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Note

The O-specific polysaccharide structure from the lipopolysaccharide of the Gram-negative bacterium *Raoultella terrigena*

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Abstract—The structure of the repeating unit of the O-specific polysaccharide from the lipopolysaccharide of the enterobacterium *Raoultella terrigena* was determined by means of chemical and spectroscopical methods and was found to be a linear tetrasaccharide containing a cyclic acetal of pyruvic acid (Pyr) as depicted below.

→2)-β-D-Man*p*-(1→3)-α-D-Man*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→
4,6-(*S*)-Pyr┘

Keywords: *Raoultella terrigena*; Lipopolysaccharide; O-Specific polysaccharide; Pyruvic acid; NMR

The genus *Raoultella* belongs to the family of the *Enterobacteriaceae*. This new genus has been recently introduced after a reclassification of the genus *Klebsiella*, during a phylogenetic study based on the 16S rDNA and *rpoB* gene sequence, and is comprehensive of the three described species: *Raoultella planticola*, *Raoultella ornithinolytica* and *Raoultella terrigena*.[1] Bacteria belonging to this genus are all Gram-negative, oxidase-negative, aerobic, non-motile, capsulated rods and are able to grow at 10 °C.[1] As all Gram-negative bacteria, they produce typical glycolipids termed lipopolysaccharides (LPSs) as the major components of the outer leaflet of their outer membrane. These molecules typically comprehend within their structure a glycolipid portion, the lipid A, that allows the molecule to embed into the membrane, an oligosaccharide region, the core and eventually, in their so-called "smooth form" (S-LPS), a polysaccharide termed O-side chain or O-specific polysaccharide (OPS), that represents the antigenic determinant of the molecule and is recognised by the acquired immune system.[2] In particular, *R. terrigena* expresses a S-LPS, and the structure elucidation of the polysaccharide moiety of this molecule represents the subject of the present paper.

Cells of *R. terrigena* were extracted using the hot phenol–water procedure.[3] The LPS was detected by silver stained SDS-PAGE in the water phase and further purified by enzymatic digestion with DNase, RNase and proteinase K. In order to define the polysaccharide structure, two aliquots of the sample were hydrolysed using either mild acidic (**PS1**) or alkaline (**PS2**) conditions, and in both cases we obtained a polysaccharide fraction that was further purified by gel permeation chromatography on a Sephacryl S-300 column. Compo-

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sitional analysis, carried out on **PS1** by GC–MS of the acetylated *O*-methyl, *O*-oct-2-yl glycoside and of the alditol acetate derivatives, yielded four different monosaccharides, D-mannose (Man), L-rhamnose (Rha), 2-amino-2-deoxy-D-glucose (GlcN) and 2-amino-2-deoxy-D-mannose (ManN). Moreover, the *O*-methyl glycoside analysis performed on **PS2** showed a very low amount of Man and the appearance of a further peak at higher retention time of GC–MS run attributed to a hexose residue carrying a cyclic acetal of the pyruvic methyl ester. Methylation analysis on **PS1** showed the presence of the derivatives of 3-substituted Rha*p*, 3-substituted Man*p*N, 4-substituted Glc*p*N and 2-substituted Man, replaced by 2,4,6-tri-substituted Man*p* in **PS2**. The pyranose form of all residues was definitely established by ^{13}C NMR data that lacked signal over 80 ppm diagnostic of furanose forms.

Both samples were subjected to extensive NMR analysis in order to combine the slightly different chemical data and to establish the complete structure of the polysaccharide.

In the anomeric region of the ^{1}H NMR spectrum of **PS1** (Fig. 1a), between 5.2 and 4.6 ppm, four signals (**A–D**) were present. A fifth signal was present at 4.55 ppm, identified as H-2 of spin system **B**. Chemical shifts of each spin system were assigned utilizing DQF-COSY, TOCSY, ROESY, ^{1}H, ^{13}C–HSQC and ^{1}H, ^{13}C–HMBC experiments (Table 1). The anomeric configuration of each monosaccharide unit was assigned on the basis of the $^{3}J_{H\text{-}1,H\text{-}2}$, $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants and the *intra*-residual NOE contacts observable in the ROESY spectrum, whereas the values of the vicinal $^{3}J_{H,H}$ coupling constants allowed the identification of each residue.

