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Characterization of the *O*-polysaccharide of *Pasteurella haemolytica* serotype A1 *

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ABSTRACT

Lipopolysaccharide was isolated from *Pasteurella haemolytica* biotype A, serotype 1 by using the phenol–water extraction procedure. Hydrolysis with mild acid afforded a high-molecular-weight antigenic *O*-chain. On the basis of 1D and 2D NMR spectral studies and microanalytical chemical methods, the *O*-polysaccharide was determined to be a linear polymer of a trisaccharide repeating unit having the structure

→ 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Gal*p*-(1 →

This *O*-polysaccharide antigen is expressed by several *P. haemolytica* biotype A serotypes.

INTRODUCTION

Pasteurella haemolytica is a Gram-negative pathogen which is associated with several diseases of domestic animals. Two distinct biotypes of this pathogen are recognised (A and T), which are further subdivided into sixteen serotypes[1]. Biotype A, serotype 1 (i.e., serotype A1) is most frequently associated with pneumonic pasteurellosis (shipping fever) in feedlot cattle, a complex disease state initiated by stress and predisposing viral infections of the respiratory tract[2]. Shipping fever pneumonia is a source of considerable economic loss in the North American cattle industry[3], and several vaccines containing *P. haemolytica* or components of the bacterium are currently marketed as an aid in the prevention of the disease.

The pathogenesis of *P. haemolytica* infections is not well understood, although several virulence factors including capsular polysaccharide[4], leukotoxin[5,6], and lipopolysaccharide (LPS)[7–9] have been identified. Serotype specificity is related to

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differences in the capsular polysaccharide antigens[10]. *P. haemolytica* LPS is also implicated as a major antigenic determinant[11], and antigenic similarities among the LPS of several *P. haemolytica* serotypes have been observed[12–17].

There would appear to be significant differences in the LPS expressed by biotypes A and T. *P. haemolytica*. Biotype A serotypes have been reported[10] to produce predominantly low-molecular-weight, rough-type LPS, whereas biotype T strains elaborate high-molecular-weight, smooth-type LPS having extensive *O*-chains[15,18,19]. Recently a high-molecular-weight *O*-polysaccharide component was detected in the LPS from serotype A1[20], and immunological studies have indicated several biotype A strains (including serotype A1) to share common *O*-polysaccharide epitopes[17]. In this paper we report the structure of the LPS *O*-polysaccharide of *P. haemolytica* serotype A1.

RESULTS AND DISCUSSION

Isolation and purification of the O*-polysaccharides of* P. haemolytica *Serotype A1.*—Extraction of *P. haemolytica* serotype A1 cells (240 g, wet weight) by the modified hot aqueous phenol method[21] afforded, after ultracentrifugation, a pure LPS (1.99 g) from the dialyzed and concentrated aqueous phase. SDS-PAGE analysis of the LPS, visualized by silver staining[22], showed heavily stained fast-migrating bands representative of low-molecular-weight rough LPS containing a lipid A-core oligosaccharide complex. The characteristic ladder pattern of high-molecular-weight smooth LPS was very faint, indicating the preparations to be predominantly R-type LPS.

The LPS (100 mg) was partially hydrolysed with hot dilute acetic acid to afford an insoluble lipid A (~ 23%) and a water-soluble product. The latter was concentrated and subjected to gel-filtration chromatography on Sephadex G-50 (Fig. 1), and three fractions were collected. Colorimetric analysis of the eluent indicated that the low-molecular-weight fraction (K_{av} 0.97, 34%) contained 3-deoxy-D-*manno*-2-octulosonic acid (Kdo)[23], whereas the core oligosaccharide (K_{av} 0.65, 23%), contained neutral aldose[24] and Kdo.

The high-molecular-weight fraction, eluting in the void volume of the column (K_{av} 0.03, 5%), contained both neutral aldose and aminoglycose[25] which were identified as galactose and 2-amino-2-deoxy-galactose residues by GLC of the alditol acetate derivatives of the acid hydrolysis products[26]. ^{1}H NMR spectroscopy revealed contamination of this fraction with capsular polysaccharide, which was removed by anion-exchange chromatography. Pure *O*-polysaccharide eluted from a column of DEAE-Sephacel as a single peak with the neutral buffer (pH 7.2) in ~ 1% yield.

