Complete Structural Elucidation of a Novel Lipooligosaccharide from the Outer Membrane of the Marine Bacterium *Shewanella pacifica*

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Shewanella pacifica is a Gram-negative microrganism that is able to grow in sea water. A novel lipooligosaccharide (LOS) has been isolated from the outer membrane of this bacterium and its primary structure fully characterised. For the first time, the presence of a 2,3-dihydroxypropanoic acid residue (glyceric acid) has been identified in the core region. The complete structure of the LOS was determined by compositional and methylation analyses, by MALDI mass spectrometry, and by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy on the oligosaccharides formed by selective degradation of the LOS. Strong alkaline treatment, aimed at recovering and identifying the complete carbohydrate backbone, was carried out by hydrazinolysis followed by de-*N*-acylation with hot KOH, whereas mild hydrazinolysis (de-*O*-acylation) allowed us to gain information about the nature of the phosphate and other non-carbohydrate substituents on the core oligosaccharide. Mild acid hydrolysis was employed to obtain a lipid A moiety with which further degradation and mass spectrometry experiments were carried out in order to determine its primary structure.

Introduction

The genus *Shewanella* was created in 1985 to house an abundant group of Gram-negative, facultative anaerobic, readily cultivated γ-Proteobacteria. Bacteria of this genus are mainly associated with severe aquatic habitats (e.g. cold, high pressure and deep-sea, etc.).[1,2] During the last few years bacteria of the genus *Shewanella* have been under intensive investigation due to their ability to reduce a variety of electron acceptors, including iron, manganese, nitrate, nitrite, thiosulfate, DMSO, trimethylamine *N*-oxide (TMAO), glycine fumarate and elemental sulfur.[3–5] Because of their metabolic versatility and wide distribution in a variety of aquatic habitats,[6] *Shewanella*-like organisms are thought to play a significant role in the cycling of organic carbon and other bionutrients.[7] Other remarkable peculiarities of *Shewanella* bacteria are the high production of polyunsaturated fatty acids (PUFA)[8,9] and the high content of acidic polysaccharides structures in their outer membrane.[10]

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In this study we report the complete structural elucidation of a novel lipooligosaccharide from three recently isolated mesophilic PUFA-producing bacteria of the genus *Shewanella* (strains KMM 3601, KMM 3605 and KMM 3772). These strains were isolated from seawater samples collected in Chazhma Bay in the Sea of Japan, Pacific Ocean, during the taxonomic survey of free-living microbial populations of the bay in the North-West Pacific Ocean contaminated by radionuclides. During the course of this work seventy *Shewanella* strains of different phenotypes were isolated,[11] and majority of these strains were found to have particular metabolic features, for example agar-digesting and haemolytic activities and production of PUFA, and showed high levels of 16S rRNA gene sequence identity (99%) to *S. japonica*.[12] The analysis of the strains KMM 3601, KMM 3605 and KMM 3772 allowed the assignment of this group of *Shewanella*-like strains to a new species, *Shewanella pacifi*ca.[13]

As a Gram-negative bacterium, *Shewanella pacifica* possesses lipopolysaccharides (LPSs) in the external leaflet of its outer membrane. Lipopolysaccharides are crucial amphiphilic constituents of the outer membrane of the Gram-negative bacterial cell wall.[14,15] Structurally, they comprise three regions in their smooth form (S-LPSs): 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 and may be divided into two regions: the inner core, constituted by typical sugars such 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.[16] 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 bacteria living in peculiar environments frequently show unusual chemical features, most likely due to the external environment the bacterium has to face.[17]

Since *S. pacifica* possesses an outer membrane in which LOSs reside, and given that this molecule is deemed important in the adaptation of this organism to its peculiar external surroundings and nothing was known about its structural features, we started a structural investigation of the LOS.

Results

Compositional Analysis

The lipooligosaccharide fraction of *S. pacifica* was extracted from dried cells with phenol/chloroform/petroleum ether and purified by 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 showed the presence of L-*glycero*-D-*manno*-heptose (L,D-Hep), D-*glycero*-D-*manno*-heptose (D,D-Hep), 2-amino-2-deoxy-D-glucose (D-GlcN), D-glucose (D-Glc) and 8-amino-8-deoxy-*manno*-oct-2-ulosonic acid (Kdo8N). The presence of this last residue was established by GC-MS of its *O*-methyl ester *O*-methyl glycoside derivative, which showed two characteristic fragments (*m*/*z* = 374 and 402, oxonium cations). Moreover, D-glyceric acid was also detected. Methylation analysis of the dephosphorylated product showed the presence of terminal-Glc, terminal-Hep, 6-substituted-GlcN, 2-substituted-Hep and 2,6-di-substituted-Hep. Fatty acids analysis revealed the presence of (*R*)-3-hydroxytridecanoic acid [C13:0 (3-OH)], both in amide and in ester linkages, and tridecanoic acid (C13:0), exclusively in an ester linkage, as the major components. C14:0 (3-OH), C12:0 (3-OH), C14:0, C12:0 and C11:0 fatty acid residues, as well as *iso*- and *anteiso*-type carbon skeleton C13:0 (3-OH) fatty acids, were also found in minor amounts. The above procedures were carried out on the three *Shewanella* strains KMM 3601, KMM 3605 and KMM 3772 and, since compositional analysis and the ^{1}H NMR spectrum were identical, further analyses were carried out only on the LOS of strain KMM 3772.

Primary Structure Determination of the Oligosaccharide (OS1) Obtained by Alkaline Degradation

Alkaline degradation of the LOS yielded a pure oligosaccharide, **OS1**, which was submitted to full 2D NMR and MS analyses. The primary structure of oligosaccharide **OS1** was established by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. Chemical shifts were assigned from DQF-COSY, TOCSY, ROESY, HSQC and HMBC experiments (Table 1). Anomeric configurations were assigned on the basis of the chemical shifts, the $^3J_{H\text{-}1,H\text{-}2}$ values determined from the DQF-COSY experiment and the $^1J_{C\text{-}1,H\text{-}1}$ values derived from the coupled ^{1}H,^{13}C-HSQC spectrum. All sugars were identified as pyranose rings, based on ^{1}H and ^{13}C NMR chemical shifts and on the HMBC spectrum, which shows intra-residual scalar connectivity between H-1/C-1 and C-5/H-5 of the residues (for Kdo8N from C-2 and H-6). The anomeric region of the ^{1}H NMR spectrum (Figure 1) contains six major anomeric signals relative to six different spin systems

Table 1. ^{1}H, ^{13}C and ^{31}P NMR chemical shifts (ppm) of the oligosaccharide OS1 derived from strong alkaline treatment of the LOS from *S. pacifi*ca.

