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Structural analysis of the carbohydrate backbone of *Vibrio parahaemolyticus* O2 lipopolysaccharides

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Abstract

A structural investigation has been carried out on the carbohydrate backbone of *Vibrio parahaemolyticus* O2 lipopolysaccharides (LPS) isolated by dephosphorylation, O-deacylation and N-deacylation. The carbohydrate backbone is a short-chain saccharide consisting of nine monosaccharide units i.e., 1 mol each of D-galactose (Gal), D-glucose (Glc), D-glucuronic acid (GlcA), L-*glycero*-D-*manno*-heptose (L,D-Hep), D-*glycero*-D-*manno*-heptose (D,D-Hep), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (NonlA), and 2 mol of 2-amino-2-deoxy-D-glucose (D-glucosamine, GlcN). Based on the data obtained by NMR spectroscopy, fast-atom bombardment mass spectrometry (FABMS) and methylation analysis, a structure was elucidated for the carbohydrate backbone of O2 LPS. In the native O2 LPS, the 2-amino-2-deoxy-D-glucitol (GlcN-ol) at the reducing end of the nonasaccharide is present as GlcN. The lipid A backbone is a β-D-GlcN-(1→6)-D-GlcN disaccharide as is the case for many Gram-negative bacterial LPS. The lipid A proximal Kdo is substituted by the distal part of the carbohydrate chain at position-5. In the native O2 LPS, D-galacturonic acid, which is liberated from LPS by mild acid treatment or by dephosphorylation in hydrofluoric acid, is present although its binding position is unknown at present.

α-D-Glc*p*A-(1→2)-L-α-D-Hep*p*-(1→3)-D-α-D-Hep*p*-(1→5)-α-Kdo*p*-(2→6)-β-D-Glc*p*N-(1→6)-D-GlcN-ol
↑4
↑1
α-Nonl*p*A-(2→6)-β-D-Glc*p*
↑4
↑1
β-D-Gal*p*

Keywords: Vibrio parahaemolyticus; Non-2-ulosonic acid; Lipopolysaccharides

1. Introduction

Vibrio parahaemolyticus is a halophilic marine bacterium that is responsible for food poisoning primarily associated with sea products. *V. parahaemolyticus* is presently divided into 11 or 13 O-serotypes[1,2] based on differences of its heat-stable somatic antigen that are lipopolysaccharides (LPS) located on the outer leaflet of the cell wall outer membrane. All serotypes of *V. parahaemolyticus* produce low-molecular-weight LPSs[3] which correspond to rough (R)-type LPS and lack the O-specific polysaccharides. Despite the absence of O-specific polysaccharides from LPS, *V. parahaemolyticus* still harbors serological O-specificity as observed in cell agglutination and agglutinin absorption tests. Therefore, in the case of *V. parahaemolyticus* LPS, structural

Abbreviations: NonlA, 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid; LPS, lipopolysaccharides; HF, hydrofluoric acid; HF-LPS, dephosphorylated LPS.

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variation of the oligosaccharide portion corresponding to the core region of the usual smooth (S)-type LPS might be responsible for the serological O-specificity of the parental strains as is the case for non-enteric mucosal pathogens.[4]

The chemical compositions of *V. parahaemolyticus* LPSs have been elucidated,[3,5–7] and based on their sugar content, they have been classified into ten chemotypes. In spite of these investigations, and in contrast to the LPSs of other Gram-negative bacteria, the chemical structure of the carbohydrate portion of *V. parahaemolyticus* LPS has only been elucidated for one (O12)[8] out of 13 serotypes. In this paper we describe a structural investigation of the carbohydrate backbone isolated from LPS of *V. parahaemolyticus* O2.

2. Results

2.1. Chemical analysis of LPS and chemically modified LPS

Table 1 shows the chemical composition of LPS and deacylated, dephosphorylated LPS (HF-LPS). The native LPS contained D-glucose (Glc), D-galactose (Gal), L-*glycero*-D-*manno*-heptose (L,D-Hep), D-*glycero*-D-*manno*-heptose (D,D-Hep), 2-amino-2-deoxy-D-glucose (D-glucosamine, GlcN), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and non-2-ulosonic acid (NonlA) as sugar components. In addition to the sugar components, the LPS contained fatty acids comprising the lipid A moiety and relatively large amounts of phosphate. The NonlA was previously identified as 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glyccero*-D-*galacto*-non-2-ulosonic acid.[9]

After O-deacylation of the LPS by treatment with anhydrous hydrazine, the product (LPS_{OH}) was subjected to matrix-assisted laser/desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. In the negative-ion mode spectrum (Fig. 1), the major ion peak at m/z 2698.8 corresponds to the calculated molecular weight (2700.3 Da) of the following nonasaccharide carrying five phosphate groups, two ethanolamines (EA) and 2 mol of fatty acid: Hex_2/Hep_2/HexA/$HexN_2$/Kdo/NonlANAc/P_5/EA_2/3-OH-C14:O_2. The ion peak at m/z 2874.8 is 176 Da larger than the former ion, indicating that this compound contains an additional HexA unit. In addition to these ions, several ion peaks were observed, which corresponded to the molecular mass originated by lose of phosphate and/or EA groups from the respective molecules described above.

