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Structural elucidation of the core-lipid A backbone from the lipopolysaccharide of *Acinetobacter radioresistens* S13, an organic solvent tolerant Gram-negative bacterium

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Abstract—The structure of the core oligosaccharide of the lipopolysaccharide from an organic solvent tolerant Gram-negative bacterium, *Acinetobacter radioresistens* S13, was investigated by chemical analysis, NMR spectroscopy and MALDI-TOF mass spectrometry. All the experiments were performed on the oligosaccharides obtained either by alkaline degradation or mild acid hydrolysis. The data showed the presence of two novel oligosaccharide molecules containing a trisaccharide of 3-deoxy-D-*manno*-octulopyranosonic acid in the inner core region and a glucose rich outer core whose structure is the following:

α-Glc-(1→6)-α-Glc-(1→6)-β-Glc-(1→4)-β-Glc-(1→3)-α-GlcNAcA-(1→4)-α-Kdo-(2→5)-α-Kdo-(2→6)-β-GlcN4P-(1→6)-α-GlcN1P
4 (R); 4 (α-GalN); 7 (β-GlcN); 4 (α-Kdo)

R = H in the main oligosaccharide and β-Glc in the minor product.

The bacterium was grown on aromatic (phenol and benzoic acid) and nonaromatic carbon sources and the core oligosaccharide resulted to occur always with this novel structure.

Keywords: *Acinetobacter radioresistens*; Carbohydrate; NMR spectroscopy; Lipopolysaccharide

1. Introduction

The finding of bacteria that are able to grow in pollutant enriched environments has led, in the last years, to a mounting interest in discovering ways of employing such microorganisms in the field of environmental biotechnology and in bioremediation strategies.[1,2] In particular, some Gram-negative bacteria belonging to the genera *Pseudomonas*[3–6] and *Acinetobacter*[7] have shown the ability to grow on media where the only carbon source is provided by aromatic compounds, conventionally classified as recalcitrant pollutants because of their resistance to degradation. In the present investigation, we have carried out the structure elucidation of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter radioresistens* S13, a solvent tolerant Gram-negative bacterium isolated from the soil surrounding an activated sludge pilot plant in Torino, Italy, that shows efficient catabolic activity towards phenol and benzoate.[8]

As for all Gram-negative bacteria, *A. radioresistens* possess an outer membrane whose external leaflet surface is mainly composed of lipopolysaccharides (LPSs). These are amphiphilic macromolecules composed of a

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hydrophilic hetero-polysaccharide (formed by core oligosaccharide and O-specific polysaccharide, often named 'O-chain') covalently linked to a lipophilic moiety named lipid A, which anchors these macromolecules to the outer membrane. LPSs not containing O-chain are termed rough (R) LPSs or lipooligosaccharides (LOSs); LOSs may occur in both wild and laboratory strains possessing mutations in the genes encoding for the O-specific polysaccharide biosynthesis or transfer. LPSs and LOSs are unique and vital molecules that are involved in the interaction between bacterial cells and external environment,[9] thus, in case of bacteria able to live in harsh environments, it is of high interest to look for particular structural features that can occur in these molecules. *A. radioresistens* S13 was found to produce a novel R-LPS with the same structural features independently from the culture growth conditions, that is, if exposed or not to aromatic compounds. This result differs from previous data obtained in our laboratories on other bacterial LPSs (*Pseudomonas stutzeri* OX1) where chemical modifications were found for such structures when the growth of the bacterium was carried in aromatic compounds.[10–12] Moreover, the structure shows common features with other R-LPSs isolated from other *Acinetobacter* strains, in particular, phosphorylation only occurs on the lipid A disaccharide backbone and this latter is connected to the glucose rich outer-core by a Kdo trisaccharide, lacking heptose residues.[13–16]

2. Results and discussion

The R-LPS fraction was extracted by phenol–chloroform–petroleum ether procedure[17] from the dried cells of *A. radioresistens* S13 grown supplying acetate, phenol or benzoate as the only carbon source and in all cases it was identified by SDS-PAGE, where, after silver staining, the typical migration to the bottom of the gel was visible. Monosaccharide analyses of the R-LPS extracted from *A. radioresistens* grown in phenol as carbon source revealed the presence of D-GlcN, D-GalN, D-Glc, Kdo and HexNA, this latter subsequently identified as D-GlcNA by NMR. Methylation analysis[18,19] of the core region revealed the presence of 6-substituted-GlcN, 6-substituted-Glc, 4-substituted-Glc, terminal-Glc, terminal-GlcN and terminal-GalN and, in minor amount, 4,6-disubstituted-Glc. Methylation analysis of the Kdo region showed the presence of 4,5-disubstituted-Kdo, 4,7-disubstituted-Kdo and terminal-Kdo.

