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Structural Characterization of the Core Oligosaccharide Isolated from the Lipopolysaccharide of the Psychrophilic Bacterium *Colwellia psychrerythraea* Strain 34H

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Dedicated to the memory of Ernesto Fattorusso

Keywords: Bacteria / Lipopolysaccharides / Carbohydrates / Oligosaccharides / Structure elucidation / NMR spectroscopy

Cold-adapted bacteria are microorganisms that thrive at very low temperatures in permanently cold environments (0–10 °C). Their ability to survive under these harsh conditions is the result of molecular evolution and adaptations, which include the structural modification of the phospholipid membrane. To give insight into the role of the membrane in the mechanisms of adaptation to low temperature, the characterization of other cell-wall components is necessary. Among these components, the lipopolysaccharides are complex amphiphilic macromolecules embedded in the outer leaflet of the external membrane, of which they are the major constituents. The cold-adapted *Colwellia psychrerythraea* 34H bacterium, living in deep sea and Arctic and Antarctic sea ice, was cultivated at 4 °C. The lipooligosaccharide (LOS) was isolated and analysed by means of chemical analysis. Then it was degraded either by mild hydrazinolysis (*O*-deacylation) or hot KOH (4 M; *N*-deacylation). Both products were investigated in detail by ^{1}H and ^{13}C NMR spectroscopy and by ESI FT-ICR mass spectrometry. The oligosaccharide portion consists of a unique and very short species with the following general structure: α-L-Col-(1→2)-α-D-GalA-(1→2)-α-D-Man-[3-*P*-D-Gro]-(1→5)-α-D-Kdo-4-*P*-Lipid-A.

Introduction

Microorganisms can thrive in what we call extreme environments on Earth. Macelroy named these lovers ("philos" to Greeks) of extreme environments "extremophiles".[1] They had to adapt to one or several extreme physicochemical parameters: thermophiles and hyperthermophiles live above 60 °C near geysers and hydrothermal vents; halophiles thrive in hyper-saline environments; alkaliphiles prefer high pH; and acidophiles thrive at low pH.[2] Cold-adapted microorganisms include both steno-psychrophilic (formerly "true psychrophile") and eury-psychrophilic (formerly "psychrotolerant" or "psychrotrophic") organisms. The former show an optimal growth temperature of 15 °C, and a maximum temperature for growth of 20 °C; the latter have the ability to grow at temperatures below 15 °C, but have maximum growth rates at temperature optima above 18 °C.[3,4] To survive extremes of pH, temperature, and salinity, microorganisms have been found to develop unique defenses against their environment, leading to the biosynthesis of unusual molecules ranging from simple osmolytes to complex secondary metabolites. In addition, the cell envelope shows adaptive changes in the face of the extreme environmental conditions, particularly in its lipid composition.

Microorganisms that thrive in permanently or seasonally very cold habitats solve the "freezing risk" by adopting heterogeneous strategies, such as the maintenance of membrane fluidity by increasing the synthesis of unsaturated fatty acids, and by changing the chain length of the fatty acids,[5–7] and also the quality and quantity of phosphorylation.[8,9] The outer membrane of Gram-negative bacteria forms a barrier for the cell, and it is made up of phospholipids, outer-membrane proteins (OMP), and lipopolysaccharides (LPS). The lipopolysaccharides are complex amphiphilic macromolecules embedded in the outer leaflet of the external membrane of which they are the major constituents. Smooth-form lipopolysaccharides (S-LPS) consist of three covalently linked regions, the glycolipid lipid A,

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also known as the endotoxin for human pathogens, the oligosaccharide region (core region), and the O-specific polysaccharide (O-chain, O-antigen). Rough-form lipopolysaccharides (R-LPS), also named lipooligosaccharides (LOS), lack the polysaccharide portion.[10,11] In order to check whether a role is played by the lipopolysaccharides in the molecular mechanism of adaptation to low temperatures, the complete structural determination of these molecules must be undertaken.

Colwellia psychrerythraea strain 34H, a Gram-negative bacterium isolated from Arctic marine sediments, is an intensively investigated steno-psychrophilic bacterium.[12] It has cardinal growth temperatures (optimum of 8 °C, maximum of 19 °C, and extrapolated minimum of –14.5 °C)[12] that rank among the lowest of all characterized bacteria, which makes this bacterium an attractive model to study the adaptive strategies of the cellular envelope to a sub-zero lifestyle.

In this paper, we report the structural characterization of the carbohydrate backbone of the LOS of *Colwellia psychrerythraea* 34H. The lipooligosaccharide was degraded both by mild hydrazinolysis (*O*-deacylation) and hot KOH (4 M; *N*-deacylation). Both products were investigated by chemical analysis, by ^{1}H and ^{13}C NMR spectroscopy, and by electrospray-ionization Fourier transform ion cyclotron resonance mass spectrometry.

