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Phospholipids of *Azotobacter agilis*, *Agrobacterium tumefaciens*, and *Escherichia coli**

Tsuneo Kaneshiro and Allen G. Marr

Department of Bacteriology,
University of California at Davis,
Davis, California

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SUMMARY

The principal lipid of *A. agilis*, *A. tumefaciens*, and *E. coli* extractable with ethanol and methanol-chloroform was a phosphatidyl ethanolamine. A phosphatidyl choline was detectable only in *A. tumefaciens*. The fatty acids of phospholipid from *A. agilis* were found to be myristic (7%), palmitic (35%), palmitoleic (41%), and octadecenoic (17%) acids. The fatty acids from *A. tumefaciens* were myristic (1%), palmitic (15%), hexadecenoic (1%), methylene hexadecanoic (6%), octadecenoic (30%), and lactobacillic (47%) acids. The neutral lipid was a minor fraction (5% to 10%) of the extractable lipids and was found to consist of free fatty acids, esters, and coenzyme Q.

Only a few studies have been made of the kinds of phospholipids present in gram negative bacteria. Cephalins were detected in bacterial endotoxins by Morgan and Partridge (1) and by Westphal and Lüderitz (2). Similarly, phospholipids extracted directly from whole cells (3, 4) or envelopes (5) of gram negative bacteria were identified as phosphatidyl ethanolamine by the classical methods of differential solubility, functional group analyses, and elemental analyses. The amount of phosphatidyl ethanolamine relative to other lipids and the types of phospholipids other than phosphatidyl ethanolamine have not been established.

Chromatography of intact phospholipids and gas-liquid chromatography of fatty acid esters permit a more thorough and precise characterization of bacterial lipids than is possible with traditional methods. Our investigation of *Azotobacter agilis*, *Agrobacterium tumefaciens* and *Escherichia coli* has indicated that phosphatidyl ethanolamine is the dominant lipid in these gram-negative microorganisms.

EXPERIMENTAL METHODS

Growth of Bacteria. A laboratory strain of *E. coli* was cultured in a medium containing 0.64 g $MgCl_2 \cdot 6H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 1.0 g KCl, 12.2 g K_2HPO_4, 9.4 g KH_2PO_4, 1.5 g $(NH_4)_2SO_4$, and 10.0 g glucose per liter. *A. tumefaciens* strain TT10 was grown in the synthetic medium of Starr (6), and *A. agilis* (*A. vinelandii* strain O) was grown in Burk's synthetic medium (7). The cultures were grown aerobically at 30° and were centrifuged soon after the end of exponential growth. The cells were washed twice with distilled water, once with cold 0.1 N HCl and suspended in distilled water.

Extraction and Purification of Lipids. Aliquots of the cell suspensions were dried to constant weight at 60° and 15 cm Hg. The remaining suspension was centrifuged, and the pellet was extracted twice with 95% ethanol at 60° for 10 min and twice with 30% methanol in chloroform. Combined extracts were pooled, and solvents were removed at 50° and 15 cm Hg.

About 1.5 g of the total lipids were fractionated by chromatography on a 1.6 × 60-cm column containing 30 g of silicic acid (Mallinckrodt Chemical Works, St. Louis, Missouri, 100 mesh). Fractions were eluted with 6-column volumes of (I) 10% diethyl ether in petroleum ether (v/v), (IIa) 25% methanol in chloroform, (IIb) 50% methanol in chloroform, and (III) 100% methanol. For most of the subsequent analyses, fractions IIa and IIb were pooled (fraction II).

Water-soluble products of hydrolyzed phospholipids were separated by descending paper chromatography on Whatman No. 4 filter paper with *n*-butanol—propionic acid—water 2:1:1 (v/v) and *n*-propanol—ammonium hydroxide—water 6:3:1 (v/v) as solvents. The chromatograms were developed with ninhydrin or

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molybdate (8). Choline and primary amines were differentiated by the multiple spray technique of Lea, Rhodes, and Stoll (9).

Dinitrophenyl (DNP-) phospholipids were prepared by reacting with 1-fluoro-2,4-dinitrobenzene (10). The DNP-lipids were purified by chromatography on Celite with 50% ethanol in chloroform. The DNP-lipids were hydrolyzed in acid (11) or deacylated in 0.2 N KOH in methanol at 37° for 30 min. DNP-amino acids and DNP-ethanolamine were prepared by the method of Sanger (12).

