Characterization of the Antigenic Lipopolysaccharide O Chain and the Capsular Polysaccharide Produced by *Actinobacillus pleuropneumoniae* Serotype 13

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*R. A. pleuropneumoniae* is a gram-negative bacterium causing contagious pleuropneumonia in pigs. Based on NAD requirements, *A. pleuropneumoniae* can be divided into biobar 1 strains, which are NAD dependent, and biobar 2 strains, which are NAD independent. To date, 15 serotypes, based on carbohydrate antigens, have been described (5), and serotype 1 and 5 have been subdivided into 1a and 1b and 5a and 5b, respectively (17, 23).

The chemical structures of the carbohydrate antigens found in the capsules and the O-polysaccharide components (O-PSs) of the lipopolysaccharides (LPSs) of *A. pleuropneumoniae* serotypes 1 to 12 (27) and serotype 14 (28) have been elucidated and have assisted in explaining observed serological specificities and cross-reactions. Recently, three new serotypes of *A. pleuropneumoniae* have been described, serotypes 13 and 14, and serotype 15 (biovar 1), the predominant serotype present in Australian pigs (5).

Knowledge of the serotypes prevalent in a geographic region is important (4) since current vaccines involving killed whole-cell bacterial preparations can protect only against infection by a homologous serotype present in the vaccine (7, 14, 24, 25, 30). We have undertaken the characterization of the newly described serotypes of *A. pleuropneumoniae*, and this paper describes the elucidation of the structure of the LPS O-PS and the capsular polysaccharide (CPS) of *A. pleuropneumoniae* serotype 13.

**Materials and Methods**

*A. pleuropneumoniae* serotype 13 (NRCC 6230; Fodor strain N-273 [9, 26]), the reference strain from the collection of M. Gottschalk (Laval University), was grown on chocolate agar plates (Oxoid) incubated aerobically at 37°C overnight and used to inoculate 1 liter of medium in 4-liter baffle flasks (medium, brain heart infusion broth [Difco; 57/g liter]) supplemented with hemin (Sigma H-2250) to a final concentration of 5 mg/liter. Stock D-glucose was added to the final concentration of 10% (wt/vol). The 1-liter culture was grown in a New Brunswick Scientific G62 psychrometer at 37°C and 175 rpm for 6.5 h. Twenty-three liters of medium in an MBR 30-liter fermentor was inoculated with the 6.5-h culture, and growth was continued at 37°C with dissolved oxygen controlled at 20% saturation. At 17 h the culture was killed by the addition of phenol to a 5% concentration and the cells were harvested by using a Cepa Z41 continuous centrifuge (yield, 355 g [wet paste]).

**Preparation of LPS, CPS, and O-PS**. A cell paste of *A. pleuropneumoniae* serotype 13 (355 g) was extracted with stirred hot 50% aqueous phenol (550 ml, 70°C, 15 min), and the separated phenol and aqueous phases from the cooled (4°C) extract were dialyzed against tap water until phenol free and then lyophilized. The products were resuspended in 60 ml of 0.02 M sodium acetate (pH 7.0) and were treated sequentially with RNase, DNase, and proteinase K (37°C, 1 h each). Trace solids were removed from the digest by low-speed centrifugation, the samples were subjected to ultracentrifugation (105,000 × g, 4°C, 12 h), and the precipitated gels were dissolved in water and lyophilized to yield 2.2 g (aqueous phase) and 84 mg (phenol phase) of LPS.

The supernatants were treated with cold acetone (6 volumes), and the precipitated products (crude CPS) were collected by centrifugation. The aqueous phase precipitate (1.90 g) of crude putative CPS upon Sephadex G-50 gel filtration yielded a high-molecular-mass polysaccharide (Kav, 0.03 to 0.04; 320 mg [distribution coefficient Kav = (Ve - Vb)/(Ve - V0), where Ve is the elution volume of the specific material, Vb is the void volume of the system, and V0 is the total volume of the system]) with an α1,6 N-acetylglucosamine (ε 0.3, water) that was used in subsequent analyses.

O-deacetylated CPS was prepared by treatment of native CPS (30 mg) with 0.02
M ammonium hydroxide (5 ml) at 37°C for 4 h and following dilution with water was lyophilized to yield acetate-free CPS (27 mg) with an 
\[
\frac{[\beta\text{H}]}{[\alpha\text{H}]} = 1.4 \text{ (water)}.
\]
Aqueous-phase LPS (0.5 g) was hydrolyzed with 2% (vol/vol) acetic acid (AcOH) (75 ml, 100°C, 2 h), and following the removal of precipitated lipid A (117 mg) the lyophilized water-soluble products were fractionated by Sephadex G-50 column chromatography to yield a high-molecular-mass O-PS (\(K_{av} 0.03 \text{ to } 0.04\); 224 mg), a core oligosaccharide (\(K_{av} 0.34\); 80 mg), and a low-molecular-mass fraction (\(K_{av} 0.94\); 40 mg) containing KDO (3-deoxy-D-octulosonic acid).