Results and Discussion

LPS Extraction and Preliminary Analysis

Colwellia psychrerythraea strain 34H cells were grown aerobically, and the recovered cell pellet was extracted using phenol/chloroform/light petroleum (PCP) to obtain the crude LPS.[13] When it was analysed by DOC-PAGE electrophoresis, the crude LPS showed positive silver staining. In particular, a rough LPS (LOS_{PCP}) was revealed (Figure 1). Subsequent extraction by the phenol/water method[14] yielded only a low amount of the lipooligosaccharide (LOS_W), the purity of which was lower than that of the LOS_{PCP}. The sugar composition of the LOS_{PCP} was obtained by GC–MS analysis of the acetylated methyl glycosides. Thus, the occurrence of D-galacturonic acid (GalA), 2-amino-2-deoxy-D-glucose (GlcN), D-mannose (Man), 3,6-dideoxy-L-*xylo*-hexose (colitose, Col), and 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo) was revealed. The latter residue was revealed only after dephosphorylation of the LOS_{PCP}, which was achieved by HF treatment. This result suggested the presence of a phosphate group on this residue, which prevented the detection of Kdo by GC–MS.[15] Methylation analysis indicated the presence of terminal Col, 6-substituted GlcN, 2-substituted GalA, and 2,3-disubstituted Man. The methylation data also revealed that all the residues were in the pyranose form.

The absolute configurations of the sugar residues were determined by GC–MS analysis of the corresponding acetylated 2-octyl glycosides.[16]

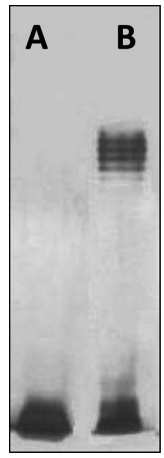


Figure 1. 14% DOC-PAGE analysis of *Colwellia psychrerythraea* strain 34H LOS_{PCP} (lane A) and *Escherichia coli* O55:B5 LPS used as standard (lane B).

GC–MS analysis of the fatty acid methyl esters revealed the presence of decanoic, dodecanoic, 3-hydroxydodecanoic, tetradecenoic, tetradecanoic, pentadecenoic, pentadecanoic, 3-hydoxytetradecenoic, esadecenoic, esadecanoic, octadecenoic, and octadecanoic acids.

Mass Spectrometric Analysis of the *O*-Deacylated LOS_{PCP}

The LOS_{PCP} was *O*-deacylated with anhydrous hydrazine,[17] and the product obtained (LOS-OH) was analysed by ESI FT-ICR MS. The charge-deconvoluted spectrum showed the presence of one main species (M) at 1844.607 Da. Other signals were attributed to potassium and sodium adducts (Figure 2). To obtain further structural information, the LOS-OH was subjected to capillary skimmer dissociation (CSD), which generated the Y and B fragments[18] resulting from the cleavage of the Kdo/lipid A linkage.[19]

The CSD spectrum (Figure 3) showed the presence of a fragment at 922.176 Da, which was assigned to the core oligosaccharide, and a second fragment at 922.423 Da, which was assigned to the lipidA-OH. In particular, the following composition was assigned to the core oligosaccharide: Gro-ColGalAManKdoP_2 (accurate mass 922.175 Da), where Gro represents a glycerol residue. To the lipid A-OH was assigned the following composition: $GlcN_2P_2$[C12:0(3-OH)][C14:1(3-OH)] (accurate mass 922.426 Da), in agreement with the information obtained by chemical analysis.

NMR Spectroscopic Analysis of the Fully Deacylated LOS_{PCP}

To characterize the core oligosaccharide, the LOS-OH was further *N*-deacylated by strong alkaline hydrolysis, and the resulting oligosaccharide (OS) was analysed by one- and two-dimensional NMR spectroscopy (Table 1, Figures 4, 5, 6, and S1).

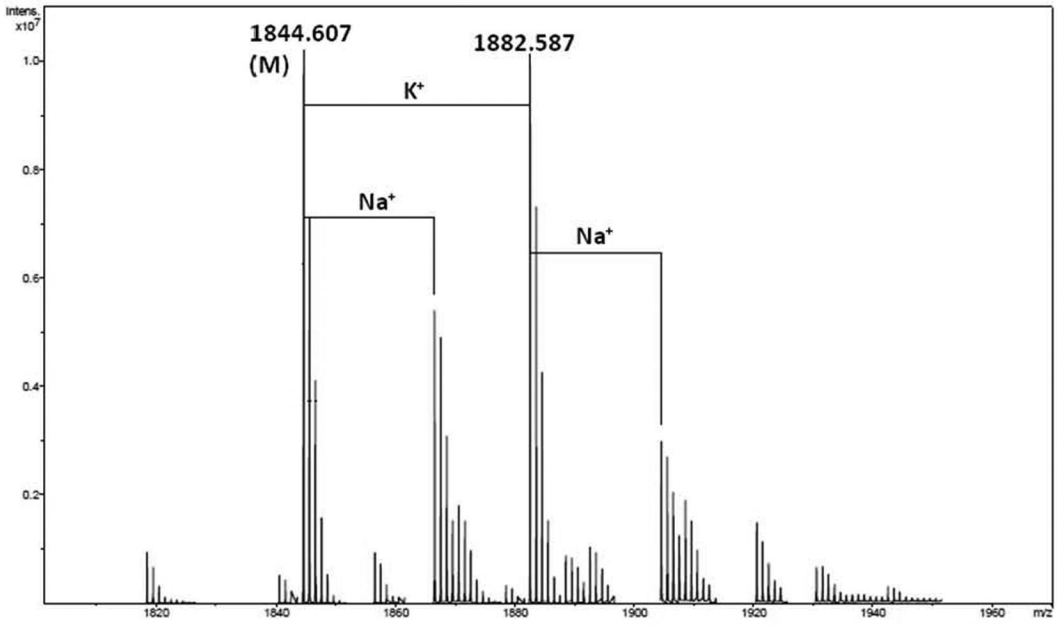


Figure 2. Charge-deconvoluted ESI FT-ICR mass spectrum of the LOS-OH isolated from *Colwellia psychrerytraea* strain 34H. The spectrum was acquired in negative-ion mode.

