

Structure of the O-antigen of *Acinetobacter lwoffii* EK30A; identification of D-homoserine, a novel non-sugar component of bacterial polysaccharides

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We established a peculiar structure of the O-specific polysaccharide (O-antigen) of a psychrotrophic strain of *Acinetobacter lwoffii*, EK30A, isolated from a 1.6–1.8 million-year-old Siberian permafrost subsoil sediment sample. The polysaccharide was released by mild acid degradation of the lipopolysaccharide and studied using chemical analyses, Smith degradation, ¹H and ¹³C NMR spectroscopy and mass spectrometry. It was found to contain D-homoserine, which is N-linked to 4-amino-4,6-dideoxy-D-glucose (Qui4N) and is N-acylated itself with acetyl in about half of the repeating units or (S)-3-hydroxybutanoyl group in the other half. The following is the structure of the tetrasaccharide repeating unit of the polysaccharide: →3)-β-D-Quip4NAcyl-(1→6)-α-D-Galp-(1→4)-α-D-GalpNAc-(1→3)-α-D-FucpNAc-(1→ where Acyl stands for either N-acetyl- or N-[(S)-3-hydroxybutanoyl]-D-homoseryl.

Introduction

Bacteria can activate the innate immune responses *via* specialized receptors present on immunocytes of all vertebrates. Gram-negative bacteria are unique in that they activate a distinct pattern-recognition receptor, TLR4, which then triggers cascades of immune and inflammatory responses. The molecular pattern that is recognized by TLR4 is lipopolysaccharide (LPS, endotoxin), a component of the bacterial cell wall, and the recognition occurs in a complex with the adaptor protein MD-2.¹ The exact chemical structure of a particular LPS is the basis of serotyping bacteria and it can also determine the strength of the signal that TLR4 delivers inside the cell. The S-form LPS consists of a lipid moiety called lipid A, a central oligosaccharide (core), and a polysaccharide chain (O-polysaccharide, O-antigen), which defines the serological O-specificity of bacteria.² Some bacteria produce an R-form LPS that lacks O-polysaccharide.

Gram-negative non-motile bacteria of the genus *Acinetobacter* from the family Moraxellaceae are important soil organisms, which participate in mineralization of various organic compounds. Nevertheless, *Acinetobacter* are a key source of hospital infections in debilitated patients.³ *Acinetobacter lwoffii* are responsible for cases of community-acquired meningitis and pneumonia *via* airborne transmission, being more commonly associated with meningitis than other *Acinetobacter* species. Recently, it has been demonstrated that *A. lwoffii* F78 isolated from a farm cowshed exhibits allergy-protecting effects, and that the LPS mediates these

beneficial effects.⁴ The LPS of *A. lwoffii* F78 is devoid of O-antigen, and structures of the core and lipid A of this strain have been established.⁵

Permafrost may contain bacteria frozen millions years ago, and some of these bacteria retained viability and can be re-grown. We were interested to find out whether ancient Gram-negative bacteria may have some unusual features that are not found in modern bacteria. Here we report on an unusual structure of the O-polysaccharide of a psychrotrophic bacterium *A. lwoffii* EK30A isolated from a 1.6–1.8 million-year-old Siberian permafrost subsoil sediment sample.⁶

Results and discussion

The LPS was isolated in a yield 8% from dried bacterial cells by extraction with hot aqueous phenol and purified by precipitation of proteins and nucleic acids with trichloroacetic acid. Mild acid degradation of LPS cleaved a lipid portion and released an O-polysaccharide, which was isolated by Sephadex G-50 gel-permeation chromatography in a yield 42% of the LPS mass. Full acid hydrolysis of the polysaccharide followed by analysis using a sugar analyzer showed the presence of galactose as the only neutral monosaccharide. Analysis using an amino acid analyzer revealed as the main components 2-amino-2-deoxygalactose (GalN), 2-amino-2,6-dideoxygalactose (FucN) and an amino acid having the same retention time as homoserine (hSer). Determination of the absolute configurations by GLC of the acetylated (S)-2-octyl glycosides showed that Gal, GalN and FucN are D. Further studies showed that the O-polysaccharide contains also 4-amino-4,6-dideoxy-D-glucose (D-Qui4N) and a residue of (S)-3-hydroxybutanoic acid (Hb). Qui4N was not detected in sugar analysis owing to its destruction in the course of acid hydrolysis.⁷

