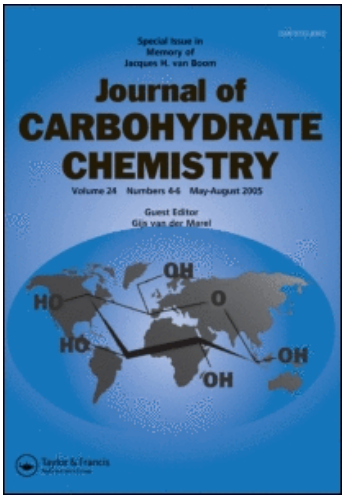

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Structural Studies on the Lipopolysaccharide Core of Bacteria of the Genus *Citrobacter:* Two Different Core Structures in *Citrobacter* O14 Serogroup

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Structural Studies on the Lipopolysaccharide Core of Bacteria of the Genus *Citrobacter:* Two Different Core Structures in *Citrobacter* O14 Serogroup

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Core oligosaccharides were released by mild acid hydrolysis of the lipopolysaccharides of 25 strains of bacteria *Citrobacter* representing 19 O-serogroups. Screening by sugar analysis and high-resolution ESI MS revealed structural heterogeneity of the core within each strain and core structure variations between strains. The core oligosaccharides from *Citrobacter werkmanii* O14 strains PCM 1548 and 1549 were further purified by anion-exchange chromatography and their full structures were elucidated by methylation analysis and two-dimensional NMR spectroscopy; similar inner core regions and significantly different outer core pentasaccharide regions were established.

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D-Galacturonic acid 3-(2-aminoethyl)phosphate was found to be a characteristic component of the *Citrobacter* core.

Keywords *Citrobacter*; Core oligosaccharide; Lipopolysaccharide structure; Electrospray ionization mass spectrometry

Abbreviations ESI FT-ICR MS, electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; Hep, L-*glycero*-D-*manno*-heptose; Hex, hexose; HexA, hexuronic acid; HexN, hexosamine; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; PEtN, phosphoethanolamine.

INTRODUCTION

Bacteria of the genus *Citrobacter* from the family Enterobacteriaceae are inhabitants of the intestinal tract and are present in sewage, surface waters, and food contaminated with fecal material. In the immunocompromised hosts they may cause outbreaks of febrile gastroenteritis and opportunistic infections, including urinary and respiratory tract disorders.[1] *Citrobacter* strains are serologically heterogeneous and, based on the O-antigens, are classified into 43 O-serogroups.[2]

The O-antigen represents a polysaccharide chain (O-polysaccharide) of the lipopolysaccharide (LPS), the major outer membrane component and a virulence factor of enteric bacteria. Most O-antigens are heteropolymers built up of oligosaccharide repeats (O-units) and some are homopolysaccharides. The so-called S-type LPS consists of three structural domains, including the O-polysaccharide, a core oligosaccharide, and lipid A. Some LPS molecules are either devoid of any O-polysaccharide (R-type LPS) or have only one nonpolymerized O-unit attached to core (SR-type LPS). The O-polysaccharide structures have been elucidated in the majority of *Citrobacter* strains (for review see ref. 3), whereas the core region of the *Citrobacter* LPS has been studied in a few selected strains and in most of them only a partial core structure has been established.[4–8]

Our recent studies[9–12] on the LPSs of the genera *Proteus* and *Providencia* have demonstrated that high-resolution ESI FT-ICR MS is a useful tool for screening of the oligosaccharides released by mild acid hydrolysis of the R- and SR-type LPS. In this work we used the MS approach for screening of the core oligosaccharides isolated from 25 *Citrobacter* strains representing 19 O-serogroups. In addition, full-core oligosaccharide structures were determined in two selected *Citrobacter* strains by methylation analysis and NMR spectroscopy.

MATERIALS AND METHODS

Bacterial Strains, Growth, and Isolation of Lipopolysaccharides

Citrobacter strains were obtained from the collection of the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Bacteria were cultivated in a liquid medium with aeration at 37°C for 24 h, harvested, and freeze-dried. LPS preparations were isolated from acetone-dried bacteria by phenol–water extraction[13] and purified from nucleic acids by Sepharose 2B gel chromatography.[14] LPSs from most strains were recovered from the water phase and those from strains PCM 1553, 1558, 1509, 1536, 1571, 2540, 1562, 1567, 1550, and 1557 from both water and phenol phases.

Mild Acid Degradation of Lipopolysaccharides and Isolation of Core Oligosaccharides

An LPS sample (200–300 mg) from each strain was hydrolyzed with aqueous 1% HOAc at 100°C for 1 to 2 h and a lipid precipitate was removed by centrifugation at 13,000 × *g* for 20 min. The carbohydrate portion was fractionated by gel-permeation chromatography on a column (100 × 2.0 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Uppsala, Sweden) in 0.05 M pyridinium acetate buffer pH 5.6 with monitoring by a phenol/sulfuric acid reaction to give an O-polysaccharide, a core oligosaccharide bearing one O-unit and an unsubstituted core oligosaccharide (fractions I–III, respectively). Core fraction III was further purified on a BioGel P-2 column (100 × 2 cm) and in some cases separated to fractions IIIa and IIIb.

Fractions III, IIIa, and IIIb were used for MS studies and sugar and methylation analyses without further purification. For NMR spectroscopic studies, oligosaccharides were additionally purified by anion-exchange chromatography on a 5-mL HiTrap Q column (Amersham Biosciences) in a gradient of 0→1 M NaCl over 1 h at a flow rate 3 mL·min^{-1}. Compounds were desalted by gel filtration on a column (50 × 1.6 cm) of Sephadex G-15 (Amersham Biosciences).

Compositional and Methylation Analyses

Heptose,[15] phosphate,[16] and free amino groups[17] (EtN) were quantified directly in oligosaccharides using colorimetric methods. For sugar analysis, oligosaccharides were hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h) or 10 M HCl (80°C, 30 min); monosaccharides were converted into the alditol acetates[18] and analyzed by GLC-MS on a Hewlett-Packard 5971 instrument equipped with an HP-1 glass capillary column (12 m × 0.2 mm) using a temperature program of 150→270°C at 8°C·min^{-1}.

