Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 2711–2719
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Structural characterization of the carbohydrate backbone of the lipopolysaccharide of *Vibrio parahaemolyticus* O-untypeable strain KX-V212 isolated from a patient

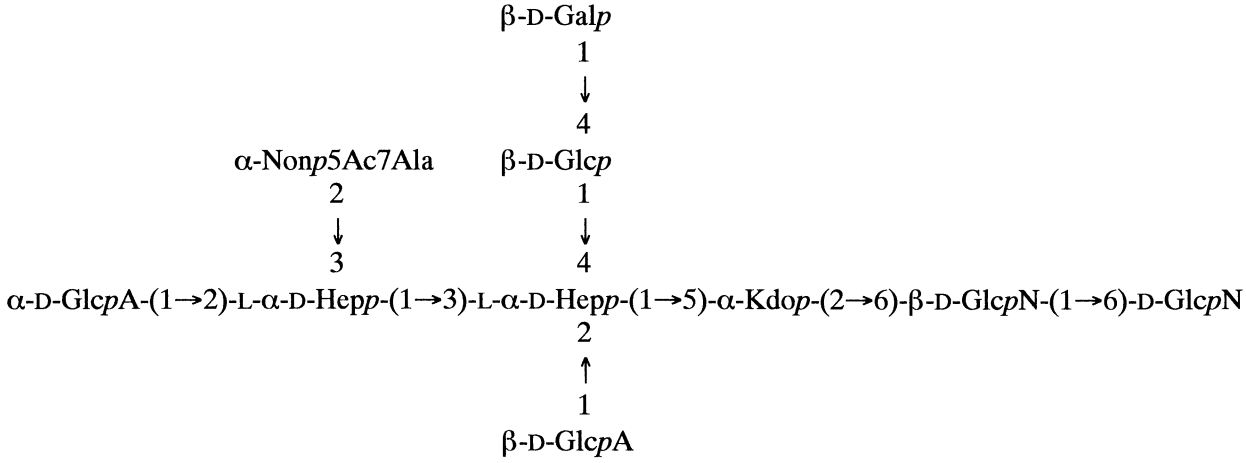
Noritaka Hashii,[a] Yasunori Isshiki,[a] Takehiro Iguchi,[b] Seiichi Kondo[a,*]

[a] *Department of Microbiology, School of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan*
[b] *Department of Clinical Dietetics and Human Nutrition, School of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan*

Received 15 March 2003; accepted 19 June 2003

Abstract

Vibrio parahaemolyticus strain KX-V212 of a novel serotype, which does not belong to any of the known 13 O-serotypes of this vibrio, was isolated from a patient. Its O-antigen harbors a unique strain-specific O-antigenic factor(s), in addition to that shared by the O-antigen of *V. parahaemolyticus* serotype O2. A carbohydrate backbone nonasaccharide was isolated from the lipopolysaccharide (LPS) of strain KX-V212 by dephosphorylation, reduction and deacylation and found to consist of one residue each of D-glucose, D-galactose, D-GlcN, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and 5-acetamido-7-(*N*-acetyl-D-alanyl)amino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Non5Ac7Ala), and two residues each of D-GlcA and L-*glycero*-D-*manno*-heptose (LD-Hep). Analysis of the isolated and deacylated lipid A showed that this oligosaccharide was an artifact resulting from a loss of one GlcN residue from the lipid A backbone. Therefore, the carbohydrate backbone of the LPS is a decasaccharide having the structure shown below. The initial LPS contains also D-GalA and phosphoethanolamine at unknown positions. Both similarity and differences are observed between the LPS of *V. parahaemolyticus* serotype O2 and strain KX-V212.



Keywords: *Vibrio parahaemolyticus*; 5,7-Diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid; Lipopolysaccharide

Abbreviations: Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; Non, 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid; Non5Ac7Ala, 5-acetamido-7-(*N*-acetyl-D-alanyl)amino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid.

* Corresponding author. Tel./fax: +81-49-2717673.
E-mail address: kondo@josai.ac.jp (S. Kondo).

0008-6215/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2003.06.003

1. Introduction

The marine bacterium *Vibrio parahaemolyticus* is a causative agent of sea-food-bone poisoning, which occurs in many countries. Pathogenic strains of *V. parahaemolyticus* are presently classified into 11 or 13 O-serotypes based on differences of their serological specificities.[1,2] The serological identification of isolated strains of *V. parahaemolyticus* has been made by cell agglutination and agglutinin absorption tests using diagnostic antisera raised against 13 O-serotype strains. However, some O-untypeable strains were found, which are usually not subjected to further serological analysis. Previously, it was demonstrated that, from seven O-untypeable strains, two strains, 90A-6611 and KX-V212, belong to a new O-serotype.[3] In addition to its own antigenic factor(s), the O-antigen of strain KX-V212 harbors antigenic factor(s) shared by the O-antigen of *V. parahaemolyticus* O2.

Lipopolysaccharides (LPS) are O-antigens of Gram-negative bacteria that determine the serological specificity of the bacterial cell. *V. parahaemolyticus* produces LPS consisting of a short-chain polysaccharide moiety,[4] which corresponds to the core oligosaccharide of representative S-type bacteria, such as enterobacteria. The structure of the core of the S-type LPS is genetically highly conserved and, therefore, it does not relate to expression of the serological O-specificity. In contrast, structural variations in the short-chain *V. parahaemolyticus* LPS does contribute to the serological O-specificity. Structural analyses of the carbohydrate backbone of the LPS from serotypes O2[5] and O12[6] demonstrated that they consist of 9 and 10 monosaccharide units, respectively, and their structures differ from each other even in the inner-core heptose region.

