The Lipopolysaccharides of Fruiting and Non-fruiting Myxobacteria

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SUMMARY

Lipopolysaccharides were obtained from a number of strains of yellow-pigmented rods identified as *Cytophaga* species and from representative strains of fruiting myxobacteria. The major monosaccharide components of the lipopolysaccharides were identified by paper chromatography and subsequent analysis. Most preparations contained ribose, mannose and galactose and no major differences were seen between the composition of fruiting and non-fruiting myxobacterial lipopolysaccharides. Other sugars detected included rhamnose, glucose, glucosamine and galactosamine, but some of the polymers may also contain small amounts of other monosaccharides which have not yet been identified. The chemical similarity of the lipopolysaccharides to analogous polymers from other Gram-negative bacteria confirms the belief from cytological studies that the cell wall of myxobacteria resembles that of typical Gram-negative prokaryotes.

INTRODUCTION

The myxobacteria differ from most other groups of bacteria in showing gliding motility on solid surfaces and, at least in some genera, in undergoing various cycles of cell differentiation. Many early reports suggested that the unusual type of motility might be accompanied by differences in the surface of myxobacterial cells from those found in the eubacteria. Various cytological investigations have now refuted this; Pate & Ordal (1967) observed a layer thought to be mucopeptide together with plasma membrane, peripheral fibrils and an outer membrane in *Chondrococcus columnaris*. They also detected material at the cell surface stainable with ruthenium red and consequently thought to be an acid polysaccharide. A similar slime was observed in the non-fruiting cultures of *Myxobacter* species (Stewart & Brown, 1969). Extensive electron-microscopic examination of another myxobacterial species, *Sporocytophaga myxococcoides*, led Holt & Leadbetter (1967) to conclude that vegetative cells showed no significant differences from the anatomy of Gram-negative Eubacteriales.

Most strains of myxobacteria produce variable amounts of slime during laboratory culture and this surface polysaccharide from *Cytophaga hutchinsonii* contained uronic acid(s) and pentoses (Norman & Fuller, 1942). Slime from *C. hutchinsonii* and *Sporocytophaga myxococcoides* showed that both polymers were anionic heteropolysaccharides (Martin, Preusser & Verma, 1968). The sugars found in both substances were glucose, mannose, arabinose, xylose and glucuronic acid. The slime from the *Sporocytophaga* strain also contained galactose. In contrast, slime from *C. columnaris* was a homopolysaccharide containing D-galactosamine, 50 % of which was N-acetylated (Johnson & Chilton, 1966). The mucopeptide of *Myxococcus xanthus* cells and microcysts, comprising about 0.6 % of the dry weight of each, closely resembled the mucopeptide of other Gram-negative bacteria, containing N-acetyl-glucosamine, acetyl muramic acid, glutamic acid, diaminopimelic acid and alanine in the ratio 0.75, 0.75, 1, 1, 1.7 (White, Dworkin & Tipper, 1968). The same type

of mucopeptide was present in *C. hutchinsonii* walls (Verma & Martin, 1967). If they conform to the same pattern of cell wall polymers as other Gram-negative bacteria, myxobacteria should also contain lipopolysaccharides. Information on these is scanty, but a recent report on the lipopolysaccharide composition of a strain of *Myxococcus fulvus* (Weckesser, Mayer & Drews, 1971) disclosed the presence of 3-*O*-methyl-D-xylose. The optical enantiomorph of this unusual methyl pentose was discovered simultaneously in the budding bacterium *Rhodopseudomonas viridis*, but whether these unusual sugars are of widespread occurrence is still uncertain.

The object of the present study was to compare the phenol-extractable polysaccharides of a number of *Cytophaga* strains with the corresponding polymers from representative species of fruiting myxobacteria and to determine the similarity or otherwise of the polymers isolated to corresponding material from eubacteria.

METHODS

Bacterial strains and methods of culture. The strains used in this study, together with their sources, are listed in Table 1. The *Cytophaga* strains were mainly isolated in our laboratory and showed the following characteristics (cf. Mitchell, Hendrie & Shewan, 1969): Gram-negative rods; mainly long and slightly filamentous, showing gliding motility on solid surfaces; no resting cells or fruiting bodies detectable; slime frequently present (see Table 1); yellow-pigmented, spreading colonies growing optimally at 30 to 32 °C; lysis of heated bacterial cells observed on bacterial-cell agar plates; gelatin liquefied and 'Azocoll' (Calbiochem-coloured protein conjugate) hydrolysed; *O*-nitrophenyl β -D-galactoside and *O*-nitrophenyl β -D-glucosaminide hydrolysed. All strains grew on a simple synthetic medium in which the carbon source was 1 % (w/v) sodium glutamate. The *Cytophaga* strain 292 of marine origin grew equally well on a similar synthetic medium with a sea-water salts base. The *Cytophaga auarantiaca* and *Sporocytophaga myxococcoides* strains grew slowly on the glutamate synthetic medium with cellulose added either as a suspension or in the form of sheets of Whatman no. 1 paper.

