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Distribution and Composition of Lipopolysaccharides from Mycoplasmas

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Polymeric carbohydrates containing glycerol and fatty acids were isolated from whole cells and membranes of mycoplasmas by hot aqueous phenol extraction and gel filtration. Lipopolysaccharides were found to occur in four species of *Acholeplasma*, two of *Anaeroplasma*, and in *Mycoplasma neurolyticum*. None were detected in *Spiroplasma citri* or in five species of *Mycoplasma*. All lipopolysaccharides contained both neutral and *N*-acylated amino sugars in ratios varying from 1:1 to 3:1. The neutral sugars found in varying distribution were glucose, galactose, and mannose. The amino sugars included fucosamine, an unidentified deoxyhexosamine, galactosamine, and glucosamine. Fucosamine and glucose were the only sugars common to all lipopolysaccharides. The fatty acids were similar to those found in the lipids of each organism.

A new class of lipopolysaccharide has been reported to occur in *Thermoplasma acidophilum* (17, 18). Structurally this molecule is a polymannosyl glucosyl glycerol diether and can be viewed as an extended oligosaccharide chain attached to a glycolipid. Glycosyl diglycerides containing from one to five sugar residues occur in many mycoplasmal species (28). These glycolipids exhibit serological specificity when used as antigens in complement fixation reactions (3, 21, 22, 30). Furthermore, this serological specificity is directly related to the structure of the sugar residues (30). Specific binding of certain lectins by various mycoplasmas suggests that sugar residues are located at or near the surface of these organisms and serve to distinguish one species from another (26). These findings prompted a search for lipopolysaccharides, i.e., glycosyl diglycerides with long polysaccharide chains, in mycoplasmas other than *Thermoplasma*. A preliminary report has suggested their existence in aqueous phenol extracts (29).

MATERIALS AND METHODS

The organisms employed for this study are listed in Table 1. The culture media, growth conditions, and method of harvesting of the different organisms have been described previously (11, 12, 15, 25, 27). In some instances (*Acholeplasma laidlawii, A. modicum, A. axanthum, Mycoplasma neurolyticum*), parallel experiments using intact organisms and membranes were carried out. Membranes of *Acholeplasma* species were prepared by osmotic lysis, and membranes of *Mycoplasma* species were prepared by sonic disintegration (30).

Freeze-dried organisms or membranes (1 to 10 g) were extracted three times with 30 or more volumes of chloroform-methanol (2:1, vol/vol) to remove lipids. The air-dried residues after lipid extraction were stirred with 45% aqueous phenol (1 g of residue per 35 ml of aqueous phenol) at 65 to 68 C for 15 min (33). The aqueous layer was collected after centrifugation at 12,000 × *g* for 30 min at 0 C. The phenol layer was reextracted with an equal volume of water. The combined aqueous layers were dialyzed for 60 h at room temperature against several changes of deionized water. Following lyophilization, the fluffy white material was dissolved (about 1 g per 100 ml) in 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 7.0, made 0.001 M with $MgCl_2$. After addition of a few milligrams each of ribonuclease A and deoxyribonuclease I (EC 3.1.4.5) (Sigma Chemical Co., St. Louis, Mo.) and a drop of toluene to prevent microbial growth, the mixture was incubated at 37 C overnight. The enzymatically digested mixture was dialyzed against deionized water at room temperature for 24 h and lyophilized. This procedure degraded contaminating nucleic acids to smaller fragments that could be separated more easily from the polymeric carbohydrates by gel filtration. The use of membranes as starting material significantly reduced nucleic acid contamination.