Chromatography. Gas chromatography was performed using a ZB-50 column (30 m by 0.25 mm; Phenomenex) in an Agilent 6850 chromatograph fitted with a flame ionization detector or a Varian Saturn 2000 ion-trap gas chromatography-mass spectrometry (MS) instrument and a temperature program of 170°C (delay, 2 min) at 2°C/min to 220°C. Retention times and mass spectra were matched with authentic reference samples. Gel filtration chromatography was done with either Sephadex G-50 or Bio-gel P2 columns as previously described (20).

NMR spectrometry. For nuclear magnetic resonance (NMR) spectrometry, 1H, 13C, and 31P spectra were recorded with a Varian 400-MHz spectrometer with samples in D2O and referenced to an internal acetone standard (1H, 2.225 ppm; 13C, 31.07 ppm). COSY, TOCSY, NOESY, HSQC, and HMBC experiments were done as previously described (31).

Periodate oxidation. Smith-type oxidation (11) of O-PS was done as previously described (1).

O deacetylation. CPS (29 mg) dissolved in 0.02 M ammonium hydroxide (5 ml) was kept at 37°C for 4 h, and following dilution with water (10 ml) and lyophilization, the void volume fraction obtained by Sephadex G-50 gel filtration was collected, lyophilized (yield, 27 mg), and used for NMR analysis.

Colorimetric analyses. Determination of O-acetyl was made as described by Hestrin (15), and phosphate was determined by the method of Chen et al. (6).

RESULTS AND DISCUSSION

Extraction of fermentor-grown cells of the reference strain of A. pleuropneumoniae serotype 13 by a modified hot aqueous phenol method (16) released into solution the LPS and CPS produced by the bacterium. The aqueous phase of the cooled extract upon ultracentrifugation yielded a precipitated gel that was dissolved in water and lyophilized to give LPS (6% yield based on dry cell mass). Dilution of the supernatant with acetone yielded a precipitate of crude CPS that upon Sephadex G-50 column gel filtration gave a high-molecular-mass fraction (\(K_{av} 0.02 \text{ to } 0.03\); 3% yield based on dry cell mass) of pure CPS.

Mild acid hydrolysis of the LPS (2% AcOH, 100°C, 2 h) yielded a precipitate of released lipid A (23%), and gel filtration column chromatography of the water-soluble products gave a high-molecular-mass O-PS (45%), a core oligosaccharide fraction (16%), and a low-molecular-mass fraction containing KDO (8%).

The O-PS had an 
\[
\frac{[\beta\text{H}]}{[\alpha\text{H}]} = 1.4 \text{ (water)}
\]
nanomeric signals (H-1) of the component glycose units: \((-4)-\alpha-L-Rha-(\rightarrow (A), \rightarrow-3)-\beta-D-Gal-(\rightarrow (B), \rightarrow-3,4)-\beta-D-GalNAc-(\rightarrow (C), and \beta-D-Gal-\rightarrow. Also indicated are the NAc methyl signal of C and the C-6 methyl signal of A.

FIG. 1. 1H NMR spectrum of the O-PS of A. pleuropneumoniae serotype 13. Indicated are the four anomeric signals (H-1) of the component glycose units: \((-4)-\alpha-L-Rha-(\rightarrow (A), \rightarrow-3)-\beta-D-Gal-(\rightarrow (B), \rightarrow-3,4)-\beta-D-GalNAc-(\rightarrow (C), and \beta-D-Gal-\rightarrow. Also indicated are the NAc methyl signal of C and the C-6 methyl signal of A.
Further proof of the above structural assignment was provided by a Smith-type periodate oxidation (11) in which Rhap residue A and \( \alpha \)-Gal residue D were oxidized. Following reduction (\( \text{NaBH}_4 \)) and mild hydrolysis of the oxidized polymer, a trisaccharide was identified by chemical and NMR analysis to have structure II, the expected degradation product from the proposed O-PS structure I:

\[
\begin{align*}
\text{CH}_3\text{OH} & | \\
\beta-\text{D-Galp-(1\rightarrow4)-\beta-}\text{D-GalpNAc-(1\rightarrow-O-C-H) & |} \\
\text{HO-C-H} & | \\
\text{CH}_3 & |
\end{align*}
(II)
\]

The accumulated experimental data provide convincing evidence that the structure of the LPS O-PS of \( A.\) \textit{pleuropneumoniae} serotype 13 is identical to that previously determined for the O antigen of \( A.\) \textit{pleuropneumoniae} serotype 7 (3). The O-PS structure is similar to that of the O-PS of \( A.\) \textit{pleuropneumoniae} serotype 4 (2), differing only in the replacement of the \( \beta-\text{D-GalpNAc} \) by a 1,3-linked \( \beta-\text{D-Galp} \) side-group residue instead of a 1,3-linked \( \beta-\text{D-Galp} \) side-group residue. It is interesting that Lebrun et al. (19) showed that monoclonal antibodies can be specific or nonspecific for the respective O-PSs of \( A.\) \textit{pleuropneumoniae} serotypes 4 and 7, a finding consistent with the chemical structures, which suggest that the O-PSs should share similar carbohydrate epitopes and yet should also have unique structural features involving single nonreducing \( \beta-\text{D-Galp} \) or \( \beta-\text{D-Galp} \) side end-group residues.

