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The structure of the carbohydrate backbone of the LPS from *Shewanella putrefaciens* CN32

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Abstract

The lipopolysaccharide (LPS) from a natural rough strain of *Shewanella putrefaciens* CN32 was analyzed using NMR and mass spectroscopy and chemical methods, and the following structure of its carbohydrate backbone is proposed:

β-Gal*f*-(1→3)-β-Gal-(1→4)-β-Glc-(1→4)-α-DDHep2*P*EtN-(1→5)-α-Kdo4*P*-(1→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*

1. Introduction

Shewanella putrefaciens CN32 is a Gram-negative bacterium that was isolated from an anaerobic subsurface core sample taken 250 feet below the Morrison Formation in northwestern New Mexico. It (and other subsurface strains) are important environmental bacteria since they have been shown to have iron-reducing power and under anaerobic conditions can convert Fe(III) to Fe(II) in iron oxides, particularly hydrous iron oxide.[1,2] As the bacterium uses Fe(III) as a terminal electron acceptor, Fe(II) is liberated from the mineral, and an ensuing series of complex biogeochemical processes can result. New external iron mineral phases, such as magnetite, goethite and vivianite, can develop, and even unique intracellular iron phases can form.[3,4] In addition, the surface of *Shewanella* also has a strong affinity for preformed fine-grained minerals.[5] These reactions require a close surface association with environmental iron phases,[6] unless soluble electron shuttles are available,[7] for adhesion or respiration to occur. Frequently, the cells become enshrouded in nanomineral phases because cellular surface components attach to them so well.[8]

As a Gram-negative bacterium, *Shewanella* possesses a cell wall consisting of an outer membrane as its surface layer with lipopolysaccharide (LPS) as an important outermost component.[9,10] In many Gram-negative pathogens, such as the Enterobacteraceae, a long flexible O-side chain (the O-antigen) is positioned above a core oligosaccharide and a lipid A, and this type of LPS is referred to as the 'smooth' variety, since these bacteria form smooth colonies when grown on agar plates. Bacteria without the O-side chain produce rough colonies, and their 'rough' LPS consists of only lipid A and core oligosaccharide. Often, enterics with rough LPS do not survive well in natural environments. *S. putrefaciens* CN32 is different in this respect since it contains rough LPS under simulated natural conditions.[10,11] Since mineral–bacterial interactions are important for growth of this environmentally important bacterium, since LPS is a main surface component of CN32's outer membrane, and since the core oligosaccharide should be important for mineral adhesion, it was deemed important to determine the core's structure.

Abbreviations: LPS, lipopolysaccharide; DDHep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; P, phosphate; *P*EtN, phosphoethanolamine; HPAEC, high-performance anion-exchange chromatography.

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2. Results and discussion

Alkaline deacylation of the LPSs from *S. putrefaciens* gave a mixture of two oligosaccharides (**1** and **2**), differing by the position of a phosphate group (Scheme 1). HPAEC analysis of the products obtained after desalting of the deacylation products showed only two peaks in the ratio of approximately 3:1. Structures **1** and **2** were determined by NMR spectroscopy, mass spectrometry and chemical analysis without further separation. Assignment of the NMR spectra (Table 1, Fig. 1) showed the presence of seven monosaccharide residues in each oligosaccharide. The identity of the monosaccharides was established on the basis of chemical shifts and vicinal coupling constants. Heptose residue E had, according to the position of its C-6 signal at 71.7–72.0 ppm, the D-glycero-D-manno configuration, which was then confirmed by GLC analysis. Low-field ^{13}C chemical shifts of the residue H indicated its furanosidic ring form; comparison of the chemical shifts with the literature data[12] showed its galacto configuration. The sequence of monomeric components was determined using NOE and HMBC data. The following NOEs were observed in **1** and **2**: B1A6w, E1C5s, E1C7s, F1E4s, F1E6w, G1F4s, G1F6m, H1G3s. HMBC spectra contained all expected transglycosidic correlations.

Oligosaccharides **1** and **2** contained phosphate substituents at A1 (^{31}P signal at 2.1 ppm), B4 (4.3 ppm), C4 (4.3 ppm), E3 in **1** (4.2 ppm), E2 in **2** (4.3 ppm), which gave HMQC correlations to corresponding protons.

Electrospray-ionization mass spectra of oligosaccharides **1** and **2** (negative-ion mode) contained a peak of doubly charged ion corresponding to a molecular mass of 1558.6 (calculated for $C_{45}H_{82}N_2O_{49}P_4$ 1559.0).