For the deacylated HF-LPS, with the exception of GalA, all component sugars detected in the LPS were retained, while most of the fatty acids and phosphate groups were removed. As previously reported,[9] the carbohydrate portion of the LPS obtained after mild acid hydrolysis did not contain GlcN, indicating that this is a component of the lipid A moiety rather than the carbohydrate portion of the LPS.

Table 1
Sugar composition of LPS and dephosphorylated, reduced and deacylated LPS (deacylated HF-LPSred) obtained from *V. parahaemolyticus* O2 LPS

Component sugar	LPS [a]	Deacylated HF-LPSred [a]
Glucose	214	578
Galactose	144	428
L-*glycero*-D-*manno*-heptose	18	89
D-*glycero*-D-*manno*-heptose	13	123
Glucosamine	88	608
Uronic acid[b]	359	559
Glucuronic acid	+	+
Galacturonic acid	+	−
Kdo[c]	+	+
NonlA[d]	+	+
Phosphate	1329	28
3-OH-C12:0	72	−
$C_{14:0}$	133	−
3-OH-$C_{14:0}$	155	−
$C_{16:0}$	127	−

[a] Values are expressed as nmole/mg of samples.

[b] Uronic acid was estimated by a colorimetric method, and the presence of glucuronic and galacturonic acid were confirmed by GC and GC–MS after reduction of their carboxyl groups.

[c] 3-Deoxy-D-*manno*-oct-2-ulosonic acid, Kdo was not detected by the periodate–thiobarbituric acid reaction, but its presence was confirmed by GC and GC–MS.

[d] 5,7-Diamino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid.

2.2. FABMS spectrometry of the carbohydrate backbone isolated from O2 LPS

A partial purification of deacylated, reduced HF-LPS (HF-LPSred) by DEAE Sephacel chromatography yielded two fractions: a major fraction that eluted at 157 mM NaCl and a minor fraction that eluted at 250 mM NaCl. The major fraction was analyzed by fast-atom bombardment mass spectrometry (FABMS). In the mass spectrum (positive-ion mode, Fig. 2), two major ion peaks were detected. The ion peak at m/z 1679.6 corresponded to the calculated molecular weight (1679.5 Da) of a nonasaccharide (PS1) consisting of 1 mol each of 2-amino-2-deoxy-D-glucitol (GlcN-ol),

