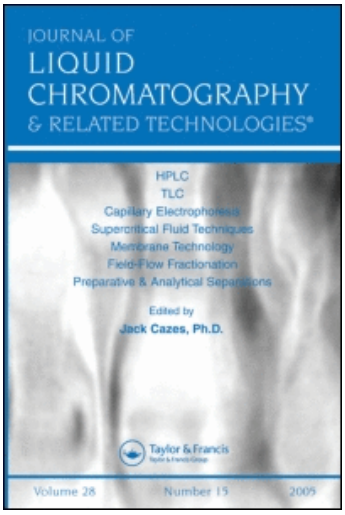

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PHOSPHOLIPID COMPOSITION AND DIFFERENTIATION OF METHANOTROPHIC BACTERIA

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ABSTRACT

A combination of TLC and extraction of native cells on the layer of sorbent was used for analysis of phospholipid composition of bacteria. The method is suitable for rapid routine differentiation of genera and species of obligate methanotrophic bacteria.

INTRODUCTION

During the past decade bacterial taxonomy has undergone essential changes which are reflected in the 8th edition of Bergey's Manual of Determinative Bacteriology (1). It was largely due to the use of modern chemotaxonomic techniques which become currently accepted in systematics of bacteria (2-3).

All organisms contain polar lipids, and their analyses often enable one to elaborate classification systems of microorganisms (3). Presently,

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researchers pay a special attention to rapid and qualitative methods of analysis which allow the study of a great number of strains and their rapid preliminary identification (express-diagnostics).

Methanotrophic bacteria attract an increasing attention due to their practical importance. However, the taxonomy of the organisms still needs further investigation. So far the questions of methanotroph classification have been tackled with help of conventional methods of bacterial taxonomy. The elaboration of progressive chemotaxonomical approaches in the taxonomy of methanotrophic bacteria is an urgent task.

Previously it was shown (4) that methanotroph types (groups) differ in their phospholipid and fatty acid composition. A more detailed analysis of fatty acids of a large group of methanotrophic bacteria (68 strains) allowed the identification of these organisms to genera and species (5). Such an analysis revealed also the differences in the phospholipid composition of different genera and types of methanotrophs (6).

In the present work the possibility of the express-diagnostics of obligate methanotrophic bacteria based on thin-layer chromatography of their phospholipids.

MATERIALS AND METHODS

Materials

Thin-layer glass plates, 20 x 20 cm (DC-Fertigplatten Kieselgel 60 F 254 from E.Merk, Darmstadt, FRG), were used. Phosphatidic acid (PA), phosphatidyl glycerol (PG), diphosphatidyl glycerol (DPG), phosphatidyl choline (PC), phosphatidyl ethanolamine

(PE), monomethyl- (PMME) and dimethylphosphatidyl ethanolamine (PDME) were obtained from Supelco (USA). Agar was purchased from Difco (USA). Solvents and mineral salts were from Khimreaktiv (USSR).

Methods

Methanotrophic bacterial cultures were grown on mineral salts medium containing (g/l distilled water): KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2HPO_4 \cdot 5H_2O$, 1.5; KH_2PO_4, 0.7; (mg/l): EDTA, 5.0; $FeSO_4 \cdot 7H_2O$, 2.0; $ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; $CoCl_2 \cdot 6H_2O$, 0.2; $CuCl_2 \cdot 5H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.02; Na_2MoO_4, 0.03; pH 6.9 $\pm$ 0.2. The medium was sterilized at 121°C (1 atm) for 1 h. Phosphates were sterilized separately, cooled and added to the other components. The medium was solidified with agar (2%).

The cultures were grown in test tubes with solid medium or in 0.7 l - flasks with 100 ml liquid medium. Flasks were sealed with rubber stoppers. A mixture of methane and air (1:1) was added through two glass tubes in the stoppers with cotton filters. For better aeration the flasks were shaken on a rotor shaker (140 ppm). In case of a solid medium the bacteria were grown in vacuum dissicators filled with a methane - air mixture (1:1).

Use was made of pure cultures of obligate methanotrophs of the culture collection of IBPhM, USSR Academy of Sciences. Strains OB3b and 5 were courteously provided by Prof. Wittenbury (University of Warwich, Coventry, UK); and strain Texas, by Prof. Quayle (University of Sheffield, Sheffield, UK).

For thin-layer chromatography the cell suspension was washed with saline (0.15 M NaCl) to a final concentration of 5 to 15 µg/ml of dry weight. 10 µl of the cell suspension was applied onto a silica gel

layer to obtain an evenly coated region of 5 x 5 mm, Then the plate was dried under nitrogen stream, and the procedure was repeated. The cells were extracted on the layer by a chloroform - methanol mixture (1:2). To do so, the plates were developed in the solvent mixture, the start line being 2-3 cm above the line of application of the cell suspension. This was coupled with the extraction of lipids from the cells and their movement along the layer. After this procedure was 5-6 times repeated the extracted lipids were concentrated in the form of a narrow strip at the front line of the extractant. Between two subsequent extractions the solvent was removed by drying under nitrogen stream. Then the silica gel layer with the extracted cells was removed by a scalpel.

