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Comparison of Bacterial Lipopolysaccharides by High-Performance Liquid Chromatography

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A comparison of lipid-free polysaccharides from gram-negative bacteria was rapidly accomplished by using high-performance liquid chromatography of underivatized hydrolysates. Examination of a number of such products revealed that, contrary to earlier reports, *Xanthomonas campestris* lipopolysaccharide contained heptose, together with rhamnose and galactose, but not mannose. The polymers from the methanotrophs "*Methylomonas albus*" and "*Methylosinus trichosporium*" contained heptose and glucose, and that from a "*Klebsiella aerogenes*" strain contained heptose, glucose, and galactose. The absence of heptose from the lipopolysaccharide of *Myxococcus xanthus* was confirmed.

Although there has been considerable study of the morphology of methylotrophic bacteria, indicating both similarities to and differences from other gram-negative bacteria (2, 3), the lipopolysaccharides (LPSs) of these species do not appear to have been analyzed. The microcysts of a type I species, "*Methylmonoas albus*," resemble other microcysts in that they lack LPS and contain instead a glucan and amino sugars (9), but the vegetative cells were not examined. The LPSs of two other groups of gram-negative bacteria, *Xanthomonas* species and *Myxococcus* species, are also of particular interest in that they are thought to differ from those of other species in lacking heptose, although the latter group contains unusual sugars, such as 3-*O*-methylxylose (16).

Traditionally, the composition of the LPSs of gram-negative bacteria has been compared by chromatography of polymer hydrolysates. The free sugars have been either examined directly by paper or thin-layer chromatography or converted to volatile derivatives, such as alditol acetates, and studied by gas-liquid chromatography. Frequently, analysis of the polysaccharides has been achieved by using various colorimetric procedures, which may be subject to different experimental errors. High-performance liquid chromatography (HPLC) offers an alternative method in which no conversion to derivatives is required. HPLC has been used for the quantitation of heptose and ketodeoxyoctonic acid in an examination of the core region of LPS from "rough" strains lacking side chains (8). During a study of LPSs from a range of gram-negative species, it was clear that rapid comparison and quantitation could be obtained by HPLC. The LPSs could not be used directly because of degradation products from lipid A, but hydrolysis of the lipid-free polymers provided a suitable starting material.

The bacterial strains used were *Xanthomonas campestris* 646, originally derived from ACTC 13951, and the non-polysaccharide-producing mutant 6D; "*M. albus*" BG8; *Enterobacter aerogenes* XM6; a nonmucoid mutant of a "*Klebsiella aerogenes*" serotype 2 strain; "*Methylosinus trichosporium*" OB3B; and *Myxococcus xanthus* FB, originally obtained from M. Dworkin. Bacteria were grown with shaking at 30°C in appropriate culture media in Erlenmeyer flasks containing 1 liter of medium. The enterobacterial species and *X. campestris* strains were grown for 48 h in a semisynthetic medium containing yeast extract, casein hydrolysate, and glucose (11). The methanobacteria were grown in a salts medium with methane as the carbon source as described by Whittenbury et al. (15) for 96 to 120 h. The *M. xanthus* strain was cultured for 72 h in C10 medium (7). Bacteria were washed free from medium components with phosphate-buffered saline (pH 7.0) and freeze-dried. LPSs were prepared from the lyophilized cells by the standard phenol water extraction procedure, purified by ultracentrifugation at 100,000 × *g* for 4 h, and lyophilized (14). Lipid A was removed by hydrolysis with 1% (vol/vol) acetic acid at 100°C for 1 h. When sufficient material was available, a portion of the polymer was oxidized with sodium periodate to provide information on the nature of the carbohydrate linkages. The final products after dialysis and lyophilization were hydrolyzed with 0.5 N H_2SO_4 at 100°C for 16 h and neutralized with Amberlite IR410 resin in the bicarbonate form. The neutralized solutions were dried under vacuum, dissolved in a small volume of distilled water, filtered, and applied to a Gilson HPLC system with a Rheodyne 7125 syringe injection valve. Analysis was performed on various columns by using a refractive index monitor for detection of the sugars. Columns were calibrated with a range of monosaccharide standards, including several heptoses

TABLE 1. Elution times of monosaccharide standards from HPLC columns

Monosaccharide	Elution time (min) from column[a]:	
	1	2
2-*O*-Methylglucose	12.54	4.59
3-*O*-Methylglucose	12.41	4.49
D-Glucose	13.22	10.74
D-Xylose	14.4	5.89
D-Galactose	15.07	11.64
D-Mannose	17.08	10.34
L-Rhamnose	15.71	7.2
L-Fucose	17.1	8.1
D-Glycero-D-galactoheptose	15.29	18.5
D-Glycero-L-guloheptose	19.49	ND[b]
D-Glycero-L-mannoheptose	21.08	16.22
D-Glycero-L-galactoheptose	18.02	19.81

[a] Column 1, HP-87P with water as the mobile phase at a flow rate of 0.6 ml/min and at 85°C. Column 2, Nucleosil-5-NH_2 amino column with acetonitrile-water (75:25) as the mobile phase at a flow rate of 1 ml/min and at ambient temperature.
[b] ND, Not done.

