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Lipid and Lipopolysaccharide Composition of *Acholeplasma oculi*

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The total lipid content of *Acholeplasma oculi* comprises 13.3% of the dry weight of the organism and is about equally distributed between the neutral lipids plus glycolipids and the phospholipids. The phospholipids were identified as phosphatidyl glycerol and diphosphatidyl glycerol. The glycolipid fraction contained *O*-α-D-glucopyranosyl-(1 → 1)-2,3-diacyl glycerol and *O*-α-D-glucopyranosyl-(1 → 2)-*O*-α-D-glucopyranosyl-(1 → 1)-2,3-diacyl glycerol. The neutral lipid contained pigmented carotenoids. Hot aqueous phenol extraction of lipid-extracted whole cells yielded a polymeric carbohydrate comprising 2.3% of the dry weight of the organism. The *A. oculi* lipopolysaccharide was found to contain only neutral sugars and no amino sugar, in contrast to other acholeplasmas. The neutral sugars consisted of fucose, galactose, and glucose in a ratio of 2:19:3.

The occurrence of *Acholeplasma oculi* as a potential pathogen in goats was reported by Al-Aubaidi et al. (1). Isolation of this organism from horses (2) and camels (3) has been reported recently. Mycoplasmal components, such as glycolipids, phosphoglycolipids, and lipopolysaccharides, have been found (15) to be of significance not only because of their role in structure and function of membranes, but also because of their antigenic reactivity. This suggested their potential importance as specific antigenic determinants which could aid in the taxonomic classification of mycoplasmas. *A. oculi* was selected for study because this species is representative of a type not yet examined. The results show that the lipopolysaccharides of this organism are distinct from other acholeplasmas examined thus far.

MATERIALS AND METHODS

Growth of cells. *A. oculi* 19L was grown in tryptose broth containing 1% yeast extract, 1% glucose, and 1% (vol/vol) PPLO serum fraction (Difco Laboratories, Detroit, Mich.). The usual batch of organisms consisted of 90 liters of an 18-h-old culture started from 10% inoculum grown in the same liquid medium for multiple transfers. Incubation was carried out statically at 37°C in 2-liter volumes contained in 3-liter flasks. Growth was followed by visual examination for turbidity. Prior to harvesting, cultures were checked to ensure purity. The organisms were harvested by concentration in a Sharples centrifuge, followed by sedimentation at 27,000 × *g* and 0°C in a Sorvall RC2B centrifuge. The sediment was washed in cold phosphate-buffered saline (0.2 M, pH 7.5) and lyophilized. Isotopic labeling was performed by growth of organisms in 100 ml of medium supplemented with 5 μCi of [2-^{14}C]mevalonic acid (specific activity, 20 mCi/mmol), 2.5 μCi of [1-^{14}C]oleic acid (specific activity, 30 mCi/mmol), or 500 μCi of [^{32}P]orthophosphate (carrier-free).

Extraction of lipids and lipopolysaccharides. Lipids were extracted from lyophilized cells by stirring with 40 volumes of chloroform-methanol (2:1, vol/vol) at room temperature for 60 min. Methods for further purification and separation into major classes have been documented previously (10).

The lipopolysaccharides were extracted from the air-dried residue after lipid extraction, using the Westphal method of hot aqueous phenol as described by Smith et al. (14). Briefly, the dried residues were stirred with 45% aqueous phenol at 70°C for 15 min. The aqueous layer was collected after centrifugation at 12,000 × *g* for 30 min at 4°C. The phenol layer was reextracted with an equal volume of water. The combined aqueous phases were dialyzed for 60 h at 25°C against deionized water and lyophilized. The nucleic acid contaminants were digested by dissolving the lyophilized aqueous phase in 0.1 M Tris buffer (pH 7.5) made 0.001 M with $MgCl_2$ and adding a few milligrams of RNase A and DNase I (Worthington Biochemicals Corp., Freehold, N.J.). The mixture was incubated overnight at 37°C after adding 1 to 2 drops of toluene to prevent microbial growth. The enzymatic digest was dialyzed against deionized water at room temperature for 24 h and lyophilized. The final purification was performed by passage of the nuclease-digested preparation, dissolved in deionized water, through a column of controlled-pore-size glass beads (CPG-10-2000; Electro Nucleonics, Inc., Fairfield, N.J.). The collected fractions were assayed for carbohydrate, using the phenol-sulfuric acid method of Ashwell (5). The fractions containing carbohydrate and showing low adsorption at 260 nm were pooled, lyophilized, and weighed.