Residue		1	2	3	4	5	6	7	8
A	^{1}H	5.531	3.070	3.780	3.434	4.152	3.872/4.290		
6-GlcN	^{13}C	92.8	52.5	70.2	70.8	73.4	70.4		
	^{31}P	2.410							
B	^{1}H	5.308	3.965	3.799	3.860	3.862	4.055	3.720	
2-Hep	^{13}C	100.1	81.2	73.1	67.6	73.1	70.3	64.2	
C	^{1}H	5.234	4.104	4.283	3.856	4.066	4.110	3.804/3.822	
2,6-Hep	^{13}C	100.7	82.2	73.7	68.2	70.5	79.2	63.1	
D	^{1}H	5.146	4.067	3.854	3.849	3.665	4.018	3.789	
t-Hep	^{13}C	103.3	71.4	71.9	68.2	73.4	70.4	64.2	
E	^{1}H	5.097	3.570	3.748	3.420	3.870	3.753/3.800		
t-Glc	^{13}C	101.8	72.8	74.9	70.9	72.9	61.7		
F	^{1}H	4.692	2.880	3.721	3.898	3.531	3.577/3.694		
6-GlcN	^{13}C	100.9	56.8	75.1	73.5	75.3	62.7		
	^{31}P				3.210				
G	^{1}H	–	–	1.980/2.282	4.544	4.342	3.926	3.977	3.196/3.504
5-Kdo8N	^{13}C	175.0	101.7	36.1	69.9	75.2	74.6	67.1	43.9
	^{31}P				2.000				

(**A**–**F**). Their identification was possible by the complete assignment of all proton signals and the determination of the $^3J_{H,H}$ vicinal coupling constant values. Residues **A**–**E** possess an α-configuration, ($^1J_{C,H}$ = 173 Hz). In particular, the **A** residue was plainly identified as the phosphorylated GlcN I of the lipid A skeleton because of its chemical shifts and the multiplicity of the anomeric signal (doublet of doublets, $^3J_{H\text{-}1,H\text{-}2}$ = 3.1 Hz and $^3J_{H\text{-}1,P}$ = 7.8 Hz). Spin systems **B**–**D** possess low $^3J_{H\text{-}1,H\text{-}2}$ and $^3J_{H\text{-}2,H\text{-}3}$ values, diagnostic of H-2 equatorial orientation, and, starting from the H-2 signals, it was possible, by measuring a TOCSY spectrum, to assign all the other resonances within the spin systems, thus allowing us to identify these three residues as heptoses. Spin system **E** was identified as α-glucose since it has the typical $^3J_{H,H}$ vicinal coupling constant values. A β-anomeric configuration was assigned to the residue **F** on the basis of $^3J_{H\text{-}1,H\text{-}2}$ and $^1J_{C\text{-}1,H\text{-}1}$ values (7.8 and 162 Hz, respectively) and, eventually, a ROESY experiment, which showed intra-residual NOE connectivity between H-1 and the H-3/H-5 signals for this sugar residue. It was identified as the β-GlcN of the lipid A backbone since H-2 is correlated to a nitrogen-bearing carbon signal in the HSQC spectrum (δ = 56.8 ppm).

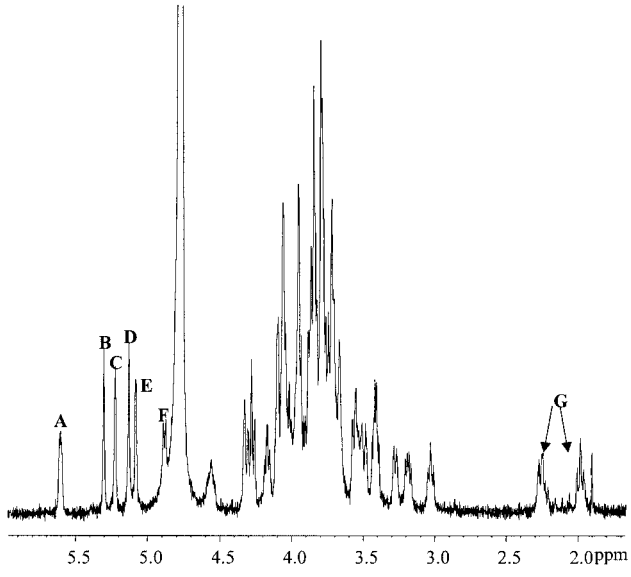


Figure 1. ^{1}H NMR spectrum of *S. pacifica* oligosaccharide (**OS1**) obtained after strong alkaline treatment. Anomeric signals of spin system are designated as in Table 1.

In addition, in the high-field region of the spectrum the characteristic diastereotopic H-3 methylene signals of a Kdo-like residue (residue **G**) are visible at δ = 1.98 (H-3*ax*) and 2.28 ppm (H-3*eq*). The α-configuration was established on the basis of the chemical shifts of the H-3 protons and by the $^3J_{H\text{-}7,H\text{-}8a}$ and $^3J_{H\text{-}7,H\text{-}8b}$ coupling constant values of 7.1 and 3.0 Hz, respectively.[18,19] For the **G** residue a ROESY experiment was particularly useful for the identification of the H-6 resonance, given the very low $^3J_{H\text{-}5,H\text{-}6}$ value (less than 1 Hz).

The ^{13}C NMR chemical shifts were assigned from an HSQC experiment, using the assigned ^{1}H NMR spectrum. Six anomeric carbon resonances (Table 1), numerous carbon ring signals and three nitrogen-bearing carbon signals were identified, two of which were assigned to the C-2 of lipid A GlcN residues, whereas the third one, an antiphase methylene signal present in the DEPT-HSQC spectrum, correlated with H-8$_a$ and H-8$_b$ of Kdo8N. By comparison with the ^{13}C chemical shifts of unsubstituted residues,[20] several low-field shifted signals suggested glycosylation, namely, at O-6 of residues **A** and **F**, O-5 of **G**, O-2 of **B** and O-2 and O-6 of **C**.

Phosphate substitution was established on the basis of ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum showed the presence of the monophosphate monoester signals (Table 1). The site of substitution was inferred from the ^{1}H,^{31}P-HSQC spectrum, which shows correlations of ^{31}P signals with H-1 **A** (GlcN), H-4 **F** and H-4 **G**.

The sequence of the monosaccharide residues was determined from the NOE contacts in the ROESY spectrum (Figure 2), and by ^{1}H,^{13}C-HMBC correlations. The typical lipid A carbohydrate backbone was eventually assigned on the basis of the NOE signal between H-1 of **F** and H-6$_{a,b}$ of **A**. Kdo8N of **G** is substituted by heptose **C**, as indicated by the NOE cross-peak found between H-1 of **C** and H-5 of **G**, and, in addition, between H-1 of **C** and H-7 of **G**. Heptose **C** is linked to heptose **D** by O-2 since NOE effects between H-1 of **D** and H-1 and H-2 of **C** are present. The **B** residue is linked at O-6 of **C**, since its H-1 signal gives a strong NOE cross-peak with H-6 of **C**. Finally, the **B** residue is substituted at O-2 by glucose **E** as H-1 and H-2 of **B** experience an NOE with H-1 of **E**.