NMR analysis was performed on both oligosaccharides obtained after alkaline treatment (compound **1**) and mild acid hydrolysis (compound **2**). Compound **1** was isolated by gel permeation chromatography after complete de-acylation of the LOS by anhydrous hydrazine and hot KOH. In the ^{1}H NMR spectrum of oligosaccharide **1** (Fig. 1) eight major anomeric protons could be identified belonging to eight distinct spin systems (**A**–**H**), beside the presence of a minor anomeric signal **N** at 4.505 ppm. In the high field region, between 1.741 and 2.791 ppm, signals were visible for three diastereotopic methylene groups of three distinct Kdo residues (**I**–**M**). For each spin system, the full assignment of the ^{1}H and ^{13}C resonances was attained from DQF-COSY, TOCSY, ^{1}H,^{13}C-HSQC (Fig. 2) and ^{1}H,^{13}C-HMBC spectra (Table 1). All the monosaccharides were present as pyranose rings, according to the methylation data and to the HMBC spectrum that showed for all residues intraresidual scalar connectivity between H-1/C-1 and C-5/H-5 atoms (for Kdo units, between C-2 and H-6). Anomeric and relative configurations for residues

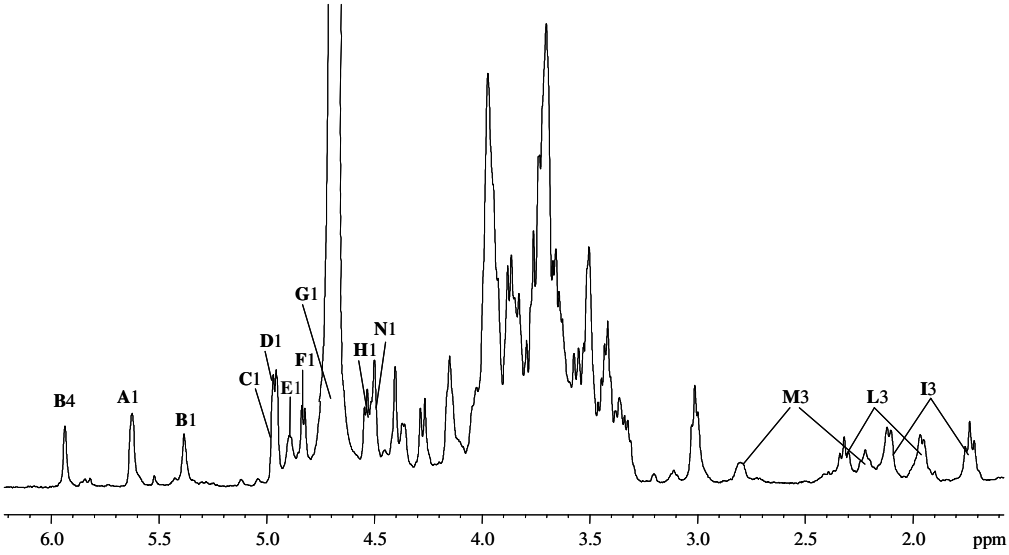


Figure 1. ^{1}H NMR of oligosaccharide **1** obtained after alkaline degradation of the LPS. The spectrum was recorded at 300 K. Anomeric signals and Kdo methylene groups are designed with capital letters.

