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Note

Structural and serological studies on the O-antigen show that *Citrobacter youngae* PCM 1505 must be classified to a new *Citrobacter* O-serogroup

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ABSTRACT

The O-polysaccharide obtained by mild acid hydrolysis of the lipopolysaccharide of *Citrobacter youngae* PCM 1505 was studied by sugar and methylation analyses along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopies. The following structure of the tetrasaccharide repeating unit of the polysaccharide was established:

$$\begin{array}{l}\rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Rha}p\text{-}(1\rightarrow \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad 4 \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \uparrow \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad 1 \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \alpha\text{-D-Gal}f\end{array}$$

Structural and serological data obtained earlier and in this work show that the strain studied is a candidate to a new *Citrobacter* O-serogroup.

Gram-negative bacteria of the genus *Citrobacter* from the family Enterobacteriaceae are opportunistic pathogens often responsible for serious enteric diseases as well as extraintestinal disorders, such as neonatal meningitis and brain abscesses.[1] Currently, the genus comprises 11 named genomospecies,[1] and known *Citrobacter* strains are classified into 43 O-serogroups[2] and 20 chemotypes based on their serological cross-reactivity and chemical composition of the lipopolysaccharide (LPS) O-antigens.[3] So far the structures of more than 30 different *Citrobacter* O-antigens (O-polysaccharides, OPSs) have been elucidated.[1,4–11] Chemical and serological studies on *Citrobacter* LPSs are carried out with the aim to understand the molecular basis for cross-reactivity between representatives of various enterobacterial genera and to improve the serological classification of this highly heterogenous group of bacteria.

Now, we report on a new structure of the OPS of *Citrobacter youngae* PCM 1505. This strain was originally classified to serogroup O6 as IHE Be 16/50 = M.W. Wright strain 'Mich 11'. Later, the pathogenic strain 'Mich 11' initially isolated from stools was reclassified as a strain of *C. youngae* serogroup O8.[12,13] The data on the OPS structure combined with serological data showed that, in fact, strain PCM 1505 belongs to neither of the serogroups.

The LPS was isolated from dry bacterial cells by extraction with hot aqueous phenol. Mild acid degradation of the lipopolysaccharide afforded an OPS, which was isolated by GPC on Sephadex G-50. Sugar analysis by GLC of the acetylated alditols derived after hydrolysis of the OPS with 2 M CF_3CO_2H revealed galactose and rhamnose in the ratio ~1:2.9. GLC of the acetylated (*S*)-2-octyl glycosides showed that rhamnose has the D configuration. Oxidation with D-galactose oxidase confirmed the D configuration of galactose. The content of galactose determined colorimetrically in the OPS hydrolysate was 27.5%. Methylation analysis demonstrated the presence of 2-substituted, 3-substituted, 3,4-disubstituted

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rhamnose and terminal galactofuranose in the ratio 0.98:0.87:1.0:0.81 or 0.7:1.0:1.0:0.75 when 2 M CF_3CO_2H or 10 M HCl were used for hydrolysis, respectively. Therefore, the OPS has a branched tetrasaccharide repeating unit with galactofuranose as a lateral monosaccharide residue.

The ^{1}H NMR and ^{13}C NMR (Fig. 1) spectra of the OPS showed, *inter alia*, signals for four anomeric atoms at δ_H 4.80–5.29 and δ_C 97.9–103.7, three major CH_3-C groups of rhamnose residues (Rha^I–Rha^{III}) at δ_H 1.33–1.35 and δ_C 18.1–18.7, and one $HOCH_2$–C group of galactose at δ_C 63.6. The four major sugar spin systems were assigned by tracing connectivities in the 2D ^{1}H,^{1}H COSY, TOCSY, and ^{1}H,^{13}C HSQC spectra (Table 1).

