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Note

The structure of the carbohydrate backbone of the LPS from *Shewanella* spp. MR-4

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ABSTRACT

The rough type lipopolysaccharide isolated from *Shewanella* spp. strain MR-4 was analyzed using NMR, mass spectroscopy, and chemical methods. Two structural variants have been found, both contained 8-amino-3,8-dideoxy-D-*manno*-octulosonic acid and lacked L-*glycero*-D-*manno*-heptose. A minor variant of the LPS contained phosphoramide substituent.

Shewanella spp. strain MR-4, a Gram-negative bacterium isolated from the Black Sea, is an important environmental microorganism participating in complex biogeochemical processes involving metal reduction.[1,2] Surface components of this organism play an important role in its adhesion to mineral surfaces.[3] *Shewanella* spp. MR-4 produces a large quantity of capsular polysaccharide (CPS), which gives the cells 'smooth' appearance. However, its LPS has no polymeric O-chain, belonging to the 'rough-type'. The CPS[4] contains a monosaccharide similar to anthrose that makes it cross-reactive with the BclA, a glycoprotein located on the surface of B. anthracis spores.[5] Here, we describe the results of structural analysis of the *Shewanella* spp. MR-4 LPS.

Monosaccharide analysis of the *Shewanella* MR-4 LPS showed the presence of two major constituents: D-galactose and D-*glycero*-D-*manno*-heptose, and three minor constituents: glucosamine (GlcN), mannose (Man), and glucose (Glc). The GlcN originated from the backbone of lipid A, Man, and Glc appeared in the GC analysis of alditol acetates as a result of the reduction of fructose, which was identified in the LPS-derived oligosaccharides by NMR. The absolute configuration of Gal, DDHep, and GlcN were determined as D using GLC of acetylated 2-butyl glycosides. Fatty acid analysis of the LPS revealed the presence of 14:0(3-OH), 14:0, and 12:0 fatty acids. O-Deacylated LPS contained 14:0(3-OH) acids, being attached as amides to the GlcN of lipid A.

Alkaline deacylation of the LPS from *Shewanella* spp. MR-4 gave a complex mixture of oligosaccharides that were separated by HPAEC. Only the three major products were analyzed. The structure of the oligosaccharide **1** as presented in Scheme 1 was determined by 2D NMR spectroscopy (Table 1). Assignment of the NMR spectra showed the presence of four monosaccharide residues: α-GlcN1*P*, β-GlcN4*P*, α-8-aminoKdo4*P*, and α-DDHep3*P*. The identity of the monosaccharides was established on the basis of proton and carbon chemical shifts and H,H vicinal coupling constants. The presence of the amino group at position 8 of the Kdo followed from the high field chemical shift of C-8 (44.3 ppm). All monosaccharides were phosphorylated, and the phosphate group position was determined using ^{1}H–^{31}P NMR correlation spectroscopy. The sequence of monomeric components was determined using NOE (correlations B1A6w, E1C5s, E1C7s) and HMBC (H–C correlations B1A6, C2B6, E1C5) data. ESI mass spectra of oligosaccharide **1** (negative mode) contained a peak of a double charged ion corresponding to a molecular mass of 1072 Da (calculated for $C_{27}H_{53}N_3O_{33}P_4$ 1071.6 Da). The second major oligosaccharide had the same structure but without phosphate at O-4 of the 8-aminoKdo. This was confirmed by NMR and MS analysis (data not shown).

Oligosaccharide **2**, eluted near the solvent front in HPAEC, contained the residues of β-fructofuranose and α-galactopyranose

Abbreviations: LPS, lipopolysaccharide; DDHep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; 8-aminoKdo, 8-amino-3,8-dideoxy-D-*manno*-oct-2-ulosonic acid; *P*, phosphate; *P*EtN, phosphoethanolamine; HPAEC, high performance anion exchange chromatography.

