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The complete structure of the lipooligosaccharide from the halophilic bacterium *Pseudoalteromonas issachenkonii* KMM 3549^T

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Abstract—Novel lipooligosaccharide components were isolated and identified from the lipooligosaccharide fraction of the halophilic marine bacterium *Pseudoalteromonas issachenkonii* type strain KMM 3549^T. The complete structure was achieved by chemical analysis, 2D NMR spectroscopy and MALDI mass spectrometry as the following:

β-Gal-(1→7)-α-Hep3*P*-(1→5)-α-Kdo4P-(2→6)-Lipid A
α-Glc-(1→4)-β-Gal-(1→4)-

All sugars are D-pyranoses. Hep is L-*glycero*-D-*manno*-heptose, Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid, P is phosphate, residues and substituents in italic are not stoichiometrically linked. In addition, by MALDI mass spectrometry of the intact LOS, the lipid A moiety was also identified as a mixture of penta-, tetra- and triacylated species.

Keywords: *Pseudoalteromonas issachenkonii*; Lipooligosaccharide; Lipid A; Marine bacteria

1. Introduction

Obligate marine bacteria of the genus *Pseudoalteromonas* belong to γ-subclass of the class *Proteobacteria* and comprise a copious group of prokaryotic organisms often recovered from the marine environments, for example, coastal, open- and deep-water of the seas and oceans. Taxonomically, this group of marine prokaryotes derives from the genus *Alteromonas*. The revised *Alteromonas* genus retains one species, *A. macleodii*, while the new genus *Pseudoalteromonas* includes the rest of species of the rRNA homology group II.[1] Members of *Pseudoalteromonas* spp. isolated from a variety of sites, that is from the surface of biological and nonbiological objects, sediments, seawater, sea ice, marine animal intestines, form complex symbiotic associations with other microorganisms and marine macrohydrocoles. Coexistence and survival in such environments might be the reason for the evolutionary development of the diverse metabolic pathways observed in *Pseudoalteromonas* that results in the production of various biologically active compounds.[2,3]

Pseudoalteromonas issachenkonii is a Gram-negative, aerobic, marine bacterium with polar flagella recently isolated from the thallus of the brown alga *Fucus*

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evanescens collected in the Kraternaya Bight of the Kurile Islands in the Pacific Ocean.[4,5] The bacterium was shown to belong to the genus *Pseudoalteromonas* according to 16S rDNA gene sequence analysis and DNA–DNA hybridisation showed 27–54% relatedness between strain KMM 3549^T and other type strains of this genus. This halophilic organism has bacteriolytic, proteolytic and haemolytic activity and degrades algal polysaccharides, producing a number of glycosyl hydrolases (fucoidanases, laminaranases, alginases, agarases, pullulanases, β-glucosidases, β-galactosidases, β-*N*-acetylglucosaminidases and β-xylosidases). As a Gram-negative bacterium, *P. issachenkonii* should possess lipopolysaccharides in the external leaflet of its outer membrane.

Lipopolysaccharides (LPSs) are crucial amphiphilic constituents of the outer membrane of the Gram-negative bacterial cell wall.[6] Structurally, they comprise in their smooth form (S-LPSs) three regions, the O-specific polysaccharide (or O-antigen), the oligosaccharide region (core region) and the glycolipid part (lipid A). Rough (R) form LPSs do not possess an O-specific polysaccharide and are named lipooligosaccharides (LOSs). LOSs may occur in both wild and laboratory strains possessing mutations in the genes encoding the O-specific polysaccharide biosynthesis or transfer. The core regions comprise oligosaccharides composed of mostly up to 15 monosaccharides[7,8] and may be divided in two regions: the inner core, constituted by typical sugars as 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep) and the outer core, which contains more common residues. Whereas Kdo, linking the core region to lipid A, is always present, the heptose residues may be lacking. In LOSs, the core oligosaccharide is the external saccharide part of the molecule and thus it is involved in the interaction with the external environment. In particular, it possesses antigenic properties and it is thought to modulate the toxic activities of the lipid A portion.

Lipopolysaccharides from halophilic bacteria frequently show unusual chemical features most likely due to external environment bacterium has to face.[9,10] Since *P. issachenkonii* produces a R-LPS and given that this should be important in the adhesion of this organism with the peculiar external surrounding and nothing is known about the cell wall composition of this halophilic bacterium, we started the structural investigation of its R-LPS. In this framework, the complete structure of the lipooligosaccharide components of this strain is reported herein.

