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Note

Structural and genetic characterization of the O-antigen of *Enterobacter cloacae* C5529 related to the O-antigen of *E. cloacae* G3054

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ABSTRACT

On mild acid degradation of the lipopolysaccharide of *Enterobacter cloacae* C5529, the O-polysaccharide chain was cleaved at the linkages of 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (di-*N*-acetylpseudaminic acid, Pse*p*5Ac7Ac). The resultant oligosaccharide and an alkali-treated lipopolysaccharide were studied by sugar analysis along with ^{1}H and ^{13}C NMR spectroscopy, and the following structure of the tetrasaccharide repeating unit of the O-polysaccharide was established:

→4)-β-Pse*p*5Ac7Ac-(2 → 3)-β-D-Gal*p*-(1 → 6)-β-D-Gal*f*-(1 → 3)-α-D-Gal*p*-(1→

It differs from a structurally related O-polysaccharide of *E. cloacae* G3045 studied early (Perepelov, A. V.; Wang, M.; Filatov, A. V.; Guo, X.; Shashkov, A. S.; Wang, L.; Knirel, Y. A. *Carbohydr. Res.* 2015; 407:59–62) in positions of substitution of β-Pse*p*5Ac7Ac (O-4 *vs.* O-8) and β-Gal*p* (O-3 *vs.* O-6) and the absence of a side-chain α-Gal*p* residue. The O-antigen gene clusters of *E. cloacae* C5529 and G3045 are organized identically and include genes with the same putative functions in the O-polysaccharide synthesis. Based on these and serological data, it is suggested to combine *E. cloacae* C5529 and G3054 in one O-serogroup as two subgroups.

Enterobacter cloacae has been isolated from a wide range of infections in hospitalized patients, particularly in neonatal critical care [1]. Extended-spectrum β-lactamases (ESBLs) and carbapenemases have been reported to be widespread in *E. cloacae* [2]. With the growing worldwide spread of *E. cloacae* strains that produce extended-spectrum β-lactamases (ESBLs) capable of hydrolyzing almost all β-lactams except carbapenems [3,4], *E. cloacae* became an emerging drug-resistant nosocomial pathogen [5].

In order to substantiate the antigenic heterogeneity and cross-reactivity on the molecular level, detailed structural studies of the O-antigens are performed, which help to improve the classification of *E. cloacae* strains. A few *E. cloacae* O-antigen-related studies have been reported, and until recently, O-polysaccharide structures have been established for four strains from different O-serogroups [6–10]. The O-polysaccharides of two strains, C6285 and G3054, include non-2-ulosonic acids, namely, di-*N*-acetyllegionaminic acid and di-*N*-acetylpseudaminic acid, respectively. In this work, we studied another pseudaminic acid-containing O-polysaccharide of a clinical isolate of *E. cloacae*, C5529, and found it to be structurally, genetically, and serologically related to that of *E. cloacae* G3054 from serogroup O1 [9].

The lipopolysaccharide (LPS) was isolated from *E. cloacae* C5529 cells by extraction with hot phenol-water. On degradation with dilute acetic acid, the LPS afforded no expected O-polysaccharide but an oligosaccharide (OS), evidently owing to cleavage of acid-labile glycosidic linkages of an O-polysaccharide component. Further studies showed that this component was 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (di-*N*-acetylpseudaminic acid, Pse5Ac7). Sugar analysis using GLC of the alditol acetates derived after full acid hydrolysis of the

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OS revealed only galactose. GLC analysis of the acetylated (*S*)-2-octyl glycosides indicated that Gal has the D configuration.

The ^{13}C NMR spectrum of the OS (Fig. 1, top) showed signals for four anomeric carbons at δ 97.9, 98.0 (quaternary carbon; data of a DEPT experiment), 104.7, and 110.5, three OCH_2—C groups at δ 62.3, 62.9, and δ 72.4 (data of a DEPT experiment), one CH_3—C group at δ 16.8, one C—CH_2—C group at δ 33.3, two nitrogen-bearing carbons at δ 49.2 and 54.2, 15 oxygen-bearing sugar ring carbons in the region δ 68.4–84.3, two *N*-acetyl groups at δ 23.4, 23.5 (both Me), and δ 175.2 (2 CO). The ^{1}H NMR spectrum of the OS contained *inter alia* signals for three anomeric protons at δ 4.44–5.13, one CH_3—CH group at δ 1.09 (d, J ~ 7 Hz), one CH—CH_2—C group at δ 2.11 and δ 1.86, and two *N*-acetyl groups at δ 1.97 and 2.02.

The ^{1}H and ^{13}C NMR spectra of the OS were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, and ^{1}H,^{13}C HSQC experiments, and spin systems for β-Gal*p* (**A**), β-Gal*f* (**B**), α-Gal*p* (**C**), and β-Pse*p*5Ac7Ac (**D**) were identified (Table 1). The last sugar was confirmed by a comparison of ^{13}C NMR chemical shifts and $^3J_{H,H}$ coupling constants with published data [11].

