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Note

Structure of the capsular polysaccharide and the O-side-chain of the lipopolysaccharide from *Acetobacter methanolicus* MB 58 (IMET B346)

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A structural study of the capsular polysaccharide (CPS) and the O-side-chain of the lipopolysaccharide (LPS) of the type strain of the bacterium *Acetobacter methanolicus* MB 58/4 (IMET 10945) demonstrated the identity of the repeating units of both polymers[1]. In order to check the assumption that such a feature could occur in more strains of this genus, we extended our investigation and determined the structures of both the CPS and the O-side-chain of the LPS of *A. methanolicus* MB 58 (IMET B346). This strain was taxonomically and biochemically described by other authors[2,3].

The CPS and the LPS were extracted from lyophilised bacterial cells by the phenol–water method[4] and separated by ultracentrifugation[1]. After RNase and DNase digestion, the LPS was purified by two further ultracentrifugation steps. The CPS was purified by ion-exchange chromatography on DEAE-Sephacel[1]. The main carbohydrate product was recovered in the neutral fraction.

Hydrolysis of the polysaccharides with 2 M hydrochloric acid, removal of the lipid precipitate from the LPS hydrolysate, and subsequent analysis using a sugar analyser and GLC of the alditol acetates demonstrated glucose and galactose to be the major components in both the CPS and the LPS (Table I). Incubation of the hydrolysates with D-glucose oxidase resulted in complete disappearance of glucose, showing that it has the D configuration.

The ^{1}H NMR spectrum (Fig. 1) of the CPS contained three signals of equal intensity for H-1 of α-pyranoses at 5.24 (d, 1 H, $J_{1,2}$ 2.2 Hz), 5.14 (d, 1 H, $J_{1,2}$ 3.2

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TABLE I

Monosaccharide composition [a] of the capsular polysaccharide (CPS) and the lipopolysaccharide (LPS) of *A. methanolicus* MB 58 (IMET B346)

Component	Rha	Man	Gal	Glc	Hep [b]
CPS	traces	0	2.2	1	0
LPS	4	3	21	14	1

[a] Molar proportions of sugar residues obtained by GLC analysis are given. [b] L-*glycero*-D-*manno*-Heptose.

Hz), and 5.04 ppm (d, 1 H, $J_{1,2}$ 3.0 Hz), and the signals for 15 other protons in the region 3.3–4.2 ppm.

The ^{13}C NMR spectrum of the CPS (Fig. 2 and Table II) contained signals for three anomeric carbons at 97.4, 97.6, and 99.4 ppm, three signals of C-6 of hexoses at 62.5, 67.4, and 68.2 ppm, all being triplets in the gated-decoupling spectrum, and signals for 12 other carbons bearing oxygen in the region 69–75 ppm. The absence from the spectrum of signals at a field lower than 78 ppm, which are characteristic for furanose rings[5], proved the three hexoses to be pyranosides. The relatively large coupling constants $^1J_{C,H}$ 170.9 Hz for all three anomeric carbons determined from the gated-decoupling spectrum of the CPS confirmed that the monosaccharide residues are α-linked[6].

In order to establish the mode of substitution of the monosaccharides, the CPS was methylated and cleaved by formolysis followed by hydrolysis with 0.25 M sulfuric acid, borohydride reduction, and peracetylation. Analysis by combined GLC–MS of the methylated sugars thus obtained in the form of alditol acetates revealed the presence of 2,3,4-tri-*O*-methylglucose, 3,4,6-tri-*O*-methylgalactose, and 2,3,4-tri-*O*-methylgalactose, which were identified using published data[7,8]. Thus, the glucose and one of the galactose residues are substituted at position 6,