2. Results and discussion

The lipooligosaccharide fraction of *P. issachenkonii* was extracted from dried cells with phenol/chloroform/petroleum ether and purified with gel permeation chromatography. The SDS-PAGE showed, after silver nitrate gel staining, a migration to the bottom of the gel in accordance with the lipooligosaccharide nature of this fraction. The compositional monosaccharide analysis of LOS identified L,D-Hep, D-Gal, D-GlcN, D-Glc and Kdo. Methylation analysis showed the presence of terminal-Gal, 5-substituted-Kdo, 6-substituted-GlcN, 7-substituted-Hep, and, in minor amount 4,7-disubstituted-Hep, 4-substituted-Gal and terminal-Glc. Fatty acids analysis revealed, as major components, the presence of (*R*)-3-hydroxydodecanoic acid [C12:0 (3-OH)] and (*R*)-3-hydroxyundecanoic acid [C11:0 (3-OH)] both in amide and in ester linkages and of dodecanoic acid (C12:0) and undecanoic acid (C11:0) exclusively in ester linkage. In minor amount C10:0 (3-OH), C13:0 (3-OH), C10:0 were found.

Alkaline degradation of LOS yielded an oligosaccharide mixture differing in the length of oligosaccharide chain and the degree of phosphorylation. The primary structure of oligosaccharides within LOS fraction was established by ^{1}H, ^{31}P and ^{13}C NMR spectroscopy. Chemical shifts were assigned utilising DQF-COSY, TOCSY, NOESY, ROESY, HSQC, HMBC and HSQC–TOCSY experiments. Anomeric configurations were assigned on the basis of the chemical shifts, of $^3J_{\text{H-1,H-2}}$ values, which were determined from the DQF-COSY experiment and of $^1J_{\text{C-1,H-1}}$ deriving from ^{1}H,^{13}C-HSQC registered without decoupling during acquisition. The data are presented in Table 1. All sugars were identified as pyranose rings, based on ^{1}H and ^{13}C NMR chemical shifts and on the HMBC spectrum that showed intraresidual scalar connectivity between H-1/C-1 and C-5/H-5 of residues (for Kdo between C-2 and H-6). The anomeric region of the ^{1}H NMR spectrum (Fig. 1) contained six major anomeric signals, in nonstoichiometric ratio, relative to six different spin systems (**A**–**F**). Their identification was possible by the complete assignment of all proton signals and the determination of the $^3J_{\text{H,H}}$ vicinal coupling constant values.

Three residues (**A**, **B** and **D**) possessed the α-configuration, as showed by a coupled HSQC ($^1J_{\text{C,H}} = 173$ Hz). In particular, **A** residue was obviously identified as the GlcN I of lipid A skeleton because of its chemical shifts and the multiplicity of the anomeric signal (double doublet, $^3J_{\text{H-1,H-2}} = 3.2$ Hz and $^3J_{\text{H-1,P}} = 8.1$ Hz). Spin system **B** had the anomeric signal at 5.15 ppm, it possessed low $^3J_{\text{H-1,H-2}}$ and $^3J_{\text{H-2,H-3}}$ values, diagnostic of H-2 equatorial orientation, and, in the TOCSY spectrum from H-2 it was possible to assign all the other cross-peaks within the spin system leading to identify it as heptose monosaccharide. Further set of signals present in minor amount were visible for this spin system in the TOCSY spectrum, they were all ascribed as heptose residues (**B1**) in different chemical/magnetic environments. Spin system **D** ($^3J_{\text{H-1,H-2}} = 3.5$ Hz) was identified as terminal α-glucose residue since it possessed all large

Table 1. ^{1}H, ^{13}C, ^{31}P chemical shifts (ppm) of oligosaccharides **1**–**3** in their tetraphosphorylated form

Unit	Chemical shift δ ($^{1}H/^{13}C/^{31}P$)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
A	5.63	3.36	3.88	3.43	4.15	4.27/3.85	
α-GlcN I	90.9	54.7	70.1	70.3	72.8	69.8	
	2.57						
B	5.15	4.08	4.46	3.82	4.18	4.10	4.20/3.81
			(4.08)				
7-α-Hep	100.08	70.86	73.0	72.2	75.8	70.0	71.0
			2.10				
B1	5.15	4.12	4.41	3.99	4.44	4.12	4.20/3.81
			(4.10)				
4, 7-α-Hep	100.08	69.8	69.7	78.7	73.0	69.5	71.0
			1.98				
C	4.94	3.047	3.84	3.826	3.71	3.50/3.70	
β-GlcN	99.8	55.8	72.2	72.79	75.2	63.7	
				2.83			
D	4.89	3.488	3.77	3.41	4.13	3.75/3.91	
t-α-Glc	100.4	72.2	73.4	70.2	69.7	61.0	
E	4.46	3.54	3.65	3.90	3.69	3.90/3.78	
t-β-Gal	103.8	71.2	73.6	68.8	76.5	61.1	
F	4.58	3.48	3.67	3.92	3.65	3.70	
t-β-Gal	103.9	72.6	74.9	70.1	76.9	60.9	
F1	4.52	3.59	3.73	4.00	3.8	3.80	
4-β-Gal	103.8	71.0	72.9	77.4	75.9	60.9	
	$3_{ax/eq}$	**4**	**5**	**6**	**7**	**8**	
G	1.97/2.22	4.56	4.29	3.83	3.88	3.89/3.70	
Kdo	34.65	70.13	72.8	72.9	69.07	63.7	
		1.96					