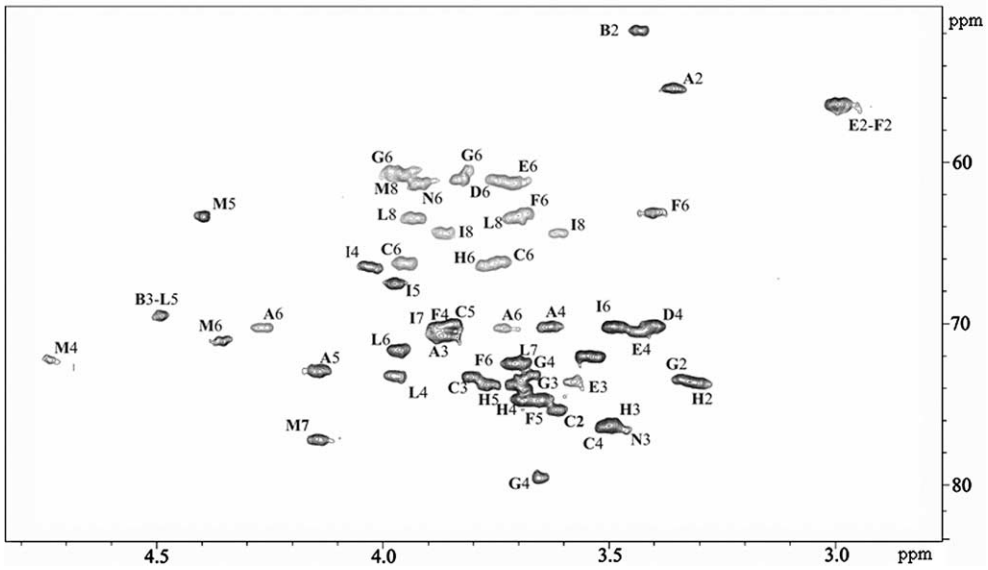


Figure 2. Zoom of the $^{1}H,^{13}C$-HSQC spectrum of oligosaccharide **1**. All the heteronuclear correlation are assigned. The experiment was recorded with carbon multiplicity editing, grey signals refer to methylene groups.

Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of oligosaccharide **1**

	1 (3_{ax-eq})	2 (4)	3 (5)	4 (6)	5 (7)	6 (8)
6-α-GlcN	5.632	3.364	3.871	3.643	4.140	4.272/3.736
A	90.8	54.9	70.3	69.9	72.5	69.8
ΔHexNA	5.371	3.428	4.490	5.932		
B	91.3	50.9	69.0	104.7	n.d.	n.d.
6-α-Glc	4.960	3.566	3.761	3.490	3.871	3.741/3.959
C	98.0	71.5	73.4	71.9	70.3	66.2
t-α-Glc	4.942	3.531	3.708	3.403	3.693	3.824
D	98.1	71.6	73.7	70.0	72.4	60.6
t-β-GlcN	4.880	3.000	3.586	3.441	3.512	3.736
E	99.3	55.9	73.2	70.1	75.9	60.6
6-β-GlcN	4.821	3.008	3.815	3.960	3.692	3.401/3.691
F	100.3	55.9	72.8	71.2	73.9	62.7
4-β-Glc	4.712	3.350	3.647	3.650	3.695	3.980/3.827
G	102.0	73.0	74.3	79.1	74.1	60.3
6-β-Glc	4.532	3.320	3.504	3.691	3.753	3.952/3.782
H	103.3	73.1	75.9	74.6	73.6	66.4
t-β-Glc	4.505	3.281	3.460	3418	3.462	3.891
N	103.3	73.6	76.5	70.3	76.6	61.1
t-Kdo	1.741/2.100	4.032	3.975	3.496	3.869	3.615/3.870
I	35.0	66.1	67.1	70.1	70.2	64.3
4,5-Kdo	2.312/1.960	3.980	4.496	3.970	3.701	3.941/3.714
L	35.0	73.3	69.0	71.6	72.4	63.4
4,7-Kdo	2.226/2.791	4.730	4.402	4.351	4.147	3.968
M	n.d.	72.2	63.4	71.2	77.0	60.3

Values are referred to internal standard acetone at 300 K (^{1}H 2.225, ^{13}C 31.45 ppm). Numbers in brackets refer to Kdo residues, n.d. is not determined.

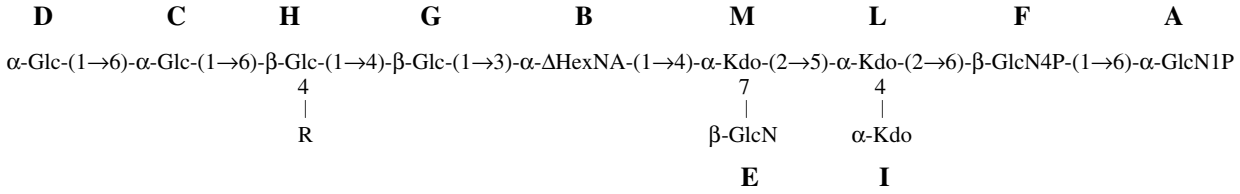
A–**H** and **N** were established on the basis of the chemical shift values and of the $^{3}J_{H,H}$ coupling constants obtained by DQF-COSY. These data allowed to assign α-*gluco*-configuration to residues **A**–**D** and β-*gluco*-configuration to residues **E**–**H** and **N**.

