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Lipid Composition of Some Species of *Arthrobacter*

N. SHAW AND D. STEAD

Microbiological Chemistry Research Laboratory, Department of Organic Chemistry, The University, Newcastle upon Tyne, NE1 7RU, England

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The lipids from *Arthrobacter crystallopoietes*, *A. pascens*, and *A. globiformis* were investigated. Each strain contained three glycolipids, a monogalactosyl diglyceride, a digalactosyl diglyceride, and a dimannosyl diglyceride, and traces of tri- and tetraglycosyl diglycerides. The phospholipids in all three strains consisted of bisphosphatidylglycerol, phosphatidylglycerol, and phosphatidyl*myo*inositol. No evidence could be obtained for the occurrence of mannophosphoinositides. Analysis of the fatty acids by gas-liquid chromatography showed that they are predominantly $C_{15:0}$ *anteiso* and $C_{17:0}$ *anteiso* compounds. No significant differences were observed in the composition of lipids extracted from homogeneous cell preparations of the rod and sphere forms of *A. crystallopoietes*.

The lipids of *Arthrobacter globiformis* contain three glycolipids: a monogalactosyl diglyceride, a digalactosyl diglyceride, and a dimannosyl diglyceride (22). Although glycosyl diglycerides are widely distributed in gram-positive bacteria (19), *A. globiformis* is unusual in containing two different diglycosyl diglycerides. In view of the known similarity in glycolipid composition between related organisms (19), it was of interest to study other members of this genus. The present paper describes the lipids of *A. crystallopoietes* and *A. pascens* and compares them with those of *A. globiformis*. An examination was also made of the differences in lipid composition between the two morphological forms (rods and spheres) of *A. crystallopoietes*.

MATERIALS AND METHODS

Materials. *A. pascens* NCIB 8910 and *A. crystallopoietes* NCIB 9499 were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. A culture of *A. globiformis* 616 and samples of digalactosylglycerol and dimannosylglycerol isolated from the glycolipids of this organism were kindly supplied by R. W. Walker, University of Massachusetts, Amherst. 1-*O*-β-D-Galactopyranosylglycerol, diglycerol phosphate, and bis(glycerolphosphoryl)glycerol were synthesized by previously published procedures (4, 5). A sample of phosphatidyl*myo*inositol was a gift from R. Letters, A. Guinness & Son Ltd., Dublin, Ireland.

Analytical methods. Hexoses were determined by the phenol-H_2SO_4 method (9), and periodate was measured spectrophotometrically (8). Paper chromatography of sugars, polyols, and glycosides was carried out by the descending method in the solvent systems butan-1-ol-pyridine-water, 6:4:3 (v/v), and butan-1-ol-ethanol-water aqueous NH_3 (specific gravity, 0.88), 40:10:49:1 (v/v), organic phase. Chromatography of phosphates was carried out by the ascending method in the system propan-1-ol-aqueous NH_3 (specific gravity, 0.88)-water, 6:3:1 (v/v). Compounds were detected by the periodate-Schiff reagents for α-glycols (1), the alkaline $AgNO_3$ reagent for sugars and polyols (20), and the acid molybdate spray for phosphoric esters (11). Lipids were examined by thin-layer chromatography (TLC) in the system chloroform-methanol-water, 65:25:4 (v/v). Components were detected by reagents specific for phosphorus (7), α-glycols (18), and amino compounds (16). Preparative TLC was carried out on plates (20 by 20 cm) coated with silicic acid 1-mm thick. Fatty acids were analyzed by gas-liquid chromatography (GLC) as their methyl esters (3) and glycosides as their trimethyl silyl derivatives (4).

Samples of lipids and glycosides were hydrolyzed by heating at 100 C for 3 hr with 2 N HCl. The hydrolysate was evaporated to dryness over KOH in vacuo, redissolved in a minimum quantity of water, and examined by paper chromatography. Lipids were deacylated with sodium methoxide (16).

