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Structural analysis of the O-specific polysaccharide isolated from *Plesiomonas shigelloides* O51 lipopolysaccharide

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ABSTRACT

Plesiomonas shigelloides strain CNCTC 110/92 (O51) was identified as a new example of plesiomonads synthesising lipopolysaccharides (LPSs) that show preference for a non-aqueous surrounding during phenol/water extraction. Chemical analyses combined with ^{1}H and ^{13}C NMR spectroscopy, MALDI-TOF and ESI mass spectrometry showed that the repeating units of the O-specific polysaccharides isolated from phenol and water phase LPSs of *P. shigelloides* O51 have the same structure: →4)-β-D-Glc*p*NAc3NRA-(1→4)-α-L-Fuc*p*Am3OAc-(1→3)-α-D-Quip*N*Ac-(1→, containing the rare sugar constituent 2,3-diamino-2,3-dideoxyglucuronic acid (Glc*p*NAc3NRA), and substituents such as D-3-hydroxybutyric acid (R) and acetamidino group (Am). The HR-MAS NMR spectra obtained for the isolated LPSs and directly on bacteria indicated that the O-acetylation pattern was consistent throughout the entire preparation. The ^{1}H chemical shift values of the structure reporter groups identified in the isolated O-antigens matched those present in bacteria. We have found that the O-antigens recovered from the phenol phase showed a higher degree of polymerisation than those isolated from the water phase.

1. Introduction

Plesiomonas shigelloides, a Gram-negative enterobacterium,[1] is responsible for cases of water- and food-borne outbreaks of intestinal infections. *P. shigelloides* are usually found in fish, crabs, prawns, mussels and oysters. It is one of the most frequent causes of travellers' diarrhoea in Japan and China (third position in rankings).[2] Incidents of extra-intestinal infections, most notably, meningitidis in neonates, bacteremia, sepsis and septic shock were reported for this bacterium. Sepsis and meningitidis caused by *P. shigelloides* are associated with the serious course and high fatality rate.[2]

The pathogenicity of *P. shigelloides* is not yet fully understood. The cholera-like toxin,[3] thermostable and thermolabile toxins,[4,5] β-haemolysin[6] and cytotoxin complex containing lipopolysaccharide (LPS, endotoxin)[7] were described as possible virulence factors of *P. shigelloides*. LPS, the main constituent of the outer cell wall, seems to be one of the least characterised virulence factors of *P. shigelloides*. To date only a few structures of *P. shigelloides* LPSs, that is, the O-specific polysaccharides from strains 22074, 12254,[8] 302-73 (serotype O1),[9] the core oligosaccharide substituted with one O-repeating unit for strain O17[10] and two complete LPSs isolated from *P. shigelloides* O54 and O74[11–15] were elucidated.

Unlike the majority of smooth type LPSs that are recovered from the water phase during the phenol/water extraction procedure,[16] the LPS of *P. shigelloides* O74 was recovered from both the phenol and water phases.[13,14] Moreover, the yield of *P. shigelloides* O74 LPS was much lower in the case of the water phase compared to that isolated from the phenol phase. Structural studies of this LPS indicated that the presence of deoxy and aminodideoxy sugars, *N*-acetyl and *N*-acyl substituents, and scarce free hydroxyl groups could contribute to the overall hydrophobic nature of this endotoxin.[13]

We now report on structural studies of the O-specific polysaccharide of the *P. shigelloides* CNCTC 110/92 LPS, isolated from the phenol (LPS_{PhOH}) and water phases (LPS_{H_2O}). The strain CNCTC 110/92 is a clinical isolate and was classified as serovar O51:H1a1c.[17] As the yield of *P. shigelloides* CNCTC 110/92 LPS recovered from the water phase was low, O-specific polysaccharides were isolated from both phenol and water phase LPSs and were investigated by chemical analysis, mass spectrometry and ^{1}H, ^{13}C NMR spectroscopy. The elucidated structures of the O-specific polysaccharide were compared with those in the isolated LPSs and their original forms directly on the surface of bacterial cells, using the high-resolution magic angle spinning NMR technique (HR-MAS NMR).

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2. Results

2.1. Isolation of lipopolysaccharides and O-specific polysaccharides

The lipopolysaccharide of *P. shigelloides* O51 (strain CNCTC 110/92) was isolated by phenol/water extraction and was purified as previously reported.[13] Both phenol and water phases were collected. Yields of LPS_{PhOH} and LPS_{H_2O} were 1.7% and 0.2% of the dry bacterial mass, respectively.

The SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed the smooth character of both LPS_{PhOH} and LPS_{H_2O}, with a pattern indicating different degrees of polymerisation of the O-antigen (Fig. 1). LPS_{PhOH} had a higher number of repeating units of the O-specific polysaccharide compared to that of LPS_{H_2O} (Fig. 1). The O-specific polysaccharides (PS_{PhOH}, PS_{H_2O}) and different oligosaccharide components were released by mild acid hydrolysis of LPS_{PhOH} and LPS_{H_2O} and separated by gel filtration on Bio-Gel P10. The isolated oligosaccharide fractions were identified as the core oligosaccharide and core oligosaccharides substituted with one and two O-repeating units, on the basis of MALDI-TOFMS analyses (data not shown). The average yields calculated for different batches of PS_{PhOH} and PS_{H_2O} were 23% and 20%, respectively. However, the ratios of the O-specific polysaccharide fractions to all released oligosaccharides were 2.7 for PS_{PhOH} and 1.4 for PS_{H_2O}, suggesting the preference of LPS with a higher degree of polymerisation within its O-antigen for phenol phase.