In the low-field region of the proton spectrum, the signal at 5.932 ppm was assigned to H-4 signal of **B** spin system that was identified as 2-amino-2,4-dideoxy-α-hex-4-enopyranosyluronic acid (ΔHexNA), in fact in the TOCSY spectrum correlations within **B** spin system ended at H-4 at low fields (5.932 ppm), that was at its turn correlated, in the $^{1}H,^{13}C$-HSQC spectrum, to an olefin carbon signal at 104.7 ppm. This residue was clearly derived from a HexNA residue that lost the

substituent at O-4 by a β-elimination, due to the alkaline treatment of **1**. In addition, in the HSQC spectrum, four nitrogen-bearing carbon signals were visible and they were identified as the C-2/H-2 correlation of residues **A**, **E** and **F** (GlcNs) and of residue **B** (ΔHexNA). Typical downfield displacement for the carbon resonances indicated glycosylation at O-6 of residues **A**, **C**, **F** and **H**, O-4 of residues **G**, **M** and **L**, O-5 of residue **L**, and O-7 of the residue **M**, whereas **D**, **E** and **I** were terminal residues (Fig. 2). One minor signal was visible in correspondence of an alternative C-4 signal of **H** (**H′**), at 79.1 ppm, suggesting nonstoichiometric substitution at O-4. The sequence of the monosaccharides within the oligosaccharide chain was deduced from the 2D-ROESY spectrum, where diagnostic cross-peaks were present for each anomeric signals with the protons on the glycosylation site of the next residue (Fig. 3). The following NOE contacts were revealed in the spectrum: H-1 **F**/H-6 **A**; H-1 **E**/H-7 **M**; H-1 **B**/H-4 **M**; H-1 **G**/H-3 **B**; H-1 **H**/H-4 **G**; H-1 **C**/H-6 **H**; H-1 **D**/H-6 **C**. The proximity of the residues was further confirmed by the scalar long range correlations of the $^{1}H,^{13}C$-HMBC spectrum, where the cross peaks H-1 **G**/C-3 **B**; H-1 **E**/C-7 **M**; H-1 **B**/C-4 **M**; H-1 **H**/C-4 **G** and C-1 **H**/H-4 **G**; H-1 **C**/C-6 **H** and H-1 **D**/C-6 **C** were visible. The nonstoichiometric β-Glc residue (**N**) was linked, when present, to O-4 of residue **H′**, as shown by NOE contact H-1 **N**/H-4 **H′**, and as expected on the basis of methylation analysis, which indicated the presence of a 4,6-disubstituted-Glc.

The sequence of Kdo trisaccharide was inferred from the dipolar couplings detected in the ROESY experiment. The presence of a cross-peak in the ROESY spectrum, in correspondence of H-3_{eq} **I** and H-6 **L** resonances, is only possible in case of 2–4 glycosidic linkage between these two residues both possessing α-configuration.[20] The 2–5 linkage between Kdo **L** and **M** was proven by the NOE contacts of H-5 **L** with H-5 **M**, H-3_{ax} **M**, H-3_{eq} **M** and H-4 **M**. By comparison with the oligosaccharide from *A. baumannii* ATCC 19606,[13] where a similar inner core skeleton is present, α-configuration was attributed to residue **M**. In fact, also in this case, the 4,5-disubstituted Kdo, residue **L**, showed an inversion in the usual chemical shift values for H-3_{ax} and H-3_{eq} (2.312 and 1.960 ppm, respectively) and unusual low-field chemical shift values for Kdo **M**, substituted by ΔHexNA residue. Only two ^{31}P NMR signals were present (3.40 and 2.87 ppm), that were plainly assigned to O-1 and O-4′ of the lipid A disaccharide backbone by $^{1}H,^{31}P$-HSQC.

All of these information suggested the following structure:



where R = H or β-Glc (**N**).

This structure was further confirmed by the MALDI-MS spectrum (Fig. 4). The main ion at *m*/*z* 2125.7 (Kdo_3-Glc_4-$GlcN_3$-ΔHexNA-P_2) was in full agreement with the molecular mass of the major oligosaccharide structure and the ion at *m*/*z* 2287.7 (Δ*m*/*z* 162) bears an additional Glc residue. Ions of shorter oligosaccharide chains, differing from the full oligosaccharide by one to four Glc units were visible. Furthermore, for each of these oligosaccharides there was also present a related ion peak lacking *m*/*z* 220, that is, without one Kdo residue. These peaks are diagnostic of partially assembled oligosaccharides where the terminal Kdo residue (**I**) was missing.