Bacteria were grown at 30 C with aeration in a medium composed of (w/v): tryptone (1.0%), glucose (1.0%), yeast extract (0.5%), and dipotassium hydrogen phosphate (0.5%). The cells were harvested after 20 hr of growth, washed twice in saline, and then freeze-dried. Under these conditions, the cells were a mixture of irregular, gram-positive rods and spheres. The medium of Ensign and Wolfe (10) was used to obtain homogeneous cell preparations of *A. crystallopoietes* in the rod and sphere form. Cultures grown at 30 C for 48 hr were composed entirely of the sphere form. The rod form was obtained by induction of an actively growing sphere culture with sterile peptone solution (0.5%, w/v). The cells were harvested 7 hr after addition of the peptone, when complete reversion to the rod form had taken place. The cells of both forms were washed twice in saline and freeze-dried.

Extraction of the lipids. The freeze-dried cells were stirred with twice their volume of chloroform-methanol (2:1, v/v) for 3 hr at room temperature. This process was repeated twice, and the combined extracts were evaporated to dryness in vacuo. The yield of lipid from all three strains was 4 to 5% dry cell weight. Each lipid extract was separated into neutral, glycolipid, and phospholipid fractions by silicic acid chromatography (21). The neutral fractions which were devoid of carbohydrate and phosphorus were not investigated further.

RESULTS

Glycolipids of A. crystallopoietes. The glycolipid fraction, which represented about 25% of the total lipid, was shown by TLC to consist of three major components, R_F 0.66 (A), 0.51 (B), and 0.43 (C), and a trace component of R_F 0.32 (D). All four gave a blue-grey color with periodate-Schiff reagents, typical of glycolipids (18). Attempts to separate the four components on a column of silicic acid by stepwise elution under a variety of conditions were unsuccessful. A pure sample of glycolipid A was obtained by preparative TLC, and the corresponding glycoside was obtained by deacylation (glycoside A). Glycolipids B and C migrated too close together on TLC to allow complete separation; they were therefore eluted together, and the glycosides obtained on deacylation (glycosides B and C) were separated by paper chromatography. Although glycolipid D was detected on preparative TLC, elution of the band did not yield sufficient material for further studies. Glycosides A, B, and C were isolated in a ratio of approximately 1:3:1, and they all gave a rapid purple color with the periodate-Schiff reagents on paper chromatograms indicative of formaldehyde produced from a 1-substituted glycerol (17).

Glycoside A gave galactose and glycerol on acid hydrolysis and had paper chromatographic properties identical to an authentic sample of 1-*O*-β-D-galactopyranosylglycerol. The retention times on GLC of the trimethylsilyl derivatives were also identical.

Glycoside B gave mannose and glycerol on acid hydrolysis in the molar proportions of 2:1. A sample of B was oxidized with 25 mM sodium metaperiodate for 114 hr, and the product was hydrolyzed with 2 N HCl. A hexose determination showed that at least 42% of the mannose was unoxidized, indicating a 1 → 3 linkage between the two mannose units. The glycoside had paper chromatographic properties identical to an authentic sample of *O*-α-D-mannopyranosyl-(1 → 3)-*O*-α-D-mannopyranosyl-(1 → 1)-D-glycerol, and the retention times of the trimethylsilyl derivatives on GLC were also identical.

Glycoside C gave galactose and glycerol on acid hydrolysis in the molar proportions of 2:1 and had properties identical to *O*-β-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl-(1 → 1)-D-glycerol both on paper chromatography and GLC.

The fatty acid composition of glycolipids A and B is shown in Table 1.

