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Structure and serological characterization of 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid isolated from lipopolysaccharides of *Vibrio parahaemolyticus* O2 and O-untypable strain KX-V212

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Abstract

Lipopolysaccharides (LPS) of *Vibrio parahaemolyticus* O2 and O-untypable (OUT) strain (KX-V212) isolated from an individual patient were shown to contain 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid (NonlA), which was readily released from LPS by mild acid hydrolysis. In the present study, we investigated the chemical and serological properties of NonlA isolated from LPS of *V. parahaemolyticus* O2 and OUT KX-V212. GC–MS and NMR analysis identified the NonlA from LPS of O2 to be 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (5NAc7NAcNonlA) and that from LPS of KX-V212 to be 5-acetamido-7-(*N*-acetyl-D-alanyl)amido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (5NAc7NAlaNAcNonlA). In ELISA inhibition analysis, 5NAc7NAcNonlA inhibited the O2 LPS/anti-O2 antiserum system, whereas, 5NAc7NAlaNAcNonlA did not show any inhibitory activity. However, after N-deacylation of 5NAc7NAlaNAcNonlA followed by N-acetylation, the product (5NAc7NAcNonlA) inhibited the O2 LPS/anti-O2 antiserum system to the same extent as that of 5NAc7NAcNonlA obtained from O2 LPS. These results suggest that 5NAc7NAcNonlA might be related to the serological specificity of O2 LPS as one of main epitope(s) involved in O2 LPS.

Keywords: 5,7-Diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid; O-Antigenic polysaccharide; *Vibrio parahaemolyticus*; ELISA

1. Introduction

Vibrio parahaemolyticus, a Gram-negative bacterium that causes food-borne poisoning frequently occurring worldwide, is presently divided into 13 (or 11) O-serotypes[1,2] and ten chemotypes[3,4] based on the serological specificities of its O-antigens and the sugar composition of its lipopolysaccharides (LPS), respectively. All of the 13 O-serotypes of *V. parahaemolyticus* produce low-molecular-mass LPSs[5] that are structurally similar to those so-called lipooligosaccharides of nonenteric mucosal pathogens, such as *Neisseria gonorrhoeae*, *N. meningitidis* and *Haemophilus influenzae*.[6] LPSs of many enteric Gram-negative bacteria consist of three chemically distinct portions, i.e., the lipid A, the core oligosaccharide (OS) and the O-antigenic polysaccharide which determines the serological specificity of the parental bacterium from which LPSs are derived. In contrast, LPSs of the lipooligosaccharide type are defective in the O-antigenic polysaccharide portion and consist of lipid A and an OS chain, which corresponds to the core OS region of other Gram-negative bacterial LPSs. LPSs are O-antigens of Gram-negative bacteria;

Abbreviations: OUT, O-untypable; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; NonlA, 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acid; 5NAc7NAcNonlA, 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; 5NAc7NAlaNAcNonlA, 5-acetamido-7-(*N*-acetyl-D-alanyl)-amido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; LPS, lipopolysaccharides; HF, hydrofluoric acid; HF-LPS, dephosphorylated LPS; OS, oligosaccharide.

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therefore, in the case of *V. parahaemolyticus*, structural variations residing in the OS portions corresponding to the core regions of normal LPSs must reflect the serological specificities of respective O-serotypes. However, the chemical structures of the OS portions from *V. parahaemolyticus* LPSs have not been elucidated, except for one O-serotype (O12)[7] out of 13 O-serotypes. We previously demonstrated[8] that an O-untypable strain (OUT KX-V212) of *V. parahaemolyticus* isolated from an individual patient had a strong serological cross-reactivity with serotype O2, but that this strain was a novel serotype that had hitherto not been reported. The sugar composition of the OS portion of the LPS from O2 was very similar to that of the OS portion from OUT KX-V212 LPS, except for the presence of D-*glycero*-D-*manno*-heptose in the former: galactose (Gal), glucose (Glc), glucuronic acid (GlcA), L-*glycero*-D-*manno*-heptose, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and glucosamine were common to both. In addition to these component sugars, N-substituted 5,7-diamino-3,5,7,9-tetradeoxy-non-2-ulosonic acids (NonlAs) were found as components of the OS portions of LPSs from both O2 and OUT KX-V212. Several isomers of NonlA, carrying different substitutions on amino groups at positions C-5 and C-7, have been reported as constituents of LPSs from Gram-negative bacteria.[9–20] In the present study, we describe chemical identification of NonlA, including the identity of their N-substituents, isolated from the LPSs of O2 and OUT KX-V212. Relations of NonlAs to serological O-specificities of these strains will also be described.