Preparation of membranes. Membranes were

prepared from washed cells by osmotic lysis (12). Membrane preparations were washed by centrifugation in water three or more times. After the final washing, membranes were suspended in deionized water to give a protein concentration of 25 mg/ml or greater.

Homogeneity or heterogeneity of lipopolysaccharides. Five methods were employed to determine the heterogeneity or homogeneity of lipopolysaccharides from *A. oculi*. These methods, which have been detailed previously (11), include permeation chromatography of deacylated lipopolysaccharides, permeation chromatography of the acetylated derivatives, permeation chromatography of the methylated derivatives, polyacrylamide gel electrophoresis in sodium dodecyl sulfate-containing gels, and Ouchterlony gel diffusion. Approximately 5-mg amounts of lipopolysaccharide were used for each analysis.

Analytical methods. Phosphorus was determined by the method of Ames (4). Nucleic acid was determined by absorption at 260 nm (7). Glycerol was determined enzymatically by glycerokinase and glycerophosphate dehydrogenase (17). Carbohydrate was estimated by the phenol-sulfuric acid procedure (5). Carbohydrate also was quantitatively estimated by gas-liquid chromatography using inositol as an internal standard (14). Amino sugar was assayed by the Elson-Morgan reaction (6). Protein was estimated by the method of Lowry et al. (8). Lipid content was determined gravimetrically after drying to a constant weight at room temperature under a stream of N_2. Fatty acids were identified as their methyl esters by gas-liquid chromatography on polar and apolar columns (14). Radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrometer (9). All materials and standards were the best grade commercially available.

RESULTS

Lipid composition. Total lipids extracted from *A. oculi* accounted for 13.3% of the dry weight of the organisms. The lipids were distributed equally between neutral lipids plus glycolipids and phospholipids (Table 1). Only those lipids that became labeled with isotopic precursors were considered integral components of the organism. Identification was limited to these lipids. Radioautographs of the thin-layer chromatogram of each lipid class are shown in Fig. 1.

Thin-layer chromatograms of the phospholipid fraction developed in chloroform–methanol–water(65:25:4, vol/vol/vol) indicated the presence of four components, A, B, B′, and C, by [^{32}P]phosphate and [1-^{14}C]oleate labeling and by spraying with phosphate reagents. Two lipid components, A and B, gave a rapid positive periodate-Schiff reaction. Analytical data on purified phospholipids are given in Table 2. Identification was achieved by assessing the behavior of the intact lipids on thin-layer chromatography and of the water-soluble deacylation products on paper chromatograms relative to authentic lipids. Components B and C, which account for greater than 95% of the total phospholipids, were identified as phosphatidyl glycerol and diphosphatidyl glycerol, respectively. The deacylation product of component B′ was identical to the deacylation product of diphosphatidyl glycerol. Component A gave a rapid periodate-Schiff reaction and a positive reaction for phosphate. Components A and B′ were identified as lysophosphatidyl glycerol and lysodiphosphatidyl glycerol, respectively.

Thin-layer chromatography of the glycolipid fraction, developed in chloroform-methanol (9:1, vol/vol), revealed four components: D, E, F, and G. All components incorporated [1-^{14}C]oleate but not [^{32}P]phosphate or [2-^{14}C]mevalonate. Each of the components gave a reaction with phenol–sulfuric acid reagent, were slow periodate-Schiff positive, but were ninhydrin and phosphorus negative. Total acid hydrolysis of the glycolipid fraction yielded fatty acids, glycerol, and glucose. Glucose was identified specifically by gas-liquid chromatography of the trimethylsilyl derivative. Analytical data on the purified glycolipids are given in Table 3. The retention times of the trimethylsilyl derivatives of the deacylation products were identical to the retention times of the mono- and diglycosyl glycerol from *Acholeplasma laidlawii* B.

