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Structural and genetic relationships of closely related O-antigens of *Cronobacter* spp. and *Escherichia coli*: *C. sakazakii* G2594 (serotype O4)/*E. coli* O103 and *C. malonaticus* G3864 (serotype O1)/*E. coli* O29

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ABSTRACT

O-Antigen (O-polysaccharide) variation is the basis for bacterial serotyping and is important in bacterial virulence and niche adaptation. In this work, we present structural and genetic evidences for close relationships between the O-antigens of the *Cronobacter* spp. and *Escherichia coli*. *Cronobacter sakazakii* G2594 (serotype O4) and *Cronobacter malonaticus* G3864 (serotype O1) are structurally related to those of *E. coli* O103 and O29, respectively, and some other members of the Enterobacteriaceae family differing in the patterns of lateral glucosylation (*C. sakazakii* G2594) or O-acetylation (*C. malonaticus* G3864). The O-antigen gene clusters of the corresponding *Cronobacter* and *E. coli* strains contain the same genes with high-level similarity, and the structural differences within both O-antigen pairs were suggested to be due to modification genes carried by prophages.

1. Introduction

Cronobacter spp. are opportunistic food borne pathogens that can cause necrotizing enterocolitis, bacteraemia, and meningitis, predominantly in neonates.[1] Currently, the genus *Cronobacter* is comprised of seven species: *C. condiment*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis*.[2–6] Recently, it had been proposed to move three original *Enterobacter* species, *E. helveticus*, *E. pulveris*, and *E. turicensis*, to the genus *Cronobacter* as *C. helveticus*, *C. pulveris*, and *C. zurichensis*, respectively.[7] However, later it was concluded that the three species constituted two new genera and proposed to reclassify them to *Franconibacter helveticus*, *Franconibacter pulveris*, and *Siccibacter turicensis*, respectively.[8] *C. sakazakii*, *C. malonaticus*, and *C. turicensis* are the three species most often isolated from infantile cases.[9]

The O-antigen (O-polysaccharide) composed of a number of oligosaccharide repeats (O-units) is a highly variable part of the lipopolysaccharide in the outer membrane of Gram-negative bacteria. Each strain expresses a particular O-antigen form, which appears to be the major target of the host immune system and bacteriophages. O-Antigen variations are important in bacterial virulence and niche adaptation.[10] By now, based on the O-antigens, 17 O-serogroups of *Cronobacter* spp. have been identified,[11–14] including 7 for *C. sakazakii*, 3 for *C. turicensis*, 2 for each of *C. malonaticus*, *C. muytjensii*, and *C. dublinensis*, and 1 for *C. universalis*. For several representatives of the *Cronobacter* spp., chemical structures of the O-polysaccharides have been established (Refs. 15–21 and refs. cited in Refs. 15,17).

In this work, structures of the O-polysaccharides of *C. sakazakii* G2594 (serotype O4) and *C. malonaticus* G3864 (serotype O1) were elucidated and found to be appropriate to their O-antigen gene clusters. The *Cronobacter* structures established were found to be closely related to those of *Escherichia coli* O103 and O29, respectively, and evolutionary relationships between the two pairs of bacteria are discussed.

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2. Results and discussion

2.1. *Cronobacter sakazakii* G2594 (serotype O4)

Structure of the O-polysaccharide: Lipopolysaccharide was obtained from cells of *C. sakazakii* G2594 by extraction with hot aqueous phenol and degraded with mild acid to give a high-molecular mass O-polysaccharide isolated by GPC on Sephadex G-50.

Full acid hydrolysis of the polysaccharide released Glc, GlcN, GalN, and 3-amino-3,6-dideoxygalactose (Fuc3N) in the ratios 3.3:1.8:1.0:0.7, which were identified using sugar and amino acid analyzers. GLC of the acetylated (*S*)-octyl glycosides showed that all sugars have the D configuration. In addition, 3-hydroxybutanoic acid (Hb) was released and its *R* configuration was established by GLC of the trifluoroacetylated (*S*)-octyl ester.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for seven anomeric carbons at δ 98.3–103.8, four nitrogen-bearing carbons (C-2 of GlcN and GalN and C-3 of Fuc3N) at δ 50.9–56.0, one C–CH_2–C group (C-2 of Hb) at δ 46.1, five CH_3–C groups (C-6 of Fuc3N at δ 16.6; H-4 of Hb and three Me of NAc at δ 23.1–23.7), and four CO groups at δ 174.6–175.8. Attached-proton test revealed signals for six $HOCH_2$–C groups, including four unsubstituted groups at δ 61.1–62.5 and two O-substituted groups at δ 66.3 and 69.3. The ^{1}H NMR spectrum of the polysaccharide contained signals for seven anomeric protons for three β-linked sugars at δ 4.54, 4.69 and 4.77 (all d, $J_{1,2}$ 7–8 Hz) and four α-linked sugars at δ 4.93, 5.12, 5.28 and 5.49 (d, $J_{1,2}$ ~3 Hz, or broadened singlet) (Fig. 2). There were also signals for one C–CH_2–C group (H-2 of Hb) at δ 2.45 and 2.51 and five CH_3–C groups at δ 1.24, 1.26 (H-6 of Fuc3N and H-4 of Hb; both d, J ~6 Hz), 2.04, 2.06, and 2.08 (Me of NAc; all s).

