Identification of ceramide phosphorylethanolamine and ceramide phosphorylglycerol in the lipids of an anaerobic bacterium

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ABSTRACT Nearly half the phospholipids isolated from the anerobic bacterium *Bacteroides melaninogenicus* are phosphosphingolipids. The two major phosphosphingolipids have been characterized as ceramide phosphorylethanolamine and ceramide phosphorylglycerol. The long-chain bases of these phosphosphingolipids appear to have branched and normal saturated carbon chains of 17, 18, and 19 atoms; the phosphate is at the 1-position of the long-chain base. The composition of the amide-linked fatty acids of the phosphosphingolipids differs from that of the ester-linked fatty acids of the diacylphosphoglycerides in having a higher percentage of 14:0, 17:0, and 18:0 acids as well as containing nearly all the monoenoic fatty acids found in the bacterial lipids.

The finding of phosphosphingolipids in bacteria is exceedingly rare and to our knowledge ceramide phosphorylglycerol has not been previously found in nature.

SUPPLEMENTARY KEY WORDS *Bacteroides melaninogenicus* · phosphosphingolipids · fatty acid composition · long-chain base composition

SPHINGOLIPIDS are exceedingly rare in bacteria (1). In this report evidence will be presented that two sphingolipids, ceramide phosphorylethanolamine and ceramide phosphorylglycerol, represent a significant proportion of the lipids of the anaerobe *Bacteroides melaninogenicus*. To our knowledge this is the first report of ceramide phosphorylglycerol in biological material.

Abbreviations: PE, phosphatidyl ethanolamine (diacyl-*sn*-glycerol-3-phosphoryl-1′-ethanolamine); GPE, glycerol phosphorylethanolamine (*sn*-glycerol-3-phosphoryl-1′-ethanolamine); LCB, long-chain base; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl. Fatty acids and LCB are denoted by number of carbon atoms:number of double bonds; br = branched.

MATERIALS AND METHODS

Carrier-free $H_3{}^{32}PO_4$ was supplied by Tracerlab Inc., Mass. in plastic bottles. Phosphatidyl ethanolamine from *E. coli* was a gift from J. H. Law. Palmitaldehyde, myristaldehyde, and the dimethylacetals of these aldehydes were supplied by Supelco, Inc., Bellefonte, Pa. Sphingosine and dihydrosphingosine were supplied by Miles Laboratories, Inc., Elkhart, Ind. Fatty acid standards, reagents for silylation, and GLC materials were supplied by the Applied Science Laboratories Inc., State College, Pa.

The strain CR_2A of *Bacteroides melaninogenicus* was supplied by R. W. Gibbons. The conditions of growth, the media, the harvesting procedure, and the determination of cultural purity have been described (2). Victor Rizza kindly provided the bacteria used in this study.

Extraction and Chromatography of Lipids

Lipids were extracted from the bacteria with chloroform–methanol as previously described (3) or by the Bligh and Dyer procedure (4). Lipids were chromatographed on Silica Gel G thin-layer plates, 0.5 mm thick, in chloroform–methanol–6.7 M ammonium hydroxide 33:15:1.25 (solvent A) for the first dimension and chloroform–methanol–acetic acid–water 42:12:3.15:1 (solvent B) for the second dimension. The lipids were detected by a combination of Rhodamine, ninhydrin and

acid molybdate (5), periodate (6), or radioautography. Lipids were recovered quantitatively after TLC by collecting the silica gel containing the component in tubes with fritted discs by means of a vacuum and eluting with chloroform–methanol–water solutions as previously described (3).

Radioautography

Radioautographs of ^{32}P-labeled lipids after chromatography were prepared with Kodak no-screen X-ray film (7).