Characterization of the O*-polysaccharide (O-PS).*—The *O*-PS of *P. haemolytica* serotype A1 was freely soluble in water and had $[\alpha]_D$ 24.5° (*c* 1.2, H_2O). Total acid hydrolysis of the polymer (2 M trifluoroacetic acid, 12 h, 100°C), followed by GLC analysis of the reduced and acetylated products[26], gave 1,2,3,4,5,6-hexa-*O*-

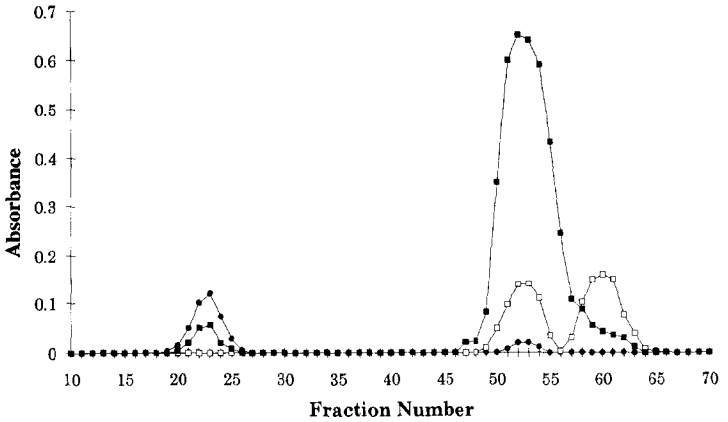

Fig. 1. Sephadex G-50 gel-filtration chromatography of the water-soluble acetic acid hydrolysis products from *P. haemolytica* serotype A1. Aliquots were analysed for neutral glycose (■), hexosamine (●), and Kdo (□).

acetylgalactitol (T_{GA} 1.01) and 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-galactitol (T_{GA} 1.41) in a molar ratio of ~2:1. The absolute configurations of the glycoses were established from the characteristic GLC retention times of their trimethylsilylated (*R*)-2-butyl glycosyl derivatives[27]. In this way the galactosyl and 2-amino-2-deoxy-galactosyl residues were each assigned to the D-series.

Glycosyl linkage analysis.—The methylated[28] *O*-polysaccharide afforded products that, after hydrolysis, reduction ($NaBH_4$), and acetylation, furnished valuable mass spectral data on the positions of the linkages of the component glycose residues (Table I). From this analysis it can be inferred that the *P. haemolytica* serotype 1 *O*-PS is composed of →3)-D-Gal*p*-(1→, →4)-D-Gal*p*-(1→, and →3)-D-Gal*p*NAc-(1→ structural units that form a linear repeating trisaccharide unit.

Glycosyl sequence determination.—High-resolution NMR techniques were employed to establish the sequence of the glycosyl residues. The general approach involved the initial complete assignment of the ^1H and ^{13}C NMR spectra of the

TABLE I

Methylation analysis data for the *P. haemolytica* serotype A1 *O*-polysaccharide.

Methylated glycose derivative [a]	T_{GM} [b]	Mole ratio [c]
2,4,6-Tri-*O*-methyl-D-galactose	1.01	1.00
2,3,6-Tri-*O*-methyl-D-galactose	1.04	1.15
2-Deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactose	1.88	0.78

[a] Determined as their [1-^{2}H]alditol acetates. [b] Retention times relative to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol-1-*d*, using GLC program *A*. [c] Uncorrected detector response relative to 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol.

