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Note

Identification of a capsular polysaccharide from *Moraxella bovis*

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Abstract—The bacterium *Moraxella bovis* is the causative agent of an economically important disease of cattle: Infectious Bovine Keratoconjunctivitis (IBK), otherwise known as pinkeye. Little is known regarding the structure of the carbohydrates produced by *M. bovis*. The structure of a capsular polysaccharide from *M. bovis* (strain Mb25) has been determined using NMR and MS analysis. From these data it is concluded that the polysaccharide is composed of the unmodified chondroitin disaccharide repeat unit.

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

Keywords: Capsular polysaccharide; *Moraxella bovis*; NMR spectroscopy; Mass spectrometry; Structural characterisation

Moraxella bovis is a Gram-negative bacillus, and is generally regarded as the etiological agent of infectious bovine keratoconjunctivitis (IBK), an economically important disease that affects cattle the world over.[1–3] Chronic IBK can result in severe keratoconjunctivitis, ocular rupture and blindness. IBK affects approximately 10% of cattle in Australian feedlots, and results in many tens of millions of dollars loss in national production.[3]

Virulence factors for *M. bovis* include pili, outer membrane proteins and secretion of hemolysin/cytotoxin.[2] The degree of strain-to-strain variation in virulence factors is unclear, although the genetic basis of the serological differences of pilus has been elucidated.[4] One epidemiological study examined variation in protein and LPS. Only three variants of LPS were observed[2] based on migration on SDS-PAGE, although no structural studies have been performed that we are aware of. Very little is known regarding the structure of *M. bovis*[2,5] LPS except that it appears to lack the repeating O-antigen structure found in bacteria such as *Escherichia coli*, and should therefore more correctly be called lipo-oligosaccharide (LOS). Similarly, very little is known, about the structure of other carbohydrates produced by *M. bovis*[5] or their involvement in pathogenicity, although there is one passing reference to a strain of *M. bovis* being capsulated,[6] but there are no reports that we are aware of that characterisation of the polysaccharide capsule has been performed.

As an initial step towards investigating the role of carbohydrates in *M. bovis* (strain Mb25) host interactions, we sought to purify and determine the structure of the LOS (to be reported elsewhere). While attempting to isolate LOS from *M. bovis* via a modified hot-phenol–water extraction process[7] polymeric carbohydrate was isolated that is consistent with expression of a capsular polysaccharide. We here describe the isolation and structural characterisation of the capsular polysaccharide associated with *M. bovis* strain Mb25.

Bacteria were harvested after overnight culture on agar and cell-associated carbohydrates were visualised

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by silver staining after electrophoretic separation using tricine-SDS-PAGE. A single band was observed in proteinase K-treated samples, indicating that *M. bovis* strain Mb25 does not express O-antigen, and would therefore be regarded as R-form or LOS. The extraction solvent of 90% phenol–chloroform–petroleum ether[8] (2:5:8) is currently being used to isolate the more lipophilic LOS R-form from *M. bovis*.

In attempting to isolate this LOS using a hot water–phenol procedure,[7,9] a polymeric carbohydrate was isolated, then heated at 100 °C for 2 h in the presence of 1% glacial acetic acid. The intended use of the mild acid hydrolysis was to cleave Lipid A at the Kdo residue (acidic sugar) from the LOS. We had already established that Mb25 does not express a polymeric O-antigen on its LPS, therefore we concluded that the carbohydrate is capsular polysaccharide. Analysis of MALDI MS methylation data indicated that the polysaccharide was present primarily as di- and tri-(Glc*p*A-Gal*p*NAc)-repeat disaccharide units (see Fig. 1). The mild acid hydrolysis had evidently resulted in specific cleavage of the Gal*p*-NAc-Glc*p*A bond to generate the fragments indicated in Figure 1. This would also explain why terminal Glc*p*A is seen and terminal Gal*p*NAc is not observed in the linkage data (Table 1). However, in light of the capsular structure, and its less lipophilic nature in relation to LOS, it is not entirely surprising that capsule was isolated by the phenol–water solvent system.[7]