2. Results

2.1. Characterization of NonlA from the LPSs of O2 and OUT KX-V212

^{1}H (Table 1) and ^{13}C NMR spectra (Table 2) of NonlA from O2 LPS (O2 NonlA) and from OUT KX-V212 LPS (OUT NonlA) were assigned using PDQF-COSY, PHSQC and gated ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of O2 NonlA displayed signals for one anomeric carbon at δ_C 96.9, one methylene group at δ_C 40.9, one methyl group at δ_C 20.4, five sugar carbons, two of which carried nitrogen (δ_C 53.9 and 54.4) and three of which bore oxygens (δ_C 67.6–70.9), one carboxyl group (δ_C 175.5), and two *N*-acetyl groups (methyl group at δ_C 23.0 and 23.4, carbonyl group at δ_C 175.0 and 175.2). The ^{1}H NMR spectrum of O2 NonlA showed signals for methylene groups at δ_H 1.85 (axial) and 2.28 (equatorial), a methyl group at δ_H 1.15 and *N*-acetyl groups at δ_H 1.98 and 2.00. The large coupling constant values for $J_{3ax,4}$ $J_{4,5}$ and $J_{5,6}$ (11.29, 10.07 and 10.38 Hz) proved that H-4, H-5 and H-6 were in an axial orientation such as those in D-*galacto*, and L-*altro* isomers. The HMBC spectrum displayed that H-5 (δ_H 3.71) and H-7 (δ_H 3.88) were correlated with carbonyl groups (δ_C 175.2 and 175.0), respectively. The ^{13}C NMR data obtained for O2 NonlA showed the presence of deoxy units at positions C-3 and C-9, and acetamido groups on positions C-5 and C-7. The ^{1}H and ^{13}C NMR data for NonlA obtained from N-deacylated and N-acetylated OUT KX-V212 LPSs completely coincided with those for O2 NonlA. Thus, both NonlAs were characterized as 5,7-diacetamido-3,5,7,9-tetradeoxy-non-2-ulosonic acid (5NAc7NAcNonlA). For determination of the stereoisomeric configuration, the ^{1}H and ^{13}C NMR data obtained for O2 NonlA were compared in detail with those reported for nine stereoisomers of 5NAc7NAcNonlA[21] as well as with those for legionaminic acid (D-*glycero*-D-*galacto* configuration),[22] isolegionaminic acid (D-*glycero*-D-*talo* configuration)[21] and pseudaminic acid (L-*glycero*-L-*manno* configuration).[9–11] On the whole, the NMR data for O2 NonlA were almost identical to those reported for synthetic D-*glycero*-D-*galacto* isomers and clearly distinguishable from those for other stereoisomers. For example, from its NMR spectra, O2 NonlA was obviously distinguishable from L- and D-*glycero*-L-*altro* isomers by signals for H-6 (δ_H 4.28) and C-6 (δ_C 70.9), both of which were shifted downfield by 0.37–0.41 and upfield by 2.8–4.8, respectively, compared with those of L-*altro* isomers. In addition, the large $J_{7,8}$ value (8.85 Hz) was quite similar to that reported (8.9 Hz) for L- and D-*glycero*-D-*galacto* isomers,[21] in contrast to the $J_{7,8}$ values for the D-*glycero*-L-*altro* (<1) and L-*glycero*-L-*altro* (5.8 Hz) isomers. On the other hand, the ^{13}C NMR signals of C-6 and C-8 of O2 NonlA were observed at δ_C 70.9 and 67.6, respectively, which were shifted upfield by approx 2.0 ppm, compared with those of the L-*glycero*-D-*galacto* isomer. Furthermore, in the NOESY spectrum (data not shown) of O2 NonlA, a strong H-6, H-8 correlation was observed, which is characteristic of the D-*glycero*-D-*galacto* but not the L-*glycero*-D-*galacto* isomer.[21] Based on these data, we concluded that O2 NonlA was 5NAc7NAcNonlA (Fig. 1a). The ratio of α to β isomers present in the purified sample was 1:16. The NMR data for OUT NonlA were quite similar to those for O2 NonlA, except for signals originating from a substituent on its amino group at position C-7 (Tables 1 and 2). FABMS (positive-ion mode) of OUT NonlA revealed the presence of a molecular ion peak (m/z 406) which was 71 Da larger than that obtained (m/z 335) for O2 NonlA, suggesting that an alanyl substituent might be present in OUT NonlA. For determination of the N-substituent, purified OUT NonlA was hydrolyzed and analyzed using an amino acid analyzer. In the amino acid area, only one peak corresponding to L-Ala (used as a standard) was detected. The D-configuration of the Ala was determined by GC–MS of its *N*-acetyl-(*S*)-(+)-2-

