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The structure of the O-antigen in the endotoxin of the emerging food pathogen *Cronobacter (Enterobacter) muytjensii* strain 3270

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ABSTRACT

Strains of the Gram-negative bacterium *Cronobacter* (formerly known as *Enterobacter*) *sakazakii* have been identified as emerging opportunistic pathogens that can cause enterocolitis, bacteraemia, meningitis, and brain abscess, and they have been particularly associated with meningitis in neonates where infant milk formulae have been epidemiologically linked to the disease. A study of the lipopolysaccharides produced by clinical isolates using chemical, 2D ^{1}H and ^{13}C NMR, and MS methods revealed that the O-polysaccharide produced by *Cronobacter muytjensii* strain 3270, isolated from powdered infant formula from Denmark, was a linear unbranched polymer of a repeating pentasaccharide unit composed of 2-acetamido-2-deoxy-D-galactose (D-GalNAc), 2-acetamido-2-deoxy-D-glucose (D-GlcNAc), 3-acetamido-3-deoxy-D-quinovose (D-Qui3NAc), L-rhamnose (L-Rha), and D-glucuronic acid (D-GlcA) in equimolar ratio, and has the structure

→3)-α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→6)-α-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→

The specific structural characteristics of the O-polysaccharides of *C. muytjensii* may be of value in the identification and tracking of the bacterial pathogen.

1. Introduction

Enterobacter sakazakii, formerly known as a yellow pigmented *Enterobacter cloacae*,[1] was renamed by Farmer et al.[2] based on DNA–DNA hybridization, biochemical reactions, and antibiotic susceptibility studies, in honor of the Japanese bacteriologist Riichi Sakazaki. Recently, the taxonomy changed again, based on the extensive phenotypic and genotypic characterizations of various strains of *E. sakazakii*.[3] A novel genus, *Cronobacter*, was proposed, with division of species based on previously described biogroups.[4] Currently, there are six different subspecies that comprise *Cronobacter sakazakii* (formerly known as *E. sakazakii*), *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter genomospecies* 1, *Cronobacter muytjensii*, and *Cronobacter dublinensis*.[4] *C. sakazakii* can cause life-threatening neonatal meningitis, sepsis, and necrotizing enterocolitis, a rather rare infection.[5–11] Immunochemical O-serotyping based on the O-polysaccharide (O-PS) constituent of lipopolysaccharides (LPS) has been a focus for major Gram-negative pathogens such as *Escherichia coli* and *Salmonella* spp., and it has proven invaluable for the identification of clinical isolates by serotyping[12,13] as well as for the detection of emerging pathogens, clonal organization, and LPS role in virulence and resistance to serum bacterial activity. A recent scheme for the O-PS classification of *C. sakazakii*, based on molecular analyses of the O-antigen *rfb* gene locus, reported that a major portion of *C. sakazakii* isolates belong to two serotype groups now designated O:1 and O:2.[14] Since a molecular level understanding of the serological specificity of O-PS antigens can only be attained from the knowledge of their fine chemical structures, we have undertaken the analysis of representative strains, formerly known as *E. sakazakii* and herein report the characterization of the O-PS of a food strain now taxonomically known as *C. muytjensii* (formerly known as *E. sakazakii* strain 3270) as a linear unbranched chain of a repeating pentasaccharide unit.

2. Experimental

2.1. Bacterial growth and LPS isolation

Enterobacter sakazakii (strain HPB3270), isolated from powdered infant formula in Denmark in 1987, was a gift from H. Muytjens. This strain has a unique ribopattern not seen in the DuPont database (data not shown), and was classified as *C. muytjensii* under a proposed new classification scheme.[3] It was grown in 3.7% brain–heart infusion broth (Difco) at 37 °C under constant aeration in a New Brunswick 25 L fermenter. The bacteria were killed with phenol (4 °C, 1% final concentration) and collected by centrifugation (284 g wet weight). The saline washed cells were extracted by stirring with 50% aq phenol at 60 °C for 10 min, and the sepa-

