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Biomass measurement of methane forming bacteria in environmental samples

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Summary

Methane-forming bacteria contain unusual phytanylglycerol ether phospholipids which can be extracted from the bacteria in sediments and assayed quantitatively by high performance liquid chromatography (HPLC). In this procedure the lipids were extracted, the phospholipids recovered, hydrolyzed, purified by thin layer chromatography, derivatized and assayed by HPLC. Ether lipids were recovered quantitatively from *Methanobacterium thermoautotrophicum* and sediments at levels as low as 8×10^{-14} moles. In freshwater and marine sediments the flux of methane to the atmosphere and the methane levels in the pore water reflects the recovery of the phytanyl glycerol ether lipid 'signature'. The proportion of the ether phospholipid to the total recoverable phospholipid was highest in anaerobic digester sewage sludge and deeper subsurface freshwater sediment horizons.

Key words: *Anaerobic microbes – Biomass – Methane bacteria – Methane formation*

Introduction

In soils and sediments approximately 50% of the total organic carbon degraded by the anaerobic microbiota [1] results in the formation of methane. The small proportion of this methane that escapes oxidation and reaches the atmosphere corresponds approximately to the anthropogenic input. Changes in the methane content of the atmosphere are expected to have profound effects on the climate [2]. In addition, the biogenesis of methane under controlled conditions has the potential of becoming a significant source of renewable energy.

A diverse group of anaerobic microorganisms appears to be exclusively responsible for the biological generation of methane. Despite the diversity, all the known methanogenic microbes are Archaebacteria which share a number of distinctive biochemical features that clearly differentiate them from all other eukaryotic and prokaryotic life [3, 4]. Among the unique features of the methanogenic bacteria

are the di- and bidiphytanylglycerol ether phospholipids [5-8]. The Archaebacteria also include the halophiles and the thermoacidophiles, *Thermoplasma* and *Sulfolobus*. The lipids contain two 20 carbon saturated isoprenoid hydrocarbons with ether linkages to glycerol (di-*O*-phytanylglycerol ether) (DPGE) or two 40 carbon isoprenoid hydrocarbons in ether linkage to glycerols (tetra-*O*-di(biphytanylglycerol ether) (biDPGE). The halophilic bacteria contain the diether DPGE [9, 10] and the thermoacidophiles the tetra-ether biDPGE [11, 12]. The methanogenic bacteria can contain both DPGE and biDPGE. The concentration of ether varies from 100% DPGE in *Methanococcus PS* and *Methanosarcina bakeri* to 37.5% in *Methanospirillum AZ* [13]. Since the halophiles and thermoacidophiles are unlikely to form a significant biomass in anaerobic sediments, these lipids could form an ideal 'signature lipid' for the determination of the biomass and growth of the methanogenic bacteria [14].

This study reports a method that can be used to estimate the biomass of the methanogenic bacteria in sediments. The phytanylglycerol ethers exist in the membranes as complex phospholipids [7, 8] which can be extracted, purified and assayed as *p*-nitrobenzoyl esters by high performance liquid chromatography (HPLC).

Materials and Methods

Materials

Glass distilled solvents were used as purchased (Burdick and Jackson, Muskegon, MI) or ChromAR grade solvents (Mallinckrodt, St. Louis, MO) were used. Chloroform was distilled just prior to use in extractions and derivatizations. Derivatizing reagents and internal standards were purchased from Sigma Chemical Co., St. Louis, MO; Aldrich Chemical Co., Inc., Milwaukee, WI. Unisil activated silicic acid 100–200 mesh was obtained from Clarkson Chemical Co. (Williamsport, PA). Whatman 2V filter papers and K6 silica gel thin layer plates (250 μ gel thickness) were purchased from Whatman Chemical Separations, Inc. (Clifton, N.J.). The radial pak B cartridges for HPLC were obtained from Waters Associates, Inc. (Milford, MA).

