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Lipids of Marine Bacteria

I. Lipid composition of marine *Achromobacter* species

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Abstract

The lipid composition of marine *Achromobacter* sp. was studied. The lipid content of this organism was 0.66% of wet weight cells. This lipid composition consisted of 22.5% of non-conjugated lipids and 77.5% of conjugated lipids. The major lipids were phosphatidylethanolamine (35.5%), phosphatidic acid (20.1%), phosphatidylglycerol (10.9%), and wax (8.0%). Lyso-phosphatidylethanolamine (3.0%), lyso-phosphatidylglycerol (2.9%), hydrocarbon (2.3%) and coenzyme Q (2.0%) were presented, but in small proportion.

The major fatty acids were $C_{15:0,15:1}$, $C_{16:0,16:1}$, $C_{17:0,17:1}$ fatty acids.

Introduction

A few studies have been made of the quantitative analyses of the lipids found in the marine bacteria.[1~3]

Hardy *et al.* reported the determination of phospholipids of two *Achromobacter* sp. isolated from the Atlantic salmon. (Hardy *et al.* Proc. Soc. Gen. Microbiol., no. 5, p. iii, 1971). DeVoe and Oginsky[4] found phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and cardiolipin in the cell envelope of marine *Vibrio* sp.. Gordon and MacLeod[5] reported the presence of phosphatidylethanolamine, cardiolipin and two unidentified phospholipids in marine bacteria. Eberhard and Rouser[6] reported the phospholipid composition of three strains of marine *Photobacterium*. Oliver and Colwell[7] examined systematically the phospholipid composition of 16 strains, isolated from marine origins, consisting of genus *Vibrio*, *Agrobacterium*, *Achromobacter*, *Spirillum*, *Pseudomonus*, *Photobacterium*, *Arthrobacter* and unidentified genera.

This paper describes the lipid composition of *Achromobacter* sp., distinguished as marine type, using the salt requiring test as proposed by Hidaka and Sakai.[8]

Materials and Methods

Growth of organisms. Cells of *Achromobacter* sp. 60–20–A–5 (A–5), kindly supplied by Prof. Dr. M. Sakai, were grown at 25°C for three days on a ZoBell 2216E plate media, containing 5 g of polypeptone (Daigo Eiyo Co., Ltd.), 1 g of yeast

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extract (Daigo Eiyo Co., Ltd.), 0.1 g of ferric phosphate, and 15 g of agar (Kyokuto Seiyaku Co., Ltd.), per liter of Herbst's artificial sea water and adjusted at pH 7.8. After a three-day incubation, the cells were scraped up from the media and washed twice with Herbst's artificial sea water.

Extraction of lipids. The lipid extraction was carried out by the slightly modified procedure of Sehgal *et al.*[9]. To the cell paste, water was added to be 95% water content. One volume of the suspension was homogenized with three volumes of methanol-chloroform (2 : 1, v/v) in a Waring blender for two minutes. Following the addition of one volume of chloroform, the mixture was homogenized for 30 seconds, and then rehomogenized for 30 seconds after the addition of one volume of distilled water. The final mixture consisting of homogenate and a two-phase solvent was centrifuged. The liquid phase was decanted into a separatory funnel and the chloroform phase was collected. The cell residue was homogenized with one volume of chloroform-methanol (2 : 1, v/v) and the homogenate was centrifuged again three times. The combined chloroform extracts were evaporated to dryness in a rotary evaporator under 35°C *in vacuo*. The residue, dissolved into a small volume of chloroform, was introduced into a cellulose column (Whatman column chromedia CF11) to remove the non-lipid materials from chloroform extracts, and then eluted with five volumes of chloroform-ethanol-water (800:200:25, v/v). The eluate, containing the total lipids, was evaporated to dryness in a rotary evaporator, and then dissolved into a small volume of chloroform and stored at –20°C until analysis.

