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Note

Structure of the O-specific polysaccharide from *Shewanella japonica* type strain KMM 3299^T containing the rare amino sugar Fuc4NAc☆

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Abstract—An acidic O-specific polysaccharide (PS) of the agar-digesting bacterium *Shewanella japonica* with the type strain KMM 3299^T was obtained by mild acid hydrolysis of the lipopolysaccharide. The polysaccharide was studied by component analysis, methylation analysis, ^{1}H and ^{13}C NMR spectroscopy, including 2D NMR experiments. The PS was determined to have the following structure involving three unusual amino sugars:

$$\rightarrow 3)\text{-}\alpha\text{-D-Fuc}p4\text{NAc-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Qui}p\text{NAc4NAc-}(1\rightarrow$$

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The genus *Shewanella* is currently comprised of 24 species of Gram-negative facultatively anaerobic *Proteobacteria* and is associated mainly with aquatic habitats. The bacteria of this genus have received a significant amount of attention due to their important roles in co-metabolic bioremediation of halogenated organic pollutants,[1] destructive oxidation of crude petroleum[2] and the dissimilatory reduction of magnesium and iron oxides.[3] Phylogenetic relationships, the taxonomy of these proteobacteria and an improved approach for identification of newly isolated wild strains have been considered elsewhere.[4] Recently, agar-digesting bacteria of the genus *Shewanella* isolated from seawater of the coastal area of the Japan Sea, have been described. Phylogenetic evidence, together with phenotypic characteristics, showed that the new isolate constituted a novel species of the genus *Shewanella*—*S. japonica* with the type strain KMM 3299^T (=LMG 1969^T = CIP 106860^T).[5]

The structures of a number of *Shewanella* polysaccharides have recently been reviewed.[6] The structures of antigenic polysaccharides from a number of *Shewanella* species containing unusual acidic *N*-acylamino sugars and non-sugar substituents have been determined by us,[7–9] and by others.[10–12] The novel *C*-branched nine-carbon sugar named shewanellose was also identified as a component of the hydrophobic antigenic polysaccharide from *S. putrefaciens* clinical strain A6.[7] In this

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paper we report the results of the structural analysis of an antigenic polysaccharide from *S. japonica* type strain KMM 3299^T containing three unusual sugar residues.

Bacterial cells were extracted with hot aqueous phenol and lipopolysaccharide (LPS) was recovered from the aqueous layer. The LPS was then degraded with dilute acetic acid followed by centrifugation and fractionation by GPC to yield the O-specific polysaccharide (PS). GLC analysis of acetylated alditol acetates after full acid hydrolysis of the PS demonstrated the presence of 2-amino-2,6-dideoxy-galactose (FucN). Moreover, methanolysis of the PS followed by GLC analysis of acetylated methyl ester methyl glycosides showed the presence of galacturonic acid (GalA). Analysis of acetylated glycosides with (*S*)-2-butanol showed that FucN has the L configuration while GalA has the D configuration.

The compositional analysis was completed by methylation data of the PS with carboxyl-reduction prior to hydrolysis. This resulted in the identification of 2,3-di-*O*-methylhexose (derived from GalA), 4,6-dideoxy-2-*O*-methyl-4-(*N*-methyl)acetamidohexose (derived from Fuc4N, vide infra), 2,6-dideoxy-4-*O*-methyl-2-(*N*-methyl)acetamidohexose (derived from FucN) and 2,4,6-trideoxy-2,4-(*N*-methyl)diacetamidohexose (derived from QuiNAc4NAc, vide infra).

The ^{13}C NMR spectrum of the PS (Fig. 1, Table 1) indicated a tetrasaccharide repeating unit with four anomeric signals at δ 98.7–102.9. It also showed three signals of CH_3–C groups for 6-deoxy sugars at δ 16.6, 16.8 and 18.2 (C-6 of Fuc4N, FucN and QuiN4N, respectively), four carbons bearing nitrogen at δ 49.0, 54.4, 56.8 and 57.6 (C-2 of FucN, C-4 of Fuc4N, C-4 and C-2 of QuiN4N, respectively), 12 other ring carbons in the region δ 67.4–80.4, carbonyl groups in the region of δ 175.2–176.1 and four *N*-acetyl signals (Me) at δ 23.1–23.6. The absence from signals of non-anomeric sugar carbons at a lower field than δ 82 in the ^{13}C NMR spectrum demonstrated the pyranoid form of all sugar residues.[13] Hence, the PS is linear and contains 3-substituted residues of QuiNAc4NAc, Fuc4NAc and FucNAc and 4-substituted GalA. The ^{1}H NMR spectrum of the PS contained four anomeric signals at δ 4.68, 4.93 and ~5.1 (double intensity), three CH_3–C groups of 6-deoxy sugars at δ 1.03 and ~1.21 (double intensity) and four *N*-acetyl groups at δ 1.94–2.06.