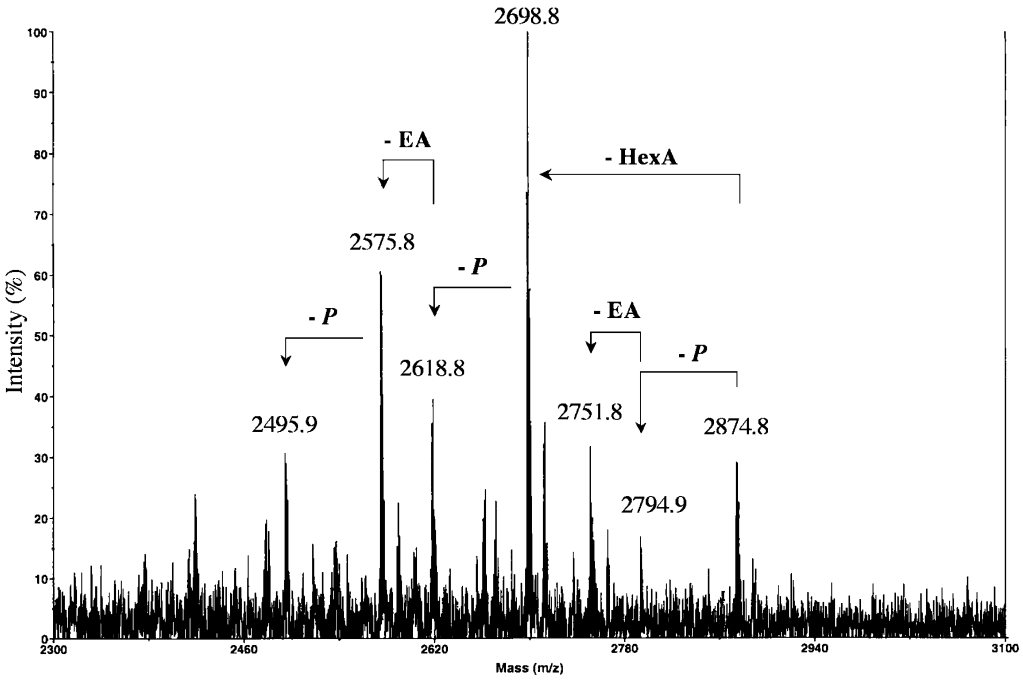

Fig. 1. MALDI-TOF mass spectrum (negative-ion mode) of O-deacylated LPS (LPS_{OH}) from *V. parahaemolyticus* O2. The main ion peak at m/z 2698.8 corresponds to the calculated molecular weight (2700.3 Da) of a nonasaccharide, $Hex_2/Hep_2/HexA/HexN_2/Kdo/NonlANAc$, containing 5 mol of phosphate, 2 mol of EA and 2 mol of 3-OH-C14:0.

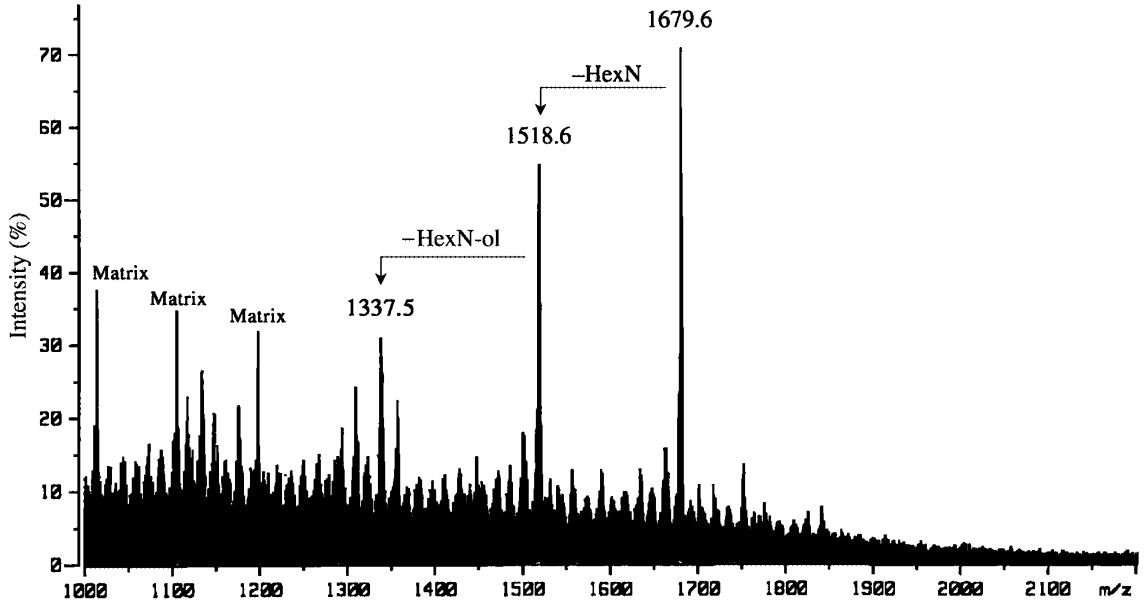

Fig. 2. FAB mass spectrum (positive-ion mode) of dephosphorylated, reduced and deacylated LPS (PS1) from *V. parahaemolyticus* O2. The main ion peak at m/z 1679.6 corresponds to the calculated molecular weight of PS1.

GlcN, Kdo, L,D-Hep, D,D-Hep, Glc, Gal, GlcA and NonlA. Another ion peak at m/z 1518.6 was 161 Da less than the calculated molecular weight of PS1, indicating that this carbohydrate chain is an octasaccharide (PS2) in which GlcN had been removed from PS1. The FABMS spectrum (data not shown) of the minor fraction revealed the presence of major ion peaks at m/z 1759.7 and m/z 1598.7 corresponding to phosphorylated PS1 and PS2, respectively. Since the minor fraction may contain carbohydrate backbones that were not fully dephosphorylated, this fraction was not subjected to further analysis. Purification of the major fraction by high-performance anion-exchange chromatography (HPAEC) yielded two main fractions, PS1 and PS2. The weight ratio of PS1 to PS2 was approx 5:1. PS1 was mainly used for further experiments.