Column chromatography. The total lipids, dissolved into a small volume of chloroform, were applied on a silicic acid column (100 mesh, Mallinckrodt Chemical Works), and eluted with the following solvents: chloroform, chloroform-acetone (1:1, v/v), chloroform-methanol (9:1, 8:2, 65:35, 45:55, v/v), and methanol. The non-conjugated lipids consisting of three fractions A, B and C were eluted with chloroform, and the conjugated lipids consisting of five fractions D, E, F, G and H were eluted with chloroform-acetone (1:1, v/v) and chloroform-methanol (9:1, 8:2, 65:35 and 45:55, v/v) (See Fig. 1). The three fractions of non-conjugated lipids were fractionated by rechromatography using a silicic acid column, respectively. Fraction A was eluted with the following solvents: hexane, hexane-ethyl ether (94:6, v/v), hexane-ethylacetate (9:1, v/v), ethyl ether and methanol. Fraction B was eluted with hexane, hexane-ethyl ether (95:5, 85:15, 70:30 and 50:50, v/v) and ethyl ether. Fraction C was eluted with hexane-ethyl ether (98:2, 90:10, 80:20, 70:30, 50:50 and 25:75, v/v). Fraction G was eluted with chloroform-acetone (1:1, v/v), chloroform-methanol (95:5 and 80:20, v/v) and methanol. Fraction H was eluted with chloroform, chloroform-methanol (9:1, 8:2 and 65:35, v/v), and methanol.

A DEAE-cellulose column (acetate type, Brown Co., Ltd.), prepared by the

procedure of Rouser *et al.*[10], was used for the complete separation of Fractions D,E, and F, respectively. In every case, 20 mg of lipids per g of silicic acid, 10 mg of lipids per g of DEAE-cellulose and 150 mg of lipids per g of cellulose were applied on the column.

Thin layer chromatography (TLC). Kieselgel G nach Stahl (Merck Co., Ltd.) spread 0.25 mm in thickness on a glass plate was used for the identification of lipids and a preparation of lipids 0.50 mm in thickness was used. The sample was applied on the plate and chromatographed by an ascending method with the following solvent system: I. diisobutyl ketone-acetic acid-water (40:25:5, v/v), II. first step, acetone-ethyl ether (1:1, v/v), second step, chloroform-methanol-acetic acid-water (60:35:2:1, v/v), III. n-hexane, IV. n-hexane-benzene (1:1, v/v), V. n-hexane-ethyl ether-acetic acid (90:10:1, v/v).

The total lipids were detected by charring after being sprayed with 50%-H_2SO_4 or, under a UV light after spraying with Rhodamine 6G. The phospholipids were detected by spraying with Dittmer and Lester's reagent. The amino lipids were detected by heating after being sprayed with 0.3% ninhydrin in butanol, and the glycolipids by heating after being sprayed with 0.2% anthrone-conc-H_2SO_4. Choline was detected with Dragendorff's reagent. Two spots were observed in Fraction H by TLC after charring, and their composition was estimated by densitometry for these spots.

Gas-liquid chromatography (GLC). The fatty acid composition of individual lipids was determined by GLC. A fatty acid methyl ester, exception of free fatty acid and wax, was prepared from individual lipids with 10% HCl-methanol by heating at 100°C for three hours in a sealed tube, respectively.

Free fatty acids were methylated with diazomethane. Wax was saponified in the mixture of xylene, ethanol and 2N-ethanolic KOH under reflex for three hours. The alcohol, obtained from wax, was acetylated with acetic anhydride, and the fatty acid methylated with diazomethane. Gas chromatography was performed on the Hitachi gas chromatograph, model F6-D, an instrument equipped with a flame ionization detector, using nitrogen as carrier gas and a 1.5 m glass column with an inner diameter of 3 mm packed with 10% diethylenglycol succinate polyester on chromosorb. The oven temperature was 170°C and injection temperature was 250°C, respectively.