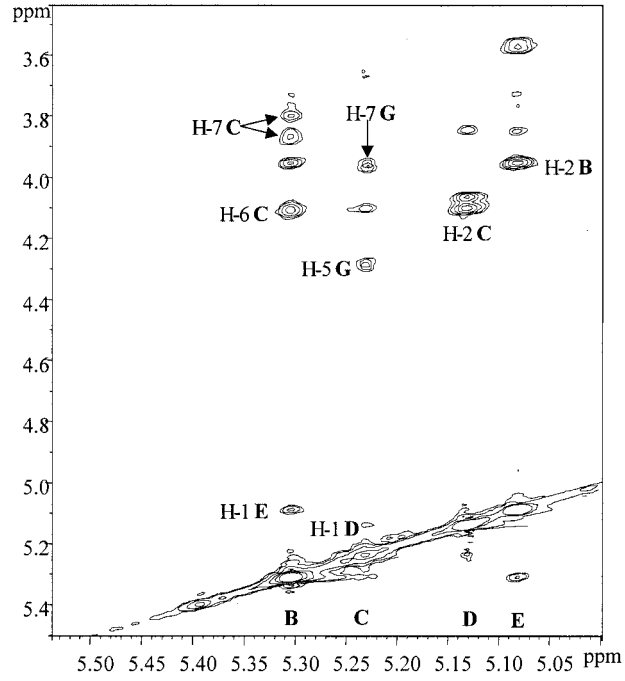


Figure 2. Section of the ROESY spectrum of oligosaccharide (**OS1**). Monosaccharide labels are as indicated in Table 1. The relevant inter-residue NOE cross-peaks are indicated.

The absolute configuration of the C-6 carbon of heptoses was established by taking into account the diagnostic C-6 chemical shift values of unsubstituted L,D-Hep and D,D-Hep occurring at around δ = 69 and 72 ppm, respectively.[21] Accordingly, the C-6 resonances at δ = 70.3 and 70.4 ppm for **B** and **D** residues are in agreement with their L,D configuration. Thus, since in the methylation data a D,D-Hep was detected, it was identified as the **C** residue, the only remaining heptose spin system. The significant low-field displacement of its C-6 signal (δ = 79.2 ppm) is in agreement with an O-6 glycosylated D-*glycero*-D-*manno*-heptose residue. Furthermore, the above NOE contacts found in the ROESY spectrum between **C** and **G** residues are only possible in the case of an identical absolute configuration of both residues;[22] thus, Kdo8N possesses a D-configuration.

The HMBC spectrum contains all the basic scalar correlations to describe the primary structure of the oligosaccharide, namely, H-1/C-1 **F** with C-6/H-6 **A**, H-1/C-1 **C** with C-5/H-5 **G**, H-1/C-1 **D** with C-2/H-2 **C**, H-1/C-1 **B** with C-6/H-6 **C** and H-1/C-1 **E** with C-2/H-2 **B**.

The MALDI mass spectrum (Figure 3) of the fully de-acylated oligosaccharide is in full agreement with the above structural hypothesis since it contains an ion peak at *m*/*z* = 1537.8 matching with the Hex Hep_3 Kdo8N Glc_2N P_3 structure. Moreover, it shows an additional informative ion peak at *m*/*z* = 1037 arising from an in-source β-elimination[23] of Kdo containing oligosaccharide ions (B-type ions, Domon and Costello nomenclature[24]), hence the bis-phosphorylated lipid A disaccharide is missing from this fragment (Δ*m*/*z* = 500). In the MALDI mass spectrum a minor ion at *m*/*z* = 1458.2 lacking phosphate is also visible and, in agreement, in the NMR analysis spin system **G** was also found with an alternative H-4 resonance at δ = 4.010 ppm, indicative of no phosphate substitution at its O-4 position. The finding of this non-phosphorylated Kdo8N residue in the alkaline treated product may be attributed to the occurrence in the intact LOS of a phosphodiester bond at its O-4 position that is cleaved under the harsh alkaline conditions (see below).

Thus, the primary structure of the oligosaccharide backbone (**OS1**) derived by alkaline degradation of the LOS from *S. pacifica* is as follows:

α-Glc-(1→2)-α-Hep-(1→6)-α-Hep-(1→5)-α-Kdo8N4P-(2→6)-β-GlcN4P-(1→6)-α-GlcN1P
α-Hep-(1→2)-┘

De-*O*-acylation of the LOS and Structural Analysis of the Obtained Lipooligosaccharide

In order to detect the presence of labile substituents in the native LOS that are lost upon harsh alkaline treatment (i.e., pyrophosphate groups), an aliquot of the sample was submitted to mild de-*O*-acylation with anhydrous hydrazine and the product obtained (**OS2**) analysed by chemical analysis, NMR spectroscopy and mass spectrometry. A fatty acids analysis revealed, as major component, the presence of (*R*)-3-hydroxytridecanoic acid [C13:0 (3-OH)] and, in minor amounts, (*R*)-3-hydroxydodecanoic acid [C12:0 (3-OH)] and (*R*)-3-hydroxytetradecanoic acid [C14:0 (3-OH)].

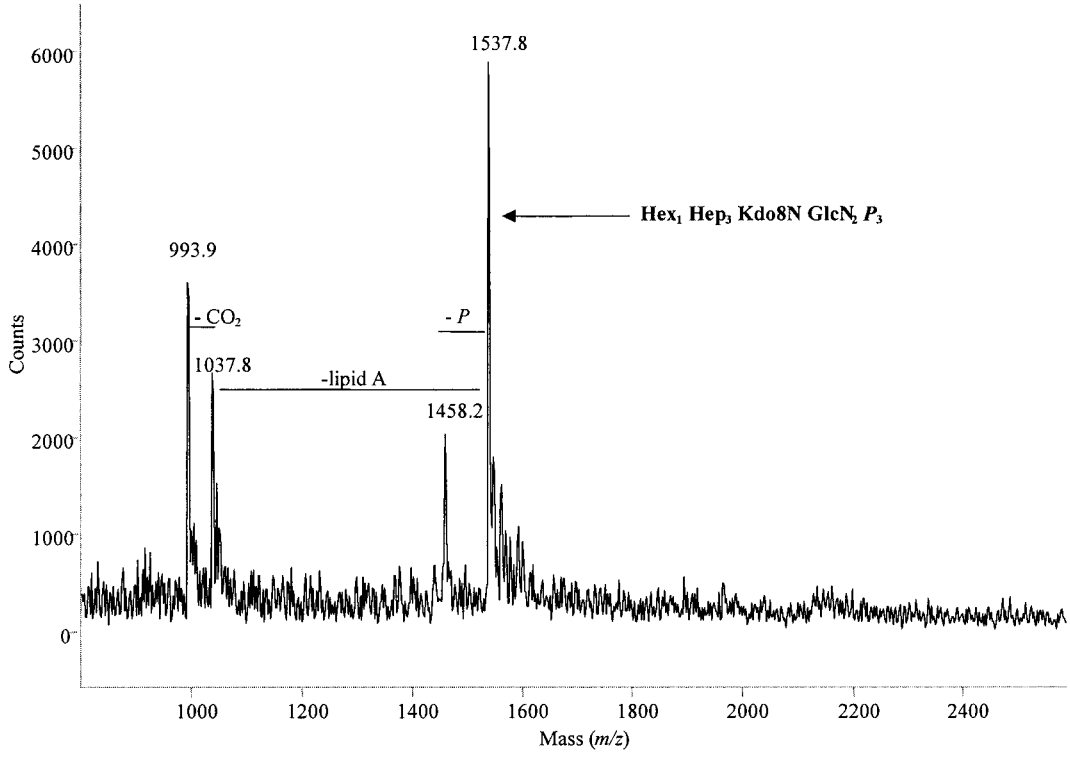


Figure 3. Negative ion MALDI-TOF mass spectrum of oligosaccharide **OS1**. Assignment of main ion peak is shown. P indicates the phosphate residue. The ion at *m*/*z* = 1458.2 is lacking a phosphate residue, while the ion at *m*/*z* = 1037.8 is related to lipid A fragment.