Cells for preparation of polysaccharides were grown in 1 l samples of the appropriate medium in 2-l Erlenmeyer flasks shaken at 200 rev./min at 30 °C on an orbital shaker (L.H. Engineering, Stoke Poges, Buckinghamshire). Cellulolytic bacteria were harvested after 7 days of incubation and other strains of non-fruiting myxobacteria after 2 to 3 days. The representative strains of higher myxobacteria were grown similarly for 4 to 5 days in shaken flasks of C/10 medium (Rechenbach & Dworkin, 1969). All bacteria were gently washed with 0.9 % (w/v) saline and then lyophilized. The cellulolytic strains were harvested as a cell pellet with residual cellulose and the entire pellet was lyophilized.

Lipopolysaccharide and polysaccharide preparation. Extraction of bacteria with 45 % (w/v) aqueous phenol was by the method of Lüderitz *et al.* (1965), followed by ultracentrifugation at 100000g for 3 to 4 h to yield a deposit of the lipopolysaccharide gel (LPS) and a supernatant solution (SF) containing soluble polysaccharides. For cellulolytic strains the cellulose was removed by filtration from the dialysed phenol extracts. No appreciable cellulose-derived material was found in the subsequent extracts. Lipid-free polysaccharides (PS) were prepared from LPS by hydrolysis in 1 % (v/v) acetic acid for 1 h at 100 °C. Degraded material of low molecular weight was removed by dialysis and precipitated lipid was deposited by low-speed centrifugation. All polysaccharide and lipopolysaccharide preparations were lyophilized.

Analytical methods. Total hexose, heptose and methyl pentose estimations were performed

Table 1. *Strains of myxobacteria and yields of polysaccharides (as % dry weight)*

Designation	Strain	Source	Yield* of		Mucoid character
			LPS† (%)	SF† (%)	
<i>Cytophaga</i>	NCIB 9336	—	0.7	3.3	+
	NCIB 9337	—	1.1	2.9	+
	G1	Soil	2.2	6.6	++
	G2	Soil	0.9	0.9	±
	G3	Soil	1.9	5.1	±
	G4	Soil	2.2	5.2	±
	G5	Soil	3.0	5.9	++
	G6	Soil	1.8	2.7	+
	G7	Soil	2.5	3.8	±
	G8	Soil	1.7	4.2	±
	C3	Lake water	2.4	3.9	++
	C4	Lake water	3.4	3.7	++
	C6	Lake water	0.7	1.9	++
	C7	Lake water	0.4	1.7	—
	H2	Soil	1.1	2.6	++
	H3	Soil	1.1	1.9	++
	NCMB 292	Sea-water	0.7	4.4	++
<i>C. aurantiaca</i>	NCIB 8628	—	—	—	—
<i>Sporocytophaga myxococcoides</i>	NCIB 8639	—	—	—	—
<i>S. cauliformis</i>	NCIB 9487	—	—	—	—
<i>Myxococcus xanthus</i>	—	Department strain	0.7	1.8	±
<i>M. virescens</i>	MXVI	(Dr Reichenbach)	1.8	4.3	±
<i>Chondrococcus exiguus</i>	ccc4	(Dr Reichenbach)	1.4	3.8	±
<i>Stigmatella aurantiaca</i>	sga1	(Dr Reichenbach)	0.5	1.0	—
<i>Polyangium ferrugineum</i>	pBr	(Dr Reichenbach)	1.5	2.9	—
<i>P. fuscum</i>	—	(Dr Kuhlwein)	0.3	1.3	—
<i>Sorangium compositum</i>	soc1	Rabbit dung‡	0.4	1.2	—

* Dry wt of bacteria. † LPS = lipopolysaccharide; SF = supernatant after ultracentrifugation.