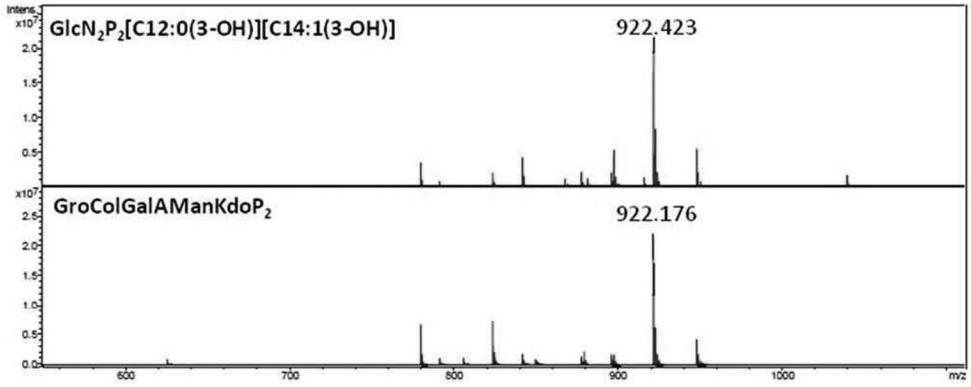


Figure 3. Charge-deconvoluted CSD mass spectrum of the LOS-OH isolated from *Colwellia psychrerytraea* strain 34H. The spectrum was acquired in negative-ion mode.

Table 1. ^{1}H and ^{13}C NMR assignments of the fully deacylated oligosaccharide of the LOS_{PCP} from *Colwellia psychrerythraea* strain 34H. The values are referenced to acetone as internal standard (^{1}H: δ = 2.225 ppm; ^{13}C: δ = 31.45 ppm). The spectra were recorded at 302 K.

Residue	1-H C-1	2-H C-2	3-H C-3	4-H C-4	5-H C-5	6a-H C-6	6b-H/7-H C-7	7b-H/8-H C-8
A	5.56	3.38	3.88	3.39	4.09	3.80	4.21	
α-Glc*p*N-1-*P*	92.8	55.3	70.6	71.1	73.9	70.3		
B	5.30	4.03	3.93	3.69	4.14	3.88	3.65	
2-α-Man*p*	100.2	80.8	71.5	68.6	73.6	62.4		
C	5.16	3.84	4.09	4.24	4.41	–		
2-α-Gal*p*A	101.7	76.9	70.0	72.3	73.4	174.8		
D	4.97	3.95	1.93	3.81	4.13	1.13		
α-Col*p*	101.5	64.7	34.2	69.6	68.2	16.8		
E	4.82	3.07	3.81	3.81	3.70	3.46	3.69	
6-β-Glc*p*N-4-*P*	99.9	56.8	72.9	75.5	75.3	63.7		
F	–	–	1.95–2.15	4.49	4.20	3.76	3.76	3.64–3.86
5-α-Kdo*p*-4-*P*	174.2	101.0	35.5	71.2	74.1	72.8	70.4	64.9

In particular, ^{1}H–^{1}H DQF-COSY (double quantum-filtered correlation spectroscopy), ^{1}H–^{1}H TOCSY (total correlation spectroscopy), ^{1}H–^{1}H ROESY (rotating-frame nuclear Overhauser enhancement spectroscopy), ^{1}H–^{13}C

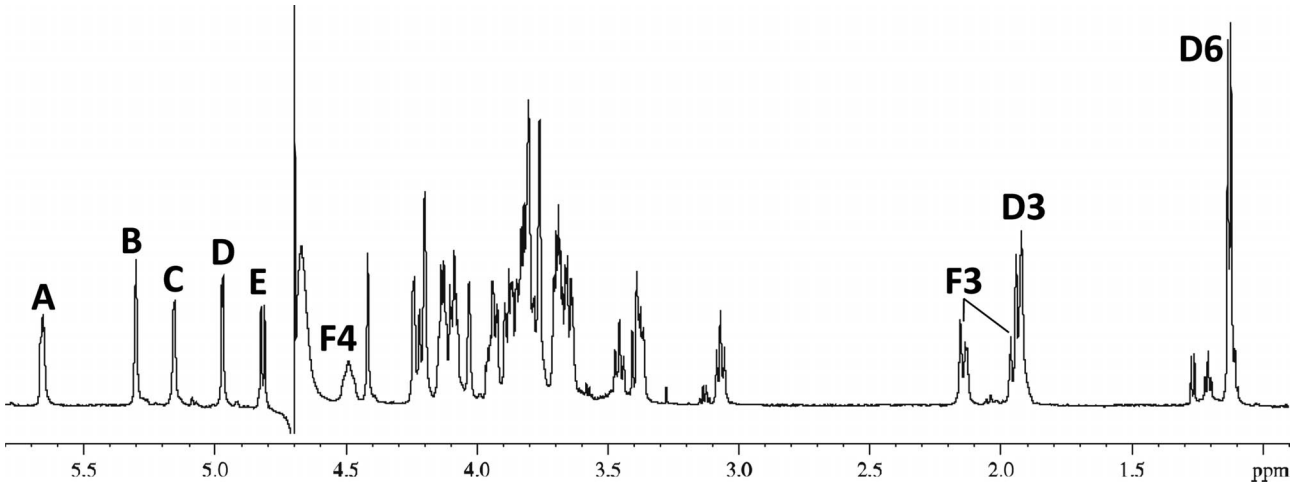


Figure 4. ^{1}H NMR spectrum of the oligosaccharide (OS) obtained by strong alkaline hydrolysis of the LOS_{PCP}. The spectrum was recorded in D_2O at 302 K at 600 MHz. The letters refer to the residues as described in Table 1 and Scheme 1.

