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Characterisation of *Archaea* in Soils by Polar Lipid Analysis

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Summary

While phospholipid fatty acid (PLFA) profiling is a well-established method used for the determination of bacterial and eukaryotic organisms in soil ecology, phospholipid etherlipid (PLEL) analyses for the characterisation of *Archaea* is a rather new approach. Analyses of PLEL derived isoprenoid side chains by GC/MS provided a broad picture of the archaeal community in a mixed soil extract, as lipids previously identified in isolates belonging to the kingdoms *Eury-* and *Crenarchaeota* were covered. Furthermore, ether-linked isoprenoid hydrocarbons, which have not been detected in archaeal isolates and monomethyl-branched alkanes which have only been found in hyperthermophilic bacteria, were detected in these soil extracts. Monomethyl-branched alkanes were the most dominant ones and accounted for 43.4% of the total identified ether-linked hydrocarbons, followed by straight chain (unbranched) and isoprenoid hydrocarbons, which accounted for 34.6 and 15.5%, respectively.

Introduction

According to moleculargenetic analyses, soil ecosystems are colonised by members of all three domains of the biosphere *Bacteria*, *Eucarya* and *Archaea* [1]. To complete the data from molecular investigations, a complementary technique is used in soil ecology [2]. This approach is based on the phenotype of the individual organisms by analysing the side chains of microbial polar membrane lipids, the so-called phospholipid fatty acids (PLFA) [2]. This approach, however, does not consider archaeal organisms because archaeal ether lipids are not hydrolysed using the standard PLFA protocol. During PLFA analysis, mild alkaline and mild acidic hydrolyses are applied to cleave ester and amid bonds, respectively [2]. The polar lipids of *Archaea* are unique and readily distinguished from the acyl phospholipids of bacteria and eukaryotes containing PLFA [2].

Archaeal polar lipids, the so-called phospholipid ether lipids (PLEL), are composed of di- and tetraethers of glycerol (archaeols and caldarchaeols, respectively) or more complex polyols with side chains consisting of C_{15}, C_{20}, C_{25} or C_{40} isoprenoids [3, 4] which can only be liberated after ether cleavage with a strong acid such as HI (hydriodic acid).
In the present study, the applicability of the phospholipid ether lipid analysis for the determination of archaeal communities in soil ecosystems is demonstrated. A mixture of different lipid extracts of hydromorphic soils was used to cover a broad diversity of archaeal lipids.

Materials and Methods

Soil Samples

A lipid mixture was prepared from different soil phospholipid extracts derived from sites varying in climate and soil type showing mostly humic and anoxic properties. Samples were taken from 0–10 cm soil depth. 50% [v/v] of the phospholipids were from Chinese rice paddies [5], 25% of the phospholipids were from hydromorphic German soils [6] and a further 25% of the phospholipids were derived from flooded peat layers from "Donaumoos", Germany [7].

Determination of Phospholipid Ether Lipids (PLEL)

Lipids were extracted from a fresh soil sample equivalent to a dry weight of 50 g according to the BLIGH-DYER method as described in [8]. The resulting lipid material was fractionated into neutral lipids, glycolipids and phospho-(polar) lipids on a silica-bonded phase column (SPE-SI; Bond Elute, ANALYTICAL CHEM INTERNATIONAL, CA, USA) by elution with chloroform, acetone and methanol, respectively. An aliquot of the phospholipid fraction equivalent to 12.5 g dry matter (dm) was subjected to acidic hydrolysis with 1.5 ml of methanol-chloroform-37% HCl (10:1:1, v/v/v) at 60 °C for 12–16 h to remove the polar head groups for obtaining ether core lipids [5]. Ether core lipids were treated with 2 ml HI (hydriodic acid, 57%) for 18 h at 100 °C to cleave the ether bonds [9]. The resulting alkyl iodides were reduced to the corresponding hydrocarbons following the procedure of PANGANAMALA *et al*. [10], with 100 mg zinc powder in 2.0 ml glacial acetic acid at 100 °C for 18 h. After the extraction and washing procedures, the dried sample was dissolved in 100 μl internal standard (0.36 nM nonadecylacid methylester) and transferred into a GC vial.
PLEL derived isoprenoid hydrocarbons were analysed by gas chromatographic mass spectrometry (HEWLETT-PACKARD 5971A mass selective detector, combined with a 5890 series II gas chromatography system, USA) equipped with an HP 5 capillary column (50 m length, 0.2 mm internal diameter; coated with a cross-linked 5% phenylmethyl rubber phase with a film thickness of 0.3 μm) with He as a carrier gas (flow rate of 0.8 ml/min) by using programmed temperature increases after 2 min from the initial temperature of 70 to 130 °C at 20 °C/min followed by 130 to 320 °C at 4.0 °C/min; the temperature was held at 320 °C for 20 min. The total run time was 74.50 min. The detector and injector were maintained at 300 °C and 320 °C, respectively. Electronic impact ionisation was performed at a voltage of 70 eV using an electron multiplier voltage between 1800 and 2000 V. Acquisition of mass spectral data was enhanced when time-depending mass ranges were applied in the SCAN modus: Between 10 to 29.9 min during the GC run, masses from 50 to 350 amu were scanned (scan rate 1.5 s^{-1}), between 30.0 to 54.9 min, the mass range was from 50 to 550 amu (scan rate 1.5 s^{-1}) and from 55.0 min to the end of the run, masses ranging between 50 and 650 amu (scan rate 1.2 s^{-1}) were considered. The gas chromatographic separation led to total ion chromatograms (TIC) after the acquisition of the mass spectral data of individual compounds.