In parenthesis chemical shift values are given for the triphosphorylated form. Spectra were recorded at 30 °C of a solution of 2 mg in 0.6 mL D_2O at pD 14 and calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. Aq 85% phosphoric acid was used as external reference (0.00 ppm) for ^{31}P NMR spectroscopy.

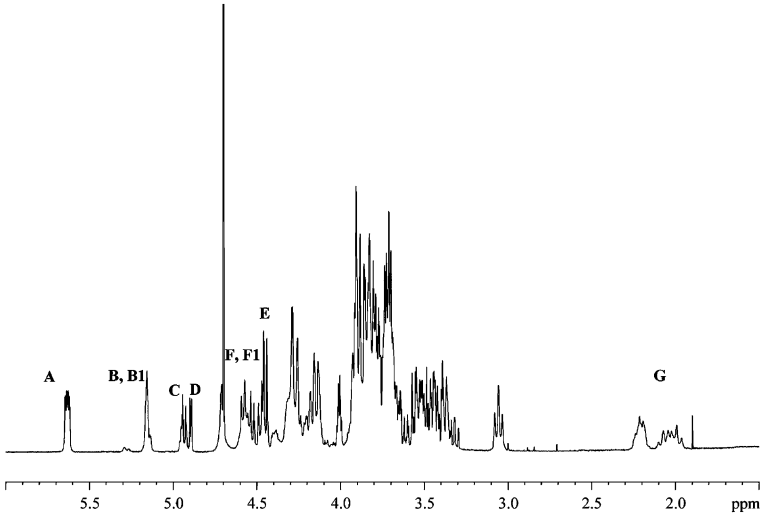


Figure 1. ^{1}H NMR spectrum of oligosaccharide mixture **1**–**3** deriving from alkaline degradation. Anomeric signals are designated by capital letters.

$^{3}J_{H,H}$ ring values. Three residues (residues **C**, **E** and **F**) had anomeric β-configuration, based on their $^{3}J_{H\text{-}1,H\text{-}2}$ values and a HSQC registered without decoupling during acquisition ($^{1}J_{C,H} = 162$ Hz). This assumption was also

supported by a NOESY experiment that showed for these sugar residues intraresidual NOE connectivity from H-1 to H-3 and to H-5. Residue (**C**) was identified as the β-GlcN (GlcN II) of lipid A backbone on the basis of its chemical shifts and coupling constants (in the range of 10 Hz) and since H-2 was correlated to a nitrogen bearing carbon signal in the HSQC spectrum (55.8 ppm). Spin systems **E** and **F** were both identified as galactose residues owing to the small $J_{H,H}$ values for H-3/H-4 and H-4/H-5. Another set of signals was present for spin system **F** that was characterised by different ring proton resonances (**F1**). The characteristic diasterotopic H-3 methylene signals of a Kdo (residue **G**) were present at 1.97 ppm (H-3_{ax}) and 2.22 ppm (H-3_{eq}). The α-configuration was established on the basis of the chemical shifts of the H-3_{eq} and H-5 protons and by the values of the $^3J_{H\text{-}7,H\text{-}8a}$ and $^3J_{H\text{-}7,H\text{-}8b}$ coupling constants of 7.1 and 3 Hz, respectively.[11,12]

The ^{13}C NMR chemical shifts could be assigned by a HSQC experiment, using the assigned proton resonances. Six main anomeric signals were apparent (Table 1), beside a large number of carbon signal relative to ring carbon, two nitrogen bearing carbon signals evidently belonging lipid A skeleton and, in addition, at high fields, a methylene carbon signal of Kdo unit was found. Low field shifted signals indicated substitutions at O-6 of residues **A** and **C** (see below), O-5 of **G**, O-7 (**B**), O-4 and O-7 (**B1**), O-4 (**F1**), while **E**, **F** and **D** were terminal residues. Phosphate substitution was inferred on the basis of ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum showed the presence of four monophosphate monoester peaks (Table 1). The ^1H,^{31}P-HSQC spectrum showed correlations for P1/H-1**A** (GlcN I), P2/H-4**C** (GlcN II) and P3/H-4**G** (Kdo), P4/H-3**B** (Hep).

