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Note

Structural characterization of the antigenic O-polysaccharide in the lipopolysaccharide produced by *Actinobacillus pleuropneumoniae* serotype 14

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Abstract—The antigenic O-polysaccharide of the lipopolysaccharide produced by *Actinobacillus pleuropneumoniae* serotype 14 was shown by chemical analysis and 1D and 2D nuclear magnetic resonance methods to be a high-molecular-mass polymer of a repeating disaccharide unit composed of a chain of (1 → 5)-linked β-D-galactofuranose (β-D-Gal*f*) residues substituted at their O-2 positions by α-D-galactopyranose residues (D-Gal*p*) (1:1):

$$\begin{array}{c}\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow \\ \quad\quad\quad\quad\quad\quad 2 \\ \quad\quad\quad\quad\quad\quad\uparrow \\ \quad\quad\quad\quad\quad\quad 1 \\ \alpha\text{-D-Gal}p\end{array}$$

Keywords: Galactan; Lipopolysaccharide; *Actinobacillus pleuropneumoniae* O:14

Actinobacillus pleuropneumoniae, an encapsulated Gram-negative bacterium, is the causative agent of porcine pleuropneumonia, a severe respiratory disease of pigs that is of economic importance where pigs are raised.[1–3] Strains of *A. pleuropneumoniae* have been allocated to 15 serotypes that are related to the unique structures of their antigenic capsular polysaccharide (CPS) and somatic lipopolysaccharide (LPS) components. Knowledge of the serotypes prevalent in a geographic region is important since killed whole-cell bacterial preparations can protect only against serotypes present in the vaccine.[4,5] It follows that structural chemical information of the LPS and CPS forms a sound basis for defining serotype differentiation, the production of specific diagnostic serotyping agents, and explanations for observed serological cross-reactions by unabsorbed polyclonal antisera.

In North America, serotypes 1 and 5 are the most commonly isolated, whereas serotypes 2 and 9 are isolated in Europe, and serotype 15 is the predominant isolate in Australian pigs.[6,7] Since the antigenic polysaccharides of *A. pleuropneumoniae* serotypes form the basis for serological typing agents and for the selection of specific vaccine components, previous studies were directed toward their chemical characterization, and the structures of the LPS and CPS components of serotypes 1 to 12 have been recorded.[8] The present work describes the characterization of the LPS O-antigen expressed by *A. pleuropneumoniae* serotype 14.

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Cells of *A. pleuropneumoniae* serotype 14 were extracted by a modified hot aqueous phenol procedure and ultracentrifugation of the respective dialyzed and concentrated separated phenol and water layers, the latter yielding essentially all the extracted LPS (ca. 6% yield based on dry cell weight).

Mild acid hydrolysis of the LPS (2% AcOH, 100 °C, 2 h) resulted in the precipitation of lipid A (21% yield), and Sephadex G-50 column chromatography of the concentrated water-soluble product afforded O-PS (46% yield, K_{av} 0.03), core oligosaccharide (6% yield, K_{av} 0.79) and a fraction (K_{av} 0.93) containing Kdo. The O-PS had $[\alpha]_D$ +74.5 (*c* 0.3, water), and on total hydrolysis (1 M H_2SO_4, 100 °C, 2 h) afforded D-galactose (94% yield), together with trace amounts (ca. 1%) of D-glucose, L-*glycero*-D-*manno*-heptose, and D-*glycero*-D-*manno*-heptose, presumed to be derived from core oligosaccharide. The D-galactose O-PS component was characterized from its specific optical rotation {$[\alpha]_D$ +78.1 (*c* 0.2, water)}, identity of its ^{1}H NMR spectrum with that of a reference spectrum, and the GLC identification of its reduced ($NaBD_4$) and acetylated product as hexa-*O*-acetylgalactitol-1-*d*.

