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Carbohydrate Research 340 (2005) 1475–1482
Carbohydrate RESEARCH

The complete structure of the core carbohydrate backbone from the LPS of marine halophilic bacterium *Pseudoalteromonas carrageenovora* type strain IAM 12662^T

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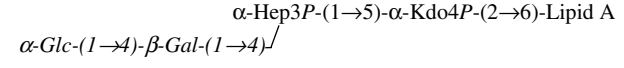
Received 2 March 2005; received in revised form 30 March 2005; accepted 31 March 2005
Available online 5 May 2005

Abstract—The complete novel structure of the components of the core oligosaccharide fraction from the LOS of the halophilic marine bacterium *Pseudoalteromonas carrageenovora* was characterized. The fully de-acylated lipooligosaccharide was studied by means of compositional analysis, matrix-assisted laser desorption/ionization mass spectrometry and complete ^{1}H and ^{13}C and ^{31}P NMR spectroscopy. The core oligosaccharide is composed by a mixture of species differing for the length of the sugar chain and the phosphorylation pattern:



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.

Keywords: *Pseudoalteromonas carrageenovora*; Lipooligosaccharide; Core oligosaccharide; Marine bacteria

1. Introduction

Marine bacteria of the genus *Pseudoalteromonas* have become a subject of intensive investigations during the last decade.[1–4] The research interest for this remarkable group of 'Gammaproteobacteria' is due to the discovery of their capabilities to produce: (i) a wide range of biologically active compounds, for example, antibiotics, cytotoxins, enzymes, bactericidal, bacteriolytic, autotoxic, antifouling, (ii) bio-controlling compounds active against invertebrate larvae, algal spores, fungi and diatoms and (iii) an array of hydrolytic enzymes.[5–7] *Pseudoalteromonas* represent one of taxonomically numerous cluster of marine cultivable prokaryote and is characterized by about 40 species. One of such species, *Alteromonas carrageenovora*, derived from a few strains hydrolyzing algal polysaccharides (alginate, agar and carrageenan) originally isolated from marine algae (Rhodophyceae).[8] Later these bacteria were identified as [*Pseudomonas*] *carrageenovora* and subsequently reclassified first as [*Alteromonas*] and eventually as *Pseudoalteromonas carrageenovora*.[2,4,9] The type strain

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doi:10.1016/j.carres.2005.03.012

of *P. carrageenovora* IAM 12662 (= IFO 12985 = NCIMB 302 = ATCC 43555) was Gram-negative, strictly aerobic, polarly flagellated bacterium isolated from seaweeds.[9]

As a Gram-negative bacterium, *P. carrageenovora* possesses lipopolysaccharides in the external leaflet of its outer membrane.[10] Lipopolysaccharides (LPSs) are amphiphilic macromolecules composed of a hydrophilic hetero-polysaccharide (formed by core oligosaccharide and O-specific polysaccharide or O-chain) covalently linked to a lipophilic moiety termed lipid A, which anchors these macromolecules to the external membrane.[11] LPSs not containing O-chain are termed Rough (R) LPSs or lipooligosaccharide (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 oligosaccharide, composed of up to 15 sugar residues, can be divided into two regions: the inner and the outer core.[12,13] The inner core is less variable and, usually, its primary structure is highly distinctive. The first residue of the core oligosaccharide is very often a Kdo residue (3-deoxy-D-*manno*-oct-2-ulosonic acid), whereas the second residue is frequently L-*glycero*-D-*manno*-heptose residue (L,D-Hep). The outer core region of LPSs is more variable and it is generally referred as the hexose region and is usually composed of neutral and common hexoses. In LOS, the core region possesses antigenic properties and it is thought to modulate the toxic activities of the lipid A portion in pathogenesis of bacteria.

Lipopolysaccharides from halophilic bacteria frequently show unusual chemical features most likely due to their external environment.[14,15] Like the phylogenetically correlated Gram-negative bacterium *P. issachenkonii*,[16] *P. carrageenovora* produces an R-LPS, which could be important in the interaction of this organism with its peculiar external surrounding. As nothing is known about the cell wall composition of this halophilic bacterium, we began the structural investigation of its R-LPS, and now report the complete structure of the lipooligosaccharide components of this strain.