Organisms

Lyophilized cells of *Methanobacterium thermoautotrophicum* were the gift of Dr. J.G. Zeikus, Department of Bacteriology, University of Wisconsin. Anaerobic sewage sludge was supplied by W.C. Leseman, Laboratory Director, City of Tallahassee, Tallahassee, Florida.

Sediment samples

Sediment samples were taken from a subtidal mudflat adjacent to the Florida State Marine Laboratory (salinity 25 mg/l, depth 1 m) (29°54.7'N; 84°29.5'W), the edge of the fresh water Lake Jackson near Tallahassee (30°37.1'N; 84°21.5'W), and a small freshwater pond near Panacea, Florida (30°3.4'N; 84°25.5'W). Samples were taken as 3.5 cm diameter cores, transported to the laboratory on ice, and quick frozen and lyophilized prior to extraction.

Methane measurements

Methane fluxes were measured at the air–water interface with a gas-filter correlative non-dispersive infrared adsorption analyzer [15, 16]. Methane fluxes can be quantified as a function of the air velocity over the water surfaces in short periods of time with this apparatus. Concentrations of methane in water samples were measured using liquid stripping gas-liquid chromatography (GLC) [17, 18]. Dissolved methane in sediment pore water was measured using an interstitial sampler as described [19, 20].

Extraction

Fig. 1 illustrates the analytical sequence used in this study. The sediments were extracted with at least 10 times the volume of the one-phase chloroform–methanol (1:2, v/v) containing a total water content (water in the sediment plus an added amount of 50 mM phosphate buffer, pH 7.4) equivalent to 0.8 times the chloroform content [21]. After at least 2 h, one volume of chloroform and one volume of water were added, and the suspension was shaken vigorously. After 24 h at room temperature, the suspension was centrifuged at 5000 × *g* for 20 min and the supernatant poured into a separatory funnel. After partitioning, the lower chloroform phase was recovered by filtration through a Whatman 2V filter into a round bottom flask and the solvent removed in vacuo on a rotary evaporator.

Column chromatography

The lipid was transferred with a minimal volume of hexane to a column of Unisil silicic acid with at least a gram of silicic acid for each 30 μmoles phospholipid. The phospholipid phosphate can be determined colorimetrically on a 5% or less aliquot

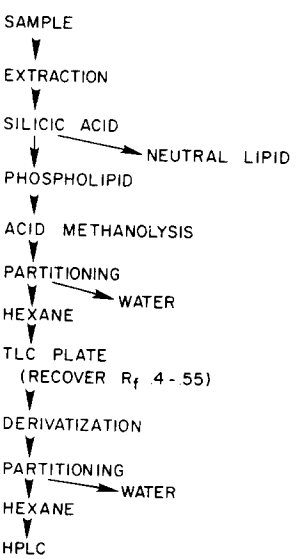

Fig. 1. Scheme for the analysis of DPGE from sediments.

of the chloroform after digestion with perchloric acid [22]. The neutral lipids were eluted with 10 column volumes each of hexane, benzene, and chloroform. The phospholipids were eluted with chloroform–methanol 2:1 followed by methanol as described [5]. The polar phospholipid fractions were pooled, dried on a rotary evaporator, and transferred to a Teflon capped test tube and dried in a stream of nitrogen.

Hydrolysis

The phospholipid fraction was dissolved in 1.5 ml methanol–chloroform–concentrated HCl (9:1:1, v/v/v), and heated to 100°C in a tightly capped tube for 2 h. This liberates the DPGE from the phospholipids. After cooling to room temperature, 1 ml of petroleum ether and 1 ml of water were added, the contents mixed on a vortex mixer for 3 min, and the tubes centrifuged at 2000 × *g* for 3 min. The ether layer was transferred to a second screw cap test tube, and the extraction repeated twice. All three combined ether extracts were dried in a stream of nitrogen. The water fraction may be utilized for the determination of the phospholipid phosphate [22].