Prior to methylation, parts of fractions IIIa and IIIb from strains PCM 1548 and 1549 were dephosphorylated by treatment with 48% HF at 4°C for 48 h. Methylation was performed according to Gunnarsson[19] as described[20]; methylated oligosaccharides were hydrolyzed as above, reduced with $NaBH_4$ or $NaBD_4$, and acetylated; and the partially methylated alditol acetates were identified by GLC-MS using the same conditions as in sugar analysis.

Mass Spectrometry

ESI FT-ICR MS of oligosaccharides was performed in the negative ion mode using an APEX II Instrument (Bruker Daltonics, Billerica, MA, USA) equipped with a 7 Tesla magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Mass accuracy has been checked through the external calibration. Samples ($\sim$10 ng·μL^{-1}) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine and sprayed at a flow rate of 2 $\mu L \cdot min^{-1}$. Capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 150°C. The capillary exit voltage was set to −100 V and in some cases increased to −200 V for gaining a better signal-to-noise ratio. The spectra, which showed several charge states for each component, were charge deconvoluted using software provided by the manufacturer (Bruker XMASS 6.0.0), and mass numbers given refer to the monoisotopic masses of the neutral molecules.

NMR Spectroscopy

Prior to the measurements, samples were freeze-dried twice from D_2O. NMR spectra were recorded using a Varian UNITY/Inova 500 spectrometer (Palo Alto, CA, USA) for D_2O solutions at 25°C using acetone as internal standard (δ_H 2.225 and δ_C 31.5 ppm). The following standard pulse sequences were used in two-dimensional NMR spectroscopy: COSY; TOCSY (mixing time 120 ms); NOESY (mixing time 300 ms); ^1H,^{13}C HSQC, and HSQC-TOCSY (mixing time 80 ms); ^1H,^{31}P HMQC (optimized for the ^1H-^{31}P coupling constant value of 11 Hz); and HMQC-TOCSY (mixing time 80 ms). Spectra were assigned with the help of the computer program PRONTO.[21]

RESULTS

Isolation and Chemical Characterization of Core Oligosaccharides

The LPSs from 25 strains representing 19 different *Citrobacter* O-serogroups were hydrolyzed under mild acidic conditions to cleave an

acid-labile glycosidic linkage of a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue between the carbohydrate and lipid A moieties. The carbohydrate material from each LPS was recovered from the water phase (46%–63% of LPS mass) or both water and phenol phase (80%–89% of LPS mass) and fractionated on Sephadex G-50. As a result, a high-molecular-mass O-polysaccharide (fractions I) and oligosaccharide fractions as well as a Kdo-containing low-molecular-mass material were obtained. MS screening showed that higher-molecular-mass oligosaccharides (fraction II) represent core bearing one O-polysaccharide repeating unit (data not shown) and they were not studied further. Lower-molecular-mass oligosaccharides (fraction III) corresponded to a nonsubstituted core, and they were fractionated on BioGel P-2 to yield one (III) or two (IIIa and IIIb) oligosaccharide fractions depending on the strain.

All isolated core oligosaccharides contained heptose, phosphorus, and ethanolamine measured by the colorimetric methods (Table 1). Composition of most of them was determined by GLC of the acetylated alditols (Table 1) and high-resolution ESI FT-ICR MS (Table 2).

Table 1: Composition of the core oligosaccharides derived from R-type LPS of *Citrobacter*.

PCM strain (serogroup)	Fraction	Relative content as determined by GLC analysis[a]				Relative content as determined by colorimetric methods		
		Glc	Gal	GlcN	GalN	Hep	PO_4	EtN
1507 (O2)	III	1	0.3			3	3.3	1.0
1509 (O3)	III	1	0.3			n.d.		
1504 (O6)	IIIa	1	0			3	3.0	2.0
1505 (O6)	IIIa	1	0.3			3	2.7	1.9
1531 (O6)	III	1	0.3		+	3	3.1	1.6
1537 (O9)	III	1	0.3	+		3	2.9	2.0
1540 (O11)	III	1	0.8	+	+	3	2.7	1.9
1542 (O12)	IIIa	1	0.4			3	4.0	2.3
1543 (O12)	III	1	0.85	+		3	2.0	1.0
1544 (O12)	IIIb	1	0.35	+		3	2.4	1.3
1545 (O13)	IIIa	1	0.9	+		3	3.1	1.9
1547 (O13)	IIIb	1	0.8	+		3	2.4	1.7
1548 (O14)	IIIb	1	0.25			3	2.8	2.0
1548 (O14)	IIIa	1	0.25			3	3.1	2.4
1549 (O14)	IIIb	1	0.85	+		3	2.3	1.5
1549 (O14)	IIIa	1	0.6	+		3	3.2	2.1
2540 (O17)	III	1	0.35		+	n.d.		
1551 (O18)	III	1	0.3		+	3	2.7	1.2
1553 (O20)	IIIa	1	0.45			3	3.6	1.6
1555 (O22)	IIIa	1	0.4			3	2.0	1.6
1558 (O25)	IIIa	1	0.45			3	3.5	1.4
1559 (O26)	III	1	0.35		+	n.d.		
1443 (O39)	III	1	0			3	2.4	1.7

[a]Glc and Gal were determined as the alditol acetates; GlcN and GalN were not clearly seen as the alditol acetates, and in most cases their identity was established from methylation analysis data.

Table 2: Composition and heterogeneity of the core oligosaccharides derived from R-type LPS of *Citrobacter*.

