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Note

The O-chain structure from the LPS of marine halophilic bacterium *Pseudoalteromonas carrageenovora*-type strain IAM 12662^T

Alba Silipo,[a] Antonio Molinaro,[a,*] Evgeny L. Nazarenko,[b] Raisa P. Gorshkova,[b] Elena P. Ivanova,[b,†] Rosa Lanzetta[a] and Michelangelo Parrilli[a]

[a]*Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, via Cintia 4, 80126 Napoli, Italy*
[b]*Pacific Institute of Bioorganic Chemistry, Far-East Branch of the Russian Academy of Sciences, 690022 Vladivostok-22, Russian Federation*

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Abstract—The O-chain polysaccharide of the lipopolysaccharide from the halophilic marine bacterium *Pseudoalteromonas carrageenovora* IAM 12662^T was characterized. The structure was studied by means of chemical analysis and 2D NMR spectroscopy of the de-*O*-acylated lipopolysaccharide and shown to be the following:

$$\begin{array}{l}\rightarrow 2)\text{-}\alpha\text{-Col}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow \\ \alpha\text{-Col}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 3)\lrcorner\end{array}$$

Col is colitose, 3,6-di-deoxy-L-*xylo*-hexose.

Keywords: *Pseudoalteromonas carrageenovora*; Lipopolysaccharide; Colitose; Marine bacteria

Marine bacteria of the genus *Pseudoalteromona*s have become a subject of intensive investigations during the last decade.[1–4] The research interest for this significant 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 and antifouling; (ii) bio-controlling compounds active against invertebrate larvae, algal spores, fungi and diatoms and (iii) an array of hydrolytic enzymes.[5–7] *Pseudoalteromona*s represent one of the taxonomically numerous cluster of marine cultivable prokaryote and is characterised by about 40 species. Within these, the type strain of *P. carrageenovora* IAM 12662^T (= IFO 12985 = NCIMB 302 = ATCC 43555) is a Gram-negative, aerobic, flagellated bacterium isolated from seaweeds.[8]

As a Gram-negative bacterium, *P. carrageenovora* possesses lipopolysaccharides (LPS) in the external leaflet of its outer membrane. Lipopolysaccharides 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 outer membrane.[9] LPS not containing O-chain are termed Rough (R) LPS or lipooligosaccharide (LOS). LOSs may occur in both wild and laboratory strains possessing mutations in the genes encoding the O-specific polysaccharide biosynthesis or transfer.

We have already elucidated the structure of the core carbohydrate backbone from the LPS of *P. carrageenovora*[10] and here we describe the structural investigation of the O-specific polysaccharide obtained from the

* Corresponding author. Tel.: +39 081 674124; fax: +39 081 674393; e-mail: molinaro@unina.it
† Present address: Swinburne University of Technology, PO Box 218, Hawthorn, Vic 3122, Australia.

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lipopolysaccharide. Sugar analysis of the lipopolysaccharide by GLC of the alditol acetates derived after acid hydrolysis (2M TFA, 120 °C, 2 h) revealed the presence of Gal, GalN and GlcN and a very low amount of a 3,6-dideoxy-hexose. When milder hydrolysis conditions were applied (1 M TFA, 100 °C, 1 h), a 3,6-dideoxy-*xylo*-hexose was identified. Glucuronic acid was detected by GLC analysis of acetylated *O*-methyl glycosides. GLC analysis of the acetylated glycosides with (+)-2-butanol showed that Gal, GalN, GlcA and GlcN have the D configuration, whereas 3,6-di-deoxy-*xylo*-hexose has the L configuration and, thus is colitose (Col). Methylation analysis of the lipopolysaccharide resulted in identification of terminal Col, 2-substituted Col, 2-substituted Gal, 4-substituted GlcA, 3-substituted GalN and 3,4-disubstituted GlcN. Colitose was identified by comparison with an authentic sample obtained by *Escherichia coli* O55:B5 LPS.