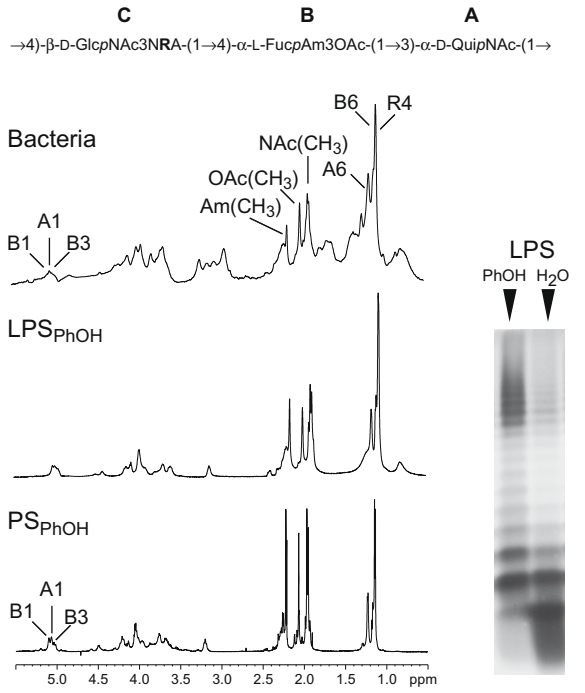

Figure 1. SDS–PAGE analysis of *P. shigelloides* O51 lipopolysaccharides and NMR spectra of the O-antigen of *P. shigelloides* O51 in situ directly on bacteria, in LPS_{PhOH} and as isolated polysaccharide (PS_{PhOH}). Structure representation of the O-PS repeating unit is presented as inset and **R** stands for D-3-hydroxybutyric acid. ^1H NMR spectra (^2H_2O suspensions, 600 MHz) of intact bacteria and LPS were acquired with the HR-MAS technique. The uppercase letters in the anomeric region refer to carbohydrate residues. LPS isolated from the phenol phase and water phase was analysed by SDS–PAGE (5 μg/lane), using a 15% separating gel, and was visualised by the silver staining method.

2.2. Chemical analysis of the O-specific polysaccharides

Sugar analysis of PS_{PhOH} and PS_{H_2O} combined with the determination of the absolute configuration demonstrated the presence of 2-amino-2,6-dideoxy-D-glucopyranose (D-Qui*p*N) and 2-amino-2,6-dideoxy-L-galactopyranose (L-Fuc*p*N). Methylation analysis indicated the presence of 3-substituted D-Qui*p*N and 4-substituted L-Fuc*p*N. Finally, no other component was identified by sugar and methylation analyses. However, NMR spectral data suggested the presence of a trisaccharide repeating unit in PS_{PhOH} (Figs. 1 and 2) and PS_{H_2O}, indicating that 2,3-diamino-2,3-dideoxy-glucuronic acid (Glc*p*N3NA) is the third constituent of the O-repeating unit. This residue was also identified among trimethylsilylated 2-butyl glycosides of the polysaccharide constituents. The absolute configuration analysis of the carboxyl-reduced PS_{PhOH}, with the use of synthetic standards of different diaminohexoses, revealed the presence of D-Glc*p*N3NA.

2.3. NMR analysis of O-specific polysaccharides

Polysaccharides PS_{PhOH} and PS_{H_2O} were analysed using 1D and 2D ^1H, ^{13}C NMR experiments. All the spin systems (Table 1) were