Significant downfield displacements of the signals for C-3 of unit **C,** C-4 of unit **D**, and C-6 of unit **B**, as compared with their positions in the corresponding non-substituted monosaccharides [11,12], indicated the linkage position in each sugar residue. A 2D ROESY experiment revealed strong interresidue cross-peaks at δ 4.44/3.77, 5.13/3.74, and 5.05/4.23, which were assigned to **A** H-1,**B** H-6a; **B** H-1,**C** H-3; and **C** H-1,**D** H-4 correlations, respectively. These data were in agreement with the ^{13}C NMR chemical shift data and defined the monosaccharide sequence in the OS, which, therefore, has the following structure:

β-D-Gal*p*-(1→6)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→4)-α-Pse5Ac7Ac

A **B** **C** **D**

To confirm that the OS represented a chemical repeat (O unit) of the O-polysaccharide and to determine the linkage mode between the oligosaccharide repeats, the LPS was O-deacylated with aqueous ammonia. The resultant polymer (LPS_{OH}) was studied by NMR spectroscopy as described above for the OS (for the ^{13}C NMR spectrum see Fig. 1, bottom; assigned ^{1}H and ^{13}C NMR chemical shifts are tabulated in Table 1). Spin systems of the same four monosaccharide residues as in the OS were identified. The ^{1}H,^{13}C HSQC spectrum revealed a downfield displacement of the H-3/C-3 cross-peak of unit **A** from δ 3.65/74.0 in the OS to δ 4.11/76.4 in the LPS_{OH}, and thus indicated substitution of unit **A** at position 3. A relatively large difference between the H-3ax and H-3eq chemical shifts of unit **D** (0.96 ppm) and a lower-field position of the C-6 signal at δ 74.4 in LPS_{OH}, as compared with its position at δ 71.6 in OS, demonstrated the β configuration of Pse*p*5Ac7Ac [11].

Therefore, the O-polysaccharide of *E. cloacae* C5529 has the structure shown in Chart 1. It is structurally related to the O-polysaccharide of *E. cloacae* G3054 studied by us earlier [9] (Chart 1).

The O-antigen gene cluster of *E. cloacae* C5529 was found between the housekeeping genes *galF* and *gnd*. 12 open reading frames (Orfs) excluding *galF* and *gnd* were identified in the gene cluster, all of which have the same transcriptional direction from *galF* to *gnd*. The cluster was organized in the same manner as that of *E. cloacae* G3054 [9] and the corresponding genes showed high-level homology (Fig. 2). Therefore, they were assigned the same functions in the O-polysaccharide synthesis: i) *pseBCFGH* genes for synthesis of CMP-Pse*p*5Ac7Ac from UPP-GlcNAc, ii) *glf* gene for mutase that converts UDP-D-Gal*p* into UDP-D-Gal*f*, iii) *wbaP* gene for D-Gal-1-P-transferase, which initiates the O-unit synthesis by transferring D-Gal-1-P from UDP-D-Gal to undecaprenyl phosphate [13], iv) three glycosyltransferase genes *orf7*, *orf9*, and *orf10*, and v) O-antigen processing genes: *wzx* for O-unit flippase and *wzy* for O-antigen polymerase.

Glycosyltransferases are specific for sugar donors, sugar acceptors, and the linkage between them. Orf9 shares 47% similarity with WcaN, a glycosyltransferase involved in the synthesis of the O9 antigen of *Klebsiella pneumoniae*, which shares the β-D-Gal*f*-(1 → 3)-α-D-Gal*p* linkage with *E. cloacae* C5529. Therefore, Orf9 was suggested to be responsible for formation of this linkage in C5529. The protein sequences of Orf7 in *E. cloacae* G3054 and C5529 are identical, and there are minor differences in the protein sequences of Orf10 in the two strains. We proposed that Orf7 is responsible for formation of the β-D-Gal-(1 → 6)-β-D-Gal linkage that is common

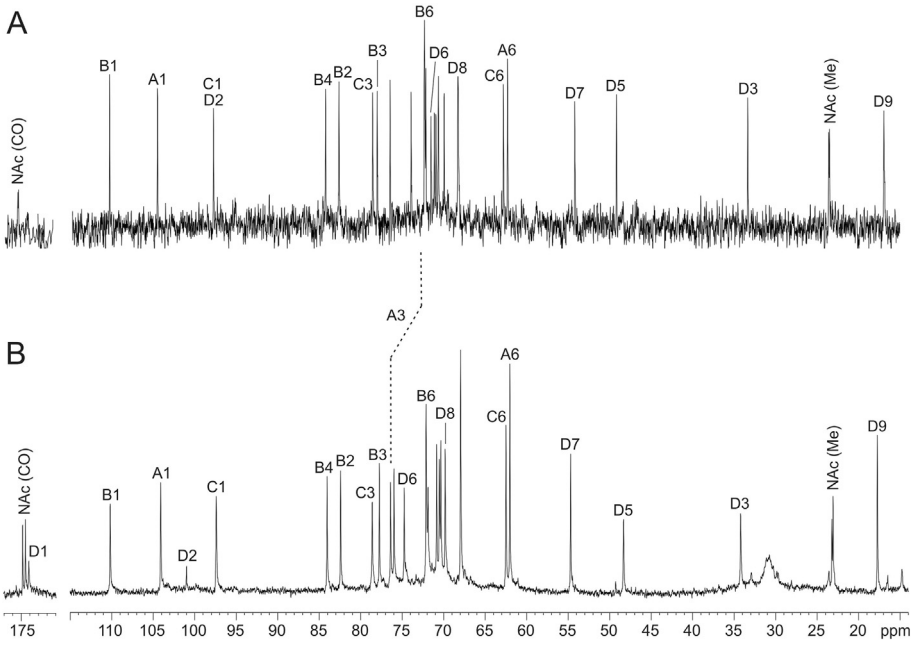

Fig. 1. ^{13}C NMR spectra of the OS (top) and LPS_{OH} (bottom) from *E. cloacae* C5529. Arabic numerals refer to carbons in sugar residues denoted by letters as shown in Table 1.