The ^{1}H and ^{13}C NMR spectra of the PS were assigned using 2D homonuclear COSY, TOCSY, ROESY and ^{1}H,^{13}C HSQC experiments (Table 1). The spin system of QuiNAc4NAc was identified by H-1/H-2 up to H-6 correlations found in the TOCSY spectrum. The COSY and TOCSY spectra showed cross peaks of H-1/H-2 up to H-4 for the other three sugar residues with the *galacto* configuration. The HMBC spectrum allowed the assignment of the remaining signals of FucNAc and Fuc4NAc using H-6/C-4, C-5 correlations at δ 1.21/72.2, 68.1 and δ

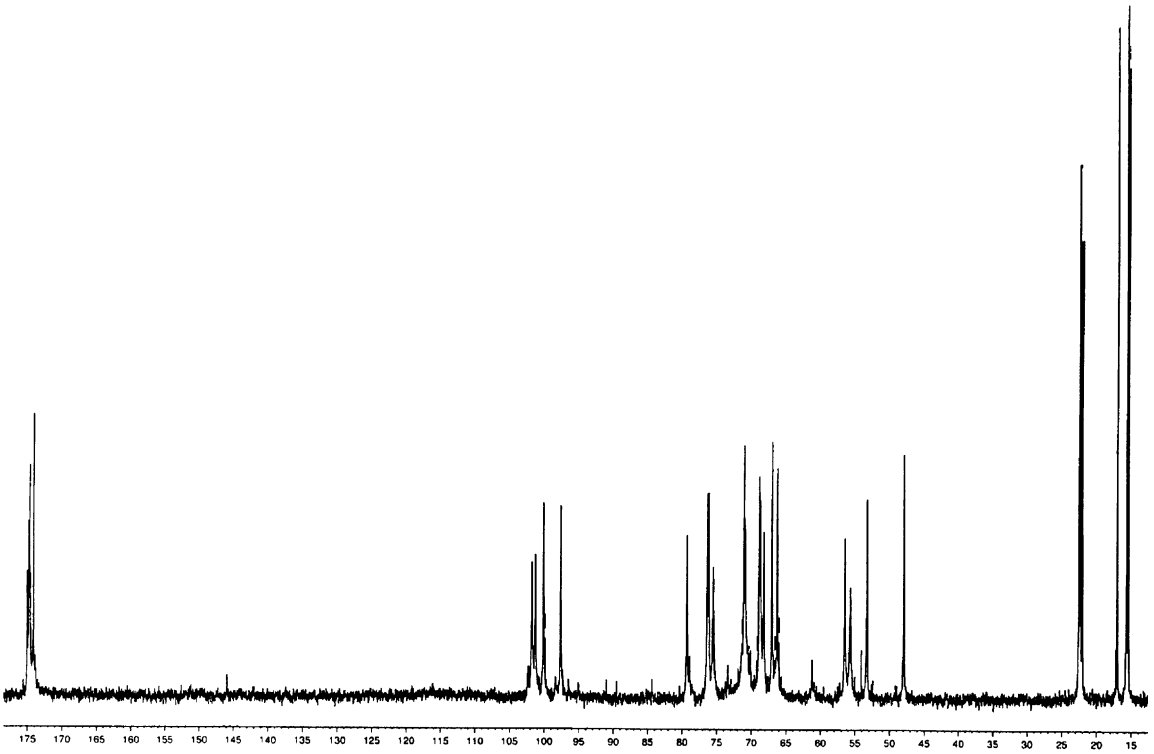

Figure 1. ^{13}C NMR spectrum (125 MHz) of the O-specific polysaccharide from *S. japonica* type strain KMM 3299^T.

