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Note

Elucidation of the structure and characterization of the gene cluster of the O-antigen of *Cronobacter sakazakii* G2592, the reference strain of *C. sakazakii* O7 serotype

Nikolay P. Arbatsky [a], Min Wang [b,c], Elena D. Daeva [a], Alexander S. Shashkov [a], Lu Feng [b,c], Yuriy A. Knirel [a,*], Lei Wang [b,c]

[a] Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russian Federation
[b] TEDA School of Biological Sciences and Biotechnology, Nankai University, TEDA, Tianjin 300457, PR China
[c] Tianjin Key Laboratory for Microbial Functional Genomics, TEDA College, Nankai University, TEDA, Tianjin 300457, PR China

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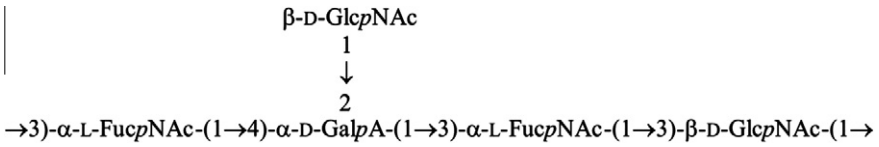
ABSTRACT

The O-specific polysaccharide from the lipopolysaccharide of *Cronobacter sakazakii* G2592 was studied by sugar analysis along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, and the following structure of the pentasaccharide repeating unit was established:



This structure is unique among the known bacterial polysaccharide structures, which is in accord with classification of strain G2592 into a new *C. sakazakii* serotype, O7. It is in agreement with the O-antigen gene cluster of this strain, which was found between the housekeeping genes JUMPStart and *gnd* and characterized by sequencing and tentative assignment of the gene functions.

Cronobacter sakazakii (formerly *Enterobacter sakazakii*) is an important food-borne pathogen, which can be isolated from a wide variety of food. It can cause invasive infections in all age groups, especially, in immunocompromised infants and elders, leading to fatality rates of 33–80% in infected children.[1,2] Recently, *C. sakazakii* strains have been typed into two[3] and, later, seven[4] O-serotypes. Aiming at creation of the molecular basis for understanding the serospecificity and classification of these bacteria to improve their detection, O-antigen (O-specific polysaccharide, O-PS) structures have been elucidated[5–9] in several strains of *C. sakazakii*, including those of serotypes O1 and O2.[5,6] In this paper, we report elucidation of a new structure of the O-PS of *C. sakazakii* G2592, the reference strain of serotype O7,[4] which was isolated from powdered infant formula of India, and characterization of the O-antigen gene cluster of this bacterium.

Lipopolysaccharide was extracted from dried cells of *C. sakazakii* G2592 with hot aqueous phenol. Mild acid degradation of the lipopolysaccharide resulted in an O-PS, which was isolated by GPC on Sephadex G-50. Full acid hydrolysis of the O-PS followed by analysis using sugar and amino acid analyzers revealed galacturonic acid (GalA), 2-amino-2-deoxyglucose (GlcN), and 2-amino-2,6-dideoxygalactose (FucN). Determination of the absolute configurations by GLC of the acetylated (*S*)-2-octyl glycosides showed that GalA and GlcN have the D configuration and FucN has the L configuration.

The ^{13}C NMR spectrum of the O-PS (Fig. 1) showed major signals for five anomeric carbons at δ 98.5–102.3, two CH_3–C groups (C-6 of FucN) at δ 16.7 (2C), two $HOCH_2$–C groups (C-6 of GlcN) at 61.2 and 63.5, one carboxyl group (C-6 of GalA) at δ 174.7, four nitrogen-bearing carbons (C-2 of FucN and GlcN) at δ 49.4 and 57.1 (each 2C), other sugar ring carbons at δ 67.6–77.0, and four *N*-acetyl groups at δ 23.3–23.7 (CH_3), and 175.2–175.8 (CO). The ^{1}H NMR spectrum contained, inter alia, signals for five anomeric protons at δ 4.52–5.39, two CH_3–C groups (H-6 of FucN) at δ 1.17 and 1.26, and four *N*-acetyl groups at δ 1.93–2.04.

* Corresponding author. Tel.: +7 499 1376148; fax: +7 499 1355328.
E-mail addresses: yknirel@gmail.com, knirel@ioc.ac.ru (Y.A. Knirel).