PCM strain (serogroup)	Fraction	Highest-molecular-mass compound			Other compound(s)
		M_{exp} (u)	M_{calc} (u)	Fragment beyond the common $Hep_3Kdo_1P_2EtN_1$ fragment	
1507 (O2)	III	1727.40	1727.40	$Glc_3Gal_1P_1$	M-P, **M-PEtN**, M-P-PEtN, M-Hex
1509 (O3)	III	1727.41	1727.40	$Glc_3Gal_1P_1$	**M-PEtN**, M-P-PEtN, M-Hex-PEtN
1505 (O6)	IIIa	1727.41	1727.40	$Glc_3Gal_1P_1$	M-P, M-PEtN, M-P-PEtN
1553 (O20)	IIIa	1727.40	1727.40	$Glc_3Gal_1P_1$	M-P, **M-PEtN**, M-PEtN-P
1558 (O25)	IIIa	1727.40	1727.40	$Glc_3Gal_1P_1$	M-P, **M-PEtN**, M-P-PEtN, M-Hex
1504 (O6)	IIIa	1823.48	1823.46	Glc_4GalA_1	**M+P-HexA** M-P, M-PEtN, M-P-PEtN
1443 (O39)	III	1823.47	1823.46	**Glc_4GalA_1**	M-PEtN, M-Hex
1543 (O12)	III	1850.52	1850.51	**$Glc_2Gal_2GlcNAc_1$**	M-PEtN, M-HexNAc+Hex-PEtN, M-HexNAc-Hex-PEtN, M-HexNAc+Hex, M-HexNAc
1531 (O6)	III	1930.50	1930.48	$Glc_3Gal_1GalNAc_1P_1$	**M-PEtN**, M-P-PEtN, M-Hex-PEtN, M-HexNAc-PEtN
1559 (O26)	III	1930.50	1930.48	$Glc_3Gal_1GalNAc_1P_1$	**M-PEtN**, M-P-PEtN, M-Hex-PEtN, M-HexNAc-PEtN
1562 (O29a)	III	1930.50	1930.48	$Hex_4HexNAc_1P_1$	**M-PEtN**, M-P, M-Hex, M-HexNAc
1567 (O30)	III	1930.50	1930.48	$Hex_4HexNAc_1P_1$	**M-PEtN**, M-P-PEtN, M-Hex-PEtN, M-HexNAc-PEtN
1555 (O22)	IIIa	1985.52	1985.52	**$Glc_4Gal_1GalA_1$**	M-HexA
1537 (O9)	III	2026.55	2026.54	**$Glc_3Gal_1GlcNAc_1GalA_1$**	M-PEtN, M-Hex, M-Hex-PEtN
1545 (O13)	IIIa	2026.55	2026.54	$Glc_2Gal_2GlcNAc_1GalA_1$	**M-PEtN**, M+Hex, M-P+Hex, M+Hex-PEtN
1547 (O13)	IIIb	2026.55	2026.54	$Glc_2Gal_2GlcNAc_1GalA_1$	M-PEtN-HexA, M-PEtN-P-HexA, M-PEtN-HexA-HexNAc, **M-HexA**, M-HexA-HexNAc
1557 (O24)	III	2026.56	2026.54	$Hex_4HexNAc_1HexA_1$	**M-PEtN**, M-PEtN-HexA
2540 (O17)	IIIa	2053.48	2053.48	**$Glc_3Gal_1GalNAc_1P_2EtN_1$**	$M-PEtN_2$+Hex, $M-PEtN_2$-P+Hex, M+Hex, M-HexNAc+Hex
1571 (O15)	IIIa	2106.50	2106.51	$Hex_4HexNAc_1HexA_1P_1$	M-P-PEtN, M-P-PEtN-HexA, M-Hex-HexA-PEtN, **M-HexA**

1551 (O18)	III	2106.51	2106.5,1	$Glc_3Gal_1GalNAc_1GalA_1P_1$	M-P, M-P-PEtN-HexA, **M-HexA**, M-Hex-HexA
1548 (O14)	IIIb	2108.53	2108.52	**$Glc_4Gal_1GalA_1P_1EtN_1$**	M-PEtN, M-$PEtN_2$,M-Hex_2, M-HexA
1548 (O14)	IIIa	2108.53	2108.52	**$Glc_4Gal_1GalA_1P_1EtN_1$**	M-PEtN, M+Heõ, M-Hex
1540 (O11)	III	2149.56	2149.55	$Glc_2Gal_2HexNAc_1GalA_1P_1EtN_1$	**M-PEtN**, M-HexA-PEtN, M-HexA-$PEtN_2$, M-HexA-HexNAc-PEtN
1544 (O12)	IIIb	2149.57	2149.55	$Glc_3Gal_1GlcNAc_1GalA_1P_1EtN_1$	**M-PEtN**, M-$PEtN_2$, M-HexNAc-PEtN, M-Hex-PEtN, M-HexA-PEtN, M-$PEtN_2$-Hex-PEtN, M-$PEtN_2$-HexNAc-PEtN, M-$PEtN_2$-HexA-PEtN, M-HexA-HexNAc-PEtN, M-HexA-HexNAc-Hex-PEtN, M-HexA-HexNAc-Hex-Hex-PEtN
1542 (O12)	IIIa	2149.55	2149.55	$Glc_3Gal_1HexNAc_1GalA_1P_1EtN_1$	M-Hex_2, M-HexNAc, **M-HexNAc-Hex**
1549 (O14)	IIIb	2149.55	2149.55	$Glc_2Gal_2GlcNAc_1GalA_1P_1EtN_1$	M-PEtN, M-$PEtN_2$, **M-HexA-PEtN**, M-HexNAc
1549 (O14)	IIIa	2149.54	2149.55	**$Glc_2Gal_2GlcNAc_1GalA_1P_1EtN_1$**	M-PEtN, M-HexA-PEtN, M-HexNAc, M-HexA

Predominant structural variants are indicated in boldface. Na- and K-adducts are not indicated. Abbreviations: EtN, ethanolamine; Hep, heptose; Hex, hexose; HexA, hexuronic acid; HexNAc, *N*-acetylhexosamine; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid (present predominantly in an anhydro form).