Table 1
^{1}H NMR data for NonlA isolated from the LPSs of *V. parahaemolyticus* O2 (O2 NonlA) and OUT strain KX-V212 (OUT NonlA) [a]

	Chemical shift ($J_{H,H}$, Hz) for												
	H-3ax ($J_{3ax.,4}$)	H-3eq ($J_{3ax.,3eq.}$)	H-4 ($J_{3eq.,4}$)	H-5 ($J_{4,5}$)	H-6 ($J_{5,6}$)	H-7 ($J_{6,7}$)	H-8 ($J_{7,8}$)	H-9 ($J_{8,9}$)	5NAc	7NAc	H-2Ala ($J_{2,3}$)	H-3Ala	AlaNAc
O2 NonlA	1.85 (11.29)	2.28 (12.82)	3.96 (4.88)	3.71 (10.07)	4.28 (10.38)	3.88 (2.14)	3.84 (8.85)	1.15 (6.10)	1.98	2.00			
OUT NonlA	1.84 (11.60)	2.28 (12.82)	3.97 (4.88)	3.64 (10.07)	4.31 (10.37)	3.87 (1.53)	3.84 (10.68)	1.08 (5.80)	2.00		4.27 (7.33)	1.41	2.00

[a] Samples were dissolved in D_2O. Acetone (δ_H 2.22) was used as an internal reference.

Table 2
^{13}C NMR data for NonlA isolated from the LPSs of *V. parahaemolyticus* O2 (O2 NonlA) and OUT strain KX-V212 (OUT NonlA) [a]

	Chemical shift for													
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	5(NAc)	7(NAc)/C-1Ala	C-2Ala	C-3Ala	AlaNAc
O2 NonlA	175.5	96.9	40.9	68.6	53.9	70.9	54.4	67.6	20.4	23.4 175.2	23.0 175.0			
OUT NonlA	175.1	96.7	40.3	68.7	53.8	70.5	54.4	67.4	20.5	23.3	176.0	51.0	18.1	22.8 175.0

[a] Samples were dissolved in D_2O. Acetone (δ_C 31.45) was used as an internal reference.