In order to prevent serological misidentification of \( A.\) \textit{pleuropneumoniae} serotype 13 due to cross-reacting antibody to common LPS O antigen, specific type 13 antibody may be made by using \( A.\) \textit{pleuropneumoniae} serotype 13 CPS or its conjugate as an immunogen. Alternatively, polyclonal antisera prepared against whole \( A.\) \textit{pleuropneumoniae} serotype 13 cells may be adsorbed out by \( A.\) \textit{pleuropneumoniae} serotype 7 cells or by insolublized LPS (8) from either \( A.\) \textit{pleuropneumoniae} serotypes 4 and 7, a process that would retain activity against \( A.\) \textit{pleuropneumoniae} serotype 13 cells.

The water phase of the phenol-extracted \( A.\) \textit{pleuropneumoniae} serotype 13 cells, after removal of LPS by ultracentrifugation and following precipitation with acetone, yielded a high-molecular-mass product which upon purification by Sephadex
FIG. 2. HSQC NMR spectrum of the O-PS of *A. pleuropneumoniae* serotype 13 showing proton and carbon correlation cross peaks for the component glycosyl units: \(\rightarrow 4\)-\(\alpha\)-l-Rha-(1\(\rightarrow\) (A), \(\rightarrow 3\)-\(\beta\)-d-Gal-(1\(\rightarrow\) (B), \(\rightarrow 3\),4\(\beta\)-d-GalNAc-(1\(\rightarrow\) (C), and \(\beta\)-d-Gal-(1\(\rightarrow\) (D).

FIG. 3. HSQC NMR spectrum of compound III from *A. pleuropneumoniae* serotype 13 CPS showing carbon and proton correlation cross peaks for the \(\alpha\)-d-galactose (Gal) and glycerol (Gro) components.
configuration of the D-Gal

G-50 column chromatography gave a fraction (K, 0.01 to
0.05) which had an [α]D +99° (c 0.3, water) and on hydrolysis
yielded D-galactose and glycerol (1:1), identified by GLC-MS
of derived glycolic acids, the D-Gal being further character-
ized by GLC of its acetylated 2-[(R)-butyl glycoside derivatives.
Colorimetric phosphate analysis (6) showed the product to
contain 7.1% phosphorus, leading to the conclusion that the
capsular material was composed of equimolecular amounts of
D-galactose, glycerol, and phosphate. Colorimetric analysis
(15) indicated that the native CPS contains a total 1.0 M
O-acetyl substitution of the D-Galp residue.

Mild basic hydrolysis or dephosphorylation of the CPS with
cold 48% aqueous HF resulted in depolymerization products and
the quantitative chromatographic isolation of compound III, composed of D-galactose and glycerol (1:1), identified by GLC-MS
of derived glycolic acids, the D-Gal being further character-
ized by GLC of its acetylated 2-[(R)-butyl glycoside derivatives.

2D NMR studies of the polymeric O-deacylated CPS (Ta-
ble 2) confirmed the above proposed basic CPS structure. Further analysis of the NMR spectra of the native CPS revealed that the O-3 and O-4 positions of the D-Galp residues were acetylated to the extent of 40 and 60%, respectively. The positions of the O-acetyl substituents were inferred from a consideration of the chemical shifts in the NMR carbon and proton resonances about the α-D-Gal O-3 and O-4 positions.

TABLE 2. NMR spectral data for compound III from the CPS and the O-deacetylated CPS of A. pleuropneumoniae serotype 13

<table>
<thead>
<tr>
<th>Glycopeptide residue</th>
<th>NMR spectral data</th>
<th>NMR spectral data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-1/C-1</td>
<td>H-2/C-2</td>
</tr>
<tr>
<td>Compound III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-D-Galp-(1→)</td>
<td>5.15 (3.8)</td>
<td>3.82</td>
</tr>
<tr>
<td>−2)-Gro</td>
<td>97.5</td>
<td>67.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-deacetylated CPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-D-Galp-(1→)</td>
<td>5.19 (−3)</td>
<td>3.82</td>
</tr>
<tr>
<td>−[P]−1-Gro-(3→[P]−)</td>
<td>99.4 (175)</td>
<td>69.3</td>
</tr>
<tr>
<td></td>
<td>4.04</td>
<td>3.97 (9.30)</td>
</tr>
<tr>
<td></td>
<td>65.6</td>
<td>78.4 (5.0)</td>
</tr>
</tbody>
</table>

a Spectra were measured at 25°C (400 MHz) in D2O with an internal acetone reference (2.225 ppm for proton and 31.07 for carbon).

b Coupling constants (JH-1,H-2), and JH-1,H-2 in hertz are given in parentheses.

c Phosphate coupling (JH,P and 3JH,P) values are given in parentheses.

The structural occurrence of 1,3 poly(glycerol phosphates) (type I teichoic acids) is not unusual. They have been found in many bacterial species (21, 22, 29), and glycosyl substitution at the C-2 position of the monosaccharide units of glucose, galactose, rhamnose, and 2-acetamido-2-deoxyhexose have been demonstrated.

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REFERENCES