Analytical Methods. For determination of ester bonds, samples of lipid (1.5 mg) were reacted with alkaline hydroxylamine, and the resulting hydroxamic acids were determined colorimetrically as the ferric hydroxamates (13). The absorbancy was measured at 530 mμ against a reagent blank. Tripalmitin was used as a standard. Since cephalin and lysocephalin give only 80% to 85% of the absorbancy per ester equivalent given by triglycerides (14), a correction factor of 1.25 was applied to the ester absorbancy obtained with phospholipids.

Total phosphorus was determined colorimetrically after digestion of samples with perchloric acid (15). Lyso compounds were estimated by hemolysis of rabbit erythrocytes (16, 17). Sterols were estimated by the Liebermann-Burchardt reaction after alkaline hydrolysis and precipitation with digitonin (18).

Samples of neutral lipid (50 to 100 mg) were chromatographed on 3-g columns of silicic acid to separate coenzyme Q from interfering materials. After elution of less polar lipids with 2% diethyl ether in petroleum ether, coenzyme Q was eluted with 3% diethyl ether in petroleum ether as a yellow-orange band. The solvent was evaporated, and the residue was dissolved in ethanol. The content of coenzyme Q was estimated by the decrease in absorbancy at 275 mμ after reduction with a small crystal of KBH_4. From the decrease in absorbancy on reduction, the concentration of coenzyme Q was calculated by assuming $\Delta E^{1\%}_{1\,cm} = 163$ (20).

Fatty aldehydes of plasmalogens were detected qualitatively by precipitation of hydrazones after reacting with a 2.7% alcoholic solution of acidic 2,4-dinitrophenyl hydrazine or were determined quantitatively by reaction with an acidic solution of p-nitrophenyl hydrazine (19).

Fatty acids were determined by gas-liquid chromatography of methyl esters on a 1/4-in. × 8-ft column of 25% diethylene glycol-succinate polyester on firebrick[1] at 189° (21). Individual methyl esters were recovered by gas-liquid chromatography and were purified by chromatography on silicic acid (21).

[1] Commercial packing purchased from Wilkins Instruments and Research, Inc., Walnut Creek, California.

TABLE 1. Results of Silicic Acid Chromatography of Lipids Extracted from Whole Cells

Organism	Percentage of Total Lipid*			Percentage Recovered
	I†	II†	III†	
A. tumefaciens	5	74	13	92
E. coli	4	80	6	90
A. agilis	9	76	9	94

* Mean of 3 determinations.

† Eluting solvents: (I), 10% diethyl ether in petroleum (v/v); (II), 25% to 50% methanol in chloroform; and (III), anhydrous methanol.

Unsaturated fatty acids were reduced by catalytic hydrogenation of the methyl ester in methanol with 5% Pt on charcoal as the catalyst at room temperature and 1 atmosphere H_2. The resulting saturated fatty acid was identified by gas-liquid chromatography.

The position of the double bond in mono-olefinic acids was established by oxidation with performic acid and periodic acid to yield fatty aldehydes (22) that were identified by gas-liquid chromatography (21).

RESULTS

Total Lipid. The lipid extracted with ethanol and methanol-chloroform accounted for 10.4%, 10.5%, and 9.2% of the dry weight of *A. agilis*, *A. tumefaciens*, and *E. coli* respectively.

Only *A. agilis* was examined for lipids not extractable in these solvents. The residue after extracting cells of *A. agilis* with ethanol and methanol-chloroform was extracted further with hot chloroform. The chloroform extract, which contained only 0.05% of the original dry weight of the cells, gave an infrared spectrum of poly-β-hydroxybutyrate (23). The residue after extraction with chloroform was hydrolyzed with 6 N HCl under reflux for 2 hours. An additional 2.3% of the original dry weight was extracted by diethyl ether after acid hydrolysis.

Composition of the Lipid. The lipid extracted with hot ethanol and with chloroform-methanol was pooled and chromatographed on silicic acid (Table 1). The "neutral" lipid (fraction I) from all three organisms represented approximately 10% of the total lipid.