Table 1. ^{1}H and ^{13}C NMR data (δ_H, ppm) of the signals from the polysaccharide of *S. japonica* type strain KMM 3299^T and inter-residue correlations from anomeric protons in the NOESY spectrum and anomeric atoms in the HMBC spectrum

Sugar residue	1	2	3	4	5	6	NOE	HMBC
→4)-α-D-Gal*p*A-(1→	5.10 [173]	3.82	4.04	4.35	4.40		H-3, **B**	
	102.4	69.7	70.0	80.4	72.2	175.7		
	(9.3)	(0.7)	(−0.3)	(8.8)	(−0.1)	(−0.7)		
→3)-α-L-Fuc*p*NAc-(1→	5.09 [172]	4.26	3.86	3.81	3.90	1.21	H-3, **D**	C-3, **D**
	98.7	49.0	77.5	72.2	68.1	16.8		
→3)-α-D-Fuc*p*4NAc-(1→	4.93 [172]	3.66	4.02	4.31	4.52	1.03	H-4, **A**	C-4, **A**
	101.2	70.0	77.3	54.4	67.4	16.6		H-4, **A**
	(7.7)	(0.2)	(7.7)	(−0.9)	(1.0)	(−0.1)		
→3)-β-D-Qui*p*NAc4NAc-(1→	4.68 [162]	3.87	3.75	3.67	3.57	1.21	H-3, **C**	C-3, **C**
	102.9	57.6	76.6	56.8	72.0	18.2		H-3, **C**
	(7.2)	(−0.9)	(3.9)	(−1.1)	(−0.1)	(0.2)		

$J_{H-1,C-1}$ values are given in square brackets. ^{13}C chemical shift differences as compared to the corresponding monosaccharides are given in parenthesis. Chemical shifts for *N*-acetyl groups are δ_H 1.94, 2.01, 2.06 (×2), δ_C 23.1, 23.4, 23.6 (×2), 175.2–176.1.

1.03/54.4, 67.4, respectively. In order to locate the position of the H-5/C-5 correlation of GalA, the pD of the sample in solution was altered from 3.5 to 1 using one drop of deuterated trifluoroacetic acid. As is typical for uronic acids with free carboxyl groups,[14] the chemical shift for C-5 of the GalA residue was observed to have shifted from δ 72.2 to 71.2. Other ring carbon resonances were changed insignificantly. Importantly, the low-field anomeric proton resonances separated at pD ~1 and were observed at δ 5.16 and 5.10.

The $J_{1,2}$ coupling constant value of ~8 Hz for the anomeric proton of QuiNAc4NAc revealed that it is β-linked, whereas the $J_{1,2}$ values of ~3 Hz showed that the other residues are α-linked. The sequence of the sugar residues in the repeating unit was determined from $^{1}H,^{1}H$ NOESY, ROESY and $^{1}H,^{13}C$ HMBC experiments. The connectivities are given in Table 1, from which the sequence →3)-α-Fuc*p*4NAc-(1→4)-α-Gal*p*A-(1→3)-α-Fuc*p*NAc-(1→3)-β-Qui*p*NAc4NAc-(1→ can be deduced. Linkage sites on all sugars were confirmed by the downfield shift of the carbon resonances in question in each case. Thus, a low-field position of the signals for C-4 of GalA[15] and C-3 of QuiNAc4NAc,[16] Fuc4NAc[17] and FucNAc[18] at δ 80.4, 76.6, 77.3 and 77.5, respectively, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides or a similar substitution pattern, confirmed the glycosylation position of each sugar residue.

Determination of the absolute configuration of a sugar residue by NMR spectroscopy is possible from analysis of ^{13}C glycosylation shifts, if the absolute configuration of one of the sugar residues is known. The ^{13}C glycosylation shifts of D-Gal*p*A indicate that the α-linked Fuc4NAc residue has the D configuration (Table 9 in Ref. 19). Furthermore, the large glycosylation shift of C-1 in α-D-GalA shows that the FucNAc residue has a different absolute configuration, namely the L configuration (Table 3 in Ref. 19). Since this result was already known from the chemical analysis, it corroborates the approach based on glycosylation shifts. The ^{13}C chemical shifts for C-2 to C-4 in β-D-QuiNAc4NAc are closely similar to those of bacillosamine residues in which an α-L-hexopyranosyl residue substitutes O-3 of a β-D-Qui*p*NAc4NAc residue.[18,20] This indicates that in the O-antigen of *S. japonica* KMM 3299^T also, QuiNAc4NAc has the D configuration. In addition, the downfield chemical shift, that is, the large glycosylation shift, for C-1 in β-D-Qui*p*NAc4NAc and the absence of a significant inter-residue $^{1}H,^{1}H$ NOE between H-1 in β-D-Qui*p*NAc4NAc and H-4 in Fuc4NAc indicates that the latter has the same absolute configuration. Thus, FucNAc has the L configuration while the other three residues have the D configuration.

