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Structural characterization of the carbohydrate backbone of the lipooligosaccharide of the marine bacterium *Arenibacter certesii* strain KMM 3941^T

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This paper is dedicated to the memory of Professor Gaspare Barone

Abstract—The structure of the carbohydrate backbone of the lipooligosaccharide (LOS) of the marine bacterium *Arenibacter certesii* strain KMM 3941^T has been elucidated. The structure was obtained by means of compositional analysis, matrix-assisted laser desorption/ionization mass spectrometry and complete ^{1}H and ^{13}C and ^{31}P NMR spectroscopy. It shows novel and interesting aspects and is the first description of *Arenibacter* lipopolysaccharides. Strong and mild alkaline treatments, to fully deacylate and only to O-deacylate the LOS were performed in order to determine the core structure. The core consists of a mixture of species differing by the presence of a non-stoichiometric α-D-rhamnose residue. The Kdo unit is substituted at O-5 by α-mannose and at O-4 by a α-galactosyluronic acid phosphate. The lipid A is constituted by a bis-phosphorylated disaccharide unit composed by a 2,3-diamino-2,3-dideoxy-β-D-glucopyranose (DAG) residue as non-reducing end and a GlcN as reducing end.

R-α-D-Rha(1→6)-α-D-Man(1→6)-α-D-Man(1→5)-α-Kdo(2→6)-β-D-GlcN3N(1→6)-α-D-GlcN
4P 4P 1P
α-D-Gal*p*A⌋

R = [α-D-Rha(1→3)] or H

Keywords: *Arenibacter certesii*; Lipopolysaccharide; Core region; Marine bacteria; 2,3-Diamino-2,3-dideoxy-D-glucose

1. Introduction

The recently described genus *Arenibacter* was established to accommodate Gram-negative, strictly aerobic, heterotrophic, dark-orange pigmented, non-motile marine

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bacteria belonging to the *Cytophaga–Flavobacterium–Bacteroides* (CFB) phylum.[1] This genus currently comprises only three species—*A. latericius*, *A. troitsensis*, and *A. certesii*[2] and forms a phylogenetic cluster with genera *Muricauda* and *Zobellia*.[3,4] The strain of *A. certesii* KMM 3941^T was isolated from a green alga, *Ulva fenestrata*, collected in the Troitsa Bay, Gulf of Peter the Great, Sea of Japan.[2]

Lipopolysaccharides, LPSs, composing about 75% of the outer membrane of Gram-negative bacteria and exposed toward the external environment, play an essential role in the adaptation of the organisms to the peculiar external surroundings. The study of LPS primary structure is important in order to understand the chemical modifications of the cell envelope to reinforce the external membrane and to ensure their survival. Lipopolysaccharides are heat-stable complex amphiphilic macromolecules indispensable for the growth and the survival of the majority of Gram-negative bacteria.[5–8] They are build up according to a common structural architecture and are composed of a hydrophilic heteropolysaccharide (formed by core oligosaccharide and O-specific polysaccharide or O-chain) covalently linked to a lipophilic moiety termed lipid A, which is embedded in the outer leaflet and anchors these macromolecules to the membrane through electrostatic and hydrophobic interactions. LPSs not containing O-chain are termed Rough (R) LPS or lipooligosaccharides (LOSs). LOSs may occur in both wild and laboratory strains possessing mutations in the genes encoding the O-specific polysaccharide biosynthesis or transfer. Lipid A possesses a rather conservative structure usually consisting of a β-(1→6)-linked glucosamine disaccharide backbone phosphorylated at positions 1 and 4′ and acylated with primary 3-hydroxy fatty acids at positions 2 and 3 of both GlcN residues; the hydroxyl groups of the primary fatty acids can be further acylated by secondary acyl moieties. In rare occasions 2,3-diamino-2,3-dideoxy-D-glucose (DAG) replaces the GlcN in the carbohydrate backbone of the lipid A.

In the core oligosaccharide, an inner and outer region are usually distinguished: the inner core, proximal to the lipid A, consists of typical monosaccharide residues as Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) and heptoses, often carrying negatively charged groups. Kdo is attached to the GlcN II of lipid A backbone and is the first sugar of the core oligosaccharide.[8] The outer core region is more variable and is usually composed by hexoses.

The LPS adaptive and dynamic changes managed by Gram-negative bacteria act on the carbohydrate backbone, on the polar heads and the acyl chain composition, and show the primary protective role that the LPSs operate in Gram-negative bacteria. The aim of this work is the elucidation of structure of the carbohydrate backbone of the LOS from the marine bacterium *A. certesii* strain KMM 3941^T.

2. Results

2.1. Isolation and compositional analysis of LOS

The LOS (R-LPS) was obtained by the phenol/chloroform/light petroleum extraction (yield 126 mg, 2.8% of dried cells) and lyophilized. The isolated LPS was a rough type (LOS), as suggested by the SDS-PAGE showing a run to the bottom of the gel and was further purified by enzymatic hydrolysis with protease, DNAse, and RNAse, followed by dialysis and Sephacryl HR-300 chromatography. Monosaccharide and methylation analyses of pure LOS revealed the presence of terminal-Rha*p*, 3-substituted Rha*p*, terminal Gal*p*A, 5-substituted Kdo*p*, 6-substituted Man*p*, 6-substituted Glc*p*N, 6-substituted DAG, all with D-configuration and in pyranose ring; the D-configuration of Kdo residue was obtained by NMR data (see below). Fatty acids analysis revealed the presence of *iso*-branched and *anteiso*-branched (*R*)-3-hydroxypentadecanoic (C15:0 (3-OH)) and (*R*)-3-hydroxyheptadecanoic (C17:0 (3-OH)) acids in both ester and amide linkages. The primary structure was achieved by chemical analysis, mass spectrometry, and NMR spectroscopy on intact and partially degraded LOS.

