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Note

Hyaluronic acid and a (1→4)-β-D-xylan, extracellular polysaccharides of *Pasteurella multocida* (Carter type A) strain 880

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Pasteurella multocida is a causative agent of enzootic pneumonia in calves and piglets, and is responsible for severe economic losses in large farms. Knowledge of the structure of the components on the surface of the bacteria will contribute to the development of an efficient vaccine.

Electron microscopy of several strains of *P. multocida* revealed capsular material that stretched far into the culture media[1]. Different fractions of the cell wall of *P. multocida*, especially proteins and lipopolysaccharides, have been examined for antigenic or immunogenic effects[2–4].

We now report on two extracellular polysaccharides isolated from *P. multocida*. Carter type A, strain 880, one of which is hyaluronic acid and the other is a (1→4)-β-D-xylan, not described previously from bacterial sources.

The polysaccharides were isolated from the culture supernatant solution, or washings of the biomass with 0.1M NaCl, by precipitation with methanol and acetone followed by treatment with dilute acetic acid and chromatography on Fractogel TSK HW-40/S. The high-molecular-weight material was fractionated by ion-exchange chromatography on DEAE-Sephacel to give a neutral polysaccharide (PS-1) and an acidic polysaccharide (PS-2).

Hydrolysis of PS-1 with 2M hydrochloric acid (100°, 4 h) gave (p.c.) xylose, whereas hydrolysis of PS-2 gave (p.c.) 2-amino-2-deoxyglucose and glucuronic acid.

The ^{1}H-n.m.r. spectrum of PS-1 contained a signal for H-1 at 4.49 p.p.m. (d, $J_{1,2}$ 7.7 Hz) and for five other protons at 3.3–4.2 p.p.m. The data in Table I are typical for a

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TABLE I

^{1}H-N.m.r. data for PS-1

Proton	*Chemical shift (p.p.m.)*	*Multiplicity*	*Coupling constant (Hz)*	
H-1	4.49	d	$J_{1,2}$	7.7
H-2	3.31	dd	$J_{2,3}$	8.7
H-3	3.57	dd	$J_{3,4}$	8.7
H-4	3.80	ddd	$J_{4,5ax}$	12.0
H-5*eq*	4.12	dd	$J_{4,5eq}$	5.3
H-5*ax*	3.38	dd	$J_{5ax,5eq}$	12.0

TABLE II

^{13}C-N.m.r. data for PS-1, methyl 4-*O*-*β*-D-xylopyranosyl-*β*-D-xylopyranoside[8], and methyl *β*-D-xylopyranoside[5,19]

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*OMe*
PS-1	103.1	74.1	75.1	77.8	64.5	
β-D-Xyl*p*-(1→	103.1	74.0	76.9	70.4	66.5	
→4)-*β*-D-Xyl*p*OMe	105.1	74.0	75.0	77.7	64.1	
β-D-Xyl*p*OMe	105.2	74.2	77.0	70.4	66.4	58.3

β-xylopyranosyl residue[5,6]. The ^{13}C-n.m.r. spectrum of PS-1 contained a signal for C-1 at 103.1 p.p.m. and four other signals in the region 64.5–77.8 p.p.m. (Table II). Thus, PS-1 is a regular homopolymer of xylose with a monosaccharide repeating unit.

The xylose was shown to be D by g.l.c. of the acetylated (*R*)- and (*S*)-but-2-yl glycosides. The retention times (11.98 and 12.36 min) were identical to those for the derivatives of D-xylose. The xylan had $[\alpha]_d$ −47° (water) and the n.m.r. data indicated the anomeric configuration to be *β* [*cf.* methyl *β*-D-xylopyranoside[7], $[\alpha]_D$ −65.5° (water)].

On the basis of ^{13}C-n.m.r. data fot methyl D-xylopyranosides and methyl *O*-D-xylopyranosyl-D-xylopyranosides[8], PS-1 must be classified as a (1→4)-*β*-D-xylan. The chemical shift of the resonance for C-1 at 103.1 p.p.m. accords with that of C-1′ in methyl 4-O-*β*-D-xylopyranosyl-*β*-D-xylopyranoside[8] (Table II). Also the *α*- and *β*-effects of glycosylation in the ^{13} C-n.m.r. spectrum of PS-1 (−2.0, +7.3, and 2.3 p.p.m.) are similar to those for C-3,4,5 (−1.8, +7.4, and 2.0 p.p.m.) of the above disaccharide glycoside (see Table II). Therefore, PS-1 has the structure →4)-*β*-D-Xyl*p*-(1→.

A polysaccharide of this structure serves as the backbone of many plant polysaccharides but has not been found hitherto in bacteria.

The ^{1}H-n.m.r. spectrum of PS-2 contained a signal at 1.91 p.p.m. (s, 3 H) for the NAc group of GlcNAc, signals for H-1 at 4.51 and 4.39 p.p.m. (2 d, $J_{1,2}$ 7 Hz), and other signals at 3.2–3.8 p.p.m. The ^{13}C-n.m.r. spectrum contained signals for C=O at 174.4

TABLE III

^{13}C-N.m.r. chemical shifts (δ, p.p.m.) of PS-2 and mammalian hyaluronic acid

Sugar unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
PS-2[a]						
→3)-β-D-Glc*p*NAc-(1→[b]	101.6	55.3	83.5	69.5	76.5	61.6
	−(101.9)	(55.8)	(83.8)	(70.0)	(76.6)	(62.7)
→4)-β-D-Glc*p*A-(1→	104.0	73.5	74.7	81.0	76.8	174.4
	(104.0)	(74.1)	(74.4)	(81.3)	(75.1)	n.c.[d]
Mammalian hyaluronic acid[c]						
→3)-β-D-Glc*p*NAc-(1→	101.6	55.5	84.2	70.0	76.8	62.1
→4)-β-D-Glc*p*A-(1→	104.2	73.8	75.0	81.2	77.6	174.7

