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Structure of the acidic polysaccharide chain of the lipopolysaccharide of *Shewanella alga* 48055

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Abstract

A lipopolysaccharide (LPS) with an acidic polysaccharide chain was isolated from the bacterium *Shewanella alga* strain 48055 and cleaved selectively at the glycosidic linkage of *N*-acetylneuraminic acid to give a tetrasaccharide. Studies of the tetrasaccharide and the *O*-deacylated LPS by ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, rotating-frame NOE spectroscopy (ROESY), and H-detected $^{1}H,^{13}C$ heteronuclear multiple-quantum coherence (HMQC) experiments, revealed the following structure of the polysaccharide repeating unit:

→3)-β-D-Gal*p*A6GroN-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*A6GroN-(1→4)-α-Neu*p*5Ac-(2→

where GroN is an amidically linked residue of 2-amino-1,3-propanediol (2-amino-2-deoxyglycerol). A similar structure, but with 2-acetamido-2,6-dideoxy-D-glucose instead of 2-acetamido-2-deoxy-D-glucose, has been reported previously for the polysaccharide chain of a non-O1 *Vibrio cholerae* H11 LPS [E.V. Vinogradov, O. Holst, J.E. Thomas-Oates, K.W. Broady, and H. Brade, *Eur. J. Biochem.*, 210 (1992) 491–498].

Keywords: *Shewanella alga*; Lipopolysaccharide; Polysaccharide; Structure; *N*-Acetylneuraminic acid; 2-Amino-2-deoxyglycerol; NMR spectroscopy

1. Introduction

Aquatic Gram-negative bacteria of the genus *Shewanella* are known as a fish pathogen, and also isolated from oil drilling, marine alga, clinical specimens, food, and other sources. They have been associated with bacteremic infections in previously reported cases [1–6] and rarely implicated as a pathogen in humans. Recently, it has been proposed that most clinical isolates of *Shewanella* from humans in Japan belonged to *Shewanella alga* [7] which was firstly isolated from red algae [8].

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Studies of cell-surface polysaccharides of *Shewanella* are scarce. Short-chain lipopolysaccharides (LPSs) which are similar to enterobacterial R-form LPSs, were characterized in some *Shewanella* spp [9,10]. Recently, we have determined a structure of an acidic polysaccharide from a *S. putrefaciens* strain which was acid-labile due to the presence of a glycosyl phosphate group [11]. Now, we report on the structure of another acidic polysaccharide which is present in LPS of *S. alga* 48055 isolated from blood of a patient with lower leg ulcers [12].

2. Results and discussion

Bacterial cells were extracted with hot aqueous phenol [13], LPS was recovered from the aqueous layer and studied without further purification. LPS contained a sialic acid, as determined by a colorimetric assay after *O*-deacylation. Mild acid hydrolysis which is conventionally used for delipidation of LPS, resulted in depolymerization of the polysaccharide chain to give an oligosaccharide (**1**) isolated by GPC. Sugar analysis of **1** using ion-exchange chromatography after full acid hydrolysis revealed galacturonic acid as well as 2-amino-2-deoxyglucose and 2-amino-1,3-propanediol (2-amino-2-deoxyglycerol, GroN) in the ratio ~1:2. GLC of acetylated (*R*)-2-butyl glycosides showed that GalA and GlcN have the D configuration.

The ^{13}C NMR spectrum of **1** contained signals for four anomeric carbons at δ 97.4–104.4, four carbons bearing nitrogen (C-2 of GlcN and two GroN residues, C-5 of Neu5Ac) at δ 51.2–56.3, one C-CH_2-C group (C-3 of Neu5Ac) at δ 37.6, six $HOCH_2$-C groups (C-1,3 of two GroN residues, C-6 of GlcN, C-9 of Neu5Ac) at δ 61.9–62.1 and 64.6, two $CONH_2$ groups (C-6 of GalA amides) at δ 171.6 and 171.8 (compare published data, e.g. [14,15]), one carboxyl group (C-1 of Neu5Ac) at *d* 177.2, 16 other sugar carbons at δ 67.8–83.1, and two *N*-acetyl groups (CH_3 at δ 23.5 and 23.7, CO at δ 175.3 and 176.2).