2.2. Structural characterization of product OS1, the fully deacylated oligosaccharide

The LOS was completely deacylated by anhydrous hydrazine followed by hot KOH and then purified by size exclusion chromatography. The compositional analysis of the isolated oligosaccharide **OS1** revealed the presence of terminal D-Rha*p*, 3-substituted D-Rha*p*, 5-substituted Kdo*p*, 6-substituted D-Man*p*, 6-substituted D-Glc*p*N, 6-substituted D-Glc*p*N3N (6-DAG), no uronic acids were detected.

A combination of homo- and heteronuclear 2D NMR experiments (DQF-COSY, TOCSY, ROESY, ^{13}P–^{1}H HSQC, ^{13}C–^{1}H HSQC and HMBC) was carried out in order to assign all the spin systems and the monosaccharide sequence. In the anomeric region of the ^{1}H NMR spectrum ten signals (residues) were identified (**A**–**G**, Table 1). Furthermore, the signals at 1.82/2.09 ppm were assigned to the H-3 methylene protons of the Kdo moiety (residue **H**, Fig. 1 and Table 1). The anomeric configurations of the monosaccharide units were assigned on the basis of the $^3J_{H1,H2}$ coupling constant obtained by the DQF-COSY and the intra-residual NOE contact observable in the ROESY experiment. The proton resonances of all spin systems were obtained by COSY and TOCSY (Fig. 2) spectra and were used to assign the carbon resonances in the HSQC spectrum. The NMR data indicated the existence of a mixture of two oligosaccharides differing by the length of the sugar backbone.

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

Unit	Chemical shift δ ($^{1}H/^{13}C/^{31}P$)					
	1	2	3	4	5	6
A	5.60	3.28	3.87	3.38	4.14	4.19/3.85
6-α-GlcN I	91.4	54.9	70.7	70.3	71.9	68.9
	3.24					
B	5.03	4.08	3.88	3.73	3.72	3.95/3.66
6-α-Man	102.1	70.6	70.7	66.8	71.9	66.1
B′	5.05	4.08	3.88	3.76	3.76	3.96/3.67
6-α-Man	102.0	70.6	70.7	66.9	72.0	66.1
C	4.85	3.97	3.85	3.63	3.76	3.98/3.64
6-α-Man	100.0	70.2	70.7	67.1	71.9	66.9
C′	4.86	3.96	3.84	3.63	3.74	3.97/3.64
6-α-Man	100.1	70.5	70.7	67.1	71.4	66.9
D	4.83	2.77	2.85	3.35	3.63	3.56
6-β-GlcN3N	100.7	55.5	56.4	70.4	75.8	62.7
D′	5.00	2.92	3.62	3.43	3.64	3.58
6-β-GlcN	99.6	56.3	n.d.	72.4	75.9	62.6
E	4.81	3.97	3.76	3.41	3.70	1.27
t-α-Rha	101.9	70.2	71.9	70.3	71.9	17.0
F	5.00	4.04	3.81	3.44	3.82	1.26
t-α-Rha	102.5	70.5	70.5	70.3	72.0	17.4
G	4.79	4.05	3.82	3.50	3.75	1.27
3-α-Rha	101.9	70.1	78.4	67.9	72.0	18.2
	$3_{ax/eq}$	4	5	6	7	8
H	1.82/2.09	4.12	4.18	3.98	3.83	3.90/3.65
5-α-Kdo	35.4	66.1	72.0	70.2	69.7	63.8

The anomeric signals of residue **A** at 5.60 ppm was attributed to the GlcN I of the disaccharide backbone of the lipid A, because of its chemical shift and multiplicity (double doublet, $^{3}J_{H1,H2} = 3.2$ Hz and $^{3}J_{H1,P} = 7.6$ Hz), in agreement with an α-D-*gluco*-anomeric configuration and a $^{4}C_{1}$ ring conformation. The ^{13}C–^{1}H HSQC spectrum showed the correlation of H-2 **A**, at 3.28 ppm, with a nitrogen bearing carbon signal at 54.9 ppm. The anomeric signals of residue **D**, at 4.83 ppm, correlated in the HSQC spectrum to a carbon at 100.7 ppm; this value, together with the $^{3}J_{H1,H2}$ coupling constant and the NOE contact of H-1 with H-3 and H-5 was diagnostic of a β-*gluco* configuration. The H-2 **D** and H-3 **D** signals at 2.77 and 2.85 ppm were correlated to high field shifted carbons at 55.5 and 56.4 ppm, indicative of the presence of two C–N linkages at position 2 and 3. In this way, **D** was identified as 2,3-diamino-2,3-dideoxy-β-D-glucose (β-DAG), representing together with the residue **A**, the disaccharide backbone of the lipid A (see below). A spin system, present in very low amount, attributed to a β-GlcN was analogously identified (residue **D′**, Table 1, Figs. 1 and 2). Residues **B**, H-1 at 5.03 ppm, and **C**, H-1 at 4.85 ppm, were both identified as α-mannose residues, as shown by their $^{3}J_{H1,H2}$ and $^{3}J_{H2,H3}$ low values (3 Hz), by the intra-residual NOE of H-1 only with H-2. Alternative spin systems were present for these residues, namely **B′** and **C′** (Table 1 and Figs. 1 and 2).

Sugar residues **E**, **F**, **G** (H-1 at 4.81, 5.00, 4.79 ppm, respectively), were recognized as three α-rhamnose residues. Actually, in TOCSY and COSY spectra, starting from the ring protons correlations were visible to methyl signals in the shielded region at 1.26-1.27 ppm. The *manno* configuration of residues **E**, **F**, **G** was established from the $^{3}J_{H1,H2}$ and $^{3}J_{H1,H3}$ values, the α-configuration by the intra-residual NOE contact of H-1 with H-2.