Paper chromatography. The water soluble materials from the acid hydrolyzed and deacylated lipids were spotted on the Whatman No. 1 or the Toyo 51A filter paper, washed with 2N-acetic acid and then chromatographed by the ascending method with the following solvent system: I. n-butanol-ethanol-water (4:1.1:1.9, v/v), II. phenol, saturated with water-acetic acid-ethanol (100:10:12, v/v), III. methanol-98% formic acid-water (80:13:7, v/v). The reagent of Hanes and Isherwood was used for the detection of phosphorus compounds. The amino acids

were detected with 0.3% ninhydrin in butanol. The sugars were detected with a $AgNO_3$-NaOH reagent.

Infrared spectrum. Infrared spectra of lipids were measured in a KBr pellet with a Nihon-Bunko DS–301 infrared spectrophotometer.

Analytical methods. Phosphorus was determined by the method of Fiske-Subbarow. Nitrogen was determined by the method of micro-Kjeldahl. Glycerol was analyzed by the procedure of Hanahan and Olley[11] and ester was estimsted by that of Rapport and Alonzo.[12]

Results

The total amount of lipids of this strain was obtained in a yield of 0.66% of the wet cell and was fractionated into non-conjugated lipids and conjugated lipids by using a silicic acid column. The non-conjugated lipids, comprising 25.5% of the total lipids, included three fractions and the conjugated lipids, consisting of five, comprised 74.5% of the total lipids. Figure 1. shows the chromatographic pattern using a silicic acid column. Since individual fraction, consisted of some components, a further purification was necessary.

The first fraction was refractionated by chromatography on a silicic acid column by a batch elution of hexane, hexane-ethyl ether (94: 6, v/v), hexane-

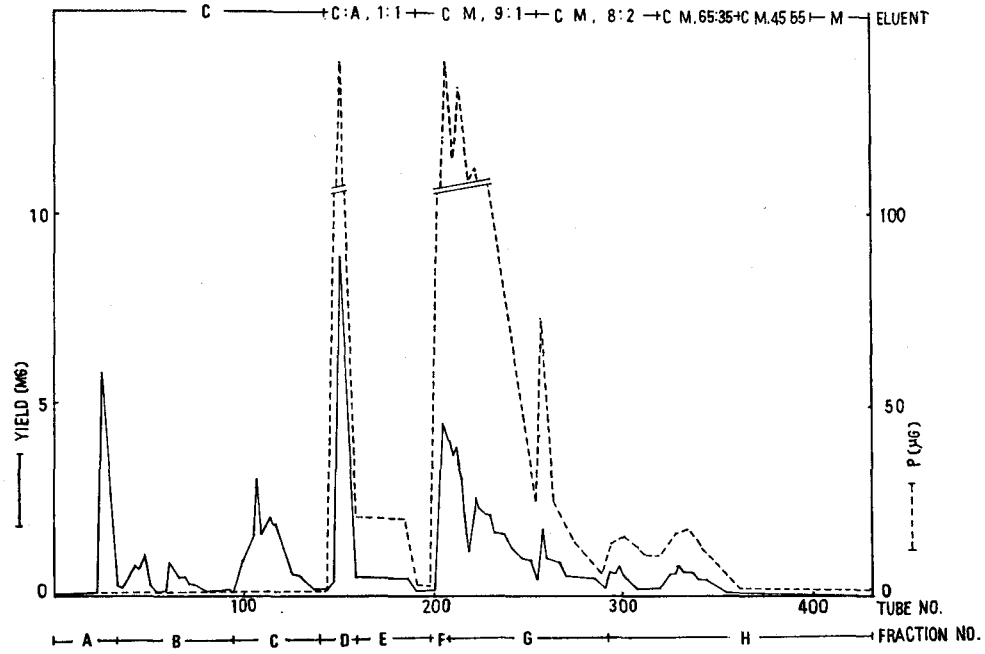

Fig. 1. Chromatography of total lipids obtaind from A-5 on silicic acid column.

ethylacetate (90:10, v/v), ethyl ether and methanol. The results with TLC in solvent systems V and VI show that the fraction eluted with hexane and hexane-ethyl ether (94:6, v/v) was hydrocarbon, comprising 2.3% of the total lipids. The fractions eluted with hexane-ethylacetate (90:10, v/v) was wax, comprising 8% of the total lipids. The fractions eluted with ethyl ether and methanol were not identified. The saponification of wax yielded fatty acids and alcohols, converting them into methyl ester and acetyl ester, respectively, and the latter two were analysed by GLC. The fatty acid and alcohol composition of wax are tabulated in Table 1.