In the present work, the chemical structure of the carbohydrate backbone of the LPS of *V. parahaemolyticus* strain KX-V212 was investigated in comparison with that of *V. parahaemolyticus* O2.

2. Results

2.1. Chemical analysis of LPS and chemically modified LPS

Table 1 shows chemical composition of the *V. parahaemolyticus* LPS and its carbohydrate backbone prepared by deacylation of the dephosphorylated and reduced LPS (HF-LPS_{red}). For chemical analyses, the carbohydrate backbone was *N*-acetylated to give NAc-HF-LPS_{red}. The initial LPS contained D-glucose, D-galactose, L-*glycero*-D-*manno*-heptose (LD-Hep), D-GlcN, D-GlcA, D-GalA, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and a 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid (Non). Previously,[7] a derivative of

Table 1
Sugar composition of the LPS and NAc-HF-LPS_{red} from *V. parahaemolyticus* KX-V212 (μmol mg^{-1})

Component	LPS	NAc-HF-LPS_{red}
D-Glucose	0.343	0.530
D-Galactose	0.221	0.304
L-*glycero*-D-*manno*-Heptose	0.026	0.183
2-Amino-2-deoxy-D-glucose	0.199	0.240
Uronic acids [a]	0.648	1.158
Glucuronic acid	+	+
Galacturonic acid	+	−
Kdo [b]	+	+
Non [c]	+	+
Phosphate	1.002	−
C14:0	0.194	−
3-OH-C14:0	0.203	−
C16:0	0.063	−

[a] Uronic acids were estimated colorimetrically and identified as glucuronic and galacturonic acids by GC and GCMS after carboxyl reduction.

[b] Kdo was not detected by the periodate-thiobarbituric acid reaction but confirmed by GC and GCMS.

[c] Non5Ac7Ala in the LPS and Non5Ac7Ac in the NAc-HF-LPS_{red}.

Non was isolated from this LPS and identified by NMR spectroscopy and GCMS as 5-acetamido-7-(*N*-acetyl-D-alanyl)amino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Non5Ac7Ala). Kdo in the initial LPS is 4-phosphorylated as demonstrated by the method of Kondo and co-workers.[6,8] In addition to the sugars and phosphate, the LPS contained fatty acids comprising the lipid A moiety. The NAc-HF-LPS_{red} contained the same sugars as the initial LPS, except for GalA. During the preparation of the NAc-HF-LPS_{red}, Non5Ac7Ala was converted into Non5Ac7Ac, i.e., the *N*-acetyl-D-alanyl group at position 7 was replaced with an acetyl group.

The LPS was *O*-deacylated with anhydrous hydrazine and subjected to MALDI-TOF MS. The negative-ion mode mass spectrum (Fig. 1) showed four major ion peaks at *m/z* 2998.0, 2875.0, 2699.0, and 2619.0. The largest ion at *m/z* 2998.0 corresponded to an undecasaccharide consisting of hexoses (Hex), heptoses (Hep), hexuronic acids (HexA), hexosamines (HexN), Kdo, Non5Ac7Ala, phosphate (*P*), fatty acids (3-OH-C14:0) and ethanolamine (EA) ($Hex_2/Hep_2/HexA_3/HexN_2$/Kdo/Non5Ac7Ala/$P_4$/EA/3-OH-$C14:0_2$; the calculated molecular mass 2999.6 Da). The ions at *m/z* 2875.0, 2699.0 and 2619.0 were from the compounds that lack *P*-EA; *P*-EA and HexA; and *P*-EA, HexA and *P*, respectively.

The oligosaccharide portion of the LPS (OS), which was prepared by mild acid hydrolysis of the LPS, contained Gal, Glc, GlcA and LD-Hep but no Non,

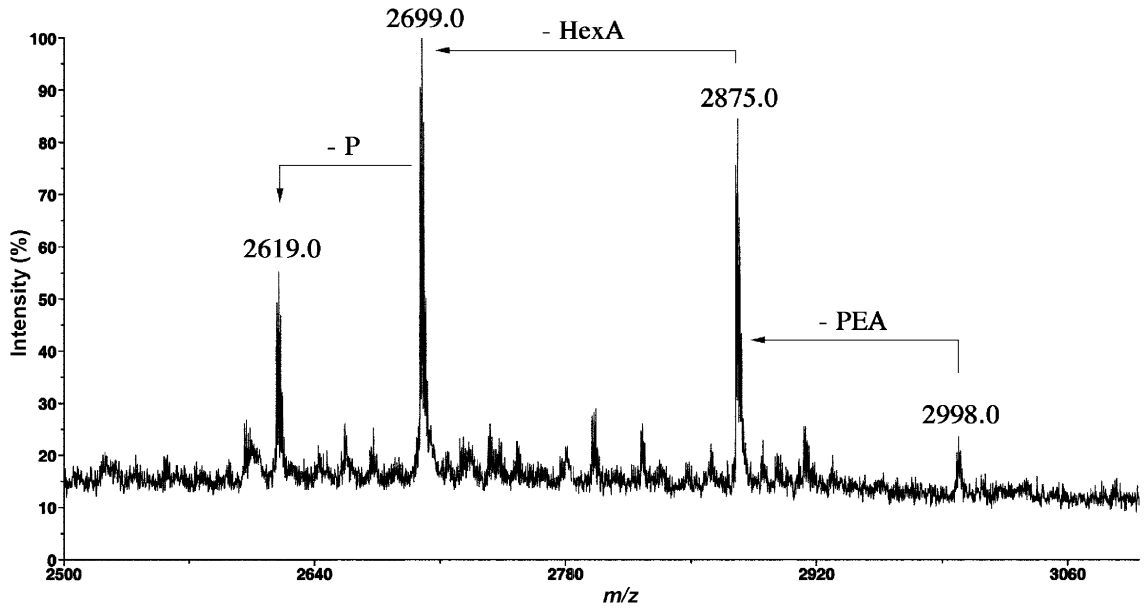


Fig. 1. Negative-ion mode MALDI-TOF mass spectrum of the *O*-deacylated LPS from *V. parahaemolyticus* KX-V212.

