Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 1750–1753
Carbohydrate RESEARCH

Note

The structure of the capsular polysaccharide of *Shewanella oneidensis* strain MR-4

Evgeny Vinogradov,[a,*] Ludmila Nossova,[a] Anton Korenevsky[b] and Terry J. Beveridge[b]

[a]*Institute for Biological Sciences, National Research Council, 100 Sussex Dr., Ottawa, ON, Canada K1A 0R6*
[b]*Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada N1G 2W1*

Received 9 March 2005; accepted 4 April 2005
Available online 4 June 2005

Abstract—Capsular polysaccharides were extracted from *Shewanella oneidensis* strain MR-4, grown on two different culture media. The polysaccharides were analyzed using ^{1}H and ^{13}C NMR spectroscopy, and the following structure of the repeating unit was established:

$$\begin{array}{l}\rightarrow 4)\text{-}\beta\text{-D-Man-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc-}(1\rightarrow 3)\text{-}\beta\text{-D-GlcNAc-}(1\rightarrow \\ \alpha\text{-D-Qui4Nacyl-}(1\rightarrow 4)\text{-}\alpha\text{-D-GlcA-}(1\rightarrow 3)\lrcorner\end{array}$$

where the residue of 4-amino-4,6-dideoxy-D-glucose (Qui4N) was substituted with different *N*-acyl groups depending on the growth media. All monosaccharides are present in the pyranose form. In the PS from cells grown on enriched medium (trypticase soy broth, TSB) aerobically it was N-acylated with 3-hydroxy-3-methylbutyrate (60%) or with 3-hydroxybutyrate (40%), whereas in the PS from cells grown on minimal medium (CDM) aerobically it was acylated mostly with 3-hydroxybutyrate (>90%).

Keywords: Capsular polysaccharide; *Shewanella*; 3-Hydroxy-3-methylbutyrate

Shewanella oneidensis is a Gram-negative bacterium associated with aquatic and subsurface environments.[1] It can attach to amorphous iron oxides and, in so doing, utilizes the Fe(II)/Fe(III) couple as a terminal electron acceptor during dissimilatory iron reduction.[2] The polysaccharides of *Shewanella* have been implicated in the adhesion of these bacteria to mineral surface (Korenevsky and Beveridge, unpublished). We have previously analyzed the structure of the core part of the rough LPS from *S. oneidensis* MR-1.[3] In contrast to MR-1, *S. oneidensis* MR-4 is a smooth strain possessing a capsule ranging from 70 to 130 nm in thickness.[4] Here, we present and discuss the results of the structural analysis of the capsular polysaccharides of *S. oneidensis* MR-4.

The capsular polysaccharides together with the LPS were obtained by phenol–water extraction of bacterial cells. The LPS contained no O-chain, as was confirmed by gel electrophoresis.[4] Although the LPS was of 'rough-type', colonies of *S. oneidensis* MR-4 cells looked smooth on agar plates due to the presence of the capsular polysaccharides. In order to remove the LPS from the preparation, mild acid hydrolysis of the extract was performed, which led to the cleavage of the lipid A-core linkage. The capsular polysaccharides fraction was isolated then by gel chromatography.

At first, the polysaccharide from cells grown on TSB were analyzed. Monosaccharide analysis (GC of alditol acetates) of the polysaccharides revealed the presence of glucose, mannose, glucosamine, and 4-amino-4,6-dideoxyglucose (Qui4N). GC analysis of the acetylated products of acidic methanolysis showed the presence of glucuronic acid.

Abbreviations: LPS, lipopolysaccharide; Qui4N, 4-amino-4,6-dideoxy-D-glucose, viosamine; HB, 3-hydroxybutyrate; HMB, 3-hydroxy-3-methylbutyrate.