Fraction I from *A. agilis* was almost free of phosphorus (0% to 0.3%) and contained no detectable sterols. The absence of significant quantities of glycerides and other carboxylic esters is indicated by a low absorption at 1740 cm^{-1} (Fig. 1a) and negative

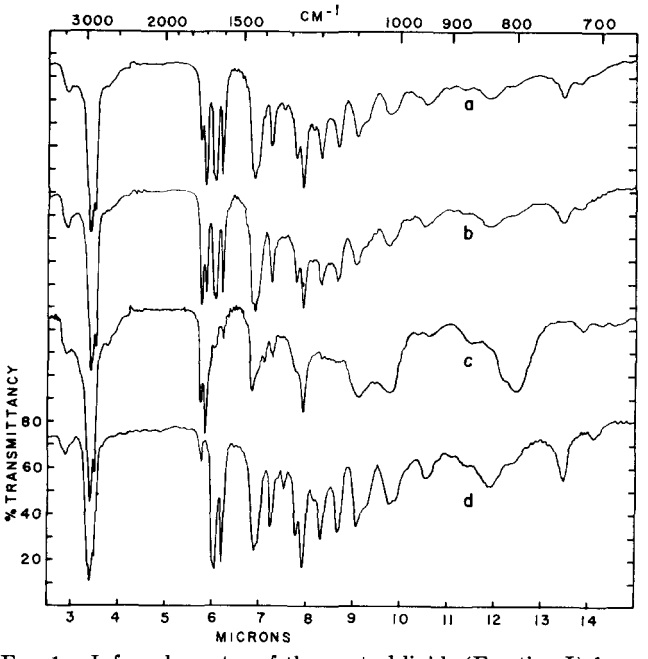

FIG. 1. Infrared spectra of the neutral lipids (Fraction I) from (a) *A. agilis*, (b) *A. tumefaciens*, and (c) *E. coli*. Spectrum d is coenzyme Q of *A. agilis* isolated by partition chromatography with silicic acid and crystallized from ethanol. The lipids are spread evenly as films over pellets of KBr.

hydroxamate reaction. The strong absorption band at 1710 cm^{-1} can be attributed to large content of free fatty acids in this fraction. The strong absorption at 1650 and 1610 cm^{-1} and weaker absorption at 1150 and 1200 cm^{-1} of fraction I from *A. agilis* are contributed by coenzyme Q (Fig. 1d). Coenzyme Q comprised 61% of the weight of fraction I.

The infrared spectrum of fraction I from *A. tumefaciens* (Fig. 1b) was quite similar to fraction I from *A. agilis*. The spectrum is again dominated by the principal absorption bands of coenzyme Q, which accounted for 39% of fraction I. The stronger band at 1740 cm^{-1} indicates a somewhat higher content of ester than was found in fraction I from *A. agilis*.

The spectrum of fraction I from *E. coli* (Fig. 1c) has a strong band at 1710 cm^{-1}, suggesting a large contribution of free fatty acid, and a weak band at 1740 cm^{-1}. Only traces of coenzyme Q were detected in fraction I from *E. coli*.

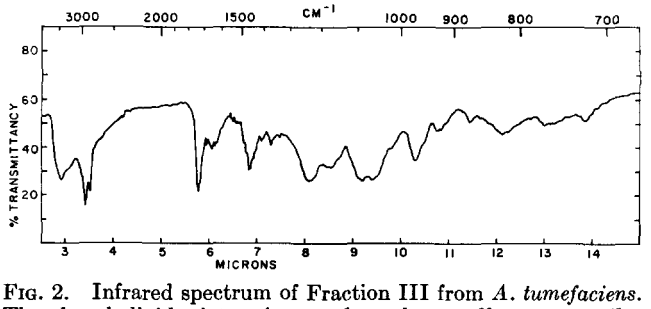

FIG. 2. Infrared spectrum of Fraction III from *A. tumefaciens*. The phospholipid mixture is spread evenly as a film over a pellet of KBr.

