Carbohydrate Research, 218 (1991) 247–251
Elsevier Science Publishers B.V., Amsterdam

Note

Structure of the extracellular polysaccharide of *Acetobacter methanolicus* MB 58/4 (IMET 10945)

H. Dieter Grimmecke*, Uwe Mamat, Michael Voges, Wolfgang Lauk,
Institute of Biotechnology, Academy of Sciences, Permoser Street 15, Leipzig O-7050 (Germany)

Alexander S. Shashkov, and Yuriy A. Knirel
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R., Moscow B-334 (U.S.S.R.)

(Received July 23rd, 1990; accepted for publication February 11th, 1991)

Acetobacter methanolicus MB 58/4 (IMET 10945) synthesises a capsular polysaccharide (CPS) and an O-side chain of a lipopolysaccharide (LPS) built up of the same Gal*f*–Gal*p* repeating unit[1]. As a part of studies of the cell envelopes and carbohydrates of this species, we now describe the structure of an extracellular polysaccharide (EPS) produced by this strain.

The polymeric fraction, isolated from the culture supernatant solution by dialysis and ion-exchange chromatography, contained rhamnose, glucose, and galactose in the ratios 1.0:1.2:3.8, determined after hydrolysis by g.l.c. of the derived alditol acetates. Incubation of this fraction with bacteriophage *Acm1* (ref. 2) gave a polymer and several oligosaccharides that were isolated by gel-permeation chromatography on BioGel P-4 (Fig. 1). Each oligosaccharide contained galactose only. These oligosaccharides and those formed by *Acm1*-mediated depolymerisation of CPS and LPS[1] were identified by p.c., gel-permeation chromatography, and sugar analysis. Part of the CPS (galactan) was liberated into the culture medium during fermentation.

Further analysis was conducted on the exopolysaccharide (EPS) eluted in the void volume of the column of BioGel P-4. For the large-scale preparation of EPS, a culture supernatant solution of an *Acm1* lysogenic strain of *A. methanolicus* MB 58/4 (IMET 10945) was used, because this prophage-harbouring strain is strongly reduced[2] in biosynthesis of the galactan CPS.

Hydrolysis of the purified EPS with 2M hydrochloric acid gave glucose and rhamnose in the ratio 1:1, identified by g.l.c. of the derived alditol acetates and with a sugar analyser. Incubation of the hydrolysate with D-glucose oxidase removed the glucose and indicated it to be D.

* To whom correspondence should be addressed.

0008-6215/91/$ 03.50

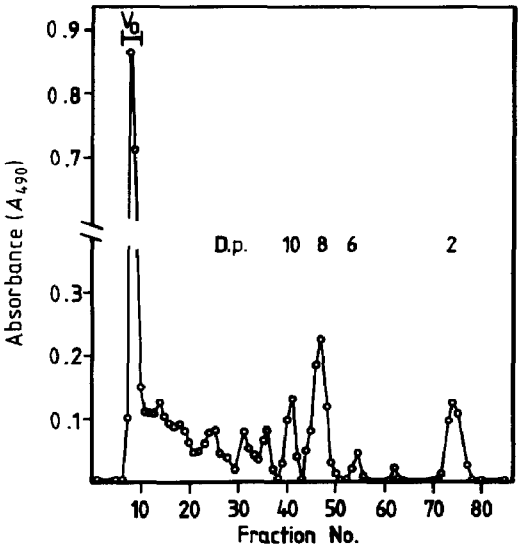

Fig. 1. Gel-permeation chromatography on BioGel P-4 of the products of degradation of the neutral exopolysaccharide fraction of *A. methanolicus* MB 58/4 (IMET 10945) with bacteriophage *Acm1*. Oligosaccharides with various d.p. values originated from the galactan CPS[1]. The void-volume peak contained the exopolysaccharide (EPS).

The ^{13}C-n.m.r. spectrum of EPS contained signals for C-1 at 101.6 and 104.7 p.p.m., a methyl group of a 6-deoxy sugar (rhamnose) at 17.9 p.p.m., a CH_2OH group of a hexose (glucose) at 61.7 p.p.m., and eight CHOH groups in the region 70–81 p.p.m. (Table I). The ^{1}H-n.m.r. spectrum of EPS contained signals for H-1 at 4.67 ($J_{1,2}$ 7.9 Hz) and 4.86 p.p.m. ($J_{1,2}$ 2.0 Hz), the Me group of rhamnose at 1.26 p.p.m. ($J_{5,6}$ 6.2 Hz), and ten other protons in the region 3.3–4.2 p.p.m. (Table II).