The identification of PLEL derived hydrocarbons was based on the comparison of retention time and mass spectral data with that obtained from the analysis of archaeal isolates [11] and mass spectral reference data [12]. Quantification was achieved using chromatography software (HP ChemStation; SOLVIT, CH) that considered the ratio between the compound of interest and the internal standard in terms of peak areas as well as the detector response for each, which was estimated experimentally using a mixed hydrocarbon standard (TRPH Mix, PROMOCHEM, USA). The detector response for a hydrocarbon, whose standard was not available, was given according to the standard used for the chemical structure that most resembled the compound of interest.

High Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionisation Mass Spectrometry (HPLC/APCI-MS)

An aliquot of the soil phospholipid mix (= 15 g dm) was subjected to acid hydrolysis to generate intact core lipids, which were analysed by a HPLC/MS device (FINNIGAN LCQ, Bremen, Germany) for the determination of their molecular weight and chemical structures. Separation was performed with a silicagel column (MERCK 250-4; length: 25 cm, internal diameter: 4 mm) by using an ethyl-acetate/hexane solvent gradient lasting for 45 min. Eluted core lipids were identified with positive ion atmospheric pressure chemical ionisation mass spectrometry (APCI-MS).

Results and Discussion

Characterisation of PLEL Derived Hydrocarbons in a Mixed Soil Extract

Isoprenoid hydrocarbons characteristic for *Eury-* and *Crenarchaeota* with chains ranging from C_{15} to C_{40} were identified with GC/MS after cleaving of the ether-linkages and subsequent reductive dehalogenation of polar lipids from the mixed soil extract (Fig. 1). The side chains of *Archaea*-specific PLEL consisted of the saturated isoprenoids i15:0, i20:0, i25:0, i40:0, i40:0-1cy, i40:0-2cy and i40:0-3cy (see Fig. 1 for explanation) and of the monounsaturated isoprenoid i20:1. Furthermore, the isoprenoids i19:0 (pristane) and i26:0 (tentatively identified as 10-ethyl-2,6,15,19-tetramethylicosane) were also detected, which eluted at 23.64 and 36.21 min, respectively. The PLEL derived isoprenoids i19:0 and i26:0 have neither been found in isolates nor in soil ecosystems before, hence their origin remains unclear. VINK *et al*. [12] detected free i26:0 in the Lower albian black shale in France, which is supposed to be derived from archaeal cells. Only the acidic hydrolysis procedure [5] was applicable to PLEL from soils rich in organic carbon to release the polar head group (GATTINGER, unpublished results), which resulted in the formation of the unsaturated isoprenoid i20:1 due to hydroxyarchaeol degradation [11]. Hence it can be assumed that i20:1 was derived from hydroxyarchaeol based PLEL rather than from PLEL with unsaturated side chains. Unsaturated PLEL have neither been detected in pure cultures nor in environmental samples so far (P. NICHOLS, personal communication), [14]. Apart from isoprenoid hydrocarbons, straight chain and monomethyl-branched hydrocarbons (e.g. iso-, anteiso-branching) were also determined at even higher concentrations than isoprenoids in the investigated soil extracts (Fig. 2). These side chains were the most dominant ones and accounted for 43.4% of the total identified ether-linked hydrocarbons, followed by straight chain (unbranched) and isoprenoid hydrocarbons, which accounted for 34.6 and 15.5%, respectively. Some of these hydrocarbons have been found as side chains in the polar lipids from hyperthermophilic members of the genus *Thermotoga* [15] and *Aquifex* [16] as well as in *Thermodesulfobacterium commune* [17]. The above findings, along with the detection of

structurally diverse tetraether membrane lipids in peat bogs, ancient sediments and marine environments [18] lead to the assumption of a ubiquitous presence of low-temperature relatives of hyperthermophiles.