* Corresponding author. Tel.: +1 613 990 0832; fax: +1 613 952 9092; e-mail: evguenii.vinogradov@nrc.ca

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.04.009

Table 1. NMR data for the *S. oneidensis* MR-4 polysaccharide from cells grown on TSB medium

Unit		1	2	3	4	5	6 (6a)	6b	Interresid. NOE from H-1	Interresid. HMBC from H-1
A, α-Qui4N	^{1}H	5.39	3.61	3.67	3.57	3.79	1.14	1.12	B4	B4
	^{13}C	100.0	73.3	71.6	57.4	68.5	17.8	17.6		
B, α-D-GlcA	^{1}H	5.22	3.60	4.00	3.80	4.32			C3	C3
	^{13}C	102.2	72.6	75.0	78.5	72.9	174.3			
C, β-D-Man	^{1}H	4.73	4.16	3.83	4.03	3.46	3.69	3.89	E4	E4
	^{13}C	100.7	71.8	81.8	73.0	76.4	61.2			
D, β-D-GlcN	^{1}H	4.59	3.79	3.81	3.45	3.54	3.76	3.98	C4	C4
	^{13}C	101.9	55.9	83.3	69.9	77.0	62.0			
E, β-D-Glc	^{1}H	4.49	3.32	3.65	3.65	3.56	3.69	3.84	D3	D3
	^{13}C	104.0	73.7	75.1	79.6	75.7	61.2			
HB	^{1}H		2.45	4.19	1.22					
	^{13}C	175.2	49.6	65.9	22.8					
HMB	^{1}H		2.43		1.29					
	^{13}C	175.0	46.0		29.2					

Acetate signals in **D**: CO 175.5; Me (^{1}H/^{13}C) 2.02/23.1 ppm. H-6/C-6 of Qui4N residue **A** in column 6 (6a) belong to the HMB-substituted residues, in the column 6b—to HB-substituted monosaccharide.

NMR data (Table 1, Figs. 1 and 2) showed that the polysaccharides have a regular structure with a repeating unit consisting of five monosaccharides. The repeating unit included also an acetamido group and the residues of 3-hydroxy-3-methylbutyrate (HMB) and 3-hydroxy-butyrate (HB). HMB was identified from the following observations: it showed singlet methyl signals at 1.29/29.2 ppm (^{1}H/^{13}C), which in the HMBC spectrum correlated with a quarternary carbon signal at 71.3 ppm. The latter gave HMBC correlation to the CH_2 signal at 2.43/46.0 (^{1}H/^{13}C) ppm, which correlated further to the carbonyl carbon at 175.0 ppm. The carbonyl group showed HMBC correlation to H-4 of Qui4N residue due to acylation of Qui4N amino group with the HMB residue. Similar observations led to identification of the HB residue, acylating the rest of Qui4N residues. The relative amount of these acyl groups was estimated as 6:4 from integration of their H-2 and H-4 signals and of the H-6 signal of Qui4N.

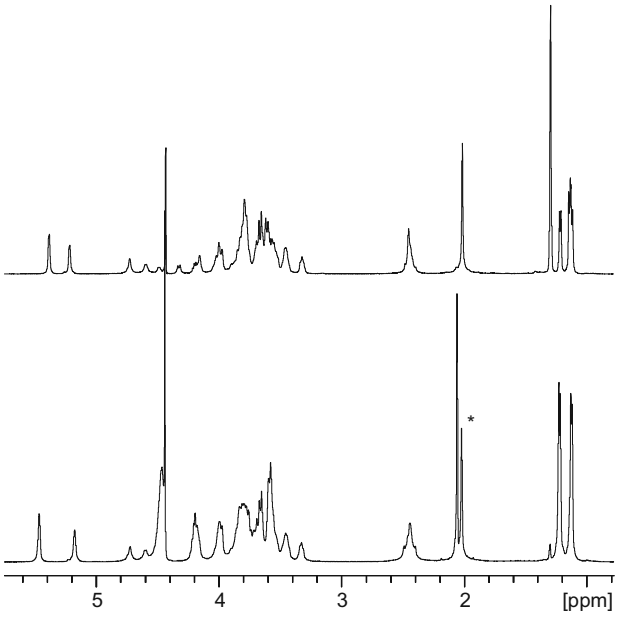


Figure 1. ^{1}H NMR spectra of the *S. oneidensis* MR-4 polysaccharides, isolated from cells grown on TSB (top) and CDM (bottom) media. Signals labeled with * belong to AcOH impurity.