The full structure of the core region of the R-LPS was achieved by performing NMR and MS analyses on the oligosaccharide **2**, obtained by 1% acetic acid hydrolysis of the acid labile glycoside bond of Kdo and following purification by GPC. The ^{1}H NMR spectrum (Fig. 5) of this product showed the presence of a heterogeneous mixture, likely due to the different arrangements of the Kdo as reducing end of the oligosaccharide. Nevertheless, it was possible to recognise the same spin systems of the core moiety already found in oligosaccharide **1** with exception of residue **B** that in this case was identified, on the basis of the $^{3}J_{H,H}$ values from the DQF-COSY spectrum, as 3,4-disubstituted 2-deoxy-2-amino-glucuronic acid (GlcNA) and of an additional anomeric signal (**O**) at 5.702 ppm. The full attribution of the ^{1}H and ^{13}C resonances of oligosaccharide **2** was carried out on the basis of the DQF-COSY, TOCSY, ROESY and $^{1}H,^{13}C$-HSQC spectra (Table 2). For spin system **O**, the ^{13}C chemical shift of C-2 (51.2 ppm), the small $^{3}J_{1,2}$, $^{3}J_{3,4}$ and $^{3}J_{4,5}$ values, the chemical shift values of anomeric proton and carbon signals led to the identification of α-GalN. In agreement, H-5 of **O** spin system was only detectable by ROESY experiment since in the TOCSY spectrum the very low $^{3}J_{4,5}$ impaired any magnetisation transfer over H-4. Furthermore, the *inter* residual NOE of H-1 **O** with H-4 **B**, indicated the attachment of residue **O** to O-4 of the GlcNA.

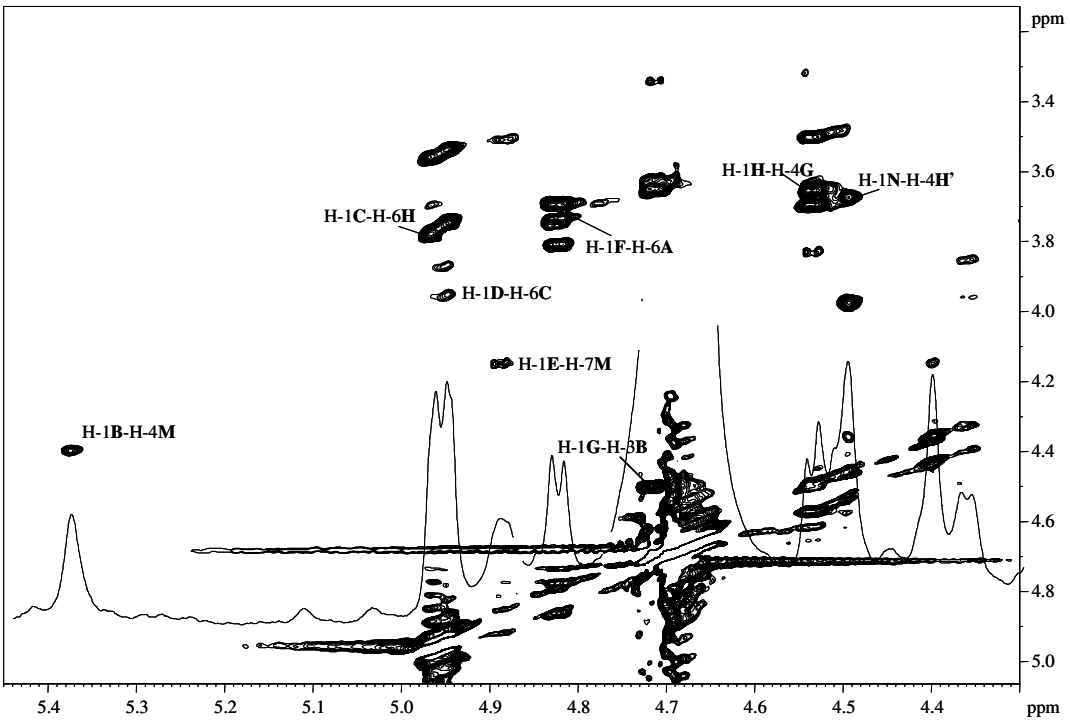


Figure 3. Zoom of the anomeric region of negative levels of the ROESY spectrum on oligosaccharide **1** obtained after alkaline degradation on the LPS from *A. radioresistens* S13. ^{1}H NMR is overlapped. Interresidual correlations diagnostic of glycosylation are indicated.