In order to improve its solubility, the sample was dissolved in 1% deuterated SDS solution (700 μL) to which 5 μL of 32% NH_4OH (pD 9.5) were added, and submitted to a complete NMR analysis (COSY, TOCSY, ROESY, ^1H,^{31}P HSQC, ^1H,^{13}C HSQC and HMBC). The carbohydrate skeleton of **OS2** was in full agreement with oligosaccharide **OS1**; seven spin-systems were identified (**A′**–**G′**; Table 2, Figure 4). In analogy with **OS1**, all the proton resonances were determined and, from these, all the carbon signals present in the HSQC spectrum were assigned. Spin system **C′**, identified as 2,6-disubstituted heptose, shows some slight divergences with respect to **C** concerning the chemical shifts of its H/C-6 and H/C-7. The sequence of the oligosaccharide chain was inferred by NOE contacts present in the ROESY spectrum (not shown), where all the diagnostic inter-residue cross-peaks allow the assignment of the above core-lipid A oligosaccharide structure. Furthermore, a few additional spin-systems attributable to non-carbohydrate substituents are present in the 2D homonuclear NMR spectra. One of these consists of a hydroxymethylene signal at δ = 3.89, 3.85/63.9 ppm correlated to a carbinolic methine signal at δ = 4.50/77.6 ppm in the COSY spectrum and, since both methylene and methine signals correlate to a carboxyl group in the HMBC spectrum, the presence of glyceric acid was deduced.

Five signals were found in the ^{31}P NMR spectrum that correlate in the ^1H,^{31}P-HSQC spectrum with different proton signals (Figure 5). Three of these were directly assigned to O-1 **A′**, O-4 **F′** and O-4 **G′**, whereas the fourth phosphate group is present as a phosphodiester group since it correlates with both the H-7 signal of heptose **C′** and the H-2 signal of glyceric acid. Thus, a phosphoglyceric moiety is present and is attached to the O-7 position of heptose **C′**. The fifth ^{31}P signal, a typical pyrophosphate signal at δ = –11.0 ppm, correlates to an H-4 **G′** proton signal and to an ethanolamine spin system, which was also detectable in the 2D homonuclear NMR spectra. On these grounds it was possible to conclude that Kdo8N possesses two alternative phosphate substituents at position O-4, either a simple phosphate group or, in minor amounts, a pyrophospho-

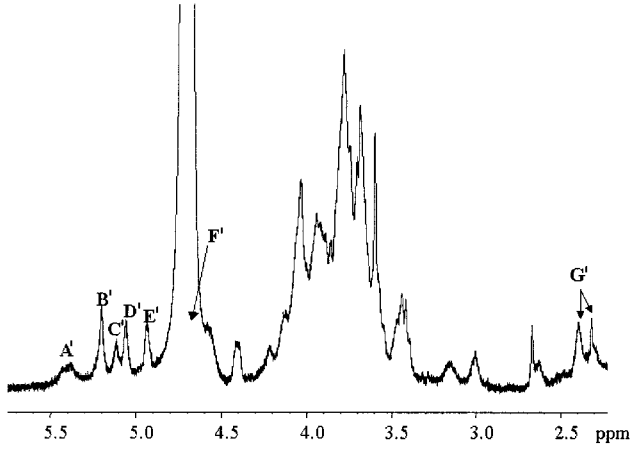


Figure 4. Section of the ^1H NMR spectrum of *S. pacifica* oligosaccharide (**OS2**) obtained after mild alkaline treatment. The spectrum was recorded for a solution of 1% deuterated SDS with 5 μL of 32% NH_4OH (pD 9.5, uncorrected value).

Table 2. ^1H, ^{13}C and ^{31}P NMR chemical shifts (ppm) of the oligosaccharide product OS2 derived from mild alkaline treatment of the LOS from *S. pacifi*ca.[a]

Residue		1	2	3	4	5	6	7	8
A′	^1H	5.498	3.870	3.841	3.522	4.017	3.770/4.172		
6-GlcN	^{13}C	93.0	54.7	70.2	69.7	71.0	68.0		
	^{31}P	4.49							
B′	^1H	5.298	3.909	4.155	3.877	3.871	4.043	3.791	
2-Hep	^{13}C	96.7	80.8	70.1	66.7	72.1	68.9	63.7	
C′	^1H	5.249	4.149	4.039	3.871	3.996	4.152	3.990/4.220	
2,6-Hep	^{13}C	100.7	78.3	69.8	66.7	73.3	76.0	64.2	
	^{31}P							0.58	
D′	^1H	5.155	4.129	3.965	3.813	3.705	4.051	3.853	
t-Hep	^{13}C	102.4	70.4	70.7	66.5	73.2	68.6	63.9	
E′	^1H	5.034	3.662	3.771	3.500	3.898	3.810/3.747		
t-Glc	^{13}C	100.8	71.8	73.0	69.8	72.2	60.9		
F′	^1H	4.639	3.923	3.914	4.031	3.691	3.560/3.680		
6-GlcN	^{13}C	100.8	55.2	73.3	73.7	74.2	62.5		
	^{31}P				3.85				
G	^1H	–	–	2.340/2.400	4.613	4.275	3.872	3.947	3.057/3.293
5-Kdo8N	^{13}C	175.0	101.7	34.4	69.3	75.2	72.1	70.4	43.5
	^{31}P				2.30				
GroA	^1H	–	4.502	3.850/3.890					
	^{13}C	173.8	77.6	63.9					
	^{31}P			0.58					
EtN*PP*	^1H	4.093	3.008						
	^{13}C	64.0	43.0						
	^{31}P	-11.0							
		–	2.481	4.010	1.510				
C13:0 (3-OH)		174.6	44.2	67.9	36.9				

[a] The chemical shifts for C/H-5 to C/H-12 of C13:0 (3-OH) fatty acid residues are in the region of δ = 1.28/30.0 ppm, while the methyl signal is found at δ = 0.860/14.0 ppm.