Accordingly, the ^1H NMR spectrum of **1** contained, inter alia, signals for three anomeric protons at δ 4.54–5.16, H-4,5 of GalA amides at δ 4.26–4.51, H-3 of Neu5Ac at δ 1.88 (axial) and 2.37 (equatorial), and two *N*-acetyl groups at δ 2.00 and 2.04.

Therefore, **1** is a tetrasaccharide containing two residues of a galacturonamide (most likely, with GroN which is unsubstituted at O-1,3) and one residue each of GlcNAc and Neu5Ac. As judged by the absence from the ^1H NMR spectrum of signals for anomeric protons of a reducing sugar residue and from a relatively low chemical shift difference between H-3*a* and H-3*e* (0.49 ppm) [16], the reducing end of **1** is occupied by a sialic acid with the equatorial carboxyl group (β-Neu5Ac).

The ^1H NMR spectrum of **1** was assigned using 2D COSY and TOCSY experiments (Table 1). The spin-systems for two GroN residues and four sugar residues, all present in the pyranose form, were distinguished by tracing connectivities in the 2D spectra. Despite a small $^3J_{4,5}$ coupling constant, an H-4/H-5 cross-peak for one of the GalA residues was clearly observed in both COSY and TOCSY spectra. Signals for H-4 and H-5 of the other GalA residue were coincident, and the position of the

Table 1
^1H NMR chemical shifts[a] (δ in ppm)

Sugar residue	Proton								
	H-1	H-2	H-3*a* H-3*e*	H-4	H-5	H-6a H-6b	H-7	H-8	H-9a H-9b
Tetrasaccharide **1**									
β-D-Gal*p*A6GroN-(1→	4.54	3.58	3.73	4.26	4.26				
→3)-β-D-Glc*p*NAc-(1→	4.75	3.92	3.89	3.64	3.54	3.96 3.84			
→3)-α-D-Gal*p*A6GroN-(1	5.16	3.90	3.88	4.51	4.30				
→4)-β-Neu*p*5Ac			1.88 2.37	4.11	4.09	4.08	3.55	3.77	3.85 3.63
Polysaccharide **2**									
→3)-β-D-Gal*p*A6GroN-(1→	4.58	3.58	4.19	4.28	4.22				
→3)-β-D-Glc*p*NAc-(1→	4.77	3.90	3.89	3.62	3.53	3.94 3.82			
→3)-β-D-Gal*p*A6GroN-(1→	5.19	3.92	3.87	4.48	4.27				
→4)-β-Neu*p*5Ac-(1→			1.76 2.93	3.81	4.04	3.79	3.45	3.62	3.85 3.67

[a]Chemical shifts for GroN are δ 3.68–3.73 (H-1,3) and 4.05–4.07 (H-2), for NAc δ 1.97–2.04.

H-5 signal was confirmed by an HMQC experiment. The monosaccharide residues were identified based on the $^3J_{H,H}$ coupling constant values, those for the aldulosonic acid, $J_{3a,4}$ 11, $J_{3e,4}$ 3, $J_{4,5}$ 10, $J_{5,6}$ 10, $J_{6,7}$ 2.5, $J_{7,8}$ 9, being typical of Neu5Ac (compare published data [17]). The $J_{1,2}$ value of 7.5 Hz demonstrated that one of the GalA residues and GlcNAc are β-linked, while the $J_{1,2}$ 3.4 Hz showed that the second GalA residue is α-linked. No significant shift for the H-5 signals of either α-GalA or β-GalA was observed on a change of pD of a solution of **1** in D_2O from 6 to 2, thus confirming amidation of both GalA residues.