Hydrolysis Procedures

Mild alkaline methanolysis to deacylate diacyl phosphoglycerides was performed at 0°C as previously described (7). The methanolysis is complete in 120 min. The reaction mixture was neutralized with the weak acid Biorex 70 resin (Bio-Rad Laboratories, Richmond, Calif.) and the mixture was extracted three times with equal volumes of diethyl ether and finally with an equal volume of chloroform. This procedure minimizes emulsion formation. The fatty acid methyl esters are quantitatively recovered in the organic phase and the glycerol phosphate esters are quantitatively recovered in the aqueous phase. Mild alkaline methanolysis at 37°C, followed by mild acid hydrolysis in 0.05 N HCl with 0.025 M $HgCl_2$ to split any plasmalogens, was performed as described by Wells and Dittmer (8).

Fatty acid methyl esters were separated from unhydrolyzed phospholipids on a 1 g silicic acid column (11 × 50 mm, Unisil, 100–200 mesh) that had been packed in chloroform. The methyl esters were eluted with 5 ml of chloroform and the phospholipids with 5 ml of chloroform–methanol 1:1 followed by 5 ml of methanol.

Strong Alkaline Hydrolysis. The "alkali-stable" lipids, which were not hydrolyzed by the mild alkaline methanolysis and which were later shown to be phosphosphingolipids, were split into long-chain bases and fatty acids (that had been amide linked) as follows. The lipid was heated in 3 M KOH in ethanol–water 1:1 at 100°C for 3 hr. The alkaline mixture was cooled, and extracted four times with an aqual volume of petroleum ether (this extracts most of the LCB). The mixture was then acidified to pH 2.0 with HCl and the fatty acids were recovered in petroleum ether. The aqueous layer was then extracted with an equal volume of chloroform to ensure complete removal of the LCB and any LCB phosphate. The first petroleum ether extract and the chloroform extract were combined; they contained the LCB fraction.

Strong Acid Methanolysis. The phosphosphingolipid was heated with 1 ml of methanol–12 N HCl 5:1 (9) at 100°C for 3 hr. After cooling, 1 ml of water was added and the mixture was extracted twice with two volumes of petroleum ether. The aqueous phase was then made to pH 12 with concentrated KOH and extracted twice with two volumes of petroleum ether.

The combined organic phases were dried under nitrogen, dissolved in chloroform, and applied to a 1 g silicic acid column (11 × 50 mm, Unisil, 100–200 mesh) that had been packed in chloroform. Fatty acid methyl esters were eluted with 5 ml of chloroform and LCB with 5 ml of chloroform–methanol 1:1 followed by 5 ml of methanol. The LCB fraction eluted from the silicic acid column was then applied as a band to a thin-layer plate and chromatographed with solvent B. The central portion of the plate was covered with Saran Wrap and the exposed ends were sprayed with ninhydrin (10). The LCB protected by the Saran Wrap was recovered from the silicia gel G (3). Part of the LCB was treated with periodate (11) and the fatty aldehydes were recovered in hexane as described by Carter and Hendrickson (11). Part of the aldehyde fraction was subjected to GLC directly, part was used to make dimethylacetals (12), and part was oxidized to fatty acids with alkaline silver oxide (13) and esterified. Dimethyl acetals and fatty acid methyl esters were analyzed by GLC.

Paper Chromatography

Glycerol phosphate esters derived from the phospholipids by any of these hydrolysis procedures were identified in several paper-chromatographic systems, for which details are given in the table footnotes. A modified Wawszkiewicz solvent was made up for chromatography on acid-washed paper (No. 589 of Schleicher & Schuell Co., Keene, N. H.) and on aminocellulose paper (Whatman AE-81) as follows: 1.15 M ammonium acetate, 11.8 mM with respect to EDTA, was made to pH 5.0 with acetic acid and then diluted 3:7 with 0.26 M NH_4OH in 95% ethanol. Detection was by periodate spray followed by *o*-tolidine (3).

Gas–Liquid Chromatography

GLC was performed with an F & M Model 402 gas chromatograph (Hewlett-Packard Co., Avondale, Pa.) using techniques previously described (14). Glass columns 3 mm O.D. × 1.8 m were packed with 3.5% (v/v) SE-30 on 80–100 mesh Diatoport S or 15% ethylene glycol succinate on 60–80 mesh Gas-Chrom P.