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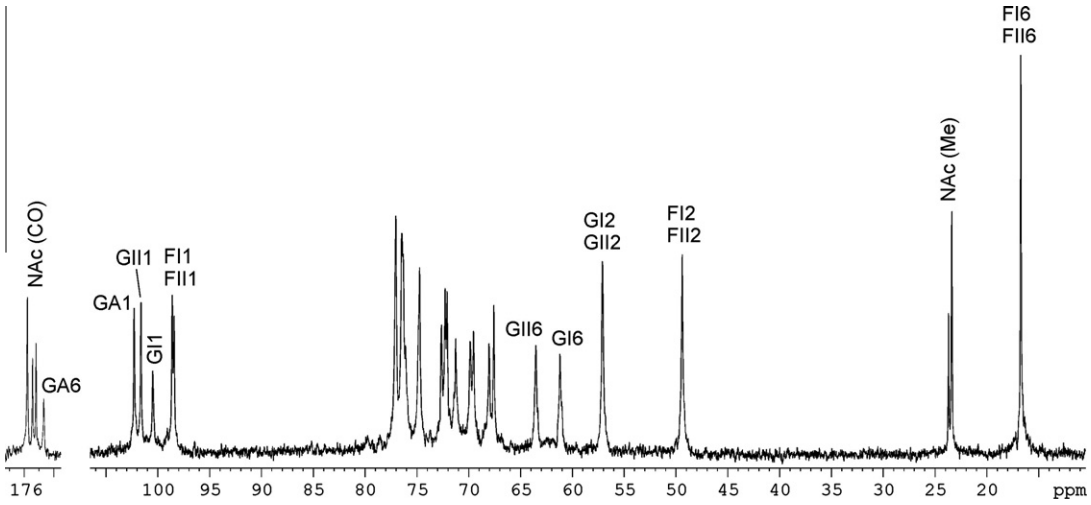

Figure 1. ^{13}C NMR spectrum of the O-PS of *C. sakazakii* G2592. Numbers refer to carbons in sugar residues denoted as follows: FI, FucNAc^{I}; FII, FucNAc^{II}; GI, GlcNAc^{I}; GII, GlcNAc^{II}; GA, GalA.

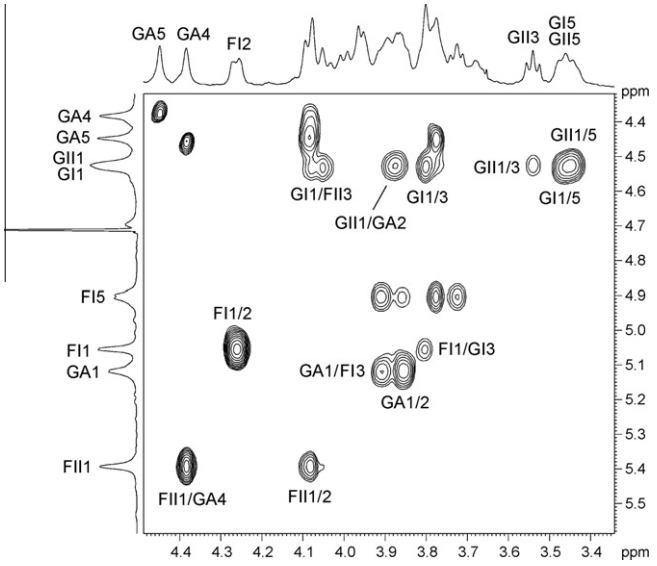

Figure 2. Part of a ROESY spectrum of the O-PS of *C. sakazakii* G2592. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Numbers refer to protons in sugar residues denoted as follows: FI, FucNAc^{I}; FII, FucNAc^{II}; GI, GlcNAc^{I}; GII, GlcNAc^{II}; GA, GalA.

Therefore, the O-PS has a pentasaccharide repeating unit containing two residues each of D-GlcNAc and L-FucNAc and one D-GalA residue.

