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Structure of the Core Region from the Lipopolysaccharide of *Plesiomonas shigelloides* Strain 302-73 (Serotype O1)

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Plesiomonas shigelloides is a Gram-negative pathogenic bacterium belonging to the Enterobacteriaceae family. To date, only few lipopolysaccharide (LPS) structures from *P. shigelloides* strains are known. In particular, three core oligosaccharides have been found. Recently, we elucidated the structure of the O-antigen of *P. shigelloides* 302-73 (serotype O1) and in this paper we present the characterization of the core structure from the LPS of the same strain. The LPS was hydrolyzed under both alkaline and mildly acidic conditions. In both cases, a mixture of oligosaccharides was obtained, which was purified by gel filtration and HPAEC. The oligosaccharides were characterized by chemical analysis, 2D NMR spectroscopy and MALDI-TOF mass spectrometry. A new core structure was found for *P. shigelloides*. In particular, from the analysis of the acid hydrolysed product it was possible to reveal the presence of a of D-*glycero*-D-*talo*-2-octulopyranosonic acid (Ko) residue, which substitutes in part the terminal 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) unit. The Ko residue is not frequently found in core structures.

Introduction

Plesiomonas shigelloides is a polarly flagellated, rod-shaped and capsulated Gram-negative pathogenic bacterium and is the only species in the genus *Plesiomonas*.[1,2] Among Enterobacteriaceae, *P. shigelloides* was recognised as a significant cause of gastrointestinal infections. Diarrhoea is the major symptom although extraintestinal infections, including septicaemia, are known to occur with predisposed individuals.

P. shigelloides was isolated from a variety of sources, including fresh water, surface water and soil, but oysters are the major food incriminated in outbreaks in the United States. Recently, quantitative assays to reveal the presence of *P. shigelloides* in oysters were developed.[3,4] *P. shigelloides* is capable of adhering to and entering into the human colon carcinoma Caco-2 cells, inducing apoptotic cell death.[5,6] Recently, the heat-shock protein GroEL from *P. shigelloides* was found to promote adhesion to Caco-2 cells, and in this way, most likely starts the invasion mechanism.[7]

To date, various virulence factors have been described to determine the pathogenic mechanism of *P. shigelloides*, which included enterotoxins,[8–10] cholera-like toxin,[11] β-haemolysin[12] and a cytotoxic complex.[13] The latter is a complex constituted by proteins and lipopolysaccharides (LPSs) (ACRP–LPS complex)[13] and has been reported to have a significant role in the enteropathogenesis of *P. shigelloides*.

LPSs represent the major virulence determinants in Gram-negative bacteria. Smooth-form LPSs typically consist of a hydrophobic domain named lipid A, a core oligosaccharide, and a distal polysaccharide (O-antigen). These molecules are involved in maintaining the selective permeability barrier function of the outer membrane (OM) and in the interaction between the microorganism and its environment as a result of their surface exposure.[14] In particular, the negative charges of the phosphate groups and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) of the core oligosaccharide, in addition to possible uronic residues, are important in mediating interactions between LPS and the positive charges on OM proteins (OMPs). To this regard, the biological role of galacturonic acids in the core of *Klebsiella pneumoniae* was investigated,[14–17] revealing the importance of this residue for the pathogenicity of this microorganism.

We recently characterised the O-chain structure of *P. shigelloides* strain 302-73 (serotype O1),[18] which is constituted by the following repeating unit:

→3)-α-L-PneNAc4*O*Ac(1→4)-α-L-FucNAc(1→4)-α-L-FucNAc(1→4)-α-L-FucNAc(1→3)-β-D-QuiNAc4NHb(1→

Up to now, only the structures of the core oligosaccharides from strains O74:H5,[19] O54:H2[20] and O17[21] were known. To increase the data of the structures of *Plesi-*

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omonas LPS core we now report the core oligosaccharide structure of *P. shigelloides* strain 302-73 (serotype O1), which was studied by using chemical analysis, ^{1}H and ^{13}C NMR spectroscopy and MALDI-TOF mass spectrometry.

Results and Discussion

Isolation and Purification of the LPS

P. shigelloides strain 302-73 was grown on tryptic-soy-broth at 37 °C, as already reported.[18] The LPS was recovered from dried bacteria cells by a PCP (phenol/chloroform/light petroleum) extraction (LPS_{PCP}), as well as by a phenol/water extraction (LPS_{PhOH}).[18] The SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) of the LPS_{PCP} revealed a high concentration of the LPS with few O-antigen repeating units; therefore, the core structural determination was performed on this sample.

Compositional Analysis

Sugar analysis by GC–MS of acetylated methyl glycosides prepared after methanolysis of the LPS for 16 h, together with absolute configuration determination, indicated, by comparison with authentic standards, the presence of D-glucose (Glc), D-galactose (Gal), D-glucosamine (GlcN), L-*glycero*-D-*manno*heptose (Hep), D-galacturonic acid (GalA), Kdo, L-2-amino-2,6-dideoxygalactose (fucosamine, FucN) and trace amounts of L-2-amino-2,6-dideoxytalose (pneumosamine, PneN). When methanolysis was performed for a shorter time (45 min, 0.5 M HCl/MeOH), two additional peaks were present in the chromatogram. The first was identified as a derivative of a HexN–HexA disaccharide, whereas the second was recognised to be the Ko–Kdo disaccharide (Ko = D-*glycero*-D-*talo*-2-octulopyranosonic acid), as it showed the typical fragment ions at m/z = 375 for the Kdo reducing end and at m/z = 461 for the Ko nonreducing end.[22]

Fatty acid methyl esters were analysed by GC–MS after methanolysis of the LPS and indicated the occurrence in the LPS sample of dodecanoic, 3-hydroxydodecanoic, tetradecanoic and 3-hydroxytetradecanoic acids.