$^3J_{H,H}$ coupling constants estimated from the 2D NMR spectra demonstrated that all rhamnose residues are in the pyranose form and confirmed that galactose is in the furanose form. The C-5 chemical shifts of δ 69.1 and 70.7 indicated that Rha^{II} and Rha^{III} are α-linked, whereas Rha^I characterized by the value for C-5 of δ 74.2 is β-linked (compare published data[14] δ 70.0 for α-Rha*p* and δ 73.7 for β-Rha*p*). The β configuration of Rha^I was confirmed by intraresidue correlations of H-1 with H-3 and H-5 in the 2D ROESY spectrum of the OPS. The C-1 chemical shift of δ 103.2 showed that Gal is α-linked (the values for C-1 of δ 103.8 and 109.9 have been reported[15] for methyl α- and β-galactofuranosides, respectively).

Low-field positions of the signals for C-2 of Rha^I, C-3 of Rha^{III}, and C-3 and C-4 of Rha^{II} at δ 78.1–81.3, as compared with their positions at δ 71.3–73.5 in the spectra of the corresponding anomers of non-substituted rhamnopyranose,[14] revealed the glycosylation pattern in the repeating unit and showed that Rha^{II} is located at the branching point. In accordance with the terminal position of Gal, the C-2 to C-6 chemical shifts of this residue were close to those in the non-substituted monosaccharide.[15]

In the 2D ROESY spectrum of the OPS, there were a number of interresidue correlations, from which the correlations between the transglycosidic protons were assigned as follows, taking into account the positions of substitution of the monosaccharide residues: Rha^{II} H-1, Rha^I H-2; Rha^{III} H-1, Rha^{II} H-3; Rha^I H-1, Rha^{III} H-3, and Gal H-1, Rha^{II} H-4 at δ 5.07/4.13; 5.08/4.15; 4.80/4.03, and 5.29/3.66, respectively. Accordingly, the ^{1}H,^{13}C HMBC spectrum showed the following correlations between the anomeric protons and linkage carbons and vice versa: Rha^{III} H-1, Rha^{II} C-3; Rha^I H-1, Rha^{III} C-3; Rha^{II} C-1, Rha^I H-2; Rha^I C-1, Rha^{III} H-3, and Gal C-1, Rha^{II} H-4 at δ 5.08/81.3; 4.80/78.2; 102.5/4.13; 97.9/4.03, and 103.2/3.66, respectively. These data defined the sequence of the monosaccharides in the repeating unit, and hence the OPS of *C. youngae* PCM 1505 has the structure shown in Chart 1.

The OPS studied shares the rhamnose backbone structure with that of *C. braakii* (formerly *C. freundii*) PCM 1536 from serogroup O8.[16] The two OPSs differ only in the lateral sugar residue (Chart 1), which is D-galactofuranose in strain PCM 1505 or D-xylofuranose in strain PCM 1536.

Immunoblotting was carried out to check if there is any serological relationship between *C. youngae* PCM 1505 and *C. braakii* PCM 1536 (serogroup O8). No cross-reaction was observed between the LPSs of the two strains and either anti-*C. youngae* PCM 1505 or anti-*C. braakii* PCM 1536 sera (Fig. 2). Earlier, it has been demonstrated that *C. youngae* PCM 1505 and PCM 1504 must not be classified to serogroup O6 together with *C. braakii* PCM 1531.[6] Therefore, the chemical and serological data show that *C. youngae* PCM 1505 does not belong to any existing *Citrobacter* O-serogroups and must be reclassified to a new serogroup.

1. Experimental

1.1. Bacterial strain, cultivation, isolation of the lipopolysaccharide and O-polysaccharide

C. youngae PCM 1505 and *C. braakii* PCM 1536 were from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Strain PCM 1505 was classified originally as a member of serogroup O6 (IHE Be 16/50 = M.W. Wright strain 'Mich 11' = PCM 1505 O6:16) but later was reclassified to serogroup O8 as *C. youngae* O8:32,33,[13] and is present in the Lanyi collection as serotype O8a,1c;32,33.[12] Strain PCM 1536 (IHE Be 64/57) was classified as *C. braakii* O8a,8b:35,37. Bacteria were cultivated in a liquid medium as described,[17] then harvested and freeze-dried.