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rated phenol and water phases of the cooled (4 °C) extract were dialyzed against tap water until free from phenol and were then lyophilized. The residues were dissolved in 0.02 M sodium phosphate buffer (pH 7.0), treated sequentially with deoxyribonuclease, ribonuclease, and protease K (2 h each at 37 °C) and the centrifuge cleared solutions were subjected to ultracentrifugation (105,000 *g*, 10 h at 4 °C). The precipitated gels were dissolved in distilled water and lyophilized to yield 2.8 and 0.32 g LPS from the respective aq and phenol phases.

The aq phase LPS (400 mg) was hydrolyzed with 2% (v/v) acetic acid for 2 h at 100 °C, and insoluble lipid A (54 mg) was removed from the cooled hydrolysate by low-speed centrifugation. The lyophilized water-soluble products dissolved in 0.05 M pyridinium acetate buffer (pH 4.5, 5 mL) were subjected to Sephadex G-50 column (3 × 90 cm) chromatography using the same buffer. The eluate was monitored by refractive index detection, and the collected fractions were lyophilized to yield O-PS (K_{av} 0.1, 275 mg), core oligosaccharide (K_{av} 0.41, 25 mg), and a Kdo-containing fraction (K_{av} 0.95, 6 mg).

2.2. Monosaccharide analysis of O-PS and oligosaccharides

Neutral glycoses and aminoglycoses were analyzed by GLC as alditol acetates following hydrolysis of samples (2–4 mg) with 4 M trifluoroacetic acid (0.5 mL at 115 °C, 2 h), the concentrated residues were reduced ($NaBH_4$) and acetylated (Ac_2O) as previously described.[15] Samples 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 Saturn 200 ion-trap instrument with the same column specifications. Mobilities of derivatives are quoted relative to hexa-*O*-acetylglucitol (T_G = 1.0).

Individually isolated O-PS sugars components were obtained by preparative chromatography on water-washed Whatman 3MM filter paper using 10:3:3 1-butanol–pyridine–water as eluent. Detection of glycoses was made on excised strips using spray reagents of *p*-anisidine HCl (2% in EtOH) and ninhydrin (2% in acetone), and mobilities are quoted relative to D-galactose (R_{Gal} = 1.0).

Colorimetric analyses of glycoses in chromatography column eluates were made using (a) the phenol–sulfuric acid method for neutral sugars,[16] (b) the procedure of Gatt and Berman for 2-amino-2-deoxyglycoses,[17] and (c) the procedure of Blumenkrantz and Asboe-Hansen for hexuronic acid.[18]

2.3. Smith-type periodate oxidation of O-PS and oligosaccharides[19]

O-PS (120 mg) was dissolved in a water soln (20 mL) containing sodium metaperiodate (400 mg) and kept in the dark at 20 °C for 18 h. Following the addition of ethylene glycol (0.4 mL), the reaction mixture was dialyzed against changes of distilled water and the retentate was then treated with $NaBH_4$ (100 mg, for 4 h), followed by acidification with acetic acid and further dialysis to remove low mass material. The lyophilized retentate was subjected to Sephadex G-50 gel filtration, and the collected high molecular weight fraction (K_{av} 0.10–0.22) was lyophilized (yield 101 mg). The above product was hydrolyzed with 2% (vol/vol) AcOH (15 mL, 100 °C, for 2 h) and was fractionated by Sephadex G-15 column gel filtration to yield a chromatographically pure oligosaccharide **I** (K_{av} 0.42, 82 mg).

In turn, oligosaccharide **I** (70 mg) was oxidized ($NaIO_4$) and reduced ($NaBH_4$) under similar conditions to those described above, and the product was recovered from the reaction solution after removal of ionic material by passage through Rexyn 101(H^+) and Duolite A4 (OAc^-) ion-exchange resins. Sephadex G-15 column chromatography of the concentrated product afforded a pure oligosaccharide **II** (K_{av} 0.64, 42 mg).