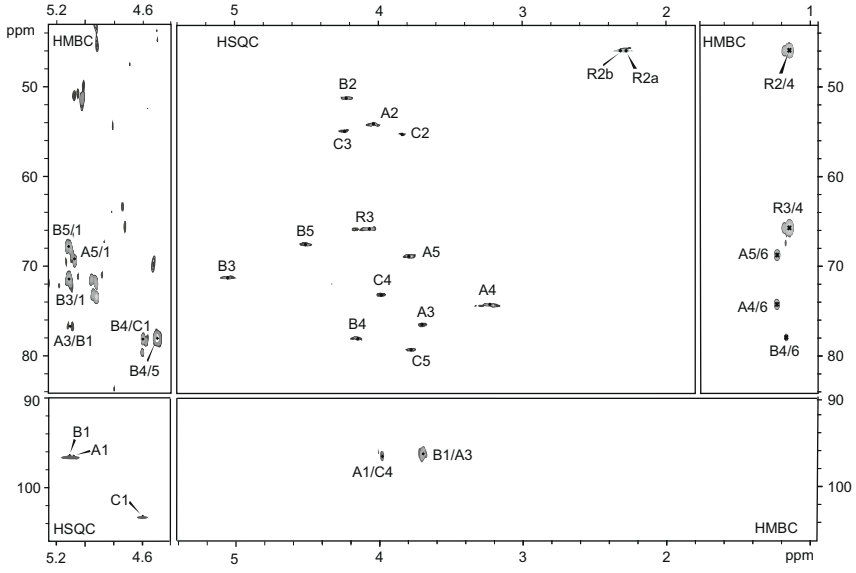

Figure 2. Selected parts of the ^1H, ^{13}C HSQC-DEPT and HMBC spectra of the O-specific polysaccharide of *P. shigelloides* O51 (strain CNCTC 110/92) obtained from LPS_{PhOH}. The cross-peaks are labelled as shown in Figure 1 (inset structure). Unassigned signals originate from the spin systems of core oligosaccharide residues.

Table 1
^{1}H and ^{13}C NMR chemical shifts of the O-specific polysaccharides (PS_{PhOH}, PS_{H_2O}, $PS_{O\text{-}deAc}$) isolated from phenol and water phases *P. shigelloides* O51 (strain CNCTC 110/92) LPSs

Residue		Chemical shifts (ppm)							
		H-1/C-1	H-2/C-2 (H2a, H2b/C-2)	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	NAc (OAc) [Am[a]]	C=O [C=N]
A →3)-α-D-Qui*p*NAc-(1→	$\mathbf{A}_{PhOH}$	5.08	4.03	3.69	3.22	3.78	1.24	1.96/22.9	174.2
$^{1}J_{C\text{-}1,H\text{-}1}$ 176 Hz		96.5	54.0	76.5	74.2	68.8	17.4		
	$\mathbf{A}_{H_2O}$	5.08	4.03	3.69	3.22	3.77	1.24	1.96/23.0	174.4
		96.3	54.0	76.3	74.1	68.6	17.4		
	$\mathbf{A}_{O\text{-}deAc}$	5.08	4.00	3.60	3.18	3.65	1.24	2.00[c]/22.8	174.6
		96.4	53.7	75.8	74.1	68.7	17.1		
B →4)-α-L-Fuc*p*Am3OAc-(1→	$\mathbf{B}_{PhOH}$	5.11	4.22	5.05	4.14	4.50	1.17	(2.08/21.0)	173.9
$^{1}J_{C\text{-}1,H\text{-}1}$ 173 Hz		96.4	51.1	71.2	78.0	67.4	15.9	[2.24/19.7]	[166.8]
	$\mathbf{B}_{H_2O}$	5.10	4.21	5.04	4.15	4.51	1.17	(2.08/21.0)	174.0
		96.2	50.9	71.0	77.9	67.4	16.0	[2.24/19.7]	[166.9]
	$\mathbf{B}_{O\text{-}deAc}$	4.90	4.07	3.82	3.95	4.45	1.16		
		97.8	50.5	67.7	81.2	67.3	15.8	2.00[c]/22.8[b]	174.6[b]
C →4)-β-D-Glc*p*NAc3NRA-(1→	$\mathbf{C}_{PhOH}$	4.60	3.84	4.24	3.98	3.76	—	1.98/23.0	175.2
$J_{C\text{-}1,H\text{-}1}$ 163 Hz		103.2	55.0	54.8	73.1	79.2	175.0		
	$\mathbf{C}_{H_2O}$	4.59	3.87	4.23	3.98	3.76	—	1.98/23.0	175.4
		103.1	54.8	54.7	73.1	79.1	—		
	$\mathbf{C}_{O\text{-}deAc}$	4.77	3.84	4.32	3.99	4.05	—	2.00[c]/22.8	174.6
		102.0	55.0	54.4	72.4	76.6	173.3		
R D-3-Hydroxybutyric acid	$\mathbf{R}_{PhOH}$	—	(2.27, 2.29)	4.06	1.15				
		174.0	45.8	65.7	22.8				
	$\mathbf{R}_{H_2O}$	—	(2.27, 2.29)	4.06	1.15				
		174.0	45.8	65.6	22.7				
	$\mathbf{R}_{O\text{-}deAc}$	—	(2.27, 2.31)	4.07	1.16				
		173.6	45.4	65.4	22.5				

Spectra were recorded for $^{2}H_2O$ solutions at 30 °C. Acetone (2.225, 31.05 ppm) was used as internal reference. The $J_{C\text{-}1,H\text{-}1}$ values obtained from a non-decoupled HSQC experiment confirmed the α-pyranosyl configuration for residues **A** and **B**, and β-pyranosyl configuration for residue **C**.
[a] Am—acetamidino group.
[b] Conversion of an *N*-acetamidino group to an *N*-acetyl group due to alkaline hydrolysis.[24]
[c] Average shift, the signals were not resolved.

assigned using COSY, TOCSY with different mixing times, HSQC-DEPT (Fig. 2), HSQC-TOCSY and HMBC spectra (Fig. 2). Chemical shift values were compared with previously published NMR data for respective monosaccharides.[18–20] Anomeric regions of HSQC-DEPT spectra obtained for PS_{PhOH} and PS_{H_2O} contained three signals (Figs. 1 and 2, Table 1). In addition signals characteristic for methyl groups of deoxyhexoses, *O*-acetyl, *N*-acetyl and a spin system, comprising methylene, hydroxymethylene and methyl groups were also identified. The spectra indicated a trisaccharide structure for the O-repeating unit. The chemical shift values were virtually identical for PS_{PhOH} and PS_{H_2O} (Table 1) and the data for the PS_{PhOH} were used for the detailed description of the identified residues. The sugar residues of the trisaccharide are indicated by capital letters throughout the entire text, tables and figures (Fig. 1, inset structure).