The ¹³C NMR spectrum of the O-polysaccharide (Fig. 1) showed signals for four anomeric carbons at δ 94.5–103.7, six CH₃ groups at δ 16.9, 17.8 (C-6 of FucN and Qui4N) and 23.0–24.0 (C-2 of

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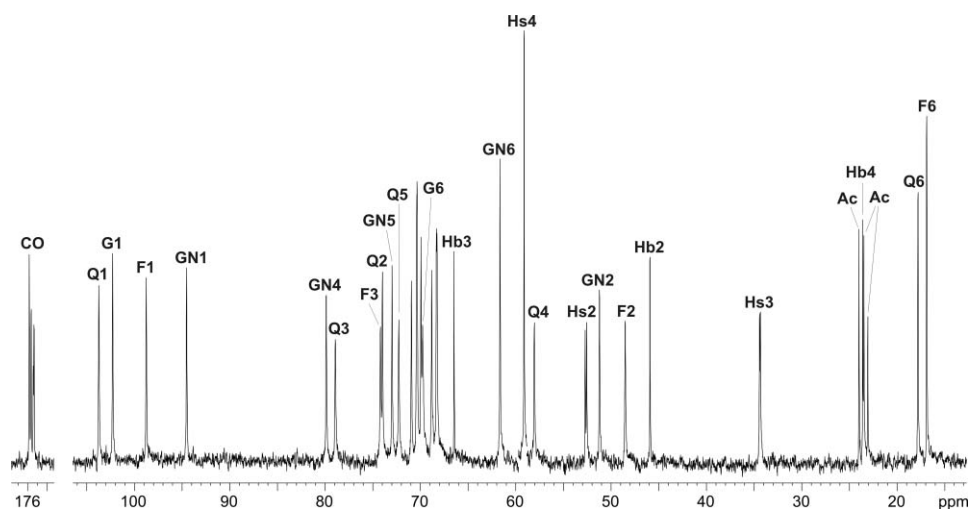


Fig. 1 ^{13}C NMR spectrum of the O-polysaccharide. Numbers refer to carbons in sugar residues and acyl groups denoted as follows: F, FucN; G, Gal; GN, GalN; Q, Qui4N; Ac, acetyl; Hb, 3-hydroxybutanoyl; Hs, homoseryl. Signals for C-2 and C-3 of homoserine are split as it carries either an acetyl or a 3-hydroxybutanoyl group.

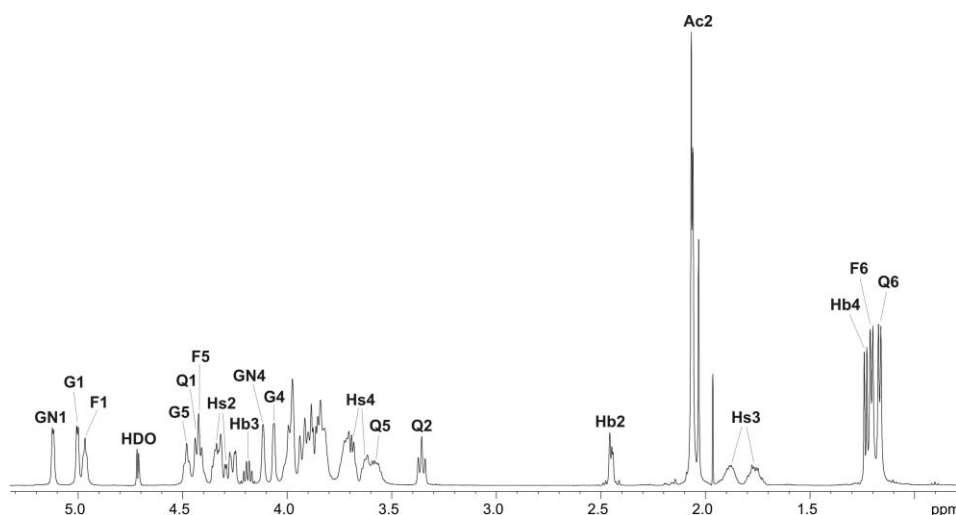


Fig. 2 ^1H NMR spectrum of the O-polysaccharide. Numbers refer to protons in sugar residues and acyl groups denoted by letters as indicated in the legend to Fig. 1. Signals for homoserine are split as it carries either an acetyl or a 3-hydroxybutanoyl group.