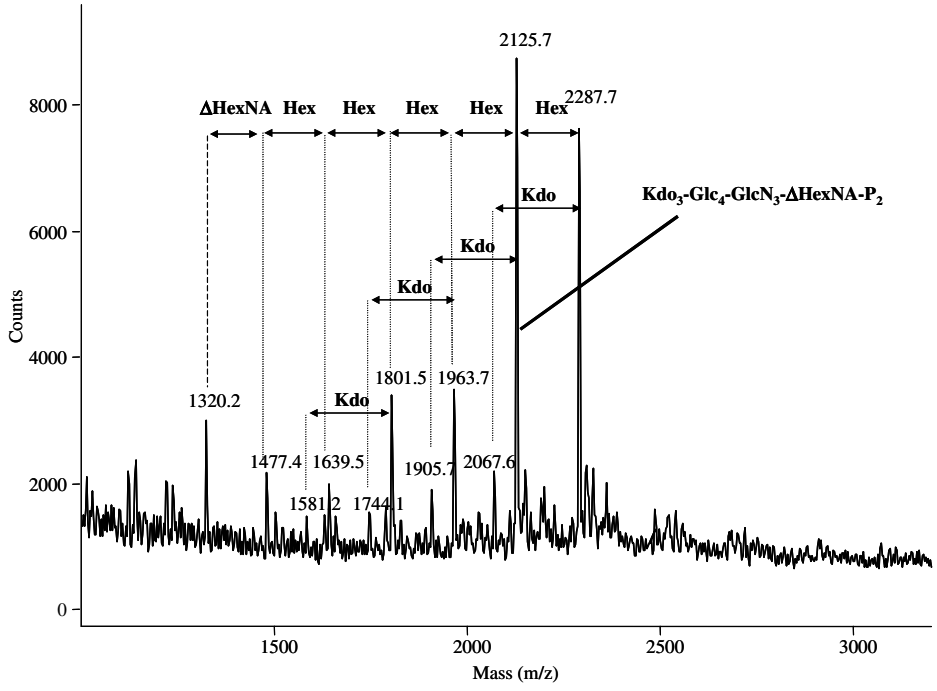


Figure 4. Negative ion MALDI-TOF spectrum on oligosaccharide **1**. Molecular ions are visible at *m*/*z* 2125.7 and 2287.7. Molecular ions for partially assembled structures are also visible.

Moreover, at high fields of proton NMR spectrum an acetyl signal was present. Acetylation shifts downfield the NMR signal of the geminal proton, and the only ^{1}H NMR signal which showed downfield displacement due to acetylation was observed for H-2 **B**, whereas all other proton resonances of the oligosaccharide did not show any significant shift attributable to acetylation.

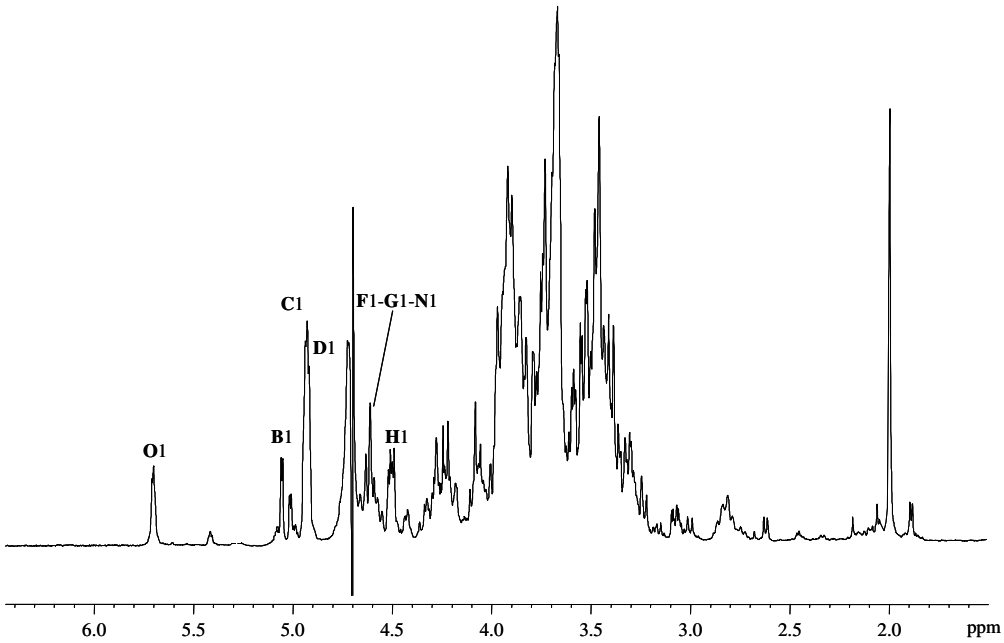
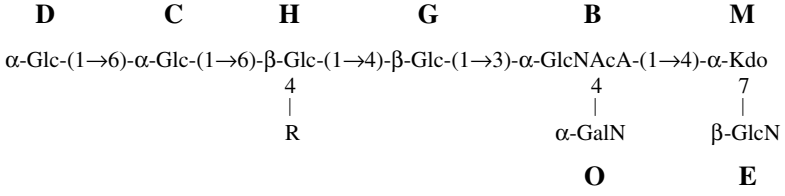