Methylation analysis revealed the presence of 3,4-disubstituted heptose, 2,3,7-trisubstituted heptose, 3,7-disubstituted heptose, 7-substituted heptose, 4-substituted galacturonic acid, terminal glucose, 6-substituted glucosamine, terminal glucosamine, terminal galactose, 4-substituted fucosamine, 4,5-disubstituted Kdo and terminal Kdo. No derivatives from Ko were detected in this analysis.

Deacylation of the LPS

To determine the primary structure of the core oligosaccharide, the LPS_{PCP} was deacylated by mild hydrazinolysis followed by alkaline hydrolysis with KOH. Because this last treatment determines the cleavage of glycosyl linkages to the 4-positions of uronic acids it was suitable to obtain the isolation of the core structure lacking most of the O-chain repeating units. The alkaline hydrolysis product was purified by HPAEC (high-performance anion-exchange chromatography), and the chromatogram obtained under alkaline conditions identified two fractions, named OS1 and OS2, respectively. Both fractions were characterized by homo- and heteronuclear 1D and 2D NMR experiments (DQF–COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; ^{1}H–^{13}C DEPT–HSQC, distortionless enhancement by polarization transfer-heteronuclear single quantum coherence; ^{1}H–^{13}C HMBC, heteronuclear multiple bond correlation), which allowed the assignments of all the spin systems and the monosaccharide sequence.

The ^{1}H NMR spectrum of OS1 is shown in Figure 1. In the region between 4.4–6.0 ppm, 11 main signals were present, of which only 9 were identified as anomeric protons from their correlations with anomeric carbon signals in a DEPT–HSQC experiment (**A**–**I**, Table 1). The two downfield shifted signals at δ = 5.82 and 5.80 ppm were both assigned to H-4 of two different *threo*-hex-4-enuronopyranosyl units, as they were correlated to the unsaturated C-4 signals at δ = 109.6 and 108.6 ppm, respectively. Starting with the signal of the anomeric proton at δ = 5.54 ppm, the spin system named **A** was recognized to be the proximal GlcN unit of the lipid A, as its proton anomeric signal showed the typical coupling constants with ^{31}P ($^{3}J_{H,P}$ = 6.0 Hz) and it is correlated with a carbon signal at δ = 92.5 ppm in the DEPT–HSQC experiment. The α anomeric configuration for residue **A** was deduced from the anomeric carbon chemical shift value at δ = 92.5 ppm and confirmed by the value of the $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constant value of 178 Hz,[23] whereas the substitution at the C-6 position of this residue was inferred from the glycosylation shift of its C-6 value at δ = 71.2 ppm. Spin systems **B**, **D** and **F** were assigned to three *manno*-configured heptose residues, as they showed small vicinal coupling constant $^{3}J_{H\text{-}1,H\text{-}2}$ and $^{3}J_{H\text{-}2,H\text{-}3}$ values, diagnostic of H-2 equatorial orientation. The anomeric configuration was found to be α for all the heptoses by measuring the $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants in a 2D *F2*-coupled HSQC experiment (Table 1). Residues **B** and **D** were identified as 2,3,7-substituted and 3,4-substituted heptoses, respectively, as their proton and carbon chemical shifts (Table 1) were found to be very similar to that of the same residues in the inner core of *P. shigelloides* O54:H2[20] and O17.[21] The two *threo*-hex-4-enuronopyranosyl units were assigned to **C** and **E** on the basis of both ROESY (Table 2) and HMBC experiments (see below). The α anomeric configuration for both residues was inferred from the value of the $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants of 176 Hz. The α *manno*-heptose **F** with H-1/C-1 at 4.89/105.0 ppm was found to be 7-substituted, as its C-7 resonance occurred at δ = 73.7 ppm. The remaining three proton anomeric signals at δ = 4.69, 4.54 and 4.47 ppm, respectively, were all attributable to β-configured residues by virtue of their $^{3}J_{H\text{-}1,H\text{-}2}$ (≈8 Hz) coupling constants. In particular, residue **G** (H-1 at δ = 4.69 ppm, C-1 at δ = 101.5 ppm)

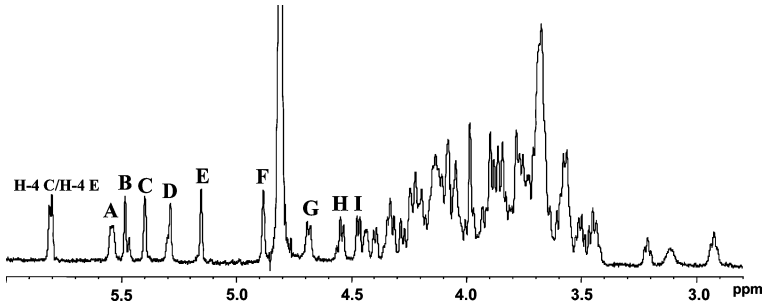


Figure 1. ^{1}H NMR of the fully deacylated LPS from *P. shigelloides* 302-73 strain (serotype O1) performed at 298 K. The spectrum was recorded in D_2O at 600 MHz. The letters refer to the residues as described in Table 1.