N-acetyl groups and C-4 of Hb), two C-CH₂-C groups (C-3 of hSer and C-2 of Hb) at δ 34.4 and 45.9, two non-substituted and one O-substituted HOCH₂-C groups (C-4 of hSer, C-6 of Gal and GalNAc) at δ 59.2, 61.7 and 69.8 (data of a DEPT experiment), four nitrogen-bearing carbons (C-2 of amino sugars and hSer) at δ 48.5–58.1, 14 oxygen-bearing carbons, including sugar ring at δ 68.3–79.9 and C-3 of Hb at δ 66.5, and a number of CO groups (C-1 of acyl groups) at δ 175.4–175.9. The ^1H NMR spectrum of the O-polysaccharide (Fig. 2) contained, *inter alia*, signals for six CH₃ groups at δ 1.17–1.23 (H-6 of FucN and Qui4N, H-4 of Hb) and 2.03–2.07 (C-2 of *N*-acetyl groups) and two C-CH₂-C groups at δ 1.75, 1.78 (H-3a of hSer), 1.88 (H-3b of hSer) and 2.46 (2 H, H-2 of Hb). Some signals of hSer and FucN were split (Table 1), and the signals for Hb and one of the *N*-acetyl groups were about twice less intense as the other signals in the spectra.

These data indicate that the O-polysaccharide has a tetrasaccharide repeating unit containing one residue each of Gal,

GalN, FucN and Qui4N as well as homoserine, acetyl and 3-hydroxybutanoyl groups, which, most likely, are *N*-acetyl substituents of the amino sugars. The identity of homoserine and 3-hydroxybutanoic acid was confirmed by GLC of the acetylated or trifluoroacetylated (*S*)-2-octyl esters, respectively, which were derived after full acid hydrolysis of the O-polysaccharide. The GLC data also showed that homoserine has the *D* configuration and 3-hydroxybutanoic acid has the *S* configuration.

The ^1H and ^{13}C NMR spectra of the O-polysaccharide were assigned using 2D ^1H , ^1H COSY, TOCSY, ROESY, ^1H , ^{13}C HSQC and HSQC-TOCSY experiments (Fig. 3 and 4, Table 1). Tracing connectivities in the TOCSY and ROESY spectra revealed four sugar spin systems as well as those for homoserine and the 3-hydroxybutanoyl group. The TOCSY spectrum showed correlations of anomeric protons H-1 with H-1–H-6 for Qui4N and H-1–H-4 for Gal, GalN and FucN (Fig. 3A). The interruption of the polarization transfer after H-4 in the last three monosaccharides having the *galacto* configuration is accounted for by a small $J_{\text{H-4,H-5}}$

Table 1 ^1H and ^{13}C NMR chemical shifts (δ , ppm) of the O-polysaccharide

Unit	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	NH
$\rightarrow 3\text{-}\beta\text{-Qui}p4\text{N}\text{-(1}\rightarrow$	4.43	3.36	3.71	3.82	3.58	1.17	8.27
homoseryl ^a	103.7	74.0 4.30/4.35	78.9 1.75/1.78; 1.88	58.1 3.62/3.69	72.3	17.8	8.22/8.25
3-hydroxybutanoyl		52.7/52.6	34.4/34.5	59.2			
acetyl		2.46 45.9 2.03 23.0	4.18 66.5	1.23 23.5			
$\rightarrow 6\text{-}\alpha\text{-Gal}p\text{-(1}\rightarrow$	5.00	3.87	3.97	4.05/4.08	4.48	3.71; 3.98	
$\rightarrow 4\text{-}\alpha\text{-Gal}p\text{N}\text{-(1}\rightarrow$	102.3 5.12	70.0 4.27	70.4 3.82/3.83	70.4 4.12	71.0 3.84	69.8 3.87; 3.91	8.19
acetyl	94.5	51.2 2.07 23.6	68.8	79.9	73.0	61.7	
$\rightarrow 3\text{-}\alpha\text{-Fuc}p\text{N}\text{-(1}\rightarrow$	4.96/4.98 98.8	4.32 48.5	3.93 74.2	3.98 68.3	4.42 68.3	1.21 16.9	7.58/7.60
acetyl		2.06 24.0					

Signals for CO (C-1 of *N*-acyl groups) are at δ 175.4–175.9.^a Data of *N*-acetylhomoserine and *N*-(3-hydroxybutanoyl)homoserine are given before and after slash.