Thin layer chromatography

The hydrolized lipid was dissolved in hexane and applied in a band 3 cm from the bottom of a Whatman K6 silica gel TLC plate. Standards recovered from *M. thermoautotrophicum* lipids were applied in lanes at each side of the TLC plate. The components were separated in an ascending solvent of hexane–diethyl ether–acetic acid (80:20:1, v/v/v). After about 25 min at room temperature the plate was removed and the end lanes sprayed with rhodamine 6G for visualization of the bands with ultraviolet light. The DPGE was at R_f 0.475 and the biDPGE at R_f 0.113. Areas ± 0.8 cm on each side of the DPGE and biDPGE bands were scraped with a razor blade and put into a glass column plugged with glass wool. The lipids were eluted from the pipette with 5–7 ml of chloroform–methanol (1:1, v/v). The DPGE and biDPGE in the eluent from each band was dried in a stream of nitrogen and stored in the dark at −20°C.

Derivatization

Preparation of the derivatizing agent required addition of 5 ml molecular sieve-dried benzene to 200 mg *p*-nitrobenzoyl chloride in a test tube. After vortex mixing, 5 ml of dry pyridine was added and mixed lightly. A 1.5 ml portion of this reagent was added to the dried ethers, plus an internal standard of 10 nmoles of docosanol (Aldrich Chemical Co, Milwaukee, WI), the test tube tightly capped and heated for 45 min at 60°C. After cooling the solution was partitioned by addition of 1 ml hexane and 1 ml of aqueous 4% sodium carbonate (w/v). The contents were mixed with the vortex mixer, centrifuged, and the water layer discarded. The extraction, mixing and centrifugation was repeated after adding a second 1 ml of 4% sodium carbonate. The extraction, mixing and centrifugation was repeated a third, fourth and fifth time substituting 1 ml of 10% tartaric acid for the carbonate. The hexane was then carefully removed to a second test tube

with a Pasteur pipette and fresh hexane was added to the last aqueous wash in the original test tube. After mixing and centrifugation, the hexane was removed and added to the hexane from the initial extraction. This was repeated with a second portion of fresh hexane. The three pooled hexane fractions were dried with a stream of nitrogen.

HPLC

The lipid derivatives were loaded with 100 µl of hexane onto a Waters radial Pak-B silica cartridge 8 mm in diameter, 10 cm long with 10 µ spherical particles in a Waters RCM-100 radial compression cartridge. The two-solvent gradient was generated with the Hewlett-Packard model 1084A HPLC. The chromatograph was operated at a pressure of 100 kg/cm^2 with a flow rate of 120 ml/h using a solvent of 100% hexane (solvent A) and 80% hexane–20% diethylether, v/v (solvent B) with a program of 5 min 100% solvent A, 5–25 min from 0 to 50% B, 25–45 min from 50 to 100% B, 100% B for 45–50 min. The *p*-nitrobenzoyl esters were detected with the Waters model 440 absorbance detector (254 nm) set to 0.2 AUFS for usual operation. The output of the detector was integrated by a Hewlett-Packard 3352 laboratory data system. A chromatogram of DPGE from freshwater sediment is illustrated in Fig. 2. The position of the DPGE was established by adding the authentic material derived from *M. thermoautotrophicum*.