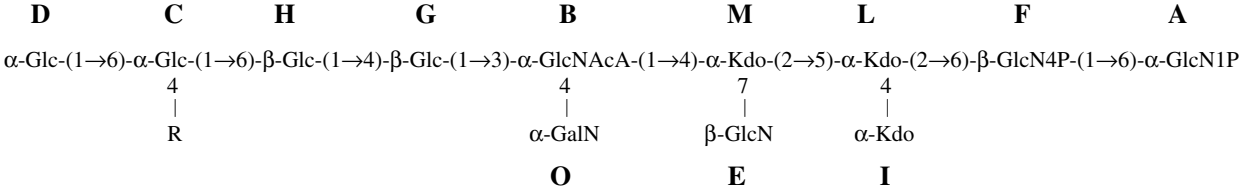
Figure 5. ^{1}H NMR of oligosaccharide **2** obtained after mild acid hydrolysis of the LPS. The spectrum was recorded at 300 K. Anomeric signals are designed with capital letters. Additional minor signals occur because of the heterogeneity due to the presence of Kdo at the reducing end of the oligosaccharide.

Thus, the structure of oligosaccharide **2** can be depicted as follows:



where R = H or β-Glc (**N**).

All the above information taken together allowed to identify the complete structure of the fully de-acylated core oligosaccharide from *A. radioresistens* S13, that can be represented as follows:



where R = H or β-Glc (**N**).

The identical oligosaccharide structure was isolated and characterised from the R-LPSs extracted from cells of *A. radioresistens* S13 grown supplying acetate and benzoate as only carbon sources in culture media.

To date, four core oligosaccharides from *Acinetobacter* LPS have been characterised,[13–16] in particular from *A. haemolyticus* NCTC 10305,[13,16] *A. baumannii* NCTC 10303[15] and *A. baumannii* ATCC 19606:[14] in all circumstances the oligosaccharide backbone was found to possess particular chemical features that noticeably differentiate it from other LPSs. In all cases and even in this one, it is a core region lacking heptose residues with a peculiar Kdo oligosaccharide arrangement. In *A. baumannii* ATCC 19606 LPS and in the present study, the Kdo residue linking the core region to lipid A is at its turn linked by two Kdo residues, one of which is present in the main chain and not in the side chain,

Table 2. ^{1}H and ^{13}C chemical shifts (ppm) of oligosaccharide **2**

	1	2	3	4	5	6
3,4-α-GlcNAcA	5.012	4.229	4.301	3.949	4.063	
B	95.2	53.6	77.6	74.4	73.7	n.d.
6-α-Glc	4.944	3.525	3.743	3.458	3.838	3.768/3.741
C	98.3	72.0	73.4	71.9	70.9	66.4
t-α-Glc	4.921	3.508	3.667	3.385	3.842	3.799
D	98.0	72.0	73.7	70.3	70.5	61.0
t-β-GlcN	4.662	2.814	3.461	3.453	3.472	3.800/3.630
E	100.0	56.5	74.1	70.0	77.2	61.3
4-β-Glc	4.624	3.317	3.578	3.686	3.512	3.896
G	103.3	73.3	74.8	79.4	72.0	61.2
6-β-Glc	4.511	3.305	3.471	3.476	3.465	3.931/3.717
H	103.2	73.7	76.2	74.1	77.6	66.4
t-β-Glc	4.604	3.339	3.519	3.671	3.521	3.874
N	103.2	73.1	75.5	73.1	75.0	60.8
t-α-GalN	5.702	3.452	3.938	3.782	3.999	3.645/3.758
O	95.7	51.2	71.1	68.3	69.1	61.1

Values are referred to internal standard acetone at 300 K (^{1}H 2.225, ^{13}C 31.45 ppm). Additional signals for *N*-acetyl group at 1.997/22.46 and 175.02 ppm.

that is, in the linear oligosaccharide chain two Kdo residues are present. The novel structure of the carbohydrate backbone of the LPS from *A. radioresistens* S13 closely resembles the one from *A. baumannii* ATCC 19606 LPS but it differs in the glucose oligosaccharide present in the outer core.

