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Structure of the capsular polysaccharide and the O-side-chain of the lipopolysaccharide from *Acetobacter methanolicus* MB 70, and of oligosaccharides resulting from their degradation by the bacteriophage *Acm6*

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ABSTRACT

Acetobacter methanolicus MB 70 was shown to be related to the type strain of this species MB 58/4 (IMET 10945) having the same galactan →2)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→ as the capsular polysaccharide (CPS) and the O-side-chain of the lipopolysaccharide (LPS). Additionally, a glucan built up of the disaccharide repeating unit →6)-α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→ was identified in strain MB 70. In the CPS, the polymers were present in the ratio ~1:1, whereas the glucan preponderated in the LPS. Bacteriophage *Acm6* specific to *A. methanolicus* MB 70 hydrolysed selectively the glucan component of both CPS and LPS. Structural elucidation of the resulting oligosaccharides led to the identification of the phage-associated depolymerase as an endo-α-(1→6)-D-glucopyranoside hydrolase.

INTRODUCTION

Bacteriophage-associated depolymerases serve as a tool for the elucidation of bacterial polysaccharide structures. This technique has been applied to K-antigens of *E. coli*, using bacteriophages with endoglycanase activity, as well as to those of *Klebsiella*, using phages with both lyase and hydrolase activity[1,2].

In a previous paper[3], we described the structure of a galactan identified for both the CPS and the O-side-chain of the LPS in the type strain of *Acetobacter methanolicus* MB 58/4 (IMET 10945). Bacteriophage *Acm1* was shown to adsorb to this strain and to degrade the CPS in the initial phase of phage infection[3,4]. Phage *Acm1* did not show any reaction with *A. methanolicus* MB 70, but

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bacteriophage *Acm6* lyses this strain macroscopically with the same plaque morphology[4]. Therefore, this specificity of the bacteriophages makes them suitable as tools for typing *Acetobacter* strains.

We now report the structures of the CPS and the O-side-chain of the LPS of *A. methanolicus* MB 70, and the oligosaccharides that resulted from polysaccharide–phage interaction.

RESULTS AND DISCUSSION

The LPS and CPS were isolated from the phenol–water extract of lyophilised bacteria[5] and purified by digestion with RNase and DNase[3]. Purification of the CPS on DEAE-Sephacel gave only one sugar-containing substance in the neutral eluate. Monosaccharide analysis revealed it to contain glucose and galactose in the ratio 1:1. Treatment of the hydrolysate with D-galactose oxidase or D-glucose oxidase resulted in disappearance of the respective monosaccharide, and, hence, both have the D configuration.

The ^{1}H NMR spectrum of the CPS contained the signals for four anomeric protons at 5.44 (broad singlet), 5.14 (doublet, $J_{1,2}$ 3.5 Hz), 5.09 (doublet, $J_{1,2}$ 3.7 Hz), and 4.63 (doublet, $J_{1,2}$ 7.6 Hz) ppm, as well as those of other protons in the region 3.3–4.5 ppm. Its ^{13}C NMR spectrum showed the presence of signals for four anomeric carbons at 108.8, 103.3, 97.7, and 96.9 ppm, four hexose C-6 carbons at 67.1, 63.9, 62.0, and 61.8 ppm, and 16 other carbons in the region 69.5–90.1 ppm. Thus, the polymer has a regular character and includes four different hexose residues.

The CPS was subjected to degradation by two different bacteriophages *Acm1* and *Acm6*. As a result of the action of *Acm6*, a polymeric fraction and an oligosaccharide with dp 12 were the major products, together with small amounts of oligosaccharides with dp 4 and 6 and a negligible amount of that with dp 8. The polymeric fraction was composed only of galactose residues. Its ^{1}H NMR spectrum contained two signals for anomeric protons at 5.44 (broad singlet) and 4.63 ppm (doublet, $J_{1,2}$ 7.6 Hz), which were also present in the spectrum of the initial CPS. Moreover, this spectrum turned out to be identical to the spectrum of the galactan isolated by us formerly from *A. methanolicus* MB 58/4 (IMET 10945) and shown[3] to possess structure **1**.

The ^{13}C NMR spectrum of the initial CPS material contained all the signals for galactan **1** (Table I) and, therefore, the CPS represents a mixture of approximately equal amounts of the galactan **1** and, most probably, a glucan. Indeed, when treated with *Acm1*, which is specific to the galactan **1**[3], the CPS afforded a glucose-containing polymeric material and galactose-containing oligosaccharides, the latter being identical to those obtained by analogous treatment of the pure galactan **1**[3].