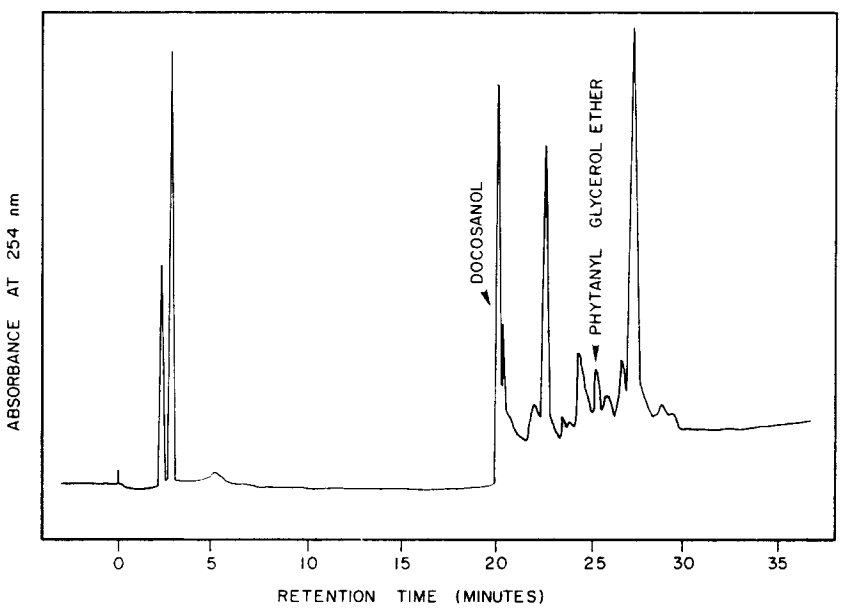

Fig. 2. Absorbance at 254 nm of the *p*-nitrobenzoyl esters after elution with a gradient of hexane–diethyl ether, by HPLC with a 2 ml/min flow rate. DPGE (8.3×10^{-10} moles) and the internal standard of docosanol (1.1×10^{-8} moles) are indicated.

Infrared spectra

IR spectra of the bands from the TLC plates and the eluates from the HPLC columns were examined in a minimum volume of carbon tetrachloride with the Perkin-Elmer 1320 IR spectrometer.

Results

Recovery of DPGE from M. thermoautotrophicum

Lyophilized *M. thermoautotrophicum* yielded 1.67 ± 0.09 (X ± S.D., n = 4) μmoles DPGE/g dry wt. and a total phospholipid of 14.6 ± 0.64 μmoles lipid phosphate/g dry wt. when analyzed by the scheme illustrated in Fig. 1. This preparation yielded 23% DPGE and 77% biDPDG which accounted for 88% of the lipid phosphate. This organism is reported to contain 18 mg/g dry wt. of polar lipid with a phosphorus content of 1.65% [5]. This calculates to a total lipid content of 9.9 μmoles of phospholipid phosphate/g dry wt. of which 4.4 μmoles (44.5%) would be expected to be DPGE [6]. This compares to our measured value of 1.67 ± 0.09 μmoles DPGE/g dry wt.

Identification of DPGE

The IR spectrum of the DPGE and biDPGE eluted from the TLC or recovered after HPLC showed broad absorbance bands centered at 3300 cm^{-1} of OH, 2940, 2860, and 2465 cm^{-1} of CH_2 and CH_3, a doublet 1380 and 1370 cm^{-1} of gem-dimethyl C-CH_3, 1730 cm^{-1} of carbonyl, and the definitive ether at 1105 cm^{-1}. This is consistent with the structure of DPGE and biDPGE [6].

Sensitivity

The molar extinction coefficient of the mono-*p*-nitrobenzyl ester of DPGE calculated from an absorbance of 0.362 in hexane–diethyl ether, 4:1, v/v at 254 nm for 0.206 μmoles was 1.76×10^6. The sensitivity, measured by diluting the sample until the signal was at least 2.4 times the noise, was 7.4×10^{-14} moles using the 0.1 AUFS scale. This gives a detection limit of 4.4×10^{-8} g of cells (4×10^4 cells the size of *E. coli*).

Recovery of DPGE from sediments

To measure the recovery of DPGE from sediments, 12.5 g wet wt. (1.5 g dry wt.) of anaerobic sewage sludge or 0.25 g of lyophilized *M. thermoautotrophicum* was added to 50 g of acid washed, ignited sand (Fisher Chemical Co., St. Louis, MO) and after thorough mixing, the sediments were processed as illustrated in Fig. 1. The sand contained $< 7.0 \times 10^{-14}$ moles of DPGE. The recovery of DPGE from the sediment plus sewage sludge was 105 ± 10%. The recovery of extractable phospholipid and DPGE from the *M. thermoautotrophicum* plus sand was 19.7 ± 4.6 μmoles phospholipid and 1.52 ± 0.25 μmoles DPGE/g dry wt. added bacteria compared to 14.6 ± 0.64 μmoles phospholipid and 1.67 ± 0.09 μmoles DPGE/g dry wt. from the lyophilized bacteria itself for a recovery of 134 ± 22% (phospholipid) and 91 ± 11% (DPGE).