The ^{13}C NMR spectrum of **1** was assigned using an HMQC experiment (Table 2). Downfield displacements of the signals for C-3 of GlcNAc and α-GalA and C-4 of Neu5Ac to δ 83.1, 80.2, and 74.9, as compared with their positions at δ 75.1, 70.6, and 68.4, respectively, in the corresponding unsubstituted monosaccharides [18], were due to the α-effects of glycosylation and revealed the substitution pattern in the linear tetrasaccharide. No such displacement was observed for β-GalA, thus showing the terminal position of this sugar at the non-reducing end. A relatively large β-effect of glycosylation (−2.3 ppm) on C-3 of Neu5Ac caused by its glycosylation at position 4 with α-GalA demonstrated different absolute configurations at C-6 of Neu5Ac and C5 of GalA [19], i.e. the L configuration at C-6 in Neu5Ac which, thus, has the expected D-*glycero*-D-*galacto* configuration.

Two interresidue proton correlations in **1** were revealed by a 2D ROESY experiment, namely, β-GalA H-1,GlcNAc H-3 at δ 4.54/3.89 and α-GalA H-1,Neu5Ac H-4 at δ 5.16/4.11, which demonstrated two partial sequences β-GalA→GlcNAc and α-GalA→Neu5Ac. Although no expected cross-peak GlcNAc H-1,α-GalA H-3 was observed, these and above data were sufficient for determination of the following complete structure of **1**:

β-D-Gal*p*A6GroN-(1→3)-β-D-Glc*p*NAc-(1→3)-
α-D-Gal*p*A6GroN-(1→4)-β-Neu*p*5Ac

1

Treatment of LPS with aqueous ammonia resulted in a polysaccharide (**2**) attached to the core-*O*-deacylated lipid A moiety of LPS. In the ^{13}C NMR spectrum of this product (Fig. 1), signals for the repeating unit of **2** were clearly observed. The spectrum demonstrated the presence of the same components as in **1** and, thus, tetrasaccharide **1** is a chemical repeating unit of polysaccharide **2**. A relatively large chemical shifts difference between H-3*a* and H-3*e* (1.17 ppm) indicated that the carboxyl group in Neu5Ac is axial [16] and, hence this sugar is α-linked.

Assignment of the ^1H and ^{13}C NMR spectra (Tables 1 and 2) and linkage and sequence analysis were performed for **2** as described above for **1**, except for that a NOESY experiment was applied instead of the 2D ROESY experiment. This revealed two cross-peaks β-GalA H-1,GlcNAc H-3 at δ 4.58/3.89 and α-GalA H-1,Neu5Ac H-4 at δ 5.19/4.04 as in **1** and, in addition, a cross-peak at δ 4.77/3.88 which was evidently a superposition of an interresidue cross-peak GlcNAc H-1,α-GalA H-3 and an intraresidue cross-peak GlcNAc H-1,H-3.

An HMQC experiment with **2** revealed low-field positions of the signals for C-3 of GlcNAc,

Table 2
^{13}C NMR chemical shifts[a] (δ in ppm)

Sugar residue	Proton								
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
Tetrasaccharide **1**									
β-D-Gal*p*A6GroN-(1→	104.4	71.7	73.6	70.3	75.8	171.6[b]			
→3)-β-D-Glc*p*NAc-(1→	103.8	56.3	83.1	69.9	76.8	62.1			
→3)-α-D-Gal*p*A6GroN-(1→	97.4	67.8	80.2	70.8	72.3	171.8[b]			
→4)-β-Neu*p*5Ac	177.2	99.9	37.6	74.9	51.2	71.5	69.9	71.9	64.6
Polysaccharide **2**									
→3)-β-D-Gal*p*A6GroN-(1→	104.2	70.2	76.8	69.0	75.6	171.3[b]			
→3)-β-D-Glc*p*NAc-(1→	103.7	56.3	83.0	69.9	76.8	62.2			
→3)-α-D-Gal*p*A6GroN-(1→	97.4	67.9	79.9	70.3	72.3	171.8[b]			
→4)-α-Neu*p*5Ac-(1→	177.2	101.1	38.5	75.6	50.8	73.9	69.6	73.1	64.0

[a]Chemical shifts for GroN are δ 61.9–62.0 (C-1,3) and 54.1–54.2 (C-2), for NAc δ 23.5–23.8 (CH_3) and 174.6–176.2 (CO).
[b]Assignment could be interchanged.