The sequence of the monosaccharide residues was determined using NOE effects of the ROESY and NOESY spectra (Fig. 2) and by means of ^1H,^{13}C-HMBC. The typical lipid A carbohydrate backbone was assigned on the basis of the NOE signal between H-1 **C** and H-$6_{a,b}$ **A**. In the case of Kdo unit **G**, it was substituted by heptose **B** as indicated by the NOE effect found between H-1 **B** and H-5 and H-7 **G**, and, in addition, between H-5 **B** and H-3_{ax} **G**. Moreover, these NOE effects implied same absolute configuration for both residues involved in the NOE contact and thus, indicative of the sequence α-L-D-heptose-(1 → 5)-α-D-Kdo.[13,14] Heptose **B** was, in turn, substituted at O-7 by galactose **E** as demonstrated by the NOE cross-peak between H-1 **E** and H-$7_{a,b}$ **B**. As for **G** Kdo location, its linkage to unit **C** was deduced by exclusion. In particular, the linkage to O-6 of **C** was inferred by taking into account the slight down field displacement of the carbon signal C-6 (63.7 ppm, Table 1) indicating its involvement in a glycosydic linkage with a ketose residue. The ^1H,^{13}C-HMBC spectrum confirmed either attachment points of residues, deduced by glycosylation shifts, and sequence proposed for the oligosaccharide, determined by NOE data, as it contained all the significant long range scalar correlations. In fact, together with intraresidual long range cross-peaks, interresidual long range connectivity were found among

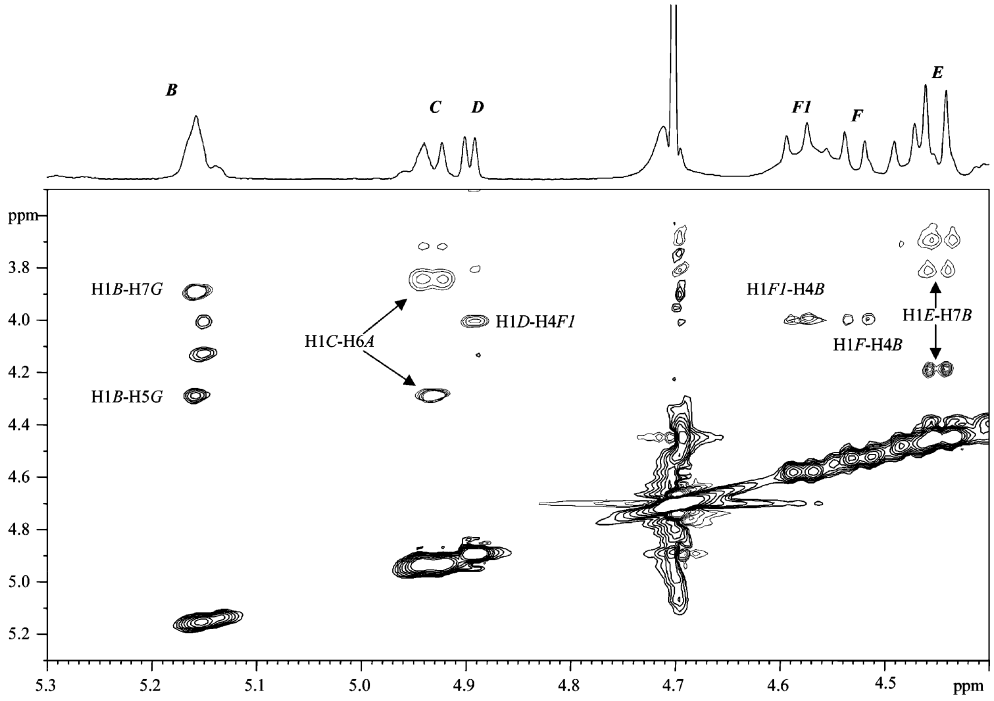
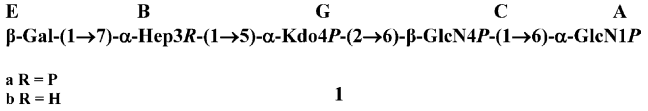


Figure 2. Part of a NOESY spectrum of oligosaccharide mixture **1**–**3**. Annotations refer to interresidual cross-peaks. The capital letters refer to residues as denoted in Table 1.

others between H-1/C-1 **C** and C-6/H-6 **A**, H-5/C-5 **G** and C-1/H-1 **B**, H-7/C-7 **B** and C-1/H-1 **E**.

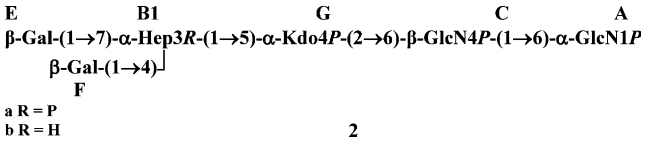
Thus, the major core glycoform (**1a**) of the lipooligosaccharide from *P. issachenkonii* could be identified as the following:



1

On the basis of this first oligosaccharide sequence other minor core glycoforms slightly differing from the first one could be identified. One of these (**1b**) was only lacking phosphate group to O-3 of heptose while all the rest of the chain was unaltered.