The ^{13}C NMR spectrum of the O-PS showed 12 signals comprised of two anomeric resonances at 106.0 ($J_{C\text{-}1,H\text{-}1}$ 180 Hz) and 97.9 ppm ($J_{C\text{-}1,H\text{-}1}$ 172 Hz), together with 10 ring-carbon resonances (61–87 ppm), consistent with the O-PS being formed of a repeating disaccharide unit composed respectively of D-galactofuranose and pyranose residues. The corresponding ^{1}H NMR spectrum (Fig. 1) similarly showed two anomeric proton signals at 5.33 ($J_{1,2} \sim 1$ Hz) and 5.11 ppm ($J_{1,2}$ 2.2 Hz) in the ratio of 1:1, together with ring protons (4.2–3.8 ppm, 10H), further confirming the predicted disaccharide nature of the repeating unit and establishing the pyranosyl component as an α-D-galactopyranosyl residue with the absence of any glycosyl residue substituents. The methylated O-PS on sequential hydrolysis, reduction ($NaBD_4$), and acetylation, afforded two equimolar derivatives identified by GLC–MS as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol-1-*d* (T_{GM} 1.09) and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylgalactitol-1-*d* (T_{GM} 1.82) (1:1). The combined initial NMR and methylation data indicated that the O-PS is composed of either a (1→5)-linked D-Gal*f* linear backbone polymer substituted at O-2 by α-D-Gal*p* residues or a (1→2)-linked D-Gal*f* backbone substituted at O-5 by α-D-Gal*p* residues.

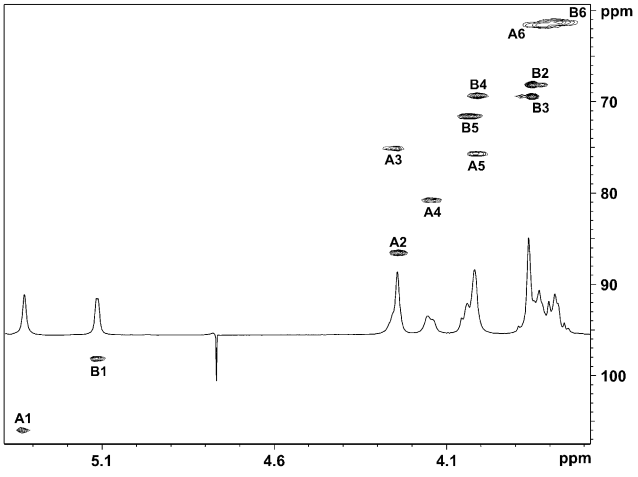

Figure 1. HSQC spectrum of the LPS O-polysaccharide of *A. pleuropneumoniae* serotype 14.

Two-dimensional homonuclear NMR proton analysis (COSY, TOCSY) were used to confirm a unique O-PS structure and fully assign the proton chemical shifts (Table 1). The residues were labeled **A** (D-Gal*f*) and **B** (D-Gal*p*), and the ring proton resonances were assigned from cross-peak identification and coupling constants observed from H-2 to H-6. Glycose linkages were established from transglycosidic NOEs in which H-1**A** showed strong interresidue connectivity to H-5**A** at 4.02 ppm along with expected weak enhancement to H-6**A** and H-1**B**. The α-D-Gal*p* H-1**B** showed enhancement of its own H-2**B** and interresidue connectivity to H-2**A**, leading to the conclusion that the O-PS was a (1→5)-linked polymer of β-D-Gal*f* residues to which α-D-Gal*p* residues are linked at position C-2. A heteronuclear 2D HSQC (Fig. 1) experiment allowed complete assignment of carbon chemical shifts (Table 1), and a long-range HMBC experiment confirmed interresidue correlations from H-1**A** to its own C-5**A**, and from H-1**B** to C-2**A**, consistent with the proposed O-PS structure.