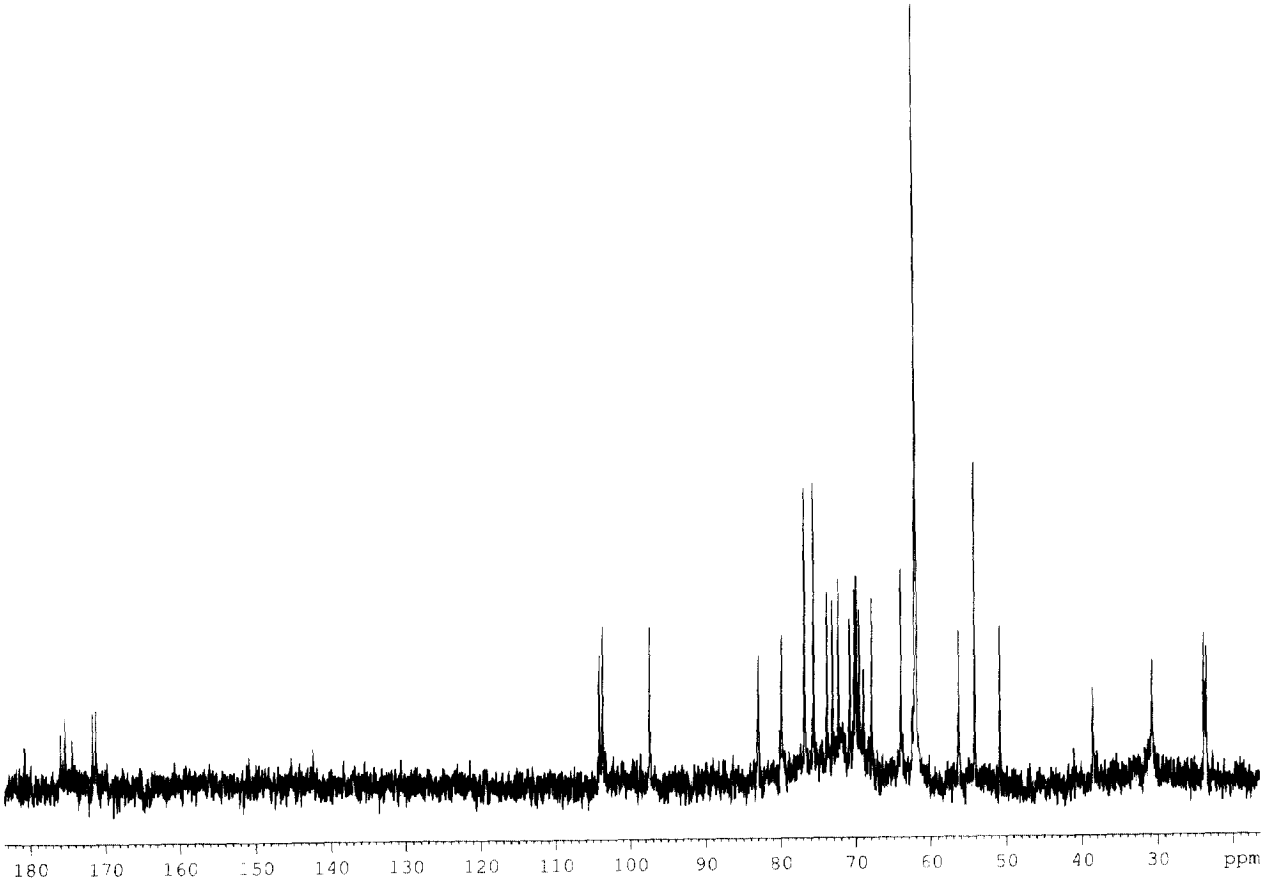

Fig. 1. ^{13}C NMR spectrum of the *O*-deacylated LPS of *S. alga* 48055. A signal for C-1 of Neu5Ac at δ 101.1 is poorly seen because of its broadening.