Table 2
Methylation analysis of carbohydrate backbone (PS1) of *V. parahaemolyticus* O2 LPS

Derivatives	Molar ratio [a]	Derived from
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol	0.54	*t*-Gal*p*
1,5-di-*O*-acetyl-6-carboxy-methyl-2,3,4-tri-*O*-methyl-glucitol	0.65	*t*-Glc*p*A
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	0.18	1,4-Glc*p*
1,5,6-tri-*O*-acetyl-2-methyl-acetamido-2-deoxy-3,4-di-*O*-methylglucitol	0.82	1,6-Glc*p*N
1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol	1.37	1,4,6-Glc*p*
1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol	0.60	1,2-L,D-Hep*p*
1,3,4,5-tetra-*O*-acetyl-2,6,7-tri-*O*-methyl-D-*glycero*-D-*manno*-heptitol	1.00	1,3,4-D,D-Hep*p*
6-*O*-acetyl-2-methylaceta-mido-2-deoxy-1,3,4,5-tetra-*O*-methylglucitol	0.73	6-GlcN-ol

[a] Molar ratios were expressed in the relative amounts to 1,3,4,5-tetra-*O*-acetyl-2,6,7-tri-*O*-methyl-D-*glycero*-D-*manno*-heptitol (1.00 mol).

2.3. Methylation analysis of PS1

The results of methylation analysis (Table 2) of PS1 revealed the presence of the following pyranosidic sugar residues: the nonreducing terminus of Gal and GlcA, 6-substituted GlcN, 4,6-substituted Glc, 2-substituted L,D-Hep and 3,4-substituted D,D-Hep. In addition to the derivatives originated from these residues, the derivatives from the 6-subsituted GlcN-ol and 4-substituted Glc residues were also determined. The latter may be an artifact from the 4,6-substituted Glc residue by release during the methylation reaction of a substituent that is possibly NonlA at position-6. Derivatives of Kdo and NonlA were not detectable by standard methylation analysis since they are unstable in acid hydrolysis.

2.4. Structural analysis of the carbohydrate backbone of O2 LPS

The ^{1}H and ^{13}C NMR spectra were assigned by using $^{1}H,^{1}H$-COSY, TOCSY, NOESY, HMQC and HMBC. PS1 was confirmed to be a nonasaccharide (Fig. 3) by NMR spectroscopy. In the ^{1}H NMR spectrum of PS1 (Fig. 4 and Table 3) , the signals of six anomeric protons were displayed around δ_H 4.3–5.4, and the COSY spectrum of PS1 clearly demonstrated the presence of six pyranosidic ring systems. On the basis of vicinal coupling constant values of the ring protons, the signals were assigned to H-1 for Hep (I), GlcA, Hep (II), Gal, Glc and GlcN in order of decreasing chemical shift value. The large $J_{H1,H2}$ values (7.3–8.2 Hz) indicated that Gal, Glc and GlcN residues were in the β-configuration. In contrast, the GlcA residue was shown to be in the α-configuration on the basis of a small $J_{H1,H2}$ coupling constant value. ^{13}C NMR experiments (Table 4) clearly demonstrated that two heptose residues have an α-configuration that was defined by $J_{C1,H1}$ coupling constant values (163–164 Hz). The α-configuration of the Kdo was indicated based on the chemical shift value for H-3 (equatorial) and the $J_{H7,H8\text{-}a}$

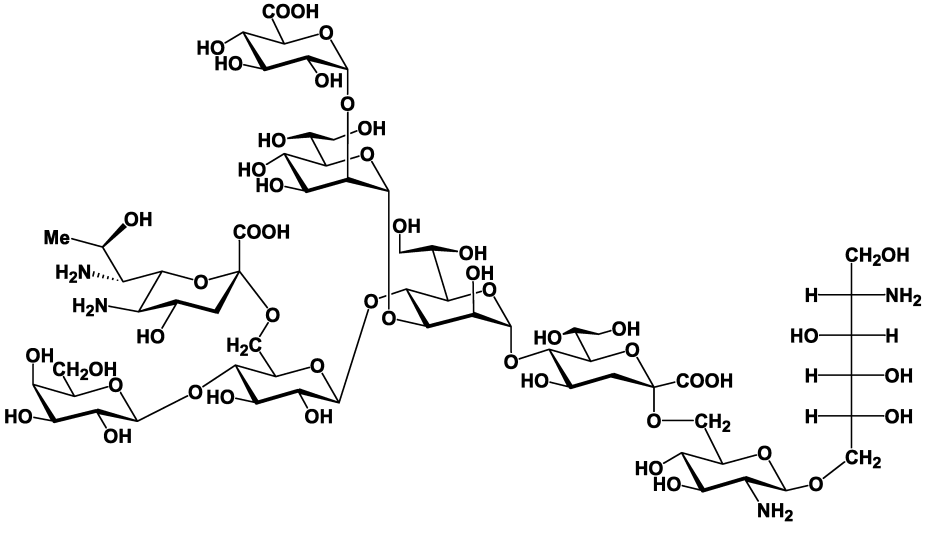

Fig. 3. Proposed structure of the carbohydrate backbone of *V. parahaemolyticus* O2 LPS. In the LPS molecule, the 2-amino-2-deoxy-D-glucitiol (GlcN-ol) at the reducing terminus is present as 2-amino-2-deoxy-D-glucose (glucosamine, GlcN).