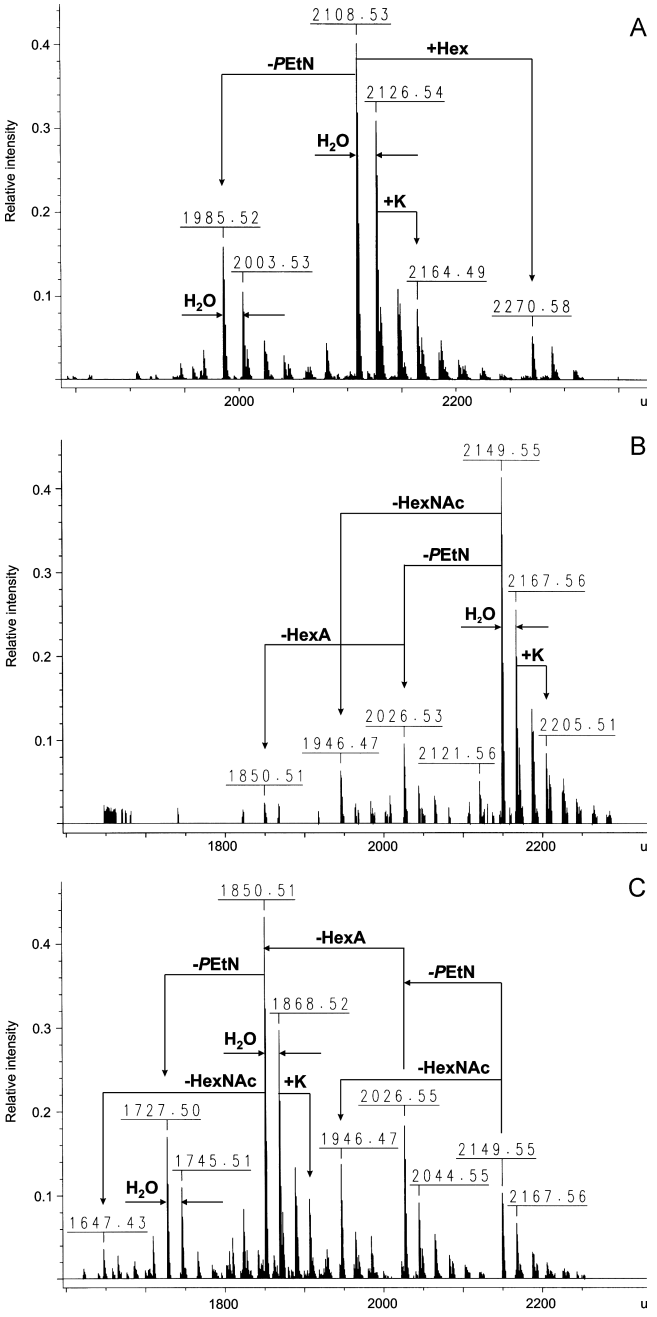

Figure 1: Negative ion ESI FT-ICR mass spectra of the core oligosaccharides derived from R-type LPSs of *Citrobacter*. **(A)** Fraction IIIa from *C. werkmanii* PCM 1548. The major peaks with 2126.54 u and 2108.53 u correspond to oligosaccharide **1**. **(B C)** Fractions IIIa and IIIb, respectively, from *C. werkmanii* PCM 1549. The peaks with 1868.52 u and 1850.51 u correspond to oligosaccharide **2a**, and those with 2167.56 u and 2149.55 u to oligosaccharide **2b**. A mass difference of 18 u corresponds to the replacement of Kdo with anhydro-Kdo.

Mass Spectrometric Studies of Core Oligosaccharides

The charge deconvoluted ESI FT-ICR mass spectra obtained in the negative ion mode contained a number of mass peaks indicating different core oligosaccharide glycoforms and different degrees of phosphorylation (for instance, see mass spectra shown in Fig. 1). In addition to Na- or/and K-adduct ions and those for compounds with Kdo in an anhydro form, there were ions for oligosaccharides differing in the presence or absence of phosphate (Δm 79.97 u) and/or phosphoethanolamine (PEtN) (Δm 123.01 u) as well as monosaccharide residues: Hex (Δm 162.05 u), HexNAc (Δm 203.08 u), and/or HexA (Δm 176.03 u) (Table 2). A high degree of heterogeneity in respect to monosaccharide composition observed in most samples suggests the occurrence of incomplete core glycoforms, particularly those lacking HexA. The lack of phosphate and PEtN could result from both intrinsic heterogeneity and partial loss by cleavage of diphosphate groups during mild acid degradation of the LPS.

Based on high-resolution MS molecular mass determination, composition of all 25 samples was inferred neglecting the monosaccharide configurations. For most samples, exact Hex and HexNAc composition was determined based on combined MS and GLC sugar and methylation analyses data (Table 1). The data of the most complete core glycoforms are summarized in Table 2. They all contain a common Hep_3Kdo_1tetrasaccharide fragment, which is typical of the inner region of core oligosaccharides released by mild acid from enterobacterial LPS.[22] In addition, the oligosaccharides include Hex residues (1 to 5), HexNAc (0 to 1), HexA (0 to 1), *P* (2 to 4), and EtN (1 to 3). There are several typical hexose regions (outer core), such as Glc_3Gal_1, Glc_4Gal_1, $Glc_3Gal_1GlcNAc_1$, and $Glc_2Gal_2GlcNAc$.

The core oligosaccharides from *Citrobacter werkmanii* PCM 1548 and PCM 1549 belonging to the same serogroup O14 were selected for further detailed studies. They have the most typical composition with respect to the presence of one HexA, three phosphate, and two PEtN groups and significantly different outer core regions, Glc_4Gal_1 and $Glc_2Gal_2GlcNAc$, respectively.

Full Structure Elucidation of the Core Oligosaccharides

For NMR analysis, the core oligosaccharides were additionally purified by HiTrap Q anion-exchange chromatography and the major oligosaccharides were studied by sugar and methylation analyses and two-dimensional ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, including $^{1}H,^{1}H$ DQF COSY; TOCSY; NOESY; $^{1}H,^{13}C$ HSQC; HSQC-TOCSY, gradient-enhanced HMBC, $^{1}H,^{31}P$ HMQC, and HMQC-TOCSY experiments. Assignment of the spectra was performed essentially as described,[23] and chemical shifts are tabulated in Table 3.