An attempted Smith-type periodate oxidation of **II** (12 mg) under the above described conditions yielded unchanged oligosaccharide **II** (10 mg).

2.4. NMR Spectroscopy and mass spectrometry

^1H and ^{13}C NMR spectra were recorded using a Varian Inova 400 MHz spectrometer with samples dissolved in 99% D_2O at 25 °C and internal acetone standard (2.225 ppm for ^1H and 31.07 ppm for ^{13}C) employing standard parameters for COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, and heteronuclear correlation *g*HMBC, for 8 Hz long-range coupling constants. CE–MS was accomplished using a Prince CE system (Prince Technologies, The Netherlands) coupled to a 4000 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Canada) employing a sheath soln of 2:1 isopropanol–MeOH and a 5 kV electrospray-ionizing voltage for the positive ion detection mode.

2.5. Gel electrophoresis

Deoxycholate–polyacrylamide gel-electrophoresis (DOC–PAGE) was performed on separating gels of 14% acrylamide, and detection was made by silver staining after oxidation with periodate.[20]

2.6. Specific optical rotation

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

3. Results and discussion

Cells grown in a fermenter were extracted by a modified hot aqueous phenol method[21] and precipitated by ultracentrifugation to afford chemically identical LPS in ~8% and 1% yields (based on dry cell weight) from the respective water and phenol phases of the cooled and dialyzed extract. DOC–PAGE analysis of the LPS showed a typical pattern of a smooth-type product in which the spacing of the bands in the silver stained gel was consistent with that of a LPS composed of a high molecular mass O-PS chain composed of repeating pentasaccharide units.

Mild hydrolysis (2% AcOH, 100 °C, 2 h) of the LPS resulted in the precipitation of an insoluble lipid A fraction (~8%) and Sephadex G-50 column fractionation of the concentrated water-soluble fraction afforded lyophilized O-PS ~69% yield, K_{av} 0.10 and ($[\alpha]_D$ +116 (*c* 0.14, water)), core oligosaccharide ~9% K_{av} 0.42%, ($[\alpha]_D$ +77 (*c* 0.22, water)), and Kdo (K_{av} 0.93, ~2%). Colorimetric analysis of the eluting O-PS collected fractions showed that they were homogeneous with respect to neutral glycose, aminoglycose, and hexuronic acid.

Hydrolysis of the O-PS with 4 M trifluoroacetic acid afforded the component glycoses that were tentatively identified by GLC analysis of their reduced ($NaBD_4$) and acetylated alditol derivatives: rhamnitol (T_G 0.65), 3-amino-3-deoxyquinivitol (T_G 0.90), 2-amino-2-deoxyglucitol (T_G 1.22), and 2-amino-2-deoxygalactitol (T_G 1.25) in approximately equal molar ratio. The O-PS hydrolysate was separated into neutral and basic glycose components by passage through columns of Rexyn 101 (H^+) ion-exchange resin for adsorption of basic glycoses followed by neutralization of the eluate with Duolite A4(OH^-) and subsequent concentration to yield neutral glycoses. Basic sugars retained on the Rexyn resin were eluted by 0.25 M hydrochloric acid and concentrated to dryness

by desiccation in vacuo over phosphorous pentoxide and sodium hydroxide.