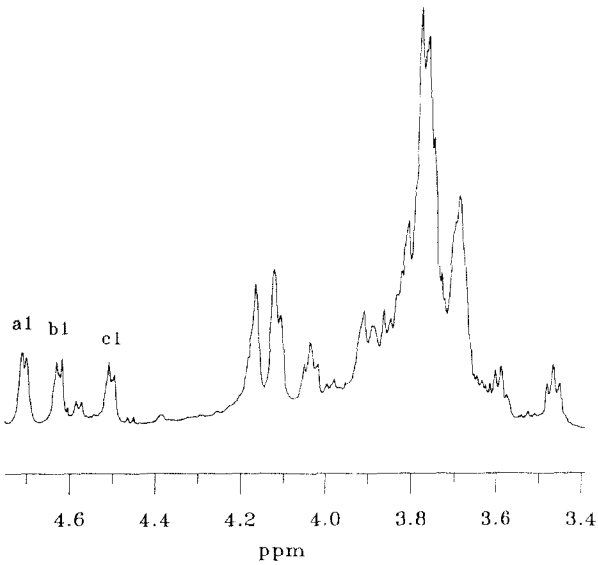

Fig. 2. ^{1}H NMR spectrum of the ring proton region for *P. haemolytica* serotype A1 LPS *O*-polysaccharide.

intact polysaccharide from which the relative stereochemistry, ring size, and anomeric configuration of the component glycoses were identified.

The ^{13}C NMR spectrum showed three resonances for the anomeric carbons of the constituent monosaccharides, clearly indicating the polysaccharide to be composed of regular trisaccharide repeating units. Diagnostic ^{13}C resonances were observed at 52.2 ppm from C-2 of the 2-amino-2-deoxy-D-galactosyl residues, and signals at 176.2 and 23.0 ppm were indicative of the aminoglycosyl residues being present as their acetamido derivatives[29].

Correspondingly, the ^{1}H NMR spectrum of the *O*-polysaccharide revealed three major resonances of equal signal area for the anomeric protons in the low-field region at 4.70 (d, ~ 1 H, $J_{1,2}$ 8.6 Hz), 4.62 (d, ~ 1 H, $J_{1,2}$ 7.6 Hz), and 4.50 ppm (d, ~ 1 H, $J_{1,2}$ 7.9 Hz) (Fig. 2), together with the characteristic high-field resonance at 2.04 ppm (s, 3 H) from the methyl protons of the *N*-acetyl groups of the 2-acetamido-2-deoxy-D-galactosyl residues. Assignment of the proton resonances was made from two dimensional homonuclear chemical shift correlation experiments (COSY[30]) and NOE measurements[31]. From the COSY experiment, subspectra corresponding to each of the three glycosyl residues were identified, the chemical shift[32] and the vicinal $^{1}H-^{1}H$ coupling constant values[33] being typical of pyranosyl units possessing the *β*-D-*galacto* configuration. The residues were labelled **a**, **b** and **c** according to decreasing order of the chemical shifts from the H-1 resonances[34]. The observed ^{1}H chemical shifts and coupling constants for the polysaccharide are given in Table II. The cross-peaks relating the H-4 and H-5 resonances in the COSY were of very low intensity and difficult to identify;

TABLE II

^{1}H NMR chemical shifts and ring proton coupling constants [a] (Hz) for the *P. haemolytica* serotype A1 *O*-polysaccharide

Residue	Glycosyl residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6	H-6′
a	→3)-β-D-Gal*p*NAc-(1→	4.70 (8.6)	4.03 (9.7)	3.90 (3.1)	4.16 (≤1)	3.67	3.74	3.82
b	→4)-β-D-Gal*p*-(1→	4.62 (7.6)	3.46 (9.3)	3.76 (3.4)	4.10 (≤1)	3.72	3.75	3.80
c	→3)-β-D-Gal*p*-(1→	4.50 (7.9)	3.67 (9.6)	3.77 (3.5)	4.12 (≤1)	3.68	3.75	3.80

[a] Observed first-order chemical shifts and coupling constants (Hz) measured at 27°C in D_2O.

however, the respective H-5 assignments were verified from NOE measurements (see below). Unambiguous assignment of the corresponding ^{13}C resonances was effected by correlation with the ^{1}H resonances of the directly attached protons by heteronuclear ^{13}C–^{1}H chemical shift correlation experiments[35] and the data is recorded in Table III.