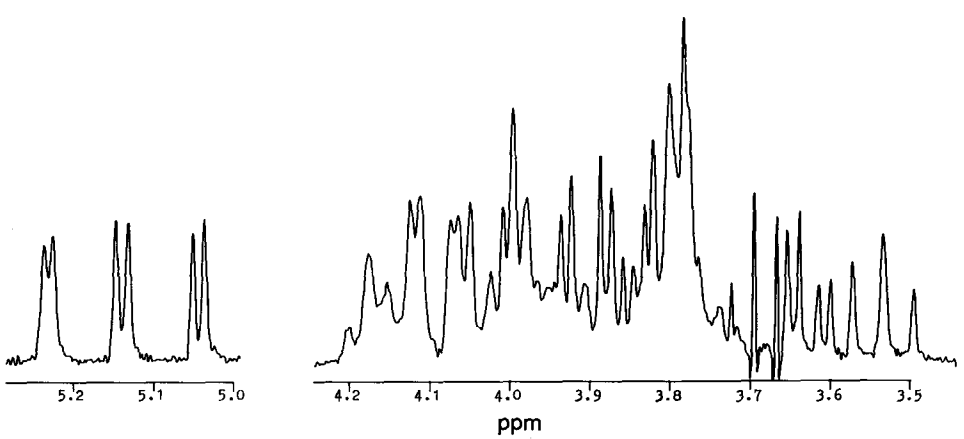

Fig. 1. ^1H NMR spectrum (300 MHz) of the capsular polysaccharide of *A. methanolicus MB* 58 (IMET B346).

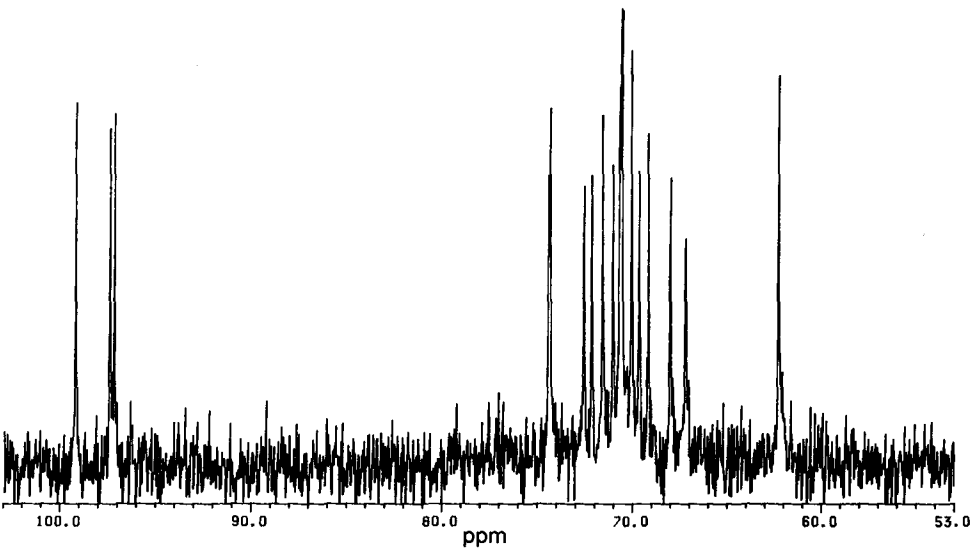

Fig. 2. ^{13}C NMR spectrum (62.89 MHz) of the capsular polysaccharide of *A. methanolicus MB* 58 (IMET B346).

whereas the second galactose residue is substituted at position 2. The substitution of two hexose residues at position 6 is in agreement with the downfield shift of their C-6 signals in the ^{13}C NMR spectrum of the CPS at 67.4 and 68.2 ppm (Table II) due to the α-effects of glycosylation[9].

For selective cleavage of the CPS, acetolysis was applied using a mixture of acetic acid, acetic anhydride, and sulfuric acid[10,11]. The products were *O*-deacety-

TABLE II

Chemical shifts (δ, ppm) in the ^{13}C NMR spectra

Sugar unit	C-1	C-2	C-3	C-4	C-5	C-6
CPS						
→6)-α-D-Glc*p*-(1→ **A**	97.6	72.6	74.6	71.1	71.6	67.4
→2)-α-D-*Galp*-(1→ **B**	97.4	74.4	70.7	69.2	72.2	62.5
→6)-α-D-Gal*p*-(1→ **C**	99.4	69.7	70.6	70.7	70.1	68.2
Dissacharide **1**						
α-D-Glc*p*-(1→	97.7 [a] 99.1 [b]	72.6 [a] 72.7 [b]	74.0	70.7	72.7	61.7
→2)-α-D-Gal*p*	90.8	74.6	70.6	68.8	71.6	62.3
→2)-β-D-Gal*p*	97.5	78.4	73.1	70.2	76.2	62.1
1-*O*-Me-α-D-Glc*p*[12]	100.3	72.5	74.2	70.6	72.7	61.7

