GLYCOLIPIDS OF A HALOTOLERANT MODERATELY HALOPHILIC BACTERIUM

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1. Introduction

Kates et al. [1] showed that the lipids of seven extreme halophilic bacteria (requiring 4 M NaCl for optimal growth) consisted almost entirely of glycerol diether derivatives which were identified as 2,3-di-*O*-dihydrophytylglycerol. Moderately halophilic bacteria (requiring 1 M NaCl) in contrast, contained glycerol diester derivatives.

Recently a moderately halophilic-halotolerant bacterium which could grow in liquid media containing 0.2 to 4.0 M NaCl was isolated by Rafaeli-Eshkol from crude salt samples of the Dead Sea evaporation ponds [2]. Considering the observations of Kates, it seemed of interest to us to study the effect of extreme salt concentrations on its lipid composition.

This communication describes the lipid composition of the halotolerant bacterium when grown in 2 M NaCl. Phosphatidylglycerol and phosphatidylethanolamine were found to be the major lipid components. In addition two glycolipids tentatively identified as glucuronosyl-diglyceride and glycosyl-phosphatidylglycerol were found. The most remarkable feature of the lipids of this organism was the unusual high content of cyclopropane acids: 11,12-methyleneoctadecanoic acid accounted for 50–60% of the fatty acids and 9,10-methylenehexadecanoic acid for additional 5 to 10%.

2. Materials and methods

2.1. *Organism and growth conditions*

An unidentified, moderately halophilic, halotolerant gram negative rod which was isolated by Rafaeli-Eshkol from crude salt samples of the Dead Sea evaporation ponds, was given to us by Prof. Y. Avidor. The organisms were grown for 48 hr at 37° in a liquid medium which contained Nutrient broth (Difco) 8 g per l, NaCl 1.5 M, KCl 0.5 M and $MgCl_2$ 0.1 M; at pH 6.8. After harvesting, the cells were washed twice with cold 2 M NaCl.

2.2. *Extraction and fractionation of lipids*

The washed cells were suspended in a small volume of water and extracted according to Bligh and Dyer [3]. The crude lipid mixture was fractionated on silicic acid (Unisil) columns. Neutral lipids were eluted with chloroform and polar lipids with increasing concentrations of methanol in chloroform (2, 10, 20 and 30%). The composition of each fraction was determined by thin layer chromatography on Silica Gel G (Merck) coated plates with chloroform–methanol–acetic acid water (100:20:12:5, by vol.) as developing solvent. The spots were visualized with iodine vapor and then identified by specific spray reagents: ninhydrin, anthrone [4] and periodate-Schiff's reagent [5]. Pure lipids were prepared by thin layer chromatography on 0.5 mm plates using the above mentioned solvent. Identical spots were pooled and the lipids eluted with chloroform–methanol (2:1, v/v).

2.3. *Preparation of ^{32}P-labelled lipids*

The organisms were grown in the presence of ^{32}P (100 μCi per 100 ml). Extraction and purification of lipids were done as described above.

2.4. *Degradation of lipids and identification of products*

Mild alkaline hydrolysis was done according to Wilkinson [6]. To identify sugar residues, the lipid was hydrolyzed for 1 hr in 2 N HCl; uronic acid was obtained by hydrolysis in 1 N HCl for 1.5 hr. The sugar residues were separated by paper chromatography employing butanol–pyridine–water (6:4:3, by vol.) as developing solvent. Glucose was visualized with glucose oxidase spray [7]. The other sugars and uronic acid were visualized by subsequently spraying the chromatograms with aniline-phthalic acid [8].

Fatty acids were isolated after alkaline hydrolysis, methylated with diazomethane and identified by gas liquid chromatography (GLC) as previously described [9]. A sample of dihydrosterculic acid was kindly given to us by Dr. J. Law. To further identify cyclopropane acids, pure methyl esters emerging from the column, were collected, hydrogenated according to McCloskey and Law [10], and the products identified by GLC.

2.5. *Enzymatic hydrolysis*

Hydrolysis by phospholipase A (EC 3.1.1.4) of *Crotalus adamanteus* venom (Worthington) was done as previously described [9]. Hydrolysis by phospholipase C (EC 3.1.4.3) of *Bacillus cereus* was carried out according to Haverkate and Van Deenen [11]. A partially purified sample of phospholipase C was kindly given to us by Dr. Z. Selinger.

2.6. *Analytical methods*

Phosphate was determined according to King [12]. Glucose and glucuronic acid were determined after acid hydrolysis by means of anthrone reagent and carbazole reagent [4, 13] respectively. Glycerol was determined enzymatically [14] after 48 hr hydrolysis in 2 N HCl. Fatty acids were determined according to Duncombe [15] after alkaline hydrolysis.

3. Results

3.1. *Identification of polar lipids*

Thin layer chromatography of the fractions which were eluted from silicic acid columns revealed the presence of 5 different lipid components the R_f values of which were: 0.94, 0.77, 0.45, 0.3 and 0.2 respectively.

Spots 1, 2 and 3: were identified as cardiolipin, phosphatidylethanolamine and phosphatidylglycerol respectively. The complete data will be published shortly.