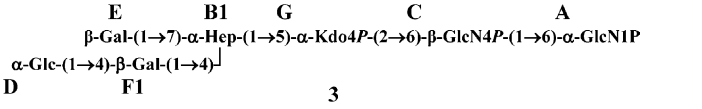
A different and longer core glycoform (**2a**) was characterised by the presence a further terminal residue (β-Gal **F**) linked to O-4 of heptose (**B1**). This was testified by the NOE cross-peak between H-1 of **F** and H-4 of **B1** in the NOESY spectrum and furthermore, by the long range correlation between H-1/C-1 **F** and C-4/H-4 **B1**. Hence, a further different core glycoform was found for the LOS from *P. issachenkonii* as follows:



2

Even for this oligosaccharide, an alternative form, devoid of the phosphate at O-3 of heptose, was found (compound **2b**).

Another oligosaccharide variant was also found. This oligosaccharide chain was characterised by the presence of a further glucose unit (**D**) linked to O-4 of galactose (**F1**). Actually, in the NOESY spectrum, a cross-peak between H-1 of **D** and H-4 of **F1** was detectable and corroborated by the long range correlation between H-1/C-1 **D** and C-4/H-4 **F1**. Thus the third and last core glycoform (compound **3**) for the LOS from *P. issachenkonii* was identified as the following:



3

Interestingly, this oligosaccharide chain was present only in its trisphosphorylated form, where the three phosphate residues on lipid A and Kdo were plainly evident, thus, with no phosphate residue at O-3 of the heptose unit.

A MALDI mass spectrum (Fig. 3) of the oligosaccharide mixture obtained by alkaline treatment confirmed the above structural hypotheses, as all ion peaks corresponding to the above compounds **1**–**3** were present. In fact, ion peaks characteristic of oligosaccharides built up of two HexN, one Hep, one Kdo, one/two/three Hex residues and three/four phosphate groups were present.

In order to reveal the correct structure of lipid A, namely, the fatty acids substitution, an aliquot of intact LOS was analysed by MALDI MS spectrometry. The MALDI mass spectrum showed molecular ions in the mass range 1700–2400 Da, and ion peaks related to fragments arising from the very labile glycosydic bond cleavage between Kdo and the lipid A moiety. Therefore the

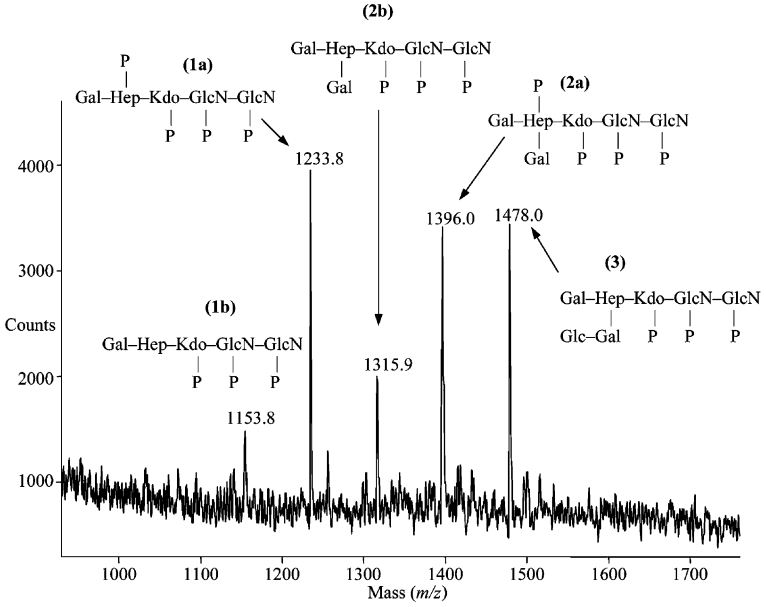


Figure 3. Negative ion MALDI-TOF-mass spectra of oligosaccharides **1**–**3** obtained in linear mode. The main ion peaks are assigned.

