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Structural Studies of the O-Chain Polysaccharide from *Plesiomonas shigelloides* Strain 302–73 (Serotype O1)

Giuseppina Pieretti,[a] M. Michela Corsaro,*[a] Rosa Lanzetta,[a] Michelangelo Parrilli,[a] Rocìo Canals,[b] Susana Merino,[b] and Juan M. Tomás[b]

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Plesiomonas shigelloides is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. It has been found in an aquatic environment in the tropical and subtropical regions and is responsible for many gastrointestinal infections in humans, which take place from drinking untreated water or eating uncooked shellfish. *Plesiomonas shigelloides* has also been reported to provoke extraintestinal infections such as meningitis and bacteremia in immunocompromised adults and neonates. Despite the emerging importance of this pathogenic microorganism, only three different O-antigens have been characterised so far. The structure of the O-chain of the lipopolysaccharide (LPS) from *Plesiomonas shigelloides* strain 302–73 (serotype O1) was determined by chemical analysis, 1D and 2D NMR spectroscopy and MALDI-TOF mass spectrometry. The polysaccharide was constituted by a linear pentasaccharidic repeating unit as follows: →3)-α-L-PneNAc4OAc(1→4)-α-L-FucNAc(1→4)-α-L-FucNAc(1→4)-α-L-FucNAc(1→3)-β-D-QuiNAc4NHb(1→ (PneNAc = 2-acetamido-2,6-dideoxy-talose, Hb = (*S*)-3-hydroxybutanoyl) PneNAc *O*-acetylation was not stoichiometric and was found to be about 75 %. The position of the *O*-acetyl group and the amount of acetylation were deduced by NMR spectroscopic analysis. All the monosaccharides included in the repeating unit were deoxyamino sugars, which most probably, together with the presence of *O*-acetyl groups, were responsible for the recovery of the LPS in the phenol layer of the phenol/water extract of dried bacteria cells.

Introduction

Diarrhoeal disease affects a huge number of people and it is a serious health problem throughout the world. This pathology is frequently caused by Gram-negative bacteria, such as *Enterobacteriaceae* and *Vibrionaceae*.[1] Among *Enterobacteriaceae*, *Plesiomonas shigelloides* has been recognised as a significant cause of gastrointestinal infections. In Japan, it was ranked third as a cause of diarrhoea in travellers.[2]

Plesiomonas shigelloides is a flagellated, rod-shaped, capsulated microorganism and is the only species in the genus *Plesiomonas*.[3] It has been isolated from a variety of sources, including fresh water, surface water, soil and shellfish. Plesiomonad infections in humans presumably derive from exposure to contaminated water sources or from eating contaminated food. *Plesiomonas shigelloides* is capable of adhering to and entering the human colon carcinoma Caco-2 cells, inducing apoptotic cell death.[1,4] Recently, the heat-shock protein GroEL from *P. shigelloides* has been found to promote the adhesion to Caco-2 cells, most likely starting in this way the invasion mechanism.[5]

To date, various virulence factors have been described to determine the pathogenic mechanism of *Plesiomonas shigelloides*, which included enterotoxins,[6–8] cholera-like toxin,[9] β-haemolysin[10] and cytotoxic complex.[11] The latter is a complex constituted by proteins and lipopolysaccharides (LPSs) (ACRP-LPS complex)[11] and has been reported to have a significant role in the enteropathogenesis of *Plesiomonas shigelloides*.

LPS (endotoxin) represents the main component of the outer leaflet of the external membrane of Gram-negative bacteria and plays a key role during severe Gram-negative infection, sepsis and septic shock.[12,13] Smooth-form lipopolysaccharides (S-LPSs) consist of three covalently linked regions: lipid A, also known as the endotoxin for human pathogen microorganisms, the oligosaccharide region (core region) and the O-specific polysaccharide (O-chain). Chemically, lipid A, which anchors the LPS to the outer membrane, consists of a glucosamine disaccharide β-GlcN-(1′-6)-α-GlcN, which is *N*- and *O*-acylated with fatty acids at the C-2, C-3, C-2′ and C-3′ positions and phosphorylated at the O-1 and O-4′ positions. The core region is made up of several sugar units such as neutral hexoses, heptose and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Finally, the O-chain can be a homo- or a heteropolysaccharide. The latter