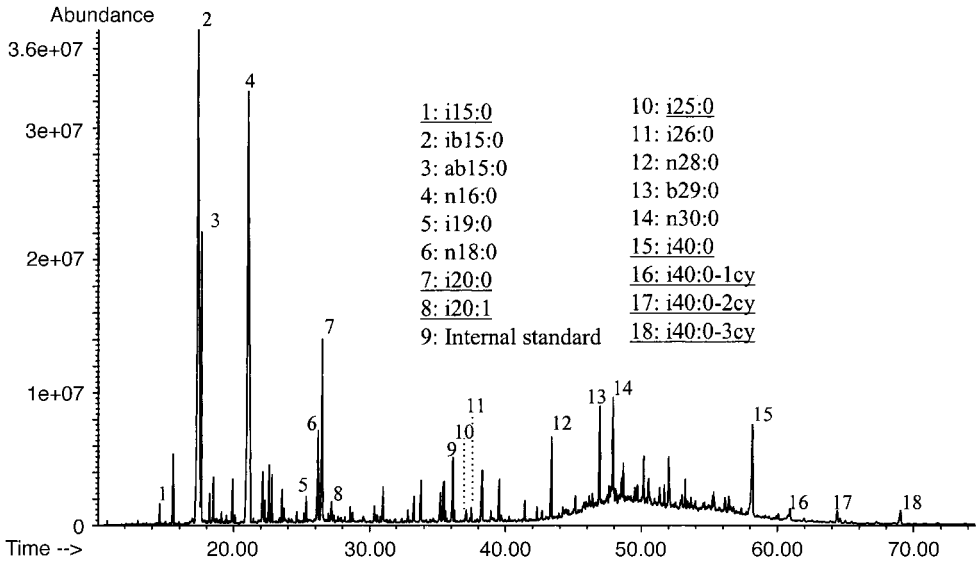

Fig. 1. Total ion chromatogram (TIC) of ether-linked hydrocarbons from soil extracts obtained by GC/MS
Archaeal markers are underlined. The following functional groups of hydrocarbons were detected: i: isoprenoid-branching, ib: monomethyl-branching in iso-position, ab: mono-methyl-branching in anteiso-position, n: *n*-alkane, b: monomethyl-branching, i20:0 indicates a saturated and i20:1 an unsaturated hydrocarbon chain; i40:0-1cy is a C_{40} isoprenoid with 1 cyclo-pentane ring.

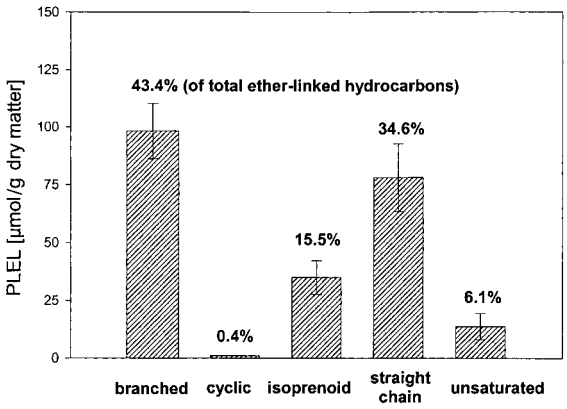

Fig. 2. Absolute and relative concentrations of ether-linked hydrocarbons from a mixed soil extract
The error bars denote standard deviation.