Table 3
^{1}H NMR data of the carbohydrate backbone (PS1) prepared by dephosphorylation and deacylation from *V. parahaemolyticus* O2 LPS

Chemical shifts of the component sugars ppm ($J_{n,n+1}$)									
Residue proton	Gal	Glc	GlcA	L,D-Hep	D,D-Hep	Kdo	NonlA	GlcN	GlcN-ol
H-1a	4.61 (7.3)	4.42 (7.3)	5.09 (3.7)	5.48 (<1)	5.01 (1.4)			4.48 (8.2)	3.77 (6.1; 11.3)
H-1b									3.88
H2	3.55 (10.1)	3.42 (9.2)	3.60 (9.2)	4.20 (2.8)	4.10 (3.4)			2.76 (9.8)	3.52 (6.1)
H-3 (axial)	3.64 (3.7)	3.55 (10.1)	3.75 (9.6)	4.03 (9.6)	4.13 (10.1)	1.91 (11.9; 12.2)	1.66 (11.9; 12.3)	3.42 (9.2)	4.10 (2.7)
H-3 (equatorial)						2.15 (5.2)	2.86 (4.7)		
H-4	3.91 (<1)	3.77 (10.1)	3.44 (10.6)	3.99 (10.1)	4.13 (10.5)	4.14 (3.7)	3.53 (10.3)	3.45 (9.6)	3.67 (7.3)
H-5	3.66	3.60	4.14	3.65 (3.7)	4.13 (3.7)	4.15 (10.4)	2.96 (10.4)	3.60	3.96
H-6a	3.66 (11.5)	3.61 (2.4; 12.4)		4.04	4.10	3.72 (8.5)	3.74 (2.1)	3.55 (11.4)	3.82 (6.4; 11.6)
H-6b	3.82	3.95 (5.8)						3.65	4.14 (2.7)
H-7a				3.69 (7.0; 11.5)	3.72 (12.0)	3.86	3.14 (8.5)		
H-7b				3.81 (4.1)	3.84 (4.6)				
H-8a						3.65 (6.4; 12.5)	4.04 (6.7)		
H-8b						3.96 (3.7)			
H-9							1.34		

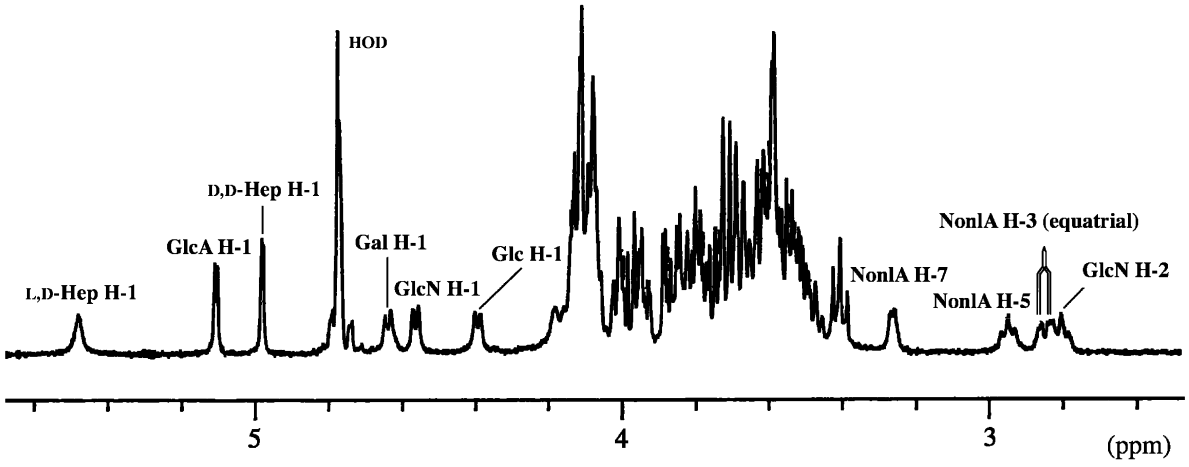

Fig. 4. ^{1}H NMR spectrum (600 MHz) of the carbohydrate backbone (PS1) isolated from *V. parahaemolyticus* O2 LPS. The spectrum was recorded for the sample (PS1) solution in D_2O (8.3 μg/μL) at pD 6.9, 30 °C.

