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Structure of the capsular polysaccharide from *Actinobacillus pleuropneumoniae* serotype 10*

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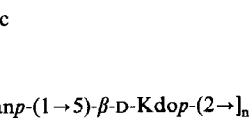
ABSTRACT

The capsular polysaccharide of *A. pleuropneumoniae* serotype 10 was found to be composed of a linear disaccharide repeating unit. By use of methylation analysis, partial hydrolysis, g.l.c.– and f.a.b.-m.s., and 1D and 2D n.m.r. studies, the polysaccharide was determined to be a polymer of a repeating disaccharide unit composed of D-mannose and 3-deoxy-D-*manno*-octulosonic acid (Kdo) having the structure:



INTRODUCTION

A. pleuropneumoniae is the etiologic agent of swine pleuropneumonia, meningitis, and arthritis[1]. It is a facultative anaerobic, gram-negative, coccobacillary rod-shaped bacterium which can be divided into biovar I, which is NAD-dependent, and biovar II, which is NAD-independent[2]. The bacteria are encapsulated by negatively charged carbohydrate polymers that are serotype specific[3]. Capsular polysaccharides are recognized as important bacterial virulence factors enhancing bactericidal serum resistance and preventing phagocytosis in the absence of specific antibodies.

To aid in the understanding of the pathogenesis, serology, and immunobiology of this bacterial infection, we have undertaken the structural characterization of the carbohydrate-containing antigens of the twelve known serotypes of *A. pleuropneumoniae*[4–8]. The structures of the capsular and *O*-specific polysaccharides from serotypes 1–8 (Refs. 9–14) and 12 (Refs. 15 and 16) have been reported. Recently the *O*-antigen of the serotype 10 was elucidated[17], and this paper reports the results of the structural analysis of the associated capsular polysaccharide of *A. pleuropneumoniae* serotype 10.

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RESULTS AND DISCUSSION

Isolation and characterization. — *A. pleuropneumoniae* serotype 10 capsular polysaccharide was obtained *via* its precipitated cetyltrimethylammonium complex from the 2.5% saline wash of fermentor-grown cells. On Sephadex G-50 gel-filtration chromatography the polysaccharide eluted as a broad peak at the void volume of the system (K_{av} 0.03) and, on DEAE-Sephacel ion-exchange chromatography, as a discrete homogeneous fraction in a sodium chloride gradient. The purified polysaccharide had $[\alpha]_D$ −17.9 (*c* 0.3, water) and was homogeneous with respect to neutral glycose and 3-deoxyoctulosonic acid. *Anal.* Found: C, 34.9; H, 4.7; N, 0.0; and ash, 0.0%.

Complete acid hydrolysis of the polysaccharide and g.l.c.–m.s. of the derived alditol acetates showed only 1,2,3,4,5,6-hexa-*O*-acetyl-D-mannitol, whereas trimethylsilylation of the reduced hydrolysis products revealed two additional components identified as 3-deoxy-D-*glycero*-D-*talo*-octono-1,4-lactone and 3-deoxy-D-*glycero*-D-*galacto*-octono-1,4-lactone[18], indicating the polysaccharide to be composed of D-mannose and Kdo residues.

The absolute configuration of D-mannose was established by g.l.c.–m.s. of its acetylated (*R*)-2-butyl glycosides[19] and that of 3-deoxy-D-*manno*-octulosonic acid from the specific optical rotation of its ammonium salt[20].

The ^{1}H-n.m.r. spectrum of the type 10 polysaccharide (Fig. 1) had signals for the geminal H-3 protons of Kdo at 2.51 (broad, 1 H) and 1.89 p.p.m. (broad, 1 H), and for the CH_3 of an *O*-acetyl group at 2.15 p.p.m. (s, 3 H). Three signals were present in the low-field region of the spectrum at 4.81 (s, 1 H), 4.50 (d, 1 H, J_{obs} 10 Hz), and 4.23 p.p.m. (d, broad, 1 H). After *O*-deacetylation of the polysaccharide, only one signal for an