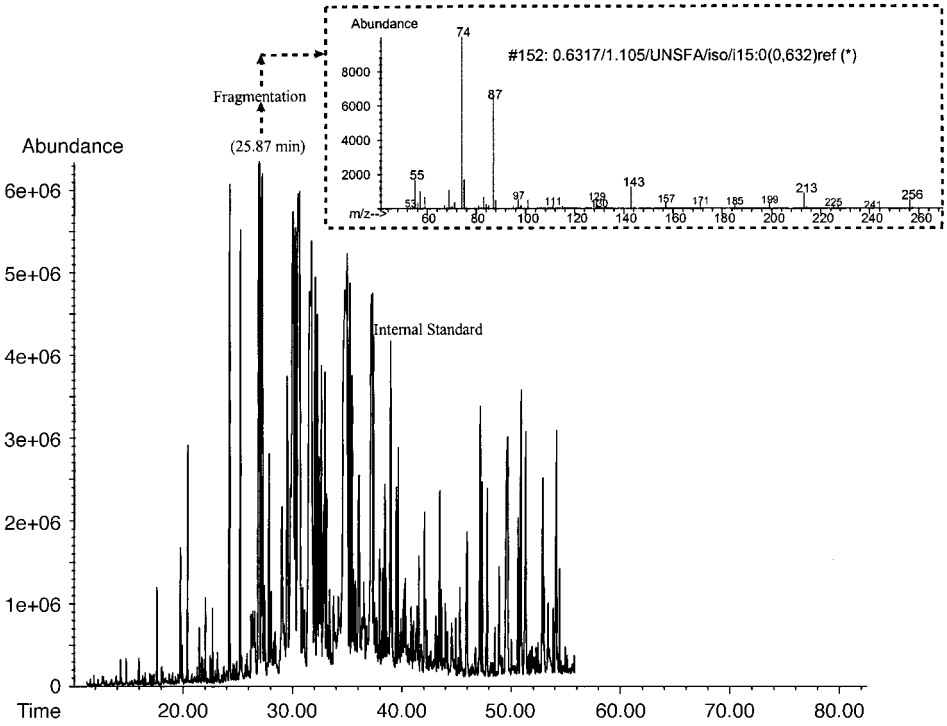

Fig. 3. Total ion chromatogram (TIC) of soil phospholipids after mild acid hydrolysis and reductive dehalogenation

The peaks were exclusively identified as non-esterlinked fatty acid methylesters (FAME) such as the one eluted at 25.87 min. FAME were also liberated when mild alkaline hydrolysis and reductive dehalogenation were applied to the same phospholipid extract (data not shown).

Specificity of Hydrocarbon Side Chains

To exclude that hydrocarbon side chains detected in Fig. 1 derived from lipids other than heavily hydrolysable ether lipids, several experiments were carried out. *(i)* It was possible to confirm by direct injection of an aliquot equivalent to 5 g dry matter (dm) of the phospholipid fraction that there were no free hydrocarbon compounds in the polar fraction even after two years of storage at – 18 °C (Data not shown). *(ii)* Aliquots of the mixed phospholipid fraction (= 5 g dm) were subjected to mild alkaline or to mild acidic hydrolysis [8] and both were treated with the reductive dehalogenation procedure [10] to determine whether linkages other than ether linkages can also liberate hydrocarbon side chains. The resulting total ion chromatogram (TIC) in Fig. 3 illustrated that no hydrocarbon side chains other than homologues for fatty acid methyl esters (FAME) were detected after applying the two different mild hydrolysis procedures. For example, at a retention time of 25.87 min, a fragmentation pattern was obtained, which matched with the entry *152* (iso-branched FAME with 15 C atoms) of the FAME spectra library

established in our laboratory for PLFA analyses [2]. These experiments confirmed that the ether lipids are the only source of released unpolar hydrocarbon chains from soil phospholipids when strong hydrolysation conditions and unpolar extractants were applied. For the further characterisation of the intact molecules, aliquots of core lipids from the mixed soil extracts were analysed by HPLC/MS and could be separated into approx. 80 peaks (Fig. 4). Ten of these compounds were further identified by additional ionisation (ms^2), along with comparison of core lipids isolated from *Methanosarcina barkeri* and *Sulfolobus solfataricus* (Tab. 1).

After 5.85 min, a C_{15}-hydroxymonoether was eluted, which could be identified according to two fragments showing the loss of H_2O in alkyl and in the glycerol moiety. The compound at retention time 6.52 min is considered to be a C_{16}-diether after comparison with a reference compound and the detection of the $[M+H]^+$ fragment with *m/z* 541. C_{20}–C_{20} archaeol was eluted after 11.57 min, showing the typical monoether fragment in the ms^2 modus (Fig. 3). Cyclic and acyclic caldarchaeols were obtained after 23.89 min, which did not show fragmentation after additional ionisation. However, the identification was possible because molecular ions were obtained, and their retention times were similar to the caldarchaeols from *Sulfolobus solfataricus*.