The repeating unit of the O-chain polysaccharide was identified by 2D NMR spectroscopy after de-*O*-acylation of the lipopolysaccharide. The standard approach to cleave lipid A moiety, that is 1% acetic acid treatment (100 °C, 2 h) of lipopolysaccharide was not helpful, since the supernatant of the hydrolysis contained a complicated polymer, presumably due to the non-stoichiometric presence of colitose that is partially hydrolysed in these conditions. On the other hand, milder acid conditions (100 °C, 1.5 h) did not completely hydrolyse lipid A. Therefore, the lipopolysaccharide was de-*O*-acylated by anhydrous hydrazine in THF. The resulting *N*-acylated polymer had a good solubility in water and ^{1}H NMR spectrum (Fig. 1) showed anomeric signals in stoichiometric ratios.

The anomeric region of ^{1}H NMR spectrum contained signals for six spin systems, which were completely assigned with COSY, TOCSY, NOESY and HSQC. Anomeric configurations were assigned on the basis of the chemical shifts, of $^{3}J_{\text{H-1,H-2}}$ values which were determined from the DQF-COSY experiment and of $^{1}J_{\text{C-1,H-1}}$ values deriving from a coupled ^{1}H, ^{13}C-HSQC. All sugars were identified as pyranose rings based on ^{1}H and ^{13}C NMR chemical shifts and on HMBC spectrum that showed intra-residual scalar connectivity between H-1/C-1 and C-5/H-5 of residues.

Spin systems **A** and **B** were identified as two different units of colitose: the H-3 ring proton signals of both residues correlated to a diasterotopic methylene signal around 2 ppm, whereas H-5 of both residues correlated to a methyl signal at 1.16 ppm. The coupling constant values of the ring proton signals were in agreement with a *xylo* configuration, which implies H-4 equatorial and H-2 axial orientations of ring protons; anomeric $^{1}J_{\text{C-1,H-1}}$ $^{3}J_{\text{H-1,H-2}}$ values of 175 and 3.1 Hz clearly indicated α-anomeric configuration. Spin system **C** and **D** were identified as two *galacto* configured residues on the basis of coupling constant values of ring protons, in particular, $^{3}J_{\text{H-3,H-4}}$ (3 Hz). The H-5 of both spin systems was only detectable by NOESY experiment since in the TOCSY spectrum the low $^{3}J_{\text{H-4,H-5}}$ value, <1 Hz, impaired any magnetisation transfer over H-4.

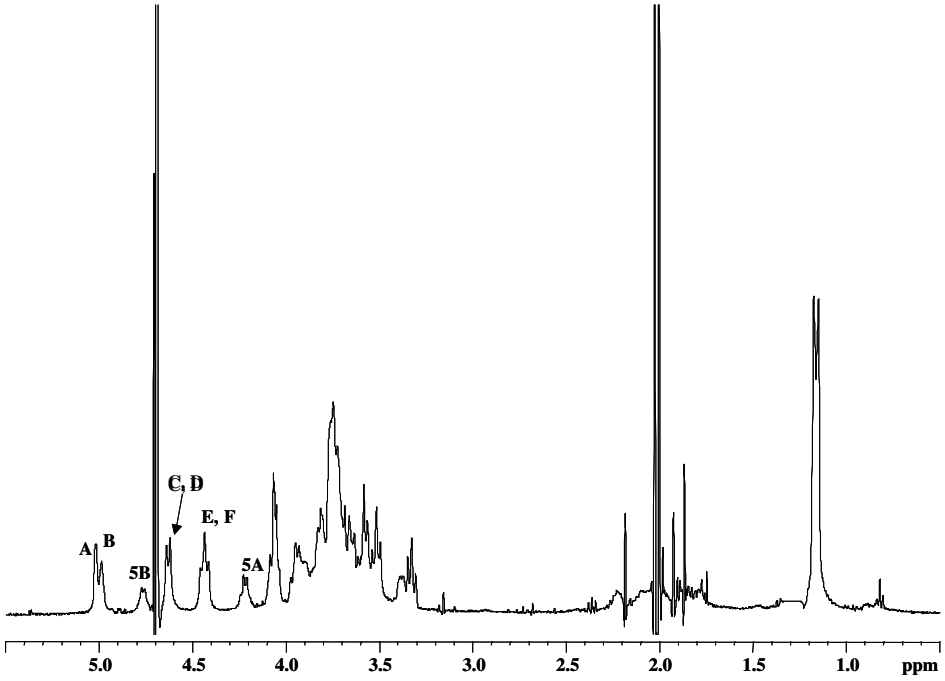

Figure 1. ^{1}H NMR of the de-O-acylated lypopolysaccharide. Indicated are anomeric resonances as well as H-5 resonances of resides **A** and **B**. The spectrum was recorded at pD 7 and 300 K.