MALDI-TOF MS of partially hydrolysed, permethylated capsule clearly showed consistent mass intervals of 463 mu at *m/z* 1921.9, 1458.7, 995.4 and 532.2 $[M+Na]^+$, consistent with -HexA-HexNAc- disaccharide repeat units (Fig. 1). Satellite signals indicative of HexA lactones are observed at *m/z* 1889, 1426 and 963 $[M+Na]^+$. Reduced disaccharide repeats are also present at *m/z* 1011 and 548 $[M+Na]^+$. Sugar analysis of the capsular polysaccharide clarified this further, indicating the presence of Glc*p*A and Gal*p*NAc. Linkage analysis of the partially hydrolysed, permethylated capsule identified terminal and 4-linked GlcA and 3-linked GalN (Table 1). The absolute configurations of these two sugars were determined by GC-MS of the trimethylsilylated (+)-2-butyl glycosides,[10,11] and were D for both sugars.

Table 1. GC–MS analysis of partially methylated alditol acetates obtained from partially hydrolysed *M. bovis* (Mb25) capsular polysaccharide

Elution time (min)	Characteristic fragment ions (*m/z*)	Assignment
19.62	102, 118, 129, 159, 175, 219	Terminal Glc*p*A
20.93	102, 118, 127, 187, 247	4-linked Glc*p*A
24.33	117, 159, 273	3-linked Gal*p*NAc

Extensive ^1H and ^{13}C NMR analysis of the *M. bovis* strain Mb25 capsular material, along with comparison to published data[12,13] revealed the repeating unit →4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→. The ^1H NMR spectrum (298 K, 600 MHz) of the capsular material from *M. bovis* strain Mb25 between 1.5 and 4.8 ppm is shown in Figure 2. Examination of the anomeric region of the ^1H NMR spectrum together with gradient COSY and gradient TOCSY spectra clearly indicated the presence of only two types of saccharide units in the capsular sample. The presence of glucuronic acid was further confirmed by the appearance of a carboxylic acid carbon at 174.51 ppm in the ^{13}C-attached proton test (APT) spectrum. Further, the presence of *N*-acetylgalactosamine was indicated by the appearance of an *N*-acetamide methyl resonance at 1.99 and 25.09 ppm in the ^1H and ^{13}C NMR spectra, respectively. Intra-saccharide connectivities were determined by examination of a combination of gradient COSY and TOCSY, HSQC and HSQC-TOCSY spectra. Inter-saccharide correlations were determined by a combination of 400 ms NOESY and HSQC-NOESY spectra. Full ^1H and ^{13}C chemical shift assignments for →4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→referenced to 3-(trimethylsilyl)-1-propane-sulfonic acid, sodium salt hydrate (DSS) are provided in Table 2. Noteworthy is that the reducing

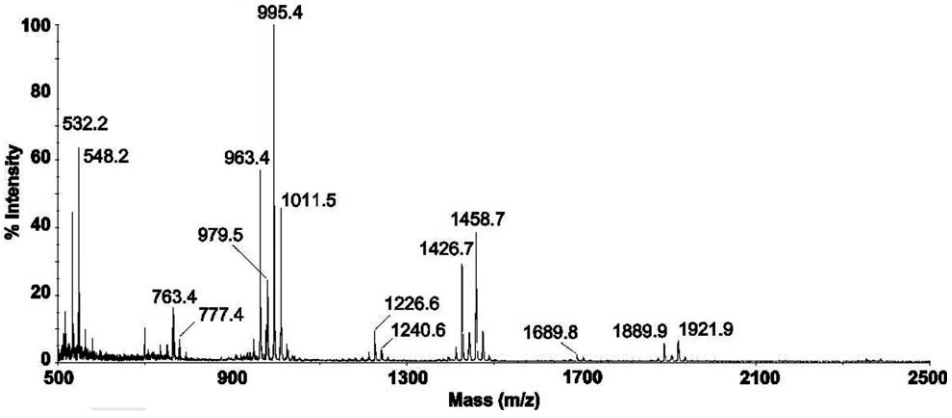

Figure 1. MALDI-TOF MS spectrum of partially hydrolysed, permethylated *M. bovis* capsular polysaccharide.