The ^{1}H and ^{13}C (Fig. 1) NMR spectra of the O-PS were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY (Fig. 2), and ^{1}H,^{13}C HSQC experiments (Table 1). Tracing connectivities in the TOCSY and ROESY spectra revealed five sugar spin systems. Those for the sugars having the *galacto* configuration (FucNAc and GalA) were distinguished by relatively low $^{3}J_{3,4}$ and $^{3}J_{4,5}$ coupling constants (<3 Hz), for FucNAc by H-6,H-5 and H-6,H-4 correlations in the ROESY spectrum, and for the amino sugars (GlcNAc and FucNAc) by correlations between protons at the nitrogen-bearing carbons (H-2) and the corresponding carbons (C-2) in the ^{1}H,^{13}C HSQC spectrum. Relatively small $J_{1,2}$ values (<4 Hz) and H-1 chemical shifts (δ 5.06–5.39) indicated that GalA and both FucNAc residues (FucNAc^{I} and FucNAc^{II}) are α-linked. The H-1 signals of two GlcNAc residues (GlcNAc^{I} and GlcNAc^{II}) were superimposed and their splitting was not seen but the H-1 chemical shifts of δ 4.52–4.53 suggested that both residues are β-linked. The linkage configurations were confirmed by H-1,H-2 correlations for α-glycosides (GalA and FucNAc) and H-1,H-3 and H-1,H-5 correlations for β-glycosides (GlcNAc), which were observed in the ROESY spectrum (Fig. 2).

The 2D ROESY spectrum (Fig. 2) showed also correlations between the following anomeric protons and protons at the linkage carbons: FucNAc^{I} H-1,GlcNAc^{I} H-3; GalA H-1,FucNAc^{I} H-3; FucNAc^{II} H-1,GalA H-4; GlcNAc^{I} H-1,FucNAc^{II} H-3; and GlcNAc^{II} H-1,GalA H-2 at δ 5.06/3.80; 5.12/3.91; 5.39/4.38; 4.53/4.05; and 4.52/3.87, respectively. These data defined the full structure of the branched O-unit with GalA at the branching point and GlcNAc^{II} as the lateral monosaccharide. The glycosylation pattern was confirmed by low-field positions at δ 74.8–77.0 of the signals for

Table 1
^{1}H and ^{13}C NMR chemicals shifts (δ, ppm) of the O-PS of *C. sakazakii* G2592

Unit	Nucleus	1	2	3	4	5	6 (6a, 6b)
→3)-β-D-Glc*p*NAc^{I}-(1→	^{1}H	4.53	3.89	3.80	3.86	3.47	3.77, 3.95
	^{13}C	100.5	57.1	76.5	69.6	76.3	61.2
→3)-α-L-Fuc*p*NAc^{I}-(1→	^{1}H	5.06	4.27	3.91	3.78	4.90	1.26
	^{13}C	98.6	49.4	77.0	72.7	67.6	16.7
→2,4)-α-D-Gal*p*A-(1→	^{1}H	5.12	3.87	4.08	4.38	4.45	
	^{13}C	102.3	74.8	71.3	76.5	72.3	174.7
→3)-α-L-Fuc*p*NAc^{II}-(1→	^{1}H	5.39	4.08	4.05	3.80	3.96	1.17
	^{13}C	98.5	49.4	76.1	69.9	68.0	16.7
β-D-Glc*p*NAc^{II}-(1→	^{1}H	4.52	3.72	3.54	3.18	3.44	3.68, 4.00
	^{13}C	101.7	57.1	74.8	72.1	77.0	63.5

Signals for the *N*-acetyl groups are at δ_H 1.93, 2.01, and 2.04 (2CH_3); δ_C 23.3 (2C), 23.4, 23.7 (all CH_3), 175.2, 175.4, and 175.8 (2C) (all CO).

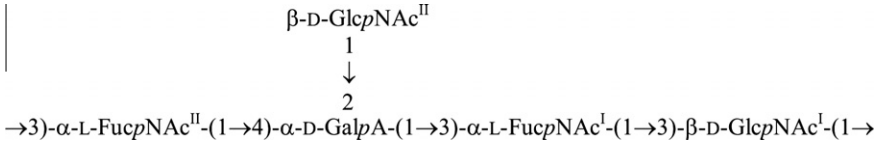

Chart 1. Structure of the O-PS of *C. sakazakii* G2592 (serotype O7).

the linkage carbons (C-2 and C-4 of GalA, C-3 of GlcNAc^{I}, FucNAc^{I} and FucNAc^{II}), as compared with their positions in the corresponding non-substituted monosaccharides.[10,11]