butylester. The peak of the derivative from D-Ala (7.90 min) was clearly separated from that (8.20 min) of the same derivative of L-Ala. In the HMBC spectrum of OUT NonlA (Fig. 2), cross-peaks between H-5 and the carbonyl group of the *N*-acetyl substituent on position C-5, as well as between H-2′ and the carbonyl group of the *N*-acetyl substituent on position C-2′ of the D-alanyl residue were observed. Moreover, the presence of a correlation peak between H-7 and C-1′ indicated that the D-Ala was linked to an amino group on position C-7. From these results, the OUT NonlA was identified as to be 5-acetamido-7-(*N*-acetyl-D-alanyl)amido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (5NAc7NAlaNAcNonlA) (Fig. 1b). The ratio of α to β isomers present in the purified sample was 1:32. The substitution of the amino group on position C-7 by a D-alanyl group was also supported by GC–MS of 7 - (*N* - acetylmethylalanyl)amido - 3,5,7,9 - tetradeoxy-1,2,4,6,8 - penta - *O* - methyl - 5 - (methylacetamido)nonitol (1-2D, 2-1D) derived from purified OUT NonlA by the sequential derivatization procedures. Upon GC–MS, prominent fragment ions at *m*/*z* 235 (22.7), 279 (6.5), 449 (4.8), 358 (41.6) and 273 (12.4) (the values in parentheses were percent intensities of the base peak *m*/*z* 128, 100%) were assigned as C-1–C-5, C-1–C-6, C-1–C-7, C-5–C-9 and C-6–C-9 fragments, respectively.

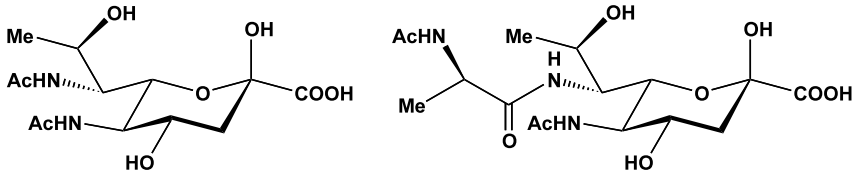

Fig. 1. Structure of non-2-ulosonic acids detected in the LPS of *V. parahaemolyticus*: (a) 5NAc7NAcNonlA isolated from O2 LPS; (b) 5NAc7NAlaNAcNonlA isolated from the OUT strain KX-V212 LPS.