Purification of the lipopolysaccharides was achieved by passage of the nuclease-digested preparations through columns of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) or controlled-pore-size glass beads, CPG-10-2000 (Electro-Nucleonics, Inc., Fairfield, N.J.). Both types of columns gave similar results: the lipopolysaccharides were eluted in the void volume and the nucleic acid fragments were eluted after the void volume. Void volume was determined with blue dextran of molecular weight about 2×10^6. In a typical operation, 150 mg or less of the enzymatic digest was applied in a volume of 3 to 5 ml of water onto a column (2.5 by 70 cm) of either Sephadex or glass beads. Deionized water served as eluant. Ten-milliliter fractions

were collected and assayed by the phenol-sulfuric acid method (2) for carbohydrate and absorption at 260 nm for nucleic acids. The fractions containing carbohydrate but lacking significant absorption at 260 nm were pooled and lyophilized. This fraction derived from each organism was employed in all subsequent analyses. Since these preparations were very hygroscopic, care was taken to dry the material to constant weight.

Unhydrolyzed preparations were assayed for protein by the method of Lowry et al. (14), for nucleic acid by absorption at 260 nm, for phosphorus by the method described by Ames (1), for neutral sugars using phenol-sulfuric acid (2), and for free amino groups by reaction with ninhydrin (24). Two hydrolysis procedures were used to determine the kinds and amounts of neutral and amino sugars, glycerol, and fatty acids. In each case an internal standard of C19 fatty acid was added to known weights (approximately 5 mg) of lipopolysaccharides prior to hydrolysis. One milliliter of 6 M trifluoroacetic acid (23) or 2 N HCl was added and the hydrolysis was carried out in Teflon-lined screw-cap tubes for 18 to 22 h at 100 C. Destruction curves for neutral and amino sugars showed that only galactose was lost, in the amount of about 10%, when trifluoroacetic acid was used. Each hydrolysate was extracted twice with 3 ml of hexane to separate the fatty acids. The pooled hexane layers of each sample were dried under a stream of N_2, esterified with 10% perchloric acid in methanol, and assayed for fatty acid methyl esters by gas-liquid chromatography. The aqueous phase was dried under N_2 and applied to a column (1 by 8 cm) of Dowex-50-X8, H^+ form in 2 ml of water (4). Two additional wash volumes of 2 ml each were added to the column, followed by 5 ml of water. The water eluate containing the neutral sugars was collected in a tube to which *m*-inositol was added as an internal standard. The amino sugars were eluted with 5 ml of 2 N HCl followed by 5 ml of water into a tube containing inositol. The eluates were dried in vacuo and dissolved in 1.0 ml of water. Known portions of these fractions were used for assays of neutral sugars by reaction with phenol-sulfuric acid (2) and for amino sugars by the Elson-Morgan reaction (7). The remainder of each fraction was used for assays of neutral and amino sugars by gas-liquid chromatography. Glycerol was determined on neutralized 2 N HCl hydrolysates by measuring the reduction of nicotinamide adenine dinucleotide by glycerol-3-phosphate dehydrogenase after phosphorylation with glycerokinase (34).

Neutral and amino sugar standards, except fucosamine, were obtained from commercial sources. Fucosamine was purified from the lipopolysaccharide of *Chromobacterium violaceum* (NCTC 7917) (6). Hydrolyzed lipopolysaccharide was subjected to ion exchange chromatography on a column (1 by 45 cm) of Dowex 50-X8, H^+ form. Neutral sugars were eluted with several bed volumes of water. Amino sugars were eluted with 0.33 M HCl (5). Fifty-milliliter fractions were collected, dried in vacuo, and checked for composition and purity by thin-layer chromatography (13) on Eastman Chromagram 13255 cellulose (no. 6064) using ethyl acetate-pyridine-water-glacial acetic acid (5:5:3:1, vol/vol) as developing solvent. Fucosamine eluted in high purity in the 100- to 150-ml fraction.