Acetic acid hydrolysis of the LPS gave, after gel chromatograhic separation, a mixture of two major products (**3** and **4**), differing by galactofuranose residue H, partially lost during hydrolysis, in the ratio of ~3:1. Both of them had several Kdo derivatives (4,7- and 4,8-anhydro-Kdo products of dephosphorylation under acidic conditions, and smaller amount of Kdo-4-phosphate) at the reducing end. The phosphate from O-4 of the Kdo residue was mostly lost during acetic acid hydrolysis, as observed previously.[13] The presence of multiple Kdo derivatives prevented complete assignment of the signals of heptose residue E. Oligosaccharides contained a phosphoethanolamine substituent at O-2 of heptose residue E (^{31}P signal at 0.3 ppm, correlating to H-1 of EtN and to H-2 of heptose E).

Electrospray-ionization mass spectra of oligosaccharides **3** and **4** (negative-ion mode) contained peaks corresponding to molecular masses of 1021.4 (compound **3**, major, calculated for $C_{35}H_{60}N_1O_{31}P_1$ 1021.9), 859.2 (**4**), 1101.4 (**3** with phosphate on Kdo), 940.3 (**4** with phosphate on Kdo).

Monosaccharide analysis (GLC of the alditol acetates) confirmed the composition of the isolated compounds. Analysis of the mixture of oligosaccharides **1** and **2** showed the presence of Glc, Gal, GlcN, and DDHep in the molar ratio of ~1:2:1:1 (underestimate for GlcN is due to the presence of free amino groups and phosphorylation). The same components in the ratio of ~1:3:2:1 were determined in the analysis of the whole LPS. Analysis of the mixture of compounds **3** and **4** showed the presence of Glc, Gal, and DDHep in the molar ratio of ~1:2:1. Methylation analysis of the dephosphorylated and reduced (48% HF, 4 °C, 24 h, dialysis, $NaBH_4$, 25 °C, 2 h, dialysis) O-deacylated LPS led to the identification of the partially methylated alditol acetates, derived from terminal Gal*f* (H), 3-substituted Gal*p* (G), 4-substituted Glc*p* (F), 4-substituted Hep*p* (E), 6-substituted Glc*p*N (B) and 6-substituted GlcN-ol (A).

The absolute D configuration of Gal, Glc, DDHep, and GlcN were determined as D using GLC of acetylated 2-butyl glycosides. The absolute D configuration of the terminal Gal (H) was also determined by optical rotation measurement of the sample of free galactose obtained after mild acid hydrolysis of the LPS.

β-Gal*f*-(1→3)-β-Gal-(1→4)-β-Glc-(1→4)-α-DDHep3*P*-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P* **1**
(main)
H G F E C B A

β-Gal*f*-(1→3)-β-Gal-(1→4)-β-Glc-(1→4)-α-DDHep2*P*-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P* **2**
(minor)
H G F E C B A

β-Gal*f*-(1→3)-β-Gal-(1→4)-β-Glc-(1→4)-α-DDHep2*P*EtN-(1→5)-Kdo **3**
H G F E C

β-Gal-(1→4)-β-Glc-(1→4)-α-DDHep2*P*EtN-(1→5)-Kdo **4**
G F E C

Overall structure of the carbohydrate backbone of the LPS from *S. putrefaciens* CN32:

β-Gal*f*-(1→3)-β-Gal-(1→4)-β-Glc-(1→4)-α-DDHep2*P*EtN-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P* **5**

Scheme 1. Structures of the isolated oligosaccharides and proposed structure of the carbohydrate backbone of the LPS from *S. putrefaciens* CN32.

Table 1
^{1}H and ^{13}C NMR data for the isolated oligosaccharides [a]