Phospholipids of A. crystallopoietes. The phospholipid fraction, which represented about 55% of the total lipid, was examined by TLC. Three components were observed, R_F 0.63, 0.32, and 0.17, and these were separated by preparative TLC. The chromatographic properties of the two major components, R_F 0.63 and 0.32, corresponded to those of bisphosphatidylglycerol and phosphatidylglycerol, respectively. Their identification was confirmed by deacylation to the water-soluble phosphate esters, bis(glycerylphosphoryl)glycerol and diglycerol phosphate, and comparison with authentic materials. Both compounds gave a mixture of glycerol and glycerol phosphates on acid hydrolysis. Their fatty acid composition is shown in Table 1. On acid hydrolysis, the third phospholipid (R_F 0.17) gave glycerol, glycerol phosphates, inositol phosphates, and a trace of mannose. The identification of inositol was confirmed by GLC of its hexaacetyl derivative (6). TLC of the lipid showed behavior corresponding to phosphatidyl*myo*-inositol, and deacylation gave glycerylphosphorylinositol. The presence of small amounts of mannose in acid hydrolysates suggested the possible occurrence of mannosylphosphoinositides. A sample (100 mg) of the total phospholipids was hydrolyzed in 2 N NaOH at 100 C for 3 hr. The solution was adjusted to *p*H 1 with 2 N HCl, and the fatty acids were removed by extraction with petroleum ether (boiling point, 60 to 80 C, 3 × 5 ml). The aqueous solution was deionized by passage through a column of Dowex 50 (NH_4^+ form) resin, and the eluate was introduced into a column of diethylaminoethyl cellulose (HCO_3^- form). The column was eluted with water to

TABLE 1. *Analysis of the fatty acid composition of glycolipids and phospholipids of Arthrobacter crystallopoietes and of total lipids of A. pascens*

Fatty acid	Composition (%, w/w)				
	A. crystallopoietes[a]				*A. pascens*
	GA	GB	PG	BPG	Total lipid
$C_{15:0}$ *anteiso* ..	66.7	70.4	86.7	84.1	83.3
$C_{16:0}$ *iso*	2.4	2.3	3.4	1.7	1.3
$C_{16:0}$	2.4	1.3	5.1	8.7	3.8
$C_{17:0}$ anteiso ..	28.5	26.0	4.8	5.5	11.6

[a] GA, glycolipid A, monogalactosyl diglyceride; GB, glycolipid B, dimannosyl diglyceride; PG, phosphatidylglycerol; BPG, bisphosphatidylglycerol.

remove the neutral components (25 ml), and the aqueous eluate was evaporated to a small volume and examined by paper chromatography. No mannosylinositol glycosides, which would be produced from mannosylphosphoinositides under these conditions (2), could be detected; only glycerol and inositol were present.

Lipids of A. pascens. The lipids of *A. pascens* were separated and identified by the methods described above for *A. crystallopoietes*. The overall composition was very similar, the same components being observed. The fatty acid composition is shown in Table 1. The ratio of the two diglycosyl diglycerides was almost 1:1, and sufficient glycolipid D was isolated to enable mannose and glycerol to be identified as products of acid hydrolysis. Even smaller amounts of another glycolipid (R_F 0.16) were detected in this organism, but in the initial separation on silicic acid it was eluted only very slowly with acetone, presumably because of its high polarity. Consequently, small quantities were present in the phospholipid fraction and were eluted with the phosphatidyl*myo*inositol during the separation of the phospholipids by preparative TLC. Thus the fraction containing phosphatidyl*myo*inositol gave traces of mannose on acid hydrolysis, but no evidence could be obtained for the presence of mannosylphosphoinositides by the hydrolysis procedure described above for *A. crystallopoietes*. In both organisms, it therefore seems likely that the mannose arose from a glycolipid, possibly a tetramannosyl diglyceride.

Lipids of A. globiformis. The lipids of *A. globiformis* were also separated and identified by the methods described above. The lipid composition did not differ significantly from that found in *A. crystallopoietes* and *A. pascens*.