A Smith-type degradation[9] of the O-PS involving sequential periodate oxidation and reduction ($NaBH_4$) afforded a residual polymer that on complete hydrolysis yielded D-galactose and glycerol (1:1), the glycerol arising from the single nonreducing D-Gal*p* substituents. Mild acid hydrolysis (2% AcOH, 100 °C, 2 h) of the

Table 1. ^{1}H and ^{13}C NMR chemical shifts of *A. pleuropneumoniae* serotype 14 LPS O-polysaccharide[a]

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
A →2,5)-β-D-Gal*f*-(1→	5.33 (~1)	4.24	4.25	4.14	4.02	3.82
	106.0 (180)	86.6	75.8	80.7	75.2	61.6
B α-D-Gal*p*-(1→	5.11 (2.2)	3.86	3.87	4.01	4.04	3.77
	97.9 (172)	68.0	69.1	69.3	71.4	61.3

[a] Spectra measured at 25 °C in D_2O with internal acetone reference (2.225 ppm for proton and 31.07 ppm for carbon). Coupling constants ($J_{H\text{-}1,H\text{-}2}$ and $J_{C\text{-}1,H\text{-}1}$) are given in parentheses in Hertz.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of *A. pleuropneumoniae* serotype 14 β-(1→5)-D-Gal*f* backbone polymer[a]

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
A′ →5)-β-D-Gal*f*-(1→	5.19(~2)	4.15	4.11	4.16	3.95	3.80
	107.1(179)	81.5	76.5	81.5	75.7	61.2

[a] Spectra measured at 25 °C in D_2O with internal acetone reference (2.225 ppm for proton and 31.07 ppm for carbon). Coupling constants ($J_{H\text{-}1,H\text{-}2}$ and $J_{C\text{-}1,H\text{-}1}$) are given in parentheses in Hertz.

oxidized and reduced O-PS product, followed by Sephadex G-50 column chromatography, afforded a high-molecular-mass D-galactan (38% yield) having $[\alpha]_D$ −107 (*c* 0.3, water) that on methylation analysis gave 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol-1-*d* (T_{GM} 1.41), consistent with the polymer being an unbranched chain of (1→5)-linked β-D-Gal*f* residues. ^{1}H and ^{13}C NMR analysis of the polymer gave chemical shift data (Table 2) in agreement with the proposed structure and with that reported for a β-(1→5)-D-galactofuranan produced by *Eupenicillium crustaceum*,[10] thus providing further confirmation and unambiguous proof for the branched structure of the O-PS repeating unit as:

[A]
→5)-β-D-Gal*f*-(1→
2
↑
1
α-D-Gal*p* **[B]**

The *A. pleuropneumoniae* serotype 14 structure is unusual, similar in nature to the comb-like D-glucan produced by *Streptococcus pneumoniae* type 37[11] and to the galactomannan of *Streptomyces* sp. VKM Ac-2125,[12] and distinct from that of the previously determined polysaccharide antigens of *A. pleuropneumoniae* serotypes. It is, however, interesting to note that D-Gal*f* residues occur in the LPS O-PSs of serotypes 3,[13] 6,[14] 8,[15] 10,[16] and 15 (unpublished results). Serological cross-reactions between *A. pleuropneumoniae* serotypes 3, 6 and 8 have been reported,[17] and it has been suggested that cross-reactions may involve D-Gal*f* containing epitopes in their somatic antigens.[8]

The core oligosaccharide fraction had $[\alpha]_D$ +67.2 (*c* 0.4, water) and on hydrolysis (2 M TFA, 110 °C, 4 h) and GLC analysis was indicated to be composed of glucose, galactose, L-*glycero*-D-*manno*-heptose, and D-*glycero*-D-*manno*-heptose in the molar ratio of 2:1:1:1, a composition found to characteristic of the inner core of the LPSs produced by *A. pleuropneumoniae* serotypes 1, 2, 5a and 5b.[18]

Analysis of the putative CPS obtained in very low yield from the ultracentrifugates of the aqueous and phenol phase cell extracts proved, by chemical and NMR analysis, to be identical with that of the isolated O-PS. At this time it is not possible to define the material as capsule or free O-PS. In previous studies it was found that in the serotypes 1–12, the homologous CPS and LPS O-antigens of each serotype had different compositions and structures.[8]