Paper chromatography of the basic glycose fraction and detection with a ninhydrin reagent revealed pink spots corresponding in mobility to 2-amino-2-deoxygalactose (R_{Gal} 0.63), 2-amino-2-deoxyglucose (R_{Gal} 0.71), and 3-amino-3-deoxyquinovose (R_{Gal} 1.44). Preparative paper chromatographic fractionation, involving triple solvent development, provided chromatographically pure samples of the three aminoglycoses that were identified as 2-amino-2-deoxy-D-glucose HCl ($[\alpha]_D$ +70 (*c* 0.2, water)), 2-amino-2-deoxy-D-galactose HCl ($[\alpha]_D$ +90 (*c* 0.2, water)), and 3-amino-3-deoxy-D-quinovose HCl ($[\alpha]_D$ +25 (*c* 0.2, water)). The characterizations were confirmed from the identity of their individual proton NMR spectra with those of equilibrated water solutions of reference compounds, and by the GLC mobilities of their acetylated glycitol derivatives with those of reference samples. The neutral glycose fraction was subjected to preparative paper chromatography to yield chromatographically pure L-rhamnose ($[\alpha]_D$ +7 (*c* 0.3, water)) that was identified by its paper chromatographic mobility, its equilibrated proton NMR spectra, and GLC of its penta-*O*-acetyl-L-rhamnitol derivative. The putative uronic acid component of the O-PS was identified as D-glucuronic acid from the identification of D-glucose-6,6-d_2 in the O-PS methanolysis and reduction ($NaBD_4$) products[22] and subsequent establishment of the D-configuration by GLC of the derived trimethylsilylated 2-(*S*)-butyl glycoside derivatives.[23] The presence of the uronic acid in the O-PS in its free carboxylic acid form was indicated by the observed carboxyl carbon shift of 174.5 ppm in the NMR spectrum with a downfield shift to 176.2 ppm (pD 9.5).

The complete structural analysis of the O-PS was made through the application of 1D and 2D ^1H and ^{13}C NMR spectroscopic methods. The spectra (Fig. 1, Table 1) revealed five distinct anomeric proton signals (5.40–4.52 ppm) having $J_{1,2}$ values consistent with four α and one β linkages, and five anomeric carbon signals located in the 104.1–97.3 ppm region with measured $J_{C\text{-}1,H\text{-}1}$ coupling constants indicative of one β and four α linkages. In addition, there were two proton signals (1.3, 1.34 ppm, 3H each) with corresponding carbon shifts (18.0 and 16.7 ppm), arising from methyl functions of 6-deoxyhexoses, three methyl proton signals (2.04, 2.02, 1.98 ppm, 9H) with corresponding carbon signals (22.5, 22.2 22.0 ppm) characteristic of three *N*-acetyl functions, and the corresponding carbonyl shifts (175.4, 175.2 and 175.1 ppm). Also present were three carbon ring signals (53.9, 53.6 and 48.0 ppm) arising from the acetamido-substituted carbon atoms of three aminohexoses components.

Two-dimensional homonuclear proton correlation (COSY) and TOCSY experiments allowed complete assignment of all the proton signals (Table 1) and confirmed the chemical characterization of the five component sugars of the O-PS. For the determination of the anomeric configurations, sequence and linkage positions of the glycoses component, the residues were labeled **A** to **E** in the order of the decreasing anomeric proton chemical shifts.

Residues **C**, **A**, and **E** were assigned the *gluco* configuration from consideration of the characteristic large coupling constants observed for $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ (9–9.8 Hz). Residue **C** was identified as α-D-Qui3NAc from anomeric coupling constants $J_{1,2}$ (3.0 Hz) and $J_{C\text{-}1,H\text{-}1}$ (173 Hz) and from a direct correlation of H-3**C** (4.33 ppm) to the corresponding C-3**C** (53.9 ppm) of the acetamido substituent from an HSQC (Fig. 1) and from cross-peak assignments to the corresponding H-6 methyl signal (1.30 ppm) and C-6 (18.2 ppm). Identification of residue **A** followed from the H-2**A** correlation to the C-2**A** (53.6 ppm) of an acetamido function and $J_{1,2}$ (3 Hz) and $J_{C\text{-}1,H\text{-}1}$ (180 Hz) allowed residue **A** to be assigned as an α-D-GlcNAc. Residue **E** was assigned as a β-D-GlcA residue from its observed anomeric coupling constants of $J_{1,2}$ (7.2 Hz) and $J_{C\text{-}1,H\text{-}1}$ (164 Hz). The third acetamido function was assigned to **B**, identified as an α-D-GalNAc residue, and **D** was established as an α-L-Rha residue from ring cross-peak proton assignment to the methyl signal of H-6 and measured C-1, H-1 coupling constant (174 Hz).

