A method for the separation and characterization of archaebacterial signature ether lipids

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Abstract A reproducible high performance liquid chromatography (HPLC) method for the separation of diethers and tetraethers isolated from archaebacterial phospholipids is reported. Fourier transform infrared spectroscopy was used for structural confirmation of these signature lipids. A mixture of tetraethers from a thermoacidophilic archaebacteria was resolved into three major components by the normal phase separation. These components were differentiated by Fourier self-deconvolution of infrared spectra. The application of the HPLC technique to environmental samples may provide an accurate assessment of archaebacterial biomass in various microbial communities. — **Mancuso, C. A., P. D. Nichols, and D. C. White. A method for the separation and characterization of archaebacterial signature ether lipids.** *J. Lipid Res.* **1986. 27: 49–56.**

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The kingdom Archaebacteria includes methanogens, halophiles, and thermoacidophiles. The methanogenic bacteria are responsible for one of the major end products from the decomposition of organic matter (1, 2) and are ubiquitous in most anaerobic sediments (3). In extremely saline habitats, halophilic bacteria are prominent among the microorganisms (4). Thermoacidophilic bacteria, in contrast, are the only known inhabitants of acidic hot springs and self-heating coal refuse piles where temperatures reach higher than 55°C (5, 6). A measure of the viable biomass of these organisms is essential to the study of the microbial community in each of these ecosystems.

Archaebacteria lack the ester-linked phospholipid fatty acids found in the cellular membranes of Eubacteria (7–9). Fatty acid signature profiles allow for the identification and quantification of specific microbial types (10–12). Membrane lipids of archaebacteria, however, consist of two isoprenoid-branched, hydrocarbon side chains bound to the glycerol phosphate backbone in ether linkages (13, 14). In some cases, the side chains of diether lipids are linked head-to-head to form tetraether lipids (15). These span the membrane creating a monolayer which contrasts the typical bilayer membrane form (16). Tetraethers make up the majority of the cell membranes of thermoacidophiles and occur in varying ratios with diethers in methanogens (7, 17, 18). Halophiles possess only diethers (8, 19).

This report describes a high performance liquid chromatography (HPLC) technique to separate these archaebacterial signature lipids from several monocultures. Fourier transform infrared spectroscopy is used to verify and characterize the various ether lipid components. This separation represents, to our knowledge, the first instrumental separation procedure that can be used routinely to assess the diether and tetraether components of microbial samples. In the future, this technique could be applied to environmental sample analyses to estimate archaebacterial biomass of various microbial communities.

MATERIALS AND METHODS

Solvents and reference compounds

Solvents were all distilled in glass and of residue analysis grade or better (J. T. Baker Chemical Co., Phillipsburg, NJ). The diether lipid standard, 1,2-di-O-hexadecyl-*rac*-glycerol, was purchased from Sigma Chemical Co., St. Louis, MO.

Bacteria and bacterial lipids

Lyophilized cells of *Methanobacterium thermoautotrophicum* strain Hveragerdi were a gift from J. P. Kaiser and Dr. K. Hanselmann of the Department of Microbiology, University of Zurich, Zurich, Switzerland. Lyophilized cells of *Methanosarcina barkeri* strain Jolich were obtained from Dr. P. A. Scherer of Scherpunkt Biotechnologie, Fachhochschule Weihenstephan Lowentorgebaude, Friesing, FRG.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; FT-IR, Fourier transform-infrared; FSD, Fourier self-deconvolution.

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Halobacterium cutirubrum was obtained from Dr. J. G. Zeikus of the Department of Bacteriology, University of Wisconsin, Madison, WI. Cultures of *H. cutirubrum* were grown on the following medium consisting of (in amounts per liter): NaCl (150 g), $MgSO_4 \cdot 7H_2O$ (6.4 g), KCl (1 g), trace vitamin solution (10 ml) (20), trace mineral solution (10 ml) (21), glucose (1 g), peptone (2 g), and yeast extract (2 g). The medium pH was adjusted to 7.5. Cells were harvested by centrifugation and the pellet was washed with 15% saline water. Cells were lyophilized before extraction.

A sample containing previously identified tetraether lipids with acyclic, monocyclic, and bicyclic side chains, isolated from *Thermoplasma acidophilum,* was provided by Dr. T. A. Langworthy of the Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, SD.

Extraction

Lipids were extracted from 50–100 mg of lyophilized cells of each strain by a modification of the Bligh and Dyer method (10). Cells were sonicated in test tubes with Teflon-lined screw-caps with 10 ml of methanol for 30 min. Five ml of chloroform and 4 ml of 50 mM phosphate buffer (pH 7.4) were added, and the samples were extracted at room temperature for 18 hr. Tubes were centrifuged and the supernatant was transferred to a 50-ml separatory funnel. Sufficient chloroform and buffer were added for a final ratio for chloroform–methanol–buffer of 2:2:1.8 (v/v/v). After separation of the two phases, the lower chloroform layer was collected through a Whatman 2V filter into a screw-cap test tube under a dry stream of nitrogen at less than 40°C.