Residue **A** with the H-1/C-1 signals at 5.08/96.5 ppm, $J_{C\text{-}1,H\text{-}1} \sim 176$ Hz was recognised as a 3-substituted α-D-Qui*p*NAc residue based on the chemical shift of the C-2 signal (54.0 ppm), the downfield shift of the C-3 signal (76.5 ppm) as compared with the corresponding non-substituted deoxypyranose[21] and the signal for an exocyclic CH_3 group (1.24 ppm, 17.4 ppm).

Residue **B** with H-1/C-1 signals at 5.11/96.4 ppm, $J_{C\text{-}1,H\text{-}1} \sim 173$ Hz was assigned as a 4-substituted α-L-Fuc*p*Am3OAc residue based on the characteristic signal of the exocyclic CH_3 group (1.17 ppm and 15.9 ppm), the chemical shift of the C-2 signal (51.1 ppm) and the downfield shift of the C-4 signal (78.0 ppm) as compared with the corresponding non-substituted deoxypyranose.[21] The HMBC spectrum showed connectivities between H-2 of **B** (4.22 ppm), the C=N (166.8 ppm) and CH_3 (2.24 ppm) resonances of the acetamidino group. Additionally, the chemical shift values of the H-3, C-3 resonances (5.05 ppm, 71.2 ppm) showed a downfield shift characteristic for the O-acetylation at C-3 (Fig. 2). The substitution at C-3 by an *O*-acetyl group was further supported by the two- and three-bond heteronuclear connectivities, observed as cross-peaks in the HMBC spectra.

Residue **C** with H-1, C-1 at 4.60/103.2 ppm, $J_{H\text{-}1,H\text{-}2} \sim 7.8$ Hz; $J_{C\text{-}1,H\text{-}1} \sim 163$ Hz was identified as a 4-substituted β-D-Glc*p*NAc3NRA due to the characteristic signals of C-2 (55.0 ppm) and C-3 (54.8 ppm), C-6 (175.0 ppm) and the downfield shift of the C-4 signal (73.1 ppm) as compared with the corresponding non-substituted glucosamine.[22] The chemical shift values of residue **C** were similar to those previously reported for 4-substituted β-D-Glc*p*NAc3NAcA.[20,23]

As several signals, including anomeric and carbonyl resonances, were not clearly resolved, PS_{PhOH} was O-deacetylated ($PS_{O\text{-}deAc}$) and analysed by NMR spectroscopy to simplify the assignments which confirmed the elucidated structure (Table 1). The observed conversion of the *N*-acetamidino group of residue →4)-α-L-Fuc*p*Am to a *N*-acetyl group during alkaline hydrolysis (O-deacetylation procedure) was reported previously by Knirel et al.[24]

A non-sugar component **R** was recognised as a D-3-hydroxybutyryl group based on characteristic signals for the CH_3 group (1.15 and 22.8 ppm), CH_2 group (2.27, 2.29 and 45.8 ppm) and the presence of a carbonyl group in this spin system (174.0 ppm) (Table 1). The absolute configuration of the 3-hydroxybutyryl group was determined enzymatically according to Kenne et al.[25] D-3-Hydroxybutyric acid was identified in PS_{PhOH}, PS_{H_2O}, but also in $PS_{O\text{-}deAc}$, suggesting that it is amide linked.

Since substitution of all amino groups in the O-repeating unit by acetamidino group, acetyl groups and D-3-hydroxybutyric acid led to signal overlaps in HMBC spectra (data not shown), the location of the D-3-hydroxybutyric acid (**R**) was established using the set of 1D selective-NOESY (Fig. 3b) and 2D NOESY experiments (data not shown). NOE connectivities observed for exchangeable protons of amide groups were investigated using PS_{H_2O} in a solution of 95% H_2O/5% $^{2}H_2O$ at low temperature (10 °C). The ^{1}H NMR spectrum (Fig. 3a) showed resonances for NH protons, and these were assigned by COSY and TOCSY experiments (data not shown). The signals at 8.41, 8.44, 8.51, 8.73 ppm were assigned as (N-2)H and (N-3)H of residue **C**, (N-2)H of residue **A** and (N-2)H of residue **B**, respectively (Fig. 3a) since in a COSY spectrum,