The phospholipids were separated in corresponding solvents. For unidimensional chromatography the solvent system containing chloroform, methanol, acetone, acetic acid a water (10:3:8:2:1) was used. Two-dimensional chromatograms were obtained using the solvent systems of chloroform-methanol - 26% NH_4OH solution (65:25:4 the first separation) and chloroform-acetone-methanol-acetic acid-water (8:2:8:2:1, the second separation). To visualize the lipid spots, the plates after separation were sprayed with 10% $CuSO_4$ solution in 8% orthophosphate acid and heated for 10 min at 175-200°C. (7).

RESULTS AND DISCUSSION

Fig.1A and 1B present the unidimensional chromatograms of phospholipids of methanotrophic bacteria of different genera and types. The chromatogram 1B was developed once in the solvent system to a length of 12 cm, whereas the chromatogram 1A was primarily

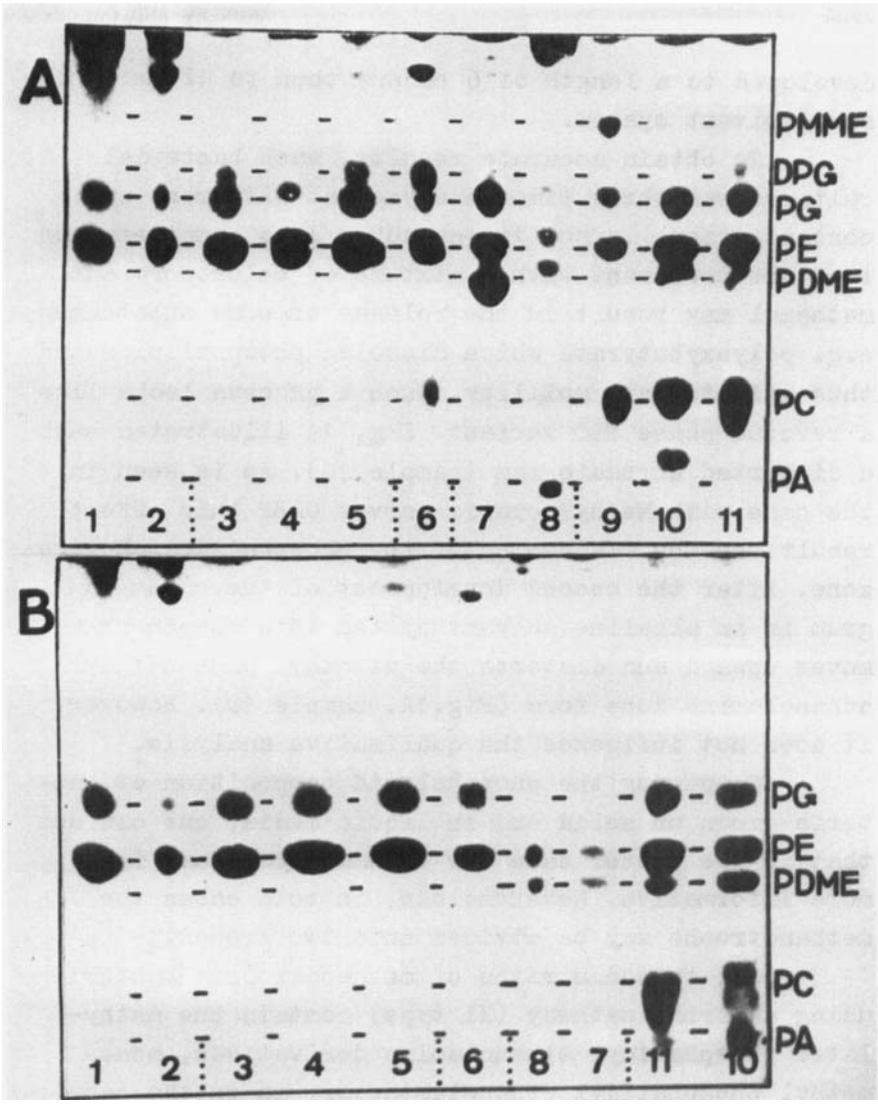

FIGURE 1. One-dimentional chromatograms of phospholipids of obligate methanotrophic bacteria grown in liquid media (A) and on solid media (B).
(1) Methylomonas methanica 12; (2) Mm.albus BG8 ;(3) Methylobacter vinelandii 87; (4) Mb.bovis 89; (5) Mb.chroococcum 90; (6) Methylococcus capsulatus "Texas"; (7) Methylosinus trichosporium OB3b; (8) Ms.sporium 5; (9) Methylocystis minimus 82; (10) Mcs.parvus OBBP; (11) Mcs.echinoides 2.

developed to a length of 6 cm and then to 12 cm in the same solvent system.

