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Structure of the repeating oligosaccharide from the lipopolysaccharide of the nitrogen-fixing bacterium *Acetobacter diazotrophicus* strain PAL 5

José O. Previato [a], Christopher Jones [b], Marilia P. Stephan [c], Lucia P.A. Almeida [a], Lucia Mendonça-Previato [a,*]

[a] *Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, 21944 970, Cidade Universitária, Rio de Janeiro, RJ, Brasil*

[b] *National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK*

[c] *Embrapa / CTAA, Guaratiba, Rio de Janeiro, RJ, Brasil*

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Abstract

Acetobacter diazotrophicus is an acid-tolerant nitrogen-fixing bacterium found in roots, rhizosphere, stems, and leaves of sugar cane (*Saccharum officinarum*) cultivated in Brazil. The *O*-polysaccharide from the lipopolysaccharide of the root isolate strain PAL 5 has been determined by a combination of methylation analysis and two-dimensional high field NMR spectroscopy. The pentasaccharide repeat has the structure:

$$\begin{array}{l}\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \beta\text{-D-Glc}\end{array}$$

Minor resonances in the NMR spectra are consistent with the presence of a proportion of repeating units which lack the β-D-Glc side-chain.

Keywords: *Acetobacter diazotrophicus*; Lipopolysaccharide; Nitrogen fixation

* Corresponding author. Tel.: +55-21-5903093; fax: +55-21-2708647; e-mail: immglup@microbio.ufrj.br.

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1. Introduction

The microaerobic, Gram-negative, acid-tolerant nitrogen-fixing bacterium *A. diazotrophicus* is a natural endophyte reported as occurring in sugarcane stems, roots, and rhizosphere [1] in Brazil and sugarcane roots in Australia [2]. This bacterium has also been isolated from other sucrose-rich plants, such as sweet potato and Cameroon grass which are propagated vegetatively [3]. Field experiments using N-balance and ^{15}N isotope dilution techniques suggest that up to 80% of the N incorporated into Brazilian sugarcane cultivars may be obtained from biological nitrogen fixation (BNF) [4,5]. However, it must be emphasized that other diazotrophic bacteria, including *Herbaspirillum seropedicae* and *H. rubrisubalbicans* [6], are also found in sugarcane. Chemical characterization of the polysaccharides on the cell surface of *A. diazotrophicus* is important for taxonomic reasons and to elucidate the role of these molecules in the differential localization of *A. diazotrophicus* strains in the sugarcane plant [1]. A variety of polysaccharides produced by bacteria of the genus *Acetobacter* have been characterized, including β-glucans [7], α-glucans [8], exopolysaccharides [9,10], capsular (CPS) and lipopolysaccharide (LPS) [8,11]. In *Rhizobium* the involvement of these carbohydrate-rich molecules in establishing the interaction between the nitrogen-fixing bacterium and the legume host has been reported [12–14]. Despite the fact that *A. diazotrophicus* has never been isolated from soil, as normally happens for *Rhizobium*, James et al. [15] described the ability of strain PAL 5 (root isolate) to infect roots and colonize the shoots of sugarcane seedlings in vitro.

We have previously reported the compositions of the CPSs from strain PAL 5 and a number of other *A. diazotrophicus* strains [16] and shown that they are composed primarily of rhamnose, mannose, galactose, and glucose. We now describe the structure of the repeating unit of the LPS from *A. diazotrophicus* strain PAL 5, determined by a combination of NMR spectroscopy and methylation analysis.

2. Experimental

Bacterium strain and growth conditions.—*Acetobacter diazotrophicus* strain PAL 5 (ATCC 49037), isolated from roots of sugarcane cultivated in northeast Brazil, was obtained through the culture collection of Embrapa/CNPAB, and was maintained on agar plates of mannitol-salts-yeast extract medium [17]. The cellular mass for LPS extraction was obtained following the same conditions utilised for capsular polysaccharide, in a synthetic medium containing 0.2% glutamate and 2% mannitol [16].