The bulk of the lipid (75% to 80%) was eluted by methanol-chloroform in fraction II. The infrared spectra of fraction II from all three organisms were identical with the spectrum of phosphatidyl ethanolamine. An ester absorption at 1740 cm^{-1}, P-O stretch at 1225 cm^{-1}, P-O-C stretch at 1075 and 1030 cm^{-1}, and absence of a band at 968 cm^{-1} were noted and are characteristic of phosphatidyl ethanolamine (24, 25).

Fraction III from *A. tumefaciens* (Fig. 2) gave an infrared absorption spectrum typical of phosphatidyl choline with a prominent band at 968 cm^{-1} (25). Phosphatidyl choline was not detected in the lipid from *A. agilis* and *E. coli*. Fraction III from *A. agilis* and from *E. coli* gave strong, broad absorption bands of $-OH$ at 3500 cm^{-1}.

Identification of Phospholipids. The phospholipids (fractions II and III) were washed with distilled water to remove a contaminating reducing sugar and were hydrolyzed in 0.2 N HCl in methanol at 60° for 2 hours. The excess HCl was removed *in vacuo*, and the residues were extracted with water and chromatographed. The products of hydrolysis of fraction II from all three organisms were those expected for phosphatidyl ethanolamine. The principal ninhydrin-positive spot had the same R_f as ethanolamine. A component with the same R_f as glucosamine and one unidentified component were the only additional ninhydrin-positive products detected. A spot corresponding to glycerophosphate was detected as a product of acid hydrolysis of fraction II from all three organisms. Choline was not detected in fraction II.

Fraction III from *A. tumefaciens* gave choline on acid hydrolysis confirming the infrared spectrum of phosphatidyl choline. Fraction III from *A. agilis* and *E. coli* contained no detectable choline. The hydrolysates of fraction III from all three organisms gave strong spots of the same R_f as hexosamine and numerous other primary amines, presumably amino acids. Moderate spots corresponding to glycerophosphate and ethanolamine were also detected.

At least in *A. agilis*, fraction II appeared heterogeneous. The phospholipid eluted with 25% methanol in chloroform (IIa) had a lower P:ester ratio than the phospholipid eluted with 50% methanol in chloroform (IIb). Both IIa and IIb behaved similarly on rechromatography; however, IIa gave a P:ester ratio of approximately 0.6 and IIb a ratio of approximately 0.9 (Table 2). Both phospholipids gave the infrared absorption spectrum of phosphatidyl ethanolamine.

TABLE 2. RESULTS OF RECHROMATOGRAPHY OF PHOSPHOLIPIDS FROM *A. agilis* ELUTED WITH 25% METHANOL (IIa) AND 50% METHANOL IN CHLOROFORM (IIb)

Eluent	Percentage of Total Recovered	P/Carboxylate Ester
Fraction IIa		0.5
Chloroform	1	Not determined
10% MeOH in $CHCl_3$	13	0.6*
20% MeOH in $CHCl_3$	72	0.6†
Anhydrous MeOH	14	5.6
Fraction IIb		0.7
Chloroform	3	Not determined
10% MeOH in $CHCl_3$	2	0.5*
20% MeOH in $CHCl_3$	73	0.9†
Anhydrous MeOH	22	22.4

* Mean of 3 determinations.
† Mean of 6 determinations.

Neither IIa nor IIb contained lyso compounds or plasmalogens.

Fractions IIa and IIb were deacylated in base, and the products were chromatographed. A strong spot with the same R_f as glycerophosphorylethanolamine was detected with both ninhydrin and molybdate. No other ninhydrin-positive products were detected, and only small amounts of phosphorus-containing products other than glycerophosphorylethanolamine were detected.

Since fraction IIb had a P:ester ratio higher than that expected for phosphatidyl ethanolamine, this fraction was examined further by preparing the dinitrophenyl derivative. After purification by chromatography on Celite, the DNP-derivative of fraction IIb in petroleum ether had an absorption maximum at 345 mμ. The DNP-derivative of IIb was hydrolyzed and chromatographed on paper in *t*-pentanol—pyridine—water 5:1:5 (v/v) and in aqueous sodium phosphate (26). After acid hydrolysis, DNP-ethanolamine was the only colored product. After basic hydrolysis, a single colored product was detected, which was presumably DNP-glycerophosphorylethanolamine.