Table 4
^{13}C NMR data of the carbohydrate backbone (PS1) prepared by dephosphorylation and deacylation from *V. parahaemolyticus* O2 LPS

Chemical shifts of the component sugars ppm ($J_{C1,H1}$)									
Residue carbon	Gal	Glc	GlcA	L,D-Hep	D,D-Hep	Kdo	NonlA	GlcN	GlcN-ol
C-1	102.1 (163)	102.5 (164)	100.7 (171)	99.8 (173)	101.1 (174)	174.4	170.9	102.6 (163)	59.0
C-2	71.3	73.7	74.4	79.6	71.1	99.8	101.1	56.3	55.4
C-3	72.8	74.2	73.2	71.8	74.3	35.1	41.0	73.6	66.1
C-4	68.9	76.1	72.8	67.6	75.8	66.1	70.0	70.6	71.1
C-5	73.6	74.9	72.5	71.0	72.6	75.8	53.5	74.9	70.5
C-6	63.6	71.1	172.3	68.8	69.3	73.1	73.0	62.3	72.1
C-7				61.5	61.4	70.1	55.5		
C-8						63.3	68.8		
C-9							18.4		

(6.4 Hz) and $J_{H7,H8\text{-}b}$ (3.7 Hz), which were characteristic for Kdo of the α-configuration.[10] The chemical shift (δ_H 2.86) for the H-3 (equatorial) of the NonlA residue was shifted downfield by 0.58 ppm compared with that for a purified β-configuration monomer. Thus the NonlA residue possessed the α-configuration.[9,11]

Characteristic correlation peaks were observed for Kdo and NonlA between their H-3 (axial) and carbonyl groups (δ_c 174.4 and 170.9), respectively. A similar correlation peak was also observed for GlcA between its H-5 proton and carbonyl group (δ_c 172.3). By the two-dimensional NOESY spectrum (Table 5), important interresidual NOE effects were observed between the H-3 (axial) of Kdo and the H-6a of GlcN, H-1 of GlcN and H-6a,b of GlcN-ol, respectively. These NOE effects indicate the presence of a partial structure α-Kdo-(1→6)-β-GlcN-(1→6)-GlcN-ol at the reducing end site of PS1. This was further supported by the long-range coupling between GlcN H-1 and GlcN-ol C-6 observed in the HMBC spectrum of PS1. The ^{13}C NMR data of GlcN and GlcN-ol were very similar to those previously reported for β-D-GlcNAc-(1→6)-D-GlcNAc-o1 disaccharide.[12] A correlation peak was observed between H-3 (equatrial) of the NonlA residue and H-6b of Glc. In addition, the Glc residue was substituted with β-Gal at position-4 as shown by the correlation peak between H-1 of β-Gal and H-4 of β-Glc in the NOESY spectrum, indicating the presence of a branched structure β-Gal-(1→4)-[α-NonlA-(1→6)]-β-Glc-(1→. Previously, Kondo and co-workers[13] reported the presence of the trisaccharide α-GlcA-(1→2)-L-α-D-Hep-(1→3)-D,D-Hep as a partial structure in the LPS isolated from *V. parahaemolyticus* O2. A trisaccharide part consisting of GlcA and two Heps, was also confirmed in a sequential analysis carried out using NOESY and HMBC experiments on PS1. For the Hep (I) residue a strong interresidual NOE was observed between H-1 of Hep (I) and H-3 of

Hep (II), and also between H-2 of Hep (I) and H-1 of α-GlcA residues. Thus the Hep (I) and (II) were assigned to L-α-D-Hep and D-α-D-Hep, respectively. For β-Glc and D-α-D-Hep residues, interresidual NOEs were observed between H-1 of β-Glc and H-4 of D-α-D-Hep, and H-1 of D-α-D-Hep and H-5 of α-Kdo residues, respectively. The linkage sites of the sugar residues displayed by NOESY and HMBC experiments were in good agreement to the results obtained by methylation analysis of PS1. From these results we propose the structure shown in Fig. 3 for the carbohydrate backbone of *V. parahaemolyticus* O2 LPS, although in the native O2 LPS the GlcN-ol at the reducing terminus is present as GlcN.

The ^{1}H and ^{13}C NMR spectra (data not shown) for PS2 were quite similar to those for PS1. However, in both spectra, signals that originate from the GlcN residue were absent. These data, combined with those obtained by FABMS, suggest that PS2 might be an artifact produced by cleavage of the β-(1→6) linkage of the lipid A backbone, possibly during the HF treatment of O2 LPS.