Because of the absence of the anomeric proton signal, the spin system of Kdo **G** was endorsed starting from the diastereotopic H-3 methylene protons resonating in a shielded region at 1.82 and 2.09 ppm (H-3_{eq} and H-3_{ax}, respectively). The α-configuration at C-2 was attributed by the chemical shift values of $H3_{eq}$ and by the values of $^{3}J_{H7,H8a}$ and $^{3}J_{H7,H8b}$.[9,10]

The down-field shift of carbon resonances allowed to identify the glycosylated positions, at O-6 of residues **A**, **B**/**B′**, **C**/**C′**, **D**, at O-5 of **H**, at O-3 of **G**, whereas residues **E** and **F** were terminal sugars, in full accordance with the methylation data. The inter-residual NOE contacts (Fig. 2) and the long-range correlations present in the HMBC spectrum yielded the sequence of monosaccharides. The inter-residue NOE contact of H-1 **D** (4.83 ppm) with H-$6_{a,b}$ **A** (4.19/3.85 ppm), together with the down-field shift of C-6 **A**, validated the β-(1→6) linkage between the α-GlcN **A** and the β-GlcN3N **D** of the lipid A backbone. The long-range correlation in the HMBC spectrum of C-2 **H** with H-6 **D**, and the

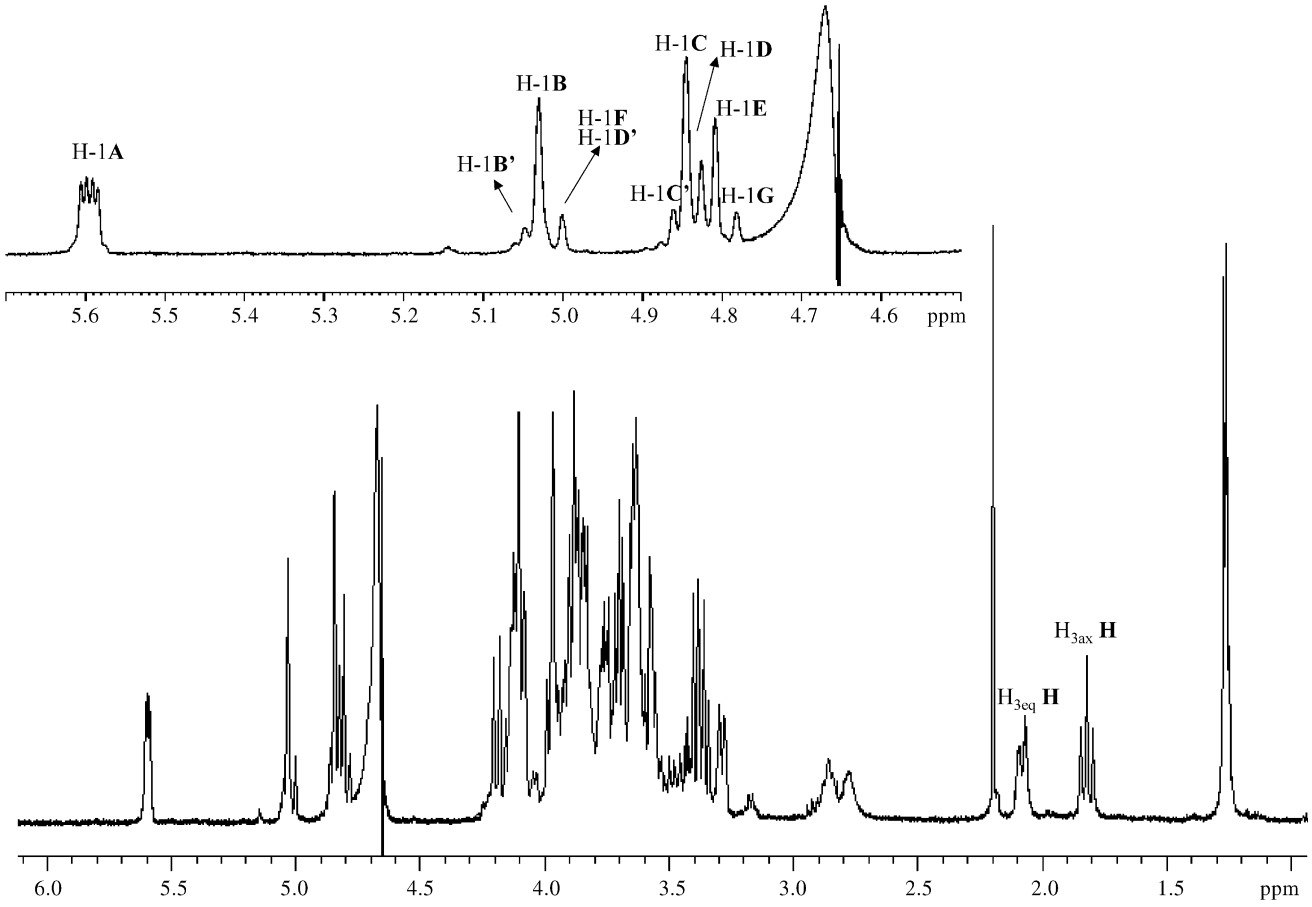

Figure 1. ^{1}H NMR spectrum of fully deacylated LOS (product **OS1**). Anomeric signals of spin system are designated as in Table 1. In the inset the expanded anomeric region is shown.

weak down-field shift of C-6 **D** (62.7 ppm) were diagnostic of the α-(2→6) ketosidic linkage of Kdo **H** with residue **D** of β-DAG. The NOE correlation of H-1 **B** (5.03 ppm) with H-5 **H** (4.18 ppm) indicated the α-(1→5) linkage of α-Man **B** to Kdo **H** residue. In addition, the existence of a NOE contact of H-1 **B** with H-7 **H** (Fig. 2) is only possible in case of an identical absolute configuration of both residues,[11] thus, both residue **B** and Kdo **H** possess the D-configuration.