The ^{1}H NMR spectrum of the glucan thus obtained showed the presence of the signals for two anomeric protons at 5.14 (doublet, $J_{1,2}$ 3.5 Hz) and 5.09 ppm

TABLE I

^{13}C NMR chemical shifts (δ, ppm) for the galactan **1** and glucan **2** from *A. methanolicus* MB 70 capsular polysaccharide

Residue	C-1	C-2	C-3	C-4	C-5	C-6
Galactan **1**						
→2)-β-D-Gal*f*-(1→	108.8	90.1	77.0	84.0	71.8	63.9
→3)-β-D-Gal*f*-(1→	103.3	71.0	81.1	69.5	76.2	62.0
Glucan **2**						
→2)-α-D-Glc*p*-(1→	97.7	77.2	72.5	70.8	72.9	61.8
→6)-α-D-Glc*p*-(1→	96.9	72.5	74.4	70.8	71.8	67.1

(doublet, $J_{1,2}$ 3.7 Hz). The same signals were observed in the initial CPS. Both chemical shifts and coupling constants indicated[6] the α configuration of the D-glucose residues.

The twelve ^{13}C NMR signals for the glucan were found by subtraction of all signals of the galactan **1** from the spectrum of the initial CPS, and their chemical shifts are listed in Table I. These data clearly demonstrated that the glucan **2**, like the galactan **1**, is built up of disaccharide repeating units. Both glucose residues are in the pyranoid form since there were no signals of non-anomeric carbons at a field lower than 77 ppm.

The structural determination of the glucan **2** was carried out by using the computer-assisted ^{13}C NMR approach[7]. To rule out a branched structure, methylation analysis[8] was carried out additionally. GLC–MS analysis led to identification of 1,2,5-tri-*O*-acetyl-3,4,6-methylglucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, and, hence, the glucan component of the CPS has the structure **2**.

→2)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→(**1**)

→2)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→(**2**)

Acid hydrolysis of the LPS gave Rha, Man, Gal, and Glc in the ratios 4:2:2:16. Exactly as for *A. methanolicus* MB 58/4 (IMET 10945)[3], the LPS was resistant towards acetic acid degradation. In order to obtain structural information, the LPS was saponified with alkali and the resulting polymer (LPS-AD) was purified by gel-permeation chromatography. Its ^{13}C NMR spectrum contained two series of signals indicating the occurrence of a major and a minor component, which were identified as the glucan **2** and the galactan **1**. Judging from the intensities of the anomeric protons in the ^{1}H NMR spectrum, the ratio of the glucan and the galactan was ~3:1, respectively. Other monosaccharides identified in the LPS did not give clear signals in the ^{1}H and ^{13}C NMR spectra and seem to originate from the LPS core (cf. the data in ref. 3).

Oligosaccharides with dp 4, 6, 8, and 12 obtained from the CPS and LPS-AD by action of bacteriophage *Acm6* were purified by gel-permeation chromatography on TSK HW-40(S). Sugar analysis indicated that they contained only glucose. The

^1H and ^{13}C NMR spectra of the major product with dp 12 were very similar to those of the glucan **2**, thus proving that it originated from this polymer.

In the ^{13}C NMR spectra of the oligosaccharides with dp 4 and 6, the signals for C-1 of the reducing α- and β-glucopyranose residues were at 90.9 and 97.3 ppm, respectively. These chemical shifts proved that the reducing residue was substituted at position 2 [cf. the chemical shifts 90.8 and 97.5 ppm of the corresponding carbons in kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose), and 93.8 and 97.7 ppm in isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose[9]]. It is evident, therefore, that the $(1 \rightarrow 6)$ linkages were susceptible to the phage depolymerase. This fact was confirmed also by the relative intensities of the signals for C-6 of the 6-substituted and 6-unsubstituted residues at 67.3 and 61.8–62.1 ppm, respectively. In the spectrum of the glucan **2**, their ratio was 1:1; in that of the dodecasaccharide, it was somewhat smaller; and in that of the hexasaccharide and the tetrasaccharide, it was 1:2 and 1:3, respectively. These data are consistent with the proposed structures of degradation products **3–6** derived from action of the bacteriophage *Acm6* on the CPS and LPS of *A. methanolicus* MB 70.

$$\alpha\text{-D-Glc}p\text{-}(1\text{-}[\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1-]_n \rightarrow 2)\text{-D-Glc}$$

$n = 1$ (**3**), 2 (**4**), 3 (**5**), and 5 (**6**)

The absence of kojibiose among these products, the low yield of tetra-, hexa-, and octa-saccharide, and the formation of dodecasaccharide as the major oligosaccharide reflect the extended catalytic site of the enzyme. One can, thus, characterise the phage *Acm6*-associated depolymerase as an endo-α-D-glucopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranoside hydrolase.