was assigned to the vicinal 6-substituted β-GlcN of lipid A from its C-2 and C-6 resonances at δ = 56.9 and 63.9 ppm, respectively, according to the characteristic values of this residue.[24] Moreover, the H-4 and C-4 downfield shifts are diagnostic for the presence of a phosphate group linked to O-4. The *gluco*-configuration for residue **H** (H-1 at δ = 4.54 ppm, C-1 at δ = 104.5 ppm) was inferred from the large vicinal coupling constants $^3J_{H\text{-}2,H\text{-}3}$, $^3J_{H\text{-}3,H\text{-}4}$ and $^3J_{H\text{-}4,H\text{-}5}$ of the spin system (8–10 Hz) measured in a DQF–COSY experiment. The final residue **I** with H-1/C-1 at 4.47/103.9 ppm was identified as a *galacto*-pyranosyl residue on the basis of the correlation in the TOCSY experiment of its anomeric proton with only four densities. Both residues **H** and **I** were unsubstituted, as none of their carbon resonances were shifted by glycosylation.[23] In the ^{1}H NMR spectrum region around 2 ppm, four signals attributable to H-3 diastereotopic protons of two different Kdo units were found. The difference between the proton chemical shifts of H-3ax and H-3eq suggested an α-configuration for both residues.[25] In particular, residue **L** with H-3ax/H3-eq at 1.87/2.11 ppm was assigned to a 4,5-substituted Kdo residue from the glycosylation shift of its C-4 and C-5 signals at δ = 71.8 and 69.1 ppm, respectively. No glycosylation shift was observed for carbon signals of residue **M**.

Table 2. Nuclear Overhauser enhancement interresidue connectivities (ROESY) for the anomeric protons of OS1 and OS2.

H-1 of sugar residue	NOE correlations
GlcN **G**	**A** H-6a,b
Hep **D**	**L** H-5
Hep **B**	**D** H-3, **H** H-1
Hep **F**	**B** H-7a,b
Gal **I**	**D** H-3, H-4
Glc **H**	**B** H-1, H-2
ΔHexA **C**	**B** H-3
ΔHexA **E**	**F** H-7a,b

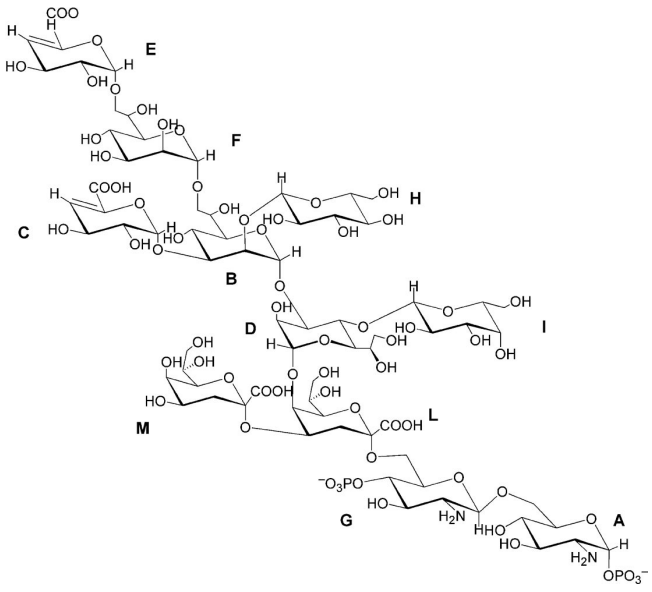
The sequence and the attachment points of the residues were deduced from a ^{1}H–^{13}C HMBC NMR experiment, which indicated the following correlations: H-1 **E** with C-7

Table 1. ^{1}H–^{13}C NMR assignments of OS1 and OS2. All the chemical shifts values are referred to acetone as internal standard (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). $^1J_{C\text{-}1,H\text{-}1}$ are reported in parentheses and given in Hertz. Spectra were recorded at 298 K.