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KOH products:

α-DDHep3*P*-(1→5)-α-8-aminoKdo4**R**-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P* **1a, R = *P*; 1b, R =** H
E **C** **B** **A**

β-Fru*f*-(2→6)-α-Gal-(1→6)-Xxx **2**
M **H** **G***

Reduced AcOH product:

α-Gal-(1→6)-α-Gal-(1→3)-Gal-ol **3**
H **G** **F**

LPS-Hy variant 1:

β-Fru*f*-(2→6)-α-Gal-(1→6)-α-Gal-(1→3)-α-Gal-(1-*P*-
M **H** **G** **F**

Y
α-GalA-(1-*P*-4)
|
→3)-α-DDHep-(1→5)-α-8-aminoKdo-(2→6)-β-GlcNac4*P*-(1→6)-α-GlcNac1*P*
E **C** **B** **A**

LPS-Hy variant 2:

Y
α-GalA-(1-*P*-4)
|
α-DDHep2*PPEtN*3*PN*-(1→5)-α-8-aminoKdo-(2→6)-β-GlcNac4*P*-(1→6)-α-GlcNac1*P*
E' **C'** **B** **A**

Scheme 1. Structures of the isolated oligosaccharides derived from the LPS from *Shewanella* spp. MR-4. *PN* is phosphoramide. ac = 14:0(3-OH) acyl group. GalA Y is present in about 50% on each of the structural variants of LPS-Hy. Residue Xxx (G^*) is a product of alkaline degradation of the Gal G.

(Scheme 1), and many variants of the degradation products of the reducing-end monosaccharide (6-substituted galactose). The structure of the oligosaccharide **2**, except for the reducing end monosaccharide, was determined by NMR (Table 1). The β-fructofuranose ring size and anomeric configuration were identified by ^{13}C chemical shift comparison with the known values.[6] Its position was determined from HMBC correlation between C-2 of Fru and H-6 of Gal H. This oligosaccharide was obtained by hydrolysis of the phosphodiester bond between Gal F and Hep E, and the subsequent destruction of the Gal residues F and partially G.

Acetic acid hydrolysis of the LPS gave a mixture of mono- and oligosaccharides. They were separated by gel filtration and anion exchange chromatography. The major products were galactotriose and free galacturonic acid. Products originating from Hep-(1-5)-8-aminoKdo disaccharide were not analyzed because they gave many forms with variable Kdo degradation products at the end and different phosphorylations.

NMR analysis of the O-deacylated LPS (LPS-Hy) showed the presence of two structural forms (variants **1** and **2**). Variant **1** contained all components that are required to produce compounds **1**–**3** upon KOH or AcOH treatment. Thus, alkaline treatment cleaves the phosphodiester bond between Gal F and phosphate group, and destroys Gal F completely and Gal G partially, resulting in oligosaccharide **2**. The rest of the molecule remains as the oligosaccharide **1**. The connection between Gal G and DDHep E via a phosphodiester bond can be traced in the ^{1}H–^{31}P HMQC correlation (Fig. 1). The Kdo in the LPS-Hy was partially substituted by α-Gal*A**P*** (residue Y) at O-4, which is visible in the ^{1}H–^{31}P HMQC correlation spectrum. Cleavage of α-Gal*A**P*** in KOH gave an analog of the oligosaccharide **1** without the phosphate group at O-4. The LPS which had phosphate group at C-4 of 8-aminoKdo without galacturonic acid retained this phosphate and gave the oligosaccharide **1** after KOH treatment. ESI MS data of the LPS-Hy (Fig. 2) showed structural variants with HexA ($Hex_4HexA_1Hep_1HexN_2Kdo_1C14OH_2P_4$, observed molecular mass of 2350.6 Da, calculated 2350 Da) and without HexA ($Hex_4Hep_1HexN_2Kdo_1C14OH_2P_4$, observed molecular mass of 2174.5 Da, calculated 2173.9 Da), as well as a peak of 2297.6 molecular mass corresponding to an additional *P*EtN substituent to the structure without GalA, and a minor peak of −28 Da probably due to the replacement of 14:0(3-OH) acyl groups by 12:0(3-OH) acyl groups.

The NMR spectra of the LPS-Hy contained the signals of another DDHep residue E′, linked to the 8-aminoKdo (LPS-Hy variant **2**). DDHep E′ was phosphorylated at positions 2 and 3 (Fig. 1), and most interestingly the ^{31}P signal of the substituent at O-3 appeared at 16.5 ppm, which indicates the presence of a phosphoramide group. A similar substituent has been identified recently in *Xanthomonas campestris* LPS.[7] Unfortunately, no derivatives originating from variant **2** were identified in the LPS hydrolysates; this variant also showed no signal in the mass spectrum and thus its structure as presented here should be considered tentative.