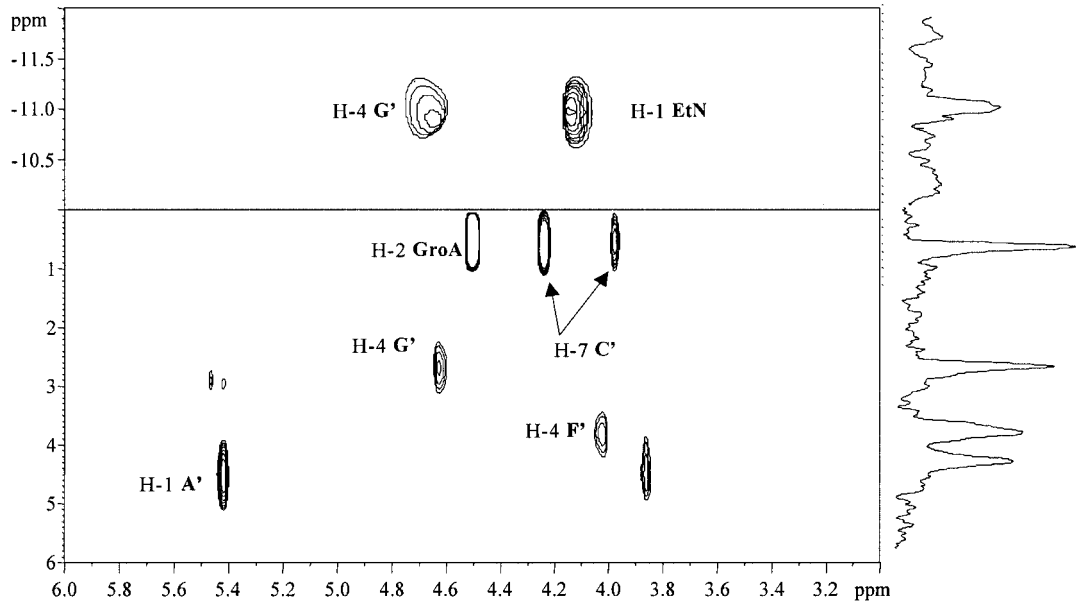


Figure 5. Section of the $^{1}H,^{31}P$-HSQC and projection of the ^{31}P NMR spectra in the F1 dimension of the oligosaccharide **OS2**. The spectrum shows cross-peaks relevant for the localisation of the phosphate groups. Monosaccharide labels are as indicated in Table 2. EtN is the ethanolamine residue.

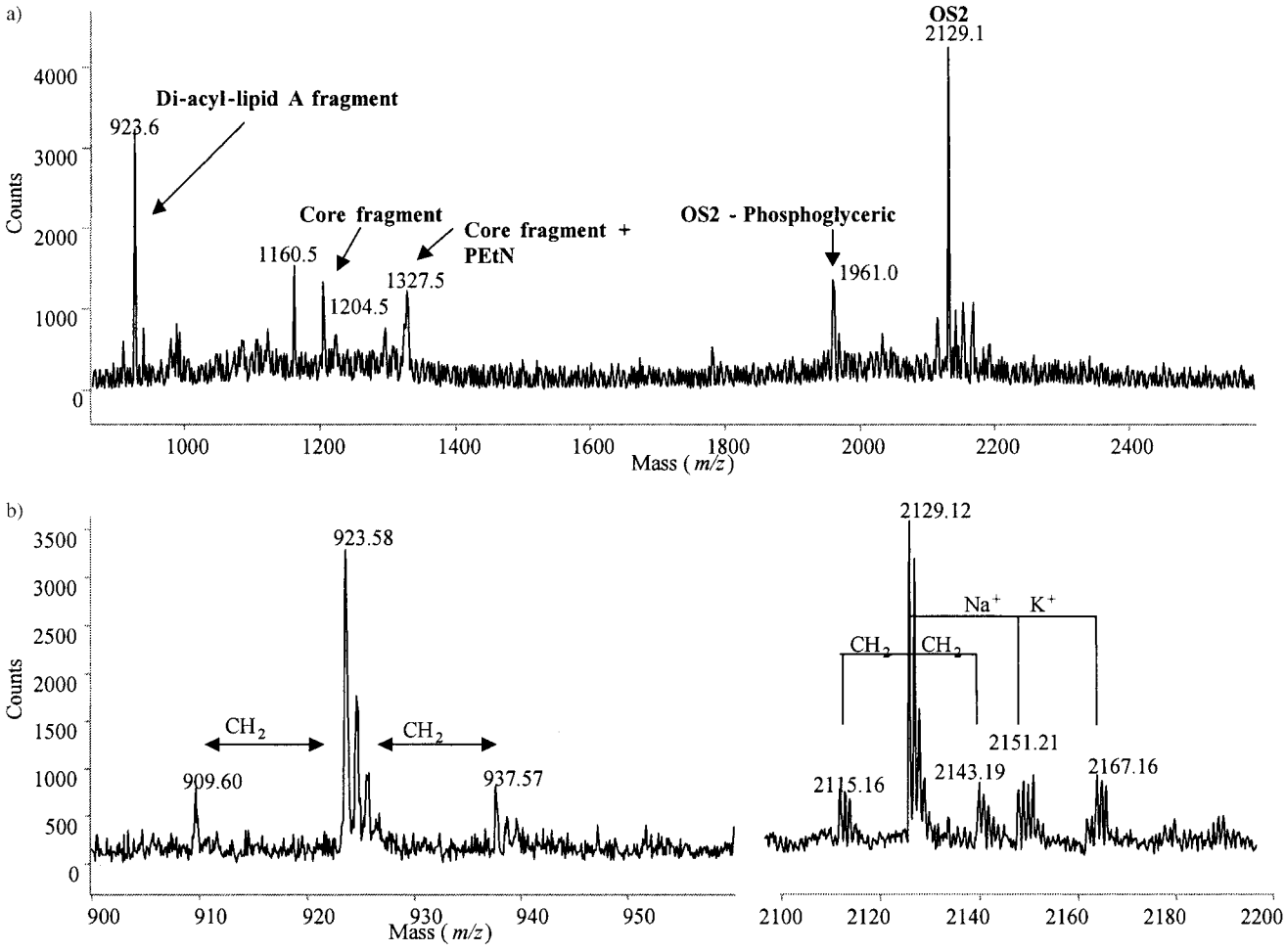


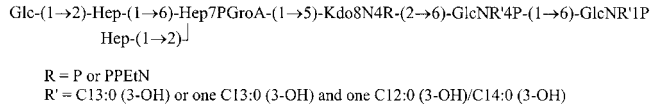
Figure 6. Total negative ion MALDI-TOF mass spectrum of a) de-*O*-acylated lipooligosaccharide (**OS2**) obtained in linear mode and b) sections of the spectrum recorded in reflector mode in which the molecular ions (right) and lipid A fragments (left) are visible. PEtN is the phosphoethanolamine residue.

ethanolamine group. In addition to the NMR signals discussed above, the spectra also show spin-systems attributable to the two acyl chains amide linked to a GlcN disaccharide (Table 2).

The MALDI mass spectrum (Figure 6a) of **OS2** shows various diagnostic ion peaks, in agreement with the above structural hypotheses. The ion at *m*/*z* = 2129.1 is in agreement with the above structure bearing two *N*-linked C13:0 (3-OH) fatty acid residues, whereas the ion at *m*/*z* = 1961.0 could be attributed to the same structure lacking the phosphoglyceric moiety. Interestingly, there are no ion peaks attributable to the lack of glyceric acid, suggesting that the phosphate group on the heptose unit, when present, always carries a glyceric acid moiety.