2. Results and discussion

2.1. Isolation and compositional analysis of LOS

The LOS (R-LPS) was precipitated after the phenol/chloroform/petroleum ether extraction and lyophilized. The SDS-PAGE showed, after silver nitrate gel staining that it migrated at the bottom of the gel in accordance with the lipooligosaccharide nature of this fraction. In order to separate LOS from glucans and other cell contaminants, the sample was purified by enzymatic hydrolysis with DNAse, RNAse and protease followed by dialysis and gel permeation chromatography. Monosaccharide and methylation analyses of intact LOS revealed the presence of 6-substituted-Glc*p*N, 5-substituted-Kdo*p*, 4-substituted-Hep*p*, terminal-Hep*p*, terminal-Gal*p*, 4-substituted-Gal*p* and terminal-Glc*p*. All hexose residues were found to be in the D-configuration while heptose residues were in the L, D-configuration. Fatty acid 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. C10:0 (3-OH), C13:0 (3-OH), C10:0 were found in minor amount.

2.2. Structural characterization of fully de-acylated oligosaccharide

The LOS was completely de-acylated by anhydrous hydrazine and hot KOH and analyzed by mass spectrometry. The negative ion MALDI-TOF mass spectrum showed various ions differing in chain length and phosphorylation pattern. The prominent ions were represented by **W** and $\mathbf{W^0}$ (Fig. 1). Species **W** at *m*/*z* 991.7 was consistent with a tetrasaccharide possessing one Kdo, two hexosamines, one heptose and three phosphate groups, species $\mathbf{W^0}$ at *m*/*z* 911.7 lacked a phosphate group whereas species **W′** at *m*/*z* 1071.6 ($\Delta m/z$ = 80 from **W**) carried a further phosphate group. At higher mass, core glycoform $\mathbf{X^0/X/X'}$, at *m*/*z* 1073.8 and 1153.7 and 1233.5, respectively, carried one additional hexose residue with respect to $\mathbf{W^0}$, **W** and **W′** ($\Delta m/z$ = 162), while the hexasaccharide ion species $\mathbf{Y^0/Y/Y'}$, at *m*/*z* 1235.3 and 1315.4 and 1395.4, carried two additional hexose residues.

The primary structure of oligosaccharides within LOS fraction was established by a combination of homo- and heteronuclear 2D NMR experiments (DQF-COSY, TOCSY, NOESY, ^{13}P–^{1}H HSQC, ^{13}C–^{1}H HSQC, HMBC) to assign all the spin systems and to identify the carbohydrate sequence (Table 1). Anomeric configurations were assigned on the basis of the chemical shifts and of $^{3}J_{H1,H2}$ values, which were determined from the DQF-COSY experiment. All sugars were identified as having 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, from C-2 to H-6).

In the ^{1}H NMR, the region of anomeric proton signals showed several spin systems (**A**–**H**; Table 1, Fig. 2) suggesting the presence of a mixture of oligosaccharides. The anomeric signals of **A** and **D** spin systems at 5.54 and 4.85 ppm were rapidly recognized. In particular, **A** residue was identified as the GlcN I of lipid A skeleton because of its chemical shifts and the multiplicity of the anomeric signal (double doublet, $^{3}J_{H1,H2}$ = 3.2 Hz and $^{3}J_{H1,P}$ = 7.6 Hz). Likewise, **D** residue was