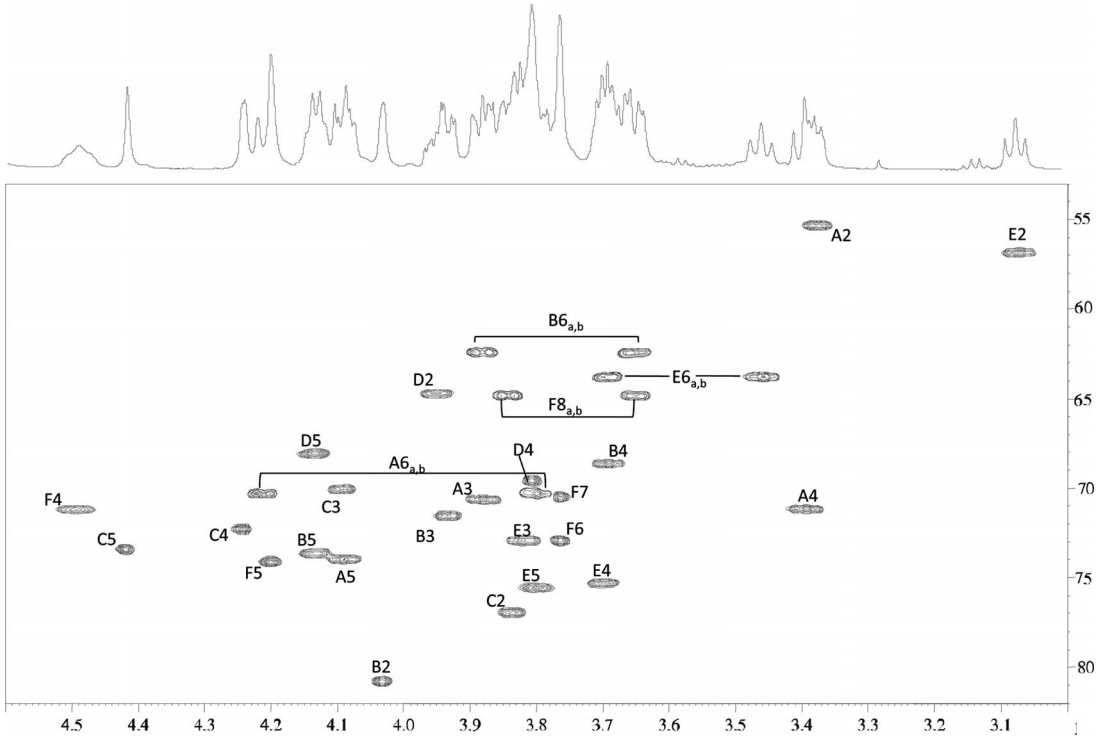


Figure 5. Alcohol signals region (δ = 3.0–4.6 ppm) of the ^{1}H–^{13}C DEPT-HSQC spectrum of OS. The spectrum was recorded in D_2O at 302 K at 600 MHz using acetone as an internal standard (δ_H = 2.225 ppm and δ_C = 31.45 ppm). The letters refer to the residues as described in Table 1 and Scheme 1.

DEPT-HSQC (distortionless enhancement by polarization transfer-heteronuclear single quantum coherence), ^{1}H–^{13}C HSQC-TOCSY, ^{1}H–^{13}C HMBC (heteronuclear multiple bond correlation), 2D *F*2-coupled HSQC, and ^{31}P NMR spectroscopy were performed.

The ^{1}H NMR spectrum (Figure 4) of the fully deacylated LOS_{PCP} showed the presence of five anomeric proton signals (A–E) between δ = 4.7 and 5.7 ppm. By taking into account all the 2D NMR experiments, the spin systems of all the monosaccharides were identified.

Residue A was assigned as the 6-substituted α-Glc*p*N-1-*P* of lipid A on the basis of the multiplicity of the anomeric proton signal due to its phosphorylation ($^3J_{H,P}$ = 6.1 Hz). Moreover, the C-2 resonance occurred at δ = 55.3 ppm, which indicated a nitrogen-bearing carbon atom, and the C-6 resonance was shifted downfield by glycosylation to δ = 70.3 ppm.

Residue E, with C-1/1-H signals at δ = 99.9/4.82 ($^1J_{C\text{-}1,1\text{-}H}$ = 166.3 Hz), was assigned as the lipid A 6-substituted β-Glc*p*N-4-*P* residue, as a result of its C-2 chemical