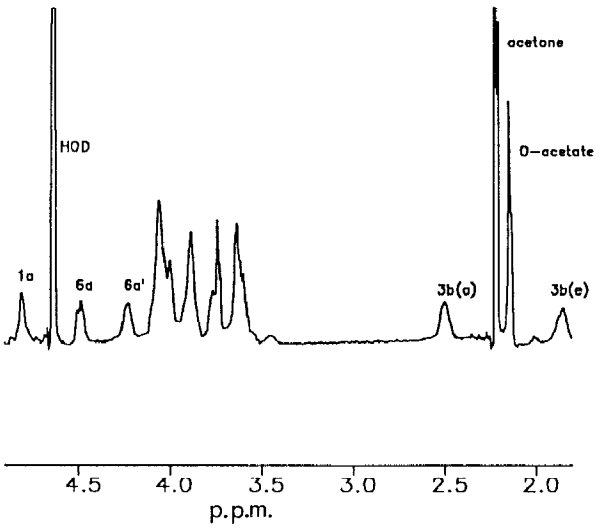


Fig. 1. ^{1}H-N.m.r. spectrum of the capsular polysaccharide of *A. pleuropneumoniae* serotype 10 recorded at 37°.

anomeric proton was observed in the low-field region of the ^{1}H-n.m.r. spectrum at 4.97 p.p.m. (s, 1 H); the two low-field doublets were absent from this spectrum suggesting the occurrence of specific deshielding effects due to substitution by *O*-acetate in the native polysaccharide.

The ^{13}C-n.m.r. spectrum of the native polysaccharide showed two resonances at 99.7 and 100.9 p.p.m. for the C-1 of mannose and the C-2 of Kdo, respectively. Diagnostic signals were also observed at 174.5 and 35.5 p.p.m. from C-1 and C-3 of Kdo, respectively, and at 21.1 p.p.m. from CH_3 of the *O*-acetate group. A ^{13}C-DEPT experiment on the native polysaccharide showed resonances for two methylene carbons at 62.4 and 64.8 p.p.m. On *O*-deacetylation of the polysaccharide, an upfield shift of the latter resonance to 62.7 p.p.m. suggested that the *O*-acetate substituent was located on either the C-6 of mannose or the C-8 of Kdo. This result is in agreement with the ^{1}H-n.m.r. data which showed the upfield shift of two proton resonances on *O*-deacetylation of the polysaccharide.

Methylation analysis of the native polysaccharide revealed two neutral components identified as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol (1:0.2) by g.l.c.–e.i.-m.s. analysis. The presence of the latter component suggested that some cleavage of the labile Kdo linkage had taken place, prior to methylation analysis, during isolation.

The combined analytical results were consistent with the type 10 polysaccharide being composed of a disaccharide repeating unit consisting of a 3-linked D-mannopyranosyl and a D-Kdo residue.

Isolation and characterization of the disaccharide repeating unit. — Mild hydrolysis of the polysaccharide with 1% acetic acid (2 h, 100°), followed by fractionation of the product on Bio-Gel P2, afforded a disaccharide **1**, for which t.l.c. showed it to be a mixture of two components; R_F 0.39 (major) and R_F 0.33 (minor). The f.a.b.-m.s. of **1**, in the positive-ion mode, showed three major peaks in the molecular-ion region at *m/z* 465 $[M + Na]^+$, 460 $[M + NH_4]^+$, and 433 $[M + H]^+$, together with a corresponding group of less abundant ions, 42 mass units lower, at *m/z* 423 $[M' + Na]^+$, 418 $[M' + NH_4]^+$, and 401 $[M' + H]^+$. An abundant A-type fragment ion at *m/z* 205 (ref. 21) indicated that the *O*-acetate group was located on a non-reducing terminal hexose residue, whereas cleavage at the other side of the glycosidic oxygen gave a fragment ion at *m/z* 221 indicative of a non-acetylated Kdo residue. These results are consistent with **1** being a mixture of the acetylated and non-acetylated forms of the disaccharide. However, since the presence of a single acetyl group in an oligosaccharide is known to enhance the sensitivity in f.a.b.-m.s.[21], the relative proportions of the two components of **1** could not be determined from the f.a.b.-m.s.

G.l.c.–m.s. analysis of methylated, carbonyl-reduced **1** gave an e.i.-m.s. consistent with the presence of a terminal non-reducing mannopyranosyl residue; *m/z* 219 (aA_1), and 187 (aA_2), and a fragmentation pattern which suggested the Kdo moiety was substituted at O-5; *m/z* 381, 162, and 130 (Fig. 2). On c.i.-m.s. analysis, methylated reduced **1** gave an abundant peak for the $[M + NH_4]^+$ ion at *m/z* 561, indicating a molecular weight of 543. Hydrolysis of methylated reduced **1** gave 1,5-di-*O*-ace-