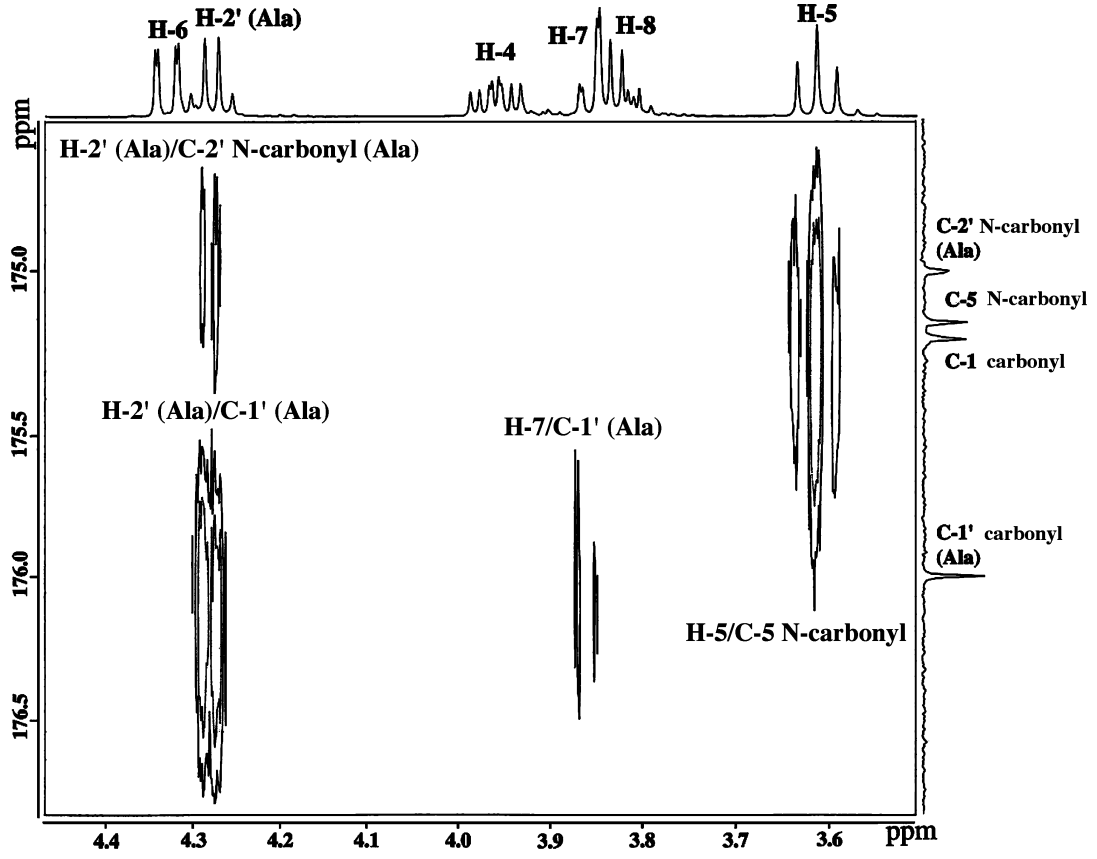

Fig. 2. HMBC spectrum of 5NAc7NAlaNAcNonlA.

Table 3
Inhibition of ELISA using antisera raised against *V. parahemolyticus* O2 and OUT strain KX-V212

Inhibitor	Inhibitory dose (μg/mL) against ELISA system	
	O2 LPS/anti-O2 antiserum	KX-V212 LPS/anti-KX-V212 antiserum
V. parahaemolyticus O2		
LPS	3.2	–
HF-LPS	0.56	–
OS	–[d]	–
5NAc7NAcNonlA [a]	200	–
V. parahaemolyticus KX-V212		
LPS	–	7.9
HF-LPS	–	20
OS	–	–
5NAc7NAlaNAcNonlA [b]		–
5NAc7NAcNonlA [c]	210	–
β-Gal-(1→4)-Glc	–	–
GlcA	–	–

[a] 5,7-Diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid isolated from O2 LPS.

[b] 5-Acetamido-7-(*N*-acetyl-D-alanyl)amido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid from KX-V212 LPS.

[c] 5,7-Diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid isolated from N-deacylated and N-acetylated KX-V212 LPS.

[d] More than 1000 μg/mL.

2.2. Serological studies

In ELISAs using polyclonal anti-whole cell antisera, strong cross-reactivities were shown between O2 LPS and OUT KX-V212 LPS. For instance, anti-O2 antiserum reacted with OUT KX-V212 LPS, giving a titre of 1430, which was almost equivalent to that (1930) observed for O2 LPS. Anti-OUT KX-V212 antiserum also reacted with OUT KX-V212 LPS and O2 LPS giving titres of 182 and 270, respectively. The serological specificity of both LPSs was examined by an ELISA inhibition test (Table 3). The sugar compositions of the LPSs and chemically modified LPSs used as inhibitors are given in Table 4. The hydrofluoric acid (HF) treatment of O2 and OUT KX-V212 LPS caused release of not only phosphate, but also galacturonic acid present in both native LPSs. In contrast, 5NAc7NAcNonlA and 5NAc7NAlaNAcNonlA were still present in the dephosphorylated LPS (HF-LPS) of O2 and OUT KX-V212, respectively. The OS portions of LPS (OS) lacked 5NAc7NAcNonlA and 5NAc7NAlaNAcNonlA in addition to galacturonic acid, all of which were completely released by the mild acid treatment of LPS used for preparation of OS. In the ELISA systems, using O2 LPS/anti-O2 antiserum and OUT KX-V212 LPS/anti-OUT KX-V212, LPS and HF-LPS from respective homologous strains, all revealed similarly strong inhibitory activities. In contrast, OS did not show any inhibitory activity. In addition, 5NAc7NAcNonlA isolated from O2 LPS and N-deacylated and N-acylated OUT KX-V212 LPS showed weak, but significant inhibitory activities against the ELISA system using O2 LPS/anti-O2 antiserum, compared with those of β-Gal-(1→4)-Glc or GlcA, which were present at non-reducing terminals of the polysaccharide portion of O2 LPS (unpublished data). On the other hand, 5NAc7NAlaNAcNonlA isolated from OUT KX-V212 LPS did not inhibit the ELISA systems using O2 LPS/anti-O2 antiserum and OUT KX-V212 LPS/anti-OUT KX-V212 antiserum. These results suggested that phosphate and galacturonic acid were not involved in the epitope structures of both LPSs and that, in the case of O2 LPS, 5NAc7NAcNonlA might be one of a number of major immunodeterminat group(s) related to expression of the serological specificity of O2 LPS. On the other hand, 5NAc7NAlaNAcNonlA might be a minor antigen factor of the OUT KX-V212, which did not cross-react with 5NAc7NAcNonlA because of its D-alanyl substituent at position C-7.