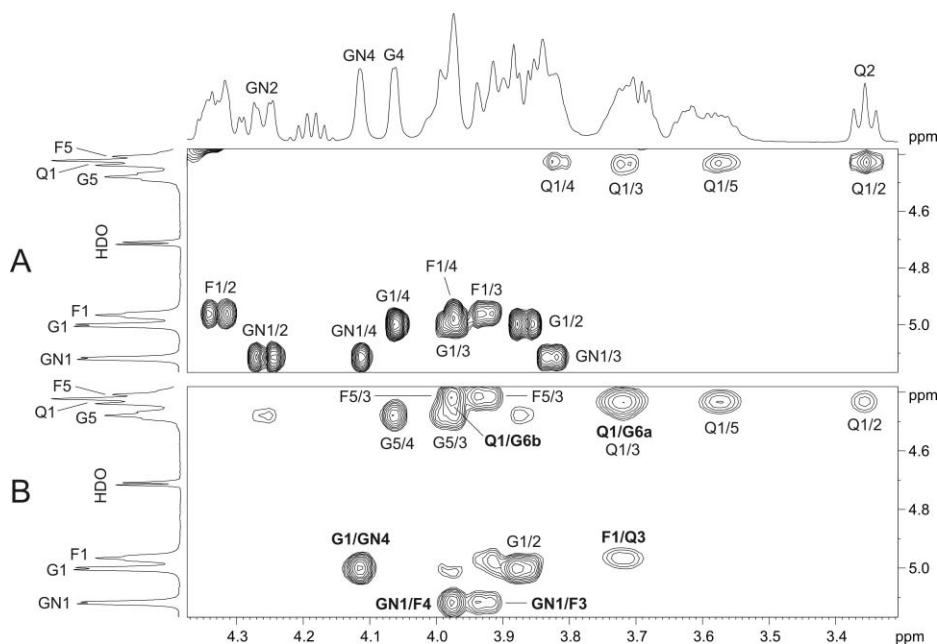


Fig. 3 Parts of ^1H , ^1H TOCSY (A) and ROESY (B) spectra of the O-polysaccharide. The corresponding parts of the ^1H NMR spectrum are displayed along the axes. Numbers refer to protons in sugar residues denoted by letters as indicated in the legend to Fig. 1. Inter-residue cross-peaks in the ROESY spectrum are shown in bold face.

coupling constant of <1 Hz. Their assignment was completed using the ROESY spectrum, which showed correlations of H-6 with H-5 and H-4 for FucN; H-4 with H-5 and H-6 for Gal and GalN. The signals within each system were assigned using the COSY spectrum. The positions of the amino groups at C-4 of Qui4N and C-2 of homoserine were confirmed, and GalN and FucN were distinguished from Gal, by correlations in the ^1H , ^{13}C HSQC spectrum between protons at the nitrogen-bearing

carbons (H-4 and H-2) and the corresponding carbons (C-4 and C-2) resonating at δ 58.1, 51.2 and 48.5, respectively (Fig. 4).

The absence from the ^{13}C NMR spectrum of any signals for non-anomeric sugar carbons at a lower field than δ 80 characteristic for furanosides⁸ indicated that all monosaccharides occur in the pyranose form. A relatively large $J_{1,2}$ coupling constant value of 8 Hz indicated that Qui4N is β -linked, whereas small $J_{1,2}$ values of 3–4 Hz showed that Gal, GalN and FucN are α -linked.