GalA and GlcN. Therefore, Non5Ac7Ala and GalA were completely released from the LPS under these conditions. GlcN was a component of lipid A, which was cleaved by partial hydrolysis. Neither Kdo nor phoshorylated Kdo was detected because 4-phoshorylated Kdo present in the LPS was converted into an anhydro derivative(s) during preparation of the OS.

Table 2 shows methylation analysis data of the OS. The partially methylated alditol acetates derived from the following pyranosidic sugar residues were detected: terminal Gal, terminal Glc-6-d_2 (from carboxyl-reduced GlcA), 4-substituted Glc, 2-substituted LD-Hep and 2,3,4-trisubstituted LD-Hep.

Table 2
Partially methylated alditol acetates derived from the OS from *V. parahaemolyticus* KX-V212

Derivative	Position of substitution	Content [a]
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol		1.4
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	4	1.2
1,2,5-Tri-*O*-acetyl-3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol	2	1.0
1,2,3,4,5-Penta-*O*-acetyl-6,7-di-*O*-methyl-L-*glycero*-D-*manno*-heptitol	2,3,4	1.0
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol-6-d_2		1.9

[a] Related to 1,2,3,4,5-penta-*O*-acetyl-6,7-di-*O*-methyl-L-*glycero*-D-*manno*-heptitol.

2.2. Structural analysis of deacylated HF-LPS$_{red}$

Upon DEAE Sephacel anion-exchange chromatography using a 0–300 mM NaCl linear gradient, the deacylated HF-LPS$_{red}$ yielded two fractions, which were eluted at 150 (minor) and 170 (major) mM NaCl. The ^{1}H NMR spectrum of the minor fraction revealed no signals for Non. Therefore, this fraction was an artifact produced by loss of Non during the chemical treatment of the LPS and was not studied further. The major fraction was purified by high-performance anion-exchange chromatography and used for detailed structural analysis.

The ^{1}H and ^{13}C NMR spectra of the deacylated HF-LPS$_{red}$ were assigned using ^{1}H,^{1}H COSY, TOCSY, NOESY, ^{1}H,^{13}C HMQC, HSQC-TOCSY, and HMBC experiments. The ^{1}H NMR spectrum of the deacylated HF-LPS$_{red}$ (Fig. 2, Table 3) displayed signals for six anomeric protons at δ 4.3–5.4, and the COSY spectrum clearly demonstrated the presence of six pyranoside spin systems. Based on the vicinal coupling constant values of the ring protons, the H-1 signals were assigned to the residues of LD-Hep^{I}, LD-Hep^{II}, GlcA^{I}, Gal, Glc, and GlcA^{II} enumerated in order of decreasing of the H-1 chemical shifts. Using the HMBC spectrum, two carboxyl signals at δ 177.1 and 177.2 were assigned to C-6 of GlcA^{I} and GlcA^{II}, respectively.

A relatively large $J_{1,2}$ value of 7.8 Hz indicated that Glc, Gal, and GlcA^{II} are β-linked, whereas a small $J_{1,2}$ value of 3.7 Hz showed that GlcA^{I} is α-linked. The anomeric configurations were confirmed by ^{13}C NMR data (Table 4). Relatively lower $J_{C1,H1}$ values of 161–163 Hz, determined by the INEPT method, demonstrated the β configuration of Gal, Glc, and GlcA^{II},

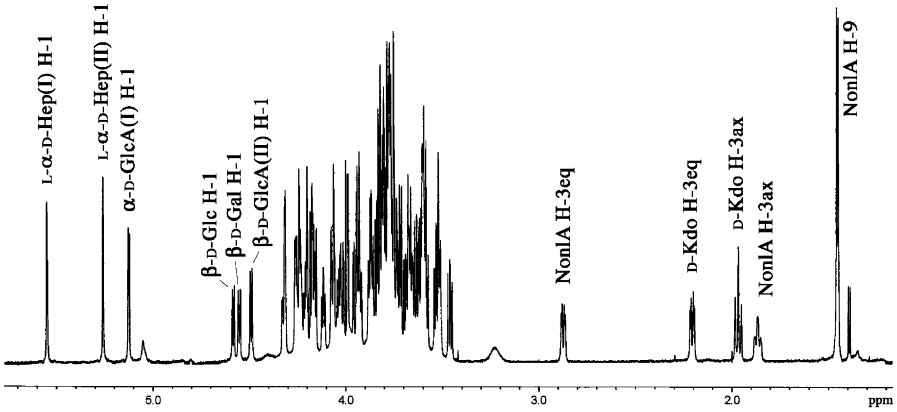


Fig. 2. ^{1}H NMR spectrum of the deacylated HF-LPS$_{red}$ from *V. parahaemolyticus* KX-V212.

whereas higher values of 170–173 Hz indicated the α configuration of GlcA^{I}, LD-Hep^{I}, and LD-Hep^{II}.