To obtain accurate results, each bacterial cultures was three times analyzed at different cell concentrations, since it was taken into consideration that the treatment with a mixture of chloroform and methanol may result in the release of some substances, e.g. polyoxybutyrate which dissolve phospholipids and thus affect their motility. Such a process looks like a reverse-phase TLC variant. Fig. 1A illustrates such a distorted chromatogram (sample 10). As is seen in the case with Methylocystis parvus OBBP this effect results in the flattening of the phosphatidyl choline zone. After the second development of the chromatogram in an alkaline solvent system this component moves upward and distorts the dimethyl phosphatidyl ethanolamine zone form (Fig.1A, sample 10). However, it does not influence the qualitative analysis.

Comparing the phospholipid composition of bacteria grown on solid and in liquid media, one can see that in the latter case the chromatograms are far more informative. Nevertheless, in both cases the methanotrophs may be divided into two groups.

All tested strains of methanotrophic bacteria using a serine pathway (II type) contain the methylated phosphatidyl ethanolamine derivatives, monomethyl phosphatidyl ethanolamine or, as in the case of Methylocystis minimus 82, dimethyl phosphatidyl ethanolamine, and phosphatidyl choline. The said phospholipids are not contained in the lipid pool of methanotrophs with ribulose monophosphate cycle (I type).

Methylococcus capsulatus is an exception to this rule. As is seen on Figures 1B and 2C, the bacteria of this species have small amounts of phospha-

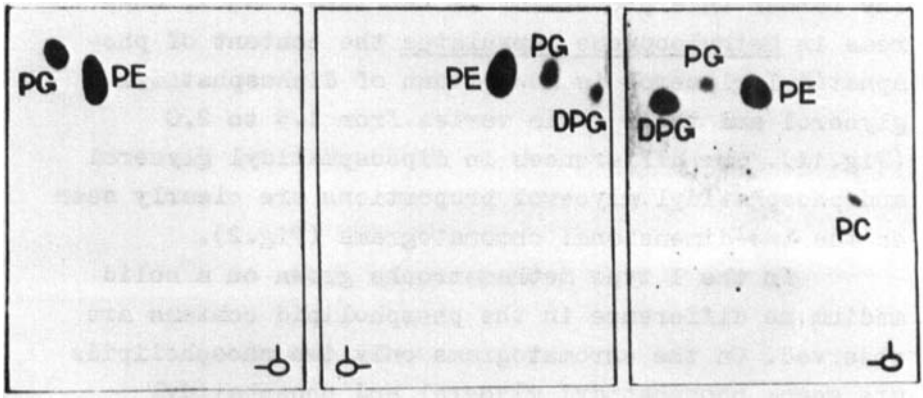

FIGURE 2. Two-dimensional chromatograms of phospholipids of obligate methanotrophic bacteria. (A) Mm.methanica 12; (B) Mb.vinelandii 87; (C) Mc.capsulatus "Texas".

tidyl choline. Methylococcus capsulatus is a I type methylotroph and uses a ribulose monophosphate partway of methane carbon assimilation. But as it was shown (8) this organism displays some characteristics of II type methanotrophs (e.g. possesses some activities of key enzymes of the serine cycle of methane carbon assimilation). This must have somehow affected its phospholipid composition as well: like the II type methanotrophs, Methylococcus capsulatus has phosphatidyl choline.

Based on the differences in the phospholipid spectra the methanotrophs within the said groups (types) can be differentiated to genera. For example, Methylomonas differs from Methylobacter by the absence of diphosphatidyl glycerol whose content in the latter in substantial. Methylobacter can easily be differentiated from Methylococcus by the ratio of diphosphatidyl glycerol and phosphatidyl glycerol. In

the former this proportion is far less than 1, whereas in Methylococcus capsulatus the content of phosphatidyl glycerol is lower than of diphosphatidyl glycerol and their ratio varies from 1.5 to 2.0 (Fig.1A). The differences in diphosphatidyl glycerol and phosphatidyl glycerol proportions are clearly seen on the two-dimensional chromatograms (Fig.2).

In the I type methanotrophs grown on a solid medium no difference in the phospholipid contens are observed. On the chromatograms only two phospholipids are seen: phosphatidyl glycerol and phosphatidyl ethanolamine (Fig.1B).

The phospholipid composition permits also to differentiate the representatives of the II type methanotrophs. Methylosinus bacteria contain small amounts of phosphatidyl choline, which is one of the major components in Methylocystis.

When using the phospholipid composition for differentiation of methanotrophic bacteria, purity of cultures is a must, because even slight contamination, e.g. with molds, results in the unproportional increase of the phosphatidyl choline content.

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