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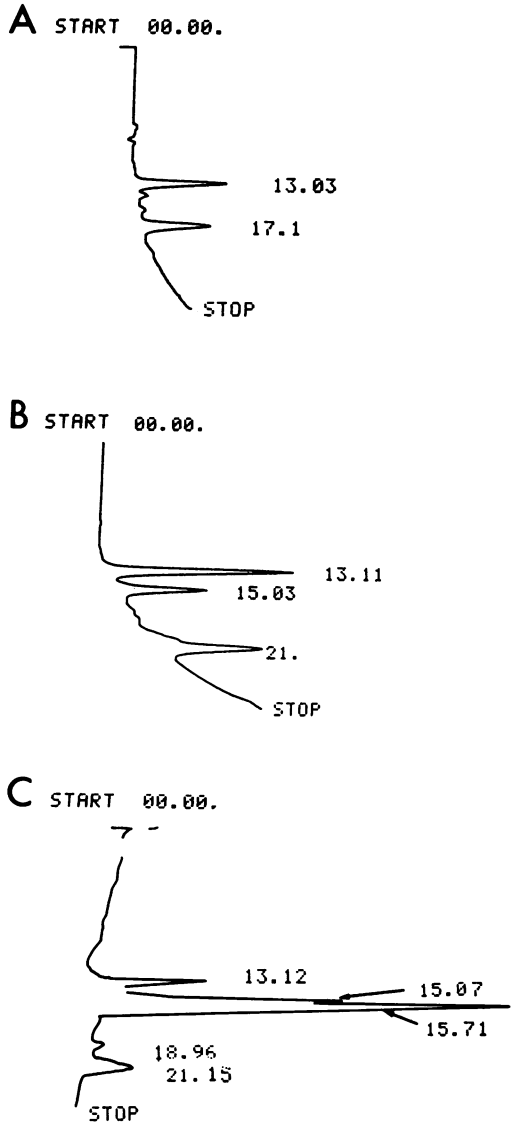

FIG. 1. Typical chromatograms on an HP-87P column of hydrolysates from *M. xanthus* FB (A), "*K. aerogenes*" 10B (B), and *X. campestris* 6D (C). The approximate elution times (in minutes) of monosaccharides were as follows: glucose, 13.1; galactose, 15.07; rhamnose 15.71; fucose, 1' 1; and glyceromannoheptose, 21.1. The material eluting at 18.96 min in panel C was not identified.

which were kindly provided by N. K. Richtmyer. Typical elution times under the conditions used in the present study for a range of monosaccharides on the two column systems adopted for analysis of the polysaccharide hydrolysates are provided in Table 1. All hydrolysates were analyzed at least four times on each column. When possible, more than one batch of LPS was prepared and analyzed. In calculations of the molar ratios of the sugars, a correction was made for the differing responses of the monosaccharides to the refractive index monitor by using a series of calibration curves for different sugars and mixtures.

Preliminary experiments showed that amino-bonded columns, which have been used in other carbohydrate separations, provided considerable information on the composition of LPS hydrolysates. They suffered from the disadvantage that certain hexoses and deoxyhexoses were not well separated. However, these sugars could be separated well on strong cation-exchange columns loaded with Pb^{2+}. The protocol finally adopted used Nucleosil-5-NH_2 amino columns (HPLC Technology, Macclesfield, England) with acetonitrile-water (75:25) as the mobile phase at a flow rate of 1 ml/min and at ambient temperature and HP-87P columns (Bio-Rad Laboratories, Richmond, Calif.) with water as the mobile phase at a flow rate of 0.6 ml/min and at 85°C. These methods could not be satisfactorily applied to amino sugars or *N*-acetylamino sugars, and no attempt was made in the present study to identify and quantify these monosaccharides.