Fractionation and hydrolysis of phospholipids

Total lipids were fractionated on a 1-g column of Unisil (Clarkson Chemical Co., Inc., Williamsport, PA, USA) by an elution sequence with 10 ml each of chloroform, acetone, and methanol (10). The methanolic phospholipid fractions were then hydrolyzed by adding 1 ml of chloroform–methanol–conc. HCl 10:1:1 (v/v/v) and by heating to 100°C for 2 hr. Glycerol diethers were formed by the acidic hydrolysis of the phospholipids and were extracted by the addition of 1 ml of hexane and 1 ml of water. Extraction was repeated twice and the pooled organic fractions were dried under a stream of nitrogen.

Thin-layer chromatography

Thin-layer chromatography (TLC) was used to isolate the glycerol diethers. The plates of silica gel K6, size: 20 cm × 20 cm × 250 μm (Whatman Chemical Separations, Inc., Clifton, NJ) were precleaned in hexane–diethyl ether–acetic acid 70:30:1 (v/v/v). 1,2-Di-O-Hexadecyl-*rac*-glycerol and authentic tetraethers and diethers prepared from *Methanobacterium thermoautotrophicum* strain Hveragerdi were applied to end lanes as reference compounds. After development, the end lanes were sprayed with Rhodamine 6G (0.01% w/v) and exposed to UV light for visualization. In this system, tetraethers and diethers were found to have an R_f of 0.07 and 0.30, respectively. Bands 2-cm wide were scraped. The silica gel was collected in Pasteur pipettes plugged with glass wool and the ether lipid components were eluted with 5 ml of chloroform–methanol 1:2 (v/v). The samples were dried under a stream of nitrogen. This TLC step, used in preliminary method development, was eventually replaced by the high performance liquid chromatography (HPLC) procedure system described below.

High performance liquid chromatography

The samples were dissolved in 100 μl to 2 ml of hexane–n-propanol 99:1. Using a 500-μl syringe (Hamilton Co., Reno, NV), 50-μl aliquots containing 3.34 μg of diether standard and between 10 and 65 μg of archaebacterial ether lipid were injected into the system which consisted of a Valco (Houston, TX) injector fitted with a 20-μl sample loop (Rainin Instrument Co., Woburn, MA) and a loop filler port (Rheodyne, Cotati, CA). The system also included a 10-μm silica absorbant precolumn (5 cm × 4.6 mm i.d., Rainin) and a Spherisorb 5-μm amino column (25 cm × 4.6 mm i.d., Phenomenex, Palos Verdi Estates, CA). The analytical column was surrounded by a plastic water-jacket (Rainin) for thermal stability.

A premixed and degassed solvent system of hexane–n-propanol 99:1 was pumped at a rate of 0.5 ml/min by a Beckman model 110A pump fitted with a pressure filter (Beckman Instruments, Inc., Fullerton, CA). The system also included a low pressure filter assembly and a restrictor assembly (Waters Assoc., Milford, MA) for additional pulse dampening.

Ether lipids were detected by a refractive index detector, model ERC-7510 (Erma Optical Works, Tokyo, Japan). Precautions were taken against baseline drift caused by ambient temperature fluctuations by covering the detector totally with a styrofoam-insulated, inverted cardboard box. The detector signal was integrated by an HP 3352 laboratory data system (Hewlett Packard, Palo Alto, CA). HPLC fractions for Fourier transform infrared spectroscopy (FT-IR) analysis and ether lipid identification were collected and dried under a stream of nitrogen.

Diffuse reflectance Fourier transform–infrared (DRIFT) spectroscopy

Infrared grade KBr (Mallinckrodt Inc., St. Louis, MO) was ground for 1 min (Wig-L-Bug, Spectra Tech. Inc., Stamford, CT), transferred to a 3 mm × 2 mm sample cup, and leveled (without compression, using a spatula) prior to DRIFT analysis. HPLC fractions were dissolved in hexane (100 μl) and transferred to the sample cup using

a 250-μl glass syringe (Hamilton Co., Reno, NV). The sample cup was then placed under a light source (ca 5 min) to enhance evaporation of the solvent.

Infrared spectra were collected using a DRIFT accessory (Spectra Tech. Inc., Stamford, CT) on a Nicolet 60SX FT-IR spectrometer equipped with a liquid nitrogen-cooled, high sensitivity, mercury-cadmium-tellurium detector (range 5500–800 cm^{-1}), a mid-IR Globar source, and a KBr beam splitter (Nicolet Instrument Corp., Madison, WI). Interferograms were zero-filled and apodized by the Haap-Genzel function prior to the Fourier transformation utilizing Nicolet SX software (TMON version 1.5). The sample chamber was evacuated for 2 min and purged with dry nitrogen for 2 min prior to sample or background data collection. The optical bench remained under a constant dry nitrogen purge throughout the analyses.