Trimethylsilyl ethers of the LCB were prepared as described by Carter and Gaver (15), and separated on the SE-30 columns at 210°C. The efficiency of the column was 2580 theoretical plates for TMS-dihydrosphingosine.

The same column had an efficiency for palmitaldehyde or its dimethyl acetal of 2600 theoretical plates when operated isothermally at 170°C. Dimethylacetals of the aldehydes were prepared by refluxing the aldehyde

in 5% anhydrous methanolic HCl for 2 hr. The mixture was cooled, neutralized with excess anhydrous sodium carbonate, and extracted with petroleum ether as described by Gray (12). Part of the reaction mixture was refluxed in 0.5 M methanolic NaOH for 2 hr to hydrolyze the methyl esters. The mixture was then cooled, diluted with water, and extracted with petroleum ether. There was essentially no change in the chromatogram of the dimethylacetals after alkaline hydrolysis.

Fatty acid methyl esters prepared in methanolic HCl in the presence of dimethoxypropane (14) were separated on SE-30 and ethylene glycol succinate columns at 150°C as described previously (14).

Analysis

Lipids were analyzed for phosphate, glycerol, hexose, amino nitrogen, and total fatty acid as previously described (3). Total aldehyde was determined after acid hydrolysis with Schiff reagent as described by Sloane Stanley and Bowler (16), with butyraldehyde as standard, and after formation of *p*-nitrophenylhydrazones as described by Rapport and Alonzo (17). Sphingosine was determined colorimetrically with the methyl orange reagent of Lauter and Trams (18). Acyl ester was determined colorimetrically (19).

IR Spectra

A Perkin–Elmer (model 237) IR spectrometer was used. The lipids were dissolved in carbon tetrachloride at concentrations of 20–40 μmoles/ml and examined in sodium chloride cells of 0.125 mm light path.

RESULTS

Total Lipid

B. melaninogenicus contains 50 μmoles of lipid phosphate per g dry weight. A sample of total lipid from *B. melaninogenicus* (29.0 μmoles of phosphate) contained less than 1.25 μmoles of carbohydrate as assayed with the anthrone reagent (3) with a glucose standard.

The phospholipids of *B. melaninogenicus* were separated by TLC in two dimensions. A radioautogram of phospholipids isolated from bacteria grown with ^{32}P after separation by TLC is illustrated in Fig. 1. The proportions of the lipid P in each spot and the percentage that is hydrolyzable by mild alkaline methanolysis are given in Table 1. Phospholipids resistant to mild alkaline methanolysis are rare in bacteria (7). The fact that a significant proportion of the phosphate from the lipid in spots 1, 3, and 4 is not made water soluble indicates that this organism contains unusual phospholipids. About 44% of the lipid phosphate of the total lipid extract was resistant to mild alkaline methanolysis. Hitherto, plasmalogens and alkyl ethers were the only