LPS of strain PCM 1505 was isolated from bacterial cells by the phenol–water procedure,[18] recovered from the water phase, and purified as described.[19] The yield of the LPS was 1.7% of dry bacterial mass. A sample of the LPS (270 mg) was heated with 1% HOAc (27 mL) for 1.5 h at 100 °C, and a lipid precipitate was removed by centrifugation. The carbohydrate-containing supernatant (61% of

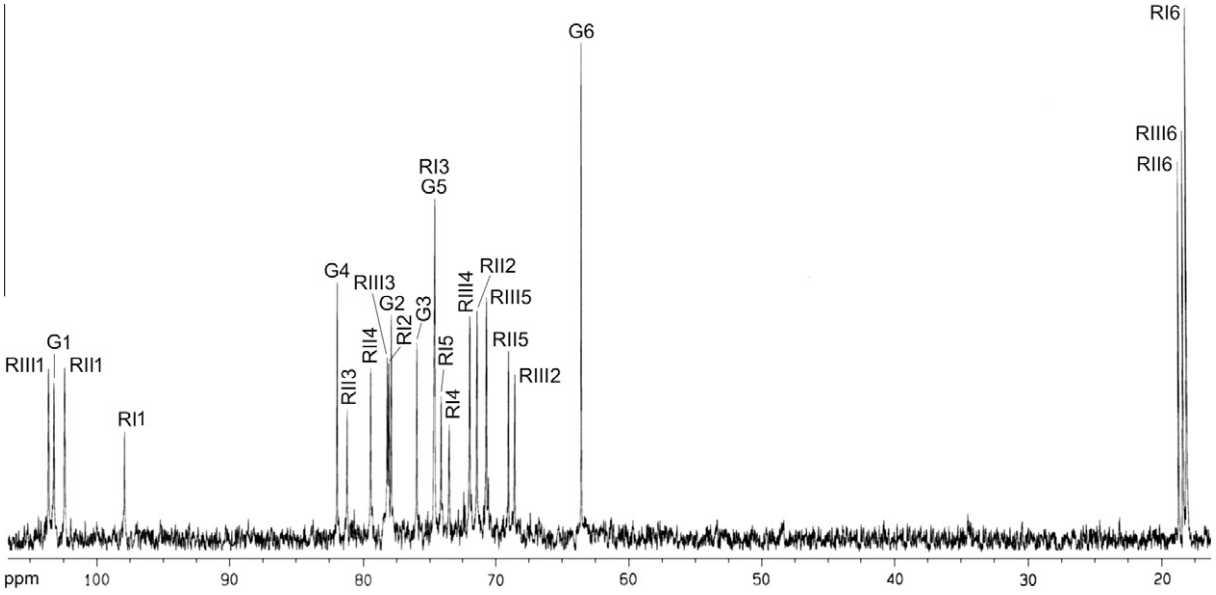

Figure 1. ^{13}C NMR spectrum of the OPS from *C. youngae* PCM 1505. RI-RIII indicate Rha^I–Rha^{III}, G indicates Gal.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the O-polysaccharide of *C. youngae* PCM 1505

Sugar residue	Nucleus	1	2	3	4	5	6 (6a, 6b)
→2)-β-D-Rha*p*^{I}-(1→	^{1}H	4.80	4.13	3.73	3.45	3.42	1.33
	^{13}C	97.9	78.1	74.7	73.6	74.2	18.1
→3,4)-α-D-Rha*p*^{II}-(1→	^{1}H	5.07	4.19	4.15	3.66	4.34	1.35
	^{13}C	102.5	71.5	81.3	79.5	69.1	18.7
→3)-α-D-Rha*p*^{III}-(1→	^{1}H	5.08	4.17	4.03	3.55	3.90	1.33
	^{13}C	103.7	68.6	78.2	72.0	70.7	18.4
α-D-Gal*f*-(1→	^{1}H	5.29	4.05	4.07	3.71	3.79	3.60, 3.73
	^{13}C	103.2	77.9	76.0	82.0	74.7	63.6

→3)-α-D-Rha*p*^{III}-(1→3)-α-D-Rha*p*^{II}-(1→2)-β-D-Rha*p*^{I}-(1→
4
↑
1
α-D-Gal*f*

→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-β-D-Rha*p*-(1→
4
↑
1
α-D-Xyl*f*