Residue	OS1	OS2	1	2	3	4	5	6	7	8
6-α-GlcN*1P*	●	●	5.54 (178)	3.18	3.90	3.60	4.14	4.34,3.78		
A			92.5	56.2	70.1	70.8	73.1	71.2		
2,3,7-α-Hep	●		5.48 (181)	4.19	4.23	4.18	3.58	3.93	3.65,3.93	
B			100.7	80.6	79.2	69.0	73.4	67.0	75.3	
α-ΔHexA	●	●	5.40 (176)	3.73	4.43	5.80	–			
C			101.7	72.4	67.0	108.6	146.3	169.5		
3,4-α-Hep	●	●	5.29 (178)	4.08	4.13	4.35	4.29	4.03	3.68,4.01	
D			99.1	71.7	72.9	74.2	73.2	71.9	64.2	
α-ΔHexA	●	●	5.15 (176)	3.82	4.40	5.82	–			
E			101.0	71.7	67.5	109.6	142.2	169.5		
7-α-Hep	●	●	4.89 (174)	3.98	3.88	3.93	n.d.	3.91	3.98	
F			105.0	71.4	71.1	67.0		67.1	73.7	
6-β-GlcN*4P*	●	●	4.69 (171)	2.96	3.77	3.68	3.70	3.45,3.69		
G			101.5	56.9	74.5	73.7	75.2	63.9		
t-β-Glc	●		4.54 (165)	3.21	3.50	3.45	3.55	3.75,3.85		
H			104.5	74.7	75.8	70.3	76.4	61.8		
t-β-Gal	●	●	4.47 (166)	3.52	3.67	3.90	3.66	3.79,3.68		
I			103.9	72.0	73.6	70.0	74.0	62.8		
4,5-α-Kdo	●	●	–	n.d.	1.87,2.11	4.12	4.24	3.91	3.70	3.75,3.95
L					35.7	71.8	69.1	72.9	70.6	64.9
t-α-Kdo	●	●	–	n.d.	1.79,2.13	4.08	4.06	3.74	4.15	3.86,4.10
M					35.7	67.2	67.8	72.5	70.3	65.3
3,7-α-Hep		●	5.39 (181)	4.22	4.08	4.19	4.12	3.87	3.95	
N			102.0	70.3	80.8	69.3	73.0	67.0	73.5	

F, H-1 **F** with C-7 **B**, H-1 **C** with C-3 **B**, H-1 **H** with C-2 **B**, H-1 **B** with C-3 **D**, H-1 **I** with C-4 **D** and H-1 **G** with C-6 **A**. NOE interresidue contacts (Table 2) confirmed the above results. In particular, a strong NOE between H-1 **D** and H-5 **L** indicated the O-5 substitution of Kdo.

All these experimental data revealed the structure shown in Scheme 1 for OS1.



Scheme 1.

In the NMR spectra of OS2, the spin system of the terminal β-Glc residue (**H**) was absent, whereas the O-2 position of residue **B** was not substituted (Table 1), which is thus indicative of a nonstoichiometric substitution of this residue for the inner core structure.

Acid Hydrolysis of LPS

In order to reveal the residues lost during hot alkaline treatment by β-degradation of both galacturonic acids, the LPS was hydrolysed under mild conditions with acetic acid, and the product, obtained after elimination of lipid A by centrifugation, was fractionated on a Biogel P-10 column. Four main fractions were collected, of which the first three were identified as O-chain with different numbers of repeating units,[18] whereas the fourth was recognised to be a mixture of core oligosaccharides by reflector positive ions MALDI-TOF (matrix-assisted laser-desorption ionisation time of flight) (Figure 2) and ^{1}H NMR spectra. The mass spectrum was quite complex and five clusters of sodium adduct signals were identified. The pseudomolecular ion $[M + Na]^+$ displayed at m/z = 1673.512 (calcd. mass 1673.501 u) revealed a molecular species M1, the composition of which was in agreement with the presence of one hexose, two aminohexoses, two uronic acids, three heptoses and one Kdo unit. Two signals at 162 u higher (M2) and 161 u lower (M3) masses indicated molecular species with one more hexose and one less hexosamine residue, respectively. Finally, the signals at m/z = 1909.518 (calcd. mass 1909.555 u, M4) and m/z = 2071.558 (calcd. mass 2071.607 u, M5) confirmed the presence of a Ko unit.

Methylation analysis of this oligosaccharide mixture showed the presence of the same residues obtained from the analysis of the entire LPS, except for the Kdo units.

The fraction was purified on a Biogel P-2 column to obtain one main peak named OS3. The MALDI-TOF spectrum of the OS3 fraction showed the presence of all the species found in the oligosaccharide mixture except for the M2 species (data not shown).

The complete assignment of all NMR signals from OS3 was achieved by 2D homonuclear and heteronuclear experi-