Fatty Acid Composition of the Phospholipids. The fatty acids from fraction II of both *A. agilis* and *A. tumafaciens* were converted to the methyl esters and analyzed by gas-liquid chromatography (Fig. 3, Table 3). Components A and B were identified as the methyl esters of myristic and palmitic acids by comparing retention volumes with authentic compounds; their retention volumes were not affected by catalytic hydrogenation.

Components C and E had retention volumes corresponding to methyl esters of hexadecenoic and octadecenoic acids respectively; both C and E disappeared on catalytic hydrogenation. Component C from *A. agilis* and component E from both *A. agilis* and *A. tumefaciens* were recovered by gas-liquid chromatography in sufficient quantity for further analysis. All of the compounds recovered failed to absorb at 965 cm^{-1}, indicating the absence of *trans* olefins. The position of the double bond was established by peroxidation of the olefin to the corresponding vicinal-glycol followed by cleavage of the glycol with periodate

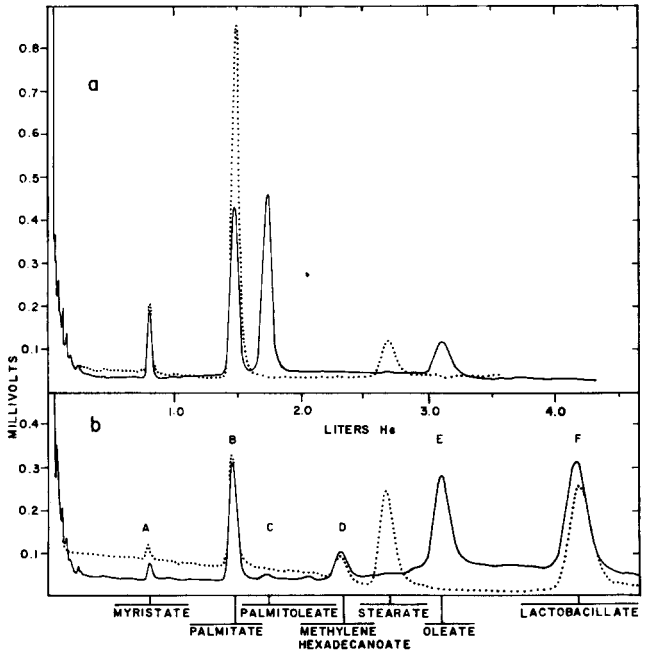

FIG. 3. Gas-liquid chromatograms of methyl esters from the phospholipids of (a) *A. agilis* and (b) *A. tumefaciens* before (——) and after (. . .) mild hydrogenation with Pt-charcoal catalyst. Retention volumes of known methyl esters are indicated. Components of retention volume greater than methyl lactobacillate were not detected although the chromatography was continued up to the eluting volume of C_{25} esters. The column was 1/4-in. x 8-ft. of 25% diethylene glycol-succinate polyester on firebrick operated at a column temperature of 189°; flow rate, 115.5 ml He per minute; detector current, 100 ma.

TABLE 3. FATTY ACID COMPOSITION OF THE PHOSPHOLIPID

Fatty Acid	Source: *A. tumefaciens*	Source: *A. agilis*
	wt. %	*wt. %*
Myristic	1.1	7.0
Palmitic	15.4	35.4
Hexadecenoic	0.6	40.9
Methylene hexadecanoic	6.2	Not detected
Octadecenoic	29.7	16.7
Lactobacillic	47.0	Not detected

TABLE 4. POSITION OF THE DOUBLE BOND IN MONO-UNSATURATED ACIDS

Fatty Acid	Source	Fatty Aldehydes Recovered*	
		Heptanal	Nonanal
Hexadecenoic	*A. agilis*	328	0
Octadecenoic	*A. agilis*	78	18
Octadecenoic	*A. tumefaciens*	311	45
Oleic		2	111

* μg of aldehyde per mg fatty acid oxidized.

to a fatty aldehyde and aldehydo-acid (22). The fatty aldehydes were identified by gas-liquid chromatography (Table 4). The hexadecenoic acid from *A. agilis* yielded heptanal exclusively; thus, it is *cis*-9, 10-hexadecenoic acid (palmitoleic acid). The quantity of hexadecenoic acid from *A. tumefaciens* was insufficient to establish the position of the double bond. The octadecenoic acids from both *A. agilis* and *A. tumefaciens* yielded mainly heptanal with smaller quantities of nonanal; thus, the octadecenoic acids are mixtures of *cis*-11,12-octadecenoic acid (*cis*-vaccenic acid) and oleic acid, with *cis*-vaccenic acid predominating. The octadecenoic acids from *E. coli* were previously reported to be a mixture of *cis*-vaccenic and oleic acids (21).