Moreover, only H-2 of spin system **C** correlated to a nitrogen-bearing carbon in the HSQC spectrum and in the HMBC spectrum scalar correlations were found between H-2 of **C** and carboxyl group signals around 176 ppm and between this latter and methyl signals at 2.01 ppm. Anomeric $^{1}J_{C\text{-}1,H\text{-}1}$ $^{3}J_{H\text{-}1,H\text{-}2}$ values of 164 and 8.0 Hz indicated β-anomeric configuration that was further corroborated by *intra*-residual NOE connectivity between H-1 and H-3/H-5 signals found in the NOESY spectrum. Thus, spin system **C** was identified as GalNAc and spin system **D** was identified as Gal.

Spin systems **E** and **F** both possessed *gluco* configuration given the high value (10 Hz) of the ring proton coupling constants, and both residues were in β-anomeric configuration as shown by anomeric $^{1}J_{C\text{-}1,H\text{-}1}$ $^{3}J_{H\text{-}1,H\text{-}2}$ values, approximately 165 and 7.9 Hz, respectively, and by *intra*-residual NOE connectivity between H-1 and H-3/H-5 signals. Residue **E** was identified as GlcNAc since its H-2 signal correlated to a nitrogen bearing carbon in the HSQC spectrum and to a carboxyl group in the HMBC spectrum and, in addition, this latter signal also correlated to methyl signals at 2.00 ppm. Residue **F** was recognized as a glucuronic acid unit since either in the COSY or in the TOCSY spectra no traces of H-6 proton signals were found for this monosaccharide. In addition, in the HMBC spectrum a carboxy group signal correlated to both H-5 and H-4 proton signals of this residue.

C-4 of **E**, while **A** was terminal residue. The sequence of the monosaccharide residues was determined using NOE dipolar correlations from the NOESY spectrum and by HMBC scalar correlations. Colitose **A** was attached at C-2 of Gal residue **D**, as shown by interresidual NOE connectivity H-1 **A**/H-1 and H-2 **D** and by HMBC scalar correlations H-1, C-1 **A**/C-2, H-2 **D**. Galactose **D** was linked at C-3 of nodal glucosamine **E**, as revealed by NOE H-1 **D**/H-3 **E** and long range correlations H-1, C-1 **D**/C-3, H-3 **E**. This latter residue was substituted by colitose **B** at C-4, as shown by NOE effect between H-1 **B** and H-4 **E** and HMBC correlation between H-1, C-1 **B** and C-4, H-4 **E**. Residue **E**, in turn, was linked to C-4 of uronic acid **F**, given the NOE correlations between H-1 **E** and H-4 **F** and the HMBC correlations between H-1, C-1 **E**/C-4, H-4 **F**. Glucuronic acid was linked to C-3 of galactosamine as demonstrated by H-1 **F**/H-3 **C** dipolar correlations and H-1, C-1 **F**/C-3, H-3 **C** scalar correlations. This last residue was attached to C-2 of colitose as confirmed by NOE contact among H-1 and H-2 of **C** residue and H-1 and H-2 of **B** residue; in addition, this was proved by HMBC scalar correlation between H-1, C-1 **C**/C-2, H-2 **B.**

These NMR data perfectly matched the compositional and methylation analysis, thus it can be concluded that the O-chain polysaccharide of the lipopolysaccharide from the bacterium *P. carrageenovora* possesses the following structure:

→2)-α-Col*p*-(1→4)-β-Glc*p*NAc-(1→4)-β-Glc*p*A-(1→3)-β-Gal*p*NAc-(1→
α-Col*p*-(1→2)-β-Gal*p*-(1→3)┘

^{13}C NMR chemical shifts were assigned by HSQC experiment, using the assigned ^{1}H NMR spectrum. Six anomeric carbon resonances at δ 96.2–105.0 numerous carbon ring signals and two nitrogen-bearing carbon signals were identified (Table 1). By comparison with ^{13}C chemical shifts of unsubstituted residues,[11] several low-field shifted signals suggested glycosylation, namely, at C-2 of residue **B** and **D**, C-3 of **C**, C-4 of **F**, C-3 and