The identity of the constituent monosaccharides was determined by sugar analysis using GLC-MS of the alditol acetates (Table 1) as well on the basis

Table 3: NMR chemical shifts (δ, ppm) of the purified core oligosaccharides.

Residue	H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6a	H/C 7/6b	H/C 7b/ 8a,b
Oligosaccharide **1** (*C. werkmanii* PCM 1548)								
Kdo **C**		1.90	2.26	4.13	4.16			3.52, 3.90
		96.2	34.3	66.5	73.8			
Hep **E**	5.18	4.01	4.03	4.63	4.22	4.11	3.72	3.72
	100.5	71.8	78.5	72.5	72.2	69.5	64.1	
Hep **F**	5.12	4.38	4.00	4.02	3.65	4.22	3.60	3.79
	103.6	70.4	80.0	66.2	73.5	68.2	70.5	
Hep **G**	4.95	3.94	3.86	3.87	3.64	4.28	3.85	4.03
	101.0	71.0	71.7	67.1	72.8	68.3	73.3	
Glc **H**	5.26	3.58	4.04	3.73	3.99	3.79	3.96	
	101.2	72.7	74.5	76.0	71.8	61.4		
GalA **K**	4.55	3.71	4.20	4.48	4.43			
	103.2	70.1	78.0	69.8	74.7	172.6		
Glc **L**	5.69	3.68	3.82	3.47	3.73	3.79	3.88	
	97.5	77.2	72.0	70.3	73.6	61.3		
Glc **M**	5.18	3.61	3.79	3.58	4.12	3.90	4.15	
	97.8	72.6	73.7	70.0	72.0	69.1		
Glc **N**	4.91	3.52	3.74	3.46	4.14	3.76	3.82	
	101.0	72.9	73.8	70.4	73.0	61.0		
Gal **O**	4.49	3.59	3.74	4.02	3.74	3.83	3.90	
	104.1	71.9	73.3	78.1	73.3	61.4		
Oligosaccharides **2a,b** (*C. werkmanii* PCM 1549)								
Kdo **C**		1.91	2.25	4.12	4.16	3.84	3.71	3.53, 3.89
		96.6	34.5	66.6	74.2	72.7	72.5	64.8
Hep **E**	5.17	4.03	4.12	4.62	4.20	4.12	3.72	3.72
	100.8	71.8	78.4	72.4	72.5	69.6	64.1	
Hep **F**	5.14–5.18	4.42	4.01	4.06	3.68	4.18	3.63	3.76
	103.5	70.3	80.8	66.3	73.5	69.3	70.4	
Hep **G** in **2a**	4.95	3.95	3.87	3.86	3.61	4.04	3.67	3.73
	101.0	71.1	71.8	67.2	72.3	69.8	64.0	
Hep **G** in **2b**	4.92	4.01	3.87	3.87	3.67	4.27	3.88	4.06
	101.5	71.0	71.7	67.2	72.8	68.3	73.1	
Glc **H**	5.25	3.58	4.12	3.85	4.04	3.79	3.94	
	101.2	73.2	75.3	71.7	71.7	61.5		
Glc **I**	5.26	3.70	3.80	3.48	3.89	3.79	3.91	
	96.7	72.5	74.2	70.4	73.3	61.6		
Gal **J**	6.10	4.23	4.12	4.26	3.89	3.75	3.75	
	95.0	69.2	71.5	65.4	71.7	62.2		
Gal **P**	5.58	4.01	4.06	4.07	4.07	3.81	3.81	
	92.9	73.3	69.0	70.0	72.5	62.3		
GlcNAc **R**	5.11	4.01	3.63	3.58	3.94	3.78	3.91	
	93.4	54.3	72.6	70.7	73.1	61.7		
GalA **K** in **2b**	4.54	3.70	4.19	4.46	4.33			
	103.1	70.3	78.1	70.0	75.2	173.9		

Additional signals for EtN are at δ_H 3.30 (CH_2N) and 4.16–4.21 (CH_2O), δ_C41.0 (CH_2N) and 61.1–61.4 (CH_2O); NAc at δ_H 2.03, δ_C 22.9 (Me) and 175.6 (CO). ^{31}P NMR chemical shifts are: GalA **K** P3 and EtN P δ 0.20–0.24, Hep **E** PP4 δ −10.6 and EtN PP δ −9.9 to −10.0.

of characteristic ^{3}J coupling constants and NMR chemical shifts, which were in agreement with expected values for the sugar pyranosides.[24] All *Citrobacter* strains studied were found to possess a conserved inner core region containing one Kdo **C**, three Hep **E–G**, one GalA **K** and one Glc **H** residues, which were designated as in our earlier studies of related *Providencia* core structures.[11]

Citrobacter werkmanii *PCM 1548 (O14)*

Fraction IIIa from *C. werkmanii* PCM 1548 comprised 61% of the total carbohydrate material eluted from BioGel P-2. The Hep:P:EtN molar ratio measured by colorimetric methods was 3.0:3.1:2.4 and the Glc:Gal molar ratio determined by GLC of the alditol acetates was ~4:1. These data are essentially in agreement with the results of ESI FT-ICR MS analysis (Table 2). Methylation analysis of fraction IIIa revealed terminal Glc; 2-, 4-, and 6-substituted Glc; 4-substituted Gal; and 7-substituted and 3,7-disubstituted Hep. A similar analysis after dephosphorylation of fraction IIIa revealed additionally 3-substituted Hep (Table 4).

The major oligosaccharide **1** was isolated from fraction IIIa by HiTrap Q chromatography and studied by NMR spectroscopy. In addition to the common monosaccharides (see above), it contained three more Glc residues and one Gal residue, which were designated as **L–N** and **O**, respectively.