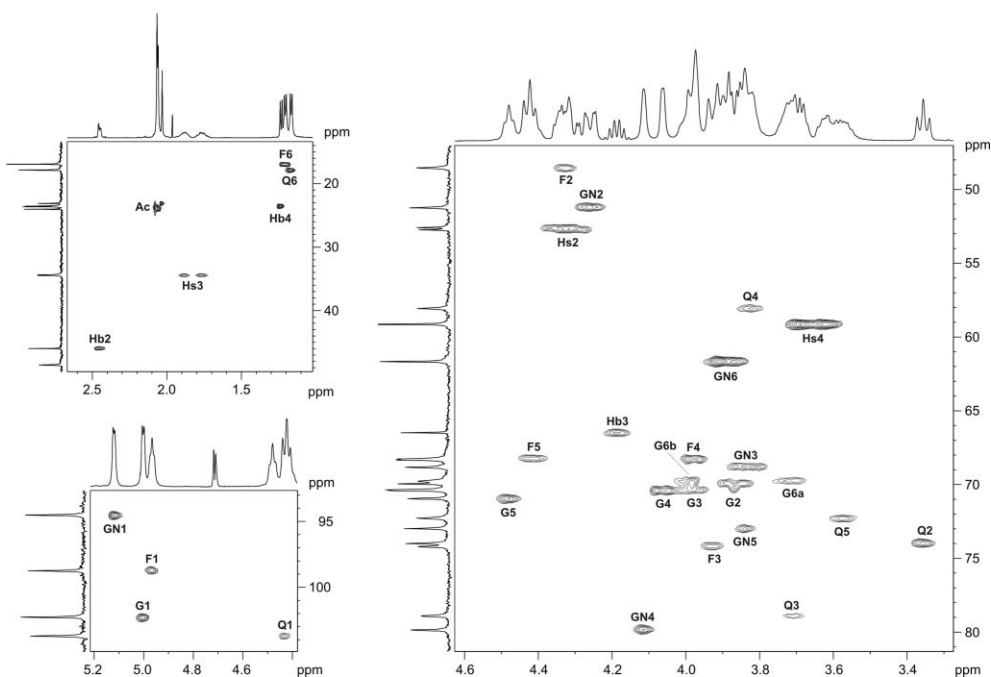


Fig. 4 Parts of a ^1H , ^{13}C HSQC spectrum of the O-polysaccharide. The corresponding parts of the ^1H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Numbers refer to H/C pairs of sugar residues and acyl groups denoted by letters as indicated in the legend to Fig. 1.

Downfield displacements of the signals for C-3 of FucN and Qui4N, C-4 of GalN and C-6 of Gal to δ 74.2, 78.9, 79.9 and 69.8 in the ^{13}C NMR spectrum of the O-polysaccharide (Fig. 4) from their positions at δ 68.7,⁹ 74.5,¹⁰ 69.9¹¹ and 62.4,⁹ respectively, in the spectra of the corresponding non-substituted monosaccharides demonstrated the sites of glycosylation of the monosaccharide residues. These data showed that the polysaccharide is linear, FucN and Qui4N are 3-substituted, GalN is 4-substituted and Gal is 6-substituted.

In the ROESY spectrum (Fig. 3B), there were inter-residue cross-peaks between the following anomeric protons and protons at the linkage carbons: Qui4N H-1/Gal H-6; Gal H-1/GalN H-4; GalN H-1/FucN H-3; and FucN H-1/Qui4N H-3 (Table 2). GalN H-1 showed also a correlation with FucN H-4 at δ 5.12/3.98, which is typical of α -1 \rightarrow 3-linked disaccharides with the *galacto* configuration of the glycosylated sugar and the same absolute configuration of the constituent monosaccharides.¹² Therefore, the ROESY data were in agreement with the positions of substitution

of the monosaccharide residues revealed by the ^{13}C NMR chemical shifts (see above) and defined their sequence in the repeating unit. The linkage pattern and the monosaccharide sequence were confirmed independently using a ^1H , ^{13}C HMBC experiment, which revealed correlations between anomeric protons and linkage carbons and *vice versa* (Table 2).

With the known sequence of the monosaccharides, the absolute configuration of Qui4N was established by a β -effect on the C-4 chemical shift caused by its glycosylation with D-FucN at position 3. A small β -effect of glycosylation of -0.2 ± 0.4 ppm is characteristic of the same absolute configuration of the constituent monosaccharides in α -1 \rightarrow 3-linked disaccharides with the *gluco* configuration of the glycosylated sugar whereas a significantly larger negative β -effect of -1.4 ± 0.2 ppm would be observed in case of their different absolute configurations.¹³ In the O-polysaccharide, the β -effect on C-4 of Qui4N is close to 0¹⁰ and, hence, FucN and Qui4N has the same, *i.e.* the D configuration. The α -effect of glycosylation (+4.4 ppm) on C-3 of Qui4N

Table 2 Interresidue homonuclear (ROESY) and heteronuclear (^1H , ^{13}C HMBC) correlations for anomeric atoms in the O-polysaccharide of *A. lwoffii* EK30A