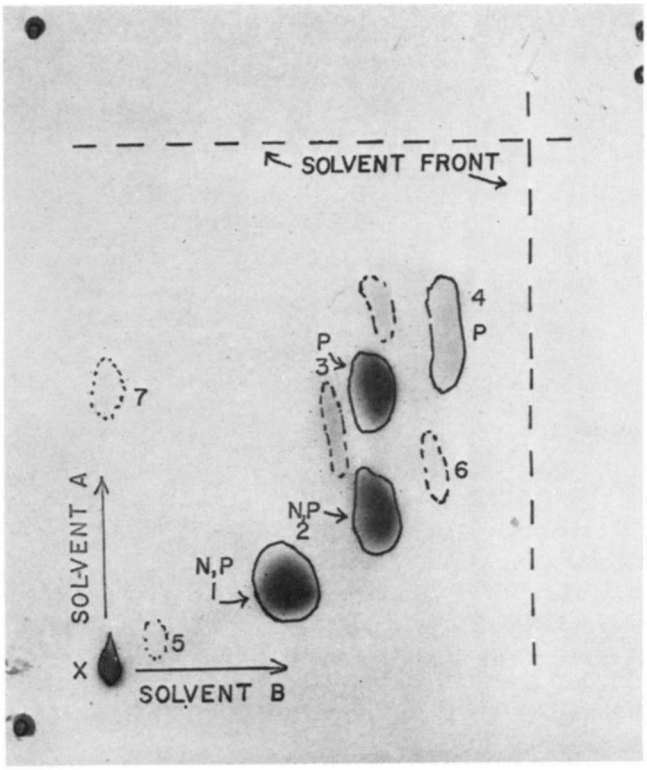

FIG. 1. Radioautogram of the lipids from *B. melaninogenicus* grown with ^{32}P after separation by TLC in ammoniacal chloroform–methanol (first dimension,solvent A) and acetic acid–chloroform–methanol (second dimension). Exact proportions given in Methods. N indicates reactivity with ninhydrin; P indicates reactivity with acid molybdate for phosphate. Spot 1 was later identified as ceramide phosphorylethanolamine, spot 2 as phosphatidyl ethanolamine, and spot 3 as ceramide phosphorylglycerol.

two classes of phospholipids known in bacteria that are not hydrolyzed in 0.1 M KOH to water-soluble phosphate esters. The lipid that was stable to mild alkali treatment was examined for these phospholipids as follows.

Lipid Stable to Mild Alkali

Mild alkaline methanolysis followed by mild acid hydrolysis in the presence of Hg^{++} quantitatively releases fatty aldehyde from any plasmalogens as well as releasing water-soluble glycerol phosphate esters (8). When the products of mild alkaline methanolysis of the lipid of *B. melaninogenicus* were subjected to mild acid hydrolysis in the presence of 25 mM $HgCl_2$, less than 0.2 μmole (as measured by the Schiff reagent) or less than 0.12 μmole (measured as the *p*-nitrophenylhydrazone) of fatty aldehyde were released from 11.6 μmoles of lipid. Plasmalogens could, therefore, account for less than 2% of the methanolysis-resistant phospholipids.

The lipids stable to mild alkali were not completely hydrolyzed after refluxing in anhydrous 0.7 M methanolic HCl for 24 hr as recommended for alkyl ethers by Kates

TABLE 1 PROPORTIONS OF THE PHOSPHOLIPIDS FROM *B. melaninogeniscus* SEPARATED BY TLC

Spot	Total P	Water-Soluble after Hydrolysis
	%	
Origin	1.2	—
1	19.7	2.0
2	45.4	100.0
3	16.7	16.0
4	11.2	51.0
5	1.2	—
6	1.7	—
7	1.2	—

A total of 1.15 μmoles of lipid was subjected to TLC as illustrated in Fig. 1. The lipids were located by radioautography and eluted from the silica gel, and determinations were made of total phosphorus and water-soluble phosphorus after mild alkaline hydrolysis for 2 hr at 0°C.

(20) and their IR spectra showed no prominent absorption bands in the 1100–1110 cm^{-1} (C–O–C) region typical of alkyl ethers. The IR spectra did show absorption at 3300 cm^{-1} (N–H stretching) and 1645 and 1550 cm^{-1} (secondary amide), which suggested that the lipids were phosphospingolipids.

Strong acid methanolysis of a total lipid sample recovered after mild alkaline methanolysis rendered the phosphate water soluble, and both the aqueous and organic phases contained ninhydrin-reacting components. These data and the IR absorption bands at 1645 and 1550 cm^{-1} strongly suggested that the unusual lipids were phosphosphingolipids. To further establish the identity of the mild alkali-stable lipids we identified the LCB.

Long-Chain Bases of the Phosphosphingolipids

The LCB and the methyl esters of the fatty acids recovered in the organic phase after strong acid methanolysis were separated by silicic acid column chromatography, and the LCB fraction was further purified by TLC. The LCB from *B. melaninogenicus* and authentic sphingosine both moved with an R_f of 0.52. The LCB fraction was recovered from the silica gel G and TMS derivatives were prepared for GLC. Three major peaks were obtained on GLC with retention times corresponding to TMS derivatives of 17:0br, 18:0, and 19:0br in the proportions listed in the first column of Table 2.