TABLE 1

COMPARISON OF PHOSPHOLIPID AND DPGE CONTENT OF SEWAGE SLUDGE, FRESHWATER, AND ESTUARINE SEDIMENTS TO *M. thermoautotrophicum*

Sample	Phospholipid (moles/g dry wt.)	DPGE (moles/g dry wt.)	Ratio DPGE/phospholipid
M. thermoautotrophicum	$1.46 \pm 0.06 \times 10^{-5}$	$1.67 \pm 0.1 \times 10^{-6}$	$1.12 \pm 0.02 \times 10^{-1}$
Sewage sludge	$1.21 \pm 0.48 \times 10^{-6}$	$1.36 \pm 0.69 \times 10^{-8}$	$1.1 \pm 0.4 \times 10^{-2}$
Freshwater sediment			
0 to −4 cm	$1.17 \pm 1.1 \times 10^{-6}$	$9.0 \pm 15.2 \times 10^{-9}$	$1.3 \pm 3.5 \times 10^{-4}$
−5 to −50 cm	$9.3 \pm 3.5 \times 10^{-8}$	$8.7 \pm 7.5 \times 10^{-9}$	$9.7 \pm 8.8 \times 10^{-2}$
Estuarine sediment			
0 to −10 cm	$1.8 \pm 0.2 \times 10^{-8}$	$7.02 \pm 3.6 \times 10^{-11}$	$3.9 \pm 2.1 \times 10^{-3}$

Relation of DPGE in sediments to methane content

The surface of a small pond outside Panacea, Florida yielded 93 mg methane/m^2/day. The water column contained a gradient from 0.065 mg/l to 1.711 mg/l at −36 cm at the sediment–water surface. The pore water from the 0 to −4 cm horizon contained 11.0 ± 1.73 mg methane/l and 11.6 ± 1.26 mg methane/l in the −4 to −50 cm horizon. Both horizons contained nearly the same amount of DPGE but the upper layer contained more than 10-fold more total microbial biomass as measured by the extractable phospholipid (Table 1). Near-shore sediments of large clear lake near Tallahassee (Lake Jackson) contained only $2.81 \pm 0.46 \times 10^{-10}$ moles of DPGE/g dry wt.

Estuarine salt marsh sediments like those that produced a methane flux of 5 mg methane/m^2/day contained <0.01 mg methane/l in the 0 to −10 cm horizon and 0.05 to 0.75 mg methane/l in the −10 to −20 cm horizon. The 0 to 10 cm horizon contained 100-fold less total microbial biomass as measured by the total phospholipid and methane bacterial biomass than was estimated by the content of DPGE (Table 1).

Discussion

This method of analyzing DPGE was sufficiently sensitive and reproducible to permit quantitative recovery of the DPGE of added methane-producing bacteria or sewage sludge from sands. In the same analysis the biomass of the total microbial community can be estimated as the extractable phospholipid. These lipids can be further fractionated for indications of the community structure and nutritional status of the sedimentary microbial community [14]. The assay of methane-forming bacteria by the DPGE content is expensive. A total of 64 h, 40 of which require the attention of the analyst, was required to analyze 6 sediments.

Sewage sludge from an anaerobic digester contained approximately 0.8% of the dry weight of methane bacteria like *M. thermoautotrophicum* (Table 1). Freshwater sediments near the sediment surface that produce a high flux of methane to the atmosphere contain a rich sedimentary microbiota with the expected high propor-

tion of methane-forming bacteria. However the distribution of the methane-forming bacteria as indicated by DPGE was patchy as indicated by the high variance (Table 1). Deeper horizons from this sediment showed that the DPGE contaning methane-forming bacteria formed a much larger proportion of the total microbial biomass than at the surface (Table 1). Salt water depressed both the total microbial biomass and the DPGE containing microbiota (Table 1).

Acknowledgments

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