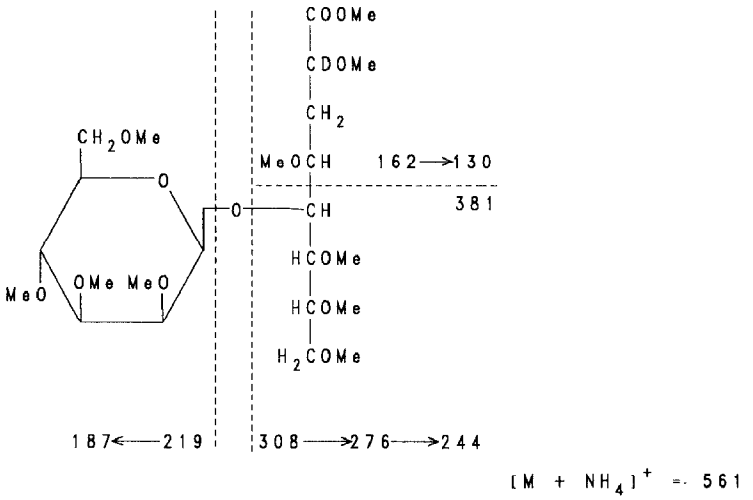


Fig. 2. G.l.c.–m.s. fragmentation patterns from the methylated reduced partial hydrolysis product **1**.

tyl-2,3,4,6-tetra-*O*-methylmannitol as the only detectable sugar by g.l.c.-m.s. analysis of the reduced and acetylated derivative.

The ^{1}H- and ^{13}C-n.m.r. spectra of carbonyl-reduced **1** were complex due to the presence of the two epimeric forms at C-2 of the Kdo residue. The ^{1}H-n.m.r. spectrum had signals for an anomeric proton at 4.87 p.p.m. (s, 1 H) and for the geminal H-3 protons of the Kdo residue at 2.26 (d, broad, 0.5 H, ^{2}J 13.0 Hz), 2.08 (d, broad, 0.5 H, ^{2}J 13.0 Hz) and 1.93 p.p.m. (dd, 1 H, ^{2}J 13.0, ^{3}J 9.4 Hz). The ^{13}C-n.m.r. spectrum had resonances at, *inter alia*, 100.2 (C-1 of mannose), 61.6 (C-6 of mannose), and twinned signals at 38.2/38.3 and 62.0/61.9 p.p.m. (C-3 and C-8 of Kdo, respectively).

The above results established **1** as a disaccharide with the structure,

D-Man*p*-(1→5)-D-Kdo
|
Ac

N.m.r. studies of the capsular polysaccharide. — The complete assignment of the ^{1}H- and ^{13}C-n.m.r. spectra of the native and *O*-deacetylated polysaccharides was achieved from COSY, relay COSY, and CHORTLE experiments (Tables I and II).

Detailed examination of the COSY spectra led to the identification of the subspectra corresponding to the mannose (residue **a**) and the Kdo (residue **b**) β-D-pyranosyl ring systems. The anomeric configurations of the Kdo*p* residues were established from the chemical shift and *J* values of the H-3 protons which indicated[22,23] the presence of β-linked Kdo*p* residues. The anomeric configurations of the mannose residues were determined as β from their $^{1}J_{C,H}$ value of 161 Hz[24]. Significant deshielding of the resonances for H-3a (4.04 p.p.m.) and C-3a (77.1 p.p.m.) indicated[25] substitution at O-3 of the mannose residue. Upfield shifts of H-6a and H-6′a (0.6 and 0.5 p.p.m.,

TABLE I

Proton chemical shifts[a] for the capsular polysaccharide of *A. pleuropneumoniae* serotype 10

Compound	*Residue b* →5)-β-D-*Kdo*p-(2→								*Residue a* 3)-β-D-*Man*p-(1→						
	H-3a	*H-3e*	*H-4*	*H-5*	*H-6*	*H-7*	*H-8*	*C-8'*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
Native capsular polysaccharide	1.889	2.508	3.766	4.073	3.610	4.059	3.886	3.886	4.814	4.007	4.042	3.634	3.642	4.504	4.233
O-Deacetylated capsular polysaccharide	1.87[b]	2.47[c]	3.77[d]	4.09	3.59	4.05	3.94	3.94	4.79	4.00	4.07	3.55	3.44	3.94	3.71

[a] Measured at 37° in p.p.m. from internal acetone (δ 2.225). [b] $J_{3a,3e}$ 13.0 Hz. [c] $J_{3e,4}$ 4.8 Hz. [d] $J_{3a,4}$ 10.0 Hz.