The present results differ from previous data obtained in our laboratories on *P. stutzeri* OX1: in that bacterial model, in fact, we found not only a constitutively modified LPS, but also chemical modifications of such structure caused by the growth in aromatic medium.[10–12] This behaviour has not been established for *A. radioresistens* S13 since in the two stimulated conditions (phenol, benzoate) the bacterium biosynthesises the same LPS structure as in the control culture (acetate). Nevertheless, in a previous report concerning proteomic studies[21] we found in the membrane proteome of *A. radioresistens* S13, a protein involved in the LPS core biosynthesis which was over-expressed (together with other glycosyl transferases) in the presence of both phenol and benzoate. Further investigations currently planned in our laboratories, will shed light on this aspect.

3. Experimental

3.1. Cells growth and isolation of LPS

The *A. radioresistens* S13 strain used was isolated and microbiologically characterised as previously described.[8,22] Cells were cultured in the Sokol and Howell minimal medium[23] supplemented with phenol (400 mg/L), benzoate (400 mg/L) or acetate (4000 mg/L) as sole carbon source. Cultures were incubated in Erlenmeyer flasks at 30 °C in a Gallenkamp oscillating shaker (210 rpm). Biomass was harvested by centrifugation (3000*g*, 15′, 4 °C) at the end of the exponential growth phase and kept at −24 °C until LPS extraction. Isolation of R-LPS was performed from the dried cells (3.520 g) with the conventional aqueous 90% phenol/chloroform/petroleum ether (50 mL, 2:5:8 v/v/v) extraction.[17] After removal of the organic solvents under vacuum, the R-LPS fraction was precipitated from phenol with water, washed first with aqueous 80% phenol, and then three times with cold acetone, each time centrifuged, and lyophilised (40 mg, 1.1% of the dry mass). The occurrence of LPS in this fraction was attained by Silver-stained SDS-PAGE performed as described.[24]

3.2. General and analytical methods

Determination of Kdo, neutral sugars, organic bound phosphate, absolute configuration of the hexoses, GLC and GLC–MS were all carried out as described.[10–15] For methylation analysis of Kdo region, LPS was carboxy-methylated with methanol/HCl (0.1 M, 5 min) and then with diazomethane to improve its solubility in DMSO. Methylation was carried out as described.[18,19]

3.3. Isolation of oligosaccharides

An aliquot of the isolated R-LPS (17 mg) was dissolved in anhydrous hydrazine solution in THF (1 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (10 mL), and allowed to precipitate.[25] The precipitate was then centrifuged (3000*g*, 30 min), washed twice with ice-cold acetone and dried. This material was successively de-N-acylated with 4 M KOH. Salts were removed using a Sephadex G-10 (Pharmacia) column (50 × 1.5 cm). The fraction eluted in the void volume constituted the partially degraded oligosaccharide comprehensive of the lipid A disaccharide backbone.

A second aliquot (18 mg) of R-LPS was hydrolysed with 1% acetic acid heating at 100 °C for 2 h. after centrifugation (8000*g*, 60 min), the precipitate composed by Lipid A was washed twice with water and then removed. The supernatants were collected and lyophilised, obtaining 12 mg of de-lipidated oligosaccharide, further purified by GPC on a TSK HW-40 in 50 mM NH_4HCO_3.

3.4. NMR spectroscopy

1D and 2D ^{1}H NMR spectra were recorded on a solution of 6 mg in 0.6 mL of D_2O at 30 °C. ^{1}H and ^{13}C NMR experiments were carried out using a Bruker DRX-600 equipped with a cryogenic probe whereas for ^{31}P NMR spectra, a Bruker DRX-400 spectrometer was used. Spectra were calibrated with internal acetone δ_H 2.225, δ_C 31.45. Aqueous 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 ($t1 \cdot t2$) of 4096·1024 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 100 ms, using data sets ($t1 \cdot t2$) 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).[26,27] Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048·512 points, and 64 scans were acquired for each $t1$ value. Experiments were carried out in the phase-sensitive mode according to the method of States et al.[28] A 60 ms delay was used for the evolution of long-range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048·1024 points using forward linear prediction extrapolation.[29]

3.5. Mass spectrometry

MALDI mass spectra were carried out in the negative polarity in linear or in reflector mode on a Voyager STR instrument (Applied Biosystems, Framingham, MA, USA) equipped with a nitrogen laser ($\lambda = 337$ nm) and provided with delayed extraction technology. Ions formed by the pulsed laser beam were accelerated through 24 kV. Each spectrum is the result of approximately 200 laser shots. Saturated solution of 2,4,6-trihydroxyacetophenone (THAP) was used as the matrix.

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