A direct ^{1}H–^{13}C correlation between the resonances of H-2**a** (4.03 ppm) and C-2**a** (52.2 ppm) permitted **a** to be identified as the 2-acetamido-2-deoxy-D-galactose unit[36], while the residues **b** and **c** correspond to the two D-galactose residues revealed by chemical analysis. In agreement with the methylation analysis data, significant deshielding of the ^{13}C resonances assigned to C-3**a**, C-4**b**, and C-3**c** (Table III) indicated those positions as the sites of the glycosyl linkages[29].

The arrangement of three glycosyl residues within the repeating unit was determined from interresidue ^{1}H–^{1}H NOE measurements[31], which also served to confirm the positions of the glycosidic linkages (Table IV). NOE measurements were made using a 2D phase-sensitive NOESY experiment[37].

The NOEs were employed qualitatively to establish short (≤ 3 Å) through-space connectivities between the anomeric and aglyconic protons of the adjacent glycosidically linked residues. In addition, the occurrence of intraresidue NOEs between H-1 and H-3, and between H-1 and H-5 within the pyranose ring systems of

TABLE III

^{13}C NMR chemical shifts for the *P. haemolytica* serotype A1 *O*-polysaccharide

Residue	Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
a	→3)-β-D-Gal*p*NAc-(1→	103.2	52.2	80.7	68.7	75.4	61.4
b	→4)-β-D-Gal*p*-(1→	105.2	70.6	73.2	76.8	74.0	61.9
c	→3)-β-D-Gal*p*-(1→	105.3	70.7	82.5	69.3	75.4	61.9

[a] Determined by ^{13}C–^{1}H chemical shift correlation at 27°C in D_2O.

TABLE IV

Proton NOE data for the *P. haemolytica* serotype A1 *O*-polysaccharide [a]

Anomeric proton	Observed proton		Partial sequence	Linkage site
	Intraresidue NOE	Interresidue NOE		
1a	**3a**, **5a**	**4b**	**a** → **b**	O-4 (**b**)
1b	**3b**, **5b**	**3c**	**b** → **c**	O-3 (**c**) [b]
1c	**3c**, **5c**	**3a**	**c** → **a**	O-3 (**a**)

[a] Measured from the NOESY spectrum of the *O*-polysaccharide at 27°C in D_2O. [b] Confirmed from methylation analysis data in Table I.

each of the glycosyl residues was indicative of their assigned β-configurations. Thus, the anomeric proton resonance (4.70 ppm) of the 2-acetamido-2-deoxy-β-D-galactopyranose residue **a**, showed strong NOE connectivities within the pyranose ring system to H-5**a** (3.67 ppm) and H-3**a** (3.90 ppm), and across the glycosidic bond to H-4**b** (4.10 ppm) of the D-galactopyranosyl residue **b**. The connectivity established by the transglycosidic NOE (H-1**a**–H-4**b**), indicates the partial sequence **a** → **b** and further suggests that the 2-acetamido-2-deoxy-β-D-galactopyranosyl residue is linked to the O-4 position of the β-D-galactopyranosyl unit. Because of the overlapping H-3**b** and H-3**c** resonances (~ 3.76 ppm), interpretation of the observed NOE connectivity to H-1**b** (4.62 ppm) was ambiguous. However, the occurrence of interresidue NOE relating the H-1**c** (4.50 ppm) and H-3**a** (3.90 ppm) resonances was sufficient to establish the linear sequence of glycoses within the repeating trisaccharide unit as, → **c** → **a** → **b** → .

The accumulated evidence from compositional, methylation and NMR analyses permit the repeating unit of the *P. haemolytica* serotype A1 *O*-polysaccharide to be assigned the structure,

→ 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Gal*p*-(1 →

A recent comparison of the electrophoretic profiles of *P. haemolytica* LPS using specific immunoblotting detection techniques has indicated that most biotype A serotypes produce a common, highly immunogenic *O*-polysaccharide[17]. In this study, the *O*-polysaccharide components from three serotypes (serotypes A1, A6, and A9) were also shown to be identical from comparison of their ^1H NMR spectra. The results of the present investigation establish the structure of this common *P. haemolytica* antigen.