Isolation of LPS.—Cells of *A. diazotrophicus* grown on 10 L medium were washed twice with 50 mM PBS (pH 7.2). After removal of CPS from the cells in a Waring Blendor [16], LPS was extracted by the phenol–water method [18] as follows: approximately 30 g (wet weight) of *A. diazotrophicus* PAL 5 cells were suspended in distilled water (400 mL) and heated to 70 °C. An equal volume of 90% (w/v) aqueous phenol at 70 °C was added and the mixture stirred for 15 min at 70 °C. After cooling, the liquid phases were separated by centrifugation at $3000 \times g$ for 30 min. The upper aqueous phase was dialysed against water for 72 h, concentrated, and freeze-dried. The dry residue containing LPS was suspended in water to 3% (w/v) and washed four times by ultracentrifugation at $105{,}000 \times g$ for 16 h at 4 °C. Nucleic acids and CPS were removed in the washing procedure, leaving the LPS in the pellet. The LPS was suspended in water and precipitated with 3 vol ethanol. Approximately 5 mg of the LPS was desalted on an FPLC system (Pharmacia, Uppsala, Sweden) by passage through an HR10/10 fast desalting column (Pharmacia) eluted with de-ionised water at 0.5 mL/min. The high molecular weight material was collected and lyophilised.

Analytical procedures.—For sugar analysis, purified LPS was methanolyzed with 0.5 M HCl in methanol containing myo-inositol as internal standard (18 h at 80 °C), neutralized with silver carbonate, re-*N*-acetylated with acetic anhydride, and the trimethylsilyl derivatives prepared. The products were analysed by gas–liquid chromatography (GLC) on a DB-5 fused silica column (30 m × 0.25 mm i.d.), using hydrogen (0.7×10^5 Pa) as the carrier gas. The column temperature was programmed from 120 to 240 °C at 2 °C/min. The absolute configuration of the sugars was determined by GLC of their trimethylsilylated 2-butylglycosides as previously described [19]. Fatty acids were identified by GLC and GLC–mass spectrometry (GLC–MS) after methanolysis and trimethylsilylation [20]. For the analysis of ribose content, purified LPS was suspended in Tris EDTA buffer (pH 7.5), containing 50 KUI of RNase (Sigma), and maintained overnight at 37 °C. After boiling and dialysis against water, the preparation was submitted to methanolysis and sugar analysis, as described above.

Table 1
Methylation analysis of the *Acetobacter diazotrophicus* PAL 5 LPS

O-Methyl sugar	Structure	Molar ratio [a]
3,5-Rib*f*	2-*O*-substituted Rib*f*	0.7
3,4-Rha*p*	2-*O*-substituted Rha*p*	0.8
2,4-Rha*p*	3-*O*-substituted Rha*p*	1.0
2,3,4,6-Glc*p*	terminal Glc*p*	1.0
4-Rha*p*	2,3-di-*O*-substituted Rha*p*	1.0

[a] For molar ratios the value for 2,3-di-*O*-substituted Rha*p* is taken as 1.0.

Methylation analysis.—The LPS was methylated by the procedure of Ciucanu and Kerek [21]. The methylated LPS was hydrolysed with TFA acid (4 M, 4 h, 100 °C), reduced with sodium borohydride, acetylated, and the partially methylated alditol acetates (PMAAs) were analysed by GLC and GLC–MS, as previously described [22].

NMR spectroscopy.—NMR spectra were obtained on a Varian Unity 500 spectrometer equipped with a 5 mm triple resonance or broadband probehead, as previously described [23] at an indicated probe temperature of 50 °C. ^{1}H NMR spectra were referenced to internal acetate anion at 1.908 ppm and ^{13}C NMR spectra to internal 1,4-dioxane at 67.4 ppm. The spectrometer software was VNMR version 4.3, and standard pulse sequences were used throughout apart from the introduction of an echo sequence into the TOCSY experiment. The TOCSYECHO spectrum had a mixing time of 80 ms and the NOESY [24] of 100 ms. The mixing time in the HMQC-TOCSY experiment [25] was 20 ms. The HMBC experiment [26,27] was tuned for a long-range ^{1}H–^{13}C coupling constant of 10 Hz. All heteronuclear correlation spectra were obtained in the ^{13}C-coupled mode, and $^{1}J_{C,H}$ coupling constants were determined from the HMQC experiment.