A Hewlett-Packard model 420 biomedical gas chromatograph with flame ionization detectors and a model 3370A digital electronic integrator were used for gas-liquid chromatography. The procedure used for chromatography of fatty acid methyl esters has been documented previously (15). Neutral sugars were identified as the trimethylsilyl derivatives of both free sugars and methyl glycosides, and amino sugars as trimethylsilyl derivatives of both free and *N*-acetylated sugars. Neutral sugars were converted to methyl glycosides by incubation at 100 C overnight in anhydrous 0.5 M HCl in methanol. Reagent was removed under N_2. Amino sugars were converted to the *N*-acetyl derivatives by incubation overnight in methanol-acetic anhydride (3:1, vol/vol) with a small amount of solid silver acetate as catalyst. Reaction mixtures were filtered through glass wool and dried under N_2. All carbohydrate samples were converted to the trimethylsilyl derivatives by incubation for at least 30 min at room temperature in a reagent composed of pyridine-hexamethyl disilazane-trimethylchlorosilane-bistrimethylsilyl trifluoroacetamide, 2:2:1:1 (vol/vol). Reagent was removed by evaporation under N_2 and the residues were reconstituted in chloroform for gas-liquid chromatography on a glass column (183 by 0.6 cm) packed with 5.5% SE-30 on GasChrom Q. Column temperature was 185 C, carrier gas was helium, and flow rate was nominally 60 ml per min (15).

RESULTS

A typical chromatographic pattern on controlled-pore-size glass beads for a mycoplasma containing lipopolysaccharide is shown in Fig. 1. A peak containing carbohydrate as measured by the phenol-sulfuric acid method appears at the void volume. This peak is followed by a second containing nucleic acid. The carbohydrate appearing in the second peak is accounted for by the pentoses found in nucleic acids. Those mycoplasmas yielding aqueous phenol-extractable carbohydrate with chromatographic behavior similar to that shown in Fig. 1 are listed in Table 1 together with those mycoplasmas that apparently contain no such carbohydrate. In the case of the latter group of organisms only the nucleic acid peak is seen in the chromatographic patterns. To ensure that the nucleic acid peak did not contain polysaccharide, samples from this peak were hydrolyzed and examined for hexoses and amino sugars by gas-liquid chromatography. Only trace amounts or no hexoses or amino sugars could be detected.

The yields of polymeric carbohydrate from lipid-extracted whole-cell residues are tabulated in Table 2. Polymeric carbohydrate accounts for about 1% of the dry weight for the species of *Acholeplasma* and *M. neurolyticum*,