Residue **B** was in turn substituted at O-6 by residue **C**, according to the NOE (Fig. 2) of H-$6_{a/b}$ **B** with H-1 **C**. Furthermore, the NOE correlations of H-$6_{a/b}$ **C** with H-1 **E** (Fig. 2) gave evidence of glycosylation of residue **C** at O-6 by α-rhamnose **E**. The HMBC spectrum confirmed the structure assigned for oligosaccharide **OS1**, since it contained all the required long-range correlations to demonstrate the spatial proximity of the residues.

The ^{31}P NMR spectrum showed a single signal resonating at 3.24 ppm in the region typical of monophosphate monoester groups. In the ^{31}P–^{1}H HSQC experiment this ^{31}P signal correlated to H-1 of α-GlcN **A** of the lipid A backbone at 5.60 ppm. Thus, these results can be summarized in the following oligosaccharide structure:

E C B H D A

α-D-Rha(1→6)-α-D-Man(1→6)-α-D-Man(1→5)-α-D-Kdo(2→6)-β-D-GlcN3N(1→6)-α-D-GlcN1P

A minor oligosaccharide was identified by the analysis of the minor set of signals in the NMR spectra. Actually, the NOE contact between H-1 of residue **F** of terminal Rha and H-3 of Rha residue **G** gave evidence of the α-(1→3) linkage between these two monosaccharides (Fig. 2). Furthermore, residue **C′** of 6-Man was in turn glycosylated at O-6 by residue **G**, as inferred by the NOE connectivity of H-1 **G** with H-$6_{a/b}$ **C′**. The anomeric proton of **C′** showed NOE contact with H-6 **B′**, validating the following structure

F G C' B' H D A
α-D-Rha(1→3)- α-D-Rha(1→6)-α-D-Man(1→6)-α-D-Man(1→5)-α-D-Kdo(2→6)-β-D-GlcN3N(1→6)-α-D-GlcN1P

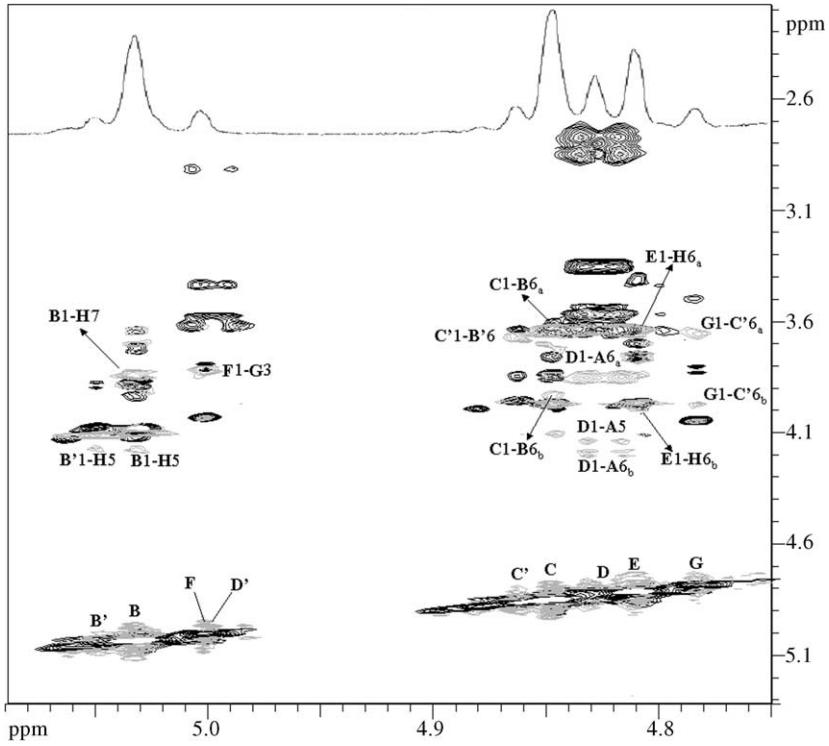

Figure 2. Section of the ROESY (gray) and TOCSY (black) spectrum of oligosaccharide (**OS1**). Monosaccharide labels are as indicated in Table 1. The relevant *inter*-residue ROE cross-peaks are indicated.

Thus, the minor oligosaccharide differed in the presence of an additional residue of rhamnose. A MALDI mass spectrum (Fig. 3) of the oligosaccharide mixture obtained by alkaline treatment confirmed the above structural hypotheses. The ion peaks corresponding to the two above oligosaccharides identified by NMR were present at *m*/*z* 1109.2 and 1255.2. The species at *m*/*z* 1109.2 matched with an oligosaccharide built up of one DAG, one HexN, two Hex, one Kdo, one deoxy-hexose residues and one phosphate group. The ion at *m*/*z* 1255.2 (Δ*m*/*z* 146) differed by the presence of an additional deoxy-hexose.

2.3. Structural characterization of product OS2, the O-deacylated LOS

In order to detect labile groups cleaved after the harsh alkaline treatment, that is, to investigate the origin of the GalA unit, lacking in the **OS1** product but detected in the compositional analysis of the intact lipooligosaccharide, the LOS was O-deacylated by mild hydrazinolysis[12] (product **OS2**). The **OS2** product was analyzed by methylation analysis and NMR spectroscopy. Compositional analysis revealed the presence of terminal D-Rha*p*, 3-substituted D-Rha*p*, terminal D-Gal*p*A, 5-substituted D-Kdo*p*, 6-substituted D-Man*p*, 6-substituted D-Glc*p*N, 6-substituted D-Glc*p*N3N (6-DAG).

The sample was dissolved in 1% deuterated SDS with 5 μL of NH_4OH 32% (pD 9.5) and underwent a complete NMR analysis (COSY, TOCSY, ROESY, ^{1}H–^{31}P HSQC, ^{1}H–^{13}C HSQC, and HMBC). Despite the line broadening of the signals visible in the ^{1}H NMR spectrum (Fig. 4), due to micelles originated by the presence of acyl moieties on the sugar backbone, a full assignment of the sugar backbone was possible.