3. Discussion

In the present study, N-acylated NonlA has been demonstrated to be an acidic constituent of polysaccharide portions of the LPS from *V. parahaemolyticus* O2 and OUT KX-V212. The stereoisomeric configuration of the NonlA was identified as the D-*glycero*-D-*galacto* configuration, based on ^{1}H and ^{13}C NMR data obtained in this study, as well as on those reported for synthetic NonlA isomers.[21] Isomers of NonlA are known to distribute as constituents of O-polysaccharides in the LPSs of Gram-negative bacteria such as genus *Pseudomonas*,[9–11,14,21] *Shigella*,[9] *Legionella*,[12,13] *Vibrio*,[15,17] *Acinetobactor*,[16] *Salmonella*,[17] *Yersinia*[19] and *Proteus*[20]. Their originally described stereoisomeric configurations have been revised[22] by using chemically synthesized D-*glycero*-D-*galacto* and D-*glycero*-D-*talo* isomers. According to these data, Gram-negative bacteria containing NonlA in their O-polysaccharides can be classified into four groups, i.e., those containing the D-*glycero*-D-*galacto* isomer, the L-*glycero*-D-*galacto* isomer, the D-*glycero*-D-*talo*- isomer or the L-*glycero*-L-*manno* isomer. In the present study, it was demonstrated that *V. parahaemolyticus* belong to the first, the D-*glycero*-D-*galacto* isomer-containing group in which taxonomically related members of genus *Vibrio*, *V. salmonicida* and *V. alginolyticus* are also included.

Some of the NonlAs hitherto reported carry N-substituents other than the acetyl group, for instance, a 3-hydroxybutyryl group at N-5 (*A. baumannii* O24, *P. fluorecens*, *L. pneumophila* and *V. salmonicida*) and formyl at position N-7 (*P.aeruginosa* O5).[23] The amino groups on C-5 and C-7 of NonlA isolated from LPS of *V. parahaemolyticus* O2 are both acetylated; however, in NonlA present in the LPS from OUT KX-V212, the amino group on C-7 is acylated by a D-alanyl residue. This is the first demonstration of the existence of a NonlA derivative carrying an amino acid on its amino group as a constituent of the polysaccharide portion of LPS. The contribution of NonlA to the serological and physicochemical properties of LPS has also been discussed. In this study, we examined the relationship of NonlA to the serological specificities of LPS by means of an ELISA inhibition test using NonlA-containing HF-LPS, NonlA-defective OS and isolated NonlA itself. Against the ELISA system O2 LPS/anti-O2 whole cell antiserum, both LPS and HF-LPS exhibited similarly strong inhibitory activities, whereas OS did not show any inhibitory activity. In particular, 5NAc7NAcNonlA monomer isolated from O2 LPS, as well as from N-deacylated and N-acetylated OUT KX-V212 LPS, also exhibited weak, but significant inhibitory activity against the same ELISA system. Therefore, in *V. parahaemolyticus* O2 LPS, 5NAc7NAcNonlA must relate to its serological specificity as one of the main epitopes. On the other hand, 5NAc7NAlaNAcNonlA did not inhibit the ELISA system of O2, indicating that the acetyl group on the amino group at C-7 is essential for expressing the serological specificity of O2 LPS. LPS and HF-LPS from KX-V212 also exhibited a strong inhibitory activity against the homologous ELISA system. However, in this case, it was suggested that 5NAc7NAlaNAcNonlA might form part of the epitope(s), but not a main epitope that determines the serological specificity of this strain. 5NAc7NAcNonlA is a component of LPS of serotype O2, only, out of 13 O-serotypes of *V. parahaemolyticus*. Therefore, a monoclonal antibody raised against it will be most useful for serological diagnosis to avoid confusion caused by the serological cross-reactivity frequently observed between O2 and other serotypes of this *Vibrio*.