Each sample scan resulted in a single-sided interferogram of 4096 data points which provided a resolution of 4 cm^{-1}. Signal averaging of 250 scans per sample required 2.25 min of total measurement time. All resulting spectra were ratioed to a background of KBr, and are reported in transmittance units in order to compare data with the existing literature. Fourier self-deconvolution (22) of spectra converted to absorbance units was performed using the Nicolet IRDCON · FTN program (23).

RESULTS

Fig. 1 shows chromatograms of a) the 1,2-di-O-hexadecyl-*rac*-glycerol standard and the archaebacterial ether lipids and standard compounds from b) *Halobacterium cutirubrum*, c) *Methanosarcina barkeri* strain Jolich, d) *Methanobacterium thermoautotrophicum* strain Hveragerdi, and e) *Thermoplasma acidophilum*. All five chromatograms were produced by the normal phase HPLC operating at a flow rate of 0.5 ml/min, a chart speed of 10 cm/min, and a detector setting of R=1/4 (in a range of 32 to 1/64). The detector response was linear for the diether standard in the concentration range used.

Two components, visible in all chromatograms, are associated with the solvent. The presence of these two components is best demonstrated in Fig. 1a in which two peaks precede that of the diether standard. The first, with the retention time of 7.0 min, occurs as either a positive or negative deflection. The second, always positive, is seen after 13.0 min, close to the ether lipid standard. These results are due to slight differences between the refractive indices of the solvent dissolving the sample and that found in the reference cell of the detector. Also occurring in all chromatograms is the peak associated with the standard compound (retention time = 13.5 min) which was added to act as a visual reference.

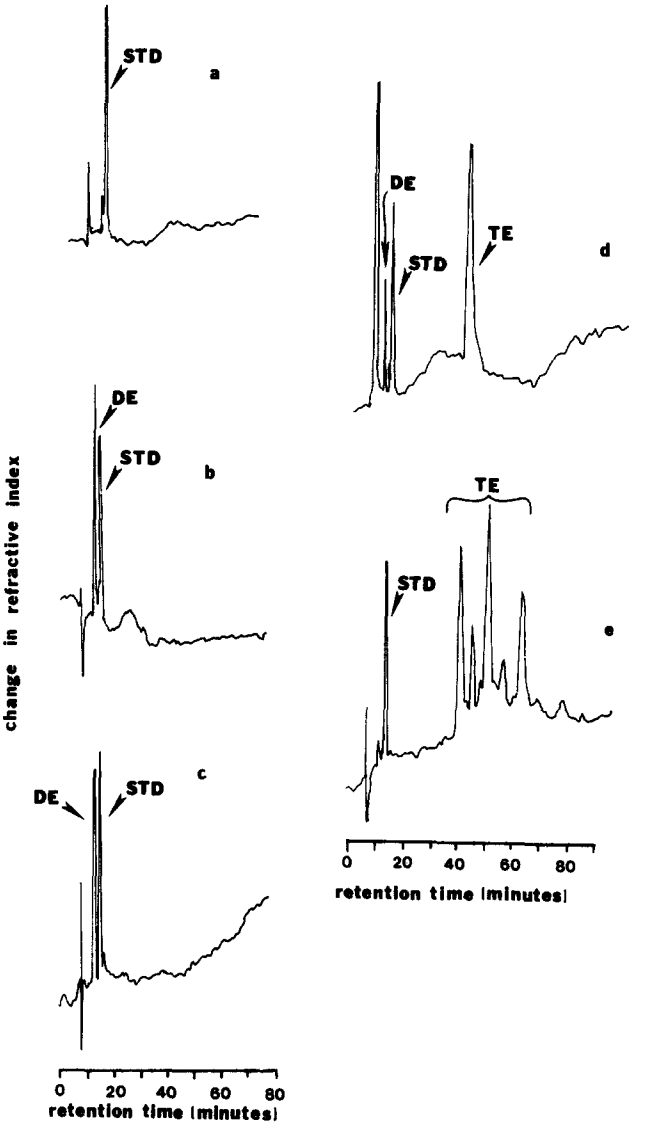

Fig. 1. HPLC traces illustrating separation of archaebacterial ether lipids: a) glycerol diether standard, b) *Halobacterium cutirubrum*, c) *Methanosarcina barkeri* strain Jolich, d) *Methanobacterium thermoautotrophicum* strain Hveragerdi and e) *Thermoplasma acidophilum*. Standard (6 nmol) was added to all samples. Chromatographic conditions were: flow 0.5 ml/min, chart speed 10 cm/hr, RI detector at R=1/4. Abbreviations used are archaebacterial diether (DE), archaebacterial tetraether (TE), and diether standard (STD).