Neutral lipids. Thin-layer chromatograms of the neutral lipid fraction were developed in a two-step solvent system, isopropyl ether–acetic acid (96:4, vol/vol) for 10 cm, followed by petroleum ether–diethyl ether–acetic acid (91:10:1, vol/vol/vol) for 13 cm in same direction. These chromatograms indicated the presence of three components by [^{14}C]oleate labeling (H, I, J) and two components by [2-^{14}C]mevalonate labeling (H, I). These components were identified as carotenoids and triglyceride. These lipids are common in all acholeplasmas including *A. axanthum* (13). Pigmented carotenoids were visualized easily by the yellow coloration of cell pellets. Unfractionated carotenoids exhibited the typical absorption spectrum with the major absorption peak at 468 nm.

Each phospholipid and glycolipid was treated

TABLE 1. *Lipid composition of A. oculi*

Lipid class	Dry wt of organism (%)	Total lipids
Total lipids	13.23	100
Neutral lipids	0.14	1.1
Glycolipids	6.10	46.1
Phospholipids	6.99	52.8

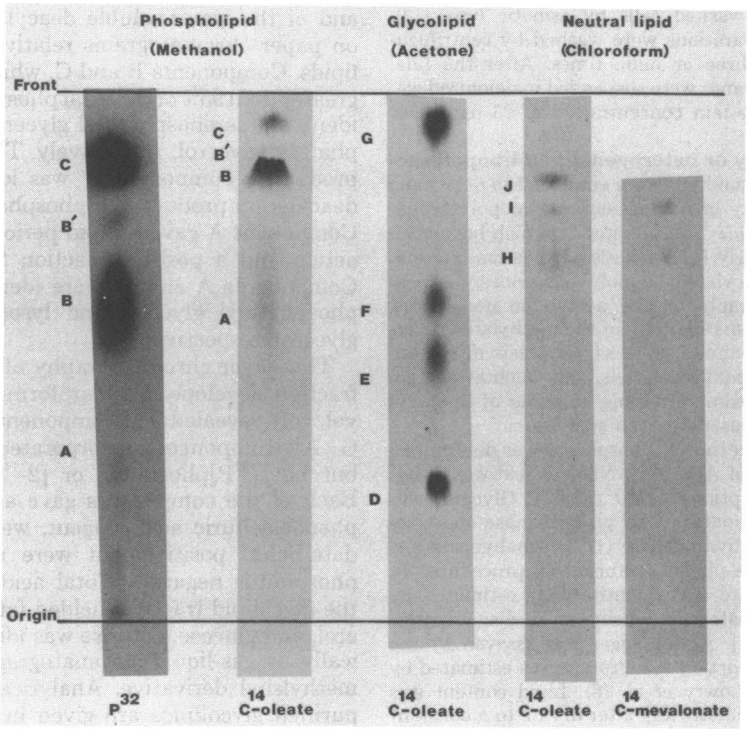

FIG. 1. *Autoradiographs of neutral lipid, glycolipid, and phospholipid fractions from A. oculi grown with $KH_2[^{32}PO_4]$, potassium [1-^{14}C]oleate, or [2-^{14}C]mevalonic acid.*

TABLE 2. *Identification of the phospholipids from A. oculi*

Component	Total PL[a] (%)	Water-soluble deacylation product[b]	Identity
A	1.9	GPG	Lysophosphatidyl glycerol
B	32.5	GPG	Phosphatidyl glycerol
B'	1.3	GPGPG	Lysodiphosphatidyl glycerol
C	64.3	GPGPG	Diphosphatidyl glycerol

[a] PL, Phospholipids.

[b] Abbreviations: GPG, glycerophosphoryl glycerol; GPGPG, glycerophosphoryl glycerophosphoryl glycerol.