Table 1. *Fatty acid and alcohol composition of wax obtained from A-5.*

$C_{m:n}$	Fatty acid	Alcohol
12:0	—	1.1(%)
12:1	—	—
13:0	0.3(%)	4.6
13:1	0.2	—
14:0	0.4	5.3
14:1	0.6	—
15:0	8.3	6.6
15:1	20.8	7.4
16:0	7.2	7.7
16:1	26.0	19.2
17:0	7.8	9.2
17:1	26.7	25.3
18:0	0.4	5.5
18:1	1.2	—
UK.	0.1	8.1

Note. m: Carbon number, n: Number of unsaturation
UK.: Unknown material, —: Trace, (<1.0%)

The second fraction, Fraction B, was rechromatographed on a silicic acid column by a batch elution of hexane-ethyl ether solvent at various ratio.

The fraction eluted with hexane-ethyl ether (95:5, v/v) showed a bright orange yellow color, and was identified as coenzyme Q according to Lester *et al.*[13]. The spectrum of the orange yellow material dissolved in ethanol was analyzed before and after reduction with KBH_4. The characteristic absorption maximum at 277 nm disappeared after reduction, and a new absorption maximum appeared at 290 nm, which showed an intensity 1/3.5 of the former. The content of coenzyme Q was found to be 2.0%.

The fraction eluted with hexane-ethyl ether (85:15, v/v) proved to consist of seven components from the results of TLC in solvent system VI. Sterols were presumed to exist from the two positive spots on the Liebermann-Burchard reaction.

The results with TLC in solvent systems V and VI showed that Fraction C exhibited identical TLC chromatographic behavior with an authentic free fatty

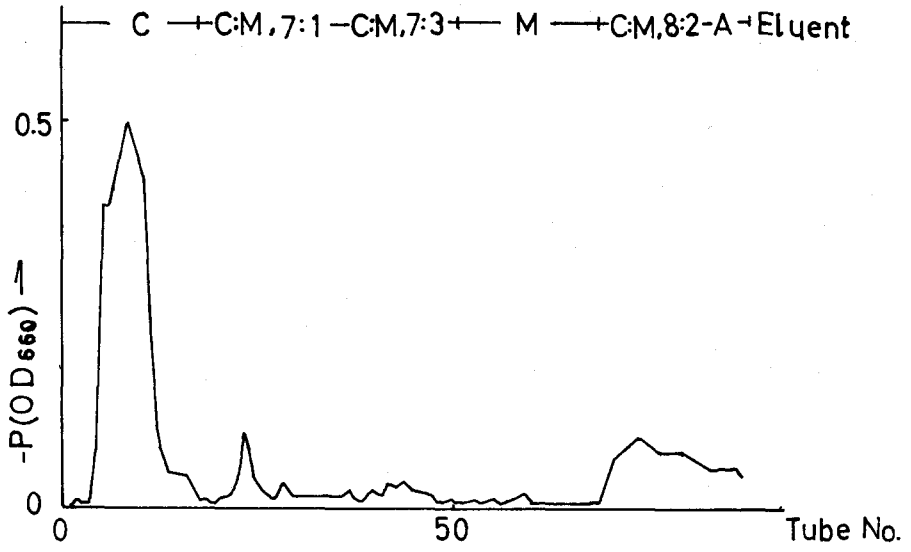

Fig. 2. Rechromatography of Fraction D on DEAE-cellulose.

acid, however, a little quantity of contaminants was detected. The purification of this fraction on silicic acid was achieved by a batch elution of hexane-ethyl ether solvent at various ratio. The free fatty acid eluted with hexane-ethyl ether (90: 10, v/v) comprised 92.6% of this fraction and 10.0% of the total lipids. The infrared spectrum and neutral value agreed with the authentic fatty acid.