Unit	^1H ^{13}C (anomeric)	Correlations to		
		Unit	ROESY	HMBC
\rightarrow 3)- β -Qui4N-(1 \rightarrow	4.43 103.7	\rightarrow 6)- α -Galp-(1 \rightarrow	H-6 3.71	C-6 69.8 H-6 3.71
\rightarrow 6)- α -Galp-(1 \rightarrow	5.00 102.3	\rightarrow 4)- α -GalpN-(1 \rightarrow	H-4 4.12	C-4 79.9 H-4 4.12
\rightarrow 4)- α -GalpN-(1 \rightarrow	5.12 94.5	\rightarrow 3)- α -FucpN-(1 \rightarrow	H-3 3.93	H-3 3.93
\rightarrow 3)- α -FucpN-(1 \rightarrow	4.96/4.98 98.8	\rightarrow 3)- β -Qui4N-(1 \rightarrow	H-3 3.71	H-3 3.71

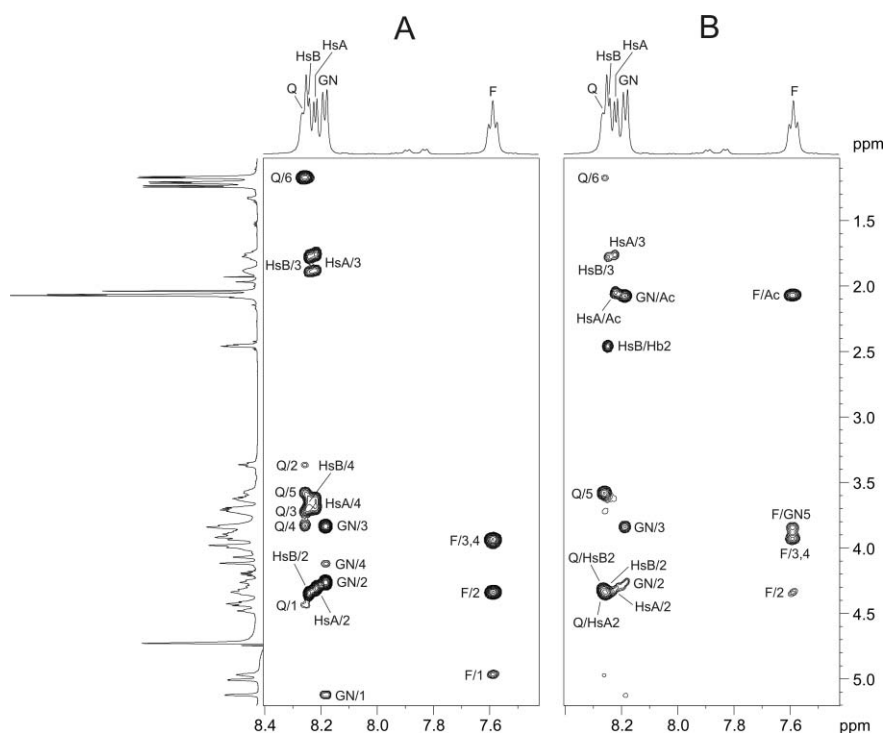


Fig. 5 Parts of ^1H , ^1H TOCSY (A) and ROESY (B) spectra of an O-polysaccharide solution in a $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixture showing correlations for NH protons. The corresponding parts of the ^1H NMR spectrum are displayed along the axes. Letters without numbers refer to NH protons; numbers refer to protons in sugar residues and acyl groups denoted by letters as indicated in the legend to Fig. 1; HsA and HsB stand for homoserine bearing an acetyl or a 3-hydroxybutanoyl group, respectively.

in the α -D-FucNAc-(1 \rightarrow 3)- β -Qui4NAcyl disaccharide is in the range of the reported α -effect values of +3.9–4.9 ppm in similar α -D-GlcNAc-(1 \rightarrow 3)- β -D-Qui4NAcyl disaccharide fragments of various bacterial polysaccharides.¹⁴

To determine location of the *N*-acyl groups, NMR spectra of the O-polysaccharide were measured in a 9 : 1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixture. This enabled detection of NH protons, which were assigned using the TOCSY spectrum (Fig. 5A, Table 1), and their correlation with CH protons. In the 2D ROESY spectrum (Fig. 5B), NH-2 protons of GalN and FucN showed strong correlations with CH_3 of the *N*-acetyl groups at 8.19/2.07 and 7.58, 7.60/2.06, respectively, both groups occurring in the stoichiometric amount. NH-4 proton of Qui4N gave a cross-peak with H-3 of homoserine at δ 8.27/1.8, which is thus the *N*-substituent of Qui4N. NH-2 proton of homoserine gave two peaks at δ 8.22 and 8.25, which showed correlations with the *N*-acetyl group at δ 2.03 and H-2 of *N*-(3-hydroxybutanoyl) group at δ 2.46, respectively, both groups being present in non-stoichiometric amounts. Therefore, the homoserine residue on Qui4N is *N*-acylated with acetyl group in some repeating units or 3-hydroxybutanoyl group in the other units. As judged by the intensities of the NMR signals of the *N*-acyl groups, each group is present in about half repeating units.