Moreover, as for the mass spectrum of **OS1**, ion peaks arising from an in-source β-elimination[23] at the Kdo8N level related to the core and lipid A fragments are present. The ion at *m*/*z* = 1204.5 and its de-carboxylated derivative were identifiable as the complete core oligosaccharide structure including the phosphorylated Kdo8N, while the ion at *m*/*z* = 1327.5 is due to Kdo8N carrying the additional pyrophosphoethanolamine group. As for lipid A ion peaks, in agreement with fatty acid analysis, the peak at *m*/*z* = 923.6 is due to a diacylated lipid A possessing two *N*-acyl C13:0 (3-OH) residues. The other two ion peaks, at *m*/*z* = 909.6 and 937.4, could be attributed to minor lipid A ion species possessing either a C14:0 (3-OH) or a C12:0 (3-OH) fatty acid residue (Δ*m*/*z* = 14). Accordingly, two homologous additional ion peaks are also visible as molecular ion peaks of the de-*O*-acylated LOS (Figure 6b).

Thus, the lipooligosaccharide **OS2** obtained by mild alkaline degradation possesses the chemical structure



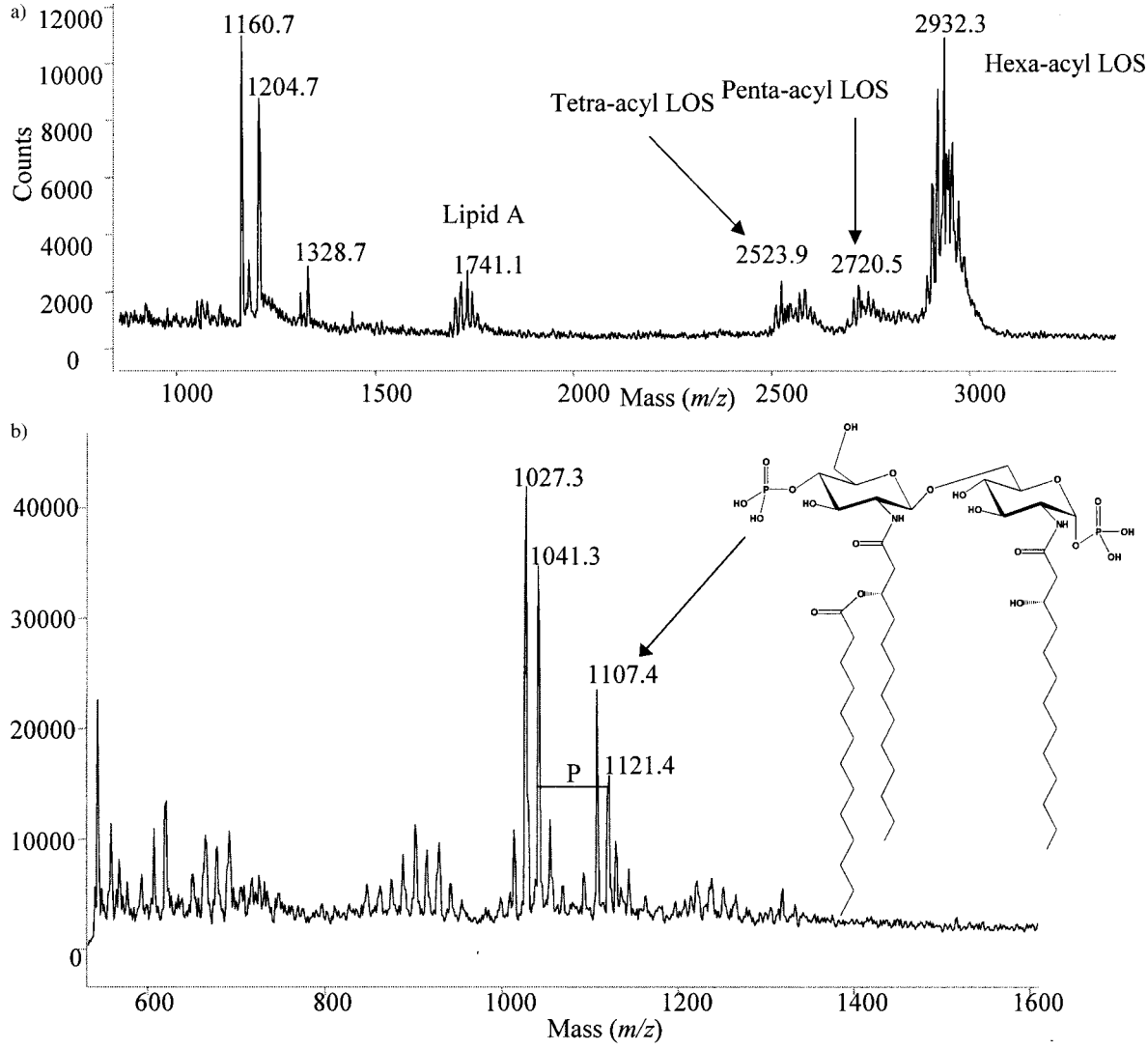


Figure 7. Negative ion MALDI-TOF mass spectrum of a) intact lipooligosaccharide, in which the fragments deriving from lipid A and core are also visible and b) ammonium hydroxide treated lipid A fraction of *Shewanella pacifi*ca. The main ion peak at *m*/*z* = 1107.4 is illustrated.

At this stage most of the structural elucidation of the lipooligosaccharide from *S. pacifica* was complete, whereas the complete fatty acid distribution on the intact LOS molecule remained to be established. The MALDI mass spectrum (Figure 7a) of the intact LOS shows three patterns of ion peaks at high molecular masses assignable to three different molecular species, one of which is present in very large amounts. Each pattern of ions contains peaks with a difference of 14 Da, in accordance with the presence of different *O*-acyl fatty acids, as already established by compositional analysis. At low molecular masses the expected fragments arising from bond cleavage between Kdo and the lipid A moiety were found. The core ion peaks are in complete accordance with the data discussed above, as an ion at *m*/*z* = 1204.7 and its decarboxylated derivative are present, and, in minor amounts, a peak for phosphoethanolamine is also visible. Beside the core oligosaccharide ion peaks, the hexaacylated lipid A pattern of ion peaks is visible at around *m*/*z* = 1741.0. This latter ion, for instance, was identifiable as a bis-phosphorylated glucosamine disaccharide containing two C13:0 (3-OH) residues in amide linkages and two C13:0 (3-OH) in ester linkages as primary fatty acids and two C13:0 as secondary fatty acids. All the other peaks differ by 14 Da and were attributable to the high heterogeneity of primary and secondary fatty acids that was also reflected in the heterogeneity of the molecular ions.

Thus, the major molecular ion peak was assigned as a hexaacylated lipid A while the minor molecular ion species were attributed to a pentaacylated lipid A lacking an *O*-linked primary fatty acid (ions around *m*/*z* = 2720.5) and a tetraacylated lipid A species lacking either an *O*-linked primary fatty acid or a secondary fatty acid (ions around *m*/*z* = 2523.9).