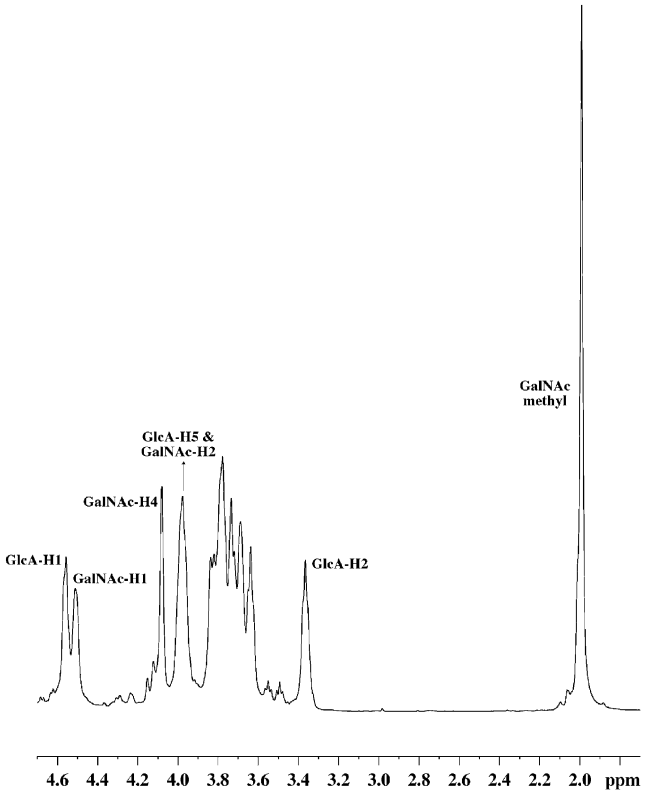

Figure 2. 600 MHz ^{1}H NMR spectrum of the capsular polysaccharide unmodified chondroitin from *M. bovis*.

end and non-reducing end residue peaks were undetected in these spectra.

Sulfates were not detected on the disaccharide repeat unit as verified by comparison with published NMR data for unmodified and sulfated chondroitin.[12,13] It is conceivable however, that subjecting the material to mild acid hydrolysis could have cleaved off any sulfate groups had they been initially present.

It was not immediately obvious from examination of the ^{1}H, 400 ms NOESY or HSQC-NOESY spectra what the anomeric configuration of the sugars at each glycosidic linkages were. However, from a ^{13}C proton-coupled spectrum we obtained $^{1}J_{C1-H1}$ coupling constants of 164 and 163 Hz for Glc*p*A and Gal*p*NAc, respectively, clearly indicate β-configurations for both sugars.[14] These assignments were further confirmed by molecular dynamics simulations with intra-residual proton–proton distances extracted and compared to observed NOE's.

In summary, extensive NMR, molecular modelling and MS analysis of the capsular polysaccharide from *M. bovis* Mb25 revealed an unmodified chondroitin disaccharide repeating unit. Whether expression of capsular polysaccharide is common to all *M. bovis* strains is unclear. We are now examining other strains for the presence of capsular polysaccharide.

1. Experimental

1.1. Bacterial strain and growth

M. bovis strain Mb25 was obtained from the collection of Dr. Pat Blackall, Department of Primary Industries, Queensland, Australia. It was imported to Australia from Omaha, Nebraska from Dr J Baldwin, Corn States Serum Company, Harney Street, Omaha, Nebraska. The lyophilised bacteria (stored in 1957) were cultured on BHI agar (Oxoid) overnight before storage at −80 °C in BHI media supplemented with 30% glycerol. From this frozen stock, starter cultures were grown overnight at 37 °C on agar with 10% defibrinated horse blood (DHB) (Biomerieux). Colonies were resuspended in PBS and used to inoculated 70 × 150 mm plates of BHI + 10% DHB. After overnight incubation at 37 °C bacteria were harvested into sterile water with 0.5% phenol. After two hours standing at room temperature, bacteria were pelleted by centrifugation at 3000 × *g*.

Proteinase-K digestion: Approximately one colony of *M. bovis* (Mb25) cells was suspended in PBS to give an absorbance reading of 5 at 280 nm. This suspension was Proteinase-K treated at 37 °C for 1 h. Tricine-SDS-PAGE gel of undigested *M. bovis* versus proteinase-K digested *M. bovis* was run for 2.5 h at 200 mA followed by Coomassie blue and silver staining.