Part of the LCB fraction was treated with periodate and the resulting fatty aldehydes were analyzed by GLC. Fatty aldehydes were detected with retention times characteristic of 15:0br, 16:0, and 17:0br. Dimethyl acetals of the fatty aldehydes were prepared and had retention times characteristic of dimethyl acetals of 15:0br, 16:0, and 17:0br fatty aldehydes. The 16:0 fatty aldehyde, the dimethyl acetal of the 16:0 fatty aldehyde, and the TMS derivative of the 18:0 LCB cochromatographed with, respectively, palmitaldehyde, palmitaldehyde dimethylacetal, and the TMS derivative of authentic dihydrosphingosine.

TABLE 2 COMPOSITION OF LCB IN THE PHOSPHOSPHINGOLIPIDS OF *B. melaninogenicus*

LCB	Total Phospho-sphingolipid	Spot A*	Spot B†
		% of the total	
17:0br	12.2	11.5	22.2
18:0	18.1	23.6	16.0
19:0br	68.6	64.9	61.8

Composition determined by GLC of the TMS derivatives.
* Later identified as ceramide phosphorylethanolamine. Corresponds to spot 1 of Fig. 1.
† Later identified as ceramide phosphorylglycerol. Corresponds to spot 3 of Fig. 1.

A portion of the aldehydes derived from the LCB fraction was oxidized to fatty acids, methylated, and subjected to GLC. The resulting fatty acid methyl esters had retention times of 15:0br, 16:0, and 17:0br. The 15:0br fatty acid methyl esters cochromatographed with a 15:0br fatty acid methyl ester derived from *Staphylococcus aureus* and the 16:0 ester cochromatographed with methyl palmitate.

Fatty Acids from Phosphosphingolipids

Fatty acid methyl esters were recovered from the diacyl phospholipids after mild alkaline methanolysis and separated from the phosphosphingolipids by silicic acid chromatography. Their composition was compared with that of the amide-linked fatty acids recovered after strong alkaline hydrolysis of the phosphosphingolipids followed by methylation. The methyl esters were subjected to GLC on polar (ethylene glycol succinate) and nonpolar (SE-30) columns; the proportions of each fatty acid determined on the two columns agreed. Normal saturated, normal monoenoic, iso branched, and anteiso branched fatty acids were detected, but the proportions were different in ester and amide linkage (Table 3). The phosphosphingolipids contain larger proportions of 14:0, 17:0, 18:0, and most of the monoenoic fatty acids found in the phospholipids. There appear to be no hydroxylated or C_{19}–C_{24} fatty acids.

Location of the Phosphate in Phosphosphingolipids

Strong alkaline hydrolysis of the phosphosphingolipids yielded LCB together with a small portion of LCB phosphate, extracted together from the reaction mixture with organic solvents and purified by TLC in solvent B. The band that reacted with both ninhydrin and molybdate was recovered from the Silica Gel G. This mixture of LCB phosphates was treated with

TABLE 3 FATTY ACID COMPOSITION OF THE DIACYL PHOSPHOGLYCERIDES AND PHOSPHOSPHINGOLIPIDS FROM *B. melaninogenicus*

Fatty Acid	Diacyl Phosphoglycerides	Phospho-sphingolipids
	% of total fatty acids	
12:0	0.2	0.2
13:0br	4.8	1.0
14:0br	0.3	4.7
14:0	4.5	12.3
15:0br	63.0	21.7
15:0	0.5	2.8
16:0br	—	7.9
16:0	10.3	4.0
16:1	—	3.1
17:0br	5.4	4.7
17:0	4.3	12.5
18:0br	—	1.8
18:0	5.3	17.1
18:1	1.4	6.5

The methyl esters of the fatty acids from the phosphoglycerides were obtained by mild alkaline methanolysis, and were separated from the intact phosphosphingolipids by silicic acid chromatography. The amide-linked fatty acids were obtained from the phosphosphingolipids by strong alkaline hydrolysis and methylation. Analysis was by GLC on polar and nonpolar phases.

periodate and the fatty aldehydes released were analyzed by GLC. From 0.06 μmole of LCB phosphate, 0.05 μmole of aldehydes was recovered, with retention volumes corresponding to 15:0br, 16:0, and 17:0br. If the phosphate ester were at the 1-position of the long-chain bases, these aldehydes would be recovered after periodate treatment.