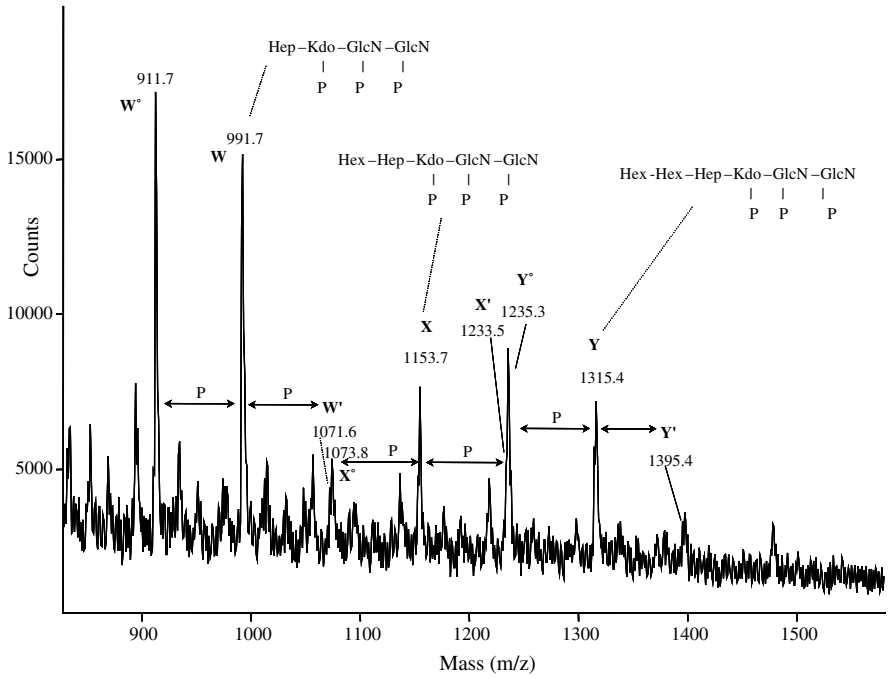

Figure 1. Negative ion MALDI-TOF mass spectrum of the oligosaccharides fraction obtained in linear mode. The main ions are assigned, the ions not specified in the spectrum correspond to sodium adducts of the molecular ions and to prompt fragments due to H_3PO_4 loss.

identified as the β-GlcN of lipid A backbone on the basis of its chemical shifts and coupling constants (in the range of 10 Hz). In agreement, both H-2 signals of **A** and **D** spin systems correlated to a nitrogen bearing carbon signals in the HSQC spectrum (Table 1). Residues **B** and **C** possessed the α-*manno*-configuration, as shown by the low coupling constant values $^3J_{H1,H2}$ and $^3J_{H2,H3}$. Starting from the H-2 signal in the TOCSY spectrum, it was possible to completely assign the spin systems, thus identifying both as heptose residues in different chemical/magnetic environment. Additional different sets of signals (**B**′ and **C**′) were present for spin system **B** and **C**. Residues **F** and **G** possessed the β-configuration as shown by $^3J_{H1,H2}$ (7.9 Hz). This assumption was also supported by a NOESY experiment that showed for these residues intra-residual NOE connectivity from H-1 to H-3 and to H-5. **F** and **G** residues were identified as galactose residues owing to the small $J_{H,H}$ values for H-3/H-4 and H-4/H-5. Spin system **E** ($^3J_{H1,H2}$ = 3.5 Hz) was identified as terminal α-glucose residue since it possessed all large $^3J_{H,H}$ ring values. Slightly different sets of signals were also present for the above described spin systems **F**, **G** and **E**, characterized by different ring proton resonances (**F**′, **G**′ and **E**′). The spin system of Kdo, residue **H**, was attributed starting from the diasterotopic H-3 methylene protons at 1.89 and 2.12 ppm (H-3_{ax} and H-3_{eq}, respectively). The α-configuration at C-2 was attributed by the chemical shift values of H-3_{eq} and by the values of $^3J_{H7,H8a}$ and $^3J_{H7,H8b}$.[17,18] A second and minor set of signals was also present for Kdo (**H**′) characterized by slightly different chemical shift values for H-3 and H-4. The proton resonances of all spin systems obtained by COSY and TOCSY spectra were used to assign the ^{13}C NMR chemical shifts in the HSQC spectrum. Low-field shifted signals indicated substitutions at O-6 of **A** and **D**, at O-4 of **C** and **G**, while **B**, **E** and **F** spin systems were non-substituted monosaccharide residues. These data were in full accordance with the methylation data.

Phosphate substitution was inferred on the basis of ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum showed the presence of monophosphate monoester peaks (Table 1), which were assigned by a ^{31}P–^1H HSQC experiment (Fig. 3). Thus, the phosphate groups were linked at O-1 of α-GlcN **A**, O-4 of β-GlcN **D**, O-3 of α-Hep **B**′, O-3 of α-Hep **C**′, O-4 of α-Kdo **H**.