Therefore, it is concluded that the structure of the repeating unit from the O-specific polysaccharide of *S. japonica* KMM 3299^T is as follows:

→3)-α-D-Fuc*p*4NAc-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Qui*p*NAc4NAc-(1→

It is interesting to note that three of the four residues present in this polysaccharide are amino sugars. The unusual diamino sugar bacillosamine has previously been found in the capsular polysaccharide of *Alteromonas* sp. KMM 155,[6,21] in the O-antigens of *Pseudomonas aeruginosa*,[17] *P. aurantica* IMB 31,[18] *P. reactans*,[22] *Vibrio cholerae* O3,[23] O5[24] and O8,[20] *Fusobacterium necroforum*,[25] *Francisella novicida* (U112),[26] *Flavobacterium psychrophilum*,[27] *Ralstonia pickettii*,[28] *Pseudoalteromonas haloplanktis* ATCC 14393[29] and in the S layer of *Bacillus licheniformis*.[30]

The sugar 4-acetamido-4,6-dideoxy-α-D-galactose has been found before in the cyclic enterobacterial common antigen from *Yersinia pestis*[31] and in the repeating unit of the O-chain polysaccharide of *P. fluorescens* biovar A strain IMV 1152.[17]

1. Experimental

1.1. Bacterial strain, growth and isolation of LPS

S. japonica type strain KMM 3299^T was isolated from a seawater sample from a depth of 0.5–1.5 m at the Pacific Institute of Bioorganic Chemistry of the Marine Experimental Station, Troitza Bay, Gulf of Peter the Great, Japan Sea. Bacteria were grown with shaking (160 rpm) on the modified Yoshimizu–Kimura medium (36 h, 20 °C).[32] Wet bacterial cells from 20 L of the cultural fluid were extracted with hot aq 45% phenol as described.[33] The aqueous layer was separated by centrifugation, dialyzed against distilled water, concentrated and freeze-dried to yield LPS (660 mg).

1.2. Degradation of LPS

The LPS was hydrolyzed with aq 2% HOAc (100 °C, 3 h), the lipid A precipitate (26%) was removed by centrifugation. The water-soluble portion was concentrated and fractionated by GPC on a column (1.0 × 100 cm) of TSK-50 (F) gel in water to give the O-specific polysaccharide (48% of LPS weight).

1.3. Sugar analysis

The polysaccharide was hydrolyzed with 2 M CF_3COOH (120 °C, 2 h). The monosaccharides were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (25 °C, 1 h), acetylated with a 1:1 (v/v) mixture of pyridine and Ac_2O (120 °C, 0.5 h) and analyzed by GLC. Methanolysis of the polysaccharide (1 mg) was carried out using 1 M HCl–MeOH (85 °C, 16 h) followed by acetylation with Ac_2O in pyridine (120 °C, 30 min) with subsequent GLC analysis. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-(+)-2-butyl glycosides according to the published methods.[34,35] GLC was performed using a Hewlett–Packard 5890 Series II instrument equipped with an HP fused silica column (0.25 mm × 30 m) using a temperature programme of 170–180 °C (1 °C/min) followed by a temperature of 180–230 °C (7 °C/min).

1.4. Methylation analysis

Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[36] A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aqueous 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in the sugar analysis, converted into alditol acetates and analyzed by GLC–MS on a TermoQuest Finnigan mass spectrometer model Trace GC 2000 series equipped with an EC-1 column (0.32 mm × 30 m). A temperature gradient of 150 °C for 2 min followed by 10 °C/min to 250 °C was used.

1.5. NMR spectroscopy

Samples were deuterium exchanged by freeze-drying three times from D_2O and then examined in solutions of 99.97% D_2O, using internal acetone as reference (δ_H 2.225, δ_C 31.45). NMR spectra were recorded at 30 °C (pD 3.5) and 25 °C (pD 1) on a JEOL Lambda 400 MHz spectrometer equipped with a DEC AXP 300 computer workstation. The mixing time for the ROESY experiment was 250 ms. For the TOCSY experiment the duration of the MLEV17 spin lock was 80 ms. Other 2D parameters were essentially the same as previously described.[37] In addition, some NMR spectra were recorded on Bruker 500 MHz (HMBC delay time = 50 ms) and Varian Inova 600 MHz (NOESY mixing time = 100 ms) spectrometers.

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