Fractionation of the Phosphosphingolipids

The total lipid extract of *B. melaninogenicus* was treated by mild alkaline methanolysis and the organic phase of the reaction mixture was subjected to TLC in two dimensions using solvents A and B. Three phosphate-containing spots were detected: spot A at R_f values 0.21 and 0.22, spot B at R_f 0.41 and 0.36, and spot C at R_f 0.63 and 0.61. Spot A reacted with ninhydrin, and spot B reacted with periodate. The proportion of phosphate in each spot was: spot A, 49.6%; spot B, 36.0% and spot C, 14%. Spot A corresponds to spot 1 in Fig. 1 and spot B corresponds to spot 3 in Fig. 1.

Identification of Ceramide Phosphorylethanolamine

The lipid from spot A was collected and subjected to strong acid methanolysis. The TMS derivatives of the LCB fraction were prepared and separated by GLC. The ratios of the three major TMS derivatives of the LCB were similar to those of the whole phosphosphingolipid (Table 2). The water-soluble portion of the hydrolysis mixture contained two substances, with the paper chromatographic properties of ethanolamine hydrochloride and *O*-phosphorylethanolamine hydrochloride

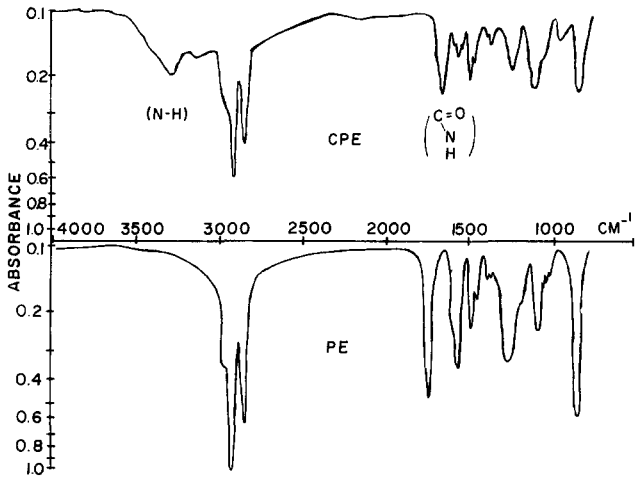

FIG. 2. IR spectra of PE and ceramide phosphorylethanolamine (CPE) from *B. melaninogenicus*. The upper curve illustrates the spectrum of CPE at a concentration of 56 μmoles/ml. The lower curve indicates the spectrum of PE at a concentration of 33 μmoles/ml. Spectra were measured in CCl_4 solution in cells with sodium chloride windows and an optical path of 0.125 mm.

in two solvent systems (Table 4). These hydrolysis products also had the same ion-exchange chromatographic properties (i.e. same elution volume) as the two reference compounds on a type C chromo-bead resin, in the short-column Technicon amino-acid analyzer using a 5 hr, five chamber gradient. The two ninhydrin-reacting components were present in approximately equal quantities.

The IR spectrum of spot A is illustrated in the upper part of Fig. 2. Besides the absorption typical of NH stretching and secondary amides, the following tentative identifications can be made: 2960 cm^{-1}, CH_3 stretching; 2925 and 2850 cm^{-1}, aliphatic CH_2 stretching; 1465, 1380, and 1370 cm^{-1}, CH_2 and CH_3 bending; 1220 cm^{-1}, P=O; and 1080 and 1025 cm^{-1}, P–O–C.