‡ Reichenbach (1970).

on unhydrolysed material by micro-modifications of the cysteine-sulphuric acid reaction under appropriate conditions (Dische & Shettles, 1948; Dische, Shettles & Osnos, 1949). Pentose was assayed by the orcinol technique. D-Glucose and D-galactose were determined, using the respective oxidase-based procedures, on samples hydrolysed in N-H₂SO₄ (100 °C for 4 h) and neutralized with Amberlite resin IR 410 (HCO₃ form). Total hexosamine was determined on hydrolysates (6 N-HCl at 100 °C for 6 h) after evaporation of the acid *in vacuo*; D-galactosamine was assayed specifically in the hydrolysates with the galactose oxidase reagent. Hydrolysates (H₂SO₄ or HCl) were examined by paper chromatography for the presence of monosaccharides. The solvents were: I, pyridine-butan-1-ol-water (4:6:3, by vol.) (Whistler & Conrad, 1954); and II, butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1946). Sugars were detected with saturated aniline oxalate solution or alkaline silver nitrate and amino sugars with ninhydrin reagent.

RESULTS

Isolation of lipopolysaccharides. The phenol extraction procedure, widely used to obtain lipopolysaccharides from whole cells of Gram-negative bacteria, has also proved successful for the family Myxobacteriaceae. Low yields were at first obtained from the fruiting species; this was due to the difficulty of suspending the cells in water and was overcome by a brief extraction with chloroform/methanol (2:1, v/vat) room temperature prior to phenol extraction. Purification by ultracentrifugation yielded clear colourless gels of LPS and clear supernatant fluids which, in some preparations, were very viscous. Although the high viscosity was partly due to the presence of RNA, it may also result from surface polysaccharide slime or capsule. The yields of LPS and non-sedimentable material resembled those described for other Gram-negative bacteria (Table 1). Because of the presence of cellulose in the material used for extraction, no realistic yields can be given for *Cytophaga aurantiaca* or *Sporocytophaga myxococcoides*.

Lipopolysaccharide composition. Preliminary examination of polysaccharide hydrolysates by paper chromatography indicated the presence of a range of monosaccharide components of which the most frequent were ribose, mannose and galactose. Rhamnose was the only 6-deoxyhexose identified and ribose the most frequent pentose. Glucosamine and galactosamine were also detected. The same sugars were observed in hydrolysates of the supernatant fluids after ultracentrifugation (SF), probably due to material of LPS origin but lower molecular weight failing to sediment in the ultracentrifuge. Subsequently, all the LPS preparations were analysed for the sugars identified in paper chromatograms (Table 2). Traces of other sugars were found in several preparations; as judged by their intensity of staining on chromatograms, the amount of these other monosaccharides probably represented less than 5% of the total sugars present and was seldom enough to permit characterization. All the lipopolysaccharides contained material giving a positive reaction in the thiobarbituric acid test as modified by Heath (Lüderitz *et al.* 1965), but the lack of satisfactory standards prevented quantitative estimation of the presumed 2-keto-3-deoxyoctonate. More surprising was the observed content of heptose as assayed by the cysteine-sulphuric acid test. All lipopolysaccharides were so tested but the range of values obtained was 0 to 2%. This may indicate a lack of heptose or, alternatively, much smaller quantities than are commonly found in the lipopolysaccharides of the eubacteria.

Many of the chromatograms of lipopolysaccharide hydrolysates carried a spot staining with alkaline silver nitrate and having R_{gle} 2.0 and R_{rhamnose} 1.2 in solvent I. The R_{gle} and R_{rhamnose} in solvent II were 3.4 and 1.3 respectively. This is similar to the mobility of 3-*O*-methyl-D-xylose (Weckesser *et al.* 1971) and the unknown substance also resembled this methylpentose in reacting with aniline oxalate to yield a red-coloured product. A small amount of the sugar was purified by preparative paper chromatography in solvent I. It did not react in either the orcinol test for pentoses or the cysteine-sulphuric reaction for 5-methylpentoses. Consequently, the presence of the 3-*O*-methylpentose does not affect the values obtained in the analyses for 5-methylpentoses and pentoses.