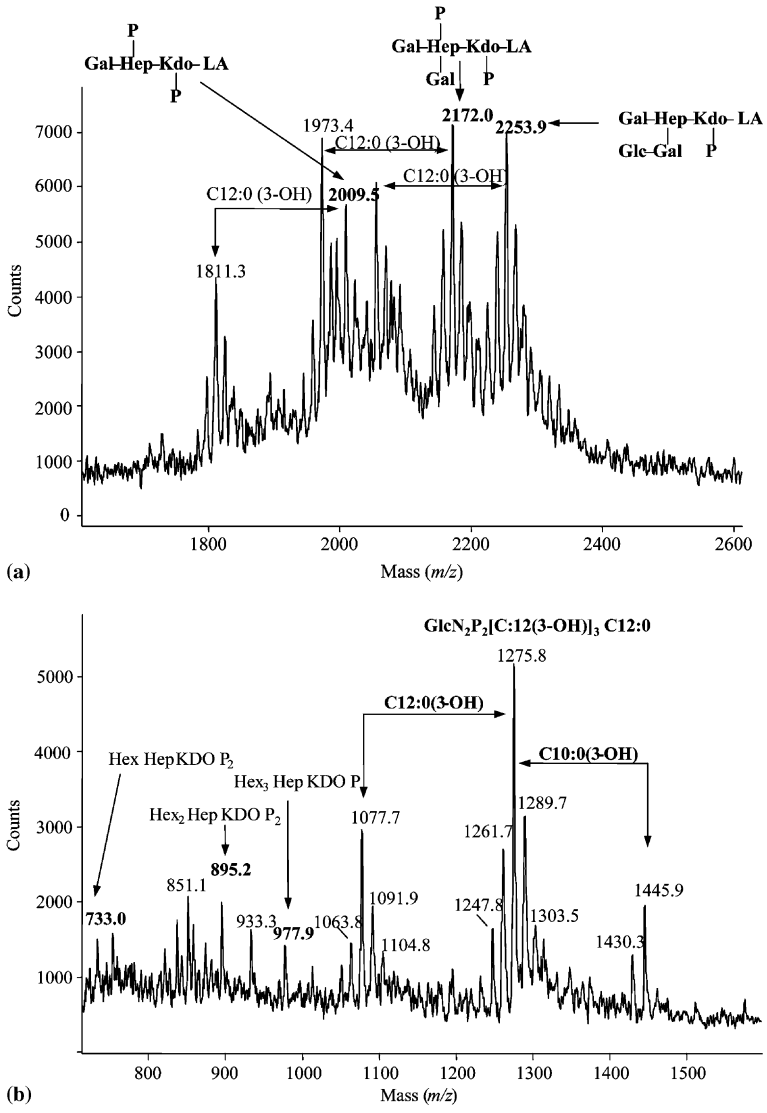


Figure 4. (a) Section at high molecular masses of negative ion MALDI-TOF-mass spectrum of intact LOS of *P. issachenkonii* obtained in linear mode at high laser intensity. The bold numbers refer to the drawn molecular species from which are visible differences attributable to the absence of fatty acid C12:0 (3-OH) ($\Delta m/z$ 198). The other ion peaks are explained by fatty acids ($\Delta m/z$ 14) and phosphate ($\Delta m/z$ 80) heterogeneity. Abbreviation, LA, lipid A. (b) Section of the same spectrum at low molecular masses. The three main series of ion peaks relative to lipid A are visible. At lower masses, ion peaks (bold numbers) relative to core oligosaccharide fragments are also evident together with peaks deriving neutral losses of a CO_2 group (m/z 933.3 and 851.1).

mass spectrum could be divided in two subspectra (Fig. 4a and b) and following interpretation was straightforward. At high molecular masses (Fig. 4a), in the range 1700–2300 Da, various series of ion peaks were present consequently to the LOS heterogeneity. Within each series, ions differing for $\Delta m/z$ 14, due to fatty acids heterogeneity, were distinguishable. The peak at m/z 2253.9 was consistent with a tetraacylated lipid A with compound **3** core oligosaccharide skeleton. The peak at m/z 2172.0 was ascribed as a tetraacylated lipid A with compound **2a** oligosaccharide skeleton, whereas at m/z 2009.5 a peak attributed to a tetraacylated lipid A with compound **1a** oligosaccharide skeleton was found. The other peaks differed for a phosphate moiety and/or fatty acid residue.

A careful look to lower molecular masses region allowed also an accurate lipid A identification. The spectrum (Fig. 4b) showed ion peaks attributable to both lipid A and core oligosaccharide forms. This fragments arise from a β-elimination[15] and yields, in the present case, either an oligosaccharide ion (B-type ions, Domon and Costello nomenclature[16]), and a lipid A ion. In particular, the ion series at m/z 1445.9, 1275.8 and 1077.7 were all identifiable with lipid A species. The ion at m/z 1275.8, was attributable to a tetraacylated lipid A carrying out three C12:0 (3-OH) and a C12:0 residues, the ion at m/z 1445.9 was consistent with a pentacylated lipid A bearing an additional C10:0 (3-OH), while the peak at m/z 1077.7 was consistent with a triacylated

lipid A carrying two C12:0 (3-OH) and a C12:0 residues. Given the fatty acid heterogeneity, several other peaks, differing for ±14 Da with respect to the above ions, were present. At lower mass range of the spectrum, B ions from core oligosaccharide were also present in full accordance with the above structural description. As already described,[15] these ions also underwent additional neutral losses of a CO_2 group.