Thus, the EPS was built up of disaccharide repeating units containing glucose and rhamnose.

The resonances in the ^{1}H-n.m.r. spectrum of EPS were assigned by using sequential, selective, spin-decoupling experiments (Table II). The ^3J values showed[3] the glucose to be β-pyranose and the rhamnose to be α-pyranose.

Irradiation of H-1 of glucose at 4.67 p.p.m. caused a marked n.O.e.[4] on H-3 of rhamnose at 3.88 p.p.m. and a smaller one on H-2 of rhamnose at 3.88 p.p.m. This result indicated that rhamnose was 3-substituted (*cf.*, ref. 5).

TABLE I

Chemical shifts (p.p.m.) of the resonances in the ^{13}C-n.m.r. spectrum of EPS

Residue	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
→4)-β-D-Glc*p*-(1→	104.7	75.1	75.6	79.2	76.2	61.7
→3)-α-L-Rha*p*-(1→	101.6	71.6	80.8	72.4	70.4	17.9

TABLE II

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

Residue	*Proton*	*Chemical shift (p.p.m.)*	*Multiplicity*	*J (Hz)*
→4)-β-D-Glc*p*-(1→				
	H-1	4.67	d	7.9
	H-2	3.37	dd	8.5
	H-3,4	3.56–3.65	m	
	H-5	3.51	m	4
	H-6a	3.69	dd	12
	H-6b	3.84	dd	2
→3)-L-Rha*p*-(1→	H-1	4.86	d	2.0
	H-2	4.14	dd	3.3
	H-3	3.88	dd	9.2
	H-4	3.61	dd	9.0
	H-5	4.03	dd	6.2
	H-6,6,6	1.26	d	

Irradiation of H-1 of rhamnose at 4.86 p.p.m. resulted in an n.O.e. on a proton that resonated at 3.6 p.p.m., either H-3 or H-4 of glucose, the resonances of which have similar chemical shifts.

Periodate oxidation of the EPS destroyed only the glucose, and indicated that the rhamnose was 3-substituted and that the glucose was 4-substituted.

The absolute L configuration of rhamnose was determined by ^{13}C-n.m.r. spectroscopy, using the known regularities in effects of glycosylation[6]. The signal at 104.7 p.p.m. in the spectrum of the EPS may belong only to C-1 of the β-glycopyranose residue, since C-1 of rhamnopyranose always resonates[7] to higher field than 104 p.p.m. The position of this signal is characteristic for the attachment of a β-D-glucopyranose residue to C-3 of L-rhamnopyranose since, for D-rhamnopyranose, C-1 of glucose would resonate in the region 100–101 p.p.m. The L configuration of the rhamnose was confirmed by calculation of the specific optical rotation of EPS by Klyne's rule[8] (Table III).

Thus, the EPS has the following repeating unit:

→3)-α-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→.

This structure accords with the chemical shifts of all the ^{13}C resonances of the EPS, which were also assigned by computer-assisted structural analysis[7] (Table I).

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of exopolysaccharide (EPS).— The reference strain for *A. methanolicus* was grown under the conditions previously described[9,10]. Nutrient broth was prepared using the low-molecular-weight fraction of

TABLE III

Calculation of the optical rotation of EPS

	$[\alpha]_D$ *(degrees)*	*Molecular weight*	$[M]_D$ *(degrees)*
Methyl β-D-glucopyranoside	−34	194	−66
Methyl α-L-rhamnopyranoside	−67	178	−120
EPS			
Calc. for			
D-Glucose and L-rhamnose	−60	308	−186
D-Glucose and D-rhamnose	+17	308	+53
Obs.	−62		

yeast extract. The EPS was isolated from the culture medium, after removal of the bacteria by centrifugation, by extensive dialysis against 10mM Tris/HCl buffer and by ion-exchange chromatography on a column (3 × 45 cm) of DEAE-Sephacel (Cl^- form). Carbohydrates were found only in the neutral eluate, and were dialysed against water, concentrated by ultrafiltration, and lyophilised.

