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Structure and genetics of the O-antigen of *Cronobacter sakazakii* G2726 (serotype O3) closely related to the O-antigen of *C. muytjensii* 3270

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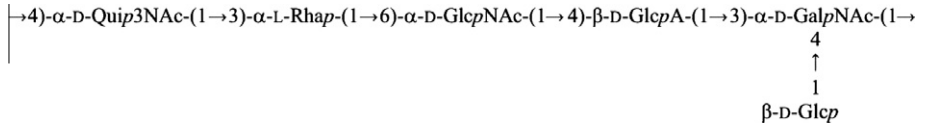
ABSTRACT

The O-specific polysaccharide (O-antigen) was isolated from the lipopolysaccharide of *Cronobacter sakazakii* G2726 (serotype O3) and studied by sugar analysis, Smith degradation, and ^{1}H and ^{13}C NMR spectroscopy. The following structure of the acidic O-polysaccharide was established:



This structure is closely related to that of the O-polysaccharide of *Cronobacter muytjensii* 3270, which has the same main chain and differs only in the lack of glucosylation. The O-antigen gene cluster of *C. sakazakii* G2726 found between the *gnd* and *galF* genes was sequenced, and the gene functions were tentatively assigned by similarity to related genes from available databases and taking into account the O-polysaccharide structure.

1. Introduction

Bacteria of the genus *Cronobacter* within the family Enterobacteriaceae are considered as emerging opportunistic pathogens and have been identified as etiological agents of bacteremia, necrotizing enterocolitis, and neonatal meningitis. Currently, the genus includes six species. *Cronobacter sakazakii* is an important foodborne pathogen, which can be isolated from a wide variety of food, such as milk, cheese, dried foods, meats, water, vegetables, rice, bread, tea, herbs, spices and powdered infant formula. 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, based on the O-antigens, *C. sakazakii* strains have been typed into seven O-serotypes.[3]

The O-antigen represents a polysaccharide chain of the lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria. Being the major target for the host immune system and bacteriophages, it becomes one of the most variable cell constituents. The O-antigen diversity is mainly due to variations in the cluster of genes for its biosynthesis, including genes for the synthesis of nucleotide-activated sugar precursors, genes for glycosyl transferases, and O-antigen processing genes encoding O-antigen flippase (*wzx*) and polymerase (*wzy*). Structural data on the O-antigens provide the molecular basis for identification of clinical isolates and are necessary for understanding their serospecificity.

Chemical structures of the O-antigens have been elucidated in *C. sakazakii* serotypes O1, O2, and O5–O7[4–7] as well as in several non-typed strains.[8–10] In addition, in the serotype strains, the O-antigen gene clusters have been sequenced, and the putative gene functions were assigned taking into account the O-antigen structures.[4–7,11] In this work, we established the structure of the O-antigen of *C. sakazakii* G2726, the reference strain of serotype O3,[3] and characterized its O-antigen gene cluster.

2. Results and discussion

2.1. Elucidation of the O-polysaccharide structure

The LPS was obtained from dried cells of *C. sakazakii* G2726 by extraction with hot aqueous phenol and degraded under mild acidic conditions to give a long-chain polysaccharide (OPS) and a

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short-chain polysaccharide, which were separated by GPC on Sephadex G-50.

Monosaccharide analysis of the OPS using sugar and amino-acid analyzers revealed rhamnose (Rha), Glc, glucuronic acid (GlcA), GlcN, GalN, and 3-amino-3,6-dideoxyglucose (Qui3N). Determination of the absolute configurations of the monosaccharides by GLC of the acetylated (*S*)-2-octyl glycosides showed that Rha has the L configuration and the other monosaccharides have the D configuration. These findings were in agreement with glycosylation effects on the ^{13}C NMR chemical shifts of the OPS (data not shown), which are sensitive to relative absolute configurations of neighboring monosaccharides.[12]

The ^{13}C NMR spectrum of the OPS (Fig. 1) contained signals for six anomeric carbons at δ 96.3–105.6, two CH_3–C groups (C-6 of Rha and Qui3N) at δ 17.9 and 19.0, three nitrogen-bearing carbons (C-2 of GlcN and GalN and C-3 of Qui3N) at δ 49.5–55.0. The ^{1}H NMR spectrum of the OPS showed signals for six anomeric protons at δ 4.47, 4.83 ($J_{1,2}$ 7–8 Hz for both), 4.87 ($J_{1,2}$ <2 Hz), 5.06, 5.27 and 5.42 ($J_{1,2}$ 3–4 Hz for all) as well as two CH_3–C groups (H-6 of Rha and Qui3N) at δ 1.31 and 1.36 ($J_{1,2}$ ~6 Hz for both).

These data together indicated that the OPS has a hexasaccharide repeating unit including one residue each of D-Glc, L-Rha, D-GlcNAc, D-GalNAc, D-Qui3NAc, and D-GlcA.

The 1D NMR spectra of the OPS were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, and ^{1}H,^{13}C HSQC experiments, and spin-systems for α-Gal*p*NAc, β-Glc*p*A, α-Glc*p*NAc, α-Rha*p*, α-Qui*p*3NAc and β-Glc*p* were identified. Low-field positions of the signals for C-3 of α-Rha*p*, C-4 of α-Qui*p*3NAc, C-4 of β-Glc*p*A, C-6 of α-Glc*p*NAc, C-3 and C-4 of α-Gal*p*NAc, as compared with their positions in the corresponding non-substituted monosaccharides,[13–15] revealed the substitution pattern in the repeating unit. The C-2–C-6 chemical shifts of β-Glc*p* were close to those of β-glycopyranose,[13] and hence, this residue occupies the terminal position in the side chain.