The NMR analysis revealed the existence of the same carbohydrate backbone of **OS1** (Table 2) whose determination was straightforward. Moreover, the NMR spectra revealed the presence of an additional spin system, residue **I**, H-1 at 5.56 ppm (Fig. 4 and Table 2). All ^{1}H resonances of spin system **I** were assigned by COSY, TOCSY, and ROESY spectra. It was identified as hexuronic acid, in fact its H-5 signal showed, in the HMBC spectrum, a scalar long-range correlation with a carboxylic group at 174.0 ppm. The carbon resonances of **I** spin system were typical of an unsubstituted residue,

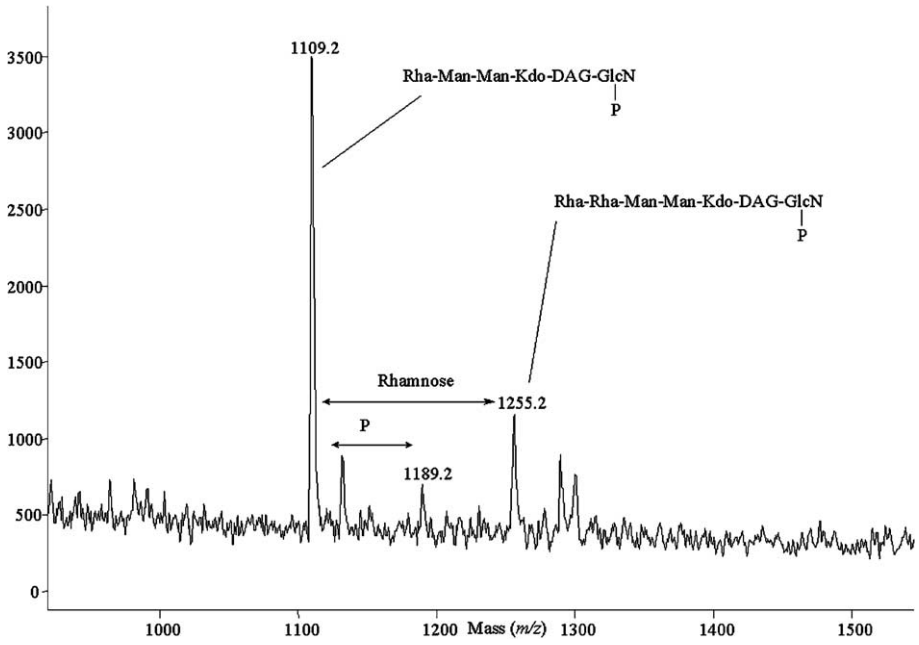

Figure 3. Negative ion MALDI-TOF spectrum of **OS1** product. Assignment of the ion peaks is shown.

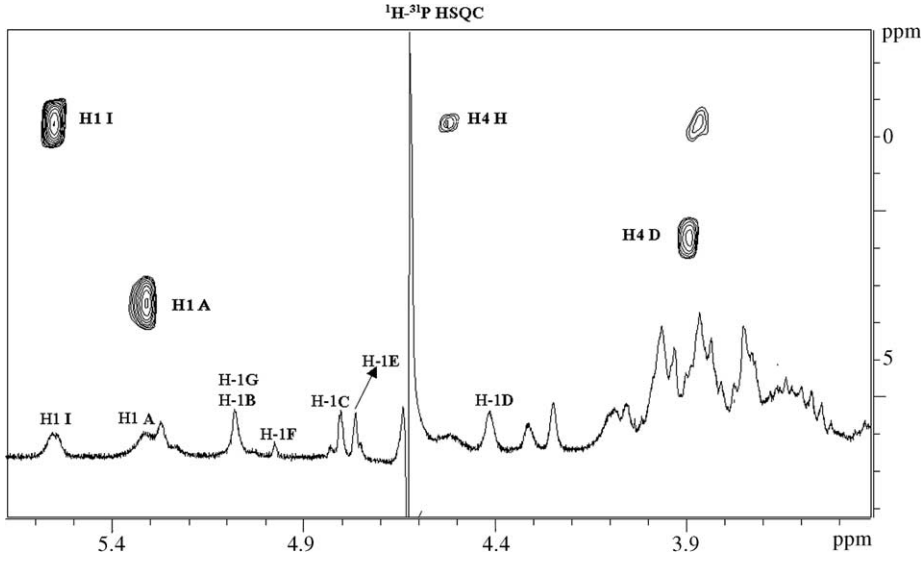

Figure 4. Section of ^{1}H NMR and ^{1}H, ^{31}P HSQC spectra of the O-deacylated LOS (product **OS2**). The localization of the phosphate groups is shown. Anomeric signals of spin system are designated as in Table 2. The spectrum was recorded in 1% deuterated SDS with 5 μL of 32% NH_4OH.

$^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ were diagnostic of a *galacto*-configuration whereas $^{3}J_{H1,H2}$, H-1, and C-1 values (5.56 and 95.7 ppm), intra-residual NOE contacts were all in agreement with an anomeric α-configuration and a $^{4}C_{1}$ ring conformation.

The ^{31}P NMR spectrum showed three signals, two of which resonated in the chemical shift region typical of monophosphate monoester group, at 3.53 and 2.02 ppm, while the third one, at −0.49 ppm, was representative of a monophosphate diester group. The location of these phosphorous signals were deduced by ^{31}P–^{1}H HSQC spectrum (Fig. 4). The phosphate groups at 2.02 and 3.53 ppm correlated to proton resonances at 5.30 and 3.97 ppm, attributed to H-1 **A** and H-4 **D**, respectively. Thus, two phosphate groups were attached to the lipid A, at O-1 of α-GlcN **A** and at O-4 of β-DAG **D**. The third phosphate signal correlated to proton resonances at 5.56 and 4.52 ppm, H-1 **I** and H-4 **H**, that is to say, the phosphodiester moiety consisted in a α-galacturonyl-phosphate located at O-4 of Kdo **I**.