Fatty acid was liberated from the amide linkage of spot A by strong alkaline hydrolysis and measuring colorimetrically (18). Its molar ratio to phosphate was 0.95. After strong acid methanolysis, the water-soluble portion contained ethanolamine and phosphate in a 0.96:1 molar ratio; the ethanolamine was determined with the amino-acid analyzer. The aldehyde derived from the

TABLE 4 R_f VALUES OF WATER-SOLUBLE HYDROLYSIS PRODUCTS OF SPOT A (CERAMIDE PHOSPHORYLETHANOLAMINE)

Component	Solvent 1	Solvent 2
O-Phosphorylethanolamine HCl	0.06	0.32
Ethanolamine HCl	0.48	0.63
Unknowns from Spot A	0.06, 0.47	0.31, 0.63

Strong acid methanolysis; ascending paper chromatography; amines detected with ninhydrin. Solvent 1, water-saturated phenol at pH 5.95. Solvent 2, methanol–pyridine–water 20:5:1.

TABLE 5 R_f VALUES OF WATER-SOLUBLE HYDROLYSIS PRODUCTS OF SPOT B (CERAMIDE PHOSPHORYLGLYCEROL)

Component	Solvent 1	2	3	4
Glycerol-2-^{14}C	0.59	0.94	0.97	0.65
sn-Glycerol-2-^{14}C 3-Phosphate	0.32	0.66	0.37	0.07
Unknowns from Spot B	0.31, 0.58	0.66, 0.94	0.36, 0.97	0.07, 0.65

Strong acid methanolysis. Solvent 1, ascending paper chromatography in isopropanol–acetic acid–water 3:1:1. Solvent 2, descending paper chromatography with modified Wawszkiewicz solvent (see Methods). Solvent 3, ascending aminocellulose paper chromatography in 3 M formic acid containing 0.4% pyridine. Solvent 4, ascending aminocellulose paper chromatography with the modified Wawszkiewicz solvent. Labeled standards were detected by radioautography. Unlabeled components were detected by spraying with periodate followed by *o*-tolidine (3).

LCB by treatment with periodate gave an aldehyde to phosphate molar ratio of 0.80. The sphingosine to phosphate molar ratio, the sphingosine being determined with the methyl orange reagent, was 0.90. Less than 0.025 μmole of acyl ester could be detected in 1.1 μmoles of spot A. The absence of acyl ester accounts for the fact that the chromatographic mobility on Silica Gel G is not affected by mild alkaline methanolysis. We conclude that spot A is ceramide phosphorylethanolamine (ceramide-1-phosphoryl-1′-ethanolamine).

Identification of Ceramide Phosphorylglycerol

Lipid was recovered from spot B (corresponding to spot 3 in Fig. 1). It reacted with periodate before hydrolysis and its chromatographic mobility on silica gel G was not affected by mild alkaline methanolysis. The lipid was subjected to strong acid methanolysis and the TMS derivatives of the LCB fraction were gas chromatographed. The LCB in this lipid contained a slightly larger proportion of 17:0br and a lower proportion of the 18:0 and 19:0br than ceramide phosphorylethanolamine (Table 2).

The water-soluble portion of the lipid after strong acid methanolysis contained glycerol and glycerol 3-phosphate as determined by chromatography in four solvent systems (Table 5).

The molar ratio of fatty acid to phosphate determined after strong alkaline hydrolysis of the lipid was 1.13. Estimation of the total LCB from the response of the hydrogen flame detector after GLC gave a molar ratio of LCB to phosphate of only 0.6, but there was no correction for loss of LCB during saponification in this determination. Glycerol was assayed, after hydrolysis in 2 M HCl in sealed tubes at 115°C for 48 hr, by a periodate reaction and measurement of formaldehyde under conditions in which sugars and aminoalcohols do not react significantly. The molar ratio of glycerol to phosphate of the intact lipid in this procedure was 0.80. The molar ratio of glycerol to phosphate measured by this procedure in the water phase after saponification was 0.90.