TABLE 3. *Gas chromatographic analysis of trimethylsilyl derivatives of water-soluble deacylation products of glycolipids*

Glycolipid	Retention time (min)	Identity
A. oculi:		
Component D	19.68	Diglucosyl glycerol
Component E	1.72, 19.68	Diglucosyl glycerol
Component F	1.72	Monoglucosyl glycerol
Component G	1.72	Monoglucosyl glycerol
A. laidlawii B:		
O-α-D-Glucopyranosyl-(1 → 1)-glycerol	1.72	Monoglucosyl glycerol
O-α-D-Glucopyranosyl-(1 → 2)*O*-α-D-glucopyranosyl-(1 → 1)-glycerol	19.68	Diglucosyl glycerol

with sodium methoxide at room temperature for 1 h (9) and then extracted twice with 3 ml of hexane to separate fatty acid esters. Both of the two components observed on thin-layer chromatograms became radiolabeled when the organism was grown with either [2-^{14}C]acetate or [2-^{14}C]mevalonate. 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, using a C-19 saturated fatty acid as the internal standard. The bulk of the fatty acids consisted of myristic, palmitic, stearic, and oleic acids (Table 4). Saturated fatty acids predominated in both phospholipids and glycolipids. Oleic acid was absent in glycolipid fraction and diphosphatidyl glycerol.

Lipopolysaccharide composition. Hot aqueous phenol extraction of lipid-extracted whole cells of *A. oculi* yielded a polymeric carbohydrate accounting for 2.3% of the dry weight

TABLE 4. *Fatty acid composition of lipids from A. oculi*[a]

Fatty acid[b]	Percent total fatty acid of:					
	Lyso PG	PG	Lyso DPG	DPG	MGDG	DGDG
Unidentified	—[c]	—	—	—	0.7	—
Unidentified	—	—	—	—	0.2	—
12:0	1.7	0.1	—	2.6	13.7	1.9
12 br	0.1	—	—	0.3	1.5	0.6
13:0	0.1	—	—	0.2	0.4	0.2
14 br	0.5	—	0.2	0.6	1.0	0.5
14:0	20.5	6.2	11.1	26.8	24.0	22.9
15 br	—	—	—	—	1.0	0.9
15:0	0.8	0.2	0.4	0.7	0.6	1.4
16:2	—	—	—	—	—	2.9
16:1	0.6	0.5	0.5	0.1	—	—
16:0	45.6	55.5	49.6	50.5	42.2	54.9
17 br	—	—	—	—	0.2	0.1
17:0	0.4	0.2	0.2	0.6	0.2	0.4
18:2	0.6	0.3	0.4	0.8	6.6	6.5
18:1	21.5	16.8	15.4	—	—	—
18:0	7.6	20.2	19.3	16.5	7.3	6.8
20 br	—	—	2.9	—	—	—
20 Δ	—	—	—	—	0.3	—
20:0	—	—	—	0.3	0.1	—

[a] Abbreviations: Lyso PG, lysophosphatidyl glycerol; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol; MGDG, monoglucosyl diglyceride; DGDG, diglucosyl diglyceride.

[b] First number represents length of carbon chain. 0, Saturated fatty acid, 1, one double bond; 2, two double bonds; br, branched chain.

[c] No fatty acid present.

of the organisms. Lipopolysaccharides from other acholeplasmas accounted for about 1% of the dry weight of the organisms (12). The chromatographic pattern on controlled-pore-size glass beads was similar to that seen in our previous study (14). A peak containing carbohydrate, as measured by the phenol-sulfuric acid method, appeared at the void volume. This peak was followed by a second, containing nucleic acid. Table 5 presents analytical data on the composition of this polymer. The simple sugars, derived by trifluoroacetic acid and HCl hydrolysis, were subjected to trimethylsilylation and analyzed by gas-liquid chromatography. The sugars present in the polymer were fucose, galactose, and glucose in a ratio of 2:19:3. That the polymer contained no hexosamine and deoxyhexosamine was confirmed by the absence of Elson-Morgan reactive material. The saturated fatty acid content was similar to that of the glycolipids. No branched-chain or hydroxy fatty acids were detected.

Although nucleic acids and protein contaminated the preparation, all of the phosphorus could be accounted for as nucleic acid.