[a] Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Napoli, Italy
Fax: +39-081-674-393
E-mail: corsaro@unina.it
[b] Departamento Microbiología, Universidad de Barcelona, Diagonal 645, 08071 Barcelona, Spain

is usually built from a variable number of an oligosaccharidic repeating unit. It is considered as an outer-cell antigenic determinant (O-antigen). Rough-form lipopolysaccharides (R-LPSs), also named lipooligosaccharides (LOSs), lack the polysaccharidic portion.[14]

Unlike other phenotypic methods, serology has more successfully been used for distinguishing different strains of *Plesiomonas shigelloides*. There are mainly two serotyping schemes, which are based on somatic (O-antigen LPS) and flagellar (H) antigens. Nowadays, 102 somatic antigens and 51 flagellar antigens have been recognised.[15]

To date, only the LPS structures and biological activity from few serotypes of *Plesiomonas shigelloides* have been investigated.[16–21] Because of the role of the LPS in *Plesiomonas shigelloides* pathogenesis mechanism, it was important to extend the information available concerning the structure of the O-chain.

In this paper, we report the complete structure elucidation of the O-chain of the LPS from *Plesiomonas shigelloides* strain 302–73 (serotype O1). The O-chain was obtained by mild acid degradation of the LPS isolated from the phenol phase after the phenol/water extraction of dried bacterial cells. The product was investigated by means of chemical analysis, ^{1}H and ^{13}C NMR spectroscopy and MALDI-TOF mass spectrometry.

Results and Discussion

Growth Bacteria, Isolation and Purification of the LPS

Plesiomonas shigelloides strain 302–73, belonging to serotype O1, was routinely grown on tryptic-soy broth at 37 °C,[22] that is, under conditions resulting in a better yield of the LPS relative to that obtained by cultivation on agar.

Dried bacteria were first extracted by phenol/chloroform/light petroleum (PCP)[23] and then by phenol/water method.[24] The crude extracts were analysed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Figure 1) and showed the presence of the LPS both in the PCP extract (LPS_{PCP}) and in the phenol layer of the phenol/water extract (LPS_{PhOH}). The LPS recovery from the phenol layer has already been reported for another strain of *Plesiomonas shigelloides*.[21] In particular, LPS_{PhOH} molecules, which showed the typical ladder banding pattern of S-LPS, contained a higher number of O-antigen repeating units than that found in LPS_{PCP} molecules.

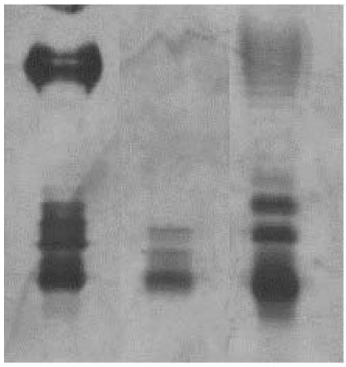


Figure 1. 18% SDS-PAGE analysis of the LPS from *Plesiomonas shigelloides* 302–73 strain (serotype O1). Lane **B** and **C** represent the PCP and phenol layer extracts, respectively. *E. coli* E55:B5 serotype (lane **A**) was used as standard.

Compositional Analysis

Fatty-acid methyl esters were analysed by GC–MS after methanolysis of the LPS. Dodecanoic, 3-hydroxydodecanoic, tetradecanoic and 3-hydroxytetradecanoic acids were present. The sugars were analysed by GC–MS as acetylated methyl glycosides as well as alditol acetates after LPS degradation and derivatisation. Both the GC–MS profiles revealed the presence of L-*glycero*-D-*manno*-heptose (L,D-Hep), glucose (Glc), galactose (Gal), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), 2-amino-2-deoxyglucose (GlcN) and 2-amino-2,6-dideoxygalactose (fucosamine, FucN). All sugar derivatives were identified by MS (EI) and GC retention times by comparison with those of authentic standards.

Moreover both analyses showed the presence of signals attributable to an additional 2-amino-2,6-dideoxyhexose and a 2,4-diamino-2,4,6-trideoxyhexose, the retention times of which corresponded with none of the standards available in our laboratories.