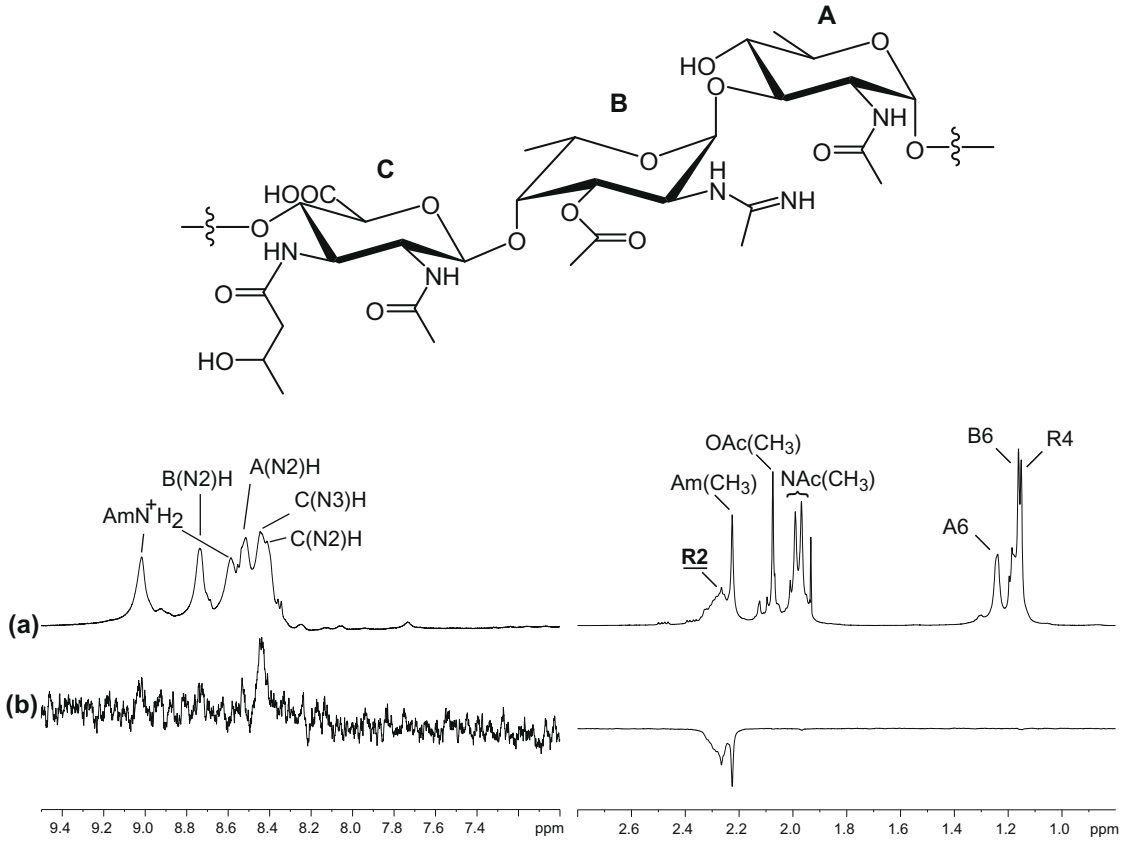

Figure 3. Structure of the O-specific polysaccharides (PS_{PhOH} and PS_{H_2O}) isolated from *P. shigelloides* O51 LPSs (strain CNCTC 110/92). (a) 1D ^1H NMR of the PS_{H_2O}. (b) One-dimensional selective-NOESY of **R**-H2 (50 ms) of PS_{H_2O}. NMR experiments were performed in 95% H_2O/5% ^2H_2O at 10 °C. For selective experiment, the excited resonance is underlined. Capital letters refer to carbohydrate residues.

connectivities between all signals for NH protons and the corresponding ring proton (H-2, H-3 of **C**, H-2 of **A**, H-2 of **B**) were found. Besides, two singlets at 9.02 and 8.58 ppm representing the protons of the amidino group were also identified (Fig. 3a). This observation was in agreement with the data expected for the amidino group at acidic pH, as reported previously.[26] 1D selective-NOESY experiments (Fig. 3b) revealed an inter-residue connectivity between **R**H2 and **C**(N-3)H, suggesting that the D-3-hydroxybutyric acid was located at N-3 of the β-D-Glc*p*NAc3NA. Additionally, 2D NOESY spectra showed inter-residue NOE connectivities between **C**(N-2)H and CH_3 protons of the *N*-acetyl group, **C**(N-3)H and **R**H2, **B**(N-2)H and N^+H_2 protons of Am (data not shown).

The sequence of sugar residues in the O-repeating unit was identified by HMBC (Fig. 2) and was confirmed by NOESY experiments, showing inter-residue connectivities between the adjacent monosaccharides (Table 2). It was concluded that the polysaccharides are composed of the trisaccharide repeating units having the structure in Figure 1 (inset structure).

Table 2
Selected inter-residue NOE and $^3J_{H,C}$-connectivities from the anomeric atoms of the fraction of the O-specific polysaccharide (PS_{PhOH}) isolated from phenol phase *P. shigelloides* O51 LPS (strain CNCTC 110/92). Identical types of connectivities were observed also for PS_{H_2O}

Residue	Atom H-1/ C-1 (ppm)	Connectivities to		Inter-residue atom/residue
		δ_C	δ_H	
A →3)-α-D-Quip*N*Ac-(1→	5.08 96.5	—	3.98	H-4 of **C**
B →4)-α-L-Fucp*A*m3OAc-(1→	5.11 96.4	76.5	3.69	C-3, H-3 of **A**
C →4)-β-D-Glc*p*NAc3NRA-(1→	4.60 103.2	78.0	4.14*	C-4, H-4 of **B**

The value marked with an asterisk represents NOE connectivity only.