4. Experimental

4.1. Bacteria and LPS

V. parahaemolyticus strains V95–269 (O2; K3) and OUT KX-V212 were kindly provided by Dr A. Kai, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan and Dr M. Nishibuchi, Center for Southeast Asian Studies, Kyoto University, Kyoto, Japan, respectively. Both strains were cultured in nutrient broth supplemented by 3% NaCl at 37 °C for 16 h. LPSs were isolated from heat-killed, acetone-dried cells by a hot phenol–water procedure[24] and purified as previously described.[3]

Table 4
Sugar composition of LPS, HF-LPS and OS isolated from *V. parahaemolyticus* O2 and OUT strain KX-V212

Component sugar	O2			KX-V212		
	LPS	HF-LPS	OS	LPS	HF-LPS	OS
Glucose	0.214	0.328	0.807	0.343	0.336	0.497
Galactose	0.144	0.168	0.604	0.221	0.240	0.643
L-*glycero*-D-*manno*-heptose	0.018	0.033	0.065	0.026	0.048	0.054
D-*glycero*-D-*manno*-heptose	0.013	0.100	0.284	−	−	−
Glucosamine	0.088	0.380	−	0.199	0.231	−
Uronic acid [a]	0.359	0.340	1.251	0.648	0.524	1.387
Glucuronic acid	+	+	+	+	+	+
Galacturonic acid	+	−	−	+	−	−
Kdo [b]	+	+	+	+	+	+
5NAc7NAcNonlA [c]	+	+	−	−	−	−
5NAc7NAlaNonlA [d]	−	−	−	+	+	−
Phosphate	1.329	0.036	0.134	1.002	0.073	0.116

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

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

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

[d] 5-Acetamido-7-(*N*-acetyl-D-alanyl)amido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid. Values were expressed as μmol/mg of samples.

4.2. Chemical treatment of LPS and isolation of NonlA

LPSs were dephosphorylated in 48% HF (50 mg/mL) at 4 °C for 48 h, and HF-LPSs were recovered by lyophilization of the reaction mixture after neutralization and successive dialysis against distilled water. For preparation of the OS of LPS and NonlA, the LPS were treated with 5% (v/v) AcOH at 100 °C for 2.5 h. After removal of the lipophilic precipitate by centrifugation, the supernatant was subjected to gel-permeation chromatography on a Sephadex G-25 column, which was eluted with 8:5:2000 pyridine–AcOH–water, and the fractions corresponding to OS and NonlA were respectively combined and freeze dried. The NonlA obtained was further purified by HPLC on YMC-pack ODS-AQ (YMC, Kyoto, Japan) (5:995 MeOH–water, 1 mL/min). Deacylation of OUT KX-V212 LPS was carried out by treatment with 4 M KOH at 100 °C for 16 h. The reaction mixture was neutralized, N-acetylated and desalted by Sephadex G-25 gel-permeation chromatography as described above. Deacylated and N-acetylated NonlA were also isolated from the deacylated and N-acetylated OUT KX-V212 LPS by means of mild acid hydrolysis, gel-permeation chromatography and HPLC as mentioned above.