Chart 1. Structures of the OPSs from *C. youngae* PCM 1505 (this work) and *C. braakii* PCM 1536 (serogroup O8).[16]

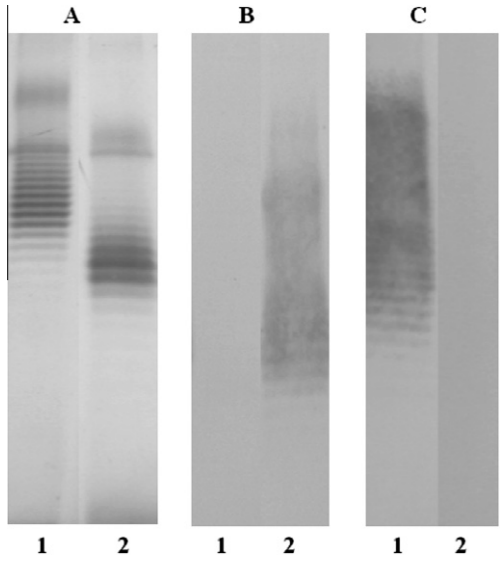

Figure 2. Silver-stained SDS–PAGE (A) and immunoblotting of the LPSs of *C. braakii* PCM 1536 (lane 1) and *C. youngae* PCM 1505 (lane 2) with anti-*C. youngae* PCM 1505 serum (B) and anti-*C. braakii* PCM 1536 (serogroup O8) serum (C).

the LPS mass) was fractionated by GPC on a column (100 × 2.0 cm) of Sephadex G-50 Fine (Sigma) in 0.05 M pyridine/HOAc buffer pH 5.6, and monitored using the phenol–sulfuric acid reaction. A high-molecular mass OPS was obtained in a yield of 43% of the total amount of carbohydrates eluted from the column.

1.2. Sugar and methylation analyses

A sample of the OPS was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), monosaccharides were converted conventionally into alditol acetates and analyzed by GLC on a Hewlett-Packard 5971A instrument equipped with an HP-1 capillary glass column (12 m × 0.2 mm) using a temperature program of 150 °C (3 min) to 270 °C at 8 °C min^{-1}. The absolute configuration of rhamnose was determined by GLC of the acetylated (*S*)-2-octyl glycosides[20] under the same conditions. The absolute configuration of galactose was established using D-galactose oxidase[21] after hydrolysis of the OPS with 2 M CF_3CO_2H (120 °C, 2 h).

Methylation of the OPS was performed as described.[22] The partially methylated monosaccharides were derived by hydrolysis of the methylated polysaccharide with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 30 min), converted into the alditol acetates, and analyzed by GLC–MS on a Hewlett-Packard 5971A instrument as in sugar analysis.

1.3. NMR spectroscopy

An OPS sample was freeze-dried twice from a 99.9 % D_2O solution and dissolved in 99.95% D_2O. ^{1}H and ^{13}C NMR spectra were recorded at 53 °C on a Bruker DRX-500 spectrometer (Germany) using internal acetone (δ_H 2.225, δ_C 31.45) as reference. 2D NMR spectra were obtained using standard Bruker software, and Bruker XWINNMR 2.6 program was employed to acquire and process the NMR data. A mixing time of 200 ms was used in ROESY and TOCSY experiments.

1.4. SDS–PAGE and immunoblotting

SDS–PAGE of the LPSs was performed by the method of Laemmli.[23] The gels were stained using the silver reagent.[24] Rabbit antisera against *Citrobacte*r strains PCM 1505 and PCM 1536 (serogroup O8) were prepared as described.[17] Immunoblotting was carried out as previously.[25] Briefly, after separation by SDS–PAGE, the LPSs were transblotted from the gel onto an Immobilon P (Millipore Corp., Bedford, MA, USA) membrane. The transblot was incubated with antiserum, washed with Tris-buffered saline (20 mM Tris/HCl, 50 mM NaCl, pH 7.5), and incubated with alkaline phosphatase-conjugated goat anti-(rabbit IgG). The immunoblot was visualized with a staining reagent (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.05 M Tris/HCl, pH 9.5, containing 5 mM $MgCl_2$).

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