2.4. Structural analysis of the O-specific polysaccharide by mass spectrometry

ESI MS/MS analysis of the partially hydrolysed PS_{H_2O} was used to confirm the sequence of sugars in the repeating unit and the presence of D-3-hydroxybutyric acid as the substituent **R** of β-D-Glc*p*NAc3NRA. PS_{H_2O} was subjected to partial acid hydrolysis with 48% aq HF. The ESI mass spectrum of the hydrolysed PS (data not shown) showed that the obtained oligosaccharides consisted of one, two and three repeating units, with two major ions $[M-H]^-$ corresponding to the N-acylated and N-acetylated repeating unit substituted with acetamidino group (*m/z* 693.29) and its O-acetylated variant (*m/z* 734.31). The ESI MS/MS spectrum of the ion at *m/z* 693.29 (Fig. 4) indicated a mixture of three trisaccharides with the sequence depending on the type of glycosidic linkage hydrolysed (data not shown). The main fragment ions (B_i, C_i, $^{0,2}A_2$, $^{2,4}A_2$, $^{2,5}A_2$) corresponded to the structure shown in Figure 4, as the α-(1→4) linkage between α-D-Qui*p*NAc and β-D-Glc*p*NAc3NRA appeared to be the most susceptible to the HF treatment.

2.5. HR-MAS NMR analysis of lipopolysaccharides and bacteria

HR-MAS NMR analysis of the intact LPS_{PhOH} and LPS_{H_2O} revealed major resonances of the O-specific polysaccharides, including the characteristic *N*-acetyl, *N*-acetamidino, *O*-acetyl, *N*-hydroxybutyryl and C-6 signals of deoxy sugars, that have been identified for the

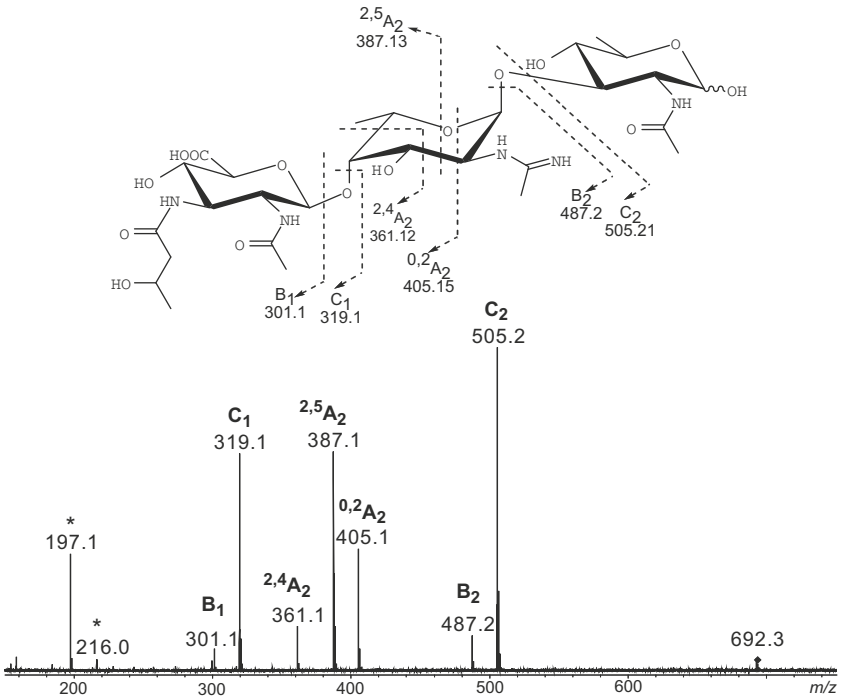

Figure 4. ESI MS/MS spectrum (negative ion mode) of the trisaccharide represented by the ion at *m/z* 693.29 (inset structure) obtained by partial acid hydrolysis of PS_{H_2O}. Interpretation of fragment ions is presented in the inset structure. All fragments, for example, $^{0,4}A_2$ have been described using the nomenclature of Domon and Costello.[53] Ions marked with an asterisk were not interpreted.

isolated PS. These structure reporter groups were then used to verify the O-antigens directly on bacterial cells. The ^{1}H chemical shift values of the O-PS in situ were in agreement with those of the isolated LPS_{PhOH} and LPS_{H_2O}. The O-acetylation pattern was preserved throughout the entire preparation.