The ROESY spectrum of the OPS (Fig. 2) showed the following inter-residue correlations for the anomeric protons: α-Gal*p*NAc H-1, α-Qui*p*3NAc H-4 at δ 5.27/3.51; β-Glc*p*A H-1,α-Gal*p*NAc H-3 at δ 4.47/3.93; α-Glc*p*NAc H-1,β-Glc*p*A H-3,4 at δ 5.42/3.67, 3.81; α-Rha*p* H-1,α-Glc*p*NAc H-6a,6b at δ 4.87/3.85, 3.90; α-Qui*p*3NAc H-1,α-Rha*p* H-2,3 at δ 5.06/4.20, 3.90; and β-Glc*p* H-1,α-Gal*p*NAc H-4 at δ 4.83/4.43. Combined with the ^{13}C NMR chemical shifts data (see above), these data define the monosaccharide sequence in the repeating unit, which was confirmed by a ^{1}H,^{13}C HMBC experiment (Fig. 3).

Therefore, the OPS of *C. sakazakii* G2726 has the structure shown in Chart 1. This structure was confirmed by Smith degradation of the LPS, which included sequential periodate oxidation, borohydride reduction, and selective hydrolysis of the linkages of the oxidized monosaccharides under mild acidic conditions. As a result, the α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→1)-Gro oligosaccharide was obtained, where Gro indicates glycerol derived from the 6-substituted GlcNAc residue. The structure of the oligosaccharide was established by 2D NMR spectroscopy essentially as described for the OPS (for assigned ^{1}H and ^{13}C NMR chemical shifts see Table 1).

NMR spectroscopic studies showed that the short-chain polysaccharide represents an LPS core with one or several repeating units attached. The NMR spectra of this mixture were too complex to be fully assigned but signals for a non-reducing sugar residue were clearly seen and were assigned to α-Qui*p*3NAc (data not shown). Therefore, this monosaccharide occupies the non-reducing end of the polysaccharide and, hence, the first sugar of the repeating unit is GalNAc as shown in Chart 1.

Derivatives of Qui3N also are present in the O-antigens of *C. sakazakii* O1 and O5. It has been suggested[7] that they occupy the non-reducing end of the OPS in these bacteria too and are responsible for the observed cross-reactivity between strains of serotypes O1, O3 and O5.[3] The OPS structure of *Cronobacter muytjensii* 3270[16] is even more closely related to that of *C. sakazakii* G2726 (serotype O3). Both O-antigens have the same main chain and differ only in the lack of glucosylation in the former (Chart 1). The close relatedness of the O-antigens of these two bacteria as well as those of *C. sakazakii* G2726 (serotype O6)[7] and *Cronobacter malonaticus* 3267[17] and a number of other *C. sakazakii* and non-*C. sakazakii* strains (authors' unpublished data) show that the creation of a combined classification scheme for all *Cronobacter* species on the basis of the existing scheme for *C. sakazakii* is advisable.

The OPSs of *C. sakazakii* G2726 (serotype O3) and *C. muytjensii* 3270 also share the α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→ trisaccharide fragment with the OPSs of *Escherichia coli* O71[18,19] and *Salmonella enterica* subsp. *enterica* serovar Dakar (serogroup O28).[20] A serological cross-reactivity of these bacteria with the closely related O-antigens is likely and must be taken into account to avoid errors in detection of strains.

2.2. Characterization of the O-antigen gene cluster

The O-antigen gene cluster (OGC) of *C. sakazakii* G2726 was sequenced and the gene functions were tentatively assigned by similarity to related genes from the available databases. A sequence of 12652 bp from the JUMPStart site to the *gnd* gene was

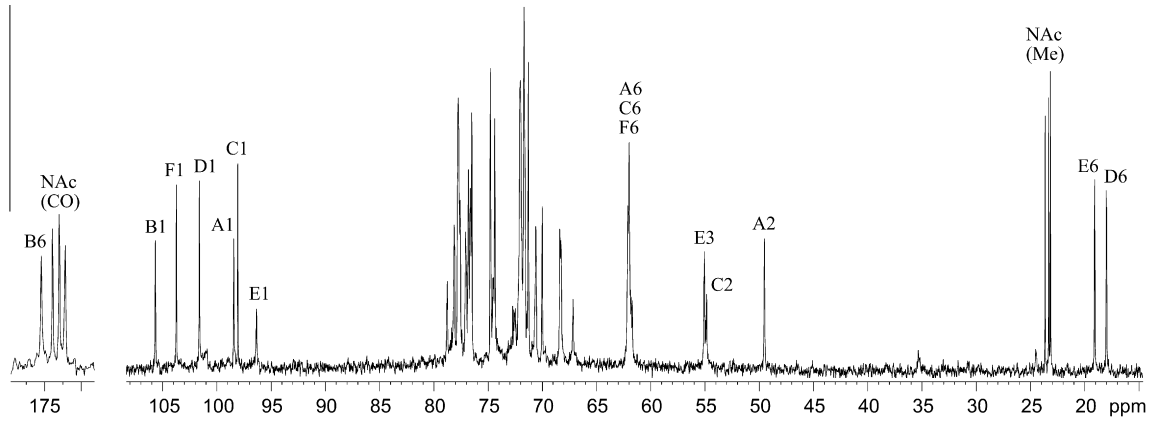

Figure 1. ^{13}C NMR spectrum of the OPS from *C. sakazakii* G2726. Numbers refer to carbons in sugar residues denoted as follows: (A) GalNAc; (B) GlcA; (C) GlcNAc; (D) Rha; (E) Qui3NAc; (F) Glc.