Isolation and Characterisation of the O-Chain Polysaccharide

In order to obtain the O-chain polysaccharide, the LPS was mildly hydrolysed with 1% aqueous AcOH. The lipidic portion of the LPS was removed by centrifugation and the supernatant was fractionated with a Sephadex G-50 column (Pharmacia), eluting with pyridine/acetate buffer. The fractions containing the O-chain were collected and freeze dried. The obtained polysaccharide (PS1) was analysed by one- and two-dimensional NMR (COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ^{1}H–^{13}C DEPT–HSQC, distortionless enhancement by polarisation transfer–heteronuclear single quantum coherence; ^{1}H–^{13}C HMBC, heteronuclear multiple bond correlation) spectroscopy.

The ^{1}H NMR spectrum (Figure 2a) showed the presence of five signals in the high chemical shift region at 5.24, 5.12, 4.95, 4.83 and 4.61 ppm. The upfield region of the spectrum revealed the presence of a broad signal at δ = 2.34 ppm, signals for the *N*- and *O*-acetyl groups in the range 1.9–2.2 ppm and methyl signals between 1.0 and 1.3 ppm. The analysis of the anomeric region of the DEPT–HSQC spectrum revealed a correlation between the proton signal at δ = 5.24 ppm and a carbon signal at δ = 72.6 ppm, which suggests the presence of an *O*-acetyl group. The integration of signals, together with the spectra heterogeneity, suggested that the *O*-acetylation was not stoichiometric. The polysaccharide was *O*-deacylated with NaOH to obtain

PS2. The lack in the ^{1}H NMR spectrum (Figure 2b) of the signal at δ = 5.24 ppm and of an *O*-acetyl signal at 2.2 ppm

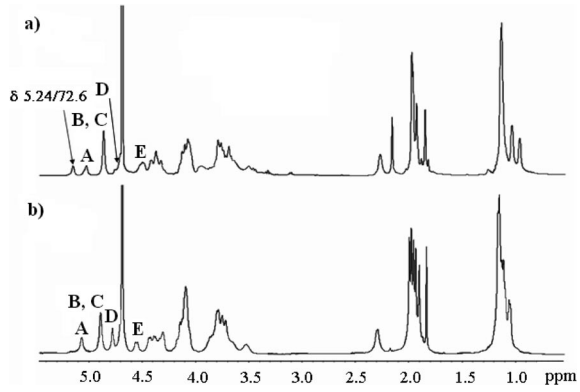


Figure 2. ^{1}H NMR spectra of PS1 and PS2 (**a** and **b**, respectively). Spectra were recorded at 400 MHz in D_2O at 298 K. The letters refer to the residues as described in Table 1.

confirmed the partial acetylation of the polysaccharide and suggested the presence of a pentasaccharidic repeating unit. Accordingly, the ^{13}C NMR spectrum (Figure 3) of PS2 contained five anomeric carbon signals at δ = 98.3–102.0, carbinolic carbon signals in the region δ = 66.2–81.5, six nitrogen-bearing carbon atoms in the range δ = 58.0–50.0, one carbon signal at δ = 46.5, one carbon signal at δ = 23.6 and methyl carbon signals at δ = 16.6–18.3. The *O*-deacylated polysaccharide was characterised by one- and two-dimensional NMR experiments (^{1}H and ^{13}C 1D NMR; COSY, TOCSY, NOESY, ^{1}H–^{13}C DEPT–HSQC, ^{1}H–^{13}C HMBC, 2D *F2*-coupled HSQC). All the proton and carbon chemical shift assignments are reported (Table 1). Starting from the anomeric proton signal at δ = 5.12 ppm (Figure 2b), letters **A**–**E** were assigned to each spin system. The anomeric configuration of all the residues was determined by a 2D *F2*-coupled HSQC NMR experiment. The $^1J_{C\text{-}1,1\text{-}H}$ values are reported in Table 1. All the sugars showed an α configuration except the bacillosamine (2,4-diamino-2,4,6-trideoxy-glucopyranose, QuiN4N*p*).