These results also lead to the conclusion that the phages specific to *A. methanolicus* studied so far (ref 3 and this paper) recognise the primary receptor in both the CPS and the O-side-chain of the LPS and destroy it by hydrolysis. It is noteworthy that the phage *Acm1*, which is specific to strain MB 58/4 and able to split its CPS and LPS, i.e., galactan **1**, does not attack strain MB 70, although this galactan is one of the components of both CPS and LPS. At the same time, the phage *Acm6*, which cleaves glucan **2** but not galactan **1**, lyses strain MB 70. This finding shows that glucan **2** and galactan **1** cover different membrane components serving as the secondary receptor.

EXPERIMENTAL

Bacteria; isolation and purification of the polysaccharides.—The conditions of bacterial growth have been described[10,11]. Freeze-dried bacteria were extracted with hot phenol–water according to a standard method[5]. Separation and purification of the CPS and LPS were performed as described earlier[3].

General methods.—Gel-permeation chromatography was conducted on columns of Sephadex G-50 (70 × 3 cm) in pyridine–acetate buffer (pH 5.4), Sephacryl S-300

(40 × 1.8 cm) in phosphate buffer (0.1 M, pH 6.8), and Fractogel TSK HW-40(S) (80 × 1.6 cm) in water. Elution was monitored with a Technicon sugar analyzer or a Knauer differential refractometer.

Descending PC was performed on FN 11 paper (Filtrak, Germany), using 6:4:3 1-butanol–pyridine–water and detection with the alkaline silver nitrate reagent.

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AM-300 and WM-250 spectrometers, respectively, for solutions in D_2O at 30°C (^{13}C) and 70°C (^{1}H) (internal acetone, δ_H 2.23 ppm; and MeOH, δ_c 50.15 ppm).

Monosaccharide analysis.—Neutral monosaccharides were identified after acid hydrolysis (M HCl, 3 h, 105°C) as alditol acetates by GLC[12] with a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector and a glass-capillary Ultra-1 column (0.2 mm × 25 m), and with a Technicon sugar analyzer working with a Durrum DA-X4 (0.6 × 14 cm) column and 0.5 M borate buffer (pH 9.0) at 60°C.

The D configuration of glucose and galactose was determined photometrically using D-glucose oxidase (EC 1.1.3.4) (Boehringer) and D-galactose oxidase (EC 1.1.3.9) (Sigma) in the peroxidase (EC 1.11.1.7)–2-toluidine system. Furthermore, oxidation of D-Glc and D-Gal by the respective enzymes was monitored by liquid chromatography in the sugar analyzer as described above.

Methylation analysis.—A standard method[8] was used for methylation and preparation of partially methylated alditol acetates. The products were identified by GLC and GLC–MS.

Bacteriophage-mediated degradation.—Bacteriophages *Acm1* and *Acm6* originated from the stock[4] of the Institute of Biotechnology (Leipzig, Germany). Degradation of the polysaccharides was performed as described[3]. Phage-resistant polysaccharide components eluted in the void volume of the Fractogel TSK HW-40(S) column were purified on Sephacryl S-300.

Alkaline degradation of LPS.—A solution of the LPS (220 mg) in 0.17 M NaOH (20 mL) was heated for 1 h at 100°C, neutralised with M HCl, and centrifuged. The supernatant solution was evaporated and subjected to gel-permeation chromatography on Sephadex G-50, to give LPS-AD (140 mg) eluted within the void volume.

REFERENCES

1 A.A. Lindberg, *Surface Carbohydrates of the Prokaryotic Cell*, Academic, New York, 1977, pp 289–356.
2 D. Rieger-Hug and S. Stirm, *Virology*, 113 (1981) 363–378.
3 H.D. Grimmeckc, U. Mamat, W. Lauk, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, and N.K. Kochetkov, *Carbohydr. Res.*, 220 (1991) 165–172.
4 B. Kiesel, U. Mamat, and L. Wünsche, *Wiss. Z. Univ. Leipzig, Nat.-wiss. Reihe*, 38 (1989) 287–295.
5 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
6 C. Altona and C.A.G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–429.
7 G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, and N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
8 B. Lindberg, *Methods Enzymol.*, 28 (1972) 178–195.

9 T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto, *J. Chem. Soc., Perkin Trans. 1*, (1973) 2425–2432.
10 H. Uhlig, K. Karbaum, and A. Steudel, *Int. J. System. Bacteriol.*, 36 (1980) 317–322.
11 M.W. Gründig and W. Babel, *Arch. Microbiol.*, 149 (1987) 149–154.
12 J.S. Sawardeker, J.H. Sloneker, and A. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.