From the interresidual NOE contacts (Fig. 4) the sequence of monosaccharides of the de-acylated LOS was determined. A mixture of tetra-, penta- and hexasaccharides present in different amounts and having differing for the phosphorylation patterns were identified (see below and Fig. 1). The β-(1→6) linkage between the GlcNs of the lipid A backbone was revealed by the interresidual NOE contact of H-1 **D** (4.85 ppm) with H-$6_{a,b}$ **A** (4.17/3.74 ppm), whereas the weak downfield displacement of C-6 of spin system **D** (62.4 ppm) was in agreement with the glycosylation by the ketose Kdo residues **H**/**H**′, and, furthermore, these residues were glycosylated at O-5. Actually, both H-1 signals of the terminal non-phosphorylated Hep **B** residue and of phosphorylated Hep unit **B**′ showed NOE correlation with the signal at 4.22 ppm assigned to H-5 of Kdo units

Table 1. ^{1}H, ^{13}C (bold), ^{31}P (italic) chemical shifts (ppm) of oligosaccharide fraction of *P. carrageenovora*

Unit	Chemical shift δ ($^{1}H/^{13}C/^{31}P$)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
A	5.54	3.27	3.78	3.34	4.06	4.17/3.74	
α-GlcN I	**90.9**	**54.7**	**72.1**	**70.8**	**72.8**	**69.5**	
	2.59						
B	5.07	4.06	3.97	3.74	3.65	4.04	3.60/3.63
t-α-Hep	**100.5**	**72.6**	**73.5**	**68.4**	**72.1**	**71.8**	**63.7**
B′	5.05	4.16	4.38	3.97	3.64	4.04	3.60
t-α-Hep	**100.5**	**70.4**	**69.5**	**70.7**	**72.1**	**71.8**	**63.7**
			3.00				
C	5.06	4.19	4.25	3.78	4.07	4.13	3.60/3.63
4-α-Hep	**100.4**	**70.4**	**71.6**	**78.7**	**69.6**	**70.5**	**63.7**
C′	5.06	4.03	4.38	3.80	n.d.	n.d.	n.d.
4-α-Hep	**100.4**	**72.5**	**69.4**	**78.5**	**n.d.**	**n.d.**	**n.d.**
			3.00				
D	4.85	2.96	3.74	3.56	3.61	3.44	
β-GlcN	**99.5**	**55.7**	**72.6**	**72.9**	**75.3**	**62.4**	
				2.31			
E	4.80	3.39	3.66	3.33	4.04	3.67	
t-α-Glc	**100.1**	**72.2**	**72.9**	**70.3**	**72.8**	**60.4**	
E′	4.77	3.41	3.66	3.33	4.05	3.67	
t-α-Glc	**100.1**	**72.2**	**72.9**	**70.3**	**72.8**	**60.4**	
F	4.35	3.45	3.55	3.81	3.59	3.68	
t-β-Gal	**103.5**	**71.2**	**72.9**	**69.1**	**75.4**	**61.2**	
F′	4.38	3.45	3.57	3.81	3.59	3.68	
t-β-Gal	**103.5**	**71.2**	**72.9**	**69.1**	**75.4**	**61.2**	
G	4.42	3.51	3.64	3.91	3.50	3.68	
4-β-Gal	**103.7**	**72.8**	**72.1**	**77.1**	**75.8**	**61.2**	
G′	4.44	3.54	3.65	3.88	3.53	3.68	
4-β-Gal	**103.7**	**72.8**	**72.1**	**76.7**	**75.1**	**61.2**	
	$3_{ax/eq}$	**4**	**5**	**6**	**7**	**8**	
H	1.89/2.12	4.47	4.22	3.80	3.70	3.81/3.61	
α-Kdo	**34.5**	**69.8**	**73.0**	**69.0**	**70.0**	**63.7**	
		1.96					
H′	1.96/2.11	4.09	4.22	n.d.	n.d.	n.d.	
α-Kdo	**34.5**	**68.8**	**70.0**	**n.d.**	**n.d.**	**n.d.**	

Spectra were recorded at 30 °C 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. n.d. not detected.