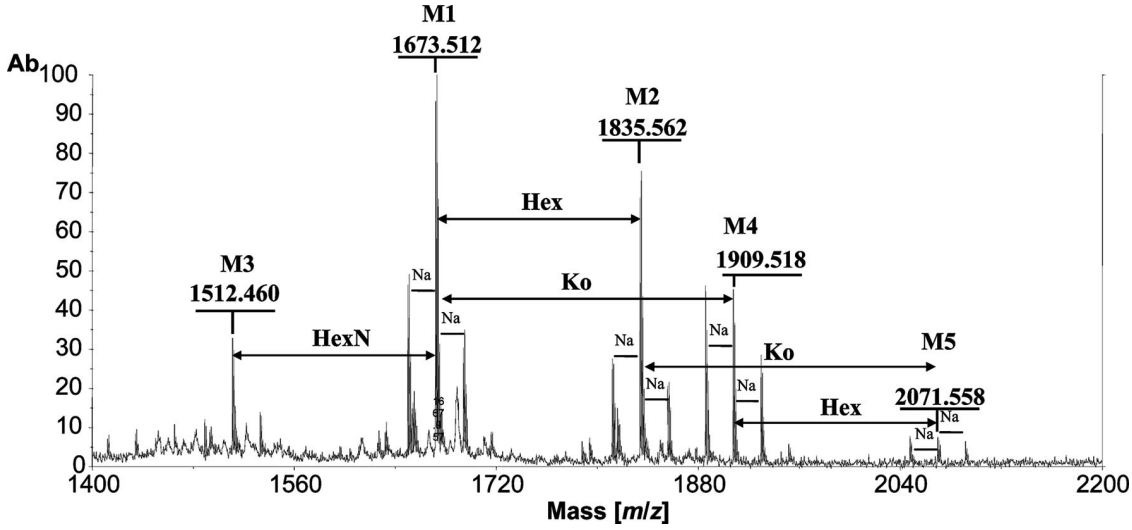


Figure 2. MALDI-TOF mass spectrum of the main fraction isolated after Biogel P-10 chromatography of the oligosaccharide mixture obtained after mild acid hydrolysis of the LPS from *P. shigelloides* 302-73 strain (serotype O1). The spectrum was acquired in positive ion mode.

ments (Table 3). The ^{1}H NMR spectrum confirmed that it was still a mixture of oligosaccharides. The identification of the monosaccharides was obtained by proton and carbon chemical shift values, supported by the proton vicinal coupling constant values of the identified multiplet patterns. Residues present in both alkaline and acid degradation products showed very similar chemical shift values (Tables 1 and 3). The region between 5.4 and 4.4 ppm of the ^{1}H NMR spectrum displayed 10 signals of which only 8 were deduced to be anomeric protons from their correlations with anomeric carbon signals in a DEPT–HSQC experiment (Table 3, Figure 3). The combination of NOE and HMBC data revealed for OS3 the same sequence as that found in OS2, except for the residues lost during the alkaline treatment. Residues **C** and **E′** were both identified as 4-substituted galacturonic acids, as their C-4 resonances occurred at δ = 80.5 and 76.9 ppm, respectively. Residues **P** and **O** were both attributed to α-configured terminal glucosamine units, owing to their small $^3J_{H-1,H-2}$ values (3.4 Hz) and to their C-2 chemical shift at δ = 55.5 ppm and 55.4 ppm, respectively. In particular, residue **P** was not present in a stoichiometric amount, as shown by integration of the H-2 signals of residues **O** and **P** in the ^{1}H NMR spectrum (data not shown). Strong NOE contacts were observed between H-1 **P** at δ = 5.19 ppm and H-4 **E′** at δ = 4.48 ppm and between H-1 **O** at δ = 5.06 ppm and H-4 **C** at δ = 4.32 ppm, which thus indicates that **P** was linked to C-4 of **E′** and **O** was linked to C-4 of **C**. Long-range scalar connectivities (Figure 3) were in agreement with the observed NOEs, as H-1 **P** and H-1 **O** were correlated with H-4 **E′** and H-4 **C**, respectively.

The low intensities of the Ko signals did not allow the complete assignment of the spin system for this residue. Nevertheless the downfield shift of C-4 of the Kdo residue suggested that the Ko unit was linked at the O-4 position of residue **L**.

Table 3. ^{1}H–^{13}C NMR assignments of OS3. All the chemical shifts values are referred to acetone as internal standard (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). Spectra were recorded at 298 K.

Residue	1	2	3	4	5	6	7	8
4-α-GalA	5.21	3.76	4.19	4.32	4.46	–		
C	99.5	69.9	69.6	80.5	72.9	176.1		
3,4-α-Hep	5.21	3.96	3.97	4.22	4.08	4.05	3.63,3.91	
D	103.1	71.6	73.7	75.3	73.1	69.9	64.2	
4-α-GalA	4.95	3.90	4.00	4.48	4.23	–		
E′	100.1	69.2	70.2	76.9	70.7	177.1		
7-α-Hep	4.87	3.85	3.80	3.83	3.58	3.88	3.76,3.48	
F	105.0	70.9	71.2	67.4	73.9	69.2	72.6	
t-β-Gal	4.45	3.46	3.58	3.84	3.62	3.63		
I	104.5	72.3	73.6	70.0	76.3	63.1		
4,5-Kdo	n.d.	n.d.	1.94	4.01	4.15	3.82	4.12	3.51,3.70
L			35.4	71.9	69.6	71.5	70.7	64.5
3,7-α-Hep	5.37	4.13	3.87	3.94	3.73	4.12	3.57,3.81	
N	101.9	69.9	83.0	65.8	73.0	68.9	75.2	
t-α-GlcN	5.06	3.17	3.79	3.46	4.16	3.76		
O	97.6	55.4	71.1	70.2	73.3	61.2		
t-α-GlcN	5.19	3.10	3.86	3.42	4.01	3.74		
P	95.2	55.5	70.9	70.4	73.3	61.2		

