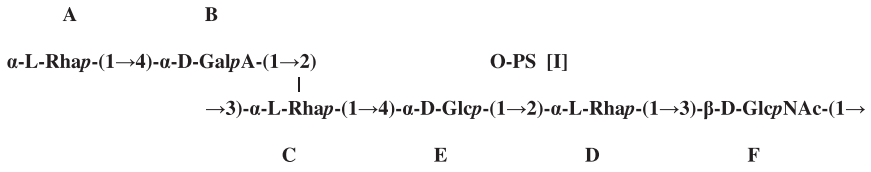


Figure 3. 1D ^{13}C NMR spectrum of the de-O-acetylated O-PS of *C. sakazakii* HPB 2855.

NMR data leads to the basic hexasaccharide-repeating unit of the *C. sakazakii* HBP 2855 O-PS [**I**] having the structure:



Consistent with the O-PS structure [**I**] the positive mode ESI mass spectrum of the *C. sakazakii* HBP 2855 de-O-acetylated O-PS (Fig. 5) contained fragment peaks corresponding to 204.0 amu (HexNAc), 350.0 amu (HexNAcdHex), 526 amu (HexNAcdHex HexA), 834.5 amu (HexNAcdHex$_2$HexAHex), and a molecular ion M+1 = 980.0 Da confirming the proposed repeating unit structure (HexNAcdHex$_3$HexAHex; calculated mass 978.9 Da).

The proposed structure was further confirmed from methylation analysis. GLC–MS analysis of the reduced ($NaBD_4$) and acetylated hydrolysis products of the reduced ($NaBD_4$/THF) fully methylated O-PS identified 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol-1d (T_{GM} 0.59), 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol-1-d (T_{GM} 0.83), 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol-1-d (T_{GM} 1.17), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol-1-d (T_{GM} 1.44), 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol-1,6,6-d_3 (T_{GM} 1.91), and 1,3,5-tri-*O*-acetyl-2-(*N*-methylacetamido)-2-deoxy-4,6-di-*O*-methyl-glucitol-1-d (T_{GM} 2.97). The identified methylated derivatives are consistent with the proposed respective linked glycose units, Rha*p*-(1→, →2)-Rha*p*-(1→, →2,3)-Rha*p*-(1→, →4)-Glc*p*-(1→, →4)-Gal*p*A-(1→, and →3)-Glc*p*NAc-(1→. The same methylated products were also identified by GLC–MS analysis in the hydrolysis products of the methylated O-PS preparation obtained by the reduction ($NaBD_4$) of O-PS by the carbodiimide method.

From integration of the methyl group resonances in the proton NMR spectra, the native O-PS was estimated to contain approximately 1.6 *O*-acetyl functions per hexasaccharide-repeat-