This structure is identical to the O-chain structure of the LPS of *Pseudoalteromonas tetraodonis*-type strain IAM 140160^{T}.[12] The finding of identical O-chain polysaccharides in *P. tetraodonis* IAM 14160^{T} and *P. carrageenovora* IAM 12662^{T} is unusual. Genotypically, both strains only share 26% of genetic identity and therefore, *P. tetraodonis* IAM 14160^{T} and *P. carrageenovora* IAM 12662^{T} are assigned to two different species.[13]

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the de-*O*-acylated polysaccharide from *P. carrageenovora.* Chemical shifts are relative to acetone (^{1}H, 2.225 ppm, ^{13}C, 31.45 ppm at 300 °K)

Sugar residue	1	2	3	4	5	6	C=O	CH_3
A	5.01	3.92	1.78/1.84	3.75	4.22	1.16		
t-α-Col	99.3	63.9	21.5	68.6	66.3	15.6		
B	4.99	4.07	2.02/2.10	3.83	4.767	1.16		
2-α-Col	96.2	73.7	22.2	69.0	66.8	15.6		
C	4.64	3.95	3.75	4.06	3.74	3.71		2.01
3-β-GalNAc	102.5	51.4	81.0	68.5	74.1	61.5	176.7	22.1
D	4.64	3.57	3.75	3.81	3.59	3.64		
2-β-Gal	101.2	76.8	68.9	69.0	76.0	61.7		
E	4.45	3.73	4.06	3.77	3.38	3.76		2.00
3,4-β-GlcNAc	101.6	55.6	75.8	72.5	75.4	61.7	176.7	22.1
F	4.43	3.32	3.52	3.68	3.58			
4-β-GlcA	105.0	72.7	73.9	80.0	76.9	173.1		

Currently, both *P. tetraodonis* IAM 14160^T and *P. carrageenovora* IAM 12662^T belong to monophyletic group of the so-called non-pigmented species within genus *Pseudoalteromonas* that comprise more than 15 species including the type strain of the genus, *P. haloplanktis*; *P. nigrifaciens* and *P. distincta* and that can produce melanin-like pigments depending on the culture medium.[14] Even though a number of studied species of the genus *Pseudoalteromonas* were to found possess a diverse and unique O-polysaccharide,[15] we believe that such phylogenetic closeness may explain the identity of O-polysaccharide structures among monophyletic species. However, a study of other analogous species from different taxa is required to confirm this observation.

1. Experimental

1.1. Bacteria and bacterial LPS

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), peptone (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. An aliquot of dried cells (2 g) was extracted by hot phenol/water as described.[16]

1.2. Isolation de-*O*-acylated lipopolysaccharide from *P. carrageenovora*

An aliquot of LPS (80 mg) was suspended in THF containing anhydrous hydrazine (3 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (20 mL), and allowed to precipitate. The precipitate was then centrifuged (3000*g*, 30 min), washed twice with ice-cold acetone, dried and then dissolved in water and lyophilised (50 mg).

1.3. General and analytical methods

Determination of neutral sugars, the determination of the absolute configuration, GLC and GLC–MS were all carried out as described elsewhere.[10] The methylation analysis was carried out by Hakomori method[17] and the sample was hydrolysed with 2 M trifluoroacetic acid (120 °C, 1 h), carbonyl-reduced with $NaBD_4$, acetylated and analysed by GLC–MS. In order to detect uronic acids, an aliquot of methylated, hydrolysed and reduced sample was carboxy-methylated with diazomethane, carboxyl-reduced with $NaBD_4$, acetylated and analysed by GLC–MS.

An authentic sample of colitose was obtained by *E. coli* O55:B5 LPS, purchased from Sigma–Aldrich.

1.4. NMR spectroscopy

For structural assignments of O-polysaccharide chain, 1D and 2D ^1H NMR spectra were recorded of a solution of 8 mg in 0.6 mL D_2O at pD 7 (uncorrected value). Experiments were carried out at 300 K using a Varian Inova 500 spectrometer. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. All the 2D NMR spectra were registered according to Varian software.

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