4.3. Chemical analysis

Neutral and amino sugars were analyzed by GC and GC–MS as described previously.[8] The total uronic acid content was estimated by the carbazole–H_2SO_4 method,[25] and glucuronic and galacturonic acids were identified by GC and GC–MS as previously described.[7] Phosphate was determined by the method of Lowry and co-workers.[26] Since the LPSs of *V. parahaemolyticus* O2 and KX-V212 were negative in the thiobarbituric acid reaction,[27] Kdo was qualitatively detected by GC and GC–MS as its O-acetylated methyl ester methyl glycoside.[28] Alanine was analyzed after hydrolysis (6 M HCl, 100 °C, 16 h) of NonlA from the LPS of OUT KX-V212 using a JLC-300 amino acid analyzer (JEOL, Tokyo). For GC and GC–MS analysis, NonlA (3 mg) was O-permethylated,[29] reduced with $NaBD_4$ in 1:1 EtOH–water at room temperature for 16 h and O-peracetylated in 1:1 pyridine–Ac_2O at 100 °C for 30 min. The absolute configuration of Ala was determined by GC–MS as the N-acetylated (*S*)-(+)-2-butylesters derived by butanolysis in 2 M HCl in (*S*)-(+)-2-BuOH at 86 °C for 2 or 3 h, followed by N-acetylation in Py–Ac_2O.

4.4. 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, Kyoto, Japan) or HR52 (Chromato Packing Center, Kyoto, Japan) using a temperature program of 180 °C (3 min) increased to 240 °C at 5 °C/min for neutral and amino sugar analysis (DB210), 100 °C (3 min) increased to 200 °C at 3 °C/min for Ala (DB210) and 150 °C (3 min) increased to 320 °C at 5 °C/min for NonlA (HR52). GC–MS and FABMS were carried out using a JMS-700 (JEOL, Tokyo) instrument.

4.5. NMR spectroscopy

One-dimensional ^1H, ^{13}C and gated ^{13}C NMR, two-dimensional PDQF-COSY, PHSQC and HMBC NMR spectra of NonlA dissolved in D_2O were recorded using a JEOLα-500 instrument at 30 °C. Acetone (δ_H 2.22, δ_C 31.45) was used as an internal reference.

4.6. ELISA

Polyclonal anti-whole cell antisera against *V. parahaemolyticus* O2 and KX-V212 were prepared by immunizing rabbits with heat-killed whole cells.[30] The ELISA was carried out basically according to the method of Bartodziejska and co-workers.[31] Maxi-sorp U-bottom microplates (Nunc, Demmark) were coated with LPS (500 ng/well) dissolved in 150 mM NaCl containing 15 mM Na_2HPO_4, pH 7.2 (NaCl/Pi) at 4 °C overnight. Plates were washed with NaCl/Pi, and non-specific binding sites were blocked with NaCl/Pi containing 2.5% casein at 37 °C for 1 h. The plates were washed four times with NaCl/Pi, and antisera (50 μL) appropriately diluted with NaCl/Pi containing 2.5% casein were added. After incubation at 37 °C for 1 h and washing with NaCl/Pi, 50 μL of peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, AL, USA) diluted 1:1000 with 5% BSA in NaCl/Pi was added and incubated for 1 h at 37 °C. The plates were washed with NaCl/Pi and 100 mM sodium citrate pH 4.5 (substrate buffer), and then 50 μL of freshly prepared substrate solution [2,2′-azino-bis(3-ethylbenzthiazolinesulfonic acid), 1 mg/mL] and 25 μL of aq 0.1% H_2O_2 were added. The reaction was stopped after 30 min incubation at 37 °C by adding 50 μL of 2% oxalic acid, and the absorbance was read at 405 nm using a microplate reader Model 550 (Bio-Rad, Japan). The end titre yielding $A_{405} > 0.2$ was taken as the reciprocal of the highest dilution of antiserum. For inhibition of ELISA, 50 μL of serially diluted inhibitor solutions in NaCl/Pi were mixed with an equal volume of anti-*V. parahaemolyticus* O2 and KX-V212 antisera which were diluted with NaCl/Pi to give A_{405} 1.0–1.2 without adding inhibitor. After incubation for 1 h at 37 °C, the inhibitor–antiserum mixture (50 μL) was added to antigen-coated plates, and further steps were performed as described above. The concentration of

inhibitor causing 50% inhibition was expressed as a 50% inhibition dose.

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