These data indicated that the polysaccharide had a heptasaccharide O-unit consisting of one residue each of D-GalN and D-Fuc3N, two residues of D-GlcN, and three residues of D-Glc as well as three *N*-acetyl groups and one (*R*)-3-hydroxybutanoyl group.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC, and HSQC-TOCSY experiments, and spin-systems for seven sugar residues and a 3-hydroxybutanoyl group were identified. The coupling constants determined from the 1D and 2D spectra showed that (i) all monosaccharides were in the pyranose form; (ii) Fuc3N, one of the GlcN residues ($GlcN^I$), and one of the Glc residues (Glc^I) were

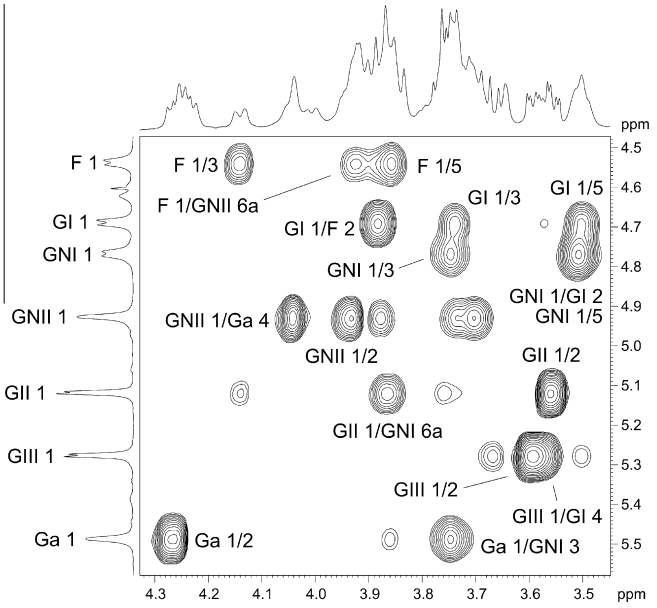

Figure 2. Part of a 2D ROESY spectrum of the O-polysaccharide from *C. sakazakii* G2594. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Numbers refer to protons in sugar residues denoted as indicated in the legend to Figure 1.

β-linked; and (iii) GalN, the second GlcN residue ($GlcN^{II}$), and two other Glc residues (Glc^{II} and Glc^{III}) were α-linked. The configurations of the glycosidic linkages were confirmed by interresidue H-1,H-2 correlations for α-linked monosaccharides and H-1,H-3 and H-1,H-5 correlations for β-linked monosaccharides, which were observed in the 2D ROESY spectrum of the polysaccharide (Fig. 2).

Low-field positions of the signals for C-2 of Fuc3N, C-4 of GalN, C-6 of $GlcN^{II}$, C-2 and C-4 of Glc^I, C-3 and C-6 of $GlcN^I$, as compared with their positions in the corresponding non-substituted monosaccharides,[22,23] revealed the substitution pattern in the O-unit. In accordance with the presence of two branched points (Glc^I and $GlcN^I$), Glc^{II} and Glc^{III} were located at the terminal position of the side chains as followed from the similarity of their C-2–C-6 chemical shifts to those of α-glucopyranose.[22]

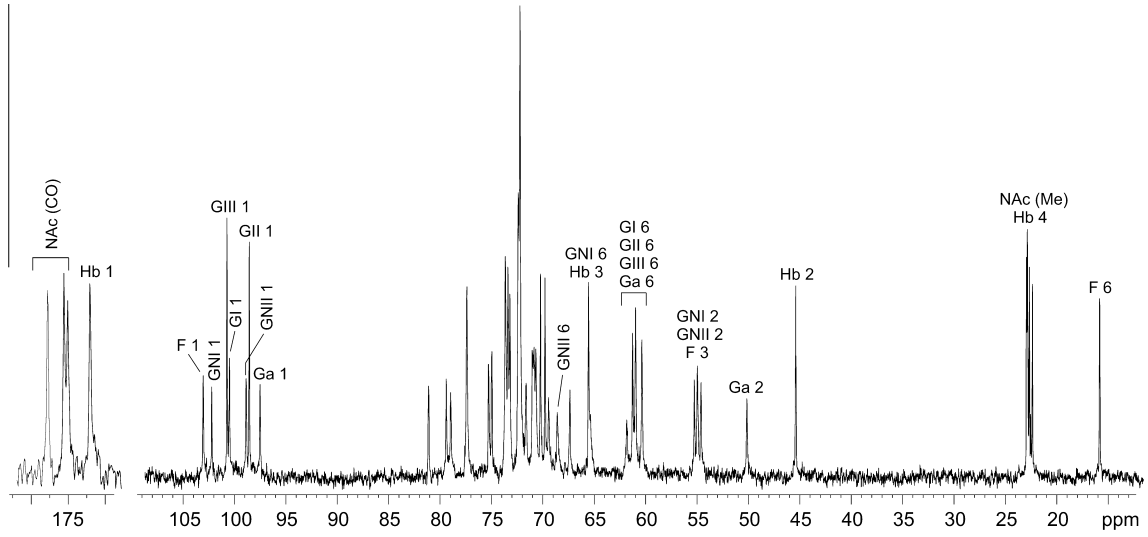

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide from *C. sakazakii* G2594. Numbers refer to carbons in sugar residues denoted as follows: GI, β-Glc^I; GII, α-Glc^{II}; GIII, α-Glc^{III}; GNI, $GlcN^I$; GNII, $GlcN^{II}$; Ga, GalN; F, Fuc3N; Hb, 3-hydroxybutanoyl.

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