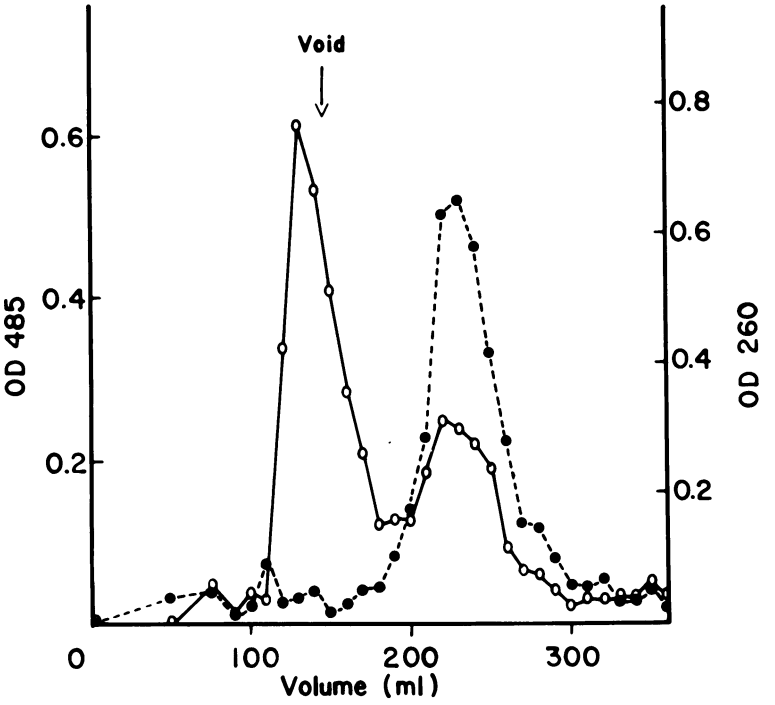

FIG. 1. *Separation of lipopolysaccharide from nucleic acids by filtration through controlled-pore-size glass beads. Column of CPG-10-2000 glass beads, 2.5 by 70 cm (exclusion limit, 1.2 × 10⁶). Ten-milliliter fractions of water eluant were collected. Solid line, Carbohydrate measured by phenol-sulfuric acid method; dashed line, nucleic acids measured by absorption at 260 nm.*

TABLE 1. *Distribution of lipopolysaccharides among various mycoplasmas*

Organisms containing lipopolysaccharides	Organisms devoid of lipopolysaccharides
Thermoplasma acidophilum strain 122-1B2	*Spiroplasma citri* strain Morocco
Acholeplasma laidlawii strain B	*Mycoplasma capricolum* strain 14
A. modicum strain 49	*M. gallisepticum* strain S-6
A. axanthum strain 743	*M. gallinarum* strain J
A. granularum strain BTS-39	*M. arthritidis* strain 07
Mycoplasma neurolyticum strain PG39	*M. hyorhinis* strain BTS-7
Anaeroplasma bactoclasticum strain 7LA	
A. abactoclasticum strain 6-1	

whereas *Anaeroplasma* and *Thermoplasma* contain elevated amounts. Table 3 presents analytical data on the composition of these polymers. Except *Thermoplasma*, which is devoid of amino sugars, all contain neutral and amino sugars, glycerol, and fatty acids. In general the molar ratio of fatty acids to glycerol approaches 2:1. The amino sugars are *N*-acylated (probably *N*-acetyl) as judged from the virtual absence of free amino groups. Variable amounts of nucleic acids and protein contaminate the preparations. The values given for protein are maximal since even the use of considerable sample gave minimal color reactions with Lowry reagent. All of the phosphorus could be accounted for as nucleic acids.

The identities and distribution of the neutral and amino sugars are shown in Table 4. Glucose was found to be the predominant neutral sugar in *A. laidlawii* and *Anaeroplasma abactoclasticum*, whereas galactose predominated in *A. modicum* and *M. neurolyticum*. *A. axanthum* and *A. granularum* contained equal amounts of glucose and galactose. Mannose occurred in *A. laidlawii* and the two anaerobic species. The 6-deoxyamino sugar, fucosamine, was common to all the organisms, being the predominant amino sugar in *A. laidlawii*, *A. axanthum*, *M. neurolyticum*, and *A. abactoclasticum*. An unidentified deoxyamino sugar, possibly quinovosamine, occurred in five of the seven organisms. Galactosamine was the major hexosamine found in *A. modicum* and *M. neurolyticum*. *A. granularum* contained mostly glucosamine. An equal distribution of glucosamine and galactosamine occurred in *A. bactoclasticum* and *A. laidlawii*. The latter organism contained very little hexosamine. The ratios of neutral to amino sugar varied from ap-

TABLE 2. *Yield of lipopolysaccharide from mycoplasmas*

Organism	Lipid-extracted residue	Lipopolysaccharide	
		g	% Residue
Thermoplasma[a] *acidophilum*	2.00	0.062	3.1
Acholeplasma laidlawii	6.30	0.046	0.7
A. modicum	12.10	0.083	0.7
A. axanthum	10.00	0.085	0.9
A. granularum	7.68	0.096	1.3
Mycoplasma neurolyticum	2.32	0.021	0.9
Anaeroplasma bactoclasticum	0.19	0.005	2.5
A. abactoclasticum	0.20	0.004	1.8

[a] Data from reference 17.

proximately 1:1 to 3:1 for the seven organisms examined.