The sequence and linkage positions of the glycose residues in the O-PS were established from NOE and long-range HMBC observed connectivities. A strong NOE cross-peak between H-1**E** (β-D-Glc*p*A) to H-3**B** (α-D-Gal*p*NAc) along with enhancement of its own H-3**E** and H-5**E** confirmed the sequence of β-D-Glc*p*A-(1→3)-α-D-Gal*p*NAc and residue **B** showed a strong NOE between H-1**B** and H-4**C** together with enhancement of its own H-2**B** defining the substitution pattern of α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc. A strong inter-residue NOE between H-1**C** to H-3**D** and also to its

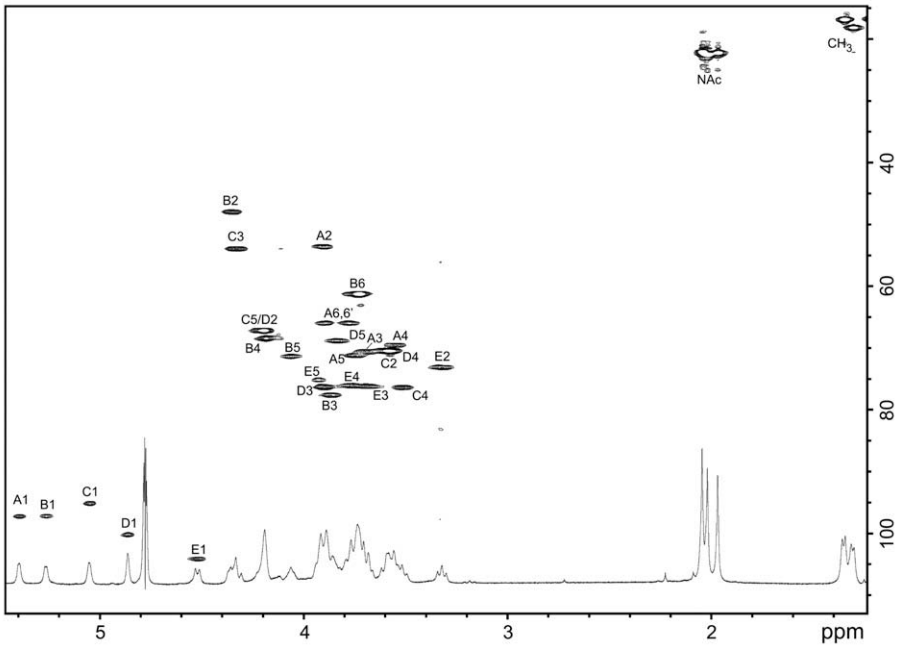

Figure 1. ^1H–^{13}C HSQC correlation of the *Cronobacter muytjensii* strain 3270 LPS O-polysaccharide.

Table 1
^{1}H and ^{13}C NMR chemical shift data for the O-polysaccharide of *C. muytjensii* strain 3270

Glycose	Chemical shift (ppm)					
	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→6)-α-D-Glc*p*NAc-(1→	5.40 (3.0) 97.3 (180)	3.90 53.6	3.70 70.6	3.55 69.6	3.75 71.2	3.90/3.78 65.9
B→3)-α-D-Gal*p*NAc-(1→	5.26 (3.8) 97.2 (178)	4.35 48.0	3.87 77.6	4.18 67.2	4.06 71.3	3.73 61.2
C→4)-α-D-Qui*p*3NAc-(1→	5.05 (3.0) 95.2 (173)	3.57 70.5	4.33 53.9	3.52 76.4	4.21 67.2	1.30 18.2
D→3)-α-L-Rha*p*-(1→	4.86 (~1) 100.3 (174)	4.19 67.2	3.89 76.4	3.59 70.4	3.85 68.8	1.34 16.8
E→4)-β-D-Glc*p*A-(1→	4.52 (7.5) 104.1 (164)	3.32 73.2	3.68 76.2	3.77 75.1	3.92 76.1	– 174.5