The structure of the O-polysaccharide was confirmed by Smith degradation, which included periodate oxidation of the 6-substituted Gal residue, borohydride reduction and mild acid hydrolysis at the linkage of the oxidized monosaccharide. Sephadex G-25 gel-permeation chromatography of the degradation products afforded a mixture of oligosaccharides **1** with the *N*-acetyl group and **2** with the *N*-(3-hydroxybutanoyl) group on the homoserine

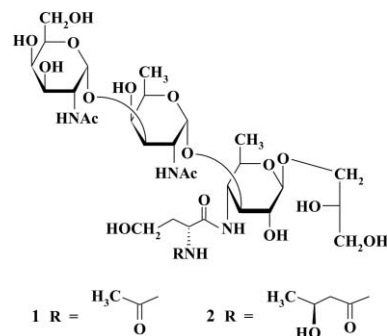


Fig. 6 Structures of the major Smith degradation products from the O-polysaccharide.

residue (Fig. 6). Their structures were confirmed by ESI MS, which showed $[\text{M} - \text{H}]^-$ ions at m/z 769.32 and 813.34 in the negative ion mode and $[\text{M} + \text{Na}]^+$ ions at m/z 793.33 and 837.36 in the positive ion mode (calculated molecular masses 770.34 and 814.37 Da for **1** and **2**, respectively). Minor smaller oligosaccharides were isolated too, which lack an *N*-acyl group or GalNAc or both GalNAc and FucNAc.

Therefore, based on the data obtained, it was concluded that the O-polysaccharide of *A. lwoffii* EK30A has the following structure: \rightarrow 3)- β -D-Quip4NAcyl-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 4)- α -D-GalpNAc-(1 \rightarrow 3)- α -D-FucpNAc-(1 \rightarrow

where Acyl stands for either *N*-acetyl-D-homoseryl in about half of the repeating units or *N*-[(*S*)-3-hydroxybutanoyl]-D-homoseryl in the other half. One can speculate that two distinctly acylated

Qui4N nucleotide precursors are synthesized in EK30A and transferred to Gal equally efficiently by the same glycosyltransferase or by two competing glycosyltransferases. Alternatively, a partial modification of the *N*-acyl group, *i.e.* conversion of the acetyl to the 3-hydroxybutanoyl, may occur in the already assembled repeating unit before, during or after polymerization.

Conclusions

Strain EK30A is the first representative of *A. lwoffii* whose O-polysaccharide structure has been elucidated in all detail. It was found to contain unique homoserine Qui4N derivatives. Although it is not uncommon that in natural glycopolymers Qui4N carries unusual N-linked non-sugar substituents, including amino acids (*e.g.*, alanine, serine, aspartic acid, derivatives of 5-oxoproline),¹⁵ homoserine has not hitherto been found on this or another amino sugar in either LPS or any bacterial polysaccharide. Also, a peculiar feature of the O-polysaccharide studied is an alternation of *N*-acyl groups on homoserine (acetyl *versus* 3-hydroxybutanoyl). In bacterial polysaccharide antigens, unusual sugars and their derivatives use to occupy the non-reducing end of the polymer chain and are therefore crucial for defining serological specificity of bacteria. High antigen diversity, especially due to variations in the nature of the terminal groups, may offer various bacterial clones a selective advantage in their specific niche. It can be expected that other so far unknown peculiarities of antigenic polysaccharides will be unravelled in further studies of ancient bacteria isolated from permafrost.