3. Results and discussion

Chemical analysis of the A.diazotrophicus *PAL 5 LPS.*—Hot phenol–water extraction of *A. diazotrophicus* cells released LPS into the aqueous phase in a yield of 1.6% of the freeze-dried weight. The LPS preparation contained virtually no protein, as determined by the method of Bradford [28]. No uronic acid was observed in the assay of Dische [29]. Sugar analysis of the purified LPS by GLC of the trimethylsilylated methyl glycosides showed the presence of ribose (Rib), rhamnose (Rha), and glucose (Glc) in a molar ratio of 0.9:3.8:1.0 together with traces of

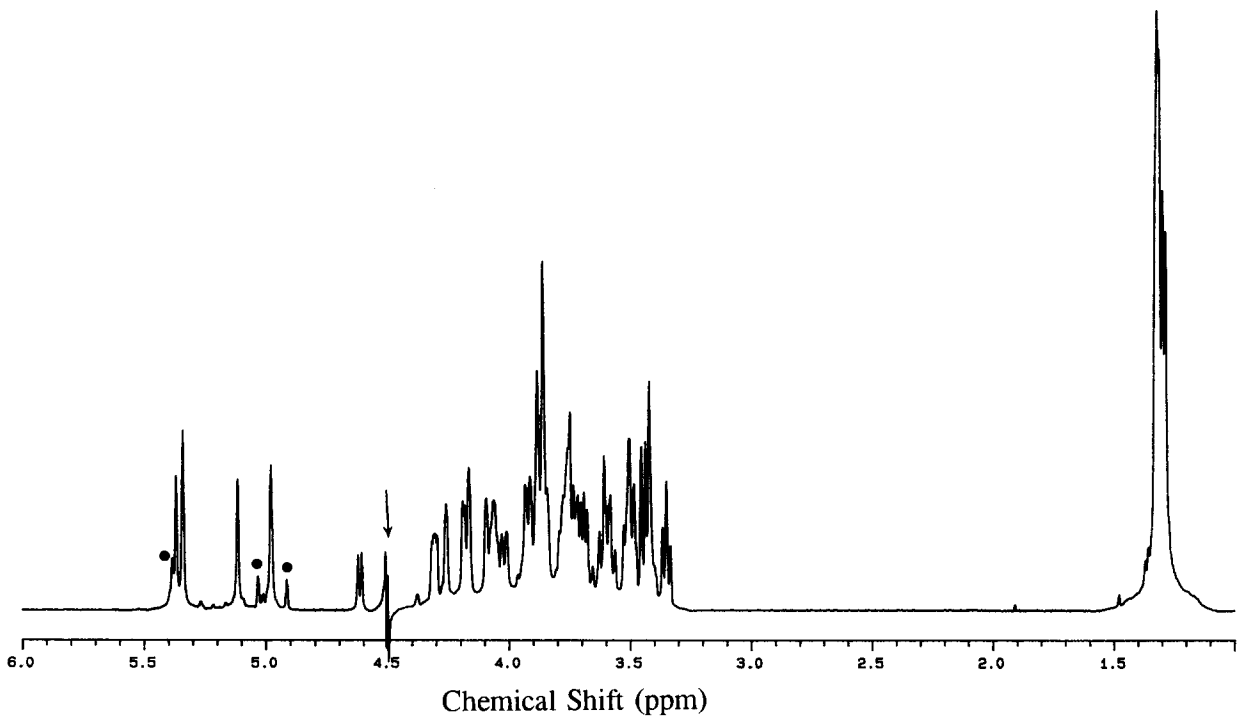

Fig. 1. One-dimensional 500-MHz ^{1}H NMR spectrum of the *Acetobacter diazotrophicus* PAL 5 LPS, obtained at 50 °C. The arrow indicates the position of presaturation of the HOD resonance and points indicate the anomeric resonances from the minor repeat unit.