TABLE II

^{13}C Chemical shifts[a] for the capsular polysaccharide of *A. pleuropneumoniae* serotype 10

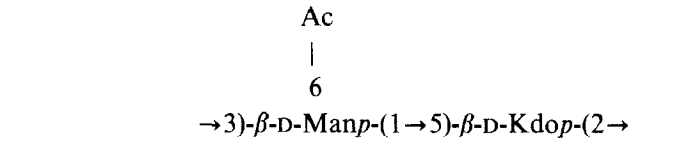
Compound	*Residue b* →5)-β-D-*Kdo*p-(2→								*Residue a* →3)-β-D-*Man*p-(1→						O-*Acetyl*	
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C=O*	CH_3
Native capsular polysaccharide	174.5	100.9[b]	35.5	67.8	76.7	73.3	65.9	62.4	99.7	70.2	77.1	66.3	74.2	64.8	175.0	21.1
O-Deacetylated capsular polysaccharide[c]	175.3	(—)[d]	35.2	68.4	76.8	73.5	66.1	62.4	99.6	70.9	77.2	66.1	74.1	62.7		

[a] Measured at 37°, in p.p.m. from internal acetone (δ 31.07). [b] Measured using a delay between pulses of 2 s. [c] Assigned with reference to the native polysaccharide and published data[25]. [d] No signal observed for C-2b (a recycle delay of only 1 s was employed).

respectively) (Table I) following *O*-deacetylation identified O-6 of the mannose residue as the position of substitution by the acetate group. The chemical shifts values for the C-1, C-2, and C-3 of the Kdo residue indicates it to be in the pyranosidic form[26,27]. Comparison of the ^{13}C-n.m.r. chemical shifts of the β-Kdo*p* residue with literature values for the unsubstituted analogue[20], confirmed the involvement of the C-5 ring atom in the glycosidic linkage since the resonance for C-5b experiences significant deshielding (~10.0 p.p.m.), while the adjacent C-4b and C-6b resonances are displaced upfield by approximately 1.0 p.p.m. The preferred $^5C_2$ conformation of Kdo*p* can also be determined from the ^{13}C chemical shifts. In this conformation C-3 of a Kdo derivative is shielded by the axially disposed hydroxyl group on C-5 (ref. 20).

The anomeric configuration of the β-D-mannopyranosyl residue and its position of linkage to the β-Kdo*p* residue was also confirmed from a 1D n.O.e. difference experiment. Thus, upon saturation of the anomeric proton of the mannosyl residue **a**, intraresidue n.O.e.'s were observed between H-1a and H-2a, H-1a and H-3a, and H-1a and H-5a. In addition, interresidue effects were observed between H-1a and H-5b and H-1a and the H-8b/H-8'b proton pair (Fig. 3). As it was evident from the ^{13}C-DEPT spectrum of the *O*-deacetylated polysaccharide that neither O-6 of the β-D-mannopyranosyl residue nor O-8 of the β-Kdo*p* residue were substituted (Table II), it can be concluded that the β-Kdo moiety is linked through position O-5.

Thus, the combined chemical and n.m.r. evidence leads to the conclusion that the capsular polysaccharide of *A. pleuropneumoniae* serotype 10 is a linear polymer of disaccharide repeating units having the structure,



The structure of the capsular polysaccharide of *A. pleuropneumoniae* serotype 10

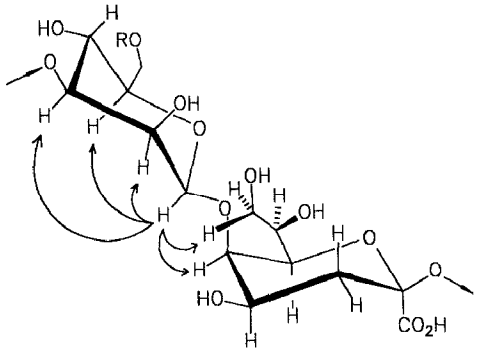


Fig. 3. Structure of *A. pleuropneumoniae* serotype 10 capsular polysaccharide repeating unit (R = Ac). Through-space connectivities were identified by a ^{1}H–^{1}H n.O.e. analysis on the *O*-deacetylated polysaccharide (R = H).

is similar to that of serotype 5a in that it contains Kdo residues and is composed solely of glycose units.

EXPERIMENTAL

Production of capsular polysaccharide. — *A. pleuropneumoniae* serotype 10 (NRCC 4265) was grown (yield 3 g wet weight/L), and its capsular polysaccharide was isolated as previously described[28]. Pure polysaccharide was obtained by gel filtration on Sephadex G-50, followed by ion-exchange chromatography on DEAE-Sephacel[28], from which it eluted as a single peak in a 0–0.5M NaCl gradient.

Analytical methods. — Quantitative colorimetric methods used were the phenol–sulphuric acid for neutral glycose[29] and the periodate oxidation–thiobarbituric acid method for 3-deoxy-2-octulosonate[30].