In the ^{13}C NMR spectrum, two signals for anomeric carbons were observed at δ 98.0 and 101.5, in addition to those of six aldopyranoses described above. The COSY spectrum revealed the presence of two pyranoside spin systems terminated with a methylene group. The ^{1}H,^{13}C HMQC and HMBC spectra showed that one of the sugar residues consists of eight carbons, including a carboxyl carbon (δ 171.3, C-1), and is thus Kdo. This conclusion was confirmed by the vicinal coupling constants of the ring protons and the ^{13}C NMR chemical shifts data. The other residue has nine carbons, including a carboxyl carbon (δ 172.9, C-1), two nitrogen-bearing carbons (δ 52.5 and 54.4; C-5 and C-7, respectively) and a methyl carbon (δ 18.2, C-9), and is thus Non. The D-*glycero*-D-*galacto* configuration of Non, which has been established earlier,[7] could not be unambiguously confirmed by the coupling constants owing to broadening and poor resolving of the signal for H-5 in the ^{1}H NMR of the deacylated HF-LPS$_{red}$. The ^{13}C NMR chemical shifts of Non were in good agreement with those of synthetic 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid.[9]

The α configuration of Kdo was determined based on the H-3eq chemical shift (δ 2.21) as well as $J_{7,8a}$ and $J_{7,8b}$ values (6.4 and 3.7 Hz, respectively), which are characteristic of α-Kdo*p*.[10] The signal for H-3eq of Non (δ 2.87) was shifted downfield by 0.59 ppm as compared with that of the β anomer, thus indicating that this sugar has the α configuration.[7,9]

The sequence of the constituent sugars in the deacylated HF-LPS$_{red}$ was established by long-range coupling and interresidual NOEs. The HMBC spectrum displayed intense cross-peaks between GlcA^{I} H-1 and LD-Hep^{I} C-2; Kdo H-5 and LD-Hep^{II} C-1; Gal H-1 and Glc C-4; and GlcA^{II} H-1 and LD-Hep^{II} C-2. A NOE between

Table 3
^{1}H NMR data of the deacylated HF-LPS$_{red}$ from *V. parahaemolyticus* KX-V212 (δ, ppm; $J_{n,n+1}$, Hz, in parentheses)

Proton	D-Gal	D-Glc	D-GlcA^{I}	D-GlcA^{II}	D-Hep^{I}	D-Hep^{II}	Kdo	Non	D-GlcN-ol
H-1a	4.55 (7.8)	4.58 (7.8)	5.12 (3.7)	4.49 (7.8)	5.55 (<1)	5.26 (<1)			3.84 (7.3; 12.3)
H-1b									3.95 (4.1)
H-2	3.51 (9.2)	3.46 (9.6)	3.68 (10.1)	3.53 (9.2)	4.31 (2.8)	4.26 (3.2)			3.61 (6.9)
H-3ax	3.73 (3.2)	3.66 (10.5)	3.82 (9.2)	3.59 (9.2)	4.07 (10.1)	4.33 (10.5)	1.97 (12.3; 12.4)	1.87 (11.0; 12.8)	4.18 (2.8)
H-3eq							2.21 (4.6)	2.87 (4.6)	
H-4	3.99 (<1)	3.59 (10.5)	3.52 (9.2)	3.71 (9.6)	3.94 (9.3)	4.24 (9.7)	4.25 (2.8)	3.75 (10.8)	3.75 (7.8)
H-5	3.80 (7.8)	3.77 (6.8)	4.16	3.76	3.81 (3.2)	4.17 (2.7)	4.19 (2.8)	3.23	4.02 (6.4)
H-6a	3.58 (11.5)	4.03 (12.4)			4.21 (7.8)	4.12 (5.5)	3.73 (9.2)	4.41	3.81 (11.5)
H-6b	3.61 (4.7)	4.07 (2.3)							3.87 (2.8)
H-7a					3.77 (11.5)	3.66 (10.5)	3.94 (6.4)	3.52 (9.2)	
H-7b					3.87 (4.6)	3.82 (6.9)			
H-8a							3.62 (11.9)	4.24 (6.4)	
H-8b							3.95 (3.7)		
H-9								1.45	

Table 4
^{13}C NMR data of the deacylated HF-LPS$_{red}$ from *V. parahaemolyticus* KX-V212 (δ, ppm; $J_{C1,H1}$, Hz, in parentheses)

Carbon	D-Gal	D-Glc	D-GlcA^{I}	D-GlcA^{II}	LD-Hep^{I}	LD-Hep^{II}	Kdo	Non	D-GlcN-ol
C-1	102.2 (163)	102.3 (162)	100.4 (172)	101.7 (161)	100.2 (173)	99.5 (170)	171.3	172.9	58.5
C-2	70.7	72.4	71.5	71.9	79.6	77.3	101.5	98.0	55.1
C-3	72.2	74.0	72.1	75.3	70.4	72.6	34.4	39.9	65.7
C-4	68.2	77.9	71.9	71.3	66.5	73.2	65.5	69.0	71.5
C-5	74.9	74.0	73.5	77.1	71.0	70.8	72.9	52.5	70.3
C-6	64.2	64.1	177.1	177.2	67.5	68.2	71.3	72.6	60.7
C-7					62.8	62.8	68.7	54.4	
C-8							62.7	65.6	
C-9								18.2	