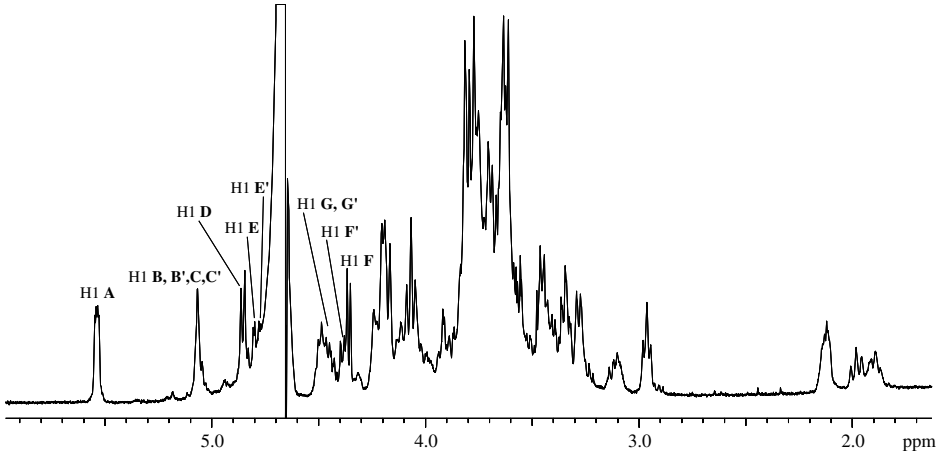

Figure 2. ^{1}H NMR spectrum of the oligosaccharide fraction deriving from alkaline degradation. Anomeric signals are designated by capital letters.

H/H′. These NOE effects indicated linkages between O-5 of Kdo **H/H′** and Hep **B** in the glycoforms **W** and **W^0** and linkage between O-5 of Kdo **H** and Hep **B′** in the minor tetra-phosphorylated glycoform **W′**. These data

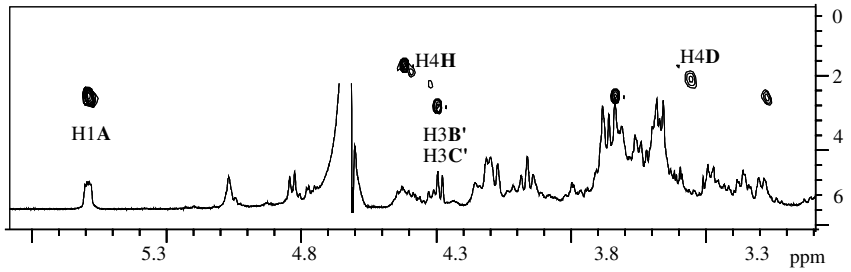

Figure 3. ^{31}P–^{1}H HSQC spectrum of the oligosaccharide fraction. The capital letters refer to residues as denoted in Table 1.