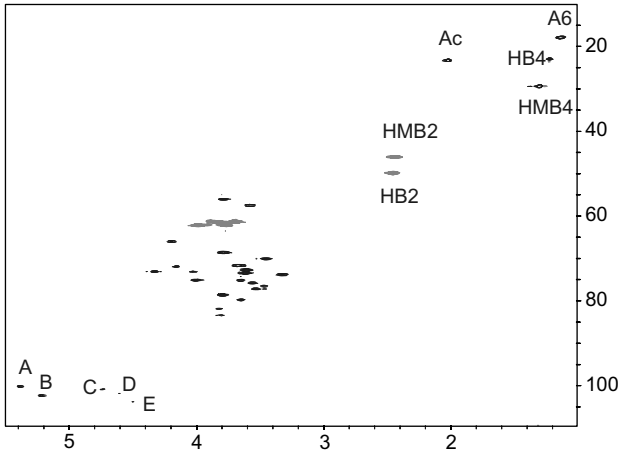


Figure 2. HSQC spectrum of the polysaccharide from *S. oneidensis* MR-4.

The identity of the monosaccharides was established on the basis of ^{1}H and ^{13}C NMR chemical shifts and $^{3}J_{H,H}$ coupling constants, which for monosaccharide residues were in agreement with expected values for the respective pyranosides. Thus, the residues of 4-amino-4,6-dideoxy-α-glucopyranose (**A**), α-glucopyranosyluronic acid (**B**), β-mannopyranose (**C**), *N*-acetyl-β-glucosamine (**D**), and β-glucopyranose (**E**) were identified. The anomeric β-configuration of the mannose residue was confirmed by the observation of intraglycosidic NOE correlations between H-1 and H-3, H-1 and H-5.

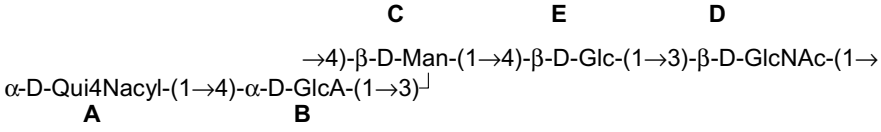
Determination of the monosaccharide sequence was achieved on the basis of NOE and HMBC data (Table 1). Observed NOE correlations between protons **A**1 and **B4**, **B**1 and **C**3, **C**1 and **E**4, **E**1 and **D**3, **D**1 and

C4, and HMBC (Table 1) corresponded to the structure presented below. HMBC correlations between acetate C-1 and H-2 of GlcN residue indicated an N-acetylation of GlcN.

The absolute D-configuration of all monosaccharides was determined by GC of the acetates of 2-butyl-glycosides or esters. The absolute (*R*)-configuration of 3-hydroxybutyrate was determined in the polysaccharide hydrolysate (4 M HCl, 100 °C, 5 h) using an enzymatic test (Sigma) according to the manufacturer's instructions.

The polysaccharide from *S. oneidensis* MR-4 grown on CDM media had the same structure except that it contained mostly HB and only a minor amount of HMB acyl groups at the Qui4N residue. NMR shifts for some signals were insignificantly different from that of the TSB grown polysaccharide (data not shown).

On the basis of experimental data, the following structure of the O-specific polysaccharides *Shewanella* MR-4 was proposed:



where *acyl* is HB or HMB, and all monosaccharides are present in the pyranose form.

Multiple attempts to use methylation analysis for both polysaccharides produced poor results; only derivatives of 3,4-disubstituted mannose and 4-substituted glucose were found, whereas no trace of methylated derivatives of GlcN and Qui4N was observed; large peaks of unidentified compounds were present.

HMB is found in most living organisms, being a metabolite of the amino acid leucine and a precursor of cholesterol. In bacteria, HB and HMB belong to the general class of alkanoates, or hydroxyacid polyesters, that are found as carbon storage compounds (e.g., poly-β-hydroxybutyrate [PHB]) where they are concentrated and packaged into cytoplasmic granules called PHB bodies. HB has been found in a number of bacterial polysaccharides and LPSs, but detection of HMB in these structures is described here for the first time. It is likely that, by being polysaccharide substituents, they affect the surface properties of the microorganisms by making their surface more hydrophobic (Korenevsky and Beveridge, unpublished). Why the change of growth conditions leads to the switching of acylation of Qui4N from HB to HMB remains to be elucidated.

The structures of the polysaccharides and LPS core parts from three other strains of *Shewanella* have been described previously;[3,5–10] they show no resemblance with each other.