Kdo H-3 and H-6a,b of D-glucosaminitol (GlcN-ol) was observed in the NOESY spectrum and indicated the presence of an α-Kdo*p*-(2→6)-D-GlcN-ol fragment. In addition, the NOESY spectrum showed the following interresidual correlations: LD-Hep^{II} H-1,Kdo H-5; GlcA^{II} H-1,LD-Hep^{II} H-2; GlcA^{I} H-1,LD-Hep^{I} H-2; Non H-3ax and 3eq,LD-Hep^{I} H-3; LD-Hep^{I} H-1,LD-Hep^{II} H-3; Glc H-1,LD-Hep^{II} H-4; and Gal H-1,Glc H-4 (Table 5). These data enabled the conclusion that the deacylated HF-LPS$_{red}$ from the LPS of *V. parahaemolyticus* KX-V212 is a reduced nonasaccharide having the following structure:

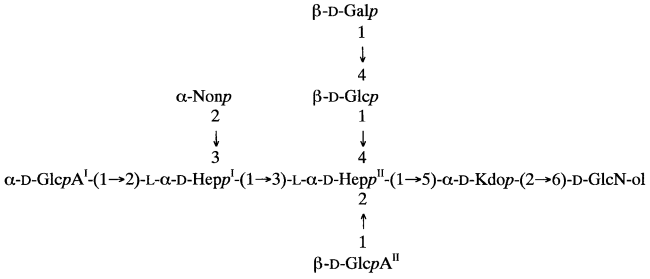


Table 5
Important NOEs observed in the NOESY spectrum of the deacylated HF-LPS$_{red}$ from *V. parahaemolyticus* KX-V212

Sugar residue	Proton	Interresidual NOE on	
		Sugar residue	Proton (intensity)
β-D-Glc*p*	H-1	L-α-D-Hep*p*^{II}	H-4 (s)
β-D-Gal*p*	H-1	β-D-Glc*p*	H-4 (s)
α-D-Glc*p*A^{I}	H-1	L-α-D-Hep*p*^{I}	H-1 (w), H-2 (s)
β-D-Glc*p*A^{II}	H-1	L-α-D-Hep*p*^{II}	H-2 (s)
L-α-D-Hep*p*^{I}	H-1	L-α-D-Hep*p*^{II}	H-3 (s)
		α-D-Glc*p*A^{I}	H-1 (w)
L-α-D-Hep*p*^{II}	H-1	α-Kdo*p*	H-5 (s), H-7 (m)
α-Kdo*p*	H-3ax,3eq	D-GlcN-ol	H-6a (w), H-6b (w)
α-Non*p*	H-3ax,3eq	L-α-D-Hep*p*^{I}	H-3 (m)

s, strong; m, medium; w, weak.

2.3. Characterization of lipid A and LPS backbone structure

Since only one GlcN residue from the lipid A backbone occurred at the reducing end of the deacylated HF-LPS, this compound might not represent the full carbohydrate backbone of the LPS. Therefore, the lipid A backbone was obtained by deacylation of the isolated lipid A and analyzed. The NMR data (Table 6) revealed that the lipid A backbone is a β-D-Glc*p*N^{II}-(1→6)-α-D-Glc*p*N^{I} disaccharide. Furthermore, the ^{1}H,^{31}P HMQC spectrum (Fig. 3) showed the presence of two phosphate groups, one being located at position 4 of β-GlcN^{II} and the other glycosidically linked to α-GlcN^{I}.

Therefore, the carbohydrate backbone of the LPS is a decasaccharide having the structure shown in Fig. 4. The phosphate groups are present at position 1 of GlcN^{I} and position 4 of GlcN^{II} in the lipid A backbone and at position 4 of Kdo in the core, as in all other vibrio LPS studied so far. In addition, the LPS and *O*-deacylated LPS contain D-GalA and phosphoethanolamine, which

Table 6
^{1}H NMR data of the deacylated lipid A from *V. parahaemolyticus* KX-V212 (δ, ppm; $J_{n,n+1}$, Hz, in parentheses)

Atom	GlcN^{I}	GlcN^{II}
H-1	5.58 (3.7)[a]	4.85 (8.5)
H-2	3.18 (9.8)	2.95 (10.1)
H-3	3.86 (9.8)	3.78 (9.8)
H-4	3.47 (10.1)	3.90 (9.8)
H-5	4.17 (7.0)	3.55 (4.3)
H-6a	3.91 (12.2)	3.84–3.89 (2.8)
H-6b	4.25 (1.8)	
C-1	92.6	101.7
C-2	56.0	57.2
C-3	72.0	74.3
C-4	71.2	73.4
C-5	73.3	77.0
C-6	69.9	61.7

[a] $J_{H-1,P}$ 7.3 Hz.