General methods. — Gel-permeation chromatography was conducted on a column (1.5 × 90 cm) of BioGel P-4 (−400 mesh) (BioRad) by elution with water. Fractions (1.5 mL) were collected and analysed by the phenol–sulfuric acid method[11].

Descending p.c. was performed on FN 11 paper (FILTRAK), using 1-butanol–pyridine–water (6:4:3) and detection with the *p*-anisidine–*o*-phthalic acid reagent. Optical rotations were measured on aqueous solutions with a JASCO Model DIP 360 digital polarimeter (Biotronic) at 20°. The ^1H- and ^{13}C-n.m.r. spectra were recorded with Bruker WM-250 and AM-300 instruments on solutions in D_2O at 30° (internal acetone for ^1H, 2.23 p.p.m., and internal methanol for ^{13}C, 50.15 p.p.m.). N.O.e. data were obtained by the t.O.e. method[12] in the difference mode; time constants used were D1 = 4 s (relaxation delay), and D2 = 0.5 s (build up of n.O.e.).

Identification of the monosaccharides. — Hydrolyses were carried out with M HCl (3 h, 105°) in sealed tubes. The products were analysed with a Technicon Sugar Analyzer and by g.l.c. of the derived alditol acetates[13]. The D configuration of the glucose was determined conventionally, using D-glucose oxidase (EC 1.1.3.4) (Boehringer).

Bacteriophage-mediated degradation. — To a solution of the exopolysaccharide fraction (250 mg) in 40mM sodium acetate (pH 4.5, 20 mL) that contained 10^{-2} M NaCl, 10^{-3} M $MgCl_2$, and 10^{-3} M $CaCl_2$ (ref. 2) was added a suspension of bacteriophage *Acm1* (0.5 mL, 10^{13} plaque-forming units/mL). The mixture was incubated at 25° for 24 h, then concentrated to 2 mL, centrifuged, and applied to the column of BioGel P-4.

Periodate oxidation. — A solution of the EPS (void-volume peak from BioGel P-4, 1 mg) in 0.1M acetic acid (1.5 mL) that contained 0.05M $NaIO_4$ was incubated for 1 day at 4°. After the addition of ethylene glycol (50 μL) and dialysis against water, $NaBH_4$ (10 mg) was added. The mixture was incubated for 2 h at room temperature, the residual borohydride was destroyed with a few drops of M acetic acid, the salts were

removed by dialysis against water, and the product was hydrolysed and subjected to sugar analysis as described above.

ACKNOWLEDGMENTS

We thank Ms. B. Nickel and Mr. P. Heilemann for expert technical assistance.

REFERENCES

1 H. D. Grimmecke, U. Mamat, W. Lauk, A. S. Shashkov, Y. A. Knirel, E. V. Vinogradov, and N. K. Kochetkov, *Carbohydr. Res.*, 220 (1991) 165–172.
2 U. Mamat, unpublished results.
3 C. Altona and C. A. G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–429.
4 R. M. Keller and K. Wüthrich, *Biol. Magn. Reson.*, 3 (1981) 1–52.
5 A. S. Shashkov, Y. A. Knirel, N. V. Tanatar, and N. K. Kochetkov, *Carbohydr. Res.*, 146 (1986) 346–349.
6 A. S. Shashkov, G. M. Lipkind, Y. A. Knirel, and N. K. Kochetkov, *Magn. Reson. Chem.*, (1988) 735–747.
7 G. M. Lipkind, A. S. Shashkov, Y. A. Knirel, E. V. Vinogradov, and N. K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
8 W. Klyne, *Biochem. J.*, 46 (1950) xxii–xxiii.
9 H. Uhlig, K. Karbaum, and A. Steudel, *Int. J. Syst. Bacteriol.*, 36 (1986) 317–322.
10 A. Steudel, D. Miethe, and W. Babel, *Z. Allg. Mikrobiol.*, 20 (1980) 663–672.
11 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Biochem.*, 28 (1956) 350–356.
12 G. Wagner and K. Wüthrich, *J. Magn. Reson.*, 33 (1979) 675–680.
13 J. Metz, W. Ebert, and H. Weicker, *Chromatographia*, 4 (1971) 345–350.