In order to assign the secondary fatty acid location, the LOS was selectively hydrolysed with ammonium[17] and the obtained product analysed by MALDI MS spectrometry. The spectrum registered in negative mode contained a main ion peak centred at m/z 1077.7 and attributable to triacylated lipid A species carrying two C12:0 (3-OH), one of which bearing an additional C12:0 residue. The positive mode MALDI spectrum (data not shown) showed the presence of oxonium ions arising from the cleavage of the glycoside linkage of nonreducing GlcN of lipid A (GlcN II) at m/z 620.9 and minor peaks at m/z 634.9 and 606.7. The fragment at m/z 620.9 could be ascribed to a diacylated GlcN II ion carrying a C12:0 (3-OH) and a C12:0 and testified the presence of the secondary fatty acid exclusively linked to the primary amide bound fatty acid on GlcN II. Thus, the complete structure (Fig. 5) of the lipid A of LOS from *P. issachenkonii* was also established.

Despite the copious number of O-polysaccharide chains elucidated from *Proteobacteria* and in particular from *Pseudoalteromonas*, few data are existing on the respective core regions.[18,19] In particular, this is the second core structure elucidated from a *Pseudoalteromonas* LPS and, in both cases, the **Hep-(1→5)-Kdo4*P*-(2→6)-GlcN4*P*-(1→6)-GlcN1*P*** skeleton is present. In two core glycoforms of the LOS from *P. issachenkonii*, a 4,7-di-substituted heptose is present and this is, at our knowledge, the first time that in a core region a heptose is found with such a substitution pattern.

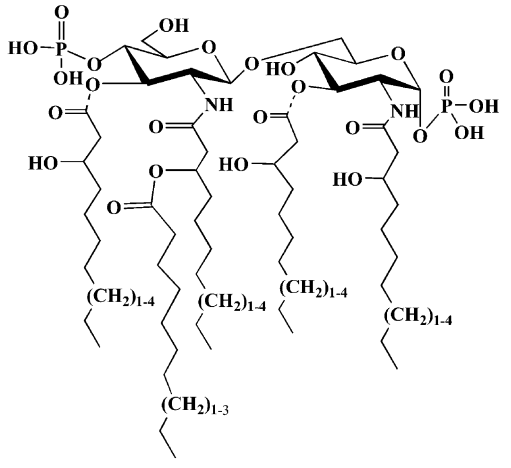


Figure 5. The complete structure of lipid A from *P. issachenkonii*. Nonstoichiometric substitutions are denoted as dotted bonds.

Even in the present core oligosaccharide, Kdo is stoichiometrically phosphorylated at O-4 as the majority of single Kdo residues present in lipopolysaccharides of *Vibrionaceae* and *Pasteurellaceae*,[7,8] since the presence of a second negative charged group is necessary in Kdo region. Furthermore, the succeeding heptose residue is nonstoichiometrically phosphorylated at O-3. The total content of negative charges plays crucial role in integrity of the external membrane and it is thought that these negatively charged groups with bivalent ions as counterparts assemble ionic bridges between LPS molecules, which contribute to the rigidity and stability of the Gram-negative cell wall. Thus, the high number of negative charges in this short oligosaccharide from *P. issachenkonii* can be important for the integrity of outer membrane that is exposed to an anomalous external surrounding.

3. Experimental

3.1. Bacteria and bacterial LOS

Bacterial strain was recovered from enrichment culture during degradation of algal thallusses as described elsewhere.[4,5] Brown algae were collected by scuba-divers in mid-summer (July 1999) at the Kraternaya Bight of the Kurile Islands in the Pacific Ocean during the 23rd scientific expedition of the RV '*Akademician Oparin*'. The strain was isolated from the resulting suspension by plating (0.1 mL) on agar plates of marine agar 2216 (Difco) and on plates with medium B [0.2% (w/v) Bacto peptone (Difco), 0.2% (w/v) casein hydrolysate (Merck), 0.2% (w/v) Bacto yeast extract (Difco), 0.1% (w/v) glucose, 0.002% (w/v) KH_2PO_4, 0.005% (w/v) $MgSO_4 \cdot 7H_2O$, 1.5% (w/v) Bacto agar (Difco), 50% (v/v) natural seawater and 50% (v/v) distilled water, pH 7.5–7.8]. After the initial isolation, strains were purified on medium B and were maintained on the same semi-solid medium B in tubes under mineral oil at 4 °C and stored at 80 °C in marine broth (Difco) supplemented with 30% (v/v) glycerol. The *P. issachenkonii* isolate was streaked on agar plates from broth cultures every 6 months to ensure purity and viability. In a preparative scale, bacteria were grown on a liquid medium containing (g/L) 1 glucose, 5 pepton, 2.5 yeast extract, 0.2 K_2HPO_4, 0.05 $MgSO_4$, sea water (750 mL) and distilled water (250 mL). Cells were collected by centrifugation, washed with water and next lyophilised obtaining 5.230 g of dried cells.