Thus, the analysis of the O-deacylated product allowed the detection of two new phosphorylation sites,

Table 2. ^{1}H, ^{13}C, and ^{31}P NMR chemical shifts of the carbohydrate backbone of the O-deacylated LOS (product **OS2**) from *Arenibacter certesii*

Unit	Chemical shift δ ($^{1}H/^{13}C/^{31}P$)					
	1	2	3	4	5	6
	5.30	3.75	3.82	3.67	4.05	3.92
6-α-GlcN I	95.7	54.4	71.0	69.0	73.8	69.9
	3.53					
B	5.08	4.05	3.84	3.75	3.76	3.81
6-α-Man	101.4	70.7	69.6	66.6	72.1	68.9
C	4.81	3.97	3.81	3.55	3.66	3.56/3.93
6-α-Man	99.9	68.6	70.8	67.2	72.3	66.9
D	4.43	3.25	3.42	3.97	3.43	3.66/3.68
6-β-GlcN3N	102.4	53.4	52.7	74.7	76.0	61.3
				2.02		
E	4.77	3.94	3.74	3.34	3.66	1.26
t-α-Rha	100.6	70.4	70.4	72.4	72.3	16.9
F	4.97	3.98	3.75	3.39	3.76	1.21
t-α-Rha	99.1	70.4	70.4	72.3	72.0	17.3
G	5.03	3.87	3.76	3.40	3.75	1.21
3-α-Rha	101.3	68.6	78.9	72.2	72.0	18.2
I	5.56	3.84	3.88	4.25	4.42	
t-α-GalA	95.7	69.7	68.6	71.0	73.3	
	−0.49					174.0
	$3_{ax/eq}$	4	5	6	7	8
H	1.91/2.18	4.52	4.30	3.76	4.09	3.97/3.70
5-α-Kdo	37.6	68.0	73.6	70.5	71.5	65.2
		−0.49				

sensitive to cleavage under other strong alkaline treatment, for example, a phosphodiester group located on the Kdo moiety and a phosphate, situated at O-4 of the β-DAG, both totally lacking in the product **OS1**. The structure of the carbohydrate backbone of the lipooligosaccharide from *A. certesii* is drawn in Figure 5.

3. Discussion

The genus *Arenibacter*, which currently comprises the three species *A. latericius*, *A. troitsensis*, and *A. certesii*, accommodates Gram-negative, strictly aerobic, heterotrophic mesophillic rod-shaped, and pigmented marine

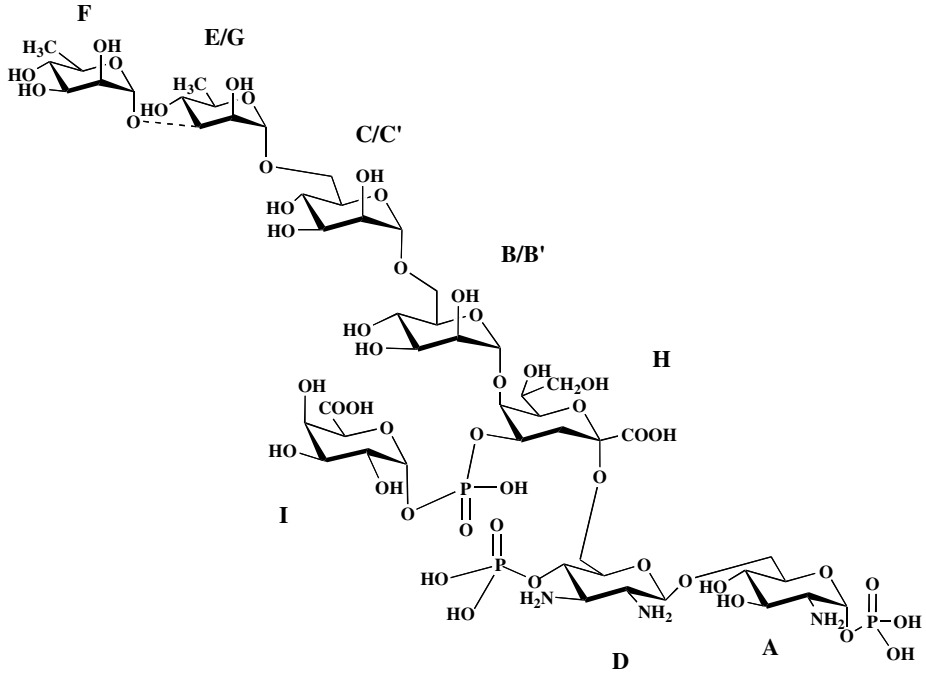

Figure 5. The structure of the carbohydrate backbone of the lipooligosaccharide from *Arenibacter certesii*. Dotted line indicate non-stoichiometric bond.

bacteria belonging to the family *Flavobacteriaceae*.[1,2] The strains of this genus are non-motile, because they commonly attach to the suitable substrates or to another cells. In this point of view, LPS, an important component of their cell-walls, which provides for an adhesion, can be considered as an adaptation factor of the organisms to surround conditions. Consequently, the study of its primary structure is important in order to comprehend the mechanisms helping to bacteria to survive in different marine environments.

In this paper, the carbohydrate skeleton of the lipo-oligosaccharide from a marine bacterium of the genus *Arenibacter* was for the first time isolated and studied by chemical analysis, NMR spectroscopy, and MALDI-MS. The lipid A moiety possesses a carbohydrate backbone characterized as [P→4-β-D-DAG-(1→6)-α-D-GlcpN-1→P] that is further glycosylated by a Kdo unit that is in turn substituted at O-5 by a α-mannose and at O-4 by α-galacturonyl phosphate. The α-mannose is linked at O-6 by a second unit of α-mannose, in turn substituted at the same position by a α-rhamnose unit. Finally, the α-rhamnose carried at O-3 a second non-stoichiometric unit of α-rhamnose.