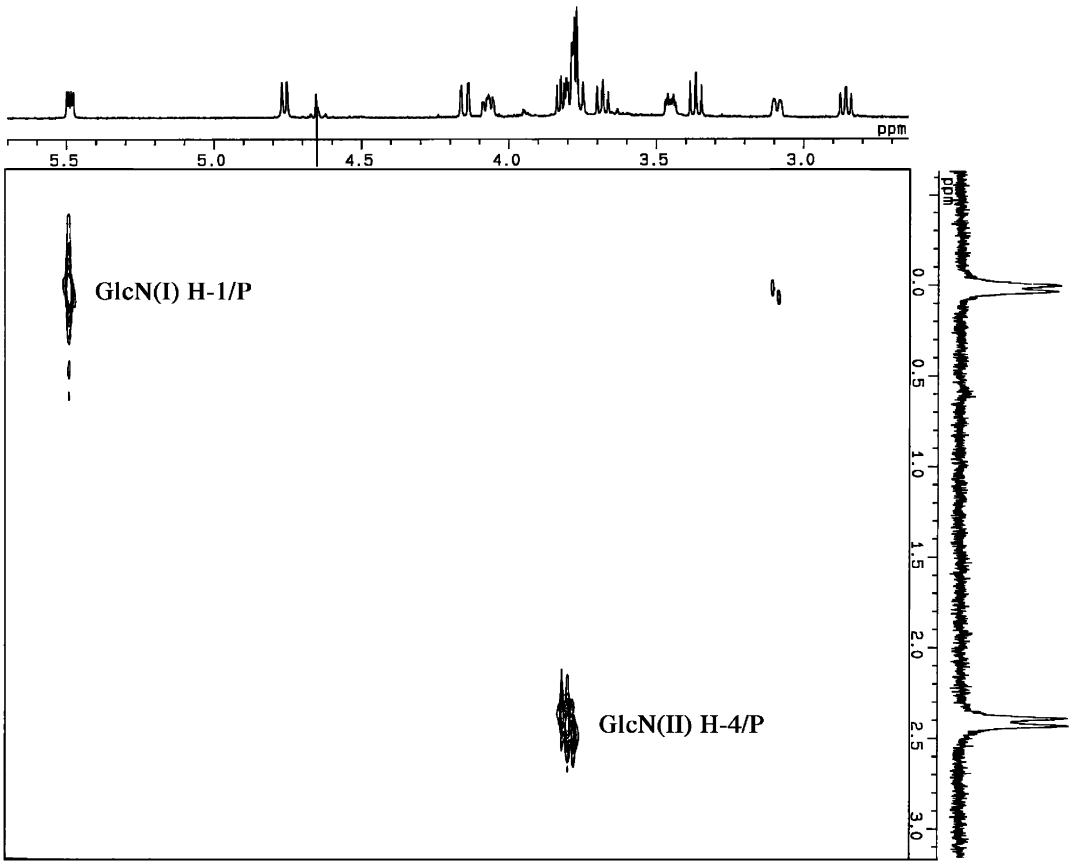


Fig. 3. $^{1}H,^{31}P$ HMQC spectrum of the deacylated lipid A from *V. parahaemolyticus* KX-V212.

were readily released by treatment of the LPS with mild acid (aq 5% AcOH at 100 °C) or aq HF. The sites of attachment of these components, which are present also in the LPS of *V. parahaemolyticus* O2,[5] were not determined.

3. Discussion

Earlier, the structures of the LPS carbohydrate backbone have been elucidated in only two from 13 O-serotypes of *V. parahaemolyticus*.[5,6] This is a nonasac-

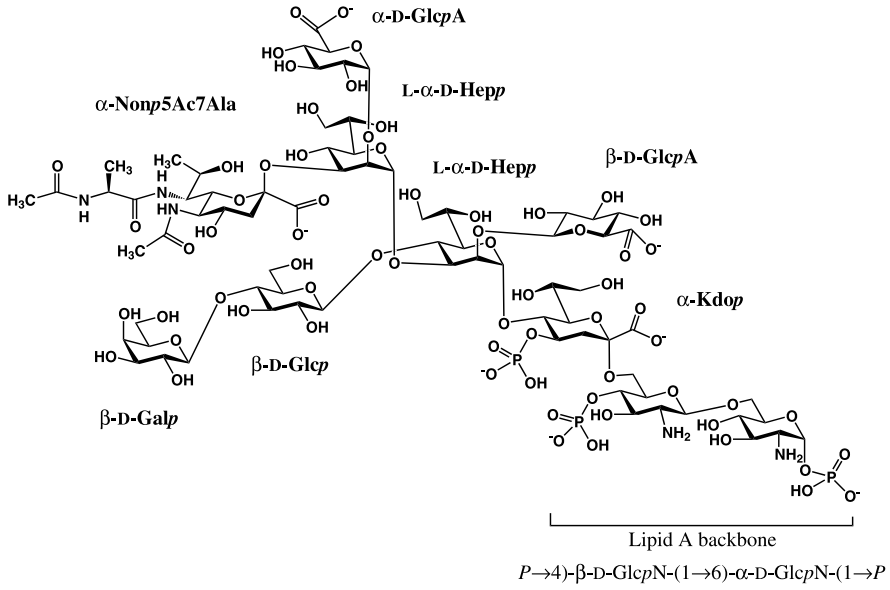
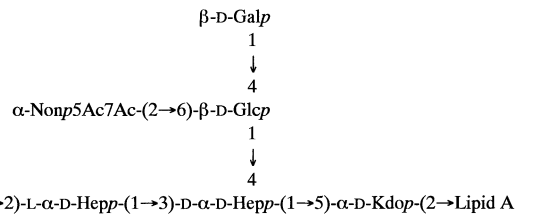


Fig. 4. Proposed structure of the carbohydrate backbone of the LPS of *V. parahaemolyticus* KX-V212.

charide and a decasaccharide in serotypes O2[5] and O12[6], respectively, which structurally correspond to the core-lipid A backbone of the LPS of other Gram-negative bacteria. The carbohydrate backbone of the short-chain LPS of strain KX-V212 studied in this work is also a decasaccharide, and its structure resembles that of *V. parahaemolyticus* O2 shown below:[5]



Remarkably, both LPS contain derivatives of Non at a non-reducing terminus, though the N-substituents at position 7 of Non are different. β-D-Glc*p*A-(1→2)-L-α-D-Hep*p* and β-D-Gal*p*-(1→4)-D-β-Glc*p* fragments at non-reducing ends of the core are also shared by both strains. These structural similarities may be responsible for the serological cross-reactivity of *V. parahaemolyticus* KX-V212 and O2. On the other hand, the LPS of strain KX-V212 differs from that of serotype O2 in the site of attachment of Non, the presence of an additional GlcA residue and the replacement of the Kdo-proximal residue of D-*glycero*-D-*manno*-heptose with L-*glycero*-D-*manno*-heptose. These structural variations, together with the different N-substituents at position 7 of Non, may relate to the expression of different serological O-specificities by strain KX-V212 and serotype O2.