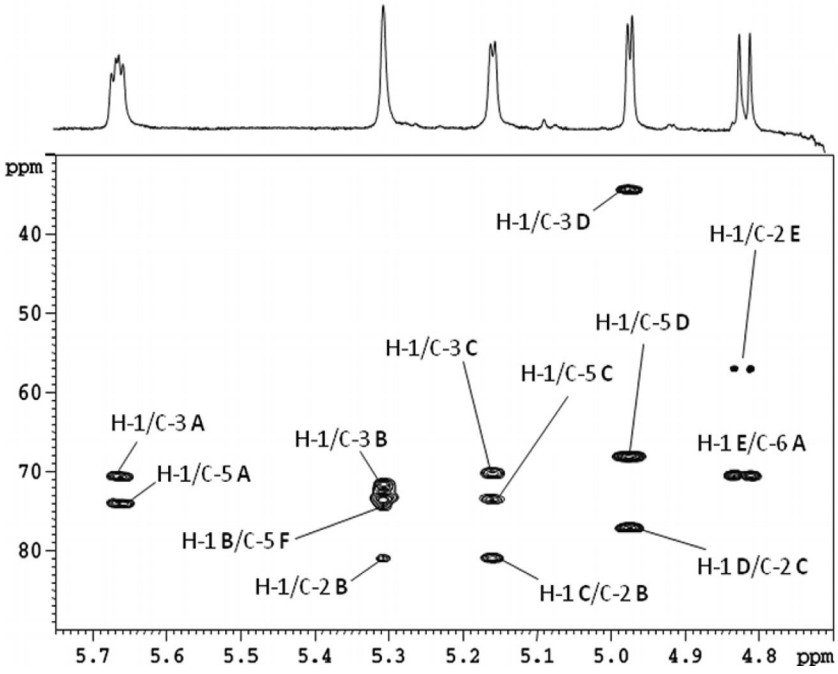


Figure 6. Anomeric region of the ^{1}H–^{13}C HMBC spectrum of OS. The spectrum was recorded in D_2O at 302 K at 600 MHz using acetone as an internal standard (δ_H = 2.225 ppm and δ_C = 31.45 ppm). The letters refer to the residues as described in Table 1 and Scheme 1.

shift at δ = 56.8 and its linkage to O-6 of residue A. In fact 1-H of residue E showed a long-range scalar coupling with C-6 of residue A in the HMBC spectrum. The β-anomeric configuration was corroborated by the intraresidue NOE correlations observed between 1-H and both 3-H and 5-H in the ROESY spectrum. Moreover, the downfield shifts of 4-H and C-4 are diagnostic for the presence of a phosphate group linked at O-4.[20]

The Kdo (residue F) proton and carbon chemical shifts were identified starting from the diastereotopic protons 3_{ax}-H and 3_{eq}-H. The chemical-shift difference between these protons depends on the configuration of the C-1 carbon atom, being different for the α and β anomers. In this case, the difference of $\Delta(3_{ax}\text{-H} - 3_{eq}\text{-H})$ = 0.2 ppm allowed us to assign an α configuration to the residue.[21] Moreover, both protons showed a correlation in the DQF-COSY spectrum with a signal at δ = 4.49 ppm, assigned as Kdo 4-H, which was in turn correlated to a carbon atom with signal at δ = 71.2 ppm in the DEPT-HSQC spectrum. Both 4-H and C-4 resonances were shifted downfield relative to reference values,[22] and the observed chemical shifts were diagnostic for the presence of a phosphate in that position.[23] The Kdo 5-H proton was identified by vicinal scalar coupling with 4-H in the DQF-COSY spectrum, and the corresponding carbon atom was downfield shifted to δ = 74.1 ppm indicating glycosylation at this position. In addition, the Kdo anomeric carbon atom showed a long-range correlation with 6-H of residue E, thus confirming its linkage to the lipid A backbone.

Residue B was assigned as a 2-substituted α-mannopyranose on the basis of the small $J_{1\text{-H},2\text{-H}}$ and $J_{2\text{-H},3\text{-H}}$ coupling-constant values. The glycosylation at the C-2 position was inferred by comparing the carbon chemical shift values with standard values,[24] and the α-anomeric configuration was established by the $J_{C\text{-}1,1\text{-H}}$ coupling-constant value, which was 176.4 Hz. The HMBC spectrum (Figure 6) showed the presence of a long-range scalar coupling between the 1-H proton of this residue and C-5 of Kdo, thus indicating that this position was substituted by residue B.

Residue C, with 1-H/C-1 signals at δ = 5.16/101.7 ppm, was identified as a 2-substituted α-galactopyranuronic acid, since its C-6 signal occurred at δ = 174.8 ppm, and its C-2 signal was shifted downfield to δ = 76.9 ppm. The α-anomeric configuration was inferred from its $J_{C\text{-}1,1\text{-H}}$ coupling-constant value of 176.3 Hz. This residue was linked to residue B at the O-2 position, as shown by the presence of a correlation between 1-H of residue C and C-2 of residue B in the HMBC spectrum (Figure 6).

The last residue (D) of the core oligosaccharidic chain was identified as a terminal α-colitose, since its 3-H/C-3 and 6-H/C-6 signals occurred at δ = 1.93/34.2 and 1.13/16.8 ppm, respectively. The α-anomeric configuration was inferred from its $J_{C\text{-}1,1\text{-H}}$ value of 173.9 Hz. Its 1-H showed an interresidue NOE correlation with 2-H of residue C in the ROESY spectrum, and a long-range scalar coupling with the signal at δ = 101.7 ppm of a carbon atom in the HMBC spectrum (Figure 6). Thus, it was shown to be linked to residue C at the O-2 position.

To sum up, the above data allowed the identification of the main carbohydrate backbone of the lipopolysaccharide from *Colwellia psychrerythraea*, as shown in Scheme 1.

