

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 $\rightarrow 2)\text{-}\beta\text{-D-Gal f-(1}\rightarrow 3)\text{-}\beta\text{-D-Gal p-(1}\rightarrow$ as the capsular polysaccharide (CPS) and the O-side-chain of the lipopolysaccharide (LPS). Additionally, a glucan built up of the disaccharide repeating unit $\rightarrow 6)\text{-}\alpha\text{-D-Glc p-(1}\rightarrow 2)\text{-}\alpha\text{-D-Glc p-(1}\rightarrow$ was identified in strain MB 70. In the CPS, the polymers were present in the ratio $\sim 1:1$, 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- $\alpha\text{-(1}\rightarrow 6)\text{-D-glucopyranoside}$ 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 paper³, 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 morphology⁴. 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 bacteria⁵ and purified by digestion with RNase and DNase³. 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 ¹H 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 ¹³C 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 ¹H 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 shown³ to possess structure 1.

The ¹³C 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 1³, 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 1³.

The ¹H 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

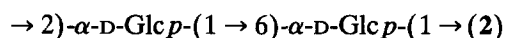
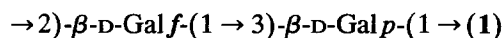
^{13}C NMR chemical shifts (δ , ppm) for the galactan 1 and glucan 2 from *A. methanolicus* MB 70 capsular polysaccharide

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 indicated⁶ 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 approach⁷. To rule out a branched structure, methylation analysis⁸ 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.

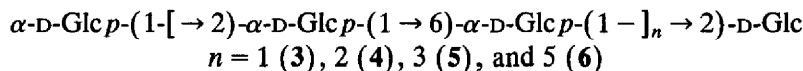


Acid hydrolysis of the LPS gave Rha, Man, Gal, and Glc in the ratios 4:2:2:16. Exactly as for *A. methanolicus* MB 58/4 (IMET 10945)³, the LPS was resistant towards acetic acid degradation. In order to obtain structural information, the LPS was saponified with alkali and the resulting polymer (LPS-AD) 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 ^1H NMR spectrum, the ratio of the glucan and the galactan was $\sim 3:1$, respectively. Other monosaccharides identified in the LPS did not give clear signals in the ^1H 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-glucose⁹]. 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.



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 method⁵. Separation and purification of the CPS and LPS were performed as described earlier³.

General methods.—Gel-permeation chromatography was conducted on columns of Sephadex G-50 (70 \times 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.

¹H NMR and ¹³C NMR spectra were recorded on Bruker AM-300 and WM-250 spectrometers, respectively, for solutions in D₂O at 30°C (¹³C) and 70°C (¹H) (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 GLC¹² 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 method⁸ 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 stock⁴ of the Institute of Biotechnology (Leipzig, Germany). Degradation of the polysaccharides was performed as described³. 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.

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