Homogeneity and heterogeneity. Five different pore-sized glass-bead columns, CPG-10-75, CPG-10-240, CPG-10-700 and CPG-10-2000, were used to examine the deacylated lipopolysaccharides and the peracetylated and permethylated derivatives. Figure 2 reveals the data for

TABLE 5. *Composition of lipopolysaccharides from A. oculi*

Component	Content (μg/mg)
Neutral sugars	472.2
Amino sugars	0
Glycerol	2
Fatty acids	4
Protein	43
Nucleic acids	142
Phosphorus	4
Free amino groups (μmol/mg)	0

deacylated lipopolysaccharides. Two peaks could be separated on both the CPG-10-240 and the CPG-10-700 columns. The peracetylated lipopolysaccharide also separated into two peaks on a CPG-10-75 column (Fig. 3). The permethylated lipopolysaccharides exhibited two peaks on a CPG-10-75 column and four peaks on a CPG-10-240 column (Fig. 4). These results suggested heterogeneity as to size.

Procion red-dyed lipopolysaccharide was prepared and subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate as previously described (11). No migration of the lipopolysaccharides from *A. oculi* into the gel occurred. The control lipopolysaccharide from *Acholeplasma granularum* migrated as shown previously (11). Presumably the presence of only neutral sugars in the lipopolysaccharide

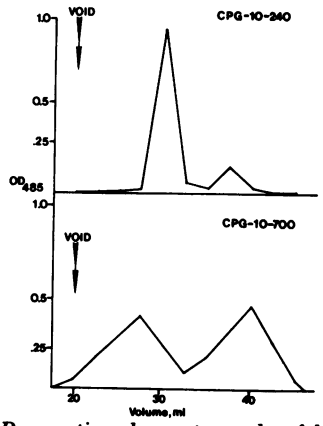

FIG. 2. *Permeation chromatography of deacylated lipopolysaccharides from A. oculi on columns of controlled-pore-size glass beads. Elution was accomplished with deionized water.*

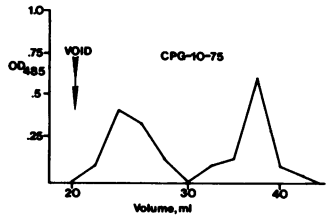

FIG. 3. *Permeation chromatography of peracetylated lipopolysaccharides from A. oculi on a column of controlled-pore-size glass beads. Elution was performed with methanol.*

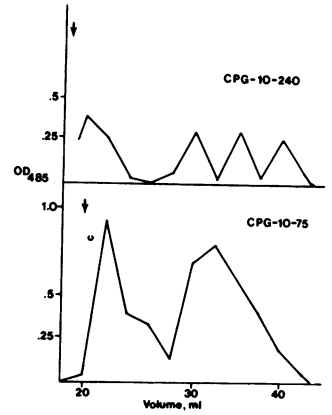

FIG. 4. *Permethylated lipopolysaccharides from A. oculi on a column of controlled-pore-size glass beads. Elution was performed with methanol.*

of *A. oculi* does not provide sufficient charge on the molecule to allow migration in an electrical field.

Ouchterlony gel diffusion analysis is shown in Fig. 5. The lipopolysaccharides produced one band when run against membrane antiserum (Fig. 5A). The membrane solubilized in 1% Triton X-100 produced two bands. The lipopolysaccharides did not produce any precipitin lines against lipopolysaccharide antiserum. Since the deacylated lipopolysaccharides gave two fractions by permeation chromatography, these were used for antibody absorption studies. Each fraction was collected and employed as an antigen. Membrane antiserum was absorbed three times with each fraction singly. Precipitates formed overnight at 4°C were removed by centrifugation. Each of the absorbed antisera was run against each fraction as well as against unabsorbed serum. No precipitin lines appeared with any antigen against absorbed sera, whereas a single line was observed against the unabsorbed serum (Fig. 5B). The line that appeared between unabsorbed serum and absorbed sera could be explained as excess antigen in adsorbed sera.