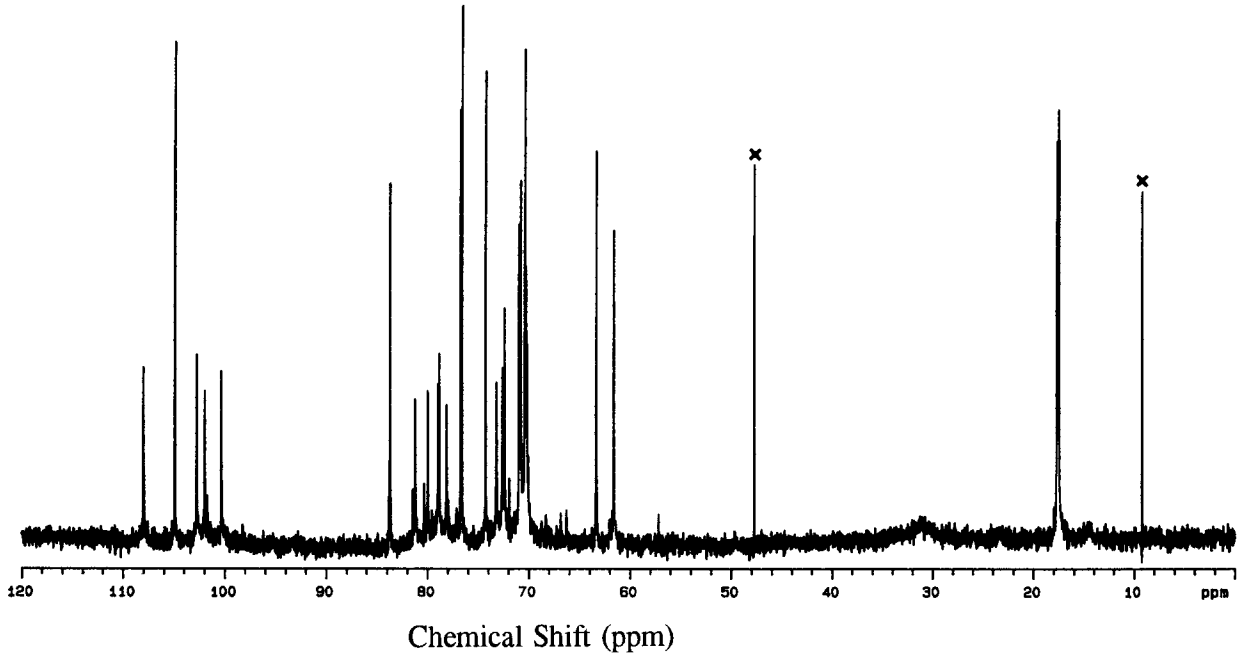

Fig. 2. One-dimensional 125-MHz ^{13}C NMR spectrum of the *Acetobacter diazotrophicus* PAL 5 LPS, obtained at 50 °C. The resonances marked '×' arise from triethylamine.

mannose and galactose. The presence of Rib was observed even after treatment of the LPS preparation with RNase, eliminating the possibility of nucleic acid contamination. The absolute configuration of the Rha and Glc residues was defined as L- and D-, respectively, by GLC of their trimethylsilylated (±)-

Table 2
^{1}H and ^{13}C NMR assignments for the repeat unit [a]

Residue	H-1 C-1 $^{1}J_{C,H}$ (Hz)	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6/H-5′ C-6	H-6′
→ 2)-β-Rib*f*	5.370 107.98 177.2	*4.189* *81.15*	4.310 70.88	4.064 83.66	3.871 63.25	3.699	
→ 2,3)-α-Rha(1)	5.340 101.92 174.6	*4.258* *79.90*	*4.019* *78.07*	3.609 72.54	3.878 70.09	1.314 17.45	
→ 2)-α-Rha(2)	5.116 100.26 173.7	*4.093* *78.78*	3.922 70.74	3.507 73.15	3.754 70.19	1.321 17.63	
→ 3)-α-Rha(3)	4.981 102.72 172.0	4.164 70.76	*3.851* *78.86*	3.581 72.32	3.780 70.12	1.290 17.50	
terminal β-Glc	4.616 104.87 161.5	3.348 74.20	3.499 76.54	3.432 70.34	3.410 76.68	3.874 61.55	3.739
Minor residues							
→ 2)-Rib*f*	5.385 107.95	*4.165* *81.38*					
→ 3)-α-Rha(1)	5.033	4.315	*4.060*	3.502			
→ 3)-α-Rha(3)	4.913	[4.300]					