Strain KX-V212 belongs to a novel O-serotype,[3] which is not among the 13 known O-serotypes of this vibrio. In addition to its own O-antigenic factor(s), this strain harbors that in common with serotype O2. Strain KX-V212 was isolated in 1996 in Japan from a patient with diarrhoeal disease (Myanmar appeared to be the country where the infection likely occurred). Previously,[3] we have reported that another *V. parahaemolyticus* strain, 90A-6611, isolated in 1990 from a patient in the USA belongs to the same O-serotype as strain KX-V212 and that sugar composition of the LPS from both strains is similar. In this study, the presence of the Non5Ac7Ala was confirmed in the LPS of strain 90A-6611 too (data not shown).

Interestingly, the strains of the new O-serotype were isolated from patients far apart in time and geography. Since not all O-untypeable isolates of *V. parahaemolyticus* have been investigated, one cannot assert that strains belonging to the new O-serotype might have caused some cases of infection in the world, but the data are suggestive. In the future, not only diagnostic antisera raised against the 13 known *V. parahaemolyticus* O-serotypes but also that against the new O-serotype should be used in serodiagnosis.

4. Experimental

4.1. Bacteria and bacterial LPS

V. parahaemolyticus strain KX-V212 (O-untypeable, K-untypeable) was kindly provided by Dr M. Nishibuchi (Center for Southeast Asian Studies, Kyoto University, Kyoto). The strain was cultured in nutrient broth supplemented with aq 3% NaCl at 37 °C for 16 h. LPS was isolated from heat-killed, acetone-dried cells as described previously.[11,12]

4.2. Preparation of dephosphorylated LPS (HF-LPS) and reduced HF-LPS (HF-LPS$_{red}$)

The LPS (800 mg) was dephosphorylated with aq 48% HF (50 mg mL^{-1}) at 4 °C for 48 h, and the reaction mixture was diluted fivefolds with ice-cold water, neutralized slowly with chilled dilute aq NH_3 under cooling in ice bath and dialyzed against distilled water. The HF-LPS (685 mg) was recovered by lyophilization, dissolved in distilled water (6.3 mg mL^{-1}) and reduced with $NaBH_4$ (12.5 mg mL^{-1}) at 55 °C for 16 h to give the dephosphorylated and reduced LPS (HF-LPS$_{red}$) (645 mg).

4.3. Deacylation of HF-LPS$_{red}$

O- and N-deacylation of the HF-LPS$_{red}$ was carried out according to the method of Holst and co-workers.[13] Briefly, the dried HF-LPS$_{red}$ (500 mg) was O-deacylated with anhyd hydrazine (20 mg mL^{-1}) at 37 °C for 1 h with stirring. The O-deacylated product recovered by precipitation with cold acetone was N-deacylated with 4 M KOH (25 mg mL^{-1}) at 100 °C for 16 h. After removal of insoluble and hydrophobic material by centrifugation and extraction with $CHCl_3$, the deacylated HF-LPS$_{red}$ (57.2 mg) was recovered from the water-soluble fraction by GPC on a Sephadex G-25 column (100 × 2.5 cm, Amersham Biosciences, UK), eluted with 8:5:2000 Py–HOAc–water buffer pH 5.0. The deacylated HF-LPS$_{red}$ was purified by anion-exchange chromatography on a DEAE Sephacel column (20 × 1.6 cm, Amersham Biosciences), using a 0–100% gradient of 300 mM NaCl in 10 mM Tris–HCl pH 8.5, followed by HPAEC on a CarboPac PA1 column (4 × 250 mm, Dionex, CA) eluted with a 20–25% gradient of 1 M NaOAc in 100 mM NaOH at 1 mL min^{-1}. Prior to sugar analysis, the deacylated HF-LPS$_{red}$ was *N*-acetylated with Ac_2O in aq 1% $NaHCO_3$ to yield the NAc-HF-LPS$_{red}$.

4.4. Preparation of the core oligosaccharide (OS) and deacylated lipid A backbone

The LPS (500 mg) was degraded with aq 5% AcOH at 100 °C for 1.5 h. After centrifugation, the supernatant was subjected to GPC on Sephadex G-25 as described above. Fractions corresponding to the OS (113 mg) were combined and lyophilized. The sediment obtained by centrifugation was washed with distilled water and lyophilized to yield lipid A (149 mg). Lipid A (36 mg) was O- and N-deacylated as described above for the LPS, and the lipid A backbone (5.9 mg) was isolated by GPC on Sephadex G-25.