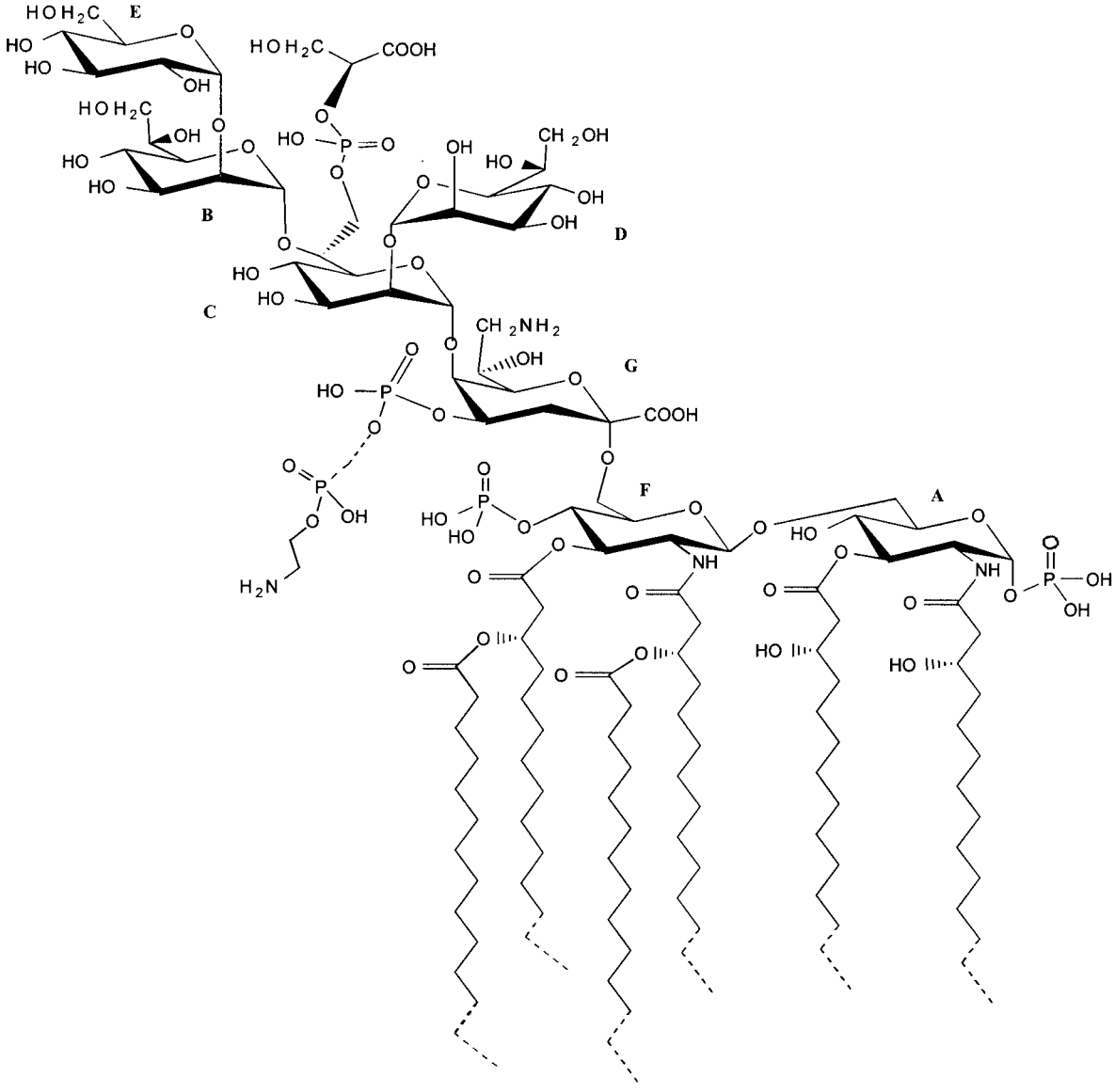


Figure 8. The complete structure of the lipooligosaccharide of *S. pacifi*ca. Dotted bonds indicate non-stoichiometric substitutions.

Mild Acid Hydrolysis of the LOS and Structural Analysis of the Obtained Lipid A

In order to determine the precise distribution of the acyl chains on the disaccharide backbone, and thus the primary structure of the lipid A, the LOS was hydrolysed with sodium acetate buffer at pH 4.4, which only cleaves the labile ketoside linkage of Kdo and provides the lipid A moiety.

An aliquot of lipid A was selectively de-*O*-acylated with 12% NH_4OH and then analysed by MALDI MS. This soft chemical approach leaves the amide-bound acyloxyacyl moieties unaltered, if present,[25] and allows the determination the secondary fatty acids' location. The negative-ion mode MALDI mass spectrum (Figure 7b) shows a pattern of ion peaks that was straightforwardly attributed. The ion at m/z = 1107.4 was ascribed to a tri-acylated bis-phosphorylated lipid A species with two amide-linked 13:0 (3-OH) residues, one of which is substituted at O-3 by a secondary 12:0 acyl chain. The other peaks again differ by 14 Da owing to the presence of fatty acids with different lengths. The ions at m/z = 1027.3 and 1041.3 were attributed to the same lipid A species but lacking the phosphate residue lost during the acid hydrolysis carried out to get the lipid A. Thus, one of the two secondary fatty acids present in the hexaacylated species is linked as an acyloxyacyl amide moiety. The other secondary fatty acid must be obviously linked at position O-3 of the ester-linked primary fatty acids.

The location of these two acyloxyacyl moieties on the disaccharide backbone was established by analysis of the positive ion MALDI-TOF spectrum of the de-phosphorylated lipid A. The spectrum (not shown) contains several pseudomolecular ions $[M + Na]^+$ with the same acylation pattern as the intact lipid A. At low molecular masses, ion peaks attributable to oxonium ions are present due to the in-source cleavage of the glycoside linkage under the high power laser settings. The ion at m/z = 963.9 is consistent with a tetraacylated oxonium fragment carrying one residue of GlcN II, two 13:0 (3-OH), one 12:0 and one 13:0, thus indicating that both the acyloxyacyl amide and the acyloxyacyl moieties are located on GlcN II. Oxonium fragments related to the presence of fatty acids with different lengths (±14 Da) are also visible.

In conclusion, the complete structure (Figure 8) of the lipooligosaccharide of the outer membrane of *S. pacifica* has now been established.

Discussion and Conclusion

The genus *Shewanella* currently comprises 12 species of Gram-negative, facultative anaerobic proteobacteria associated mainly with aquatic habitats. During the last decade, the bacteria of this genus have received a significant amount of attention due to their important roles in co-metabolic bioremediation of halogenated organic pollutants,[26] destructive souring of crude petroleum[27] and the dissimilatory reduction of magnesium and iron oxides. It is obvious that the outer membrane is highly involved in all of these peculiar metabolic capabilities of this organism, thus we have extensively studied and characterised its major component, the LOS. This class of molecules contains unique and vital components of these microorganisms and plays an important role in their survival and their interaction with the environment.