The fully deacylated oligosaccharide lacks the phosphate group on the DAG moiety. The reason of its cleavage under strong alkaline conditions needs to be further investigated. MS and NMR experiment performed on the intact LOS and on the lipid A moiety should raise this point. Work is in progress to clarify the reason of this peculiar reactivity.

DAG has been previously identified as component of lipid A backbone in other LPSs.[13–15] The DAG-Glc*p*Nβ-(1→6)-linked disaccharide was found in lipid A from *Campylobacter jejuni* and *Rhodospirillum salinarum*,[13,14] whereas the DAG-DAG β-(1→6)-linked disaccharide is present in the lipid A from LPS of *Aquifex pyrophilus*,[15] *Bordetella pertussis*, and *Legionella pneumophila*, and, as a minor component, in lipid A from *C. jejuni*.[13,14] The presence of the DAG moiety in the lipid A backbone of *A. certesii* contributes to assure the bacterium a major resistance to unusual environment. Actually, the superior resistance of the amide linkages to the hydrolysis under alkaline condition can be seen as an adaptation of the bacterium to external hostile environment.

The presence of a second negative charged group is necessary in Kdo region, as the majority of single Kdo residues present in lipopolysaccharides of *Vibrionaceae* and *Pasteurellaceae*,[6–8] bears a phosphate group at O-4. The linkage at O-4 of Kdo by α-galacturonyl phosphate found in *A. certesii* LPS represents a rather new carbohydrate scheme in lipopolysaccharide core structures and biosynthesis. Actually, a phosphodiester bond with a galactosyluronic acid, a third negatively charged residue, is only present[16] in *Xanthomonas* LPS. Another element of originality of this core oligosaccharide structure matters on the absence of heptose residues, commonly present in *Enterobacteriaceae* LPSs.[5–8]

Many different mechanisms have been described that contribute to enhance resistance, all describing structural changes in outer and inner membranes and the decoration with substituting groups able to assure protection. They are a consequence of the evolutionary bacterial adaptation to the extreme environment, and assure the bacteria a best protection and reinforcing of the external membrane. In *A. certesii*, the inner core region, close to the lipid A, carries anionic substituents such as α-galacturonyl phosphate group on Kdo residue. These negatively charged substituents are associated and stabilized by divalent cations[5] present on the surface of the external membrane and are functionally important. In fact, these electrostatic interactions are a further contribution to the adaptation to the varied environments, since they reduce the membrane permeability and enhance its stability.

4. Experimental

4.1. Bacterial growth and LPS extraction

The type strain of *A. certesii* KMM 3941^T was cultivated on a liquid medium containing glucose (1 g/L), pepton (5 g/L), yeast extract (2.5 g/L), K_2HPO_4 (0.2 g/L), $MgSO_4$ (0.05 g/L), sea water (750 mL), and distilled water (250 mL). Cells were collected by centrifugation, washed with water, and next dried with acetone (three times) resulting in ~10 g of dried cells from 20 L of the cultural fluid.

The dried cells (1.5 g) were extracted first with chloroform/light petroleum/phenol[17] and then with hot phenol/water according to the conventional procedures.[18] In details, dried cells were extracted three times with a mixture of 2:5:8 aq 90% phenol–$CHCl_3$–light petroleum ether (v/v/v) as described.[17] After removal of the organic solvents under diminished pressure, the LOS fraction was precipitated from phenol with water, washed first with aq 80% phenol, and then three times with cold acetone, and lyophilized (yield 126 mg, 2.8% of dried cells). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) was performed as described.[19] For detection of LPS and LOS, gels were stained with silver nitrate.

4.2. Isolation of OS1

An aliquot of LOS (40 mg) was dissolved in anhyd hydrazine (2 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,

dissolved in water, and lyophilized (32 mg, 80% of the LOS). This material was N-deacylated with 4 M KOH as described.[20] Salts were removed using a Sephadex G-10 (Pharmacia) column (50 × 1.5 cm). The resulting oligosaccharide **OS1** constitutes the complete carbohydrate backbone of the lipid A-core region (16 mg, 40% of the LOS).

4.3. Isolation of OS2

LOS was O-deacylated by mild hydrazinolysis (37 °C, 1.5 h) with anhyd hydrazine in tetrahydrofurane, followed by precipitation with acetone at 4 °C and centrifugation (2.750*g*, 15 min, yield: 75% of LOS).

4.4. General and analytical methods

Determination of sugar residues, including the determination of the absolute configuration, organic-bound phosphate, and the absolute configuration of the hexoses were all carried out as described elsewhere.[12,20–22] For methylation analysis of Kdo region, LOS was carboxy-methylated with methanolic HCl (0.1 M, 5 min) and then with diazomethane to improve its solubility in Me_2SO. Methylation was carried out as described.[23,24] LOS was hydrolyzed with 2 M trifluoroacetic acid (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated as described above, carboxyl-reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analyzed by GLC–MS.

Total fatty acid content was obtained by acid hydrolysis of lipid A. Briefly, lipid A was first treated with HCl (4 M, 4 h, 100 °C) and then neutralized with NaOH 5 M (30 min, 100 °C). Fatty acids were then extracted in $CHCl_3$, methylated with diazomethane, and analyzed by GLC–MS. The ester bound fatty acids were selectively released by base-catalyzed hydrolysis with 1:1 0.5 M NaOH–MeOH (85 °C, 2 h), then the product was acidified, extracted in $CHCl_3$, methylated with diazomethane, and analyzed by GLC–MS. The absolute configuration of fatty acids was determined as described.[25]

4.5. NMR spectroscopy

For structural assignments of **OS1**, 1D and 2D ^1H NMR spectra were recorded on a solution of 5 mg in 0.6 mL of D_2O, at 300 K, at pD 7; for structural assignment of **OS2**, spectra were recorded on a soln of 1% deuterated SDS with 5 μL of NH_4OH 32% at 298 K at pD 9.5 (uncorrected value).