$J_{1,2}$ coupling constants estimated from the two-dimensional NMR spectra showed that Glc residues **H** and **L–N** are α-linked, whereas Gal **O** and GalA **K** residues are β-linked. The α-configuration of Hep **E–G** residues was inferred from the presence of H1/H2 and the absence of H1/H3 and H1/H5 intraresidue NOE correlations, a pattern typical of α-linked pyranosides. The configurations of the glycosidic linkages were confirmed by the ^{13}C NMR chemical shift data (Table 3) compared to published data.

Downfield displacements of signals for the linkage carbons, including those of Kdo **C** C5 (δ 73.8), Hep **E** C3 (δ 78.5), Hep **F** C3 and C7 (δ 80.0 and 70.5), Hep **G** C7 (δ 73.3), Glc **H** C4 (δ 76.0), Glc **L** C2 (δ 77.2), Glc **M** C6 (δ69.1), and Gal **O** C4 (δ 78.1), as compared with the values for the corresponding unsubstituted monosaccharides, revealed positions of substitution of the monosaccharide residues. No such shifts were observed for carbon signals of Glc **N** and GalA **K**, and hence they occupy terminal positions in the oligosaccharide.

The following correlations between protons of neighboring monosaccharides were observed in the NOESY spectrum (Fig. 2): Glc **N** H1/Gal **O** H4, Gal **O** H1/Glc **M** H6, Glc **M** H1/Glc **L** H2, Glc **L** H1/Glc **H** H3 and H4, Glc **H** H1/Hep **F** H3, GalA **K** H1/Hep **G** H7, Hep **G** H1/Hep **F** H7, Hep **F** H1/Hep **E** H3, and Hep **E** H1/Kdo **C** H5 and H7. Combined with the ^{13}C NMR chemical shift data, the NOESY data enabled determination of the full-core carbohydrate backbone structure.

Table 4: Data of methylation analysis by GLC of the partially methylated alditol acetates derived from core oligosaccharide fractions IIIa and IIIb and dephosphorylated fractions $IIIa_{HF}$ and $IIIb_{HF}$ from *C. werkmanii* PCM 1548 and PCM 1549.

PCM strain (serogroup)	Monosaccharide	Molar ratios related to 4-substituted D-glucose					
		IIIa		$IIIa_{HF}$	IIIb		$IIIb_{HF}$
		A	B	A	A	B	A
1548 (O14)	t-Glc	0.9	1.1	1.1	0.8	0.9	0.7
	→ 2)-Glc	1.0	1.0	1.0	1.0	1.2	0.8
	→ 4)-Gal	0.7	0.6	0.7	0.9	0.1	0.4
	→ 4)-Glc	1	1	1	1	1	1
	→ 6)-Glc	1.3	1.2	1.1	1.0	1.2	0.8
	t-Hep	0.3	0.5		0.4	0.3	0.1
	→ 3)-Hep			0.7			0.5
	→ 7)-Hep	0.7	0.4	0.7	0.7	0.2	0.5
	→ 3, 7)-Hep	0.8	0.6	1.0	1.1	+	0.6
1549 (O14)	t-Glc	0.5	0.8	0.8	0.7	0.9	1.0
	→ 4)-Glc	1	1	1	1	1	1
	→ 2)-Gal	0.8	0.9	0.9	1.1	1.2	1.1
	t-Hep + →2,3)-Gal[a]	0.7	0.3	1.0	1.4	0.7	1.5
	→ 2, 6)-Gal	0.4	0.4	0.3	0.2	+	0.3
	→ 3)-Hep			0.7			0.9
	t-GlcNAc	0.5	0.3	0.8	0.8	0.3	1.0
	→ 7)-Hep	0.4	+	1.0	0.3	+	0.8
	→ 3, 7)-Hep	0.7	0.1	1.8	1.6	0.2	1.8

A, hydrolysis with 10 M HCl, 80°C, 30 min; B, hydrolysis with 2 M CF_3CO_2H, 120°C, 2 h. t-Glc stands for 2,3,4,6-tetra-*O*-methylglucose derived from a terminal glucose; →2)-Glc for 3,4,6-tri-*O*-methylglucose from a 2-substituted glucose, etc.; + for a trace amount of the methylated derivatives.

[a]The methylated derivatives were not separated in GLC.

The ^{31}P NMR spectrum of the oligosaccharide **1** showed the presence of one phosphate and one diphosphate group. A two-dimensional $^{1}H,^{31}P$ HMQC experiment revealed correlation of the phosphate group with H3 of GalA **K** and CH_2O-group of one of the EtN residue at δ_P/δ_H 0.24/4.20 and 0.24/3.30, respectively. The diphosphate group exhibited correlation between H4 of Hep **E** and the CH_2O-group of the other EtN residues at δ_P/δ_H −10.6/4.63 and −9.9/3.30, respectively. The phosphorylation pattern was additionally confirmed by downfield displacements of the resonances for GalA **K** C3 and Hep **E** C4 to δ 78.0 and 72.5, respectively.

Based on the NMR data, it was concluded that the major core oligosaccharide **1** from *C. werkmanii* PCM 1548 has the structure shown in Figure 3, which is in full agreement with sugar and methylation analyses data (see above).

ESI mass spectrum of fraction IIIa (Fig. 1, A) confirmed that oligosaccharide **1** corresponds to the most representative LPS core form of *C. werkmanii* PCM 1548. Indeed, peaks for a $Glc_4Gal_1GalA_1Hep_3Kdo_1P_3EtN_2$