Components D and F from *A. tumefaciens* were identified as cyclopropane compounds (Fig. 3). Component D has the same retention volume as methyl *cis*-9,10-methylene hexadecanoate, which is a major fatty ester in the lipids of *E. coli* (21). Component F had the same retention volume as methyl lactobacillate. The identification of component F as methyl lactobacillate was confirmed by the isolation of the ester by gas-liquid chromatography. The purified acid of the methyl ester absorbed at 1020 cm^{-1}, which is characteristic of cyclopropane compounds. The uncorrected melting point of the free acid was 24–25°. Lactobacillic acid has been previously reported to be present in large amounts in the fatty acids of *A. tumefaciens* (27). Cyclopropane acids were not detectable in the fatty acids from *A. agilis*.

Analyses of the fatty acid compositions of fractions I and III indicated that proportions of individual fatty acids were not significantly different from those reported for fraction II. Also, the fatty acid composition of fractions IIa and IIb from *A. agilis* were identical.

The fatty acids obtained by hydrolysis of the bound lipid of *A. agilis* contained significant amounts of fatty acids not present in fraction II. Methyl esters of the fatty acids peculiar to the bound lipids were highly polar. Methyl esters of the normal fatty acids eluted from silicic acid in 3% diethyl ether in petroleum ether. The esters of the polar fatty acids from the bound lipids eluted with 20% diethyl ether in petroleum ether. The structures of the polar fatty acids are now under investigation.

DISCUSSION

The principal lipid from each of the three gram-negative bacteria was phosphatidyl ethanolamine, confirming several previous reports of cephalins in gram-negative bacteria (1–5, 28). Only *A. tumefaciens* contained detectable amounts of phosphatidyl choline. The phospholipids of gram-positive bacteria have been reported to be devoid of nitrogen and are presumed to be phosphatidic acids (29, 30). Since the phospholipids are known to be located in the membrane of gram-positive bacteria (29, 30) and in the wall and membrane of gram-negative bacteria (5, 31), this difference in the composition of the main phospholipids of the two groups of organisms would be expected to result in large differences between gram-positive and gram-negative bacteria in permeability and other surface properties (32).

Sub-fractions IIa and IIb of the phospholipids of *A. agilis* both appear to be mainly phosphatidyl ethanolamine. The apparent difference in polarity of the two fractions on chromatography with silicic acid disappears when they are rechromatographed, yet the difference in P:ester ratio persists. Contamination of fraction IIb with lysophosphatidyl ethanolamine or plasmalogen would explain this difference; however, neither lyso compounds nor plasmalogens were detected. The infrared spectra and products of acid and base hydrolysis are in agreement with a phosphatidyl ethanolamine structure with the same fatty acid composition for the principal lipids in both fractions IIa and IIb. The triester structure postulated by Collins (33) might explain the initially higher retention of IIb than IIa on silicic acid, and the change in chromatographic properties of IIb could be ascribed to the instability of triester phospholipids on silicic acid. However, the expected products of decomposition of the postulated triester would include lyso compounds, which were not detected.

The fatty acid composition of the phospholipids of *A. agilis* and *A. tumefaciens* conforms to a rather simple pattern common to many bacteria (34). Palmitic acid is the dominant straight-chain, saturated fatty acid; myristic acid is the only other normal saturated fatty acid present in detectable amounts. Hexadecenoic and octadecenoic acids are the only

unsaturated fatty acids. The octadecenoic acid from both organisms is principally *cis*-11,12-octadecenoic acid but contains some oleic acid. *A. tumefaciens* forms the cyclopropane homologues of both of the monounsaturated fatty acids as does *E. coli* (21). Cyclopropane acids are not present in *A. agilis*.

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