The fatty acid distribution among the various lipopolysaccharides is presented in Table 5. In all cases, saturated fatty acids predominated. Significant amounts of hydroxy fatty acid occurred in *A. axanthum*, reminiscent of its lipids (16). Branched chained and unsaturated fatty acids comprised a significant fraction of the total fatty acids from the lipopolysaccharides of *A. granularum*, *A. bactoclasticum*, and *A. abactoclasticum*.

DISCUSSION

This study has demonstrated the existence of polymeric carbohydrate containing glycerol and fatty acids in lipid-free whole cells and membranes of mycoplasmas. All genera of the

TABLE 3. *Composition of lipopolysaccharides from mycoplasmas*

Component	Amt (μg/mg) in:							
	A. laidlawii	*A. modicum*	*A. axanthum*	*A. granularum*	*M. neurolyticum*	*A. bactoclasticum*	*A. abactoclasticum*	*T. acidophilum*[a]
Neutral sugars	556	502	308	216	409	99	104	824
Amino sugars	224	198	264	244	242	184	144	0
Glycerol	2	2	8	24	4	4	10	12
Fatty acids	4	14	18	28	8	40	33	143[b]
Protein	55	24	93	88	73	ND[c]	ND	36
Nucleic acids	86	53	90	135	74	ND	ND	1
Phosphorus	4	4	8	16	2	ND	ND	0
Free amino groups (μmol/mg)	0.054	0.034	0.019	0.080	0.033	ND	ND	0

[a] Data from reference 17.
[b] Alcohols.
[c] ND, Not done.

TABLE 4. *Neutral and amino sugar composition of lipopolysaccharides from mycoplasmas*

Sugar	Mol% of sugar in:						
	A. laidlawii	*A. modicum*	*A. axanthum*	*A. granularum*	*M. neurolyticum*	*A. bactoclasticum*	*A. abactoclasticum*
Neutral							
Glucose	98.5	9.2	45.2	52.0	5.9	47.3	89.0
Galactose		90.8	54.8	48.0	94.1	27.2	8.2
Mannose	1.5					25.5	2.8
Glucose:galactose:mannose	65.7:ND[a]:1	1:9.9:ND	1:1.2:ND	1.1:1:ND	1:15.9:ND	1.9:1.1:1	31.8:2.9:1
Amino							
Fucosamine	63.9	33.2	63.3	25.3	54.0	14.1	83.3
Deoxyamino sugar (unidentified)	30.7	11.2	30.9	14.5		17.0	
Glucosamine	2.9			60.2		34.1	16.7
Galactosamine	2.5	55.6	5.9		46.0	34.8	
Hexosamine : deoxyhexosamine	1:17.5	1.25:1	1:16.0	1.5:1	1:1.2	2.2:1	1:5.0
Neutral:amino sugar	3.05:1	3.14:1	1.45:1	1.09:1	2.06:1	0.66:1	0.89:1

[a] ND, Not done.

TABLE 5. *Fatty acid composition of lipopolysaccharides from mycoplasmas*

Fatty acid[a]	Mol% fatty acid in:						
	A. laidlawii	*A. modicum*	*A. axanthum*	*A. granularum*	*M. neurolyticum*	*A. bactoclasticum*	*A. abactoclasticum*
<12	5.3	3.5	1.0	0.8		0.3	1.6
12:0	11.4	18.2	5.8	4.0	3.8	2.2	1.7
13:0	1.3	0.4			0.4		0.2
13:br	1.7	2.6	1.9	6.2		0.4	0.7
13:h	0.6		0.3				
14:0	15.4	33.0	25.0	13.1	27.7	7.9	6.0
14:br		1.5		11.3		1.7	2.3
14:h			18.8				
15:0	0.6	1.0	0.3	0.8	0.9	7.2	5.1
15:br	9.5	2.5		14.7	0.5	8.9	16.2
15:h			0.4				
16:0	34.0	28.6	17.5	34.9	48.0	32.1	30.6
16:br				1.8	1.4	5.5	5.3
16:h	4.7		23.9				
17:0	3.0	1.1	0.5	0.1	0.6	0.9	0.5
17:br	2.3	0.5		1.0	0.1	5.4	4.7
18:0	5.1	7.0	3.6	1.6	13.1	14.2	14.6
18Δ	5.1	0.7	0.5	9.6	4.3	13.1	10.4
18:h			0.7				