3. Discussion

Since Okawa et al.[7] have found LPS as a constituent of a cytotoxin complex from *P. shigelloides,* elucidation of the chemical structures of these poorly characterised molecules became even more important. In our ongoing structural studies of LPSs of *P. shigelloides* (serotypes O54,[12] O74,[14,13] O37,[27] O51), we have stumbled upon features which seem to be characteristic for the LPS of this bacterium, that is, the lack of phosphate groups, the presence of GalA in the core oligosaccharide[11–15] and the unusual hydrophobicity of the O-specific polysaccharides.[14,13,27] Recently, another highly hydrophobic LPS from *P. shigelloides* strain 302-73 (serotype O1) was reported.[9] To date, only a few LPSs of other species were isolated from the phenol phase, for example, *Chromatium vinosum*,[28] *Yersinia enterocolitica* serotype O:9,[29] *Pseudomonas aeruginosa* O3,[24] *Azorhizobium caulinodans* ORS571,[30] *Acinetobacter* spp.,[31,32] *Stentrophomonas maltophilia* O16,[33] *Pseudomonas reactans* strain NCPPB1331,[34] *Xanthomonas campestris* strain 8004,[35] and *Pseudoalteromonas rubra* ATCC 29570,[T] [36] and *Thiobacillus* sp. IFO14570.[23]

Similarly to the O-specific PS of *P. shigelloides* O74,[13] the free hydroxyl groups in the repeating unit of the O-specific polysaccharides isolated from *P. shigelloides* O51 LPS are scarce. The O-specific PS contains aminodideoxyhexoses and diaminodideoxyuronic acids, which are substituted with *N*- and *O*-acetyl groups, an acetamidino group and D-3-hydroxybutyric acid. A rare monosaccharide: 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid N-substituted with D-3-hydroxybutyric acid has been identified among the constituents of the O-specific PS. To the best of our knowledge, it is the first time that this residue, acylated with D-3-hydroxybutyric acid has been found. However, the N-acetylated form of the D-Glc*p*N3NA residue was recognised previously in the structure of O-specific polysaccharides of *P. aeruginosa* type O6,[20,37] *Pseudoalteromonas* sp. KMM 634,[38] and *Thiobacillus* sp. IFO14570.[23] It was also found as a component of *Propionibacterium acnes* cell wall PS[39] and a polysaccharide part of the glycoconjugate from *Treponema medium* ATCC 700293.[40]

As we have demonstrated, the O-specific polysaccharides PS_{H_2O} and PS_{PhOH} of *P. shigelloides* O51 are built up of the same trisaccharide repeating units and differ only in the degree of polymerisation. Similar behaviour of LPS molecules was previously reported for other strains whose LPS was isolated from both phenol and water phases.[30] Therefore composition of the O-repeating units does not seem to be the only factor influencing the physicochemical properties of such LPSs. Haseley et al. suggested that the main solubility factor might be conformational rather than compositional.[32]

Structure determination is the first step into the understanding of the unusual physicochemical properties of such LPSs. The possible role of the LPSs associated with an increased hydrophobicity in the pathogenicity of *P. shigelloides* has not been investigated so far. The data herein presented could be used in the future to study the role of such structures for the type of aggregates formed in an aqueous environment and the biological activity of *P. shigelloides* endotoxin.

4. Experimental

4.1. Bacteria

P. shigelloides serovar O51:H1a1c (strain CNCTC 110/92) was obtained from the Collection of the National Institute of Public Health, Prague, Czech Republic. Bacteria were grown for 48 h and harvested as described previously.[41]

4.2. Lipopolysaccharide and O-specific polysaccharide

The LPS was extracted from bacterial cells by the hot phenol/water method[16] and was purified as previously reported.[13] LPSs from water and phenol phases (LPS_{H_2O}, LPS_{PhOH}) were collected and dialysed extensively against de-ionised water and purified by ultracentrifugation. LPS (200 mg) was degraded by treatment with 1.5% AcOH at 100 °C for 30 min. The reaction mixture was freeze–thaw cycled, and again incubated at 100 °C for 15 min and then centrifuged (40,000*g*, 20 min). The O-specific polysaccharides (average yield: PS_{H_2O}, 41 mg; PS_{PhOH}, 47 mg) were separated from the core oligosaccharides (LPS_{PhOH}: 3.7 mg; LPS_{H_2O}: ~1 mg) and core oligosaccharides substituted with one (LPS_{PhOH}: 13.6 mg; LPS_{H_2O}: 24.5 mg) and two (LPS_{PhOH}: 2.4 mg; LPS_{H_2O}: 4.3 mg) repeating units of the O-antigen by gel permeation chromatography, performed on Bio-Gel P10 column (1.6 × 100 cm) equilibrated with 0.05 M pyridine/AcOH buffer of pH 5.6. Eluates were monitored with a Knauer differential refractometer and all fractions were freeze-dried and checked by ^{1}H NMR spectroscopy and MALDI-TOFMS.

4.3. O-Deacetylation of the polysaccharide

The polysaccharide (PS_{PhOH}, 20 mg) was treated with 4 mL of aqueous 12.5% NH_3 for 16 h at ~22 °C followed by twofold dilution with water and lyophilisation. The sample was analysed by NMR spectroscopy.

4.4. Partial acid hydrolysis

A sample of the polysaccharide (1 mg) was hydrolysed with 48% aq HF (1 mL) at ~22 °C. Progress of hydrolysis was checked by MALDI-TOFMS. The products obtained after 24 h hydrolysis were concentrated to dryness by evaporation and were analysed by ESI MS/MS.