Coupling constants ($J_{H\text{-}1/H\text{-}2}$ and $J_{H\text{-}1/C\text{-}1}$) in Hertz are given in parentheses.

own H-2**C** confirmed the α-D-(1→3) linkage of **C** to the α-L-Rha*p* residue. A final connectivity from residue **D** (α-L-Rha*p*) H-1 to H-6,6′ of **A** (α-D-Glc*p*NAc) considered in conjunction with the downfield shift of C-6**A** (65.9 ppm) from reference unsubstituted C-6 (~62 ppm) confirmed the (1→6) linkage of α-L-Rha*p* to the α-D-Glc*p*NAc residue in the O-PS. Further 2D HMBC experiments confirmed the connectivities of H-1**D** to C-6**A**, H-1**A** to C-4**E**, H-1**E** to C-3**B**, H-1**B** to C-4**C**, C-1**C** to H-3**D**, and C-1**D** to H-6**A**. A ^{13}C DEPT experiment inverted the signal located at 65.9 ppm which was subsequently identified as the C-6 of residue **A** (α-D-Glc*p*) involved in glycosidic linkage at its *O*-6 position. Thus, the observed connectivities and anomeric configurations lead to the O-PS structure being defined as an unbranched polymer of a repeating pentasaccharide with the sequence →**B**→**C**→**D**→**A**→**E**→ having the structure

[**B**] [**C**] [**D**] [**A**] [**E**]

3)-α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→6)-α-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→

Further to the deduction of the *O*-PS structure from the results of glycose composition and 2D high resolution NMR spectral analysis, the proposed structure was confirmed using Smith-type periodate oxidation degradation involving stepwise oxidations starting from the O-PS (see Scheme 1) and subsequent characterization of the resulting oligosaccharide degradation products. Periodate oxidation of the O-PS followed by reduction ($NaBH_4$) of the product afforded a high molecular mass polymer that on hydrolysis and GLC analysis allowed to identify D-GalN, L-Rha, D-Qui3N and glycerol (1:1:1:0.7). This result indicated that the D-GlcA and D-GlcNAc components of the parent O-PS were the two residues oxidized by periodate, and that the presence of glycerol in the hydrolysate had arisen from component 6-*O*-linked D-Glc*p*NAc residues. Mild acid hydrolysis of the above oxidized and reduced ($NaBH_4$) O-PS afforded a chromatographically pure oligosaccharide **I** (K_{av} 0.85, Sephadex G-15) having

Table 2
^{1}H and ^{13}C NMR chemical shift data for the derived oligosaccharide **I** from *C. muytjensii* strain 3270 O-polysaccharide

Glycose	Chemical shift (ppm)					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
B^{1} α-D-Gal*p*NAc-(1→	5.27 (3.8) 96.8 (174)	4.20 49.3	3.86 67.2	4.06 71.5	4.00 68.4	3.75 61.2
C^{1}→4)-α-D-Qui*p*3NAc-(1→	5.02 (3.0) 94.82 (170)	3.57 70.4	4.31 53.9	3.52 75.8	4.20 67.1	1.30 18.0
D^{1}→3)-α-L-Rha*p*-(1→	4.84 (~1) 99.7 (172)	4.17 66.9	3.87 76.1	3.58 70.5	3.76 68.9	1.33 16.7
F→1-Glycerol	3.81/3.49 68.5	3.91 70.5	3.6/3.65 62.5			

Coupling constants ($J_{H\text{-}1/H\text{-}2}$ and $J_{H\text{-}1/C\text{-}1}$) in Hertz are given in parentheses.