Hydrolysates of several lipopolysaccharides prepared from fruiting myxobacteria (but not from the *Cytophaga* strains tested) contained both ribose and another substance staining red with aniline oxalate. From its position on chromatograms run in both solvent systems, the pentose was identified as xylose; as judged by the intensity of staining of LPS hydrolysates, the ratio of xylose (when present) to ribose was about 1:5, except in *Chondrococcus* LPS which contained xylose but little ribose. Weakly stained spots were observed on some

Table 2. Composition of myxobacterial lipopolysaccharides (as % dry weight)

Strain	Rhamnose	Ribose	Mannose	Glucose	Galactose	Hexo- samine	Galacto- samine
<i>Cytophaga</i>							
9336	6.7	10.6	10.5	1.6	1.2	5.6	0
9337	0	10.4	8.3	0	2.7	13.2	6.6
G1	0	12.8	11.0	0.7	5.3	13.2	5.7
G2	0	13.5	11.3	9.6	0.6	6.5	0
G3	0	12.5	10.0	0	1.0	7.3	0
G4	8.2	7.4	7.2	0.7	5.6	9.6	0
G5	4.9	7.1	8.1	4.0	4.7	11.9	3.4
G6	11.2	10.7	9.0	1.2	7.0	7.2	0
G7	8.4	9.3	5.6	9.2	0.7	11.2	7.8
G8	0	13.2	8.0	1.4	8.3	7.7	0
C3	0	9.3	10.2	0.6	0.5	10.1	5.0
C4	0	12.9	9.0	1.1	6.5	11.4	4.4
C6	0	14.4	8.7	1.6	4.8	13.9	5.9
C7	0	11.0	6.2	1.6	4.7	9.6	4.0
H2	6.8	7.7	9.3	11.5	9.3	11.6	3.9
H3	0	10.2	13.0	3.8	6.7	10.8	3.8
292	0	10.5	7.2	3.3	6.6	9.8	3.8
<i>C. aurantiaca</i>	11.6	9.1	10.5	7.5	0	7.5	0
<i>Sporocytophaga myxococcoides</i>	0	11.4	5.7	7.3	7.2	6.8	0
<i>S. cauliformis</i>	0	13.4	8.5	2.5	13.4	5.2	0
<i>Myxococcus xanthus</i>	8.6	15.2	0	7.9	5.1	15.6	5.6
<i>M. virescens</i>	0	10.7	9.3	6.4	0	11.1	6.4
<i>Chondrococcus exiguus</i>	14.1	12.4	11.0	4.4	6.6	7.6	3.8
<i>Stigmatella aurantiaca</i>	12.5	7.4	18.5	5.0	2.4	5.0	0
<i>Polyangium ferrugineum</i>	6.7	9.7	9.0	7.2	2.9	7.2	0
<i>P. fuscum</i>	7.5	11.0	9.0	12.4	1.0	6.0	0
<i>Sorangium compositum</i>	0	13.4	1.4	5.2	4.0	7.7	3.6

chromatograms developed with ninhydrin but insufficient material was available for further identification or characterization of the possible amino sugars.

Derivation of sugars found in lipopolysaccharide hydrolysates. Even after purification of lipopolysaccharide solutions by ultracentrifugation, preparations from whole cells can be associated with contaminating polymers; these may include nucleic acids and exopolysaccharides. If the ribose were derived from RNA rather than lipopolysaccharide, solutions should have an appreciable extinction at 260 nm. No such absorption was demonstrated by solutions of lipopolysaccharides, although the supernatants obtained after ultracentrifugation were rich in nucleic acids as judged by their extinction at 260 nm and the high ribose content. Further proof that the ribose in myxobacterial preparations was an integral component of the LPS was obtained by an examination of LPS prepared from cell walls of representative *Cytophaga* strains. It closely resembled the product from whole cells both in its ribose content and in the other sugars present.

The lipid A component of eubacterial lipopolysaccharides contains D-glucosamine; consequently, glucosamine is found in most lipopolysaccharides although it may not necessarily be part of the polysaccharide (Lüderitz, Staub & Westphal, 1966). Mild hydrolysis with acetic acid (1 %, v/v, at 100 °C for 1 h) splits off the lipid A, leaving a lipid-free water-soluble polysaccharide. Where sufficient material was available, *Cytophaga* lipopolysaccharides were freed from lipid A by acetic acid hydrolysis, dialysed, centrifuged and the supernatant polysaccharide solution lyophilized. Analysis of the lipid-free polysaccharides was