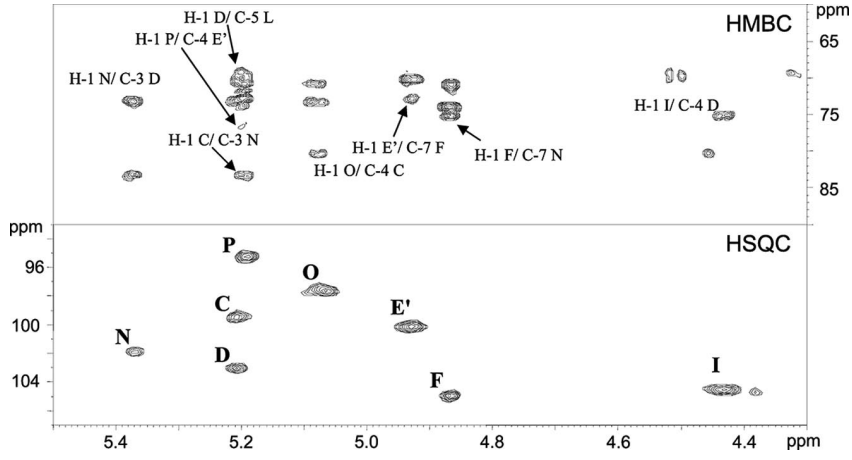
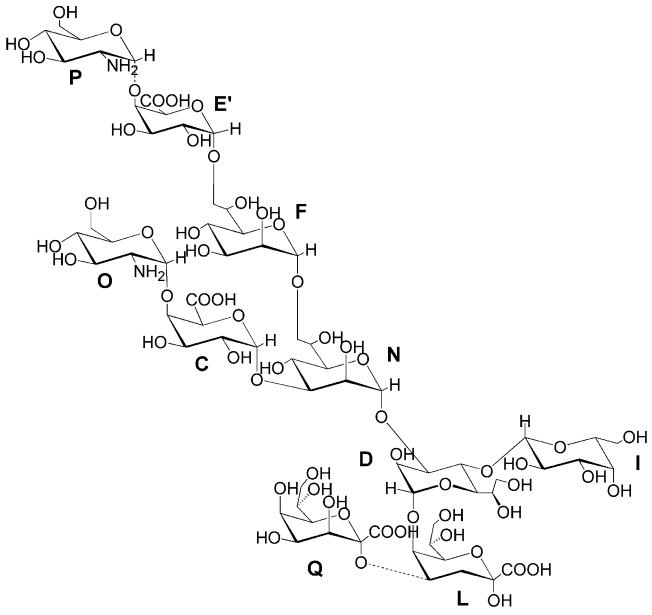


Figure 3. Anomeric region of the ^{1}H–^{13}C HMBC and DEPT–HSQC spectra of OS3 fraction from *P. shigelloides* 302-73 strain (serotype O1) performed at 500 MHz in D_2O at 298 K. The letters refer to the residues as described in Table 3.

It was concluded that the OS3 fraction has the structure reported in Scheme 2.



Scheme 2.

The results obtained from the alkaline degradation and the occurrence of terminal glucose in the GC–MS chromatogram of the partially methylated alditol acetates indicated that M2 species contained a glucose linked at the O-2 position of heptose **N**.

In conclusion, the core oligosaccharide of the LPS from *P. shigelloides* strain 302-73 (serotype O1) is shown in Scheme 3.

This structure is similar to that of serotype O54[20] and O17,[21] even if some new features are present in both the inner and outer core. A glucosamine residue was nonstoichiometrically linked to the branching galacturonic acid, and more interestingly, a Ko unit substitutes in part the terminal Kdo residue. The presence of the Ko residue is not frequent in core structures, and to date it has been described as a substitute for Kdo in the LPS of *Burkholderia*,[26] *Acinetobacter*,[27] *Tatlockia*,[28] *Serratia*[29] and *Yersinia*.[22] Finally, this new core oligosaccharide confirmed the lack of a uniform core structure for the unique species of the *Plesiomonas* genus.

Experimental Section

Bacteria Growth, Isolation and Purification of the LPS: *P. shigelloides* strain 302-73, belonging to serotype O1, was routinely grown on tryptic-soy-broth at 37 °C, and dried bacteria were extracted both by PCP and phenol/water method, as reported.[18]

Sugar and Fatty Acids Analysis: Monosaccharides were analysed as acetylated methyl glycosides, which were obtained from the crude LPS (0.5 mg). Methanolysis was performed in 1 M HCl/MeOH (0.5 mL, 80 °C, 20 h), and the sample was extracted with hexane (2×). The hexane layer was analysed by GC–MS to identify fatty acid methyl esters. The methanol layer was dried and acetylated with Ac_2O and Py (100 °C, 30 min). To reveal the presence of the Ko–Kdo disaccharide the methanolysis was performed in 0.5 M HCl/MeOH (0.5 mL, 85 °C, 45 min).

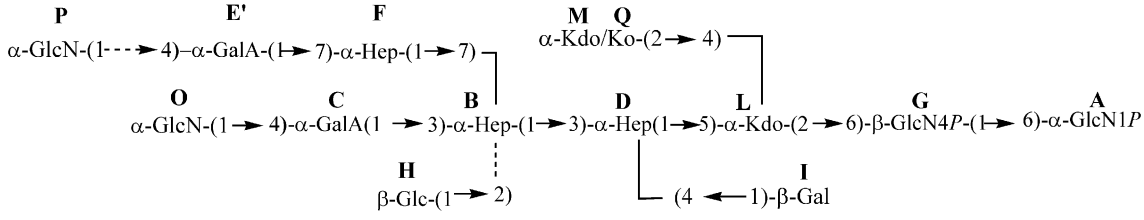
The linkage positions of the monosaccharides were determined by methylation analysis. Briefly, the LPS (1 mg) was *N*-acetylated with Ac_2O in anhydrous MeOH. Methylation was performed with CH_3I in DMSO and NaOH (2.5 h). The product was then hydrolysed with 4 M trifluoroacetic acid (100 °C, 4 h), reduced with $NaBD_4$ and then acetylated.