The bacterial samples (Fig. 1b–d) include a peak from the diether fraction of the lipids with retention time of 11.3 min. In preliminary work, the same component was present in chromatograms of methanogenic bacterial lipids isolated by TLC prior to HPLC. This substance was previously positively identified as the diether by side chain analysis using chemical ionization gas–liquid chromatography–mass spectrometry (24). The tetraether component of *Methanobacterium thermoautotrophicum* has been previously identified as the bidiphytanylglycerol tetraether, 2,3,2',3'-tetra-O-dibiphytanyl-di-*sn*-glycerol,

based on mass spectral data (7). Fig. 1d also shows the tetraether lipid demonstrated by the peak eluting at 40.5 min. Separation of the acyclic, monocyclic, and bicyclic side chain-containing tetraether lipids from *Thermoplasma acidophilum* (25) is illustrated in Fig. 1e. This chromatogram shows a component eluting at 40.5 min, as in Fig. 1d, and two other major components with retention times of 50.5 min and 63.0 min, in addition to several other minor peaks. Since the retention time of the third component in Fig. 1d corresponds to the retention time of the first of the three major components in Fig. 1e, this component was tentatively identified as the acyclic side chain containing tetraether. This lipid is known to occur in both *Methanobacterium thermoautotrophicum* and *Thermoplasma acidophilum* (7, 15). This chromatogram (Fig. 1e) demonstrates the potential of this HPLC system to resolve the various tetraether lipids, differing by the number of cyclic rings in the side chains, present in this thermoacidophilic archaebacteria (16, 26).

Confirmation of ether structures was achieved in this study by the use of FT-IR spectroscopy. The IR spectra (**Fig. 2** and **Fig. 3**) of all the HPLC fractions designated as diethers and tetraethers show peaks corresponding to hydroxyl (3450 cm^{-1}), ether (1115 cm^{-1}), primary carbinol (1051 cm^{-1}), and alkyl (1377, 1463, 2856, 2926, and 2952 cm^{-1}). Spectra are nearly identical to previously reported data obtained for ether lipids from *Sulfolobus acidophilus* (26) and *Halobacterium cutirubrum* (27). Ester C=O absorbances at 1710 to 1750 cm^{-1} (Figs. 2 and 3) were absent in all spectra, indicating that the HPLC system had