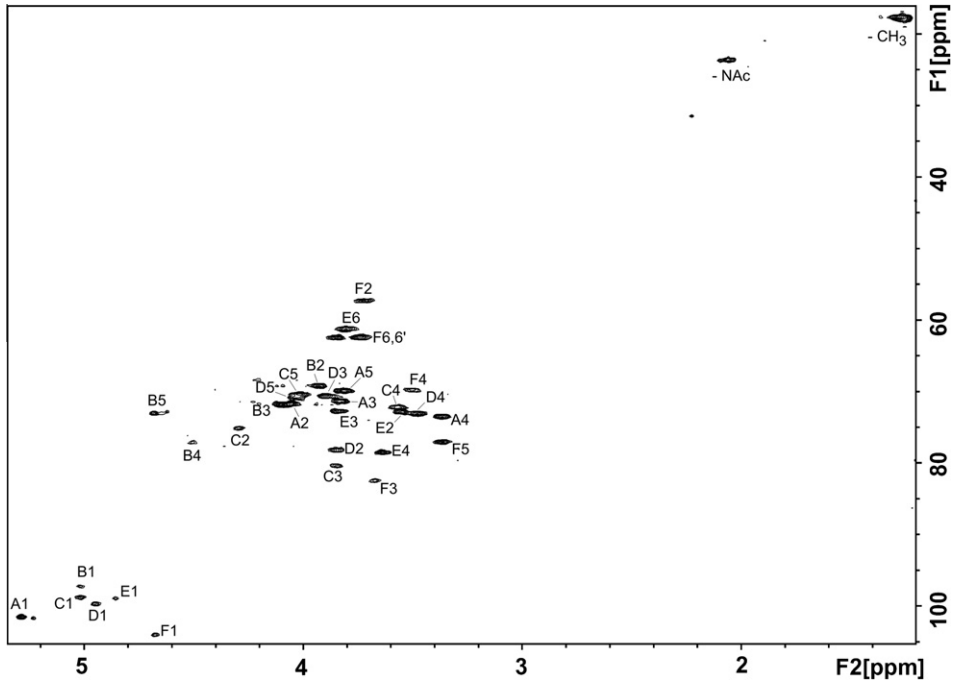


Figure 4. $^{1}H,^{13}C$-HSQC NMR spectrum of the de-O-acetylated O-PS from *C. sakazakii* HPB 2855.

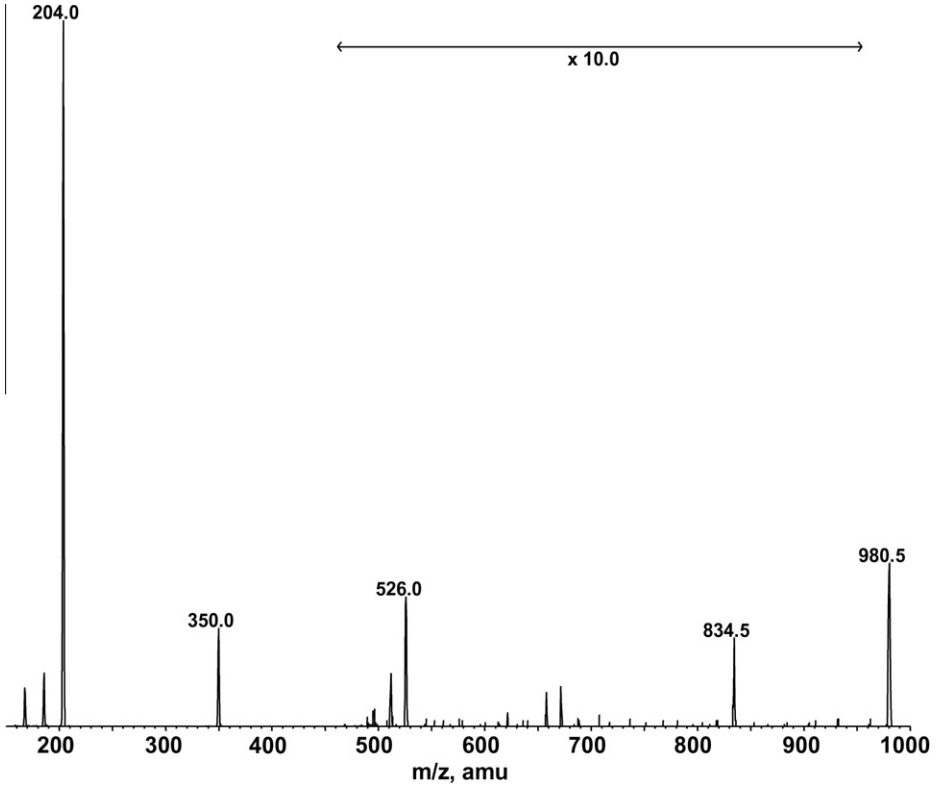
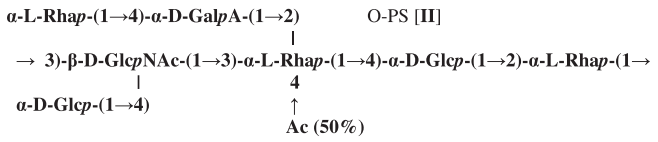


Figure 5. CE-MS spectrum of the de-O-acetylated O-PS from *C. sakazakii* HPB 2855.

ing unit. From the observed chemical resonances for the di-*O*-glycosidic-substituted branch L-Rha*p* [**C**] residue, its C-4 carbon (73.6 ppm), and H-4 (4.92 ppm) resonance chemical shifts to, respectively, 72.2 ppm and 3.57 ppm in the de-O-acetylated O-PS, it was concluded that residue **C** was fully acetylated at its *O*-4 position. Similar non-stoichemic observed proton and carbon resonance shifts indicated highly variable *O*-acetyl substitutions at the *O*-2, *O*-3, and *O*-4 positions of the terminal α-L-Rha*p* residue **A**.