[a] Chemical shifts calculated for structure **1** are given in parentheses. [b] NAc group: CH_3CO at 23.5 p.p.m., CH_3CO at 175.9 p.p.m. [c] Data from ref. 12. [d] Not calculated.

and 175.9 p.p.m., CH_3CO at 23.5. p.p.m., C-2,6 of GlcNAc at 55.3 and 61.6 p.p.m., respectively, C-1 of two sugar residues at 104.0 and 101.6 p.p.m., and for seven other carbons at 69.5–83.5 p.p.m. (Table III). Thus, PS-2 is built up of GlcNAc–GlcA repeating units.

A computer-assisted ^{13}C-n.m.r. analysis[9] of PS-2, using reported data[9,10] on chemical shifts and glycosylation effects, revealed only two structures (**1** and **2**) characterised by S values of <5 (2.3 and 2.9, respectively).

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

1

→3)-β-D-Glc*p*NAc-(1→2)-β-D-Glc*p*A-(→

2

For structure **2**, there were deviations of 0.8–1.2 p.p.m. for several signals (C-1 of GlcNAc, C-1,2,4,5 of GlcA), whereas, for structure **1**, there were two deviations of 1.7 and 1.1 p.p.m., but the remainder did not exceed 0.5 p.p.m. (Table III). The signal with the large deviation belongs to C-5 of GlcA, and the chemical shift is sensitive to pH[11,12]. The observed chemical shift for the C-5 resonance of Glc*p*A (76.8 p.p.m., see Table III) differed from those reported, namely, 75.4 (pH 1.8)[11] and 72.6 (pH 7.8)[12], and also from that in mammalian hyaluronic acid (77.6 p.p.m., pH not given).

The structure **1** of PS-2 is that of hyaluronic acid, and this was confirmed by comparison of ^{13}C-n.m.r. spectra of PS-2 and mammalian hyaluronic acid[13] (Table III). The detection of hyaluronic acid by an hyaluronidase test is used for sero-typing *P. multocida* Carter type A strains[14,15] and this fact also confirms structure **1** for PS-2.

Little is known about the biological function of PS-1 and PS-2. The purified polysaccharides had no immunogenic activity in mice[16].

EXPERIMENTAL

Bacterial growth conditions and isolation of polysaccharides PS-1 and PS-2. — The cells were grown in a chemically defined medium[17], harvested by centrifugation (6000 r.p.m., 20 min, 4°), and extracted by stirring in 0.15M NaCl (30 min, 56°). The polymer fraction was isolated after dialysis of the supernatant solution against water, and precipitation with methanol and acetone. An aqueous solution of the precipitate was lyophilised.

A similar polymer fraction was isolated from the culture supernatant solution by repeated precipitation with methanol and acetone or after evaporation of the solvent *in vacuo* and dialysis. All fractions were freeze-dried and then treated with 1% aq. acetic acid (100°, 1 h). Depending on the culture conditions, yields of 0.3–0.5% of xylan (PS-1; detected as xylose by g.l.c.) and 0.8–2.1% of hyaluronic acid[18] (PS-2) were calculated, with reference to the biomass.

The foregoing product was subjected to gel-permeation chromatography on a column (50 × 2 cm) of Fractogel TSK HW-40/S (Merck) by elution with aqueous acetic acid. The high-molecular fraction in the void volume (PS-1 and PS-2; molecular mass > 10 kDa) was separated by ion-exchange chromatography on a column (12 × 1.5 cm) of DEAE-Sephacel (Pharmacia) by elution with 10mM Tris/HCl buffer (pH 8.2) and a subsequent NaCl gradient (0→0.5M), using a Uvicord S-II system (LKB, Sweden).

PS-1 and PS-2 were desalted by elution from a column (23 × 2.5 cm) of BioGel P2 (400 mesh, BioRad). Material eluted in the void volume was lyophilised and used for structural analysis.

Monosaccharide composition. — PS-1 and PS-2 were each hydrolysed in 2M hydrochloric acid (4 h, 100°) and the products were analysed by p.c., using 1-butanol–pyridine–water (6:4:3) for neutral monosaccharides and ethyl acetate–pyridine–acetic acid–water (5:5:1:3) for amino sugars and uronic acids. The optical rotation of the xylose was determined with a Perkin–Elmer 243 polarimeter.

Butanolysis. — Samples (200 μg) of PS-1 were heated with 2M HCl–(*R*)- or (*R,S*)-*butan-2-ol* (200 μL) in screw-capped vessels for 1 h at 85°. D-Xylose (2 mg) was converted (1 h. 85°) into the (*R*)-(−)-but-2-yl glycosides in (*R*)-butan-2-ol–2M HCl (1mL). The but-2-yl glycosides were acetylated (Ac_2O–pyridine, 1:1; 1 h, 60°), and the diastereomeric products were analysed on a Varian Model 3700 gas chromatograph equipped with a fused-silica capillary column (0.3 mm × 25 m) of SE-54 and using a temperature programme of 150° for 3 min, then to 320° at 5°/min.

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250 spectrometer for solutions in D_2O at 30° (internal acetone, δ 2.24). ^{13}C-N.m.r. spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O at 60° (internal acetone, δ 31.45).

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