1.2. Isolation of capsular polysaccharide

Wet cell mass was dehydrated by successive washes with acetone, ethanol, and petroleum spirit. The cell mass was freeze dried to give 1.09 g, ground to a fine powder,

Table 2. ^{1}H and ^{13}C chemical shifts (ppm) for *M. bovis* strain Mb25 capsular, -β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→4)- in D_2O referenced to DSS recorded at 298 K on a Bruker Avance spectrometer operating at 600 and 150 MHz, respectively

Gal*p*NAc				Glc*p*A			
H-1	4.50	C-1	104.03	H-1	4.56	C-1	107.07
H-2	3.99	C-2	53.80	H-2	3.38	C-2	74.76
H-3	3.84	C-3	82.80	H-3	3.66	C-3	76.44
H-4	4.09	C-4	70.37	H-4	3.82	C-4	82.68
H-5	3.70	C-5	77.68	H-5	3.99	C-5	76.44
H-6	3.69	C-6	63.64			$\mathbf{C}O_2H$	174.51
CH_3	1.99	NH(CO)$\mathbf{C}H_3$	25.09				
		NH($\mathbf{C}$O)CH_3	177.67				

then extracted using a hot phenol–water procedure[5,10] to yield 30 mg. Carbohydrate was subjected to 1% glacial acetic acid (2 mg/mL, 100 °C, 2 h) followed by centrifugation and careful removal of the supernatant. Supernatant was freeze dried, followed by resuspension in 1.0 mL milli-Q water and centrifuge filtration (Rainin, 0.45 m membrane). Gel permeation chromatography was performed on Bio-Gel P2 using 0.02 M pyridinium acetate (pH 5.4) buffer as the eluant. Fractions were lyophilised, then carbohydrate-containing fractions were pooled following monitoring by ^{1}H NMR (D_2O) to give 20.4 mg of homogeneous material.

1.3. NMR spectroscopy

Capsular polysaccharide (5 mg) dissolved in D_2O (CIL 99.998%) was lyophilised in triplicate to remove exchangeable protons. The sample was dissolved in 500 μL of D_2O under nitrogen and ^{1}H and ^{13}C spectra were recorded at 298 K on a Bruker Avance spectrometer operating at 600 MHz and 150 MHz, respectively. Chemical shifts are reported in ppm referenced to DSS. Spectral assignment was aided by the recording of gradient COSY, gradient TOCSY (60 and 120 ms mixing time), ^{13}C attached proton test (APT), ^{1}H ^{13}C-HSQC and ^{1}H ^{13}C-HSQC-TOCSY (60 and 120 ms mixing time) spectra. All spectra were acquired using unmodified pulse sequences from the Bruker pulse sequence library.

1.4. Mass spectrometry

Trimethylsilyl derivatives: Samples were hydrolysed in 1 M methanolic hydrogen chloride at 80 °C for 16 h and the reagent was removed under a stream of nitrogen. Hexosamines were re-*N*-acetylated in 500 μL of methanol/pyridine/acetic anhydride (500:1:5 v/v) for 15 min at room temperature, then dried under a nitrogen. Trimethylsilyl derivatisation was performed in 200 μL of Tri-Sil 'Z' (Pierce) at room temperature for 30 min, after which the reagent was removed under nitrogen. Derivatised monosaccharides were resuspended in 1 mL of hexanes, centrifuged at 3000 rpm for 10 min and the supernatant transferred and dried under nitrogen for analysis by GC–MS. Samples were analysed by GC–MS using temperature program A.

Partially methylated alditol acetates: Permethylated samples were reduced in 200 μL Superhydride (Aldrich) for 1 h at room temperature. The reaction was terminated with glacial acetic acid and excess borates were removed by repeated additions (x4) of 10% acetic acid in MeOH. Samples were hydrolysed with 2 M trifluoroacetic acid for 2 h at 121 °C, reduced with 10 mg/mL sodium borodeuteride in 2 M aqueous ammonium hydroxide at room temperature for 2 h, and then acetylated with Ac_2O at 100 °C for 1 h.[15] Samples were analysed by GC–MS using temperature program B.