The lipids were extracted from homogeneous cell preparations of both morphological forms of *A. crystallopoietes*, and the extracts were examined by TLC. No qualitative differences in composition were observed between the two forms, and all the lipids present in the extracts from pleomorphic cells were present in both rods and spheres. This was confirmed by separation and identification of the individual lipid components by the methods described above. Visual estimation of the TLC suggested that any quantitative differences in composition were very small.

DISCUSSION

The lipids of both *A. crystallopoietes* and *A. pascens* contained three major glycolipid components: monogalactosyl diglyceride, digalactosyl diglyceride, and dimannosyl diglyceride. Although the precise stereochemical configuration of the glycolipids was not established, they had properties identical to the three glycolipids from *A. globiformis* previously identified by Walker and Bastl (22) as 3-*O*-β-D-galactopyranosyl-*sn*-1,2-diglyceride, 3-[*O*-β-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl]- *sn* -1 , 2-diglyceride, and 3-[*O*-α-D-mannopyranosyl-(1 → 3)-*O*-α-D-mannopyranosyl]-*sn*-1,2-diglyceride. Thus all three species of *Arthrobacter* contain the same glycolipids, although the relative amounts of each differ.

Some evidence was obtained for the presence of small amounts of a trimannosyl diglyceride and an even smaller quantity of a possible tetramannosyl diglyceride, but lack of material precluded conclusive identification. Monoglycosyl diglycerides are known to be intermediates in the biosynthesis of diglycosyl diglycerides (15), so the co-occurrence of monogalactosyl and digalactosyl diglycerides is not surprising, although the monoglycosyl diglycerides do not usually accumulate in such large amounts. No evidence could be obtained for the presence of a monomannosyl diglyceride.

The phospholipid composition of *Arthrobacter* has not been previously reported. All three strains contained bisphosphatidylglycerol, phosphatidylglycerol, and phosphatidyl*myo*inositol. The first two phospholipids are widely distributed in bacterial lipids, but the occurrence of phosphatidyl*myo*inositol is unusual (12). In *Corynebacteria* the major phospholipids are mannosylphosphoinositides, where one or more mannose units are glycosidically bound to the inositol (14). No other genus of the *Corynebacteriaceae* has yet been shown to contain phosphatidyl*myo*inositol. No conclusive evidence could be obtained for the presence of mannosylphosphoinositides in *Arthrobacter*, although acid hydrolysates of the phosphatidyl*myo*inositol preparations contained traces of mannose. A large sample of the total phospholipids was subjected to vigorous alkaline hydrolysis, and the neutral products were examined by paper chromatography. No phosphorus-free mannosylinositol glycosides, which would be produced under these conditions from mannosylphosphoinositides (2), could be detected. It seems probable that the mannose arose from the glycolipid contaminant. The larger and more polar glycosyl diglycerides are eluted very slowly with acetone from silicic acid columns, and small amounts are always eluted subsequently with the phospholipid fraction.

The fatty acid composition of *A. crystallopoietes* and *A. pascens* was very similar to that previously reported for *A. globiformis* (23); $C_{15:0}$ *anteiso* and $C_{17:0}$ *anteiso* accounted for more than 90% of the total. There was a higher con-

centration of the $C_{17:0}$ *anteiso* in the glycolipids than in the phospholipids (Table 1).

A characteristic feature of *Arthrobacter* is rod-sphere morphogenesis. A study of the cell wall composition of the two forms of *A. crystallopoietes* has revealed significant differences. Thus, in the rod form, the polysaccharide backbone of the mucopeptide is considerably longer than in the sphere form, and there is a slightly higher degree of cross-linking (13). It seemed possible that differences might also occur in lipid composition between the two forms. In particular, it was envisaged that the occurrence of either of the two diglycosyl diglycerides might be restricted to one morphological form. However, no qualitative differences either in glycolipid or phospholipid composition could be detected between the two forms of *A. crystallopoietes*, nor were there any significant quantitative differences between individual lipid components. The significance of the co-occurrence of two unrelated diglycosyl diglycerides remains unknown.

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