[a] First number represents length of carbon chain; 0, saturated; Δ, double bond; br, branched; h, hydroxy.

class *Mollicutes* except *Ureaplasma* were represented by at least one species. All species of *Acholeplasma, Anaeroplasma,* and *Thermoplasma* examined contained this type of lipopolysaccharide. None could be detected in *Spiroplasma* or *Mycoplasma* except for *M. neurolyticum*. *M. neurolyticum* is distinguishable from other species of *Mycoplasma* by its production of an exotoxin (32) and its moderate sensitivity to penicillin (35). These polymeric substances apparently represent integral components of the membranes and are not merely adsorbed from the culture media. Except for the *Anaeroplasma,* no similar material is found in the ingredients of the culture media. Rumen fluid used for the cultivation of *Anaeroplasma* (11) conceivably could contain bacterial lipopolysaccharide. However, bacterial lipopolysaccharide contains 2-keto, 3-deoxyoctonic acid, which was proven to be absent from the lipopolysaccharides of *Anaeroplasma*.

The purity of our preparations is not absolute since contaminating nucleic acids and possibly some protein could be detected. Furthermore, no attempt has been made yet to determine homogeneity or heterogeneity within each preparation. All preparations behave as large particles on gel filtration columns similar to the lipopolysaccharide of *Thermoplasma,* the structure of which has been reported (17). Preliminary experiments utilizing mild alkaline deacylation have demonstrated an increase in aqueous solubility of the treated material, suggesting the removal of covalently bonded fatty acid residues. The occurrence of glycerol and fatty acids implies one possible structure of a polysaccharide chain covalently attached to diglyceride, i.e., a glycosyl diglyceride with an extended polysaccharide chain. Resolution of structure must await further analysis.

A unique feature of all of the lipopolysaccharides from mycoplasmas, except *Thermoplasma,* is the presence of fucosamine. This amino sugar, and the variability in content of amino and neutral sugars, allows for a wide variation in structure. Such variability would permit the antigenic distinction that has already been shown with these molecules or crude aqueous phenol extracts containing these compounds (30). It is probable that these lipopolysaccharides account for the lectin-binding properties of *Acholeplasma* and *M. neurolyticum* (26). If this is the case, an explanation for lectin binding by mycoplasmas devoid of these molecules is in order. Unfortunately, no generalizations can be made because of the lack of data. However, one can speculate that these mycoplasmas may contain glycoprotein in lieu of lipopolysaccharide. The existence of glycoprotein has been demonstrated in *M. pneumoniae* (10) and has been suspected to occur in *M. hominis* (9).

Polymeric carbohydrates have been found in some mycoplasmas, but no unequivocal demonstration of covalent bonding to lipids has been reported. *M. mycoides* strains produce extracel-

lular galactans and glucans that appear to have associated fatty acid esters (19). These exhibit serological activity and appear to be polydisperse in gel diffusion tests. Several unclassified strains from goats, sheep, and poultry, as well as *A. laidlawii*, have been found to contain trace amounts of neutral sugars extractable with hot aqueous phenol (20). No amino sugars could be detected in these preparations. A polymer composed of *N*-acetyl glucosamine and *N*-acetyl galactosamine has been isolated and characterized from *A. laidlawii* (8). It appears to be weakly associated with the cytoplasmic membrane (31). This polymer differs substantially from the material reported in our study in that it contains no neutral sugar or fatty acids. Although the relation between this *N*-acetyl amino sugar polymer and the lipopolysaccharide reported here is unknown, they appear to be distinct.

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