[a] ^{1}H chemical shifts referenced to AcO^{-} at 1.908 ppm and ^{13}C chemical shifts to internal 1,4-dioxane at 67.40 ppm. Linkage positions are shown in italics. [ ] Assignment tentative.

2-butyl-glycosides. The Rib was assumed to be in the D-configuration. The fatty acid composition of the LPS was determined by GLC of the trimethylsilylated methyl esters, and showed the presence of palmitic acid and 3-hydroxylated myristic, 2-hydroxylated palmitic and 3-hydroxylated stearic acids in a peak area ratio of 6:4:1:4.

Methylation analysis.—The methylation analysis of purified LPS was carried out using PMAA derivatives which were characterized by their GLC retention times and mass spectrometric fragmentation patterns. Five major PMAAs were observed (Table 1), arising from terminal Glc*p*; 2-*O*-substituted Rib*f*; 2-O; 3-O; and 2,3-di-*O*-substituted Rha*p*. These data reflect the presence of nonreducing terminal glucose and suggest that the repeating unit of the PAL 5 strain of *A. diazotrophicus* LPS has a branched pentasaccharide structure.

NMR spectroscopy.—The lyophilised lipopolysaccharide dissolved only slowly in deuterated water at room temperature to produce a slightly hazy solution and a white residue on the surface. Two solutions were used for NMR analysis, a dilute solution (8.8 mg in 0.8 mL) for homonuclear ^{1}H experiments and a concentrated solution (29.5 mg in 0.8 mL) for heteronuclear experiments. There were no significant differences in ^{1}H chemical shifts between these two samples. The 500-MHz ^{1}H NMR spectrum at 50 °C was well dispersed and characteristic of a homogeneous sample. A small amount of triethylamine (4.7% w/w) was present as a contaminant. Approximately 5 mg of the LPS was desalted by column chromatography and the one-dimensional ^{1}H NMR spectrum repeated. The resonances assigned to triethylamine were not present in this spectrum (Fig. 1). The one-dimensional ^{13}C NMR spectrum contained five major anomeric resonances, and no low field signals from carbonyl or carboxylate groups were observed (Fig. 2). The one-dimensional ^{31}P NMR spectrum contained no significant signals, although a weak resonance was observed in the phosphodiester region.

The ^{1}H NMR spectrum was assigned by conventional methods (Table 2) — double quantum filtered COSY [30] and a TOCSYECHO spectrum with an 80 ms mixing time (Fig. 3). The ^{13}C NMR spectrum was assigned from an HMQC spectrum [26,27] and con-