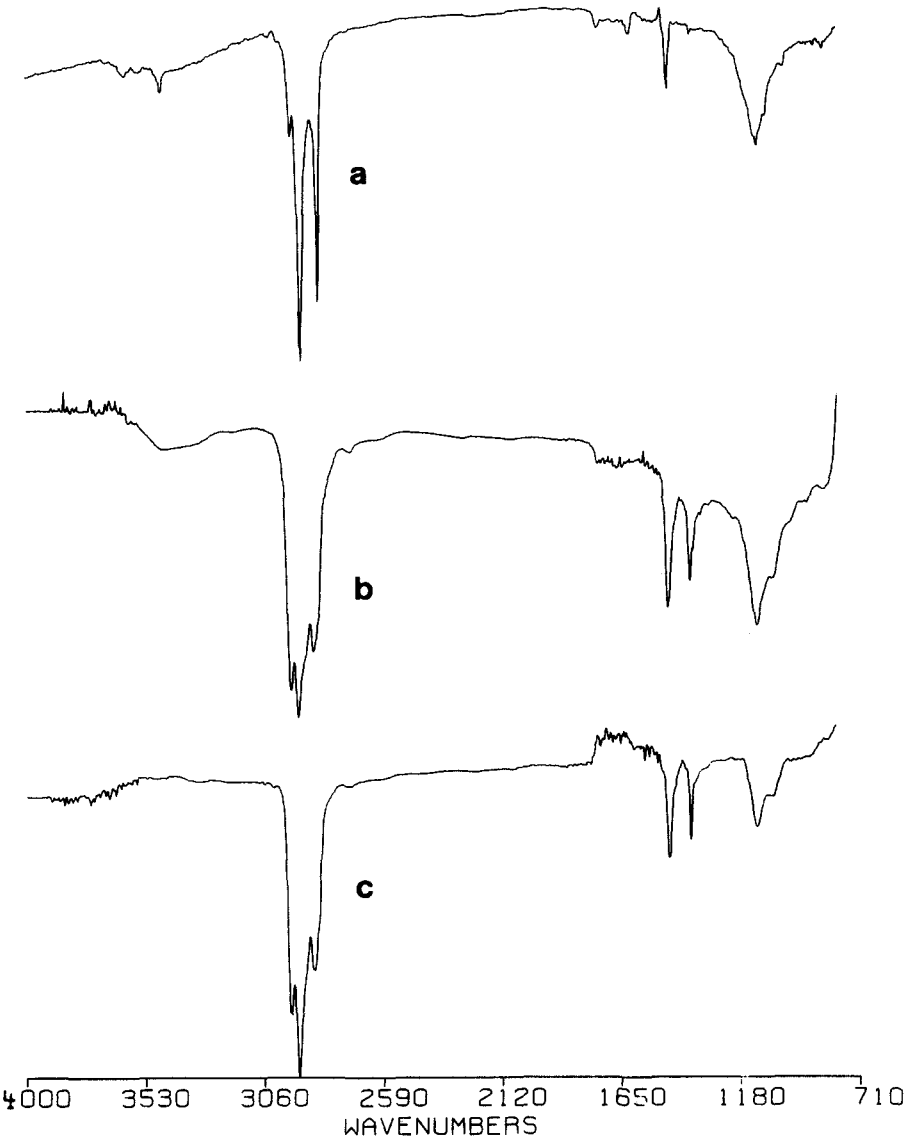

Fig. 2. FT-IR spectra (4000–800 cm^{-1}, transmittance mode) of a) glycerol diether standard, b) diether from *Methanosarcina barkeri*, and c) tetraether from *Methanobacterium thermoautotrophicum*.

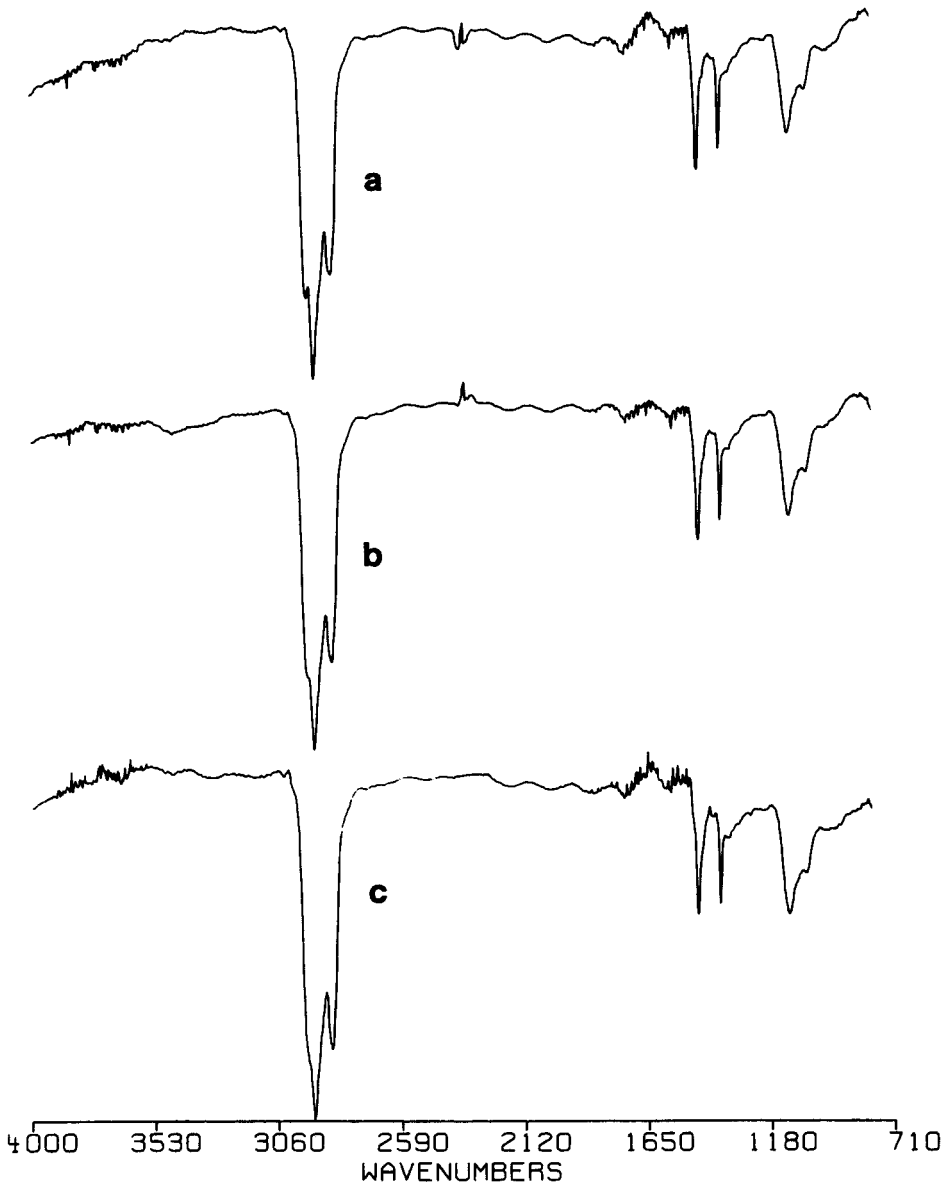

Fig. 3. FT-IR spectra (4000–800 cm^{-1}, transmittance mode) of tetraether (TE) fractions from *Thermoplasma acidophilum:* a) TE 1 from LC trace (see Fig. 1e), b) TE 2, and c) TE 3.

separated the ether lipids from any non-etheric material.