The cells were extracted three times with a mixture of aq 90% phenol/chloroform/petroleum ether (2:5:8 v/v/v) as described.[20] After removal of organic solvents under vacuum, the LOS fraction was precipitated from phenol with water, the precipitate was washed with aqueous 80% phenol and then three times with cold acetone and then lyophilised (132 mg, yield: 2.5% of the bacterial dry mass).

Sodium dodecyl sulfate poliacrylamide gel electrophoresis (SDS-PAGE 12%) was performed as described. Gel was stained with silver nitrate for detection of LPS and LOS.[21]

3.2. Isolation of oligosaccharides

An aliquot of LOS (20 mg) was dissolved in anhydrous hydrazine (1 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (20 mL) and allowed to precipitate. The precipitate was then centrifuged (3000*g*, 30 min), washed twice with ice-cold acetone, dried and then dissolved in water and lyophilised (10 mg, 80% of LOS). This material was subsequently de-*N*-acylated with 4 M KOH as described.[22] After desalting using a column (50 × 1.5 cm) of Sephadex G-10 (Pharmacia), the resulting oligosaccharide mixture represented the carbohydrate backbone of the lipid A-core region (5 mg, 25% of the LOS).

3.3. General and analytical methods

Determination of Kdo, neutral sugars, including the determination of the absolute configuration of the heptose residue, organic bound phosphate, absolute configuration of the hexoses, GLC and GLC–MS were all carried out as described elsewhere.[23–26] The methylation analysis was carried out on a de-phosphorylated sample obtained with 48% HF (4 °C, 48 h). For methylation analysis of Kdo region, LOS was carboxy-methylated with methanolic HCl (0.1 M, 5 min) and consecutively with diazomethane in order to improve its solubility in DMSO. Methylation was carried out as described.[27,28] LOS was hydrolysed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated as before, carboxyl-reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analysed by GLC–MS. Methylation of the complete core region was carried out as described,[29] and the sample was hydrolysed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated, carboxyl-reduced, acetylated and analysed by GLC–MS.

3.4. NMR spectroscopy

For structural assignments of oligosaccharide **1**, 1D and 2D ^1H NMR spectra were recorded of a solution of 2 mg in 0.6 mL ^2H_2O at pD 14 (uncorrected value). Experiments were carried out at 30 °C using a Varian Inova 500 spectrometer, and ^{31}P NMR spectra on a Bruker DRX-400 spectrometer. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. Aq 85% phosphoric acid was used as external reference (0.00 ppm) for ^{31}P NMR spectroscopy.

Nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame Overhauser enhancement spectroscopy (ROESY) were measured using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 32 scans were acquired. A mixing time of 200 ms was employed. Double quantum-filtered phase-sensitive COSY experiment was performed with 0.258 s acquisition time using data sets of 4096 × 1024 points and 64 scans were acquired. The total correlation spectroscopy experiment (TOCSY) was performed with a spinlock time of 80 ms, using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first order basis from 2D phase-sensitive double quantum-filtered correlation spectroscopy (DQF-COSY).[30,31] The intensities of NOE signals were classified as strong, medium and weak using cross-peaks from intraring proton–proton contacts for calibration.

The heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments spectrum were measured in the ^1H-detected mode with proton decoupling in the ^{13}C (or ^{31}P) domain, using data sets of 2048 × 512 points, and 64 scans were acquired for each t_1 value. The experiments were carried out in the phase-sensitive mode according to the method of States et al.[32] ^1H,^{13}C-HMBC was optimised for 6 Hz coupling constant and ^1H,^{31}P-HSQC for 8 Hz coupling constant. In all the heteronuclear experiments the data matrix was extended to 2048 × 1024 points using forward linear prediction extrapolation.[33,34]

3.5. MALDI-TOF analysis

MALDI-TOF analyses were conducted using a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology and with a reflectron. Ions formed by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated through 24 kV. Mass spectra reported are the result of 256 laser shots. The dried samples was dissolved in $CHCl_3/CH_3OH$ (50/50 v/v) at a concentration of 25 pmol μL^{-1}. The matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) in CH_3OH/0.1% trifluoroacetic acid/CH_3CN (7/2/1 by vol) at a concentration of 75 mg mL^{-1}. A sample/matrix solution mixture (1:1 v/v) was deposited (1 μL) onto a stainless steel gold-plated 100-sample MALDI probe tip, and left drying at room temperature.

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