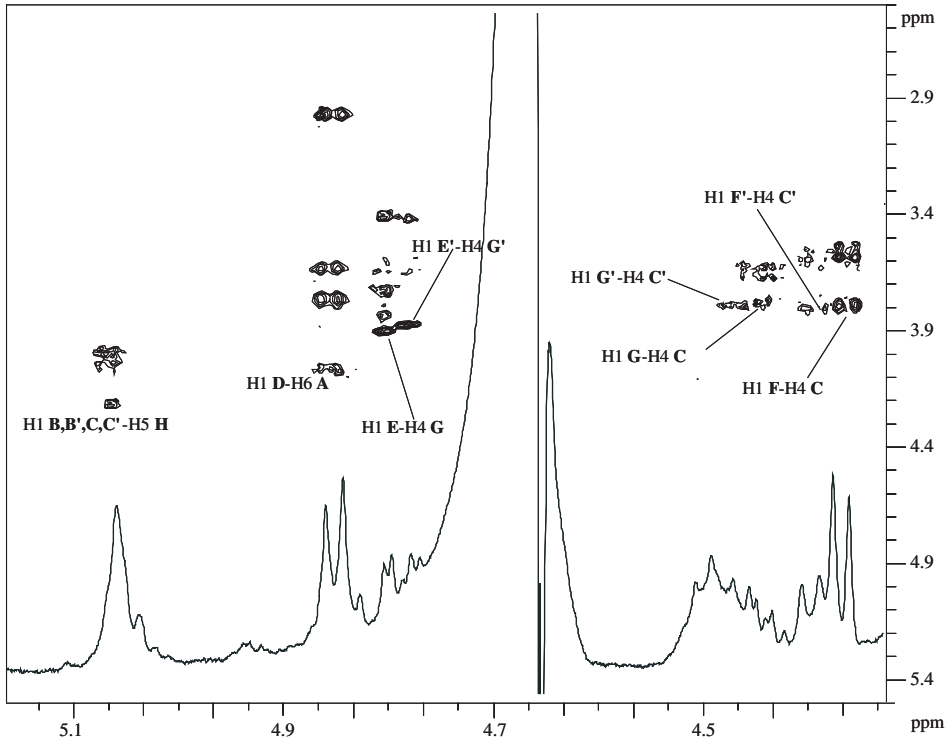

Figure 4. Part of the NOESY spectrum of the oligosaccharide fraction. Annotations refer to interresidual cross-peaks. The capital letters refer to residues as denoted in Table 1.

are in agreement with MALDI MS spectrum in which the three tetrasaccharide glycoforms **W′**, **W** and **W^0** are visible.

B/B' H/H' D A
α-Hep3*R*-(1→5)-α-Kdo4*R′*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*

W' R = P R' = P
W R = H R' = P
W^0 R = H R' = H

On the basis of this above tetrasaccharide sequence, other core oligosaccharides could be identified. From the NMR data a longer core glycoform (oligosaccharide **X^0/X/X′**) was identified, where the terminal units **B/B′** of above tetrasaccharide are substituted at O-4 position of **C/C′**. Accordingly, the H-1 signal of terminal Gal **F/F′** gave NOE contacts with H-4 **C/C′** (Fig. 3). The presence of two different spin systems for terminal Gal (**F/F′**) is most likely due to heterogeneity in phosphorylation of the Hep and Kdo residues. Also these data are in agreement with MALDI MS spectrum in which the pentasaccharide glycoforms **X^0**, **X** and **X′** are observable.

F/F' C/C' H D A
β-Gal-(1→4)/α-Hep3*R*-(1→5)-α-Kdo4*R′*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*

X' R = P R' = P
X R = H R' = P
X^0 R = H R' = H

The third and last core glycoform was characterized by an additional α-glucose unit (**E**) that was linked to O-4 of β-galactose (**G**). Actually, in the NOESY spectrum, a cross-peak between H-1 of **E**/**E**′ and H-4 of **G**/**G**′ was detectable (Fig. 3). Once more, the finding of two different spin system for terminal Glc (**E**/**E**′) and 4-substituted Gal (**G**/**G**′) is due to heterogeneity in phosphorylation of the preceding Hep and Kdo residues. MALDI MS data were in agreement with these finding and showed the two hexasaccharide glycoforms $\mathbf{Y^0}$, **Y** and **Y**′.

E/E' G/G' C/C' H D A

α-Glc-(1→4)-β-Gal-(1→4)-α-Hep3*R*-(1→5)-α-Kdo4*R*′-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*

Y' R = P R' = P
Y R = H R' = P
Y^0 R = H R' = H

In summary, we have established the complete structure of the oligosaccharide fraction from *P. carrageenovora*, shown in Figure 5.

The core oligosaccharide fraction from *Pseudoalteromonas carrageenovora* is characterized by a strong accumulation of phosphate groups in the lipid A-core portion creating a region with a high charge density. It has been demonstrated that the lipid A-inner core is usually decorated with negatively charged substituents, often present in not stoichiometric amount, which are involved in membrane assembly and stability. They are always strongly associated and stabilized by divalent cations that connect LPS molecules to each other. These electrostatic interactions contribute to reduce the membrane permeability and to enhance its stability with the formation of a strong, rigid and protective barrier. It is noteworthy that only two other core structures of LPS from *Pseudoalteromonas* have been reported to date, *P. haloplanktis* TAC 125[19] and *P. issachenkonii*.[16] They both possess, as the core oligosaccharide from *P. carrageenovora* we have described in this paper, the carbohydrate skeleton **β-Gal−(1→4)-α-Hep3*R*-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*** that can be considered a unique structural feature of the *Pseudoalteromonas* genus.