D *and* L *absolute configuration*: Samples were prepared as described for trimethylsilyl derivatives except hydrolysis was performed in 1 M (S)-(+)-2-butanolic-HCl prior to re-*N*-acetylation and TMS derivatisation. Samples were analysed by GC-MS using temperature program C.

GC–MS analysis: Was carried out using a Perkin–Elmer Clarus 500. Samples were dissolved in hexanes prior to on-column injection on a RTX-5 (30 m × 0.32 mm internal diameter, Restek Corp.). Temperature program A: The oven was held at 65 °C for 1 min before being increased to 140 °C at a rate of 25 °C/min, then to 200 °C at a rate of 5 °C/min finally to a temperature of 300 °C at a rate of 10 °C/min. Temperature program B: The oven was held at 60 °C for 1 min before being increased to 300 °C at a rate of 8 °C/min. Temperature program C: The oven was held at 65 °C for 1 min before being increased to 160 °C at a rate of 25 °C/min, then to 250 °C at a rate of 3 °C/min, finally to a temperature of 300 °C at a rate of 25 °C/min.

Chemical derivatisation for MALDI-MS: Capsular polysaccharide was partially hydrolysed in aqueous 1% acetic acid at 100 °C for 2 h, lyophilised, then reduced with $NaBH_4$ (10 mg mL^{-1}) in 2 M NH_3 for 2 h at room temperature. Excess borates were removed by repeated additions (×4) of 10% acetic acid in MeOH. Methylation using the sodium hydroxide procedure was performed as described.[16] After derivatisation, the reaction products were purified on a Sep-Pak C_{18} (Waters) as described.[16] MALDI MS was performed on the 50% acetonitrile fraction using a Perceptive Biosystems Voyager DE™ STR mass spectrometer (Foster City, CA, U.S.A.) in the reflectron mode with Delayed Extraction. Permethylated samples were dissolved in MeOH and 1 μL aliquots were premixed with 1 μL of matrix (2,5-dihydrobenzoic acid) before loading onto a metal plate.

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References

1. Brown, M. H.; Brightman, A. H.; Fenwick, B. W.; Rider, M. A. *J. Vet. Int. Med.* **1998**, *112*(4), 259–266.
2. Prieto, C. I.; Aguilar, O. M.; Yantorno, O. M. *Vet. Microbiol.* **1999**, *70*, 213–223.
3. Slatter, D. H.; Edwards, M. E.; Hawkins, C. D. *Aust. Vet. J.* **1982**, *59*(3), 69–72.
4. Atwell, J. L.; Tennent, J. M.; Lepper, A. W.; Elleman, T. C. *J. Bacteriol.* **1994**, *176*(16), 4875–4882.

5. Araujo, F.; Alviano, C. S.; Angluster, J.; Ricciardi, I. D. *Vet. Microbiol.* **1989**, *20*, 165–171.
6. Shnawa, I. M. S.; Hadad, J. J.; Karim, M. A. *Ind. J. Comp. Microbiol. Immunol. Infect. Dis.* **1989**, *10*(3), 116–119.
7. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
8. Kahler, C. M.; Carlson, R. W.; Rahman, L. E. M.; Stephens, D. S. *J. Bacteriol.* **1996**, *178*(5), 1265–1273.
9. Galanos, C.; Luderitz, O.; Westphal, O. *Eur. J. Biochem.* **1969**, *9*, 245–249.
10. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
11. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 1–7.
12. De Angelis, P. L.; Gunay, N. S.; Toida, T.; Mao, W.; Linhardt, T. *Carbohyr. Res.* **2002**, *337*, 1547–1552.
13. Belot, F.; Jacquinet, J. *Carbohydr. Res.* **2000**, *326*, 88–97.
14. Hamer, G. K.; Perlin, A. S. *Carbohydr. Res.* **1976**, *49*, 37–48.
15. Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. *Carbohydr. Res.* **1967**, *5*, 340–345.
16. Dell, A.; Reason, A. J.; Khoo, K.-H.; Panico, M.; McDowell, R. A.; Morris, H. R. In *Methods in Enzymology*; Lennarz, W. J., Hart, G. W.; Academic: San Diego, 1994; Vol. 230, pp 108–132.