Unit, compound	Nucleus	1	2 (3ax)	3 (3eq)	4	5	6(a)	7(a) (6b)	8a (7b)	8b
α-GlcN1*P* A, **1** or **2**	^{1}H	5.71	3.42	3.89	3.49	4.09	3.85	4.24		
	^{13}C	93.1	55.1	70.6	70.8	73.8	70.2			
	^{31}P	2.1								
α-GlcN4*P* B, **1** or **2**	^{1}H	4.84	3.11	3.87	3.91	3.73	3.60	3.75		
	^{13}C	100.2	56.7	72.9	75.4	75.0	63.9			
	^{31}P				4.3					
α-Kdo C, **1** or **2**	^{1}H		2.13	2.24	4.57	4.32	3.84	3.87	3.88	3.88
	^{13}C		100.5	35.0	70.8	74.0	73.2	70.3	64.5	
	^{31}P				4.3					
α-DDHep3*P* E, **1**	^{1}H	5.10	4.21	4.54	4.12	4.22	4.22	3.69	3.85	
	^{13}C	100.9	70.3	75.4	76.7	74.8	71.7	63.3		
	^{31}P			4.2						
α-DDHep2*P* E, **2**	^{1}H	5.28	4.50	4.06	3.96	4.33	4.17	3.71	3.88	
	^{13}C	99.9	75.2	69.9	78.5	74.0	72.1	63.3		
	^{31}P		4.3							
α-DDHep2*P*EtN E, **3** or **4***	^{13}C	[b]	76.1	70.4						
	^{31}P		0.3							
β-Glc F, **1** or **2**	^{1}H	4.58	3.35	3.62	3.65	3.56	3.77	3.97		
	^{13}C	103.5	74.3	75.5	79.4	76.1	61.1			
β-Glc F, **3** or **4**	^{1}H	4.58	3.37	3.65	3.65	3.64	3.78	3.96		
	^{13}C	104.0	74.6	75.9	79.9	76.2	61.8			
β-Gal G, **1** or **2**	^{1}H	4.47	3.62	3.71	4.05	3.72	3.70	3.75		
	^{13}C	103.8	71.5	81.2	69.8	76.4	62.1			
β-Gal G, **3**	^{1}H	4.47	3.62	3.71	4.05	3.72	3.73	3.73		
	^{13}C	104.4	72.0	81.8	70.3	76.9	62.7			
β-Gal G, **4**	^{1}H	4.41	3.52	3.64	3.90					
	^{13}C	104.7	72.7	74.3	70.3					
β-Gal*f* H, **1**–**3**	^{1}H	5.17	4.16	4.04	4.01	3.80	3.61	3.67		
	^{13}C	110.4	82.6	78.0	84.0	71.9	64.0			
α-Galp1P Z, O-deacylated LPS	^{1}H	5.57	3.75	3.85	4.01					
	^{13}C	97.7	70.1	71.2	70.8					
EtN	^{1}H	4.13	3.26							
	^{13}C	63.7	41.5							

[a] Chemical shifts which are given averaged for several products differ within ±0.02 ppm for ^{1}H and ±0.2 ppm for ^{13}C spectra.
[b] ^{1}H signals of E-1 at 5.19, 5.20, 5.24, 5.25, 5,42, 5.45; E-2 at 4.29, 4.34, 4.34, 4.35, 4.44, 4.45; E-3 at 3.95, 4.01–4.04; ^{13}C signals of E-1 at 97.3, 98.5 ppm.

NMR analysis of the O-deacylated LPS led to the identification of the structure **5** and additionally of a residue of α-galactopyranosylphosphate (residue Z). O-Deacylated LPS contained five phosphate groups [signals at 0.26 ppm (B4), 0.06 ppm (C4), −1.1 ppm (A1), −1.4 ppm (E2), and −3.1 ppm (Z1)]. ESIMS data corresponded to the composition $Hex_4Hep_1HexN_2$-$Kdo_1EtN_1P_5$ with two $C_{12}OH$ fatty acids (observed molecular mass 2254.5 Da), one $C_{12}OH$ and one $C_{14}OH$ (2268.3 Da), and two $C_{14}OH$ (2282.4 Da) in the ratio of approx. 1:2:1. Unfortunately no information regarding the position of the α-Gal*P* residue Z was obtained.

Consideration of the experimental data leads to the proposal for LPS core structures of *S. putrefaciens* as presented in Scheme 1. The formation of compounds **1** and **2** during alkaline treatment of the LPS is the result

of phosphate group migration from O-2 to O-3 of the heptose E residue via a 2,3-cyclic intermediate, accompanied by loss of the ethanolamine substituent and distribution of the phosphate substituent between positions 2 and 3 after ring opening. *S. putrefaciens* LPS is unusually homogenous and contains no structural variants in detectable amounts. Another feature is that Kdo is substituted with a DD-Hep residue. D-*glycero*-D-*manno*-Heptose is a biosynthetic precursor of LD-Hep; thus *S. putrefaciens* may have no mechanism for conversion of DD-Hep to LD-Hep.

3. Experimental

Bacterial strains and lipopolysaccharide isolation. S. putrefaciens.—CN32 was obtained from K. Nealson, Jet Propulsion Laboratory, Pasadena, CA and was grown aerobically in trypticase soy broth to an optical density (OD_{600}) of 1.0. Cells were harvested by centrifugation, and LPS was extracted by the Darveau and Hancock method using EDTA and SDS on cells broken in a French press.[14] DNase, RNase and protease removed contaminants, and the extracted LPS was eventually precipitated in ice-cold 95% (v/v) ethanol containing 0.375 M $MgCl_2$.