In this study, a combined chemical, MS and NMR approach has led to the complete establishment of a novel lipooligosaccharide structure from the outer membrane of a novel agar-digesting marine bacterium of the genus *Shewanella*. From the chemical point of view, this lipooligosaccharide possesses several interesting features. In the LPS from Gram-negative bacteria the lipid A-inner core region is highly conservative and always contains Kdo as the attachment point of the lipid A moiety to the core oligosaccharide. In *S. pacifica* LOS, this Kdo residue is replaced by an 8-amino derivative (Kdo8N) and this carbohydrate residue has been found in nearly all LPSs from *Shewanella* elucidated to date.[28,29] Moreover, in all *Shewanella* LPSs, and also in this case, this Kdo8N residue bears an unusual heptose residue (D,D-Hep) at the C-5 position that often carries other non-carbohydrate substituents via a phosphodiester linkage.[28,29]

The replacement of Kdo by a derivative in the inner core of LPS is rather rare, and to the best of our knowledge the only other occurrence is the presence of the 3-hydroxy-derivative of Kdo (Ko) in *Acinetobacter* LPS.[30]

An unusual and novel feature of the LPS from *S. pacifica* is the presence of glyceric acid, which is attached by a phosphodiester linkage at the D,D-Hep. Although this is a key molecule of the primary metabolism of Gram-negative bacteria, it has never been detected in the core of LPS molecules. Phosphoglyceric acid increases the total negative charge of the LPS molecule in the inner-core region and the negative charges play a crucial role in the supramolecular arrangement of LPS in the external membrane. It is deemed that these negatively charged groups allow the establishment of ionic bridges between LPS molecules due to electrostatic interactions with bivalent cations (Ca^{2+}, Mg^{2+}), and that these contribute to the rigidity and stability of the Gram-negative cell wall.[14,15] Thus, the high number of negative charges in this short oligosaccharide from *S. pacifica* could be important for maintaining the integrity of the outer membrane exposed to a peculiar external surrounding.

Experimental Section

Bacteria and Bacterial LOSs: Bacterial strains were isolated from water samples collected in October–November 2000 from a depth of 1 m, 9–13 m (salinity: 32‰; temperature: 13.6 °C) using a standard hydrological plastic bathometer at different locations in Chazhma Bay, Gulf of Peter the Great, the Sea of Japan in the Pacific ocean. The samples were kept at 4 °C and processed within 4–8 hours.

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 then dried with acetone to obtain 5 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 previously.[31] After removal of the 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 lyophilized (72 mg, yield: 3.2% of the bacterial dry mass). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) was performed as described previously. The gel was stained with silver nitrate for detection of LPS and LOS.[32]

Isolation of Oligosaccharides OS1 and OS2 and Lipid A: An aliquot of LOS (20 mg) was suspended in THF/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 (3000g, 30 min), washed twice with ice-cold acetone, dried, and then dissolved in water and lyophilized (oligosaccharide **OS2**, 15 mg, 75% of LOS). A portion of **OS2** (10 mg) was subsequently de-*N*-acylated with 4 M KOH (120 °C, 16 h) as described before.[33] After desalting on a Sephadex G-10 column (50 × 1.5 cm; Pharmacia), the resulting oligosaccharide represented the carbohydrate backbone of the LOS (oligosaccharide **OS1**, 5 mg, 50% of the **OS2**).

Free lipid A was obtained by hydrolysis of the LOS (10 mg) with 10 mM sodium acetate buffer at pH 4.4 (100 °C, 3 h). An aliquot of lipid A (250 μg) was de-phosphorylated by treatment with 48% HF (4 °C, 48 h). Mild de-*O*-acylation of lipid A was performed by treatment (200 μg) with 32% ammonium hydroxide (200 μL, 20 °C, 16 h).[25]

General and Analytical Methods: Determination of Kdo8N, neutral sugars, including the determination of the absolute configuration of the heptose residues, organic-bound phosphate, and the absolute configuration of the hexoses were all carried out as described elsewhere.[28–30,33,34] The absolute configuration of glyceric acid was deduced by comparison of it (+)-2-octyl ester with an authentic standard. The methylation analysis was carried out on a de-phosphorylated sample obtained with 48% HF (4 °C, 48 h). For methylation analysis of the Kdo region, LOS was carboxymethylated 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 before.[34] LOS was hydrolysed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxymethylated as above, 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 previously,[35,36] and the sample was hydrolysed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxymethylated, carboxyl-reduced, acetylated and analysed by GLC-MS.

GLC and GLC-MS were all carried out on a Hewlett–Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), for sugar methylation analysis and *O*-methyl glycosides derivatives the temperature program was: 150 °C for 2 min, then 2 °C min^{-1} to 200 °C for 0 min, then 10 °C min^{-1} to 260 °C for 11 min, then 8 °C min^{-1} to 300 °C for 20 min; for absolute configuration analysis it was: 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min. For fatty acids analysis the temperature program was 80 °C for 2 min, then 8 °C min^{-1} to 300 °C for 15 min; for glyceric acid derivative the temperature program was 100 °C for 25 min, then 5 °C min^{-1} to 200 °C.

NMR Spectroscopy: For structural assignments of oligosaccharide **OS1**, 1D and 2D ^{1}H NMR spectra were recorded for a solution of 2 mg of product in 0.6 mL of D_2O at pD 14 (uncorrected value). Experiments were carried out at 30 °C with a Varian Inova 500 spectrometer, and ^{31}P NMR spectra were recorded with a Bruker DRX-400 spectrometer. Spectra were calibrated with respect to internal acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm). 85% Phosphoric acid was used as external reference (δ = 0.00 ppm) for ^{31}P NMR spectroscopy.

For structural assignments of oligosaccharide **OS2**, 1D and 2D ^{1}H NMR spectra were recorded for a 700 μL solution of 1% deuterated SDS with 5 μL of 32% NH_4OH (pD 9.5, uncorrected value).

ROESY experiments was measured withg data sets of 4096 × 1024 points, and 32 scans were acquired. A mixing time of 200 ms was employed. The double quantum-filtered phase-sensitive COSY experiment was performed with a 0.258 s acquisition time with data sets of 4096 × 1024 points and 64 scans were acquired. The TOCSY experiment was performed with a spinlock time of 120 ms and data sets of 4096 × 1024 points; 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 DQF-COSY experiments.[37,38] The HSQC and HMBC experiments were measured in the ^{1}H-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.[39] ^{1}H,^{13}C HMBC was optimised for a 6 Hz coupling constant and ^{1}H,^{31}P HSQC for an 8 Hz coupling constant. In all the heteronuclear experiments the data matrix was extended to 2048 × 1024 points by forward linear prediction extrapolation.[40,41]

All NMR analyses were carried out following literature procedures[42] and the spectra were assigned with the help of the computer program Pronto,[43] which allows the simultaneous display of different two-dimensional spectra and the individual labelling of cross peaks.

MALDI TOF Analysis: MALDI-TOF mass spectra were recorded in the negative and positive polarity in linear or in reflector mode on a Perseptive (Framingham, MA, USA) Voyager STR equipped with delayed extraction technology and with a reflectron. Ions formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated by 24 kV. The 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 mL^{-1}. The matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) in CH_3OH/0.1% trifluoroacetic acid/CH_3CN (7:2:1, v/v) at a concentration of 75 mg mL^{-1}. A sample/matrix solution mixture (1:1, v/v) was deposited (1 mL) onto a stainless-steel gold-plated 100-sample MALDI probe tip, and left to dry at room temperature.

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