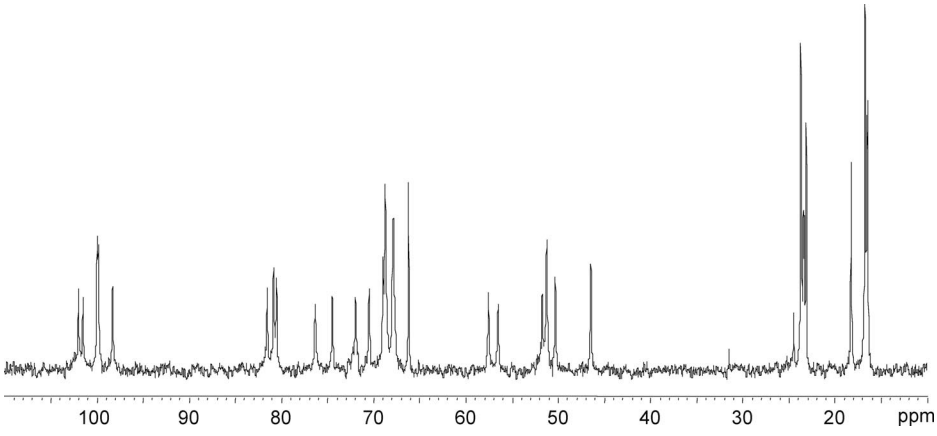


Figure 3. ^{13}C NMR spectrum of the *O*-deacylated O-chain polysaccharide (PS2) from *Plesiomonas shigelloides* 302–73 strain (serotype O1). The spectrum was recorded at 100 MHz in D_2O at 298 K.

Table 1. ^{1}H and ^{13}C NMR assignments of the *O*-deacylated O-chain (PS2) repeating unit of the LPS from *Plesiomonas shigelloides* 302–73 strain (serotype O1). All the chemical shifts values were referred to acetone as internal standard (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). Spectra were recorded at 298 K.[a]

Sugar residue	H1 C1	$J_{1\text{-}H,C\text{-}1}$ [Hz]	H2 C2	H3 C3	H4 C4	H5 C5	H6 C6
PS2							
4-α-L-FucNAc*p*	5.12	174.7	4.12	3.81	3.76	3.87	1.23
A	98.3		50.3	68.1	80.5	69.0	16.7
4-α-L-FucNAc*p*	4.95	173.6	4.22	4.11	3.83	4.43	1.11
B	100.0		51.2	67.7	80.6	69.0	16.6
4-α-L-FucNAc*p*	4.92	173.6	4.22	4.13	3.85	4.48	1.20
C	100.0		51.2	67.7	81.5	68.7	16.7
3-α-L-PneNAc*p*	4.83	173.6	4.35	4.14	3.79	4.38	1.15
D	102.0		51.5	74.5	70.5	68.7	16.6
3-β-D-QuiNAc4NHb*p*	4.61	157.7	3.91	3.77	3.71	3.57	1.17
E	101.5		57.6	76.3	56.5	72.0	18.3
(*S*)-3-Hydroxy-butanoyl	–	–	2.34	4.16	1.23		
Hb	175.8		46.5	66.2	23.6		
PS1							
3-α-L-PneNAc4*O*Ac*p*[b]	4.96	–	4.47	4.42	5.24	4.61	1.05
D′	101.9		51.5	73.0	72.6	67.1	16.2

[a] Acetyl groups: CH_3 1.95, 1.98, 2.00, 2.02, 2.04; *C*H$_3$ 23.1, 23.4, 23.3. *C*=O 174.8, 175.1, 175.8 ppm. [b] The chemical shifts for the remaining residues in PS1 were the same as those in PS2.

The analysis of the DEPT–HSQC spectrum revealed the presence of a single methylene carbon signal at δ = 46.5 ppm, which together with a methyl signal at δ = 23.6 ppm and a carbinolic signal at δ = 66.2 ppm indicated the occurrence of a 3-hydroxybutanoic acid (Hb) as the *N*-acyl substituent.[25] Moreover, the ^{1}H NMR spectrum clearly indicated the presence of five *N*-acetyl groups, which together with the Hb substituent accounted for six *N*-acylated carbon atoms.