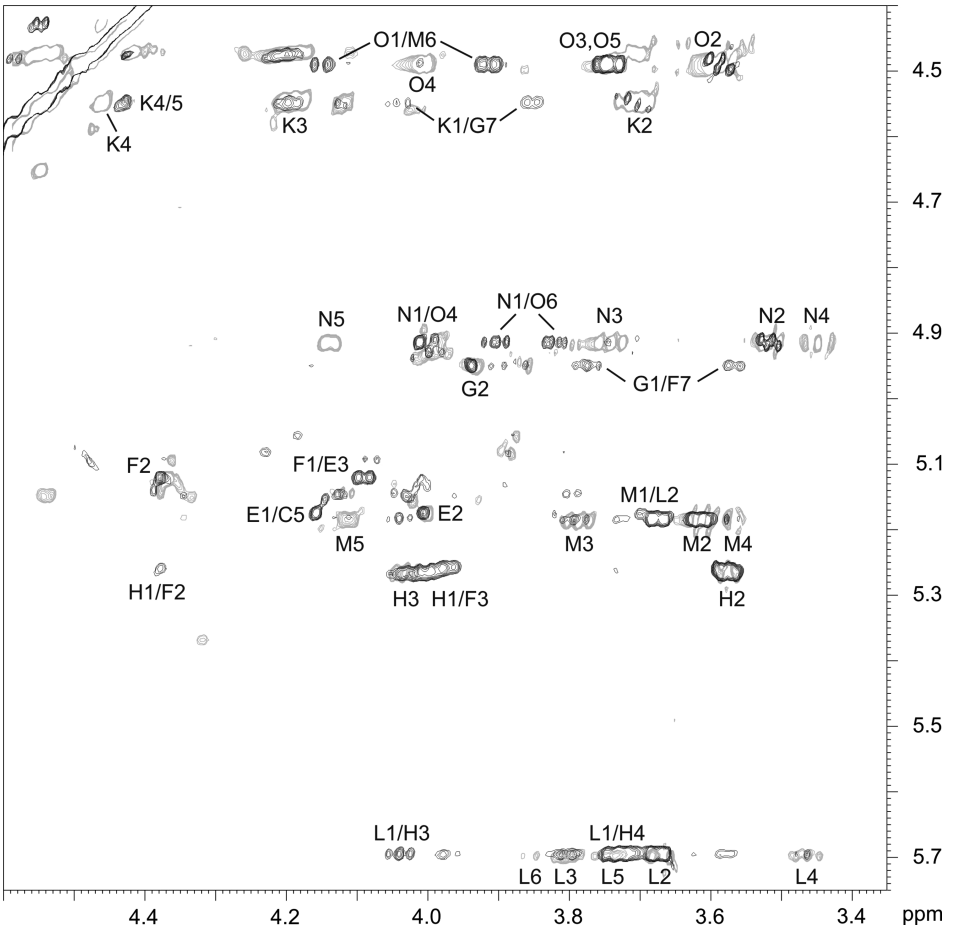

Figure 2: Parts of TOCSY (gray) and NOESY (black) spectra of the core oligosaccharide **1** from *C. werkmanii* PCM 1548. Arabic numerals refer to protons in monosaccharide residues denoted by letters as shown in Figure 3.

oligosaccharide with molecular masses 2126.54 u and 2108.53 u for compounds with Kdo in regular and anhydro forms, respectively, are major in the spectrum. In addition to **1**, the mass spectrum revealed two minor compounds, one lacking a PEtN group (most likely from the PPEtN group on Hep **E**) and the other containing an additional Hex residue. NMR studies showed the latter to be a terminal β-Glc residue but the site of its attachment could not be determined owing to its too low content.

Citrobacter werkmanii *PCM 1549 (O14)*

Fractions IIIa and IIIb from *C. werkmanii* PCM 1549 comprised 22% and 52%, respectively, of the total carbohydrate material eluted from BioGel P-2. Their Hep:P:EtN molar ratios were determined as 3.0:3.2:2.1 and 3.0:2.3:1.5,

respectively, and sugar analysis by GLC of the alditol acetates demonstrated the Glc:Gal:GlcNAc molar ratio of ~2:2:1. These and ESI FT-ICR MS data (Fig. 1B,C) suggested that fractions IIIa and IIIb differ in the content of GalA3PEtN only, a $Glc_2Gal_2GlcNAc_1GalA_1Hep_3Kdo_1P_3EtN_2$ oligosaccharide containing this component (M_r 2167.56 u and 2149.55 u for compounds with Kdo in regular and anhydro forms, respectively) being most abundant in fraction IIIa and that lacking GalA3PEtN (M_r 1868.52 u and 1850.51 u) in fraction IIIb.

Methylation analysis of fractions IIIa and IIIb (Table 4) revealed the presence of terminal and 4-substituted Glc, 2-substituted and 2,3-disubstituted Gal, terminal GlcNAc, and 7-substituted and 3,7-disubstituted Hep. After dephosphorylation, 3-substituted Hep also was identified.

Two acidic oligosaccharides **2a** and **2b** were isolated from fraction IIIb by HiTrap Q chromatography and studied by NMR spectroscopy. Analysis of two-dimensional ^1H, ^{13}C, and ^{31}P NMR spectra showed that the oligosaccharide **2b** shares the $Glc_1GalA_1Hep_3Kdo_1P_3EtN_2$ fragment with *Citrobacter* PCM 1548, whereas four other monosaccharide constituents of the outer core region are different (Table 3). They were identified by characteristic coupling constants[24] as two residues of α-Gal (**J** and **P**) and one residue each of α-Glc (**I**) and α-GlcNAc (**R**). The last monosaccharide was distinguished from Glc residues by a H2/C2 correlation at δ_H/δ_C 4.01/54.3 in the ^1H, ^{13}C HSQC spectrum.