3. Discussion

It has been demonstrated[3] by means of SDS-PAGE and compositional sugar analysis of their carbohydrate moieties that LPSs from all serotypes of *V. parahaemolyticus* are composed of low-molecular-weight carbohydrate chains. In the case of serotype O12 LPS,[8] the carbohydrate backbone has been characterized as a decasaccharide. In this study, the carbohydrate backbone of *V. parahaemolyticus* O2 LPS was shown to be a short chain consisting of nine monosaccharide units. The lipid A-Kdo region of both O2 and O12 LPS have the same structure. Similarly, the lipid A backbone of both LPS comprised a β-(1→6)-disaccharide of GlcN as is the case for many Gram-negative bacterial LPSs. In the Kdo region, only 1 mol of Kdo is present, which has a phosphate group[6] and a carbohydrate chain at positions-4 and -5, respectively. This structural feature seems to be characteristic for the LPS of not only *V. parahaemolyticus* but also several species belonging to the family *Vibrionaceae*.[6] In contrast, the distal part of the carbohydrate backbone of O2 LPS binding to Kdo is quite different from that of the carbohydrate backbone of O12 LPS. O2 LPS contains 1 mol each of L,D-Hep and D,D-Hep. Out of the 13 O-serotype LPSs,[5,7] D,D-Hep is unique to O2 LPS. D,D-Hep is bound at position-5 of the Kdo residue in the carbohydrate backbone of O2 LPS, whereas L,D-Hep is present at the equivalent position in the carbohydrate backbone of O12 LPS. Thus, O2 LPS has a different structure from that of O12 and other O-serotype LPS even in the inner core region. The chemical structure of the O-antigenic polysaccharide portion of most Gram-negative bacteria (e.g., enterobacteria) vary widely depending on their O-serotypes, and these structural variations contribute to their serological O-specificities. In contrast, the core regions are structurally highly conserved, which does not correlate with the observed serological O-specificities. A genomic or phenotypic mutation causing deletion of the O-antigenic polysaccharide portion of LPS results in conversion of the bacteria from the S-form to the R-form. In this case, the serological specificity of the parental strain disappears since the carbohydrate backbone of the LPS of R-mutants has a chemical structure that is commonly shared by R-mutants that originate from different O-serotypes. The LPSs of *V. parahaemolyticus* are chemically considered as R-type LPSs, yet they show clear serological O-specificities. The carbohydrate portions of *V. parahaemolyticus* LPS, which correspond to the core oligosaccharide of a standard LPS, are structurally different depending on their O-serotypes. Thus these sugars may play a role as O-antigens in expressing serological specificities of the parental vibrios.

In the native O2 LPS, D-galacturonic acid, which was readily released by treatment of the LPS with mild acid or HF, was found in addition to the component sugars of the carbohydrate backbone reported here. The D-galacturonic acid might be present in an acid-labile linkage via a phosphate group, although its binding position is not known at present.

Table 5
Important NOE effects observed in the two-dimensional NOESY spectrum of the carbohydrate backbone (PS1) prepared by dephosphorylation and deacylation from *V. parahaemolyticus* O2 LPS

Residue	Residue proton	Interresidual NOE	
		Sugar residue	Position (intensity [a])
β-D-Glc	H-1	D-α-D-Hep	3 (m), 4 (m)
α-D-GlcA	H-1	L-α-D-Hep	1 (w), 2 (s)
L-α-D-Hep	H-1	D-α-D-Hep α-D-GlcA	2 (m), 3 (s) 1 (w)
D-α-D-Hep	H-1	α-Kdo	5 (s), 7 (m)
α-Kdo	H-3 (axial)	β-D-GlcN	6b (w)
α-NonlA	H-3 (equatrial)	β-D-Glc	6a (w)
β-D-GlcN	H-1	D-GlcN-ol	6a (m), 6b (m)

[a] The intensity of the NOE effects were expressed as strong (s), medium (m), weak (w).

4. Experimental

4.1. Bacteria and bacterial LPS

V. parahaemolyticus strain V95–269 (O2; K3) was kindly provided by Dr A. Kai, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan. The bacterial strain was cultured in nutrient broth supplemented by 3% NaCl at 37 °C for 16 h. LPSs were isolated from heat-killed, acetone-dried cells as previously described.[5,14]

4.2. Preparation of HF-LPS

LPS (800 mg) was dephosphorylated in 48% HF (50 mg/mL) at 4 °C for 48 h, and after neutralization of the reaction mixture and successive dialysis against distilled water, HF-LPS was recovered by lyophilization (648 mg). The HF-LPS was dissolved (6.3 mg/mL) in water, reduced with $NaBH_4$ (12.5 mg/mL) at 55 °C for 16 h to give dephospholyrated and reduced LPS (HF-LPSred) (644 mg).