Residues **A**, **B** and **C**, with C-1/1-H signals at 98.3/5.12, 100.0/4.95 and 100.0/4.92 ppm, respectively, were all identified as FucNAc*p* on the basis of their C-2 and C-6 chemical shift values (Table 1). Downfield-shifted carbon signals at δ = 80.5, 80.6 and 81.5 ppm indicated substitutions at O-4 for **A**, **B** and **C** residues, respectively. Moreover, the coupling constant values for vicinal protons indicated a *galacto* configuration.

Residue **D**, with C-1/1-H signals at 102.0/4.83 ppm, showed the C-2 signal at δ = 51.5 ppm. The coupling constant values for vicinal protons ($^3J_{1\text{-H},2\text{-H}} < 2$ Hz; $^3J_{2\text{-H},3\text{-H}} = 3.5$ Hz; $^3J_{4\text{-H},5\text{-H}} < 2$ Hz) and the carbon atom chemical shifts suggested a *talo* configuration.[26,27] Particularly diagnostic was the chemical shift of the C-3 signal, which was downfield shifted at δ = 74.5 ppm in agreement with the expected value of 65.0 ppm for the unsubstituted sugar.[26] The residue was then identified as a 3-substituted 2-acetamido-2,6-dideoxytalopyranose (Pneumosamine, PneNAc*p*).

Residue **E**, with C-1/1-H signals at 101.5/4.61 ppm, was assigned to a 3-substituted QuiN4N*p* according to C-2 and C-4 signals at δ = 57.6 and 56.5 ppm, respectively. The *gluco* configuration for this residue was suggested by the $^3J_{H,H}$ values and confirmed by intraresidual NOE contacts between 3-H/5-H and 2-H/4-H. The bacillosamine was 3-substituted, as suggested by the downfield shift of its C-3 signal at δ = 76.3 ppm.[28] In addition, the analysis of the NOESY experiment revealed a contact between 4-H of this residue and 2-H of Hb, which suggests that it was amide linked to the N-4 of bacillosamine (QuiNAc4NHb*p*).

The sequence of the monosaccharide was deduced from the analysis of ^{1}H–^{13}C long-range scalar couplings (Figure 4). In the ^{1}H–^{13}C HMBC experiment, the following correlations were observed: 1-H **A**/C-3 **E**, 1-H **E**/C-3 **D**, 1-H **D**/C-4 **C**, 1-H **C**/C-4 **B**, 1-H **B**/C-4 **A**. Consistent with these data were the NOE contacts (Table 2) and the GC–MS analysis of the partially methylated alditol acetates.

The L absolute configuration for the FucN residues (**A**, **B** and **C**) was determined by gas chromatography of the acetylated (*S*)-2-octyl glycosides.[29] The 3-hydroxybutanoic acid absolute configuration was established to be *S* on the basis of the GC retention time of its trifluoroacetylated (*S*)-2-octyl ester derivative.[30]

The absolute configuration of PneNAc and QuiNAc4NHb was determined by the chemical shifts in the ^{13}C NMR spectrum.[31]

The D configuration for the β-QuiNAc4NHb residue was determined by comparison of its experimental carbon chemical shifts with those calculated for the two diastereoisomeric disaccharides α-L-FucN-(1–3)-β-L-*gluco* and α-L-FucN-(1–3)-β-D-*gluco*.[31] The ^{13}C chemical shifts of the QuiNAc4NHb unit were calculated by adding the expected shifts of glucose carbon signals in the two structures to the chemical shift values of the unsubstituted residue.[28] Particularly diagnostic were the effects of glycosylation on the C-3 and C-4 chemical shifts, which were reported to be +4.9 and –1.3 for the disaccharide L-D and +6.8 and –0.1 for the disaccharide L-L.[31] The calculated C-3 and C-4 chemical shift values for the disaccharide L-D were 76.9 and 56.7 ppm, respectively, which is consistent with the observed values of 76.3 and 56.5 ppm (Table 1).