The ^{31}P NMR spectrum of OS confirmed the presence of only three phosphomonoester signals (Kdo-4-*P* at δ = 3.9 ppm, GlcN-4-*P* at δ = 3.5 ppm, and GlcN-1-*P* at δ = 2.6 ppm), in contrast with the results obtained from the analysis of the LOS-OH mass spectra, which indicated the

D C B F E A
α-L-Col-(1→2)-α-D-GalA-(1→2)-α-D-Man-(1→5)-α-D-Kdo4*P*-(2→6)-β-D-GlcN4*P*-(1→6)-α-D-GlcN1*P*

Scheme 1.

presence of four phosphate groups. This, together with the lack of the glycerol residue in the OS structure, suggested that these groups did not withstand the alkaline treatment, which is consistent with the lability of diesters under these hydrolysis conditions.[25] In addition, the finding of a 2,3-disubstituted mannose in the methylation analysis did not fit with the above reported structure.

NMR Spectroscopic Analysis of the LOS-OH

To establish the structure of the core oligosaccharide including the labile groups lost during the harsh alkaline treatment, the LOS-OH was analysed by NMR spectroscopy (Figures 7, S2 and S3). In particular ^{1}H–^{1}H DQF-COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{1}H ROESY, and ^{1}H–^{13}C

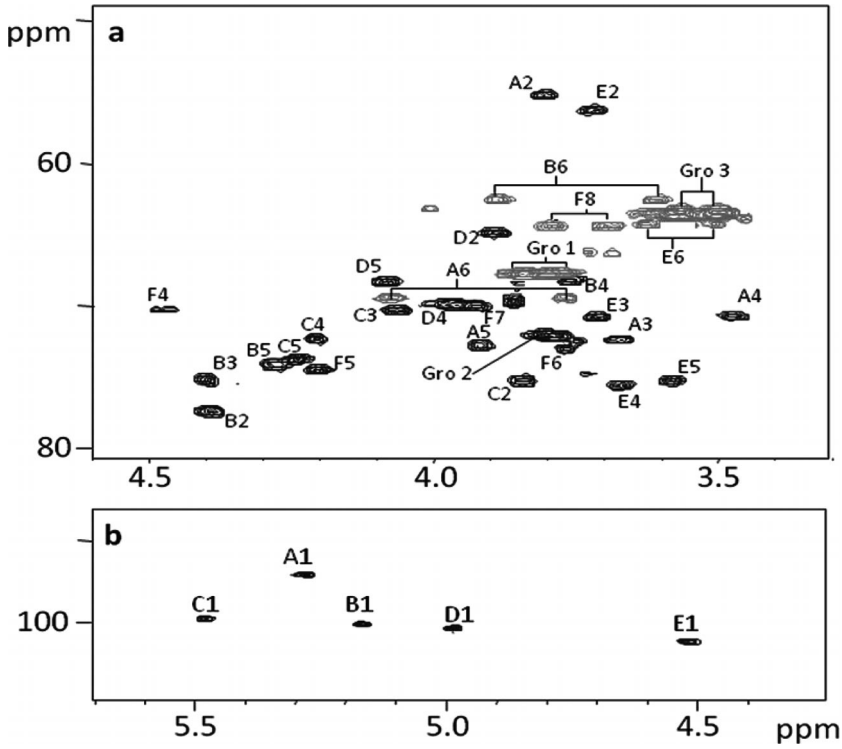
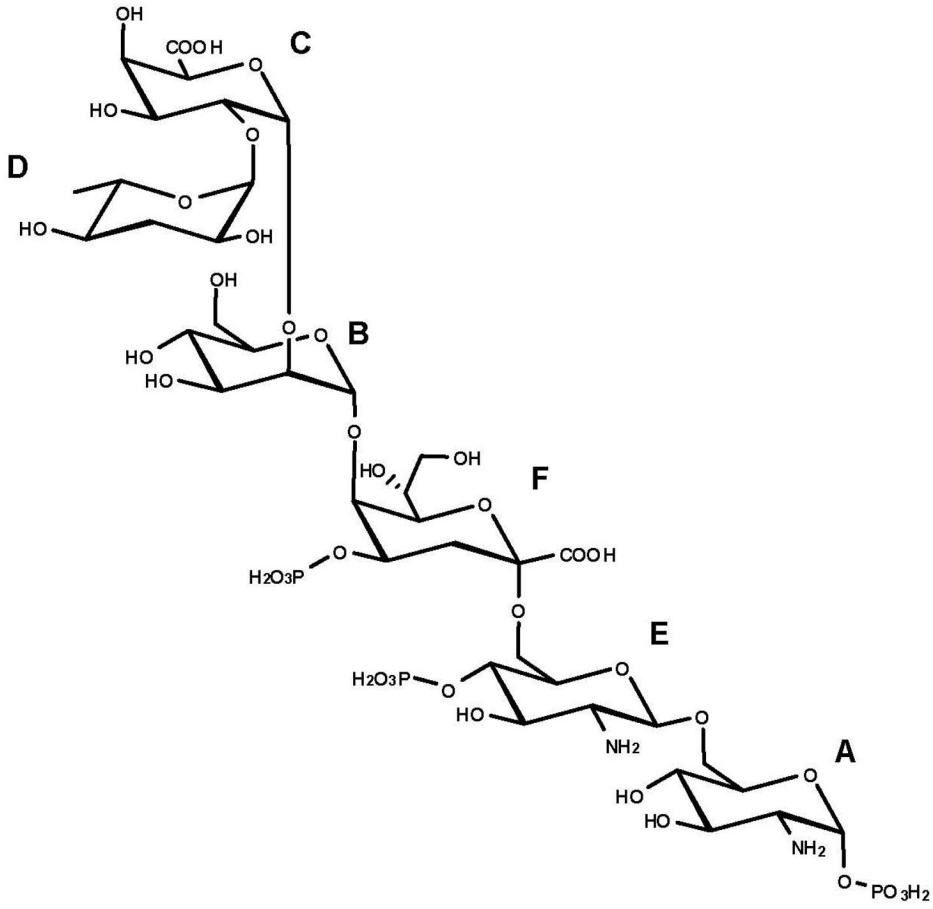