Closer examination of the alkyl absorption region at 2800–3000 cm^{-1} (**Fig. 4a–e**) shows that the spectra of the bacterial ether lipids are essentially similar (Fig. 4b–e), although subtle differences are apparent. The bands at *2926 and 2952 cm^{-1}* are the asymmetrical C–H stretching modes of CH_2 and CH_3 groups and the band centered at 2856 cm^{-1} is assigned to the symmetrical C–H stretching modes of CH_2 and CH_3 groups. The diether standard (1,2-di-O-hexydecyl-*rac*-glycerol, Fig. 4a) is readily distinguished and, consistent with the absence of methyl branches on the two side chains, shows lower absorbances in the 2950 cm^{-1} and 2850–2870 cm^{-1} regions.

Fourier self-deconvolution (FSD) has recently been applied by Griffiths and coworkers to the IR alkyl absorption bands of coal (28, 29). The elegant data obtained by those authors suggested the suitability of the FSD method for enhancing spectral information in ether lipid studies. A series of deconvoluted spectra, shown adjacent to the spectra prior to deconvolution, are presented in Fig. 4f–j. Whilst three bands were present in the spectra prior to FSD, the deconvolution process results in eight bands becoming apparent.

Band assignment for deconvoluted spectra of the alkyl region of coal has been previously attempted (29). Many of the bands noted in that study are present in our spectra