EXPERIMENTAL

Isolation and purification of the lipopolysaccharide (LPS) from P. haemolytica *serotype A1.—P. haemolytica* serotype A1 (NRCC 4121), obtained from the Veterinary Infectious Diseases Organization (VIDO), Saskatoon, SK, Canada, were grown in a fermenter (28 L, Microferm, New Brunswick Scientific) with a medium

of brain heart infusion broth (3.7%, Difco) at 37 ± 1°C for 18 h. Cells were then killed by the addition of phenol (0.75% w/v, final concentration) and were harvested using a Sharples continuous centrifuge. Collected cells (240 g) were washed with 2.5% saline, digested with lysozyme, ribonuclease, and deoxyribonuclease[14], and then extracted with hot aqueous phenol[21]. LPS was recovered from the dialyzed and concentrated aqueous layer of the extract by ultracentrifugation (105 000*g*, 4°C, 12 h) as the precipitated gel, then subjected to repeated ultracentrifugation from 2.5% saline solutions until judged to be pure by the carbocyanine dye assay[38]. Purified LPS gels were suspended in distilled water and lyophilization gave 1.99 g of LPS.

O-Polysaccharide.—A suspension of the LPS (100 mg) in aqueous acetic acid (2% v/v, 100 mL) was heated at 100°C for 2 h and the precipitated lipid A (~ 23%) was removed from the cooled solution by low-speed centrifugation. The supernatant was lyophilized, and the residue was fractionated by gel-filtration chromatography on a Sephadex G-50 column (Pharmacia, 3.5 × 100 cm) using pyridinium acetate (0.05 M, pH 4.7) as the mobile phase. The column eluant was continuously monitored for changes in refractive index using a Waters R403 differential refractometer, and fractions (10 mL) were collected and analyzed colorimetrically for neutral aldose[24], aminoglycose[25], and Kdo[23]. The gel filtration properties of the eluted materials are expressed in terms of their distribution coefficients, $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the specific material, V_0 is the void volume, and V_t the total volume of the system.

The *O*-PS was further purified by ion-exchange chromatography. The sample (3 mg), dissolved in water (1 mL), was applied to a column (1.2 × 35 cm) of DEAE-Sephacel (Pharmacia), equilibrated with 0.05 M Tris-HCl buffer (pH 7.2). The column was eluted with the same buffer (40 mL) to obtain the *O*-PS. Fractions (1 mL) were collected, assayed as above, and dialysed against distilled water to give the purified *P. haemolytica* serotype A1 *O*-PS.

Analytical methods.—Quantitative determination of constituent glycoses involved analysis of the derived alditol acetates[27]. Samples of polysaccharide (0.5 mg) were hydrolysed with trifluoroacetic acid (2 M, 0.5 mL) for 12 h at 100°C, followed by concentration under reduced pressure. The products were reduced ($NaBD_4$) and acetylated (acetic anhydride) as previously described[39], and analysed by GLC using program *A*. Absolute configurations of the glycoses were determined by GLC–MS (program *B*) of the corresponding per-*O*-trimethylsilyl (*R*)-2-butyl glycoside derivatives[27]. The identity of the glycose derivatives was established by comparison of their GLC retention times and mass spectra with those of authentic reference compounds. The galactosamine residues were *N*-acetylated prior to silylation[40].

Analytical GLC–MS was performed using a Hewlett–Packard model 5958B gas chromatograph–mass spectrometer, fitted with a DB-17 fused silica capillary column in the electron impact (EI) mode using an ionization potential of 70 eV. The following temperature programs were employed. *A*, (for alditol acetates and

partially methylated alditol acetates) 180°C for 2 min then 4°C/min to 240°C. B, (for trimethylsilylated (*R*)-2-butyl glycoside derivatives) isothermal at 175°C. Retention times are quoted relative to D-glucitol hexaacetate (T_{GA}) or 1,4,5-tri-*O*-acetyl-2,3,5-tri-*O*-methyl-D-glucitol (T_{GM}).