3. Experimental

3.1. Bacteria and bacterial LOS

The type strain of *P. carragenovora* IAM 12662^T was kindly provided by Dr. M. Akagawa-Matsushita (University of Occupational and Environmental Health, Kitakyushu, Japan). In a preparative scale, bacteria were grown 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) obtaining ~12 g of dried cells from 20 L of the cultural fluid.

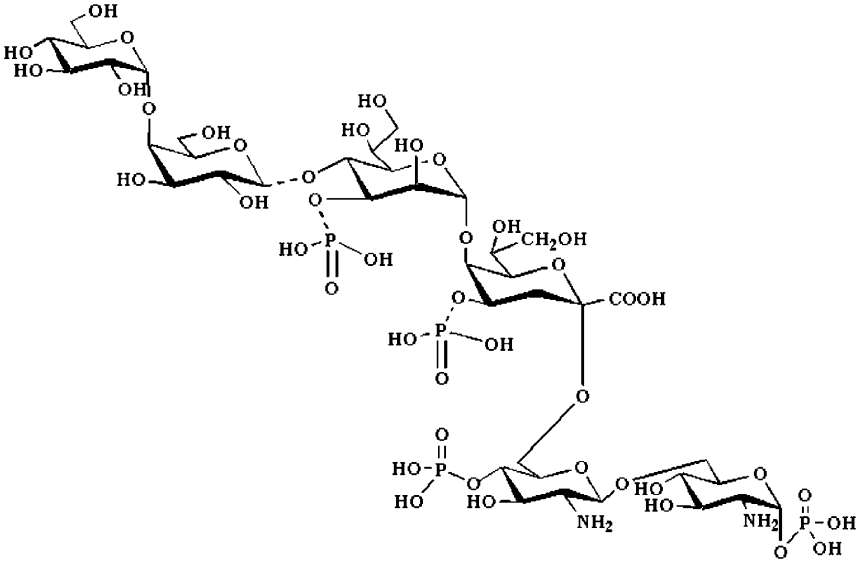

Figure 5. The structure of the components of LOS fraction isolated form *Pseudoalteromonas carrageenovora*.

An aliquot of dried cells (1 g) was 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 lyophilized (25 mg, yield: 2.5% of the bacterial dry mass). Sodium dodecyl sulfate polyacrylamide 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 lyophilized (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 fraction 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 hydrolyzed 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 analyzed by GLC–MS. Methylation of the complete core region was carried out as described,[29] and the sample was hydrolyzed with 4 M trifluoroacetic acid (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated, carboxyl-reduced, acetylated and analyzed by GLC–MS.

3.4. NMR spectroscopy

For structural assignments of oligosaccharide, 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 intra-ring 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 optimized 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 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, λ = 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 microlitres of 0.1% trifluoroacetic acid

(TFA) before MALDI analysis. The obtained sample was analyzed in negative polarity in 2,5-dihydroxybenzoic acid (DHB) 50 mg/mL TFA 0.1%-acetonitrile (80/20). Recrystallization from methanol was performed as already reported.[35]

Acknowledgements

We thank MIUR, Rome (Progetti di Ricerca di Interesse Nazionale to M.P. and legge 449/97 to D.G.) and National Council of Research (CNR), Rome for financial support. NMR experiments were carried out on a 500 MHz spectrometer of Consortium INCA (L488/92, Cluster 11) at Centro Interdipartimentale Metodologie Chimico Fisiche Università di Napoli. The work was also supported by grants of Russian Foundation for Basic Research (No. 05-04-48211) and Ministry for Industry and Science of Russian Federation (No. 2-2.16) and the grants of the Presidium of Russian Academy of Sciences 'Molecular and Cell Biology'. We gratefully thank Dr. M. Akagawa-Matsushita (University of Occupational and Environmental Health, Japan) for a gift of the *Pseudoalteromonas carrageenovora* type strain.

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