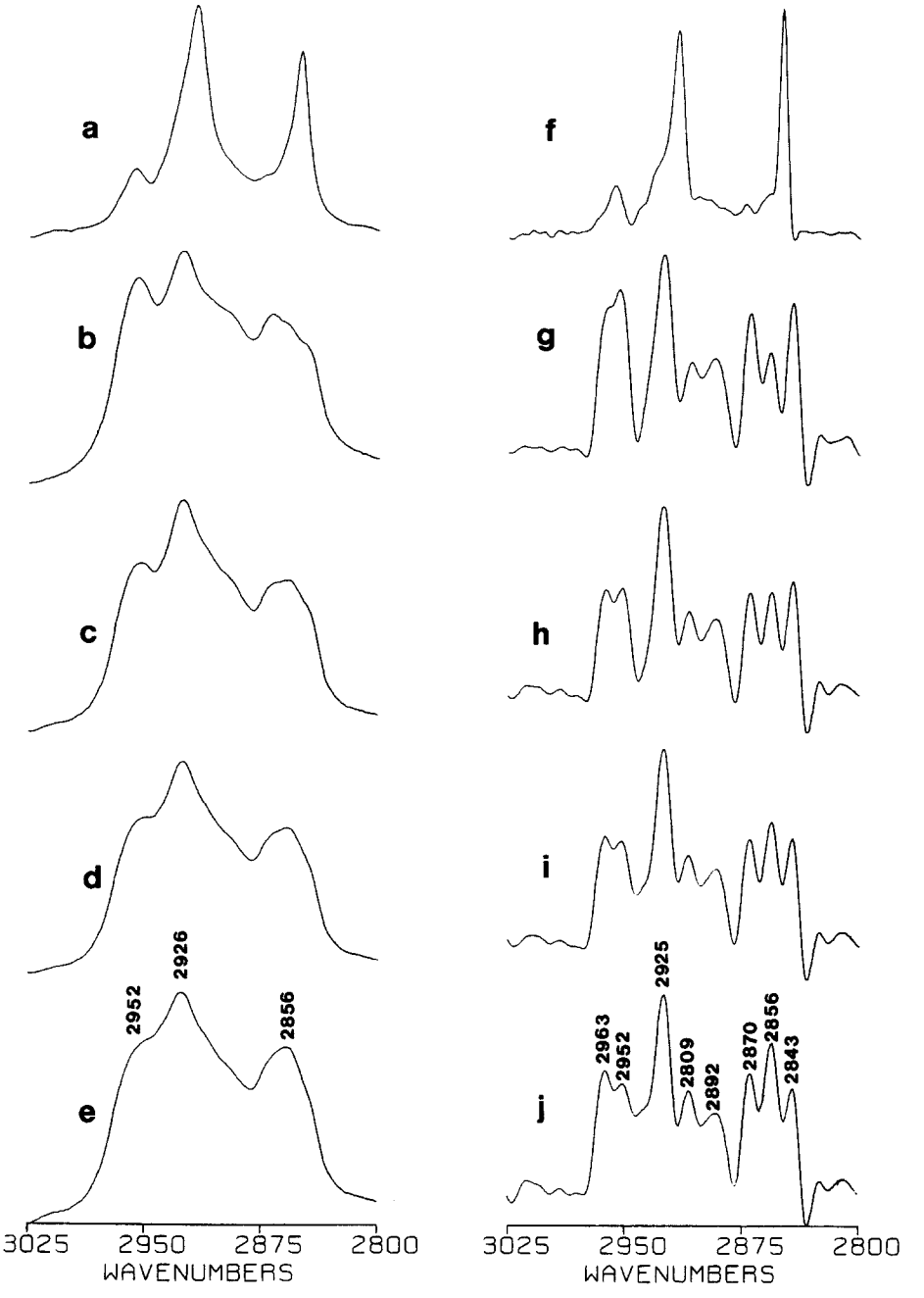

Fig. 4. Expanded FT-IR spectra of alkyl absorbance region (3025-2800 cm^{-1}) before (a–e) and after Fourier self-deconvolution (f–j): glycerol diether standard (a,f), *Methanosarcina barkeri* diether (b,g), *Thermoplasma tetraether* (TE) 1 (c,h), *Thermoplasma* TE 2 (d,i), and *Thermoplasma* TE 3 (e,j).

(Fig. 4f–j). Definitive assignment of all peaks in our spectra is not possible presently without the availability of a wide range of reference compounds. A number of trends in the spectra are apparent, however. For example, in the *Thermoplasma* tetraether fractions, the bands at 2963 and 2856 cm^{-1} become larger with increasing cyclization (Fig. 4, j>i>h). Such features may be useful in providing ether lipid structural information.

DISCUSSION

A reproducible method for the separation of diethers and tetraethers isolated from the archaebacterial phospholipids has been developed and is reported. This technique includes several improvements over a previous procedure (30). Separation of the diethers and tetraether lipids in the same chromatogram eliminates the need for

TLC. Refractive index detection of intact ether lipids after cleavage of the phosphate group to produce the alcohols, circumvents a lengthy derivatization step which introduced contamination. Ether lipids can be collected in fractions and analyzed for structural confirmation by FT-IR and can also be chemically cleaved and their side chains identified by gas-liquid chromatography-mass spectrometry (24).

Necessary precautions for a good chromatogram include maintenance of thermal stability of the column and detector, and keeping connections between the two insulated and to a minimum length and volume. In addition, dampening of pump pulses is essential when working at high sensitivity levels. Optimum separation was achieved with hexane-n-propanol 99:1 (v/v). Slight variations in this mobile phase ratio caused large changes in the relative retention time of the ether lipid solute.

One added advantage of this technique is the apparent ability to resolve a tetraether mixture from *T. acidophilum* into three major groups (Fig. 1e). These were recognizably different when analyzed by FSD of the IR spectra (Fig. 4f-j). Previously this has been achieved by TLC (18, 25, 31), which requires a larger amount of compound for visualization of the separated components. Mass spectral analysis of side chains from tetraether cleavage products is needed before structural assignments for the components resolved by liquid chromatography are made. This has been performed for the diether lipids.

The retention time for the first of the three major tetraether components corresponds to that present in the *Methanobacterium thermoautotrophicum* (Fig. 1d) sample. We have tentatively assigned this as the dibiphytanyl glycerol tetraether known to be a lipid component of *Methanobacterium thermoautotrophicum* and *Thermoplasma acidophilum* (15).

The availability of a structurally similar standard, 1,2-di-O-hexadecyl glycerol has enabled quantification of the diether to a 600-pmol detection limit. A similar standard is sought for tetraether for precise determination of the relative abundance of the ether lipid signatures. At present, an equal response factor for the diether and tetraether is assumed. The application of this technique to environmental samples should provide an accurate assessment of archaebacterial biomass in various microbial communities.