In particular, spin system **A** was identified as α-GlcNAc. Its *gluco*-configuration was inferred on the basis of coupling constant values of ring protons (all around 10 Hz), in fact, in the TOCSY spectrum, correlations were visible from H-1 to H-6s. The anomeric chemical shift and $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constant value (3.2 Hz) of residue **A** clearly indicated α-orientation. H-2 **A** correlated to a nitrogen bearing carbon that resonated at 54.1 ppm. In the ^{1}H, ^{13}C–HMBC spectrum scalar correlations were found between H-2 of **A** and carboxyl group signals around 175.0 ppm and between these latter and methyl signals at about 1.97 ppm, thus indicating that the residue is N-acetylated.

Spin system **B** was identified as α-ManNAc. In fact, the small $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constant value (about 1.8 Hz), the diagnostic H-5/C-5 chemical shift values and the characteristic shifted H-2 signal suggested α-*manno* configuration.[4] In the TOCSY spectrum, start-

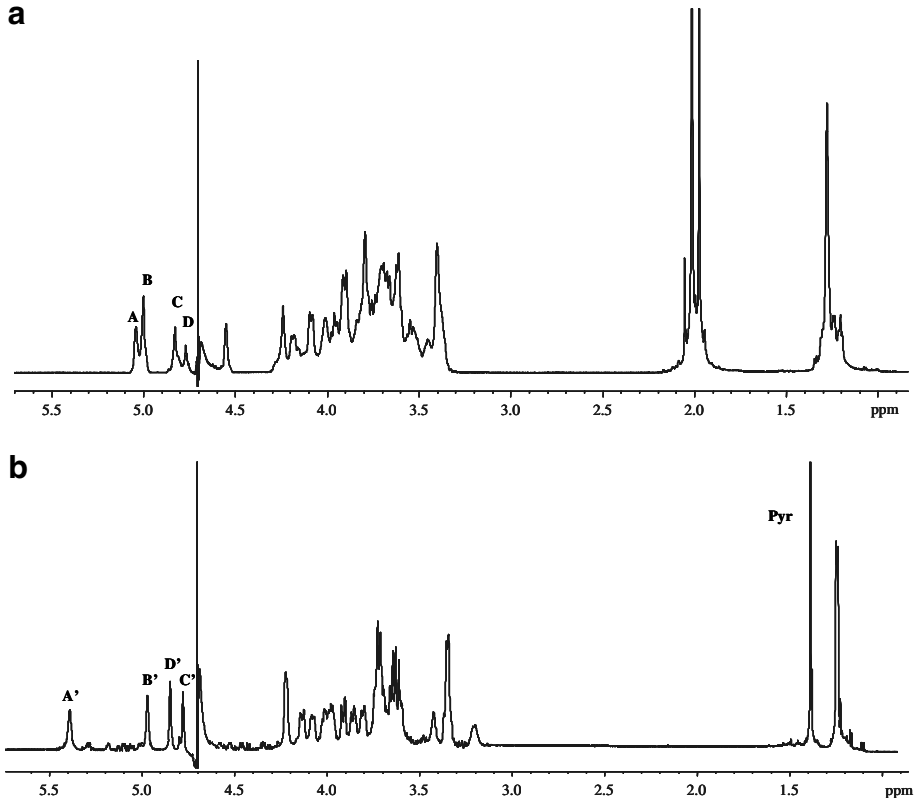


Figure 1. ^{1}H NMR of **PS1** (a) and **PS2** (b), obtained, respectively, after acid and alkaline hydrolysis of the LPS from *R. terrigena*. Capital letters refer to the identified spin systems described in Table 1.