Analytical g.l.c.–m.s. was done with a Hewlett–Packard 5885B system fitted with a flame-ionization detector and an OV-17 fused silica column (Quadrex Corp.). The following programs were employed: *A* (for alditol acetates) 180° for 2 min, then 4°/min to 240°; *B* (for partially methylated alditol acetates) 180° for 2 min, then 2°/min to 240°; *C* (for acetylated (*R*)-2-butyl glycoside derivatives) 180° for 2 min, then 6°/min to 240°; *D* (for methylated disaccharide alditols) 180° to 330° at 10°/min.

F.a.b.-m.s. analyses were carried out using a JEOL AX505H double-focusing mass spectrometer operating at an accelerating voltage of 3 kV and a mass resolution of 1500. Samples were dissolved in water and dried onto the stainless steel probe tip. A 1:1 glycerol–thioglycerol or 5:1 dithioerythritol–dithiothreitol mixture was then applied to the probe tip prior to f.a.b. analysis, and a Ze atom beam of 6 keV was used to sputter and ionize the sample. Spectra were calibrated with Ultramark 1621.

For analysis of constituent glycose, samples (1 mg) were hydrolyzed with 4M TFA (0.5 mL) for 1 h at 125° or, for Kdo residues, with 0.5M HCl (0.5 mL) for 3 h at 80°.

For the determination of the configuration of the Kdo residues, the polysaccharide (20 mg) was hydrolyzed (0.5M TFA for 6 h at 80°), and the products were separated on a column of Bio-Gel P2. The periodate oxidation–thiobarbituric acid-positive[30] fractions were pooled and purified using ion-exchange chromatography[20] (Bio-Rad AG1-X10, carbonate form). The acidic component was eluted from the column with 0.5M ammonium hydrogencarbonate, and the inorganic salt was removed by repeated lyophilization from water. The residue was recrystallized twice from ethanol–water to give ammonium 3-deoxy-D-*manno*-octulosonate which had $[\alpha]_D$ +31°. An authentic sample of ammonium 3-deoxy-D-*manno*-octulosonate[31] had $[\alpha]_D$ +38°.

T.l.c. was performed on silica gel (E. Merck) with 6:3:1 (v/v) propanol–conc. NH_3–water.

Methylation analysis. — Samples (1–2 mg) were methylated according to the Hakomori procedure[32], and the methylated products were recovered by dichloromethane extraction against water (5 × 1 mL). Methylated samples were hydrolyzed (4M TFA, 1 h, 125°), evaporated to dryness, and the residues, in water (0.5 mL), were reduced with sodium borodeuteride (1 h, 22°). After acidification with 10% acetic acid

in methanol, the solutions were concentrated, co-evaporated with methanol (5 × 1 mL) to remove borate, and acetylated with 1:1 pyridine–acetic anhydride (0.4 mL) for 30 min at 100°.

Partial hydrolysis. — Polysaccharide (27 mg) in 1% acetic acid was heated for 2 h at 100°. The solution was freeze-dried, and product **1** was fractionated on Bio-Gel P-2 (yield 13.4 mg).

N.m.r. spectroscopy. — All spectra were obtained on solutions in D_2O at 37° using a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer, operating in the pulsed F.-t. mode.

Proton spectra, recorded at 500 MHz, were obtained using a spectral width of 2.0 kHz, a 16K data block, and a 90° pulse. Chemical shifts are expressed relative to internal acetone (2.225 p.p.m.). N.O.e. difference spectra[33] were obtained using a selective low-power presaturation pulse applied to the proton resonance for 200 ms followed by a 90° observation pulse[34].

Broadband proton-decoupled ^{13}C spectra were obtained at 125 MHz with a spectral width of 31 kHz, using a 32K data set and a 90° pulse employing WALTZ decoupling[35]. The spectrum of the native polysaccharide was measured using a relaxation delay of 2 s between pulses to enhance the intensity of quaternary carbons. DEPT spectra[36] were obtained with broadband proton decoupling, a 135° proton pulse, and a 3.3 ms delay between pulses. Heteronuclear $^1J_{C,H}$ values were measured using gated decoupling[37], and all spectra were referenced relative to internal acetone (31.07 p.p.m.).

Two-dimensional homonuclear COSY and relay COSY were carried out as previously described[11], and the data were processed to give magnitude spectra[38].

General methods. — Commercial reagents and solvents were analytical grade. Concentrations were carried out under reduced pressure at $<40°$. Optical rotations were determined at 22° in 10-cm microtubes using a Perkin–Elmer model 243 polarimeter.

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