Methylation analysis[28].—To a sample of polysaccharide (2–3 mg) dissolved in Me_2SO (0.2 mL), powdered NaOH (20–30 mg) and iodomethane (0.2 mL) were added, and the mixture was stirred for 60 min at 20°C. The iodomethane was removed under a stream of N_2, and the methylated products were recovered by partitioning between CH_2Cl_2 and water (five times). Methylated and reduced samples were hydrolyzed with trifluoroacetic acid (0.5 mL, 2 M) for 12 h at 100°C, and the products were analysed by GLC–MS of their acetylated alditol derivatives.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).—LPS samples (1 μg) were analysed in 14% polyacrylamide gels by electrophoresis in the presence of 2% SDS. Bands were detected using the silver-staining method of Tsai and Frasch[22].

NMR spectroscopy.—Spectra were obtained with solutions of polysaccharides (2.5 mg/mL) in D_2O at 27°C using a Bruker AMX 600 spectrometer. ^1H spectra, recorded at 600 MHz, were obtained using a spectral width of 2.6 kHz and a 90° pulse. Chemical shifts are expressed relative to internal acetone (2.225 ppm). Broad-band decoupled ^{13}C spectra (150 MHz) were obtained using a spectral width of 33 kHz and a 90° pulse employing WALTZ decoupling[41]. Chemical shifts are expressed relative to internal acetone (31.07 ppm).

Homonuclear 2D chemical-shift-correlated spectroscopy (COSY[30]) and nuclear Overhauser enhancement spectroscopy (NOESY[31]), were carried out as previously described[39], using the conventional pulse sequences. Data was acquired over the full spectrum (sweep width 2600 Hz), or for the ring protons region (1250 Hz). Thirty-two scans were collected for each t_1 value, and a mixing time of 200 ms was employed for the NOESY experiment.

Two-dimensional heteronuclear carbon–proton chemical shift correlations were measured in the ^1H-detected mode via multiple quantum coherence [$^1H(^{13}C)$ HMQC] with a Bruker 5-mm inverse broad-band probe using reverse electronics. The HMQC experiment employed the pulse sequence described by Bax[35], and ^{13}C decoupling during ^1H acquisition was achieved using the GARP-1 composite pulse sequence[42].

General methods.—Concentrations were made under reduced pressure below 40°C. Optical rotations were determined at 20°C in 10-cm microtubes using a Perkin–Elmer 243 polarimeter.