The lipid A-core part of LPS from several *Shewanella* strains was elucidated previously.[8–12] Most of them contained 8-aminoKdo residue in place of Kdo. The absolute stereochemistry of this monosaccharide has been confirmed recently.[13] The structure described in the current publication gives one more example of 8-aminoKdo containing LPS, which seems to be a characteristic feature of *Shewanella*.

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

Unit, compound	Atom	1	2 (3ax)	3 (3eq)	4	5	6(a)	7(a) (6b)	8a (7b)	8b
α-GlcN1*P* A, 1a	^{1}H	5.74	3.44	3.93	3.52	4.12	3.91	4.26		
	^{13}C	93.2	55.4	70.9	71.1	73.8	70.4			
	^{31}P	2.0								
α-GlcN1*P* A, LPS-Hy	^{1}H	5.50	3.90	3.85	3.80	3.98	3.95	4.18		
	^{13}C	95.0	55.0	71.4	70.0	72.9	69.2			
	^{31}P	0.3								
β-GlcN4*P* B, 1a	^{1}H	4.89	3.15	3.93	3.97	3.79	3.54	3.67		
	^{13}C	100.5	56.9	72.9	75.4	75.0	63.8			
	^{31}P				3.1					
β-GlcN4*P* B, LPS-Hy	^{1}H	4.67	3.90	3.94	3.92	3.77	3.55	3.74		
	^{13}C	103.4	56.6	74.6	74.8	75.0	64.3			
	^{31}P				2.14					
α-AminoKdo C, 1a	^{1}H		2.16	2.26	4.61	4.36	3.86	4.11	3.08	3.51
	^{13}C	175.4	101.3	35.3	71.3	74.9	75.1	67.0	44.4	
	^{31}P				2.9					
α-AminoKdo C with GalA Y, LPS-Hy var. 1	^{1}H		2.12	2.34	4.65	4.40	3.92	4.13	3.24	3.55
	^{13}C			35.6	71.9	75.5	75.7	67.0	44.3	
	^{31}P				−0.6					
α-AminoKdo C no GalA Y, LPS-Hy var. 1	^{1}H		2.12	2.34	4.62	4.33	3.92	4.13	3.24	3.55
	^{13}C			35.6	71.1	75.2	75.7	67.0	44.3	
	^{31}P				1.4					
α-AminoKdo C′ with GalA Y, LPS-Hy var. 2	^{1}H		2.12	2.34	4.66	4.49	3.92	4.13	3.24	3.55
	^{13}C			35.6	71.9	75.0	75.7	67.0	44.3	
	^{31}P				−0.6					
α-AminoKdo C′ no GalA Y, LPS-Hy var. 2	^{1}H		2.12	2.34	4.62	4.43	3.92	4.13	3.24	3.55
	^{13}C			35.6	71.1	74.7	75.7	67.0	44.3	
	^{31}P				1.4					
α-DDHep E, 1a	^{1}H	5.12	4.30	4.36	3.93	4.12	4.12	3.73	3.79	
	^{13}C	102.2	70.5	77.1	67.4	74.9	73.8	63.6		
	^{31}P			2.9						
α-DDHep E, LPS-Hy var 1	^{1}H	5.11	4.32	4.47	3.92	4.07	4.04			
	^{13}C	102.2	70.9	76.2	66.5	74.6	73.2			
	^{31}P			−0.7						
α-DDHep E′, LPS-Hy var 2	^{1}H	5.39	4.72	4.55	4.10	4.18				
	^{13}C	99.0	76.5	80.6	66.9	74.4				
	^{31}P		−10.2	16.8						
α-Gal H, 3	^{1}H	4.91	3.84	3.90	4.00	3.99	3.76	3.76		
	^{13}C	99.5	69.2	70.3	70.1	72.0	62.0			
α-Gal H, 2 and LPS-Hy	^{1}H	4.96	3.82	3.89	3.99	4.03	3.72	3.82		
	^{13}C	100.2	69.9	71.0	70.9	71.4	62.4			
α-Gal G, 3	^{13}C	5.16	3.87	3.91	4.07	4.25	3.69	3.89		
	^{31}P	100.8	69.2	69.9	70.1	70.7	67.9			
α-Gal G, LPS-Hy	^{13}C	5.24	3.91	4.14	4.06	4.21	3.72	3.95		
	^{31}P	94.3	69.6	70.5	71.0	70.8	68.4			
Gal-ol F, 3	^{1}H	3.78	4.10	3.84	3.91	4.08	3.68			
	^{13}C	63.5	72.5	79.5	70.7	70.9	63.8			
α-Gal F, LPS Hy	^{1}H	5.69	4.07	3.94	4.30					
	^{13}C	97.7	70.0	73.7	65.7					
	^{31}P	−0.7								
β-Fru*f* M, 2 and LPS-Hy	^{1}H	3.68/3.74		4.17	4.10	3.86	3.67	3.82		
	^{13}C	61.7	105.2	78.2	76.0	82.6	63.8			
α-GalA Y, LPS Hy	^{1}H	5.64	3.93	4.03	4.38	4.50				
	^{13}C	96.6	69.8	70.6	72.0	74.2	176.5			
	^{31}P	−0.6								