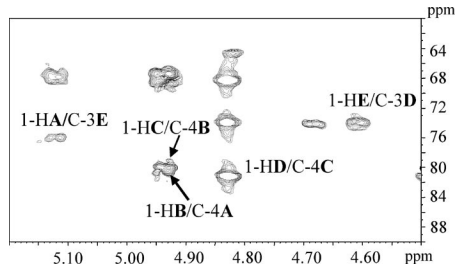


Figure 4. Anomeric region of the ^{1}H–^{13}C HMBC spectrum performed on the *O*-deacylated O-chain polysaccharide (PS2) from *Plesiomonas shigelloides* 302–73 strain (serotype O1). The spectrum was recorded at 400 MHz in D_2O at 298 K. The letters refer to the residues as described in Table 1.

Table 2. Nuclear Overhauser enhancement interresidue connectivities (NOESY) of the *O*-deacylated O-chain repeating unit of the LPS from *Plesiomonas shigelloides* 302–73 strain (serotype O1).

1-H of sugar residue	NOE correlations
FucNAc **A**	**E** H-3
FucNAc **B**	**A** H-4
FucNAc **C**	**B** H-4
PneNAc **D**	**C** H-4
QuiNAc4NHb **E**	**D** H-3

The L configuration of the 3-PneNAc (residue **D**) was deduced on the basis of the carbon chemical shifts of the 4-substituted α-L-FucNAc (residue **C**), calculated for the two diastereoisomeric disaccharides α-D-PneNAc-(1–4)-α-L-FucNAc and α-L-PneNAc-(1–4)-α-L-4-FucNAc. For the former, the C-4 glycosylation shift was reported to be +6.4, whereas that of the latter was +9.3.[31] The L configuration for PneNAc **D** was consistent with the C-4 observed value at δ = 81.5 ppm for FucNAc **C** (Table 1).[31]

The *O*-deacylated O-chain polysaccharide was analysed by reflectron positive ion MALDI-TOF MS (matrix-assisted laser-desorption ionisation-time of flight mass spectrometry) (Figure 5). The spectrum showed the presence of a complex pattern of molecular species signals. The species at *m*/*z* = 1061.9, 2082.5, 3102.8, 4123.4 and 5143.7 differed from each other in Δ ca. 1020.5 *m*/*z*, which corresponded to the molecular mass of the *O*-deacylated O-chain repeating unit (calculated mass 1020.475 u), which is in agreement with the NMR spectroscopic data. Moreover, among the main signals, additional peaks were present, the difference of which corresponded to 187 u (FucNAc/PneNAc) and/or

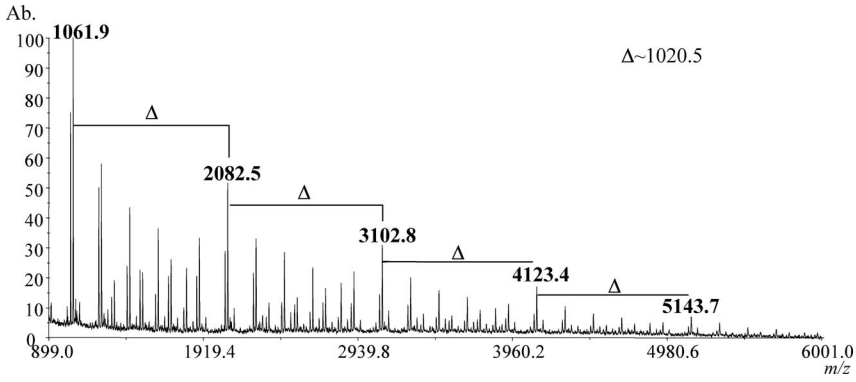
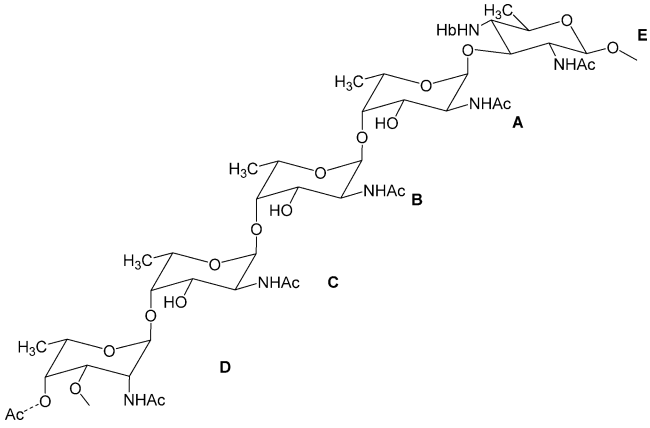


Figure 5. Positive ion mode MALDI-TOF spectrum of the *O*-deacylated O-chain polysaccharide (PS2) from *Plesiomonas shigelloides* 302–73 strain (serotype O1). The spectrum was acquired in reflector mode.

to 272 u (QuiNAc4NHb). This complex pattern of signals could be attributed to the O-chain biosynthesis mechanism.[32] However, a partial rupture of the glycosidic linkages during the spectrum acquisition could not be excluded.