4.5. Analytical methods

The LPS was analysed by SDS–PAGE according to the method of Laemmli[42] with modifications described previously[43] and LPS bands were visualised by the silver staining method.[44] Absolute configuration of the D-3-hydroxybutyric acid was determined enzymatically according to Kenne et al.[25] Monosaccharides were analysed as their alditol acetates by GC–MS.[41] Methylations were performed on native polysaccharides according to the method of Hakomori.[45] Alditol acetates and partially methylated alditol acetates were analysed with a Hewlett-Packard 5972 system using the HP-1 fused-silica capillary column (0.2 mm × 12.5 m) and a temperature program 150→270 °C at 8 °C min^{-1}. Absolute configurations of the monosaccharides were determined as described by Gerwig et al.[46,47] using (−)-2-butanol for the formation of 2-butyl glycosides. The trimethylsilylated butylglycosides produced from authentic samples were used as standards. The absolute configuration of Glc*p*N3NA was determined on the carboxyl-reduced derivative of this constituent. Briefly, uronic acid residues in the native polysaccharide (PS_{PhOH}) were activated with carbodiimide, reduced by $NaBH_4$[48] and subjected to the procedure of Gerwig et al.[46,47] The derivatives were analysed with a Hewlett-Packard 5972 system using HP-5 fused-silica capillary column (0.2 mm × 30 m) and a temperature program 100→190 °C at 5 °C min^{-1} and 190→270 °C at 2 °C min^{-1}.

4.6. NMR spectroscopy

NMR spectra of polysaccharides PS_{H_2O}, PS_{PhOH} were obtained on Bruker 600 MHz spectrometer (Laboratory of Structural Analyses, Wroclaw University of Technology). The polysaccharides were first repeatedly exchanged with ^2H_2O (99%) with intermediate lyophilisation. NMR spectra of the polysaccharides were obtained for ^2H_2O solutions at 30 °C using acetone (2.225 ppm, 31.05 ppm) as internal reference. The signals were assigned by one- and two-dimensional experiments (COSY, clean-TOCSY, NOESY, HMBC, HSQC-DEPT). The $J_{C\text{-}1,H\text{-}1}$ values were obtained from non-decoupled HSQC-DEPT experiments. In the clean-TOCSY experiments, the mixing times were 30, 60 and 100 ms. The delay time in the HMBC was 60 ms and the mixing time in the NOESY experiment was 200 ms.

To observe exchangeable protons of the amide groups in the O-specific PS, the sample of PS_{H_2O} was dissolved in 95% H_2O/5% ^2H_2O and was analysed at 10 °C. 2D NOESY, TOCSY, HSQC-DEPT and 1D selective-NOESY experiments with different mixing times (50–200 ms) were used to support the assignments of spin systems and to determine the substitution position of the D-3-hydroxybutyric acid.

High-resolution magic angle spinning (HR-MAS) ^{1}H NMR analyses of bacterial cells and lipopolysaccharides in ^2H_2O suspensions were performed as previously described.[13,49] Bacteria (~3 mg) and LPS (~2 mg) were placed into the rotor and suspended in ~10 μL ^2H_2O. All HR-MAS NMR experiments were carried out at 5 kHz spin rate at 23 °C with a Bruker 4 mm HR-MAS probe and a ZrO_2 rotor. One-dimensional spectra of bacteria and LPS were acquired with a Carr-Purcell-Meiboom-Gill, CPMG, $90°\text{–}(\tau\text{–}180°\text{–}\tau)_n$-acquisition pulse sequence (total delay time 1.2 ms) as T_2-filter to remove the broad signals from semi-solid bacterial components.[50] All HR-MAS NMR spectra were obtained with acetone (2.225 ppm) as external reference before the actual run.

All spectra were acquired and processed using a standard Bruker software. The processed 2D spectra were assigned with the help of the SPARKY program.[51]

4.7. Mass spectrometry

MALDI-TOFMS spectra (reflectron positive and negative-ion modes) were obtained on a Kratos Kompact-SEQ instrument. Samples were dissolved in water (0.5 mg/mL) and desodiated with Dowex 50 W × 8 (H^+). 2,4,6-Trihydroxyacetophenone (25 mg/mL in 1:1 MeCN–water) was used as the matrix. Angiotensin II and oxidised bovine insulin B chain were used as external or internal standards for calibration.

The MS/MS analysis of partially hydrolysed O-specific polysaccharide in negative ion mode was performed on a micrOTOF-Q spectrometer (Bruker Daltonics, Bremen, Germany). The sample was dissolved in 1:1 MeCN–water and analysed by direct infusion at a rate of 3 μL/min. Spectra were scanned in the range of *m/z* 120–2000. The ion source temperature was 180 °C, the flow rate was set at 4 L/min and the pressure of nitrogen was 0.4 bar. External calibration in the negative-ion mode was applied using the Tune mix (Bruker Daltonics, Germany) in quadratic regression mode and *m/z* range of 113–2234 Da. The isolation width for MS/MS experiments was $\Delta m/z = 10$, and the collision energy of the quadrupole was 25 eV.

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