Fractions D, E and F were separated into three spots on TLC in solvent systems I and II, and alkali hydrolysis yielded water soluble materials which resulted on spots Rf, a value indentical to, and recognized for, glycerophosphoric acid, glycerophosphorylglycerol and glycerophosphorylethanolamine by paper chromatography in solvent systems II and III.[14),15)] Fractions D, E and F were fractionated on the DEAE-cellulose column, respectively.

Rechromatography on a DEAE-cellulose column was completely separated into three components with chloroform, chloroform-methanol (7:1, 7:3, v/v), methanol, and chloroform-methanol (4:1, v/v) saturated with 29% of ammonia. Figure 2 shows a chromatographic pattern obtained from Fraction D on a DEAE-cellulose column. The fraction eluted with chloroform showed an ester/P mole ratio of 2.1 and a glycerol/P mole ratio of 1.0, being negative for ninhydrin and the anthrone-H_2SO_4 reagent, while positive for Dittmer and Lester's reagent.

Thus it was identified to be a phosphatidic acid. The fraction eluted with chloroform-methanol (4:1, v/v) staurated with 29% of ammonia showed as ester/P mole ratio of 1.8 and a glycerol/P mole ratio of 2.3, being negative for ninhydrin and the anthrone-H_2SO_4 reagent, whereas positive for Dittmer and Lester's, thus

was identified as phosphatidylglycerol. The contents of phosphatidic acid and phosphatidylglycerol comprised 20.1% and 10.9% of the total lipids, respectively. The fraction eluted with chloroform-methanol (7: 3, v/v) was identified as phosphatidylethanolamine according to the same procedure in Fraction G.

Fraction G was obtained from silicic acid column chromatography and showed to be phosphatidylethanolamine with some contaminants by TLC in solvent systems I and II. The purification of this fraction was achieved on the silicic acid column by a batch elution of chloroform-methanol solvent at various ratio. The fraction eluted with chloroform-methanol (95: 5, v/v) showed an ester/P mole ratio of 2: 1, a glycerol/P of 1.2 and a nitrogen/P of 1.0, being positive for ninhydrin and Dittmer and Lester's reagent. The water soluble materials from the acid hydrolyzate of this fraction showed two spots corresponding to glycerol and ethanolamine by paper chromatography with solvent system I. Therefore it was recognized to be phosphatidylethanolamine. The content of phosphatidylethanolamine attained 35.5% of the total lipids.

The results with TLC in solvent systems I and II showed that last Fraction H consisted of two spots, which were observed being positive for Dittmer and Lester's reagent and negative for anthrone-H_2SO_4. In regard to the ninhydrin reagent, the fast moving spot was positive and the slow moving one negative. The water soluble materials for the alkali hydrolyzate of Fraction H showed two spots corresponding to glycerophosphorylglycerol and glycerophosphorylethanolamine by paper chromatography in solvent systems II and III.[14)15)] Two kinds of lipids were fractionated from Fraction H according to the present experimental methods as mentioned above, but no complete separation was achieved, because of the appearance of an overlapped fraction. A responce of -OH was confirmed by the infrared absorption spectrum of these two lipids, respectively. Accordingly, this last Fraction H seemed to be a mixture of lyso-phosphatidylethanolamine and lyso-phosphatidylglycerol, and this composition could be estimated, using densitometry, by charring after being sprayed with 50%-H_2SO_4. The contents of lyso-phosphatidylethanolamine and lyso-phosphatidylglycerol were found to be 3.0% and 2.9% of the total lipids, respectively.

The fatty acid composition of the individual lipids, determined by GLC of its methyl esters, is tabulated in Table 2. The identification of fatty acids was achieved with the equivalent chain length value before and after the hydrogenation of various fatty acids.