[B] [C] [D] [A] [E]
→3)-α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→6)-α-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→

↓ $NaIO_4/NaBH_4$/dil. AcOH (O-Polysaccharide)

[B^{1}] [C^{1}] [D^{1}] [F]
α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→1)-glycerol (Oligosaccharide I)

↓ $NaIO_4/NaBH_4$/dil. AcOH

[C^{2}] [D^{2}] [G]
α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→1)-glycol (Oligosaccharide II)

Scheme 1. Smith-type degradation of the O-PS of *C. muytjensii* strain 3270.

Table 3
^{1}H and ^{13}C NMR chemical shift data for the derived oligosaccharide **II** from *C. muytjensii* strain 3270 O-polysaccharide

Glycose	Chemical shift (ppm)					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
C² α-D-Qui*p*3NAc-(1→	5.19 (3.2) 96.6	3.79 71.4	4.28 55.4	3.35 74.7	4.26 69.8	1.41 18.0
D²→3)-α-L-Rha*p*-(1→	5.02 (~1) 100.9	4.31 68.5	4.01 77.56	3.76 71.8	3.94 70.0	1.49 18.2
G→1-Glycol	3.99/3.78 70.1	3.92 61.9				

Coupling constants ($J_{H-1/H-2}$ and $J_{H-1/C-1}$) in Hertz are given in parentheses.

[α] +130 (*c* 0.02, water) composed of D-GalNAc, D-Qui3NAc, L-Rha, and glycerol (1:1:1:1). The glycerol product originates from a (1→6)-linked D-GlcNAc residue in the O-PS and further indicates that this D-GlcNAc residue is glycosidically linked to *O*-4 of a D-Glc*p*A residue thus showing that the O-PS contains a sequence →6)-D-Glc*p*NAc-(1→4)-D-Glc*p*A-(1→. The 2D NMR analysis of **I** (Table 2) is consistent with the above conclusions. Positive ion MS showed the expected mass weight of M + 1 + water = 629 amu, and NMR spectral analysis (Table 2) is consistent with oligosaccharide **I** being a trisaccharide having the structure

α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→1)-glycerol.

A similar Smith-type periodate oxidation procedure made on oligosaccharide **I** followed by Sephadex G-15 column chromatography afforded an oligosaccharide **II** (K_{av} 0.0.45) having ([α] +92 (*c* 0.2 water)) composed of D-Qui3NAc, L-Rha, and glycol (1:1:0.8) which is, consistent with the expected oxidation and removal of the D-GalpNAc endgroup and the production of glycol from the terminal glycerol unit in **I**. The NMR analysis (Table 3) was consistent with the disaccharide **II** having the structure α-D-Qui*p*NAc-(1→3)-α-L-Rha*p*1→glycol.

A third Smith-type periodate oxidation and reduction ($NaBH_4$) applied to oligosaccharide **II** resulted, as expected, in the quantitative recovery of the original disaccharide. The results of the sequence of Smith-type degradations were thus in agreement with the structure of the O-PS proposed from the NMR analysis.

To our knowledge, only two *C. sakazakii* O-PS structures have been reported. The first was reported by Szafranek et al.[24] on the O-antigen of *C. sakazakii* ZORB A 741, as a polymer of a repeating trisaccharide unit having the structure

α-Tyv*p*
↓
2
→3)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→
6
↑
Ac

and the second was reported for *C. sakazakii* HPB 3290[25] O-PS as a polymer of a branched pentasaccharide unit having the structure

→2)-β-D-Qui*p*3NR-(1→6)-α-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→3)-α-D-Gal*p*NAc-(1→
2
↑
R = *N*-acetyl-L-alanyl 1
α-D-Glc*p*

It appears that the two structures are antigenically unrelated to the herein reported O-antigen. The latter finding, considered in conjunction with our glycose composition and DOC–PAGE analyses made on LPS preparations from various *Cronobacter* isolates (unreported), suggests that future structural work will reveal that this group of bacteria may be serologically heterogeneous with respect to their O-antigens, supporting the proposed reclassification by Inversen and Forsythe.[8]

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