The absolute configuration of the sugars was determined by gas-chromatography of the acetylated (*S*)-2-octyl glycosides.[30]

All the derivatised monosaccharides were analysed with an Agilent Technologies gas chromatograph 6850A equipped with a mass-selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 mL min^{-1}, He as carrier gas). Acetylated methyl glycosides were analysed accordingly with the following temperature program: 150 °C for 3 min, 150 °C→240 °C at 3 °C min^{-1}. To detect the Ko–Kdo disaccharide, the temperature program was: 150 °C for 3 min, 150 °C→300 °C at 5 °C min^{-1}, 300 °C for 20 min. For partially methylated alditol acetates the temperature program was: 90 °C for 1 min, 90 °C →140 °C at 25 °C min^{-1}, 140 °C→200 °C at 5 °C min^{-1}, 200 °C →280 °C at 10 °C min^{-1}, 280 °C for 10 min. Analysis of acetylated octyl glycosides was performed at 150 °C for 5 min, then 150 °C→240 °C at 6 °C min^{-1}, 240 °C for 5 min. Analysis of fatty acids methyl esters was performed as follows: 140 °C for 3 min, then 140 °C→280 °C at 10 °C min^{-1}, 280 °C for 20 min.

Deacylation of the LPS: The LPS (80 mg) was first dried with phosphoric anhydride under vacuum and then it was incubated with hydrazine (2.7 mL, 37 °C, 1.5 h). Cold acetone was then added to precipitate the *O*-deacylated LPS. The pellet was recovered after centrifugation (4 °C, 10000 *g*, 30 min.), washed with acetone (3×) and finally dissolved in water and lyophilized (52 mg).

The *O*-deacylated LPS was dissolved in 4 M KOH (2 mL) and incubated at 120 °C for 16 h. KOH was neutralized with 4 M HCl until



Scheme 3.

pH 6, and the mixture was extracted with CH_2Cl_2 (3×). The water phase was recovered and then desalted on a column (1.5 × 100 cm) of Sephadex G-10 (17 mL h^{-1}, fraction volume 2 mL) eluted with water. The eluted oligosaccharide mixture was lyophilized (21 mg) and partially purified on a Biogel P-2 (1.0 × 120 cm, flow rate 3.8 mL h^{-1}, fraction volume 1 mL), eluted with water buffered (pH 5.0) with 0.05 M pyridine and 0.05 M sodium acetate.

Mild Acid Hydrolysis of the LPS: The LPS (58 mg) was hydrolysed with 1% aqueous CH_3COOH (6 mL, 100 °C, 3 h). The obtained suspension was then centrifuged (10000 *g*, 4 °C, 10 min). The pellet was washed with water (2×), and the supernatant layers were combined and lyophilized (33 mg). The precipitate (lipid A) was also lyophilized (24 mg). The polysaccharide portion was then fractionated first on a Biogel P-10 column (1.5 × 130 cm, flow rate 16 mL h^{-1}, fraction volume 2.5 mL) and then on a Biogel P-2 (1.0 × 120 cm, flow rate 3.8 mL h^{-1}, fraction volume 1 mL), eluted with water buffered (pH 5.0) with 0.05 M pyridine and 0.05 M sodium acetate.

HPAEC Analysis: Separation of the oligosaccharides mixture obtained after deacylation of the LPS (7 mg) was performed by HPAEC–PAD on a semi-preparative column (9 × 250 mm) of Carbopac PA-100 eluted with 53% of 1 M NaOAc/0.1 M NaOH and 47% of 0.1 M NaOH at 1 mL min^{-1} over 70 min to yield fraction A (0.5 mg) and B (1 mg). Fractions were desalted on a column (1.5 × 100 mm) of Sephadex G-10 (17 mL h^{-1}, fraction volume 2 mL, eluent water).

NMR Spectroscopy: For structural assignments of OS1 and OS2 1D and 2D ^{1}H and ^{13}C NMR spectra were recorded at 298 K with a Bruker 600 spectrometer. All 2D homo- and heteronuclear experiments (COSY, TOCSY, ROESY, HSQC–DEPT, HMBC and 2D *F2*-coupled HSQC) were performed by using standard pulse sequences available in the Bruker software. For structural assignments of OS3 1D and 2D ^{1}H and ^{13}C NMR spectra were recorded at 298 K with a Varian Inova 500 spectrometer. All 2D homo- and heteronuclear experiments (COSY, TOCSY, ROESY, HSQC–DEPT, HMBC) were performed by using standard pulse sequences available in the Varian software. Chemical shifts were measured in D_2O by using acetone as an internal standard (δ = 2.225 and 31.45 ppm for CH_3 proton and carbon, respectively).