Discussion

The writer was greatly interested in the lipids because of the membrane delimiting the cell, presumably being the origin of its characteristic of impermeability, and also probably consisting largely of lipids.

Table 2. *Fatty acid composition of the lipids obtained from A-5.*

$C_m : _n$	PA	PG	PE	Lyso-PE	Lyso-PG	Wax	F.F.A.
13:0	—	—	—	—	—	—	—
13:1	—	—	—	—	1.4(%)	—	—
14:0	—	—	—	—	—	—	—
14:1	1.9(%)	—	—	—	3.2	—	—
15:0	6.2	7.6(%)	5.2(%)	8.6(%)	3.7	8.3(%)	12.7(%)
15:1	14.1	19.7	11.5	16.7	36.2	20.8	15.1
16:0	5.8	6.6	6.1	5.9	3.5	7.2	11.8
16:1	23.7	21.7	24.9	24.6	21.3	26.0	17.2
17:0	10.1	11.0	8.1	8.9	4.2	7.8	15.1
17:1	32.1	30.1	40.1	29.7	22.4	26.7	24.4
18:0	1.9	—	—	1.0	—	—	—
18:1	3.5	1.7	3.5	2.4	2.9	1.2	2.1
Sat.	24.6	26.3	19.8	25.3	12.7	24.5	40.6
Unsat.	75.4	74.0	80.2	74.7	87.3	75.5	59.4

Note. PA: Phosphatidic acid, PE: Phosphatidylethanolamine,
PG: Phosphatidylglycerol, F.F.A.: Free fatty acid
Lyso-PE: Lyso-phosphatidylethanolamine,
Lyso-PG: Lyso-phosphatidylglcerol
Sat.: Saturated fatty acid, Unsat.: Unsaturated fatty acid,
m: Carbon number, n: Number of unsaturation
—: Trace, (<1.0%).

The purpose of this study was to investigate the lipid composition of marine bacteria which need NaCl, and either Mg, K, or Ca-salt, or sometimes all of them for their growth.

The non-conjugated lipids represent 25.5% of the total lipids and consist of hydrocarbon (2.3%) wax (8%), coenzyme Q (2%) and free fatty acid (10%). Wax hardly occurs in bacteria especially in Gram-negative bacteria, with the exception of *Corynebacterium* and *Mycobaterium*. In *Achrombacter* sp, A-5, wax has been found more abundantly than in *Acinetobacter*,[16] *E. coli*,[17] and *Serratia marcessence*.[18] Coenzyme Q of a bright orange color, represents 2% of the total lipids, agreeing with various results observed in other Gram-negative bacteria, which is generally small in amount.[19]

In the non-conjugated lipid fraction, two components, positive with the Liebermann-Burchard reaction, represent the minor ones. From this, these two substances are assumed to be sterol. Phosphatidylethanolamine is the most abundant component attaining 35.5% of the total lipids. Oliver and Colwell[7] had already shown a similar result for the amount of phosphatidylethamolamine of *Achromobacter* aquamarinus. The phosphatidic acid that was found comes next showing 20.1% of the total lipids. Phosphatidic acid must be present in all organisms as an intermediate in biosynthesis of the glycerolipids, however its content in this organism is very abundant. This phenomenon must wait for further study. Phosphatidylglycerol represents 10.9% of the total lipids. Phosphati-

dylethanolamine and phosphatidylglycerol were found extensively in bacteria, and Oliver ane Cowell,[7] and Hardy *et al.* reported similar results in the lipids obtained from *Achromobacter* isolated from their marine origin. Three percent of lyso-phosphatidylethanolamine and 2.9% of lyso-phoaphatidylglycerol are detected in this lipid, respectively.

$C_{15:0,15:1}$, $C_{16:0,16:1}$, $C_{17:0,17:1}$ fatty acids predominate in all lipids. The content of odd number fatty acids were very high.

The ratio of saturated/unsaturated fatty acid is about 1.5 in phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid and wax, respectively, whereas free fatty acid has the ratio of about 4.0.

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