The *O*-acetyl group position in the native polysaccharide was assigned to O-4 of PneNAc **D** on the basis of NMR spectroscopic analysis of PS1 (Table 1). The integration of the signals of 4-*O*-acetylated PneNAc and *O*-deacetylated PneNAc in the ^{1}H NMR spectrum of PS1 accounted for 75% of *O*-acetylation.

All the reported data allowed us to determine the structure of the O-chain polysaccharidic repeating unit of the LPS from *Plesiomonas shigelloides* strain 302–73 (serotype O1) as that reported in the Scheme 1.



Scheme 1. O-chain repeating unit structure of the LPS from *Plesiomonas shigelloides* 302–73 strain (serotype O1). Dotted lines indicate nonstoichiometric substitution.

Conclusions

Plesiomonas shigelloides is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family. It has been found in aquatic environment in the tropical and subtropical regions, and it is responsible of many gastrointestinal infections in humans. Moreover, it has also been reported to provoke extraintestinal infections such as meningitis and bacteremia in immunocompromised adults and neonates.[33–37]

Despite the emerging importance of this pathogenic microorganism, only three different O-antigens have been characterised so far.[16,17,21] Here we present the complete structure of the O-chain repeating unit of the LPS from *Plesiomonas shigelloides* 302–73 strain (serotype O1). The SDS-PAGE analysis indicated the presence of a high molecular mass LPS, which was unusually recovered from the phenol layer after the phenol/water extraction, suggesting the presence of a high hydrophobic LPS. The same behaviour was exhibited by *Plesiomonas shigelloides* O74:H5 LPS,[21] which was recovered in a large amount from the phenol phase, in agreement with the presence of deoxy-amino sugars, *N*-acetyl and *O*-acetyl groups.

The structural study of the O-chain polysaccharide was performed by chemical analysis, NMR spectroscopy and mass spectrometry and revealed that the polysaccharide was 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→

The lack of structural data concerning a suitable number of O-antigen structures prevented us from deducing anything about the structure–activity relationship. However it is tempting to speculate that the occurrence of deoxy sugars with hydrophobic substituents in all of the O-chain structures so far characterised could suggest a method adopted by *Plesiomonas shigelloides* to adhere to host cells in aqueous environment.

Experimental Section

Growth of Bacteria and Isolation of LPS: *Plesiomonas shigelloides* strain 302–73 belonging to serotype O1 was routinely grown on tryptic-soy broth at 37 °C,[22] that is, under conditions resulting in a better yield of the LPS relative to that obtained by cultivation on agar.

Dried bacterial cells (12.8 g) were extracted by using phenol/chloroform/light petroleum (2:5:8)[23] to obtain LPS (136 mg, yield 1.06% of dried cells). The residual material was then extracted with phenol/water (1:1) at 68 °C.[24] Both the water and the phenol phases

were dialysed (cut-off 3500) and lyophilised. The LPS was recovered from the phenol phase (41 mg, yield 0.3% of dried cells).

SDS-PAGE Electrophoresis: PAGE was performed by using the system of Laemmli and Favre[38] with sodium dodecyl sulfate as detergent. The separating gel contained final concentrations of 18% acrylamide, 0.1% SDS and 375 mM Tris/HCl (pH 8.8); the stacking gel contained 4% acrylamide, 0.1% SDS and 125 mM Tris/HCl (pH 6.8). LPS samples were prepared at a concentration of 0.05% in the sample buffer [2% SDS and 60 mM Tris/HCl (pH 6.8), 25% glycerol, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue]. All the concentrations were expressed as mass/vol. percentage. The electrode buffer was composed of SDS (1 g/L), glycine (14.4 g/L) and Tris (3.0 g/L). Electrophoresis was performed at a constant voltage of 150 V. Gels were fixed in an aqueous solution of 40% ethanol and 5% acetic acid. LPS bands were visualised by silver staining as described previously.[39]

Glycosyl Analysis: Monosaccharides were analysed as alditol acetates as well as acetylated methyl glycosides. Alditol acetates were obtained from the LPS (1 mg). The LPS was hydrolysed with trifluoroacetic acid (2 M, 120 °C, 2 h), reduced with $NaBD_4$ and acetylated with Ac_2O and pyridine.