Figure 7. Carbinol (a) and anomeric (b) regions of the ^{1}H–^{13}C DEPT-HSQC spectrum of LOS-OH from *Colwellia psychreritraea* strain 34H. The spectrum was recorded in D_2O at 298 K at 600 MHz using acetone as an internal standard (δ_H = 2.225 ppm and δ_C = 31.45 ppm). The letters refer to the residues as described in Table 2 and Scheme 2.

Table 2. ^{1}H and ^{13}C NMR assignments of the LOS-OH from *Colwellia psychrerythraea* strain 34H. All the values are referenced to acetone as internal standard (^{1}H: δ = 2.225 ppm; ^{13}C: δ = 31.45 ppm). The spectra were recorded at 298 K.

Residue	1-H C-1	2-H C-2	3-H C-3	4-H C-4	5-H C-5	6a-H C-6	6b-H/7-H C-7	7b-H/8-H C-8
A	5.27	3.80	3.67	3.47	3.91	3.76	4.06	
α-Glc*p*N-1-*P*	94.1	55.1	72.4	70.7	72.7	69.4		
B	5.16	4.40	4.40	3.75	4.27	3.87	3.61	
2-α-Man*p*-3-*P*	100.2	77.3	75.1	68.1	74.1	62.4		
C	5.47	3.85	4.06	4.20	4.42	–		
2-α-Gal*p*A	99.7	75.1	70.0	72.2	73.4	nd		
D	4.98	3.87	1.88–1.98	3.97	4.07	1.13		
α-Col*p*	100.3	64.9	34.3	69.7	68.2	17.1		
E	4.51	3.71	3.67	3.67	3.57	3.49	3.62	
6-β-Glc*p*N-4-*P*	102.5	56.2	72.4	75.5	75.1	64.2		
F	–	–	1.89–2.16	4.47	4.19	3.76	3.91	3.68–3.78
5-α-Kdo*p*-4-*P*	nd	nd	35.5	70.1	74.5	72.9	69.8	64.3
t-Gro-1-*P*	3.79–3.83 67.7	3.77 71.9	3.49–3.56 63.4					



Scheme 2.

DEPT-HSQC spectra were acquired. Analysis of all the spectra (Table 2) confirmed that the main carbohydrate backbone was that shown in Scheme 1, but that additional signals due to the glycerol residue were present.

In more detail, the signal of C-1 of the Gro residue occurred at δ = 67.7 ppm in the ^{1}H–^{13}C DEPT-HSQC spectrum (Figure 7), thus indicating its phosphorylation at C-1. Furthermore, the signals of 3-H and C-3 of residue B were shifted downfield to δ = 4.40/75.1 ppm, thus indicating that Gro was linked to the mannose residue at position O-3 by a phosphodiester linkage, consistent with the presence of the 2,3-disubstituted mannose in the GC–MS methylation analysis. To determine the relative configuration of the Gro residue, it was oxidized to glyceric acid. The product was hydrolysed, the free glyceric acid was esterified with chiral 2-octanol, and the resulting octyl ester derivative was analysed by GC–MS.[26] By comparison of its retention time with that of a standard sample, it was found to be D-configured.

In conclusion, the complete structure of the core lipid A saccharidic backbone of the LPS from *Colwellia psychrerythraea* strain 34H is as shown in Scheme 2.

Conclusions

In this paper, we reported the complete structure of the sugar backbone of the lipopolysaccharide fraction from *Colwellia psychrerythraea* strain 34H, a strictly psychrophilic marine bacterium isolated from deep sea and Arctic and Antarctic sea ice.[12] The molecules were extracted by the PCP method, and they had a lipooligosaccharide fraction. The complete structural determination was achieved by chemical analysis, NMR spectroscopy, and ESI mass spectrometry. It is worth noting the lack of heptose residues in the inner core of *C. psychreritraea*. Actually, in their place, an α-mannose residue is linked to the Kdo. This structural feature is commonly found in the *Rhizobiaceae* family, but has never before been found in extremophiles. Moreover, colitose and glycerol are present in the *Colwellia* LOS. Both these residues have been already found in several O-polysaccharides and K-antigens from Gram-negative bacteria, but to the best of our knowledge, this is the first time that they have been found in a core oligosaccharide. Until now, only a few structures of LPSs from other cold-adapted microorganisms have been characterised. By comparing the *C. psychrerythraea* 34H LOS structure with those obtained from *P. haloplanktis* TAC125[27,28] and TAB23,[29] and from *P. arctica*,[15] it turned out that they have in common the lack of an O-chain and the presence of a high charge density in the core region due to the presence of acidic monosaccharides and phosphate groups. In addition, the core regions have been found to be made up of only a few sugar units. These structural features seem to be com-

mon to cold-adapted microorganisms. Recently, O-chain polysaccharides were found in LPS from *Psychrobacter muricolla* and *cryohalentis*. Although the latter microorganisms had been isolated at –9 °C, the LPS was extracted from bacterial cells grown at 24 °C.[30,31] It would be worth investigating the LPS produced at a lower growth temperature.