Mass Spectrometry Analysis: Positive ion reflectron MALDI-TOF mass spectra were acquired with a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed extraction ion source. Ion acceleration voltage was 20 kV, grid voltage was 17 kV, mirror voltage ratio 1.12 and delay time 200 ns. Samples were irradiated at a frequency of 5 Hz by 337 nm photons from a pulsed nitrogen laser. Mass calibration was obtained with a maltooligosaccharide mixture from corn syrup (Sigma). A solution of 2,5-dihydroxybenzoic acid in 20% CH_3CN in water at a concentration of 25 mg mL^{-1} was used as the MALDI matrix. Spectra were calibrated and processed under computer control by using the Applied Biosystems Data Explorer software.

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[1] G. M. Garrity, J. A. Bell, T. G. Lilburn, *Bergey's Taxonomic Outline*, **2004** http://dx.doi.org/10.1007/bergeysoutline200310, p. 120.
[2] R. E. Levin, *Food Biotechnol.* **2008**, *22*, 189–202.
[3] G. Weimin, R. E. Levin, *Food Biotechnol.* **2008**, *22*, 98–113.
[4] G. Weimin, R. E. Levin, *Food Biotechnol.* **2007**, *21*, 145–149.
[5] C. Theodoropoulos, T. H. Wong, M. O'Brien, D. Stenzel, *Infect. Immun.* **2001**, *69*, 2260–2269.
[6] H. Tsugawa, T. Ono, H. Murakami, Y. Okawa, *J. Appl. Microbiol.* **2005**, *99*, 1435–1443.
[7] H. Tsugawa, H. Ito, M. Ohshima, Y. Okawa, *J. Med. Microbiol.* **2007**, *56*, 23–29.
[8] B. G. Matthews, H. Douglas, D. G. Guiney, *Microb. Pathog.* **1988**, *5*, 207–213.
[9] S. L. Abbott, R. P. Kokka, J. M. Janda, *J. Clin. Microbiol.* **1991**, *29*, 148–153.
[10] V. Manorama Taneja, R. K. Agarwal, S. C. Sanyal, *Toxicon. Suppl.* **1983**, *3*, 269–272.
[11] S. E. Gardner, S. E. Fowlston, W. L. George, *J. Infect. Dis.* **1987**, *156*, 720–722.
[12] J. M. Janda, S. L. Abbott, *J. Clin. Microbiol.* **1993**, *31*, 1206–1208.
[13] Y. Okawa, Y. Ohtomo, H. Tsugawa, Y. Matsuda, H. Kobayashi, T. Tsukamoto, *FEMS, Microbiol. Lett.* **2004**, *239*, 125–130.
[14] E. Frirdich, C. Whitfield, *J. Endotoxin Res.* **2005**, *11*, 133–144.
[15] H. Nikaido, *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 593–656.
[16] L. Izquierdo, N. Coderch, N. Pique, E. Bedini, M. M. Corsaro, S. Merino, S. Fresno, J. M. Tomás, M. Regué, *J. Bacteriol.* **2003**, *185*, 7213–7221.
[17] S. Fresno, N. Jiménez, L. Izquierdo, S. Merino, M. M. Corsaro, C. De Castro, M. Parrilli, T. Naldi, M. Regué, J. M. Tomás, *Microbiology* **2006**, *152*, 1807–1818.
[18] G. Pieretti, M. M. Corsaro, R. Lanzetta, M. Parrilli, R. Canals, S. Merino, J. M. Tomás, *Eur. J. Org. Chem.* **2008**, 3149–3155.
[19] T. Niedziela, S. Dag, J. Lukasiewicz, M. Dzieciatkowska, W. Jachymek, C. Lugowski, L. Kenne, *Biochemistry* **2006**, *45*, 10422–10433.
[20] T. Niedziela, J. Lukasiewicz, W. Jachymek, M. Dzieciatkowska, C. Lugowski, L. Kenne, *J. Biol. Chem.* **2002**, *277*, 11653–11663.
[21] J. Kluber-Kielb, R. Schneerson, C. Mocca, E. V. Vinogradov, *Carbohydr. Res.* **2008**, *343*, 3123–3127.
[22] E. V. Vinogradov, B. Lindner, N. A. Kocharova, S. N. Senchenkova, A. S. Shashkov, Y. A. Knirel, O. Holst, T. A. Gremyakova, R. Z. Shaikhutdinova, A. P. Anisimov, *Carbohydr. Res.* **2002**, *337*, 775–777.
[23] K. Bock, C. Pedersen, *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
[24] S. Müller-Loennies, B. Lindner, H. Brade, *Eur. J. Biochem.* **2002**, *269*, 5982–5991.
[25] P. K. Agrawal, C. A. Bush, N. Qureshi, K. Takayama, *Adv. Biophys. Chem.* **1994**, *4*, 179–236.
[26] Y. Isshiki, K. Kawahara, U. Zähringer, *Carbohydr. Res.* **1998**, *313*, 21–27.
[27] K. Kawahara, H. Brade, H. E. Rietschel, U. Zähringer, *Eur. J. Biochem.* **1987**, *163*, 489–495.
[28] K. Kawahara, S. Dejsirilert, T. Ezaki, *FEMS Microbiol. Lett.* **1998**, *169*, 283–287.
[29] E. V. Vinogradov, B. Lindner, G. Seltmann, J. Radziejewska-Lebrecht, O. Holst, *Chem. Eur. J.* **2006**, *12*, 6692–6700.
[30] K. Leontein, B. Lindberg, J. Lönngren, *Carbohydr. Res.* **1978**, *62*, 359–362.

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