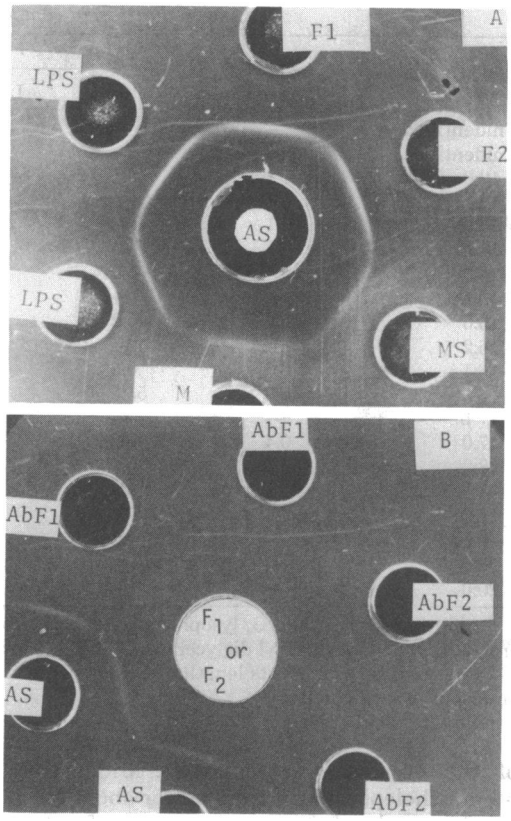

FIG. 5. *Ouchterlony gel diffusion. (A) Lipopolysaccharide (LPS) or its separated deacylated fractions (F_1, F_2) and membranes (M) or soluble membranes (MS) versus membrane-specific serum (AS). (B) Membrane antiserum absorbed with separated fractions F_1 and F_2 (AbF_1, AbF_2) versus deacylated fraction from A. oculi lipopolysaccharides.*

These two fractions derived by permeation chromatography of the deacylated lipopolysac-

charide were analyzed for sugar composition. Both fractions contained similar ratios of fucose, galactose, and glucose. These results indicated that identical antigenic determinants resided on both separated components but that a size difference existed.

DISCUSSION

The quantitative and qualitative lipid composition of *A. oculi* is similar to other acholeplasmas. Phosphatidyl glycerol and diphosphatidyl glycerol were found in all acholeplasmas, including *A. granularum*. Glycolipids, consisting of both mono- and diglycosyl diglycerides, are identical in structure to those found in *A. laidlawii* B and *Acholeplasma modicum*. The serological cross-reactivity between *A. oculi* and these organisms can be accounted for by the identity of these lipids. Pigmented carotenoids and glycerides are neutral lipids common to all acholeplasmas including *A. axanthum* (13). The occurrence of identical lipids in *Acholeplasma* species possesses significant value for differentiation between the members of *Acholeplasma* and *Mycoplasma*.

This study has demonstrated the existence of polymeric carbohydrate containing glycerol and esterified fatty acids in lipid-free whole cell and membrane of *A. oculi*. All acholeplasmas have been found to contain lipopolysaccharides in amounts varying from 0.7 to 2.3% of their dry weights. The highest amount was found in *A. oculi* and the lowest in *A. laidlawii* and *A. modicum*. The polymer from *A. oculi* contains fucose, galactose, and glucose in a ratio of 2:19:3 but no hexosamines or deoxyhexosamines. Glucose and, with the exception of *A. laidlawii*, galactose are common to all acholeplasma species. Fucose was found only in the polymer from *A. oculi*. Both the hexosamines, glucosamine and galactosamine, and the deoxyhexosamines, fucosamine and quinovosamine, occur in other *Acholeplasma* species in variable amounts. The purity of our preparation was not absolute, since contaminating nucleic acid and some protein were detected.

The results of permeation chromatography of deacylated lipopolysaccharide and the peracetylated and permethylated derivatives, and results from Ouchterlony gel diffusion, suggested that the lipopolysaccharide from *A. oculi* exists in at least two sizes of identical composition and antigenic specificity. The antigen-antibody reactions were not due to protein contaminants, since deacylated lipopolysaccharide, after passage through the glass-bead column, is completely devoid of protein, yet serves as an antigen in precipitin reactions.

The lack of migration of *A. oculi* lipopolysaccharide in sodium dodecyl sulfate-polyacrylamide gels coincides with the absence of detectable amino sugars in this polymer.

The inability of this lipopolysaccharide to elicit antibody response in rabbits might be the result of its small size (estimated molecular weight of 85,000) or the absence of amino sugars or both. Lipopolysaccharides from other *Acholeplasma* species, with the exception of *A. granularum*, have been shown to stimulate antibody production. The polymer from *A. granularum* is the smallest from acholeplasmas, having an estimated molecular weight of 20,000. The size of bacterial lipopolysaccharides is a determining factor in the immune response of B-lymphocytes.

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