1. Experimental

1.1. Growth conditions

S. oneidensis MR-4 was grown aerobically on a shaker at 24 °C on either trypticase soy broth (TSB), 30 g L^{-1} (Difco Laboratories, Detroit, MI), or a chemically defined medium (CDM). CDM contained (per liter of deionized water) 1.5 g of $(NH_4)_2SO_4$, 0.6 g of K_2HPO_4, 10 g of NaCl, 50 mg of $CaCl_2$, 75 mg of $MgCl_2$, 1.0 g of PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)), 0.2 g of glutamic acid, 5.61 mL of 60% wt/vol of sodium lactate, and 10 mL of Balch's trace-elements soln. The pH was adjusted to 7.15.

1.2. Polysaccharides isolation

The polysaccharides together with the LPS were isolated from *S. oneidensis* strain MR-4 grown aerobically on the two different media, TSB and CDM. The cultures were harvested at exponential growth phase (OD_{600} = ~0.8) by centrifugation (7000*g*) and extracted by the hot phenol–water method according to Westphal and Jann.[11] The product was hydrolyzed with 2% AcOH (100 °C, 3 h) for removal of the LPS lipid A and the polysaccharides were isolated by gel-chromatography on Sephadex G-50 column.

1.3. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 spectrometer in D_2O solns at 60 °C with acetone as standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, gHMBC (optimized for 5 Hz coupling constant), and HSQC-TOCSY. Spectra were assigned with the help of Pronto program.[12]

1.4. Monosaccharide identification

The polysaccharides (1 mg) were hydrolyzed (0.5 mL of 3 M TFA, 100 °C, 2 h), evaporated to dryness under a stream of N_2, dissolved in water (0.5 mL), reduced with $NaBH_4$ (~5 mg, 30 min), treated with AcOH (0.5 mL), dried, MeOH (1 mL) was added, and the mixture dried twice, the residue was acetylated with Ac_2O (0.5 mL, 100 °C, 30 min), dried, analyzed by GLC on a DB-17 capillary column (25 m × 0.25 mm) using a flame

ionization detector (HP 5890 instrument) in a temperature gradient of 180–240 °C at 2 °C/min, or on Varian Saturn 2000 instrument equipped with a ion-trap mass spectral detector. For the determination of the absolute configuration of the monosaccharide components of the polysaccharides, a sample (1 mg) was treated with (*R*)-2-butanol (0.5 mL) and AcCl (0.05 mL) for 3 h at 100 °C, dried, acetylated as described above, and analyzed by GLC in a temperature gradient of 120–240 °C at 2 °C/min; retention times were compared with those of standard samples prepared from the respective monosaccharides and (*R*)- and (*S*)-2-butanol.[13] As a source of *D*-Qui4N for this experiment, the O-specific polysaccharide of *Francisella tularensis* was used. The configuration of (*R*)-3-hydroxybutyric acid was determined using an enzymatic test kit (Sigma).

Acknowledgments

This work was supported by grants from the Advanced Food and Materials Network (AFMnet)—National Centre of Excellence and the USA-DOE-NABIR program to T.J.B.

References

1. Murray, A. E.; Lies, D.; Li, G.; Nealson, K.; Zhou, J.; Tiedje, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9853–9858.
2. Lower, S. K.; Hochella, M. F., Jr.; Beveridge, T. J. *Science* **2001**, *292*, 1360–1363.
3. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2003**, *338*, 1991–1997.
4. Korenevsky, A. A.; Vinogradov, E.; Gorby, Y.; Beveridge, T. J. *Appl. Environ. Microbiol.* **2002**, *68*, 4653–4657.
5. Shashkov, A. S.; Senchenkova, S. N.; Nazarenko, E. V.; Zubkov, V. A.; Gorshkova, N. M.; Knirel, Y. A.; Gorshkova, R. P. *Carbohydr. Res.* **1997**, *303*, 333–338.
6. Shashkov, A. S.; Senchenkova, S. N.; Nazarenko, E. V.; Zubkov, V. A.; Gorshkova, N. M.; Knirel, Y. A.; Gorshkova, R. P. *Carbohydr. Res.* **1998**, *309*, 103–108.
7. Shashkov, A. S.; Torgov, V. I.; Nazarenko, E. L.; Zubkov, V. A.; Gorshkova, N. M.; Gorshkova, R. P.; Widmalm, G. *Carbohydr. Res.* **2002**, *337*, 1119–1127.
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*, 385–388.
10. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2004**, *339*, 737–740.
11. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
12. Kjaer, M.; Andersen, K. V.; Poulsen, F. M. *Methods Enzymol.* **1994**, *239*, 288–308.
13. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **1978**, *62*, 349–357.