4.3. Preparation of deacylated HF-LPSred

O- and N-deacylation of HF-LPSred were carried out according to the method of Holst and co-workers.[15] Briefly, dried HF-LPSred (600 mg) was O-deacylated in anhyd hydrazine (20 mg/mL) at 37 °C for 1 h with constant stirring. The O-deacylated material recovered by precipitation in cold acetone was then subjected to N-deacylation in 4 M KOH (25 mg/mL) at 100 °C for 16 h. After removal of insoluble and hydrophobic material by centrifugation and extraction with $CHCl_3$, the deacylated HF-LPSred (56.7 mg) was obtained from the water-soluble fraction by means of gel-permeation chromatography on a column (100×2.5 cm) of Sephadex G-25 (Pharmacia), which was eluted with 8:5:2000 pyridine–AcOH–distilled water. The deacylated HF-LPSred was partially purified by anion-exchange chromatography on DEAE Sephacel (Pharmacia, 20×1.6 cm) using a 0–100% gradient of 500 mM NaCl in 10 mM Tris–HCl (pH 8.5). The partially purified deacylated HF-LPSred was further purified by HPAEC using a column of CarboPac PA1 (Dionex, 4×250 mm), which was eluted with a gradient of 13–15% NaOAc in 100 mM NaOH at 1 mL/min.

4.4. Chemical analysis

Neutral and amino sugars were analyzed by GC and GC–MS as described previously.[8] Uronic acid and Kdo were estimated by the carbazol–sulfuric acid[16] and the periodate–thiobarbituric acid methods,[17] respectively. Phosphate was estimated by the method of Lowry and co-workers.[18] For estimation of fatty acid, LPS were hydrolyzed with 4 M HCl at 100 °C for 4 h, and the fatty acids, which were extracted with $CHCl_3$, were heated in 2 M HCl in MeOH at 86 °C for 16 h to yield their methyl esters, which were then analyzed by GC and GC–MS. Non-2-ulosonic acid was detected by GC–MS as its peracetylated methyl ketoside methyl ester derived by methanolysis of LPS in 1 M HCl–MeOH at 86 °C for 1 h, followed by peracetylation with 1:1 pyridine–Ac_2O and carboxyl–remethylation with CH_2N_2. Methylation analysis was performed according to the Hakomori method.[19] Permethylated material was recovered using a SepPak C18 cartridge (SEP-PAK, Waters) and subjected to sugar analysis as mentioned above. The absolute configuration of Gal, Glc, GlcN, GlcA and GalA were determined by GC–MS as their peracetylated (*S*)-(+)- and (*R*)-(−)-2-butylglycosides,[20] which were derived by butanolysis in 2 M HCl in (*S*)-(+)- and (*R*)-(−)-2-butanol at 86 °C for 1 h, followed by peracetylation.

4.5. GC and GC–MS

GC was performed on a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column coated with DB210 (J&W Scientific, Folsom, CA) or HR52 (Chromato Packing Center, Kyoto, Japan) using a temperature program of 180 °C (3 min) raised to 240 °C at 5 °C/min for neutral and amino sugar analysis (DB210), 150 °C (3 min) raised to 320 °C at 5 °C/min for detection of non-2-ulosonic acid (HR52) and 150 °C (3 min) raised to 240 °C at 3 °C/min for fatty acid analysis (HR52). GC–MS was carried out using a JMS-700 (JEOL, Tokyo) instrument with the columns described above.

4.6. Mass spectrometry

MALDI-TOF MS was performed on an Applied Biosystems 4700 Proteomics Analyzer instrument in reflector TOF configuration in negative-ion mode at an acceleration voltage of 20 kV. A sample was dissolved in purified water at a concentration of 40 μg/μL and treated with Dowex $50W \times 8$ (H^+ form) to remove contaminating cations. An aliquot (4 μL) of the solution was mixed with 4 μL of 0.5 M 2,5-dihydroxybenzoic acid (Wako, Japan) in MeOH as a matrix solution, and 1 μL of the mixture was deposited on a metallic holder.

FABMS spectra were recorded in positive-ion mode using a JMS-700 (JEOL) instrument. Samples (30 mg/mL) were dissolved in 0.1 % TFA, and CH_3CN was added to give a final concentration of 25% (v/v).

4.7. NMR spectroscopy

NMR spectra were recorded for samples dissolved in D_2O at 30 °C by using a JEOL α-500 spectrometer (for ^1H NMR), a JEOL α-600 spectrometer (for ^{13}C NMR and gated ^{13}C NMR) and a JEOL ECA 800 spectrometer (for ^1H NMR, ^1H, ^1H-COSY, TOCSY, NOESY, HMQC and HMBC). Acetone (d_H 2.225, d_C 31.45) was used as an internal reference. Mixing times used for TOCSY and NOESY experiments were 60 and 500 ms, respectively.

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