Table 1. ^{1}H and ^{13}C (*italic*) chemical shifts (ppm) of the **PS1** and **PS2** obtained after either acidic or alkaline degradation of the LPS from *R. terrigena*

	1	2	3	4	5	6
PS1						
4-α-D-GlcNAc	5.048	3.778	3.960	3.691	4.084	3.739
A	*99.3*	*55.3*	*71.5*	*78.4*	*71.2*	*61.3*
3-α-D-ManNAc	5.004	4.556	4.187	3.548	3.901	3.706/3.901
B	*96.4*	*51.4*	*76.1*	*65.8*	*73.0*	*62.1*
3-β-L-Rha	4.836	4.242	3.615	3.405	3.405	1.285
C	*102.3*	*67.8*	*77.4*	*71.5*	*72.9*	*17.8*
2-β-D-Man	4.775	3.791	3.662	3.612	3.436	3.806
D	*97.5*	*78.7*	*74.5*	*68.2*	*77.3*	*61.6*
Acetyl[a]	—	1.97/2.00				
	175.7	*23.0*				
PS2						
4-α-D-GlcN	5.392	3.205	4.015	3.645	4.134	3.851/3.724
A′	*98.1*	*55.1*	*70.8*	*77.6*	*71.9*	*61.3*
3-α-D-ManN	4.973	3.420	4.076	3.626	3.814	3.707
B′	*96.9*	*51.5*	*77.6*	*65.2*	*73.7*	*61.3*
3-β-L-Rha	4.780	4.225	3.604	3.347	3.338	1.239
C′	*101.7*	*67.6*	*77.2*	*71.4*	*73.5*	*17.9*
2-β-D-Man	4.850	4.213	3.912	3.703	3.345	3.976
D′	*98.0*	*78.0*	*72.0*	*67.9*	*74.6*	*64.7*
(*S*)-Pyr	—	—	1.372			
	176.8	*101.9*	*24.9*			

Values are referred to internal standard acetone measured at 300 K (^{1}H 2.225, ^{13}C 31.45 ppm).

[a] The H-2 acetyl chemical shifts are relative to GlcNAc and ManNAc acetyl group, respectively, whereas carbon chemical shifts are not distinguishable.

ing from H-2 proton signals, all correlations with the ring proton were visible. In the HSQC spectrum, H-2 **B** (4.556 ppm) correlated to a nitrogen-bearing carbon signal (49.9 ppm) whereas in the HMBC spectrum both H-2 **B** and a methyl signal at 2.0 ppm correlated to a carboxyl group signal at 175.0 ppm, thus indicating that the residue is N-acetylated. **C** residue possessed β-anomeric configuration, given the $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constant value of 164 Hz and the fact that in the ROESY spectrum it was possible to find NOE contacts among H-1, H-3 and H-5. It was eventually identified as β-Rha since it possessed low $^{3}J_{H\text{-}1,H\text{-}2}$ and $^{3}J_{H\text{-}2,H\text{-}3}$ (about 1.1 and 3.2 Hz, respectively) and from H-2 signal in the TOCSY spectrum it was possible to detect H-6 methyl resonance. Likewise, the **D** residue was identified as β-Man, in fact, even for **D** it was possible to measure the $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constant value (165 Hz) and detect all the diagnostic intraresidual NOE contacts. Downfield displacement of the ^{13}C resonances due to glycosylation was observed in the ^{1}H, ^{13}C–HSQC spectrum for C-4**A**, C-3**B**, C-3**C** and C-2**D**, in full accordance with methylation analysis.

The correct sequence of the monosaccharides in the repeating unit of the polysaccharide **PS1** was inferred by the detection of the *inter*-residual scalar and dipolar correlations detected either in the ^{1}H, ^{13}C–HMBC or in the ROESY spectrum, respectively. In particular, the following diagnostic cross peaks were detected in the ROESY spectrum: H-1**A**/H-2**D**, H-1**B**/H-3**C**, H-1**C**/H-4**A** and H-1**D**/H-3**B** whereas the following scalar coupling correlations were found in the HMBC spectrum: H-1**A**/C-2**D**, C-1**A**/H-2**D**, H-1**B**/C-3**C**, C-1**B**/H-3**C**, H-1**C**/C-4**A**, C-1**C**/H-4**A**, H-1**D**/C-3**B** and C-1**D**/H-3**B**. All of these data pointed out to the following structure:

D **B** **C** **A**

→2)-β-D-Man*p*-(1→3)-α-D-Man*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→

This structure was fully confirmed by the NMR analysis of **PS2**. In the ^{1}H NMR of this sample (Fig. 1B), four spin systems (**A′**-**D′**) were identified, corresponding to spin systems **A**–**D** in **PS1**. Even in this case, the execution of a full series of 2D NMR experiments allowed the complete assignment of ^{1}H and ^{13}C resonances (Table 1). Residues **A′** and **B′** were identified as α-GlcN and α-ManN, both bearing a free amino group at C-2 since the acetyl group was removed by the alkaline treat-

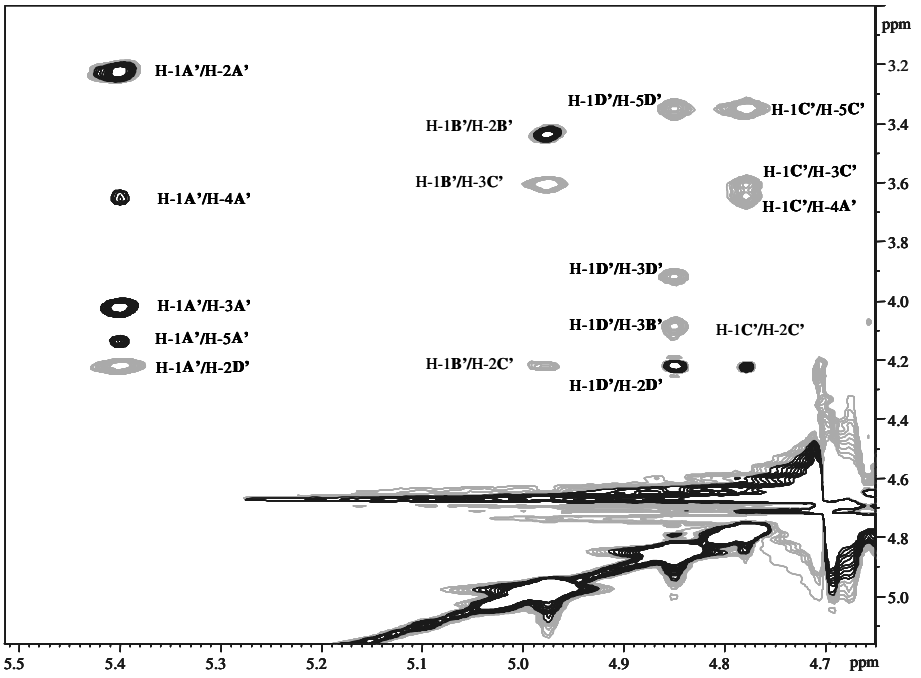


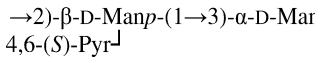
Figure 2. Anomeric region of the TOCSY (black) and ROESY (grey) spectra of **PS2**. Cross peaks are labelled according to the spin systems described in Table 1.

ment performed. Residues **C′** and **D′** were identified as the β-Rha and β-Man residues, respectively. Moreover, the ^{1}H NMR spectrum of **PS2** showed, at 1.372 ppm, a further signal for a singlet methyl group. This signal, in turn, presented, in the ^{1}H, ^{13}C–HMBC spectrum, long range scalar correlations with a carbon resonance in the anomeric region, at 101.6 ppm, and with a carboxyl group at 176.0 ppm, and was therefore identified as the methyl group of the expected pyruvic acid acetal already shown by methylation analysis and lost in the acidic treatment leading to the obtainment of **PS1**. On the basis of the methylation results and of the observation of the dipolar correlation in the ROESY spectrum between this methyl group and H-6 of the Man residue, it was possible to locate this moiety at O-4 and O-6 of this monosaccharide. Moreover, the typical proton and carbon chemical shifts of the pyruvic acid residue also allowed to establish its *S* absolute configuration.[5] The scalar and dipolar *inter*-residual correlations detected in the HMBC and in the ROESY spectra (Fig. 2) confirmed the monosaccharide sequence detected for **PS1**. In summary, we have established the structure of the OPS from the lipopolysaccharide of the Gram-negative bacterium *R. terrigena*. It consists of a linear tetrasaccharide repeating unit, in which a β-Man residue carries a cyclic acetal of pyruvic acid (Pyr), as shown below:

1. Experimental

R. terrigena cells (identified through 16S rDNA sequence analysis) were grown in batch culture at 25 °C for 14 d in sterile liquid nutrient broth (BioLab) at 16 g L^{-1} on an orbital shaker at 110 rpm in the dark. Cells were harvested by centrifugation, washed with saline solution and freeze-dried. The LPS was extracted from dried cells (4.7 g) using the hot phenol/water procedure,[3] that yielded two phases, both analysed by silver stained SDS-PAGE.[6] The LPS was detected in the water phase and underwent enzymatic purification with DNase, RNase and Proteinase K. After dialysis and lyophilisation, 344 mg of pure LPS was obtained. In order to obtain the O-polysaccharide chain, the LPS (26 mg) was hydrolysed with aq 1% AcOH for 2 h at 100 °C and centrifuged (8000*g*, 4 °C, 1 h). The supernatant thus obtained was purified by gel permeation chromatography on a Sephacryl S300-HR column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as the eluent and monitored with a Knauer differential refractometer (**PS1**, 23 mg, 90% of LPS).

Alkaline degradation of the LPS was performed on a second aliquot (20 mg). The sample was first O-deacylated with anhydrous hydrazine (1 mL, 37 °C, 2 h). The reaction was quenched with cold acetone and the



sample was collected by centrifugation (3000*g*, 4 °C, 30 min), washed twice with acetone, suspended in water and freeze-dried. After lyophilisation, the sample underwent N-deacylation with 4 M KOH (120 °C, 14 h) as described[7] and salts were removed on a Sephadex G-10 column. Further purification on a Sephacryl S300-HR, as previously described, allowed the obtainment of **PS2** (15 mg, 75% of the LPS).

The monosaccharides were identified as acetylated *O*-methyl glycoside derivatives. Briefly, samples were methanolysed with 2 M HCl/MeOH at 85 °C, 20 h, dried under reduced pressure and then acetylated with Ac_2O in pyridine at 80 °C for 30 min. After workup, the sample was analysed by GLC–MS. The absolute configuration of the monosaccharides was determined by GLC of acetylated glycosides of (+)-2-octanol, according to the published method.[8] Methylation was carried out with methyl iodide in dimethyl sulfoxide in the presence of sodium hydroxide.[9] Hydrolysis of the methylated O-polysaccharide was carried out with 2 M TFA (120 °C, 1 h). The partially methylated monosaccharides were reduced with $NaBD_4$ and converted to their alditol acetates with Ac_2O in pyridine at 80 °C for 30 min and analysed by GLC–MS.

1D and 2D ^1H NMR spectra were recorded on a solution of 3 mg in 0.6 mL of D_2O, at 300 K. ^1H and ^{13}C NMR experiments were carried out using a Bruker DRX-600 equipped with a cryogenic probe. Spectra were calibrated with internal acetone (δ_H 2.225, δ_C 31.45). ROESY experiments were measured using data sets ($t1{\cdot}t2$) of 4096·1024 points, and 32 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. TOCSY experiments were performed with a spinlock time of 100 ms, using data sets ($t1{\cdot}t2$) of 4096·1024 points, and 32 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 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).[10,11] The HSQC experiment was recorded in the ^1H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048·512 points, and 120 scans were acquired for each t1 value. Experiments were carried out in the phase-sensitive mode according to the described method.[12] The data matrix was extended to 2048·1024 points using forward linear prediction extrapolation.

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