α-GalA, β-GalA and C-4 of Neu5Ac to δ 83.0, 79.9, 76.8, and 75.6, as compared with their positions at δ 75.1, 70.6, 74.1, and 68.4, respectively, in the corresponding unsubstituted monosaccharides [18]. Therefore, **2** is a linear polysaccharide, Neu5Ac is 4-substituted and three other sugar residues are 3-substituted.

These data were in agreement with the structure of **1** and indicated that **2** has the structure shown below. Thus, **1** resulted from selective cleavage of the glycosidic linkage of Neu5Ac in **2** during mild acid hydrolysis of LPS. The lability of this glycosidic linkage is well known and distinguishes sialic acids from structurally related derivatives of 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acids which are cleaved under these conditions only when the carboxyl group is equatorial [20].

→3)-β-D-Gal*p*A6GroN-(1→3)-β-D-Glc*p*NAc-
(1→3)-α-D-Gal*p*A6GroN-(1→4)-α-Neu*p*5Ac-(2→

2 (*S. alga* 48055)

The structure of polysaccharide **2** from *S. alga* 48055 resembles much that of the polysaccharide chain (**3**) of a non-O1 *Vibrio cholerae* H11 LPS [15]. The latter differs only in the presence of 2-acetamido-2,6-dideoxy-D-glucose (QuiNAc) instead of GlcNAc and in the linkage between the amino sugar and α-GalA6GroN (1→4 instead of 1→3). This linkage was demonstrated to connect biological repeating units in the *V. cholerae* polysaccharide [15]. Three other linkages within the repeating unit are the same in both polysaccharides.

→3)-β-D-Gal*p*A6GroN-(1→3)-β-D-Qui*p*NAc-
(1→4)-α-D-Gal*p*A6GroN-(1→4)-α-Neu*p*5Ac-(2→

3 (*V. cholerae* H11 [15])

The occurrence of structurally similar and even identical O-antigens in different bacterial species, including taxonomically remote species, is not uncommon [21,22] and may result in false serological diagnosis of infectious diseases.

3. Experimental

Bacterial strain, growth, and isolation of LPS.—*S. alga* strain 48055 isolated from patient's blood in Denmark in 1994, was obtained from Dr. B.F. Vogel (Danish Technical University, Lyngby) and grown on the Youschimizu–Kimura medium [23]. Wet bacterial cells from 20 L of the cultural fluid were extracted with hot aq 45% phenol as described [13], the aqueous layer was separated by centrifugation, dialyzed against distilled water, concentrated, and freeze-dried to yield LPS (600 mg).

Chemical degradations of LPS.—LPS was hydrolyzed with aq 1% HOAc (100 °C, 2 h), a lipid precipitate (10%) was removed by centrifugation, a water-soluble portion was concentrated and fractionated by GPC on a column (1.5×100 cm) of TSK-50 (F) in water to give tetrasaccharide **1** (65%). LPS was treated with aq 12% ammonia (37 °C, 16 h), and the *O*-deacylated LPS (50%) was isolated by GPC on a column (2.5×70 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5).

Chemical analyses.—Hydrolysis was performed with 2 M CF_3CO_2H at 120 °C for 2 h. Amino components were identified using a Biotronik LC-2000 amino acid analyzer, an Ostion LG AN B cation-exchange resin and standard sodium citrate buffers at 64 °C. Uronic acids were analyzed using a Biotronik LC-2000 sugar analyzer, a Dionex A×8–11 anion-exchange resin, and 0.02 M potassium phosphate buffer (pH 2.4) at 60 °C. The absolute configurations of GalA and GlcN were determined by the published method [24] modified as described [11]. Sialic acid was determined by the resorcinol reaction [25].

NMR spectroscopy.—Prior to measurement, samples were deuterium-exchanged by freeze-drying three times from D_2O. Spectra were recorded at 60 °C on a Bruker DRX-500 spectrometer. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45). A mixing time of 300 ms was used in 2D ROESY and NOESY experiments, and that of 120 ms in TOCSY experiments.

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