Experimental

Bacterial strain and growth conditions

A. lwoffii strain EK30A was isolated from a sample of late Pliocene-early Pleistocene (Olyor) sediments (1.6–1.8 million years old).⁶ The sampling site is located in the Arctic region of Russia (Kolyma-Indigirka lowland, North-Eastern Siberia). The permafrost sampling techniques used for the collecting, storage and transportation of the sample as well as bacterial isolation methods, were as described.¹⁶

Bacteria were grown to late log phase in 7 L Luria broth using a 10-L fermentor (BIOSTAT C-10, B. Braun Biotech International, Germany) under constant aeration at 25 °C and pH 7.2–7.4.¹⁷ Bacterial cells were washed and dried as described.¹⁸

Isolation of the lipopolysaccharide and O-polysaccharide

LPS was isolated from dried bacterial mass (1.2 g) by the phenol–water procedure¹⁹ followed by dialysis of the extract without layer separation. After removal of insoluble material by centrifugation, the solution was freed from proteins and nucleic acids by treatment with cold (4 °C) aq. 50% CCl₃CO₂H, the precipitate was removed by centrifugation, and the supernatant was dialyzed against distilled water and freeze-dried to yield LPS (95 mg).

A sample of LPS (75 mg) was heated with 2% HOAc for 1.5 h at 100 °C, and a lipid precipitate was removed by centrifugation. The carbohydrate-containing supernatant was fractionated by GPC on a column (60 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in pyridinium acetate buffer pH 5.4 (4 mL pyridine and 10 mL HOAc in 1 L water) monitored

using a differential refractometer (Knauer, Germany) to give a high-molecular-mass O-polysaccharide (32 mg).

Sugar analyses

An O-polysaccharide sample (2 mg) was hydrolyzed with 3 M CF₃CO₂H (120 °C, 3 h). Neutral sugars were identified using a Biotronik LC-2000 sugar analyzer on a column (15 × 0.4 cm) of a Dionex Ax8 anion-exchange resin in 0.5 M sodium borate buffer pH 8.0 at 70 °C. Amino sugars were identified using a Biotronik LC-2000 amino acid analyzer on a column (22 × 0.4 cm) of an Ostion LC AN B cation-exchange resin in 0.2 M sodium citrate buffer pH 3 at 65 °C.

The absolute configurations of the monosaccharides and homoserine were determined by GLC of the acetylated (*S*)-2-octyl glycosides¹¹ or (*S*)-2-octyl ester, respectively, on a Hewlett-Packard 5890 instrument (USA) equipped with a capillary HP-5ms column (25 m × 0.25 mm) using a temperature gradient of 180 °C (3 min) to 290 °C at 3 °C min^{−1}. The *S* configuration of 3-hydroxybutanoic acid was determined as described.²⁰

Smith degradation

A sample of the O-polysaccharide (15 mg) was oxidized with 1% aq NaIO₄ (2 mL) at 20 °C for 48 h in dark, reduced with NaBH₄ (25 mg) at 40 °C for 3 h, an excess of NaBH₄ was destroyed with HOAc, the solution was evaporated, the residue was evaporated with methanol (5 times), dissolved in 0.3 mL water and applied to a column (110 × 1.2 cm) of Sephadex G-25. The modified polysaccharide (12 mg) was eluted with 0.1% HOAc and hydrolyzed with 0.1 M CF₃CO₂H (100 °C, 1 h). The products were fractionated on Sephadex G-25 as above to yield a mixture of oligosaccharides **1** and **2** and a mixture of smaller by-product oligosaccharides.

NMR spectroscopy

A sample of the O-polysaccharide was freeze-dried twice from a 99.9% D₂O soln and dissolved in 99.95% D₂O. ¹H and ¹³C NMR spectra were recorded at 30 °C on a Bruker AV600 spectrometer (Germany) using internal TSP (δ_{H} 0) and acetone (δ_{C} 31.45) as references. 2D NMR spectra were obtained using standard Bruker software; Bruker TOPSPIN 2.1 program was employed to acquire and process the NMR data. A mixing time of 200 and 250 ms was used in ROESY and TOCSY experiments, respectively. Other NMR experimental parameters were set essentially as described.²¹

Mass spectrometry

Oligosaccharides were analyzed by ESI MS in the negative ion mode using a micrOTOF II instrument (Bruker Daltonics). An oligosaccharide sample (~50 ng μL^{-1}) was dissolved in a 1 : 1 (v/v) water–acetonitrile mixture and sprayed at a flow rate of 3 $\mu\text{L min}^{-1}$. End plate offset voltage was −0.5 kV and capillary voltage was 3.2 kV and −4.5 kV in the negative and positive ion mode, respectively. Drying gas temperature was 180 °C.

Acknowledgements

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