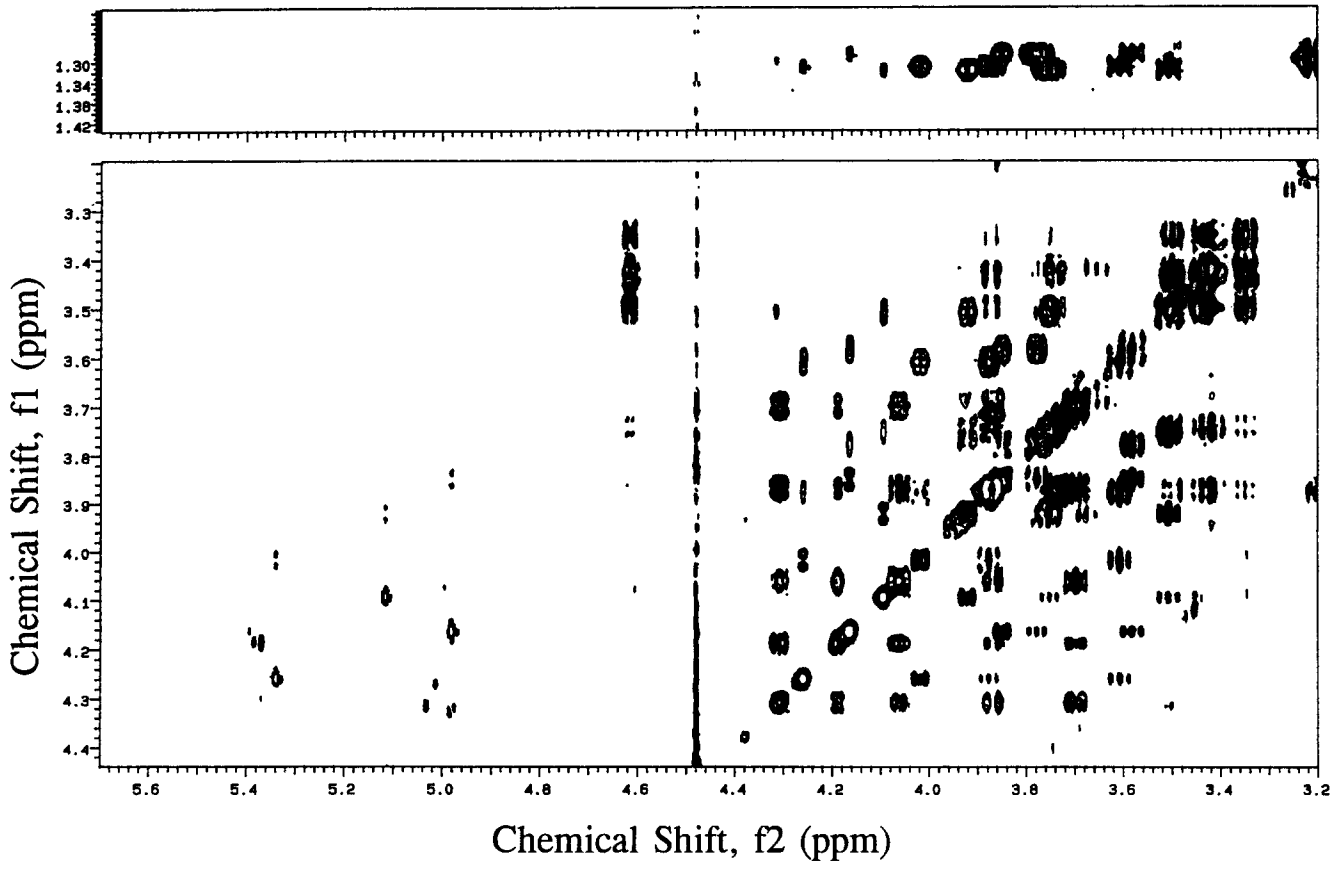

Fig. 3. Partial 500-MHz TOCSYECHO spectrum of the *Acetobacter diazotrophicus* PAL 5 LPS, obtained at 50 °C and using an 80 ms mixing time.

Table 3
Inter-residue NOEs defining the sequence and linkage of the polysaccharide

NOE from	to ppm [a]	Assigned as
β-Rib*f* H-1	4.013	α-Rha(1) H-3
	4.258	α-Rha(1) H-2
	5.120	α-Rha(2) H-1
α-Rha(1) H-1	3.845	α-Rha(3) H-3
α-Rha(3) H-1	5.112	α-Rha(2) H-1
	4.092	α-Rha(2) H-2
	3.921	α-Rha(2) H-3
α-Rha(2) H-1	5.370	β-Rib*f* H-1
	4.182	β-Rib*f* H-2
	4.979	α-Rha(3) H-1
	3.780	α-Rha(3) H-5
β-Glc H-1	4.252	α-Rha(1) H-2

[a] Underlined figures indicate most intense NOE when more than one inter-residue NOE was observed.