Spot 4: The lipid was eluted from silicic acid with 10% methanol in chloroform. It did not contain phosphate. On silica gel plates the spot stained pink when heated after spraying with anthrone reagent and showed a delayed reaction with periodate-Schiff's reagent turning grey after several hours. Acid hydrolysis of the intact or deacylated lipid yielded a uronic acid; no other sugar was found. To identify the uronic acid, the lipid was reduced by a modification of Wilkinson's method [16]. The intact lipid was dissolved in ethylenedichloride and methylated by the addition of etheral diazomethane. It was next reduced with sodium borohydride in methanol. The reduced product was hydrolyzed in 2 N HCl and the hydrolysate desalted by passage through a column of mixed bed resin (Amberlite MB-3). Paper chromatography of the dried residue revealed the presence of glucose as the only sugar, indicating that the lipid contains glucuronic acid. Quantitative analysis of glycerol–glucuronic acid–fatty acids gave a ratio of 1:1.05:2.07. Thus the lipis seems to be a glucuronosyldiglyceride. A similar lipid was isolated by Wilkinson from *Pseudomonas rubescens* [6, 16]. Dr. Wilkinson kindly sent us a sample of this lipid. A comparison of the mobility of the two lipids on thin layer plates employing our standard solvent mixture or Wilkinson's solvent [6] showed however that the lipid isolated from *Ps. rubescens* migrated much faster (just below cardiolipin) than our lipid, in both solvent systems.

Spot 5: The lipid was eluted from silicic acid with 30% methanol in chloroform. It contained phosphate. On silica gel plates the spot stained blue when heated after spraying with anthrone reagent and stained blue with periodate–Schiff's reagent. Glucose was obtained after acid hydrolysis. It contained phosphate–glycerol–fatty acids–glucose at a ratio of 1:2.05:1.98:1.15. A sample of ^{32}P-labelled glycolipid was incubated with phospholipase C of *B. cereus*: After 1 hr at 30°, 14% of the ^{32}P became water soluble (under the same conditions 88% of ^{32}P-labelled phosphatidyl glycerol was

hydrolyzed), and after 3 hr 32% of the lipid were degraded. Thin layer chromatography of the residual lipid [17] showed that diglyceride was formed. The water soluble residue was divided into two. One half was hydrolyzed in 2 N HCl. Both samples were then separated by ascending paper chromatography with isopropyl ether–90% formic acid (90:60, v/v) as developing solvent [18]. Whereas, before hydrolysis, the labelled material remained at the origin after hydrolysis 75% of the counts migrated like glycerophosphate (the residual 25% remained at the origin); inorganic phosphate was not detected. Not enough material was available to identify glucose. The lipid was also susceptible to hydrolysis with phospholipase A; 30% hydrolysis was obtained after 3 hr of incubation. A glucose containing lipid, identified as phosphatidyl glucose was isolated by Smith and Henrikson from *Mycoplasma laidlawii* [19]. Dr. Smith kindly sent us a sample of this lipid. The mobility of the two lipids on thin layers, employing our standard solvent mixture or the borate containing system of Beckman and Kenny [20], was very similar. Recently Shaw et al. [21] showed that the lipid which was isolated from *M. laidlawii* actually contained phosphate–glycerol–fatty acids–glucose at a ratio of 1:2:2:2. This lipid was not hydrolyzed by phospholipases A, C and D. Thus the lipid isolated by us must be different from the glycolipid of *Mycoplasma*. Diglucosyl derivatives of phosphatidyl glycerol were recently isolated from 3 different bacteria [22–24]. Thus, in analogy to these compounds, our lipid seems to be a monoglucosyl derivative of phosphatidylglycerol.

3.2. *Fatty acid compositions*

^{32}P-labelled phosphatidylethanolamine and phosphatidylglycerol were subjected to mild alkaline hydrolysis; 98% of the ^{32}P was recovered in the water phase indicating that the fatty acids were present as glycerol esters. The fatty acid composition of the different phospho- and glycolipids is shown in table 1. The unusual high content of cyclopropane acids is noteworthy. The structures of these acids were kindly determined for us by Dr. J. McCloskey as 11,12-methylene octadecanoic and 9,10-methylene-hexadecanoic acids. The relative proportions of the different phospho- and glycolipids is also shown in this table.

Table 1
Fatty acid composition of polar lipids.

Lipid*	% of total	Fatty acids: % of total				
		16:0	17:0	18:0	18:1	19:0
Cardiolipin	5.5	32.5	8.5	tr	tr	59.0
PE	34.5	38.1	6.1	2.2	3.6	50.0
PG	31.5	25.7	4.9	3.0	0	66.4
Glucoronosyl-DG	16.5	28.0	8.0	2.9	tr	61.1
Glucosyl-PG	12.0	38.3	9.5	tr	tr	52.2

To calculate the amount of glucuronosyldiglyceride the molar ratio:lipid phosphate to lipid uronic acid was determined. A ratio of 5 was found. The relative amounts of the glycerophospholipids were calculated from the distribution of ^{32}P in a sample of ^{32}P-labelled polar lipids

* PE : phosphatidylethanolamine
PG : phosphatidylglycerol
DG: diglyceride

4. Summary and discussion

The composition of the polar lipids of a gram negative, moderate halophilic-halotolerant bacterium was determined. When grown in a liquid medium which contained 2 M NaCl the following lipids (in reducing order) were found: phosphatidylethanolamine, phosphatidyl glycerol, two glycolipids tentatively identified as glucuronosyl-diglyceride and glucosyl phosphatidylglycerol and cardiolipin. Further work is in progress to determine the exact structure of these glycolipids. The most remarkable feature of the lipids of this microorganism is the unusually high content of 11,12-methylene octadecanoic acid.

In one experiment the same bacterium was grown in the presence of 4 M NaCl. Likewise, the fatty acids were found as glycerol esters; 11,12-methylene octadecanoic acid was the major fatty acid component. Thus, growth under extreme salt concentrations did not seem to effect the lipid composition of this bacterium.

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