4.5. Chemical analysis

Neutral and amino sugars were analyzed by GC and GCMS as described previously.[3] Uronic acids and Kdo were estimated by the carbazole-sulfuric acid[14] and periodate-thiobarbituric acid[15] methods, respectively. Phosphate was estimated by the method of Lowry and co-workers.[16] For fatty acid analysis, LPS was hydrolyzed with 4 M HCl at 100 °C for 4 h, the fatty acids were extracted into $CHCl_3$ and heated with 2 M HCl–MeOH at 86 °C for 16 h to yield the methyl esters, which were analyzed by GC and GCMS. Non was detected by GCMS as the peracetylated methyl ester methyl ketoside derived by methanolysis of the LPS with 1 M HCl–MeOH at 86 °C for 1 h followed by peracetylation with a 1:1 pyridine–Ac_2O mixture and carboxyl-methylation with CH_2N_2. Kdo phosphate was detected and the position of phosphate on Kdo determined according to the method of Kondo and co-workers.[6,8] Methylation analysis was performed by the method of Hakomori.[17] The absolute configurations of Gal, Glc, and GlcN were determined by GCMS of the peracetylated (*S*)-(+)- and (*R*)-(−)-but-2-yl glycosides,[18] which were prepared by alcoholysis with 2 M HCl in (*S*)-(+)- and (*R*)-(−)-butan-2-ol at 86 °C for 1 h followed by peracetylation. The absolute configurations of GlcA and GalA were determined after carboxy-reduction with $NaBD_4$ as described above.

4.6. GC and GCMS

GC was performed on a GC-14A instrument (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 (3 min) to 240 °C at 5 °C min^{-1} for neutral and amino sugars (DB210), 150 (3 min) to 320 °C at 5 °C min^{-1} for Kdo phosphate and Non derivatives (HR52), and 150 (3 min) to 240 °C at 3 °C min^{-1} for fatty acids (HR52). GCMS was carried out using a JMS-700 instrument (JEOL, Tokyo, Japan) on the same columns as above.

4.7. Mass spectrometry

MALDI-TOF MS was performed on a 4700 Proteomics Analyzer instrument (Applied Biosystems, Foster City, CA) in reflector TOF configuration and negative-ion mode at an acceleration voltage of 20 kV. A sample was dissolved in purified water (Wako, Japan) at 10 μg μL^{-1} and treated with Dowex 50W × 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 mixture was deposited on a metallic holder.

4.8. NMR spectroscopy

NMR spectra were recorded for samples dissolved in D_2O at 30 °C on a JEOL A-600 spectrometer (for ^{13}C NMR) and a JEOL ECA 800 spectrometer (for ^{1}H NMR and 2D NMR). Acetone (δ_H 2.225, δ_C 31.45) was used as internal reference. Mixing times in TOCSY and NOESY experiments were 60 ms and 1 s, respectively.

Acknowledgements

We thank S. Yamaguchi for excellent NMR measurements and H. Mitsuhashi for GCMS analyses. We are grateful to Dr Y. Haishima (National Institute of Health Sciences, Tokyo, Japan) for his invaluable instruction and encouragement.

References

1. Terada, T.; Yokoo, Y. *Jpn. J. Bacteriol.* **1972**, *27*, 35–41.
2. Ishibashi, M.; Kinoshita, Y.; Yanai, Y.; Abe, H.; Takeda, Y.; Miwatani, T. *Jpn. J. Bacteriol.* **1980**, *35*, 701–706.
3. Hashii, N.; Kondo, S.; Iguchi, T.; Nishibuchi, M.; Hisatsune, K. *Microbiol. Immunol.* **2000**, *44*, 229–234.
4. Iguchi, T.; Kondo, S.; Hisatsune, K. *FEMS Microbiol. Lett.* **1995**, *130*, 287–292.
5. Hashii, N.; Isshiki, Y.; Iguchi, T.; Kondo, S. *Carbohydr. Res.* **2003**, *338*, 1063–1071.
6. Kondo, S.; Zähringer, U.; Seidel, U.; Sinnwell, V.; Hisatsune, K.; Rietschel, E. T. *Eur. J. Biochem.* **1991**, *200*, 689–698.
7. Hashii, N.; Isshiki, Y.; Iguchi, T.; Hisatsune, K.; Kondo, S. *Carbohydr. Res.* **2003**, *338*, 1055–1062.
8. Kondo, S.; Haishima, Y.; Hisatsune, K. *Carbohydr. Res.* **1992**, *231*, 55–64.
9. Tsvetkov, Y. E.; Shaskov, A.; Knirel, Y. A.; Zähringer, U. *Carbohydr. Res.* **2001**, *335*, 221–243.
10. Müller-Loennies, S.; Holst, O.; Linder, B.; Brade, H. *Eur. J. Biochem.* **1999**, *260*, 235–249.

11. Hisatsune, K.; Kiuye, A.; Kondo, S. *Microbiol. Immunol.* **1980**, *24*, 691–701.
12. Westphal, O.; Lüderitz, O.; Bister, R. *Z. Naturforschung. B* **1952**, *7*, 148–155.
13. Holst, O.; Müller-Loennies, S.; Lindner, B.; Brade, H. *Eur. J. Biochem.* **1993**, *214*, 695–701.
14. Bitter, T.; Müir, H. M. *Anal. Biochem.* **1962**, *4*, 330–334.
15. Brade, H.; Galanos, C.; Lüderitz, O. *Eur. J. Biochem.* **1983**, *131*, 195–200.
16. Lowry, O. H.; Roberts, N. R.; Leiner, K. Y.; Wu, M. L.; Farr, A. L. *J. Biol. Chem.* **1954**, *207*, 1–17.
17. Hakomori, S. *J. Biochem. (Tokyo)* **1964**, *55*, 205–208.
18. Gerwig, G. J.; Kamering, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.