^1H and ^{13}C NMR spectra were measured on Varian INOVA 500 equipped with a reverse probe. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. ^{31}P NMR spectra were measured at 162 MHz on a Bruker DRX 400 spectrometer equipped with a reverse probe. Aqueous 85% phosphoric acid was used as external reference (0.00 ppm) for ^{31}P NMR spectroscopy.

Rotating frame Overhauser enhancement spectroscopy (ROESY) was measured using data sets ($t_1 \times t_2$) of 4096 × 512 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096 × 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 120 ms, using data sets ($t_1 \times t_2$) of 4096 × 512 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the *F*1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a 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).[26,27] Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^1H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 256 points, and 64 scans were acquired for each t_1 value. Experiments were carried out in the phase-sensitive mode. A 60 ms delay was used for the evolution of long-range connectivities in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048 × 1024 points using forward linear prediction extrapolation.[28,29]

4.6. MALDI TOF analysis

MALDI-TOF analyses were conducted in linear mode using a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. 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 oligosaccharide mixture containing such highly acidic species was first converted in the ammonium form by a home-made miniaturized column of cation-exchanged resin Dowex 50WX8-200 (Sigma–Aldrich). The analyte was eluted with water and dried in a centrifugal concentrator (SpeedVac Thermo Savant, USA), then was dissolved in a few microliters of 0.1% trifluoroacetic acid (TFA) before MALDI analysis. The obtained sample was analyzed in negative polarity in 4:1 2,5-dihydroxybenzoic acid (DHB 50 mg/mL) TFA 0.1%–MeCN (80/20).

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References

1. Ivanova, E. P.; Nedashkovskaya, O. I.; Chun, J.; Lysenko, A. M.; Frolova, G. M.; Svetashev, V. I.; Vysotskii, M. V.; Mikhailov, V. V.; Huq, A.; Colwell, R. R. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 1987–1995.
2. Nedashkovskaya, O. I.; Kim, S. B.; Han, S. K.; Lysenko, A. M.; Mikhailov, V. V.; Bae, K. S. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 1173–1176.
3. Bruns, A.; Rohde, M.; Berthe-Corti, L. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 1997–2006.
4. Barbeyron, T.; L'Haridon, S.; Corre, E.; Kloareg, B.; Potin, P. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 985–997.
5. Seltmann, G.; Holst, O. *The Bacterial Cell Wall*; Springer: Heidelberg, 2001.
6. Holst, O. In *Endotoxin in Health and Disease*; Brade, H., Morrsion, D. C., Opal, S., Vogel, S., Eds.; Marcel Dekker: New York, 1999; pp 115–154.
7. Alexander, C.; Rietschel, E. T. *J. Endotoxin Res.* **2001**, *7*, 167–199.
8. Holst, O. *Trends Glycosci. Glycotechnol.* **2002**, *14*, 87–103.
9. Birnbaum, G. I.; Roy, R.; Brisson, J. R.; Jennings, H. *J. Carbohydr. Chem.* **1987**, *6*, 17–39.
10. Holst, O.; Thomas-Oates, J. E.; Brade, H. *Eur. J. Biochem.* **1994**, *222*, 183–194.
11. Bock, K.; Vinogradov, V.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1994**, *225*, 1029–1039.
12. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2004**, *339*, 737–740.
13. Zärhringer, U.; Lindner, B.; Rietschel, E. T. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 211–276.
14. Zärhringer, U.; Lindner, B.; Rietschel, E. T. In *Endotoxin in Health and Disease*; Brade, H., Morrsion, D. C., Opal, S., Vogel, S., Eds.; Marcel Dekker: New York, 1999; pp 93–115.
15. Plätz, B. M.; Lindner, B.; Stetter, K. O.; Holst, O. *J. Biol. Chem.* **2000**, *275*, 11222–11228.
16. Silipo, A.; Molinaro, A.; Sturiale, L.; Dow, J. M.; Erbs, G.; Lanzetta, R.; Newman, M.-A.; Parrilli, M. *J. Biol. Chem.* **2005**, *280*, 33360–33668.
17. Galanos, C.; Lüderitz, O.; Westphal, O. *Eur J. Biochem.* **1969**, *9*, 245–249.
18. Westphal, O.; Jann, K. *Meth. Carbohydr. Chem.* **1965**, *5*, 83–91.
19. Kittelberger, R.; Hilbink, F. *J. Biochem. Biophys. Methods* **1993**, *26*, 81–86.
20. Holst, O. In *Methods in Molecular Biology, Bacterial Toxins: Methods and Protocols*; Holst, O., Ed.; Humana Press: Totowa, NJ, 2000; pp 345–353.
21. Vinogradov, E.; Korenevsky, A.; Beveridge, T. J. *Carbohydr. Res.* **2003**, *338*, 1991–1997.
22. Vinogradov, E.; Bock, K.; Petersen, B. O.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1997**, *243*, 122–127.
23. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
24. Hakomori, S. *J. Biochem. (Tokyo)* **1964**, *55*, 205–208.
25. Rietschel, E. T. *Eur. J. Biochem.* **1976**, *64*, 423–428.
26. Piantini, U.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.
27. Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 48–479.
28. de Beer, R.; van Ormondt, D. *Basic Prin. Prog.* **1992**, *26*, 201.
29. Hoch, J. C.; Stern, A. S. NMR Data Processing. In Hoch, J. C., Stern, A. S., Eds.; Wiley: New York, 1996; pp 77–101.