The LPSs chosen for study were from bacterial species thought to lack heptose (*M. xanthus* and *X. campestris*), two enterobacterial strains for which the likely LPS composition could be predicted, and two methanotrophs representing the two types recognized on morphological and physiological bases, for which no LPS composition appears to have been reported. From an examination of the behavior of authentic standards, it was clear that most of the monosaccharide LPS components could be readily separated, identified, and quantified. Thus, D-glycero-L-mannoheptose and its enantiomorph L-glycero-D-mannoheptose could be unequivocally distinguished from other sugars, including other heptoses, in both separation systems. This was also true for hydrolysates (Fig. 1). It is of interest that heptose was absent from the *M. xanthus* LPS, confirming the results of earlier studies in our laboratory (10), but was present in the *X. campestris* LPS. Earlier studies on a collection of strains of this phytopathogenic species had indicated that heptose was absent (12). Table 2 summarizes our results. As can be seen from the oxidized samples, much of the heptose can be recovered, indicating the presence of 1.3-linked residues. Structures of this type have been recognized in enterobacterial species such as *Escherichia coli* (5) and probably extend to numerous other LPSs.

Volk (13) found mannose in a *Xanthomonas* LPS preparation, while xylose was present in 12 of 20 xanthomonads examined. We failed to detect either monosaccharide in the material from the two strains of common origin but did confirm the presence of relatively large amounts of rhamnose and galactose. Surprisingly, smaller amounts of these sugars were present in the material from the wild-type strain than in that from the non-xanthan-producing mutant. The *X. campestris* preparations were also of interest because of the close relationship postulated between such bacteria and *Pseudomonas maltophilia* in terms of the lipid A composition and certain components of the LPS (6, 17). Strain differences can obviously be expected, but we did not observe arabinose or 6-deoxy-3-methyltalose, which are major components of the side-chain polysaccharides of *P. maltophilia*.

Both methanotrophs contained fucose and glucose as the major constituents; rhamnose was also present in "*M. trichosporium*" LPS, but relatively little of this material was available, and the results could not be determined to the same degree of accuracy. The rough strain of "*K. aerogenes*," as expected, contained glucose, galactose, and heptose, while the *E. aerogenes* polymer resembled those of some "*K. aerogenes*" strains in its high galactose content (1, 4). The HPLC procedure does not permit a distinction between pyranose and furanose forms.

A problem encountered in the attempt to determine whether 3-*O*-methylxylose was present in *M. xanthus* LPS was that this sugar ran very close to glucose in the systems used and was also a relatively minor component. There was no evidence for this monosaccharide, which would elute at 12.4 min on the chromatogram shown in Fig. 1. Clearly,

TABLE 2. Composition of lipid-free polysaccharides determined by HPLC

Bacterium	Column[a]	Sugar ratio of[b]:					
		Rhamnose	Fucose	Mannose	Glucose	Galactose	Heptose
"*M. albus*" BG8	1	0	0.43	0	1.0	0.05	0.15
	2	0	0.48	0	1.0	0.03	0.21
"*M. albus*" BG8 oxidized	1	0	0.71	—[c]	1.0	0	0.22
	2	0	0.74	—	1.0	0	0.26
"*M. trichosporium*" O3B3[d]	2	1.14	0.55	—	1.0	0.11	1.10
"*K. aerogenes*" 10B	1	—	—	—	1.0	0.90	0.49
	2	—	—	—	1.0	0.79	0.44
E. aerogenes XM6	1	—	—	—	1.0	8.03	0.20
	2	—	—	—	1.0	7.63	0.19
E. aerogenes XM6 oxidized	1	—	—	—	0[e]	3.7	0.10
M. xanthus FB	1	0	0	0.99	1.0	0.01	0
	2	0	0	0.81	1.0	0.01	0
X. campestris 646	1	2.80	—	0	1.0	1.95	0.11
	2	3.45	—	0	1.0	1.90	0.13
X. campestris 6D	1	5.78	—	0	1.0	2.67	0.24
	2	4.77	—	0	1.0	2.63	0.24
X. campestris 6D oxidized	1	4.17	—	0	1.0	3.50	0.25
	2	3.83	—	0	1.0	0.03	0.14

[a] See Table 1, footnote *a*.
[b] As all preparations contained D-glucose, sugar contents were all calculated relative to glucose = 1.0 on a dry-weight basis.
[c] —, Not detected.
[d] Three analyses only, as insufficient material was available for further study.
[e] All glucose was oxidized.

HPLC offers a rapid (approximately a 45-min run time on the two columns) and accurate method for studying many of the major components found in LPS hydrolysates. It is in many respects superior to older methods involving several different procedures, each with different variables. It will, however, require further development for the identification of the minor components for which authentic standards may not necessarily be available.

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