[a] α-Series. [b] β-Series.

lated with sodium methoxide and separated by gel filtration, resulting in a monosaccharide and a disaccharide fraction. Analysis of the former using a sugar analyser revealed the presence of galactose contaminated with traces of glucose. The disaccharide was shown to have the structure **1** as follows. Hydrolysis with 2 M hydrochloric acid released equal amounts of galactose and glucose identified by the sugar analyser. Borohydride reduction resulted in disappearance of galactose in the hydrolysate, and, hence, this sugar occupies the reducing terminus. This conclusion is in agreement with the presence of signals for the nonreducing α-glucopyranose residue in the ^{13}C NMR spectrum (Table II, cf. the data for methyl α-D-glucopyranoside[5]). Carbons C-1 and C-2 of the glucose residue as well as each of the carbons of the galactose residue of the disaccharide gave two signals due to the existence of the reducing sugar in two anomeric forms, the β one being predominant[9]. The position of the signals for C-6 of galactose near 62 ppm indicated that this sugar is not substituted at position 6. This result was expected since acetolysis is known to split selectively (1 → 6) linkages[10,11]. The signals for C-2 of the α-D-Gal and β-D-Gal units show a downfield shift of 5.0 and 5.2 ppm, respectively, compared to unsubstituted[9] galactose. These shifts are caused by the α-effects of glycosylation by α-D-Glc on C-2 of the reducing galactose residue. The results correspond to known data[9], and other positions of glycosidic linkages[12] can be excluded. Thus, galactose in the disaccharide is substituted at position 2. This conclusion accords with the assignments[9] of all the signals of this residue in the ^{13}C NMR spectrum of the disaccharide (Table II). Thus, the disaccharide has structure **1**.

α-D-Glc*p*-(1 → 2)-D-Gal
A **B**

1

The absolute configuration of galactose was determined by calculations of the specific optical rotation of the CPS by Klyne's rule[13]. This method gives the closest value to the experimental one when the D configuration of both galactose residues is assumed (Table III).

Thus, the data obtained demonstrated that the CPS has the structure **2**.

→ 6)-α-D-Glc*p*-(1 → 2)-α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 →
A **B** **C**

2

This structure was confirmed by complete assignment of the ^{1}H and ^{13}C NMR spectra, which was achieved by $^{1}H,^{1}H$-COSY and $^{1}H,^{13}C$-HETCOR experiments (Tables IV and II, respectively). Starting from H-1 (5.00 ppm) or H-4 (3.39 ppm) of the 6-linked α-D-glucopyranose residue in the COSY spectrum, all signals of unit **A** were localised, and the multiplicity was estimated from the better resolved ^{1}H NMR spectrum recorded at 70°C (Table IV). In contrast, the nonanomeric protons of the two α-D-galactopyranose residues (units **B** and **C**, structure **2**) could not be distinguished clearly because of strong overlapping of the signals. Their positions were found by $^{1}H,^{13}C$ shift-correlated spectroscopy.

TABLE III

Calculation of the optical rotation of the CPS

	$[\alpha]_D$	Molecular weight	$[M]_D$
Methyl α-D-glucopyranoside	+159°	194	+308°
Methyl α-D-galactopyranoside	+185°	194	+380°
Calculated for			
one α-D-glucose and two α-L-galactose residues	−93°	486	−452°
α-L-galactose, α-D-galactose, and α-D-glucose residues	+63°	486	+308°
one α-D-glucose and two α-D-galactose residues	+220°	468	+1068°
Observed	+144°		