1. Experimental

1.1. General procedures

A. pleuropneumoniae serotype 14 (NRCC 6247), reference strain 3906 from the collection of Dr. Marcelo Gottschalk, was grown on chocolate agar plates (Oxoid) incubated aerobically at 37 °C overnight and used to inoculate 1 L of medium in a 4-L baffle flask (medium: brain-heart infusion broth (Difco 37g/L) supplemented with hemin (Sigma H-2250) to a final concentration of 5 mg/L and NAD to a final concentration of 5 mg/L. Stock D-glucose was added to give a final concentration of 10% w/v. The 1-L culture was grown in a New Brunswick Scientific G26 psycrotherm at 37 °C and 175 RPM for 6.5 h. A total of 23 L of medium in a MBR 30-L fermenter was inoculated with the 6.5-h culture and growth continued at 37 °C with dissolved oxygen controlled at 20% saturation. At 17 h the culture was killed by the addition of phenol to 5% concentration, and the cells were harvested using a Cepa Z41 continuous centrifuge.

1.2. Preparation of LPS and O-PS

Cell paste of *A. pleuropneumoniae* serotype 14 (380 g) was extracted by stirring with 50% aq phenol (500 mL, 65 °C, 15 min). The separated phenol and aqueous layers from the cooled (4 °C) extract were dialyzed against tap water until phenol-free and were then lyophilized. The respective residues were resuspended in 50 mL 0.02 M AcONa, and treated sequentially with RNase, DNase and proteinase K (37 °C, 2 h each). Enzyme-treated samples were subjected to ultracentrifugation (105,000 *g*, 4 °C, 14 h), and the precipitated gels were dissolved in water and lyophilized to yield 1.92 g (aq phase) and 86 mg (PhOH phase) LPS. The ultracentrifugates were treated with acetone (6 vols), and the precipitated products (putative CPS) were collected, dissolved in water and lyophilized (yield, ~100 mg each).

Aq phase LPS (1 g) in 2% AcOH (100 mL) was heated on a boiling water bath for 2 h, precipitated lipid A was

removed by centrifugation (15,000 × *g*), and the lyophilized water-soluble products were fractionated by Sephadex G-50 column chromatography. Fractions corresponding to O-PS, core oligosaccharides and monosaccharide were collected and lyophilized.

1.3. Chromatography

Gas-chromatography (GC) was performed using a ZB-50 column (30 m × 0.25 mm) in an Agilent 6850 chromatograph fitted with a flame-ionization detector, or using a Varian Saturn 2000 ion-trap GC–MS instrument and a temperature program 170 °C (delay 2 min) at 2 °C/min to 220 °C. Retention times are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM} = 1.0).

1.4. NMR spectroscopy

^{1}H and ^{13}C spectra were recorded using a Varian 400-MHz spectrometer with samples in D_2O at 25 °C and referenced to internal acetone standard (^{1}H 2.225 ppm, ^{13}C 31.07 ppm). COSY, TOCSY, NOESY, HSQC, and HMBC experiments were used as previously described.[19]

1.5. Methylation analysis

Samples (2–3 mg) in Me_2SO (0.5 mL) were methylated by the Ciucanu–Kerek procedure[20] and were hydrolyzed (3 M TFA, 100 °C, 2 h), reduced ($NaBD_4$), converted to their alditol-1-*d* acetates and analyzed by GLC–MS.

1.6. Periodate oxidation[9]

O-PS (70 mg) in water (5 mL) containing $NaIO_4$ (300 mg) was kept for 24 h in the dark at 20 °C, treated with ethylene glycol (0.5 mL), and following dialysis against water, the retentate was reduced by the addition of $NaBH_4$ (0.3 g). After 3 h the ice-cooled solution was acidified with AcOH and dialyzed. The lyophilized retentate was fractionated by Sephadex G-50 column chromatography, and the collected void volume product (52 mg), which had $[\alpha]_D$ −42 (*c* 0.2, water), was subjected to hydrolysis with 2% AcOH (10 mL, 100 °C, 2 h). The high-molecular-mass product (22 mg, K_{av} 0.03–0.12) was obtained on Sephadex G-50 gel filtration.

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