A NOESY experiment showed a number of interresidue correlations for the outer core monosaccharides, which were interpreted taking into account methylation analysis data (see above) as follows: GlcNAc **R** H1/Gal **J** H3 and H4, Glc **I** H1/Gal **P** H1 and H-2, Gal **P** H1/Gal **J** H1 and H2, and Gal **J** H1/Glc **H** H4 and H5. These data established unambiguously the sequence of the monosaccharides, which was confirmed by ^1H,^{13}C HMBC interresidue

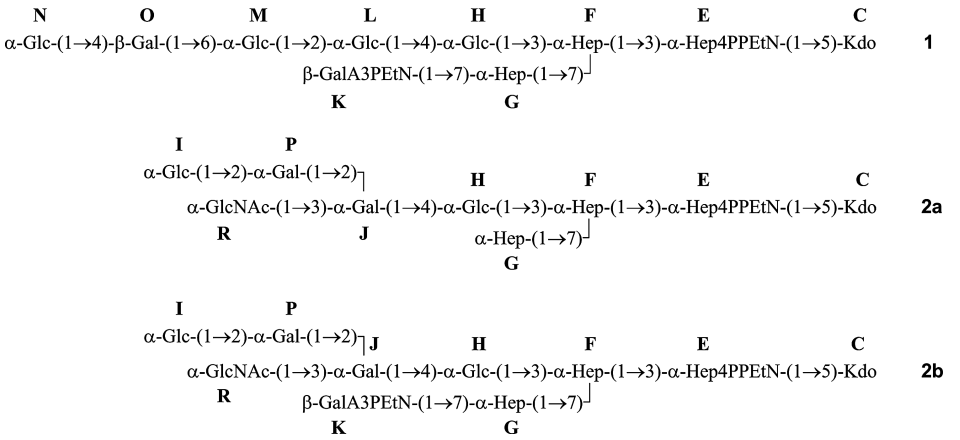

Figure 3: Structures of the core oligosaccharides from R-type LPSs of *C. werkmanii* PCM 1548 **(1)** and PCM 1549 **(2a, 2b).** All constituent monosaccharides are in the pyranose form.

correlations for anomeric atoms (data not shown). Therefore, the outer core pentasaccharide in **2b** has the structure shown in Figure 3.

In addition to the cross-peaks between transglycosidic protons, the NOESY spectrum revealed a number of correlations between protons of nonlinked residues, including Gal **J** H1/Glc **I** H1, Gal **J** H1/Gal **P** H5, Gal **P** H1/Glc **H** H3, and Glc **I** H1/Glc **H** H3 correlations. This is typical of branched oligosaccharides with a 1→2 linkage(s) and indicates strong deviations from optimal conformations in the constituent disaccharides. As a result, no typical downfield displacements are observed for signals of some linkage carbons that makes ^{13}C NMR chemical shifts inapplicable for determination of the positions of substitution of the monosaccharides.

The ^{1}H and ^{13}C NMR spectra of **2a** contained signals for most monosaccharides found in **2b** but GalA **K**. The absence of GalA **K** resulted in a higher-field position of the signal for C7 of Hep **G** at δ 64.0 in **2a** as compared with its position at δ 73.1 in **2b** (Table 3). Whereas the ^{31}P NMR and $^{1}H,^{31}P$ HMQC spectra of **2b** revealed essentially the same phosphorylation pattern as in **1** (P/GalA **K** and P/EtN at δ_P/δ_H 0.20/4.19 and 4.21; PP/Hep **E** H4 and PP/EtN at δ_P/δ_H −10.6/4.62 and −10.0/4.21, respectively), those of **2a** showed only a diphosphate group. Therefore, **2a** is devoid of GalA3PEtN that is present in **2b**. A comparison of the NMR data of the isolated oligosaccharides and ESI FT-ICR MS data of fraction IIIb (Fig. 1, C) showed that **2a** corresponds to the major $Glc_2Gal_2GlcNAc_1Hep_3Kdo_1P_2EtN_1$ oligosaccharide (M_r 1868.52 and 1850.51 u) and **2b** to the highest-molecular-mass $Glc_2Gal_2GlcNAc_1GalA_1Hep_3Kdo_1P_3EtN_2$ oligosaccharide (M_r 2167.56 and 2149.55 u).

Based on the data obtained, it was concluded that the oligosaccharides **2a** and **2b** from *C. werkmanii* PCM 1549 have the structures shown in Figure 3.

DISCUSSION

In accordance with close phylogenetic relationship to *Salmonella*, *Citrobacter* has a so-called *Salmonella*-type enterobacterial LPS core, which is characterized by substitution of the inner Hep_3Kdo_2 pentasaccharide region with a PPEtN group at position 4 of Hep **E** and with α-Glc **H** at position 3 of Hep **F**.[22] The cores of two *Citrobacter* O14 strains studied in this work and that of *Citrobacter* O23 studied earlier[8] contain also GalA **K** attached at position 7 of Hep **G**, which is known to occur in both *Salmonella*-type (*Providencia*[11]) and non-*Salmonella*-type (*Proteus*[25]) core. Mass-spectrometric screening showed that a hexuronic (galacturonic) acid is present in the majority of *Citrobacter* strains. GalA-lacking oligosaccharides present in some strains seem to correspond to incomplete core glycoforms.

A new peculiar feature of the *Citrobacter* core revealed in this work is the presence of a PEtN group at position 3 of GalA **K** (Fig. 3), which was demon-

strated in two strains studied in detail. As judged by ESI MS data, when GalA is present, at least one PEtN group and either phosphate or PPEtN group are present too, the latter being a nonstoichiometric substituent on Hep **E**. Therefore, it can be concluded that PEtN on GalA **K** is a typical *Citrobacter* LPS core constituent.

The outer core region of the *Citrobacter* LPS has a similar composition to some *Salmonella* and *Escherichia coli* strains but is structurally different. Composition and structure of this region in *Citrobacter* is rather diverse as followed from sugar analysis (Table 1) and mass-spectrometric screening data (Table 2) as well as from a comparison of two outer core structures established in this work (Fig. 3) and those established earlier.[4–8] Remarkably, significantly different outer core regions were found to occur in two *Citrobacter* strains, PCM 1548 and PCM 1549, belonging to the same O14 serogroup. Earlier, the same O-antigen, a linear homopolymer of 4-deoxy-D-*arabino*-hexose, but structurally different cores have been observed in strains belonging to *Citrobacter* O4, O27, and O36, and it has been shown that distinctions in the LPS core account for classification of these strains into different serogroups.[5,7] The O-antigen of *Citrobacter* O14 is a highly branched glycerol phosphate-containing heteropolysaccharide,[26] and, as opposite to strains of O4, O27, and O36 serogroups, in strains of O14 serogroup the different cores do not seem to comprise separate epitopes eliciting specific antibodies.

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