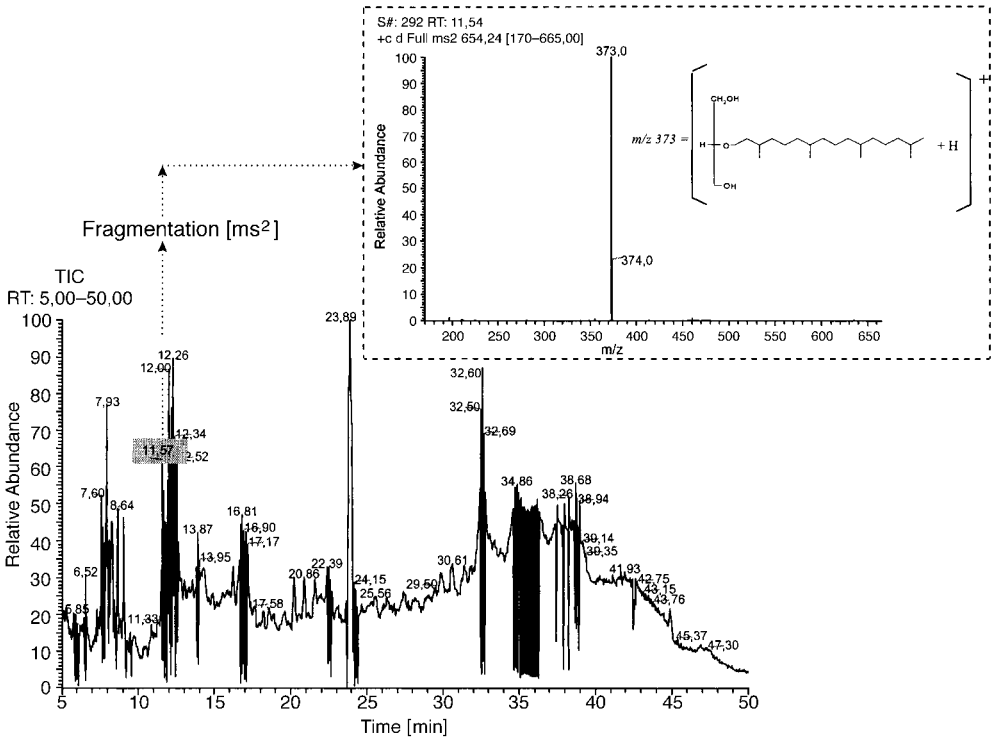

Fig. 4. TIC of core lipids from the polar fraction of soil lipids obtained by LC/MS
C_{20}–C_{20} archaeol was eluted after 11.57 min, showing the typical monoether fragment (*m/z* 373) in the ms^2 modus.

Tab. 1. List of identified ether lipids obtained by HPLC/MS

Time [min]	Chemical structure	Molecular Ion [*m*/*z*] [1)]	Fragment ions obtained by ms^2 [*m*/*z*] [1)]	Side chain	Relative abundance
5.85	Hydroxypentadecyl glycerol	321	285, 303	C_{15}	20
6.52	Dihexadecyl glycerol	541	317	C_{16}	31
11.57	Archaeol	653	373	C_{20}	61
12.52	Pentacosanyltricosanyl glycerol	863	—	C_{25} & C_{30}	64
23.89	Caldarcheol	1302	—	C_{40}	81
23.95	Caldarchaeol-1cy	1300	—	C_{40}	9
24.04	Caldarchaeol-2cy	1298	—	C_{40}	8
24.19	Caldarchaeol-3cy	1296	—	C_{40}	28
24.24	Caldarchaeol-4cy	1294	—	C_{40}	18

[1)] mass-charge ratio (= *m*/*z*) of molecular and other fragment ions, which is given as $[M+H^+]$ or $[M+H^+]$–x (x = mass of the lost functional group)

The results show that polar lipid analysis enables a cultivation-independent detection of archaeal marker molecules in complex environments such as soils and serve as a complementary tool to genetic fingerprinting methods. Both approaches, the determination of the ether-linked isoprenoids with GC/MS as well as the estimation of ether core lipids with HPLC/MS offer a great potential to study not only the composition of archaeal communities in soil ecosystems, but also the function of these communities when combined with isotopically labelled substrates [19].

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