Experimental Section

Bacteria Growth and LOS Isolation: *Colwellia psychrerythraea* 34H was grown aerobically at 4 °C in marine broth medium (DIFCO™ 2216). When the liquid culture reached the late exponential phase (OD600 = 2), cells were harvested by centrifugation at 3000 *g* at 4 °C for 20 min. Dried bacteria cells (4.8 g) were extracted by the PCP method[13] to give LOS_{PCP} (52 mg, yield 1.1% w/w of dried cells).

Sugar and Fatty Acids Analysis: A sample of LOS_{PCP} (0.5 mg) was treated first with HF (48% aq.; 100 μL); then methanolysis was performed. The monosaccharides obtained were acetylated and analysed by GC–MS as described previously,[15] while the fatty acids were analysed as methyl esters. The sugars were identified by comparison with standard samples. In particular, the colitose standard was obtained from *Escherichia coli* O55:B5 LPS (Sigma). The absolute configurations of the sugars were determined by gas chromatography of the acetylated (*S*)-2-octyl glycosides.[16] The absolute configuration of Gro was determined by GC–MS analysis of its 2-octyl ester derivative. Briefly, it was oxidized using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), then hydrolysed with TFA (trifluoroacetic acid; 2 M), and esterified with chiral 2-octanol.[26] All the sugar derivatives were analysed with an Agilent Technologies 6850A gas chromatography apparatus equipped with a mass-selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 mL min^{-1}, He as carrier gas). Acetylated methyl glycosides were analysed using the following temperature program: 150 °C for 3 min, 150→240 °C at 3 °C min^{-1}. Fatty acids were analysed as follows: 140 °C for 3 min, 140→280 °C at 10 °C min^{-1}, 280 °C for 20 min. The analysis of acetylated octyl glycosides was performed as follows: 150 °C for 5 min, then 150→240 °C at 6 °C min^{-1}, 240 °C for 5 min. The glyceric acid octyl ester derivatives were analysed with the following temperature program: 80 °C for 5 min, 80→200 °C at 5 °C min^{-1}, 200→300 °C at 10 °C min^{-1}.

Linkage Analysis: The linkage positions of the monosaccharides were determined by GC–MS analysis of the partially methylated alditol acetates. Briefly, the LOS_{PCP} (1 mg) was methylated with CH_3I (300 μL) and NaOH powder in DMSO (1.0 mL) for 20 h.[32] The product was treated with $NaBD_4$ to reduce the uronate groups, then totally hydrolysed with TFA (2 M) at 120 °C for 2 h, reduced again with $NaBD_4$, acetylated with Ac_2O and pyridine (50 μL each, 100 °C, 30 min), and the resulting product mixture was analysed by GC–MS. The temperature program used was: 90 °C for 1 min, 90→140 °C at 25 °C min^{-1}, 140→200 °C at 5 °C min^{-1}, 200→280 °C at 10 °C min^{-1}, 280 °C for 10 min.

Deacylation of the LOS: The LOS_{PCP} (20 mg) was first dried under vacuum over phosphoric anhydride and then incubated with hydrazine (1.0 mL, 37 °C, 1.5 h). To precipitate the LOS-OH, cold acetone was added. The pellet was recovered after centrifugation (4 °C, 10000 *g*, 30 min), washed three times with acetone, and finally suspended in water and lyophilized (14 mg).[17] The LOS-OH (8 mg) was dissolved in KOH (4 M aq.; 1.0 mL) and incubated at 120 °C for 16 h. The KOH was neutralized with HCl (2 M aq.) until pH = 6, and the mixture was extracted three times with $CHCl_3$. The aqueous phase was recovered and desalted on a Sephadex G-10 column (Amersham Biosciences, 2.5 × 43 cm, 31 mL h^{-1}, fraction volume 2.5 mL, eluent NH_4HCO_3 10 mM). The eluted oligosaccharide mixture was then lyophilized (3.5 mg, 44% w/w).

NMR Spectroscopy: ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe. All two-dimensional homo- and heteronuclear experiments (COSY, TOCSY, ROESY, HSQC-DEPT, HSQC-TOCSY, 2D *F*2-coupled HSQC, and HMBC) were performed by using standard pulse sequences available in the Bruker software. The mixing time for TOCSY, ROESY, and HSQC-TOCSY experiments was 100 ms. Chemical shifts were measured in D_2O at 302 K and 298 K for OS and LOS-OH, respectively, by using acetone as an internal standard (δ_H = 2.225 ppm and δ_C = 31.45 ppm).

Mass Spectrometry Analysis: Electrospray-ionization Fourier transform ion cyclotron (ESI FT-ICR) mass spectrometry was performed in the negative-ion mode with an APEX QE (Bruker Daltonics) instrument equipped with a 7 T actively shielded magnet. The LOS-OH sample was dissolved at a concentration of ca. 10 ng μL^{-1} and analysed as described previously.[33] Mass spectra were charge-deconvoluted, and the mass numbers given refer to the monoisotopic masses of the neutral molecules.

Supporting Information (see footnote on the first page of this article): The whole ^1H-^{13}C DEPT-HSQC spectra of OS and LOS-OH, as well as the proton spectra of LOS-OH are reported in this section. The main signals are described in the main text of the article. Moreover the elemental analysis of both the products are reported.

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