Table 4
Long-range $^{13}C-^{1}H$ correlations defining the sequence and linkages

Correlation from	to ppm	Assigned as
β-Rib*f* C-1	4.019	α-Rha(1) H-3
β-Rib*f* H-1	78.07	α-Rha(1) C-3
α-Rha(1) C-1	3.845	α-Rha(3) H-3
α-Rha(1) H-1	78.86	α-Rha(3) C-3
α-Rha(3) C-1	4.092	α-Rha(2) H-2
α-Rha(3) H-1	78.78	α-Rha(2) C-2
α-Rha(2) C-1	4.19	β-Rib*f* H-2
α-Rha(2) H-1	81.15	β-Rib*f* C-2
β-Glc C-1	4.252	α-Rha(1) H-2
β-Glc H-1	79.90	α-Rha(1) C-2

firmation of ambiguous resonances obtained from a HMQC-TOCSY experiment [25] with a 20 ms mixing time. Three α-Rha spin systems ($^{1}J_{H\text{-}1,C\text{-}1} > 170$ Hz), a pentose in furanose ring form ($^{3}J_{H\text{-}1,H\text{-}2}$ not resolved, $\delta_{C\text{-}1} = 107.98$ ppm), and a β-Glc residue ($^{1}J_{H\text{-}1,C\text{-}1} = 161.5$ Hz), were observed. The β-Glc was assigned as a terminal residue from the close correspondence of its ^{1}H [31] and ^{13}C chemical shifts [32] with the β-methyl glycoside model system. The sequence of the sugar residues and the linkages between them were determined from inter-residue NOEs, obtained in a NOESY experiment [24] with a 100-ms mixing time, and long-range $^{1}H-^{13}C$ correlations observed in the HMBC experiment [26]. These data are summarised in Table 3.

NOEs observed between one of the α-Rha H-1 resonances and the furanose H-2 and H-1 resonances indicated that it is → 2)-linked, and the sugar configuration was confirmed as → 2)-β-Rib*f*-(1 → by the close correspondence of its ^{1}H and ^{13}C chemical shifts with those in, for example, the *E. coli* K38 capsular polysaccharide [33]. The linkage analysis from the NOEs was confirmed by data from an HMBC experiment [26,27] and was fully consistent with the ^{13}C chemical shift data (Tables 3 and 4).

Three minor anomeric resonances at 5.385, 5.033, and 4.913 ppm were observed in the one-dimensional

Major Repeat Unit

→2)-β-D-Rib*f*-(1→3)-α-L-Rha(1)-(1→3)-α-L-Rha(3)-(1→2)-α-L-Rha(2)-(1→

2
|
1
β-D-Glc

Minor Repeat Unit

→2)-β-D-Rib*f*-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→

Fig. 4. The structure of the major and minor repeating units of the *Acetobacter diazotrophicus* PAL 5 LPS.

^{1}H NMR spectrum, at 10–20% the intensity of the major anomeric resonances. These were assigned as β-Rib*f*, α-Rha, and α-Rha H-1 resonances repectively. Limited additional data was obtained from the HMQC and TOCSYECHO spectra (Table 2), and these spin systems were assigned as arising from a minor repeating unit lacking the sidechain β-Glc residue. The structures of the repeating units are showed in Fig. 4. The main chain on this polysaccharide is identical to that determined for the O-antigen from *Klebsiella* O7 [34].

Surprisingly, no resonances attributable to a conventional LPS core were observed in the one- or two-dimensional NMR spectra, which suggests either that the LPS has a high molecular weight (> 20 repeating units) or that *Acetobacter* elaborates a core structure that is not typical of those found in other Gram negative bacteria. These possibilities are supported by the high total neutral sugar content (89% of LPS mass) detected by the phenol–sulfuric acid method [35], and by our failure to effect cleavage of the repeating polysaccharide from a putative Lipid A moiety by treatment with hot 1% acetic acid, as previously observed in *A. methanolicus* [11], and in another strain of *A. diazotrophicus* — strain PAL 3 (manuscript in preparation).

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