1. Experimental

1.1. Bacterial strains and lipopolysaccharide isolation

Shewanella spp. strain MR-4 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 followed by DNase, RNase, and protease treatment. The extracted LPS was precipitated in ice-cold 95% (v/v) ethanol containing 0.375 M $MgCl_2$.

1.2. 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 an acetone standard (2.23 ppm for ^{1}H and 31.5 ppm for ^{13}C) using

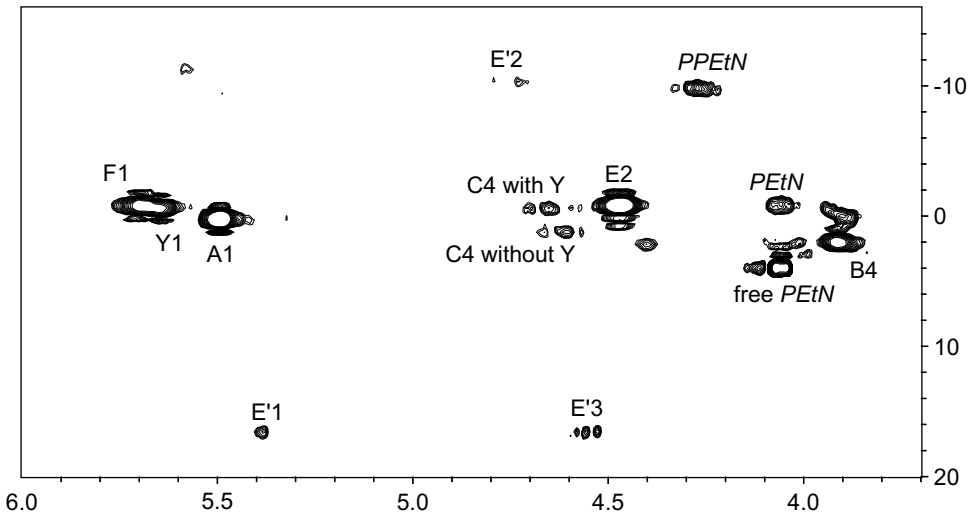

Figure 1. $^{1}H–^{31}P$ HMQC spectrum of *Shewanella* spp. MR-4 LPS-Hy.