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REFERENCES

1 L. Fodor, J. Varga, I. Hajtos, W. Donachie, and N.J.L. Gilmour, *Res. Vet. Sci.*, 44 (1988) 399.
2 G.M. Frank, R.E. Briggs, and K.G. Gillette, *Am. J. Vet. Res.*, 48 (1987) 1674–1677.
3 S.W. Martin, A.H. Meek, D.G. Davis, R.G. Thompson, J.A. Johnson, A. Lopez, L. Stephens, R.A. Curtis, J.F. Prescott, S. Rosendal, M. Savan, A.J. Zubaidy, and M.R. Bolton, *Can. J. Comp. Med.*, 44 (1980) 1–10.
4 A.W. Confer, K.R. Simons, R.J. Panciera, N.J. Mort, and D.A. Mosier, *Am. J. Vet. Res.*, 50 (1989) 98–105.
5 C.S. Baluyet, R.R. Simonson, W.J. Bemrick, and S.K. Maheswaran, *Am. J. Vet. Res.*, 42 (1981) 1920–1926.
6 P.E. Shewen and B.N. Wilkie, *Infect. Immun.*, 35 (1982) 91–94.
7 A.W. Confer and K.R. Simons, *Am. J. Vet. Res.*, 47 (1986) 154–157.
8 R.L. Rimsay, J.E. Coyle-Dennis, L.H. Lauerman, and P.G. Squire, *Am. J. Vet. Res.*, 42 (1981) 2134–2138.
9 R.F. Slocombe, M. Mulks, and C.R. Killingsworth, *Am. J. Vet. Res.*, 51 (1990) 433–438.
10 C. Adlams, in C. Adlam and J.M. Rutter (Eds.), *Pasteurella and Pasteurellosis*, Academic Press, London, 1989, pp 75–92.
11 A.W. Confer, R.J. Panciere, and D.A. Mosier, *Am. J. Vet. Res.*, 47 (1986) 1134–1138.
12 C. Burrells, H.B. Evans, and A.M. Dawson, *Vet. Microbiol.*, 8 (1983) 187–198.
13 W. Donachie, N.J. Gilmour, D.L. Mould, and I.R. Poxton, *J. Gen. Microbiol.*, 130 (1984) 1209–1216.
14 J.A. Durham, S.M. Antone, M.W. Cunningham, and A.W. Confer, *J. Clin. Microbiol.*, 26 (1989) 885–889.
15 J.C. Richards and R.A. Leitch, *Carbohydr. Res.*, 186 (1989) 275–286.
16 C.F. Wilson, A.D. Sutherland, L. Inglis, and W. Donachie, *Vet. Microbiol.*, 31 (1992) 161–168.
17 R.P. Lacroix, J.R. Duncan, R.P. Jenkins, R.A. Leitch, J.A. Perry, and J.C. Richards, *Infec. Immun.*, in press.
18 M.B. Perry and L.A. Babiuk, *Can. J. Biochem. Cell Biol.*, 62 (1984) 108–114.
19 R.A. Leitch and J.C. Richards, *Biochem. Cell. Biol.*, 66 (1988) 1055–1065.
20 R.L. Davies, Q.A.R. Parton, J.G. Cook, A. Gibbs, and J.H. Fraser, *FEMS Microbiol. Let.*, 90 (1991) 23–28.
21 K.G. Johnson and M.B. Perry, *Can. J. Microbiol.*, 22 (1976) 29–34.
22 C.M. Tsai and C.E. Frasch, *Anal. Biochem.*, 119 (1982) 115–119.
23 Y.D. Karkhanis, J.Y. Feltner, J.J. Jackson, and J.D. Carlo, *Anal. Biochem.*, 85 (1978) 595–601.
24 M. Dubois, K.A. Gillies, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
25 R. Gatt and E.R. Berman, *Anal. Chem.*, 15 (1965) 167–171.
26 S.W. Gunner, J.K.N. Jones, and M.B. Perry, *Can. J. Chem.*, 39 (1961) 1892–1895.
27 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
28 I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
29 K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
30 A. Bax, R. Freeman, and G. Morris, *J. Magn. Reson.*, 42 (1981) 164–168.
31 J.M. Prestegard, T.A.W. Koerner, Jr., P.C. Demou, and R.K. Yu, *J. Am. Chem. Soc.*, 104 (1982) 4993–4995; M.A. Bernstein and L.D. Hall, *J. Am. Chem. Soc.*, 104 (1982) 5553–5555.
32 K. Bock and H. Thogersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
33 C. Altona and C.A.G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–429.
34 M.A. Bernstein, L.D. Hall, and S. Sukumar, *Carbohydr. Res.*, 103 (1982) C1–C6.
35 A. Bax, R.H. Griffey, and B.L. Hawkins, *J. Magn. Reson.*, 55 (1983) 301–315.
36 D.R. Bundle, H.J. Jennings, and I.C.P. Smith, *Can. J. Chem.*, 51 (1973) 3812–3819.
37 A. Kumar, R.R. Ernst, and K. Wuthrich, *Biochem. Biophys. Res. Commun.*, 95 (1980) 1–6.
38 J. Janda and E. Work, *FEBS Lett.*, 16 (1971) 343–345.
39 W.B. Severn and J.C. Richards, *Carbohydr. Res.*, 206 (1990) 311–332.
40 B. Kozulic, B. Ries, and P. Mildner, *Anal. Biochem.*, 94 (1979) 36–39.
41 A.J. Shaka, J. Keeler, F. Frenkiel, and R. Freeman, *J. Magn. Reson.*, 52 (1983) 335–338.
42 A.J. Shaka and J. Keeler, *J. Prog. NMR Spectrosc.*, 19 (1987) 47–129.