NMR spectroscopy and general methods.—^{1}H and ^{13}C NMR spectra were recorded using a Varian Inova 500 spectrometer in D_2O solutions at 25 °C with acetone standard (2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C) using standard COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), gHSQC, and gHMBC (optimized for 5 Hz coupling constant) pulse sequences. ^{31}P and ^{1}H-^{31}P HMQC (optimized for 9 Hz coupling constant) spectra were recorded on Varian Inova 400 spectrometer. Spectra were assigned with the help of a PRONTO program.[15] ESI mass spectra were obtained using a Micromass Quattro spectrometer in 50% MeCN with 0.2% HCO_2H at a flow rate of 15 μL/min with direct injection. GLC, GLC–MS, methylation and monosaccharide analyses were performed as previously described.[16]

Preparation of oligosaccharides **1** *and* **2**.—LPS (100 mg) was kept at 100 °C for 4 h in 4 M NaOH (4 mL), cooled, and neutralized with 2 M HCl (7.5 mL). The precipitate was removed by centrifugation, and products were separated on a Sephadex G50 SF gel (Pharmacia) column (2.5 × 80 cm) using pyridine–acetic acid buffer (4 mL of pyridine and 10 mL of AcOH in 1 L of water) with monitoring by a Waters differential refractometer to give 30 mg of the mixture of the oligosaccharides **1** and **2**.

Preparation of oligosaccharides **3** *and* **4**.—LPS (80 mg) was hydrolyzed with 2% acetic acid (100 °C, 4 h). The resulting precipitate was removed by centrifugation, and the supernatant was fractionated on a Sephadex G50 column to give D-galactose (3 mg) and a mixture of oligosaccharides **3** and **4** (25 mg).

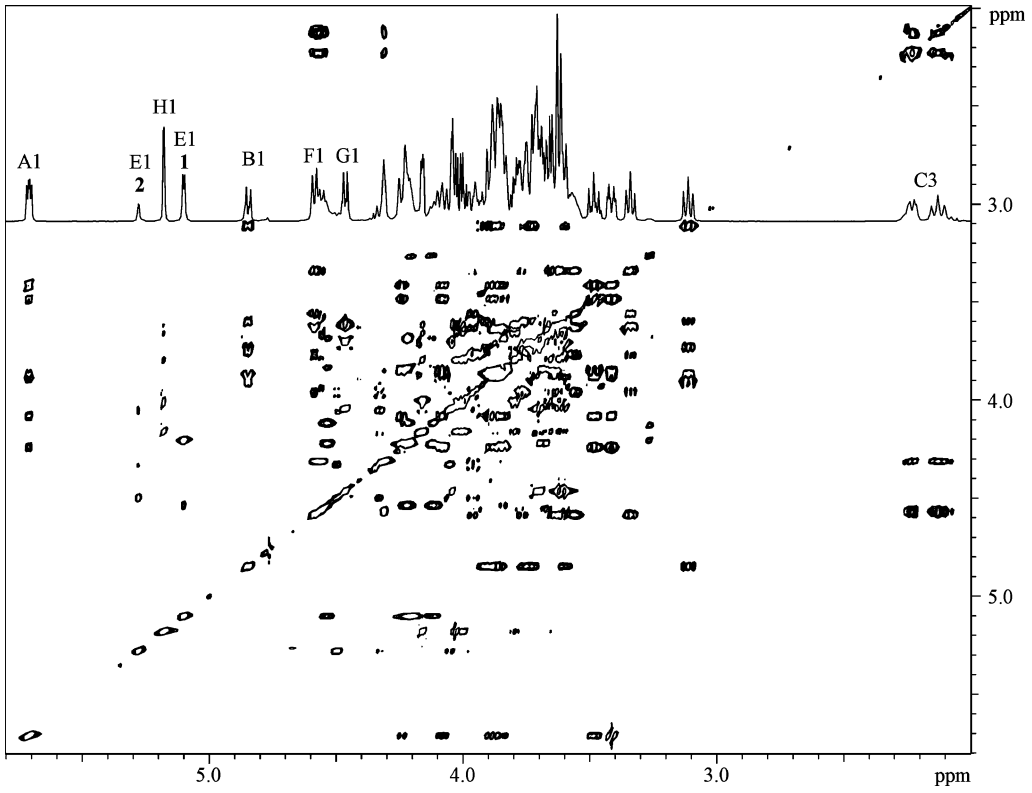

Fig. 1. TOCSY spectrum of compounds **1** and **2**.

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