Table 3. *Composition of Cytophaga lipid-free polysaccharides (as % dry weight)*

Strain	Rhamnose	Ribose	Manose	Glucose	Galactose	Hexosamine	Galactosamine
G1	—	15.0	—	—	—	—	—
G4	11.1	15.1	14.5	0	16.4	14.2	0
G5	0	10.7	18.0	14.3	6.5	16.4	5.9
G6	16.8	10.7	23.8	1.7	18.8	13.1	0
G7	16.7	12.6	14.2	9.2	16.6	17.7	10.3
G8	12.4	24.8	14.9	0.8	12.3	12.0	0
C3	0	19.3	21.3	0	5.7	13.0	6.5
C4	0	15.0	18.0	0	18.8	17.0	8.1
C6	0	14.3	22.5	0	14.8	15.9	5.1
9336	16.7	15.0	14.5	0.6	3.5	7.1	0
9337	0	13.8	21.8	0.7	3.8	16.4	5.4

performed (Table 3). All contained glucosamine and six of the ten strains tested also contained galactosamine. The other sugars detected were the same as in the LPS preparation. In the *Cytophaga* lipopolysaccharides, glucosamine thus forms an integral part of the polysaccharide as well as probably forming part of the lipid A.

DISCUSSION

From all the strains of myxobacteria examined in the present study it proved possible to isolate lipopolysaccharide which is apparently analogous to the corresponding polymer found in other Gram-negative bacteria. In those strains producing microcysts or other differentiated cell forms, the analyses presented here represent a total of the material from vegetative cells and cyst forms, no attempt having been made to separate the two. In *Myxococcus xanthus* the mucopeptide of cysts and vegetative cells is of quantitatively similar composition (White *et al.* 1968), but without separation and analysis of the microcysts it is not clear whether they actually contain lipopolysaccharide. The presence of lipopolysaccharide in the vegetative cells of myxobacteria confirms the view derived from cytological studies (e.g. Pate & Ordal, 1967) that the cell walls of myxobacteria and eubacteria are not grossly different. The amounts of lipopolysaccharide extracted from myxobacteria vary but are again comparable to yields from such groups of bacteria as the Enterobacteriaceae.

The chemical composition of the myxobacterial lipopolysaccharides differs from that of eubacterial lipopolysaccharides only in the actual sugars observed. Many strains possessed a polymer containing ribose, mannose and galactose along with one or both of the hexosamines glucosamine and galactosamine; other monosaccharides found in a number of preparations included glucose and rhamnose. Unlike most eubacterial lipopolysaccharides, heptose was either absent or present in very small amounts in myxobacterial material. Few earlier reports exist on the composition of myxobacterial wall polysaccharides. Mason & Powelson (1958) detected galactose, glucose, hexosamine and rhamnose in walls of a strain of *Myxococcus xanthus* and this is consistent with the results currently presented. The extracellular slimes from *Cytophaga hutchinsonii* and *Sporocytophaga myxococcoides* were acid heteropolysaccharides which contained several of the sugars found in the lipopolysaccharides (Verma & Martin, 1967); as there were no checks on the purity of the preparations or on the specificity of the method of extraction used, some of the sugars found might have originated in lipopolysaccharide. No uronic acids were found during the present study, so the presence

of slime in the polymers used for analysis is unlikely. Lipopolysaccharides from certain groups of bacteria are characterized by the presence of some 'exotic' sugars. Thus, 3,6-dideoxyhexoses are widely found in the Enterobacteriaceae and the Parvobacteriaceae (Lüderitz, Jann & Wheat, 1968). The presence of 3-methylxylose in *Myxococcus fulvus* was recently noted (Weckesser *et al.* 1971) and this or similar sugars have been found in several of the lipopolysaccharides from myxobacteria, although only in the *Chondrococcus exiguus* strain were the amounts of methylxylose comparable to those of other component sugars. This type of sugar also occurs in *Rhodopseudomonas viridis* (Weckesser *et al.* 1971) while 3,5-dimethylpentoses are known to occur in another photosynthetic species, *Rhodopseudomonas capsulata* (Weckesser, Mayer & Drews, 1970).

One surprising aspect of the results was the lack of appreciable differences between lipopolysaccharides prepared from the non-fruiting *Cytophaga* and *Sporocytophaga* strains, and the fruiting myxobacteria. Although all these bacteria are currently grouped in the myxobacteria, wide differences are found between the two major groups. The non-fruiting group have %GC varying from about 32 to 39, while the fruiting types form a more compact group within the range 67 to 70 (Mitchell *et al.* 1969). Considerable differences in the gross composition of the cell wall polysaccharides of the two groups might thus have been expected but none were found. The actual polysaccharide structures may still, however, differ. It should prove interesting to determine whether the lipopolysaccharide and its composition remain constant in the fruiting species, or whether, as in more complex micro-organisms, different cell wall polymers are formed during different phases of growth.

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