TABLE 6 R_f VALUES OF WATER-SOLUBLE HYDROLYSIS PRODUCTS FROM SPOT 2 OF FIG. 1 (PHOSPHATIDYL ETHANOLAMINE)

Component	Solvent 1	2	3	4
GPE	0.67	0.15	0.48	0.97
Unknown from Spot 2	0.67	0.15	0.42	0.97

Mild alkaline methanolysis. Solvent 1 (ascending), water-saturated phenol at pH 5.95. Solvent 2 (ascending), *n*-butanol–acetic acid–water 5:3:1. Solvents 3 and 4 as in Table 5. Detection was by periodate followed by *o*-tolidine.

We conclude that spot B consists of ceramide phosphorylglycerol (ceramide-1-phosphoryl-1′-*sn*-glycerol).

Identification of Phosphatidyl Ethanolamine

The lipid found in spot 2 (Fig. 1) has an IR spectrum identical with that of authentic phosphatidyl ethanolamine isolated from *E. coli*. The spectrum shows the prominent carbonyl absorption at 1745 cm^{-1} not seen in ceramide phosphorylethanolamine (Fig. 2). The water-soluble glycerol phosphate ester formed after mild alkaline methanolysis had R_f values almost identical with those of GPE in four solvent systems (Table 6).

DISCUSSION

Sphingolipids are quite rare in the bacterial lipids that have been examined to date (1). Partially purified sphingomyelin that on hydrolysis yielded 95% of the expected choline and 50% of the expected fatty acid (calculated on a molecular weight of 324) was once reported in the gonococcus (21). This lipid has not been reported in subsequent studies of this bacterium (1). Sphingomyelin was stated to be the major lipid in *Bacillus stearothermophilus* (22). Later work indicated that no sphingolipid was present in this organism (23). To our knowledge, the first well-documented occurrence of a sphingolipid in a bacterium is sphingomyelin in the

pleuropneumonia-like organism *Mycoplasma gallisepticum* (24). *M. gallisepticum* can incorporate ^{32}P into its sphingomyelin. The sphingomyelin was isolated by silicic acid chromatography and on hydrolysis yielded an LCB with the same R_f as sphingosine. The intact lipid had IR absorption bands at 1660 cm^{-1} (secondary amide) and 1735 cm^{-1} (carbonyl). A sphingolipid containing ethanolamine has been reported in the lipids of the rumen anaerobe *Bacteroides ruminicola* (25). This represents the initial finding of this lipid in a bacterium.

The present study indicates that the phosphosphingolipids of the anaerobe *B. melaninogenicus* constitute about half the phospholipids. Since the lipid does not react with anthrone there are apparently neither gangliosides nor cerebrosides in the lipids.

The two major phosphosphingolipids have been characterized as ceramide phosphorylethanolamine and ceramide phosphorylglycerol. To our knowledge, the natural occurrence of the latter has not been reported previously. Ceramide phosphorylethanolamine is not widely distributed in nature. It has been reported in the adult housefly, *Musca domestica* (26), in the rumen protozoan *Entodinium caudatum* (27), in the pupae of the green bottle fly, *Lucilia caesar* (28), and in a limited number of Mesogastropodoid snails (29).

The LCB found in the phosphosphingolipids of *B. melaninogenicus* contains branched and normal saturated sphingosines with 17–19 carbon atoms. Branched-chain LCB components have been reported in the protozoa (30). Preliminary mass-spectrometric investigation of the LCB of *B. melaninogenicus* by C. C. Sweeley has indicated that homologous series of normal and iso-branched dihydrosphingosines are also present. TMS derivatives of *N*-acetylated LCB and methyl esters of the fatty acids derived from the LCB by periodate cleavage and subsequent oxidation have been used in this study, which will be reported subsequently.

Preliminary work (by Rizza, Tucker, and White) has indicated that the diacylphosphoglycerides of *B. melaninogenicus* contain phosphatidyl glycerol, phosphatidic acid, phosphatidyl serine, and cardiolipin as well as the phosphatidyl ethanolamine reported here.

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