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REFERENCES

1. Zehnder, A. J. B. 1978. Ecology of methane. *In* Water Pollution Microbiology. R. Mitchell, editor. John Wiley and Sons, Inc., New York. 349-376.
2. Wolfe, R. S. 1971. Microbial formation of methane. *In* Advances in Microbial Physiology. A. H. Ross and J. F. Wilkinson, editors. Academic Press, Inc., New York. 107-146.
3. Zeikus, J. G. 1977. The biology of methanogenic bacteria. *Bacteriol. Rev.* **41:** 514-541.
4. Larsen, H. 1981. The family Halobacteriaceae. *In* The Prokaryotes. M. P. Starr, H. Stolp, H. G. Truper, A. Belows, and H. G. Schegel, editors. Springer Verlag, New York. 985-994.
5. Brock, T. D. 1981. Extreme thermophiles of the genus *Thermus* and *Sulfolobus*. *In* The Prokaryotes. M. P. Starr, H. Stolp, H. G. Trupel, A. Belows, and H. G. Schegel, editors. Springer Verlag, New York. 978-984.
6. Brock, T. D. 1978. The genus *Thermoplasma*. *In* Thermophilic Microorganisms and Life at High Temperature. Springer Verlag, New York. 92-116.
7. Tornabene, T. G., and T. A. Langworthy. 1979. Diphytanyl glycerol and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. *Science.* **203:** 51-53.
8. Kates, M. 1978. The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. *Prog. Chem. Fats Other Lipids.* **15:** 301-342.
9. Langworthy, T. A. 1977. Long-chain diglycerol tetraethers from *Thermoplasma acidophilum*. *Biochim. Biophys. Acta.* **487:** 37-50.
10. Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure in estuarine sediments. *FEMS Microbiol. Ecol.* **31:** 147-158.
11. Bobbie, R. J., and D. C. White. 1980. Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. *Appl. Environ. Microbiol.* **39:** 1212-1222.
12. Parkes, R. J., and J. Taylor. 1983. The relationship between fatty acid distributions and bacterial respiratory types in contemporary marine sediments. *Estuarine Coastal Shelf Sci.* **16:** 173-189.
13. Kates. M., L. S. Yengoyan, and P. S. Sastry. 1965. A diether analogue of phosphatidyl glycerolphosphate in *Halobacterium cutirubrum*. *Biochim. Biophys. Acta.* **98:** 252-268.
14. Kates, M. 1972. Ether lipids in extremely halophilic bacteria. *In* Ether Lipids: Chemistry and Biology. F. Snyder, editor. Academic Press, New York. 351-398.
15. Langworthy, T. A., T. G. Tornabene, and G. Holzer. 1981. Lipids of archaebacteria. *Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. C.* **3:** 228-244.
16. Langworthy, T. A. 1979. Membrane structure of thermoacidophilic bacteria. *In* Strategies of Microbial Life in Extreme Environments. M. Shilo, editor. Verlag Chemie, Weiheim, FRG. 417-432.
17. Langworthy, T. A. 1978. Microbial life at extreme pH values. *In* Microbial Life in Extreme Environments. D. J. Kushner, editor. Academic Press, New York. 279-315.
18. DeRosa, M., S. DeRosa, A. Gambacorta, and J. D. Bu'Lock. 1980. Structure of calditol, a new branched-chain nonitol, and of the derived tetraether lipids in thermophilic archaebacteria of the *Caldariella* group. *Phytochemistry.* **19:** 249-254.

19. Kates, M., and S. C. Kushwaha. 1978. Biochemistry of the lipid signatures of extremely halophilic bacteria. *In* Energetics and Structure of Halophilic Microorganisms. S. R. Copland and M. Ginzberg, editors. Elsevier-North Holland Publishing Co., Amsterdam. 461–480.
20. Balch, W. E., G. E. Fox, L. J. Magrum, G. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. *Biol. Rev.* **43:** 260–286.
21. Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. *J. Biol. Chem.* **238:** 2882–2886.
22. Kauppinen, J. K., D. J. Moffat, M. M. Mantsch, and D. G. Cameron. 1981. Fourier self-deconvolution: a method for resolving intrinsically overlapped bands. *Appl. Spectrosc.* **35:** 271–276.
23. Compton, D. A. C. 1983. Resolution enhancement using Fourier transforms. Part I: Fourier deconvolution. Spectral lines 5: 4–7. Reprinted as Nicolet Application Note 8311.
24. Mancuso, C. A., G. Odham, G. Westerdahl, J. S. Reeve, and D. C. White. 1985. C_{15}, C_{20}, and C_{25} isoprenoid homologues in glycerol diether phospholipids of methanogenic archaebacteria. *J. Lipid Res.* **26:** 1120–1125.
25. Langworthy, T. A. 1982. Lipids of *Thermoplasma*. *In* Methods in Enzymology. C. Parker, editor. Academic Press, Inc., New York. 396–406.
26. DeRosa, M., A. Gambacorta, and J. D. Bu'Lock. 1976. The *Caldariella* group of extreme thermoacidophilic bacteria. Direct comparison of lipids of *Sulfolobus, Thermoplasma* and the MT strains. *Phytochemistry.* **15:** 143–145.
27. H. N. M. Ross, M. D. Collins, B. J. Tindall, and W. D. Grant. 1981. A rapid procedure for the detection of archaebacterial lipids in halophilic bacteria. *J. Gen. Microbiol.* **123:** 75–80.
28. Chen. P., P. W-J. Yang, and P. R. Griffiths. 1985. Effect of preheating on chemical structure and infrared spectra of Yanzhou coal. *Fuel.* **64:** 307–312.
29. Wang, S-H., and P. R. Griffiths. 1985. Resolution enhancement of diffuse reflectance i.r. spectra of coals by Fourier self-deconvolution. 1. C–H stretching and bending modes. *Fuel.* **64:** 229–236.
30. Martz, R. F., D. L. Sebacher, and D. C. White. 1983. Biomass measurement of methane-forming bacteria in environmental samples. *J. Microbiol. Methods.* **1:** 53–61.
31. DeRosa, M., S. DeRosa, A. Gambacorta, L. Hinsle, and J. D. Bu'Lock. 1977. Chemical structure of the ether lipids of thermophilic acidophilic bacteria of the *Caldariella* group. *Phytochemistry.* **16:** 1961–1965.