Acetylated methyl glycosides were obtained from the crude LPS (0.5 mg). Methanolysis was performed in MeOH/HCl (1 M, 0.5 mL, 80 °C, 20 h) and the sample was extracted twice with hexane. The methanol layer was concentrated, and the residue was dried and acetylated.

The absolute configuration of FucNAc and 3-hydroxybutanoic acid was determined by gas chromatography of the acetylated (*S*)-2-octyl glycosides[29] and of the trifluoroacetylated (*S*)-2-octyl ester derivative,[30] respectively.

Hydrolysis of the LPS: The LPS (41 mg) was suspended in 1% AcOH and hydrolysed at 100 °C for 2 h. The reaction mixture was centrifuged (10000 × g, 10 °C, 30 min), and the supernatant and the precipitate were freeze dried to yield 33 mg of polysaccharide and 5 mg of lipid A. The supernatant was fractionated by using a Sephadex G-50 (Pharmacia) (1.5 × 100 cm, flow rate 32.4 mL/h, fraction volume 2.5 mL) eluted with water buffered (pH 4.3) with 0.4% (v/v) pyridine and 1% (v/w) sodium acetate to obtain the O-chain polysaccharide (17 mg) and the core oligosaccharide fractions (10 mg).

***O*-Deacetylation of the O-Chain Polysaccharide:** The O-chain polysaccharide (10 mg) was treated with 0.05 M NaOD for 40 min. The reaction was followed by NMR spectroscopy and stopped by neutralisation with HCl.

Methylation Analysis: The linkage positions for each monosaccharide were determined by GC–MS analysis of the partially methylated alditol acetates. Methylation was performed on the O-chain polysaccharide (1 mg) as described by Ciucanu and Kerek,[40] with CH_3I in DMSO and NaOH. The product was then hydrolysed with trifluoroacetic acid (4 M, 100 °C, 4 h), reduced with $NaBD_4$ and then acetylated by using Ac_2O and pyridine.

GC–MS Analysis: 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, He as carrier gas). Acetylated methyl glycosides and alditol acetates were analysed accordingly with the following temperature programs: 150 °C for 3 min, 150 °C→240 °C at 3 °C/min and 150 °C for 3 min, 150 °C→310 °C at 3 °C/min. For partially methylated alditol acetates, the temperature program was: 90 °C for 1 min, 90 °C →140 °C at 25 °C/min, 140 °C→200 °C at 5 °C/min, 200 °C →280 °C at 10 °C/min, 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, 240 °C for 5 min. Analysis of the octyl ester derivative of the 3-hydroxybutanoic acid was performed by using the following temperature program: 90 °C for 3 min 90 °C→150 °C at 5 °C/min, 150 °C for 5 min, 150 °C→300 °C at 25 °C/min, 300 °C for 5 min.

NMR Spectroscopy: Spectra were recorded in D_2O at 400 MHz with a Bruker DRX 400 Avance spectrometer equipped with a reverse probe in the FT mode at 298 K. The ^{13}C NMR spectrum was recorded in D_2O at 100 MHz with a Bruker DRX 400 Avance spectrometer equipped with a 5-mm BBO probe at 298 K. ^{13}C and ^{1}H chemical shifts were measured by using acetone as an internal standard (δ = 2.225 and 31.45 for proton and carbon, respectively). Two-dimensional homo- and heteronuclear experiments (COSY, TOCSY, NOESY, HSQC–DEPT and 2D *F2*-coupled HSQC) were performed by using standard pulse sequences available in the Bruker software.

Mass Spectrometry: Positive ion reflectron time-of-flight mass spectra (MALDI-TOF) 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 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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