Therefore, the O-PS of *C. sakazakii* G2592 has the structure shown in Chart 1. To our knowledge, this structure is unique among the known bacterial O-PS structures and, particularly, differs from the *C. sakazakii* O-antigen structures established earlier.[5–9] This finding is in agreement with classification of strain G2592 into a new *C. sakazakii* serotype, O7.[4]

The O-antigen gene cluster (OGC) of *C. sakazakii* G2592 has been sequenced and the gene functions were tentatively assigned by similarity to related genes from the available databases. A sequence of 8932 bp from the JUMPStart site to the *gnd* gene was obtained and eight open reading frames, excluding *gnd*, were identified. Genes for synthesis of nucleotide precursors of common sugars, such as GlcNAc, are located outside OGC.[12] The dehydrogenase gene *ugd* and the epimerase gene *gla*, which convert UDP-D-Glc to UDP-D-GalA,[12] map downstream of *gnd*. Three genes for the synthesis of UDP-L-FucNAc, *fnlA*, *fnlB* and *fnlC*,[13] were identified in OGC.

The *wecA* gene that is responsible for the transfer of the first sugar to undecaprenol phosphate to initiate the O-antigen synthesis is located outside OGC.[14] Four glycosyltransferase genes and the O-unit flippase gene, *wzx*, were identified in OGC as expected. The predicted *wzx* product has 13 well-proportioned transmembrane segments and shares 52% similarity with the putative polysaccharide biosynthesis protein Wzx of *Vibrio vulnificus*. However, no O-antigen polymerase gene *wzy* was found in OGC. In *Salmonella* serogroups A, B, and D_1, the *wzy* gene is not located in OGC but mapped far from that in the genome.[15] Since strain G2592 produced a normal S-type LPS showing a typical ladder-like SDS–PAGE pattern (data not shown), it is highly likely that it has a functional *wzy* gene outside OGC.

1. Experimental

1.1. Bacterial strain and cultivation

Strain G2592 was isolated from powdered infant formula of India in Tianjin Entry-Exit Inspection and Quarantine Bureaus of China. It was classified as *C. sakazakii* under a proposed new classification scheme[16] and used as the reference strain of serotype O7 in typing of *C. sakazakii*.[4] Bacteria were grown in 8 L Luria-Bertani medium using a 10-L fermentor (BIOSTAT C10, B. Braun Biotech International, Germany) under constant aeration at 37 °C and pH 7.0. Bacterial cells were washed and dried as described.[17]

1.2. Isolation of lipopolysaccharide and O-specific polysaccharide

Lipopolysaccharide was isolated by the phenol–water procedure[18] followed by dialysis of the extract without layer separation and purification as described.[5] The yield of the LPS was 6% of the dried cells mass. A LPS sample (95 mg) was heated with 2% HOAc for 1.5 h at 100 °C, and a lipid precipitate was removed by centrifugation. The O-PS (15 mg) was obtained by GPC of the supernatant on Sephadex G-50 Superfine as described.[5]

1.3. Sugar analyses

An O-PS sample (2 mg) was hydrolyzed with 3 M CF_3CO_2H (120 °C, 3 h) and monosaccharides were identified using Biotronik LC-2000 sugar and amino acid analyzers (Germany) as described.[5] The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides[19] on a Hewlett–Packard 5890 instrument (USA) equipped with a capillary HP-5ms column (25 m × 0.25 mm) using a temperature gradient of 180 °C (3 min) to 290 °C at 3 °C min^{-1}.

1.4. NMR spectroscopy

Samples were freeze-dried twice from 99.9% D_2O and dissolved in 99.95% D_2O. ^{1}H and ^{13}C NMR spectra were recorded at 30 °C on a Bruker AV600 spectrometer (Germany) using internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0) and acetone (δ_C 31.45) as references. 2D NMR spectra were obtained using standard Bruker software, and Bruker TOPSPIN 2.1 program was employed to acquire and process the NMR data. A mixing time of 100 and 150 ms was used in TOCSY and ROESY experiments, respectively. Other NMR experimental parameters were set essentially as described.[20]

1.5. Sequencing and analysis of genes

Sequencing of the chromosome region between JUMPStart and *gnd*, analysis of genes in OGC and search of databases for possible gene functions were performed as described.[4,21]

Acknowledgments

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