It is interesting to note that the structure of the O-antigen of *C. sakazakii* HPB 2855 has a close structural relationship to the recently determined structure [**II**] of *C. sakazakii* 767, the essential difference being the addition, in the latter strain, of an α-D-Glcp residue at the *O*-4 position of the β-D-Glc*p*NAc residue in the back-

bone chain of the repeating heptasaccharide unit in the *C. sakazakii* 767 O-PS.



It is remarkable that the discussed O-antigens of *C. sakazakii* were associated with neonatal infection in widely separated geographic locations.

It has been observed that in several O-antigens, the addition of α-D-Glc*p* and β-D-Glc*p* residues to a specific basic oligosaccharide-repeating unit forming O-PS antigens is a common occurrence. For example, the O-antigen of Salmonella O:30 contains two related structures.[15,16] One structure is a polymer of a repeating tetrasaccharide (incidentally identical of the O-PS-repeating unit in *Escherichia coli* O157 LPS[17]) while the second subtype antigen is a polymer of a pentasaccharide-repeating unit composed of the subtype tetrasaccharide by a β-D-Glc*p* attached in single non-reducing residues to the polymeric chain. A similar O-antigenic mobility is discussed in a paper on the sharing of a common repeating tetrasaccharide unit (composed of D-mannose and 2-acetamido-2-deoxy-D-glucose (3:1)) backbone in a group of *E. coli* (serotypes O17, O44, O73, O77, and O106)[18] differing from each other in the degree and substitution positions of α-D-Glc*p* residues. The O-antigen gene clusters for all members of the group encoded only proteins required for biosynthesis of the repeating tetrasaccharide unit and further analysis identified putative prophage genes mapping outside of the common O-antigen gene cluster, encoding a glucosyltransferase responsible for the side residue additions. A genetic analysis by Fitzgerald et al.[19] of the *Salmonella enterica* O:6,14 (H) that has the same basic repeating unit O-antigen described above[20] showed that the LPS O-gene cluster only encodes genes for the biosynthesis of the backbone tetrasaccharide-repeating unit and not for those of the glucose side-branch residues. In a recent analysis of the structure of the O-antigen of *E. coli* O70 serotype[21] it was found that the O-PSs prepared from LPS samples prepared from single plate colonies afforded either homogenous O-PS of a repeating tetrasaccharide or an O-PS of a repeating pentasaccharide unit in which an α-D-Glc*p* residue is linked at the 4-*O* position of α-D-Gal*p*-NAc residues contained in the backbone chain of the basic *E. coli* O70 antigen. In the light of the above-described observations we speculate that genetic analysis may reveal that the addition of the α-D-Glc*p* residue in the *C. sakazakii* 767 LPS O-antigen may involve individually acquired genes outside the O-antigen locus, encoding a putative glucosyl transferase, and that such transferase is absent in the *C. sakazakii* 2855 strain.

In our comparison of the LPS preparations of *C. sakazakii* 767 and HPB 2855 strains it was found that the yield of cell mass of *C. sakazakii* 767 was 70% greater than that of the *C. sakazakii* HPB 2855 and that the relative respective yields of O-PS from the two LPSs were approximately 2:1. Multiple preparations of O-PS from the two LPSs were consistent in composition and position of *O*-acetyl substitutions, as revealed by proton NMR spectra of the native and de-O-acetylated polymers (Fig. 1) and that the *C. sakazakii* HPB 2855 LPS did not have any α-D-Glc*p* side chain substitution. Future analysis of particularly virulent strains of *C. sakazakii* may reveal whether they belong to an O-serogroup related to the O-antigens of *C. sakazakii* 767 and HPB 2855.

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