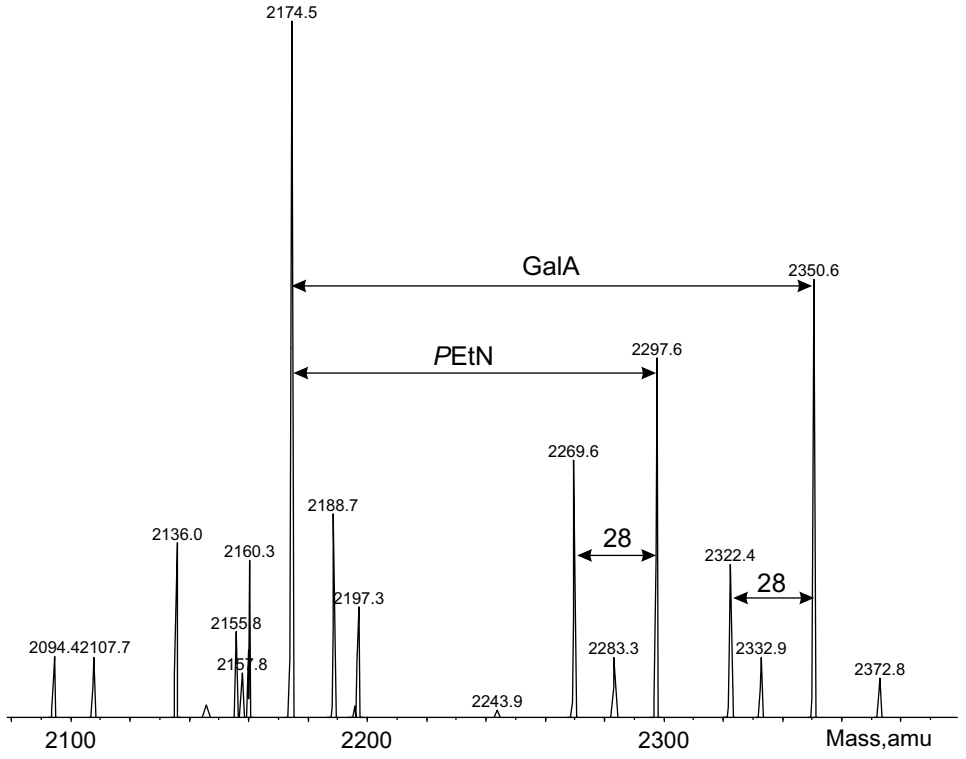

Figure 2. Reconstructed ESI mass spectrum of the *Shewanella* spp. MR-4 LPS-Hy.

standard COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, HMBC (100 ms long-range transfer delay), $^{1}H–^{31}P$ HMQC, and HMQC–TOCSY (optimized for 11 Hz coupling constant) pulse sequences. Spectra of the LPS-Hy were recorded at 60 °C in 5% fully deuterated SDS. 24% NH_4OH (10 μL) were added to the 0.6 mL D_2O sample. ESI mass spectra were obtained using a Micromass Quattro spectrometer in 50% MeCN with 0.2% HCOOH at a flow rate of 15 μL/min with direct injection. GLC, GLC–MS, methylation, and monosaccharide analyses and preparation of the oligosaccharides were performed as described.[8–10]

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References

1. Nealson, K. H.; Myers, C. R.; Wimpee, B. B. *Deep-Sea Res.* **1991**, *38*, S907–S920.
2. Marsili, E.; Baron, D. B.; Shikhare, I. D.; Coursolle, D.; Gralnick, J. A.; Bond, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 3968–3973.

3. Korenevsky, A. A.; Vinogradov, E.; Gorby, Y.; Beveridge, T. J. *Appl. Environ. Microbiol.* **2002**, *68*, 4653–4657.
4. Vinogradov, E.; Nossova, L.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2005**, *340*, 1750–1753.
5. Kubler-Kielb, J.; Vinogradov, E.; Hu, H.; Leppla, S. H.; Robbins, J. B.; Schneerson, R. *Proc. Natl. Acad. Sci. U.S.A.*, in press, doi:10.1073/pnas.0803897105.
6. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
7. Silipo, A.; Molinaro, A.; Sturiale, L.; Dow, J. M.; Erbs, G.; Lanzetta, R.; Newman, M. A.; Parrilli, M. *J. Biol. Chem.* **2005**, *280*, 33660–33668.
8. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2002**, *337*, 1285–1289.
9. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2003**, *338*, 1991–1997.
10. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2004**, *339*, 737–740.
11. Silipo, A.; Leone, S.; Molinaro, A.; Sturiale, L.; Garozzo, D.; Nazarenko, E. L.; Gorshkova, R. P.; Ivanova, E. P.; Lanzetta, R.; Parrilli, M. *Eur. J. Org. Chem.* **2005**, *2005*, 2281–2291.
12. Moule, A. L.; Galbraith, L.; Cox, A. D.; Wilkinson, S. G. *Carbohydr. Res.* **2004**, *339*, 1185–1188.
13. Leone, S.; Molinaro, A.; De Castro, C.; Baier, A.; Nazarenko, E. L.; Lanzetta, R.; Parrilli, M. *J. Nat. Prod.* **2007**, *70*, 1624–1627.