Analysis of the LPS preparation by sodium dodecylsulfate–polyacrylamide gel-electrophoresis (SDS-PAGE)[14] followed by silver staining[15] revealed numerous bands arranged in a ladderlike pattern reflecting a polydisperse O-side-chain built up of repeating units[14,16]. Attempts to split off the O-side-chain by acetic acid degradation did not result in precipitation of the lipid part as we had described for

TABLE IV

^{1}H NMR data [a] for the *Acetobacter methanolicus* MB 58 (IMET B346) capsular polysaccharide

Unit	Proton	Chemical shift (ppm)	Multiplicity [b]	Coupling constant [b] (Hz)	
→ 6)-α-D-Glc*p*-(1 →	H-1	5.00	d	$J_{1,2}$	3.2
	H-2	3.48	dd	$J_{2,3}$	9.8
	H-3	3.67	dd	$J_{3,4}$	8.9
	H-4	3.39	dd	$J_{4,5}$	10.0
	H-5	4.01	m	$J_{5,6b}$	≈ 6
	H-6a	3.68	dd	$J_{5,6a}$	≈ 6
	H-6b	3.84	dd	$J_{6a,6b}$	≈ 9
→ 2)-α-D-Gal*p*-(1 →	H-1	5.09	d	$J_{1,2}$	2.2
	H-2	3.83			
	H-3	3.87			
	H-4	3.92			
	H-5	3.91			
	H-6a, b	3.64			
→ 6)-α-D-Gal*p*-(1 →	H-1	4.90	d	$J_{1,2}$	3.0
	H-2	3.72			
	H-3	3.80			
	H-4	3.93			
	H-5	4.03			
	H-6a	3.77			
	H-6b	3.63			

[a] Data taken from the ^{1}H, ^{13}C-HETCOR experiments at 30°C. [b] Multiplicities and coupling constants were estimated from the ^{1}H NMR spectrum run at 70°C.

A. methanolicus MB 58/4 LPS[1], and, therefore, we used alkaline degradation to remove the *O*-acyl fatty acids. This procedure increases the solubility of the LPS in water and makes the O-side-chain better available for NMR analysis[1]. After saponification of the *O*-linked fatty acids and neutralisation, the polysaccharide was desalted on TSK HW-40 (S). The carbohydrate-containing peak eluted in the void volume of the column was further separated by gel filtration on TSK HW-50 (S), giving a major component of ~ 20 kD (PS-1) and a minor one of ~ 9 kD (PS-2).

The ^{1}H NMR spectrum of PS-2 contained two series of signals. One belonged to the glucorhamnan **3** found[17] by us earlier as an exopolysaccharide of the *A. methanolicus* MB 58/4 type strain. Hence, glucorhamnan **3** is also present in *A. methanolicus* MB 58.

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 4)\text{-D-Glc}p\text{-}(1 \rightarrow$$
$$\mathbf{3}$$

The second series corresponded to PS-1. Judging from the H-1 signal intensities, PS-2 contains glucorhamnan **3** and glucogalactan **2** in the ratio ~ 1:3.

The ^{13}C NMR spectrum of PS-1 (not shown) was practically identical to that of the CPS. Sugar analysis revealed galactose, glucose, and traces of rhamnose and mannose, the two last monosaccharides obviously belonging to the core portion of the LPS[1]. Hence, the O-side-chain of the LPS is built up of the same repeating units as the CPS component.

The results of this study support the view that strains of *A. methanolicus* MB series[2,3] are related to each other. This relation is reflected in the identity of the repeating units in both the CPS and the O-side-chain of the LPS, and the presence of a common glucorhamnan component.

EXPERIMENTAL

Bacterial strain, culture conditions, and polysaccharides.—The strain of *A. methanolicus* MB 58 (IMET * B346) was taken from the culture stock of the Institute of Biotechnology, Leipzig, Germany. Taxonomic implications, growth conditions, and biochemical characteristics have been described[1,2].

Polysaccharides were extracted from lyophilised bacteria by phenol–water[4] followed by ultracentrifugation for LPS sedimentation. CPS and LPS purification was carried out as described for the type strain of this species[1].

General methods.—Gel filtration was conducted on a Fractogel TSK HW-40 (S) column (1.6 × 80 cm) and a TSK HW-50 (S) column (1.6 × 60 cm), using aq 1% acetic acid as eluant and a flow rate of 45 mL/h; fractions (1.5 and 1 mL, respectively) were collected. Calibration of the gel filtration columns was performed with D-glucose, maltose, and raffinose for TSK HW-40 (S), and with the

* Type culture collection of the Institute of Microbiology and Experimental Therapy, Jena, FRG.

octasaccharide Gal_8[1], Dextrans T10 and T20, and Blue Dextran (Pharmacia) for TSK HW-50 (S). Elution curves were recorded using a differential refractometer (Knauer).

Descending PC and estimation of the optical rotation were described previously[1,17].

NMR spectra were recorded with Bruker WM-250, AM-300, and AM-360 instruments for solutions in D_2O at 70°C (^1H) and 33°C (^{13}C, ^1H,^{13}C-HETCOR, ^1H, ^1H-COSY), with internal acetone (δ_H 2.23 ppm) and methanol (δ_C 50.15 ppm). One- and two-dimensional pulse sequences were run using the standard Bruker software DISNMR version 89 11 01.0.

Identification of monosaccharides.—Hydrolysis of carbohydrates (1–2 mg) was performed with 2 M HCl (1 mL) for 3 h at 105°C in sealed tubes. After removal of acid by evaporation in vacuo, the hydrolysates were analysed with a Sugar Analyser (Technicon), using a DA-X4 resin (Durrum) column (0.6 × 14 cm) and borate buffer (pH 8.9, 0.5 M) at 0.8 mL/min flow rate, as well as by GLC of the alditol acetates[18]; both methods provided quantitative data. The configuration of glucose was determined using D-glucose oxidase[17] (Boehringer).

Methylation analysis.—CPS (10 mg) was methylated according to the Hakomori method[7]. After formolysis, hydrolysis, borohydride reduction, and acetylation, partially methylated alditol acetates were identified[7,8] by GLC on a 3% ECNSS-M column (0.3 × 150 cm) by retention times, and by GLC–MS[8].

Acetolysis of CPS.—CPS (50 mg) was peracetylated by stirring in DMF (2 mL), pyridine (3 mL), and acetic anhydride (2 mL) at 50°C for 2 h and then at room temperature (18 h). After removal of the solvents by repeated evaporation with toluene, the sample was treated with 10 : 10 : 1 acetic acid–acetic anhydride–H_2SO_4 at 40°C for 5 h[11]. The reaction was stopped by addition of pyridine (20 mL) and solvents were evaporated in vacuo. Sugar acetates were extracted by partition between water and chloroform. *O*-Deacetylation was carried out in 40 mM NaOMe (10 mL) for 30 min at room temperature[11]. After deionisation with Dowex 50 (H^+ form), the products were subjected to gel filtration on TSK HW-40 (S).

Acetic acid degradation of LPS.—LPS (100 mg) was dissolved in aq 2% acetic acid (20 mL) and heated in a boiling water bath for 2 h.

Alkaline degradation of LPS.—LPS (200 mg) in 0.1 M aq NaOH (10 mL) was heated in a boiling water bath for 90 min until the solution became clear. After neutralisation with 2 M HCl and concentration by evaporation in vacuo, the alkali-degraded LPS was desalted on TSK HW-40 (S) and the polysaccharide fraction purified on TSK HW-50 (S).

SDS-polyacrylamide gel-electrophoresis.—The LPS preparation was treated for 5 min at 95°C in 0.05 M Tris–HCl buffer (pH 6.8), 2% SDS, 10% glycerol, 0.02% Bromphenol Blue, 5 mM EDTA, and 5% mercaptoethanol, and separated on an SDS-polyacrylamide gel[14,15] (10 cm × 10 cm × 0.75 mm) containing 4 and 16% of acrylamide in the stacking and separating gels, respectively. Electrophoresis was begun at 10 mA until the Bromphenol Blue reached the resolving gel interface.

For separation, the current was increased to 20 mA and run until the tracking dye approached the bottom of the gel.

A modified[15] silver stain[16] was used for visualisation of the banding pattern.

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