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Composition of the lipids of *Nanoarchaeum equitans* and their origin from its host *Ignicoccus* sp. strain KIN4/I

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Abstract The contents and nature of the membrane lipids of *Nanoarchaeum equitans* and *Ignicoccus* sp. strain KIN4/I, grown at 90°C, and *Ignicoccus* sp. strain KIN4/I, cultivated at its lowest and highest growth temperatures (75°C and 95°C) were analyzed. Both organisms contained very simple and qualitatively identical assemblages of glycerol ether lipids, showing only differences in the amounts of certain components. LC–MS analyses of the total lipid extracts revealed that archaeol and caldarchaeol were the main core lipids. The predominant polar headgroups consisted of one or more sugar residues attached either directly to the core lipid or via a phosphate group. GC–MS analyses of hydrolyzed total lipid extracts revealed that the co-culture of *N. equitans* and *Ignicoccus* sp. strain KIN4/I, as well as *Ignicoccus* sp. strain KIN4/I grown at 90°C, contained phytane and biphytane in a ratio of approximately 4:1. Purified *N. equitans* cells and *Ignicoccus* sp. strain KIN4/I cultivated at 75°C and 95°C had a phytane to biphytane ratio of 10:1. Sugar residues were mainly mannose and small amounts of glucose. Consistent ^{13}C fractionation patterns of isoprenoid chains of *N. equitans* and its host indicated that the *N. equitans* lipids were synthesized in the host cells.

Keywords *Nanoarchaeum equitans* · *Ignicoccus* · Lipid analysis · Intact polar lipids · Archaeol · Caldarchaeol · Glycerol dibiphytanyl glycerol tetraether

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Introduction

Hyperthermophilic microorganisms having an optimal growth temperature between 80°C and 105°C (Stetter 1989) exist in the domains of Archaea and Bacteria, where they cluster around the root in 16S rRNA-based phylogenetic trees (Stetter 1995). Within the Archaea, three phyla have been identified, namely the Crenarchaeota, Euryarchaeota and "Korarchaeota" (Woese et al. 1990; Barns et al. 1996). However, members of the "Korarchaeota" have not been isolated or identified and are only known through their 16S rDNA sequences (Barns et al. 1996).

Recently, a novel member of the Archaea was isolated, *Nanoarchaeum equitans*, which represents a new phylum, the Nanoarchaeota (Huber et al. 2002). *N. equitans* is a tiny coccus (Ø 400 nm) growing on the surface of a newly discovered *Ignicoccus* species, *Ignicoccus* sp. strain KIN4/I. It was isolated from a hydrothermal system at the Kolbeinsey Ridge, north of Iceland. *N. equitans* can only be cultivated in co-culture with *Ignicoccus* sp. strain KIN4/I under strict anoxic conditions at temperatures between 75°C and 98°C (Huber et al. 2003a). With a genome size of only 490,885 bp, it has one of the smallest genomes known so far. Analysis of its gene content revealed a lack of most biosynthetic pathways, including lipid biosynthesis (Waters et al. 2003). Phylogenetic analyses confirmed that the Nanoarchaeota branch very deeply within the archaeal domain (Huber et al. 2002). Their 16S rRNA genes exhibit base exchanges even at former "universal" sequence signatures previously thought to be identical for all organisms (Hohn et al. 2002). Although very little is known about the physiology of *N. equitans*, recent studies suggested that it may be a parasite for *Ignicoccus* sp. strain KIN4/I (Jahn 2003).

The composition of archaeal cell membranes is one of their most characteristic and diagnostic features. They contain lipids with isoprenoidal hydrocarbon chains linked by ether bonds to glycerol. In contrast, the membranes of Bacteria and Eukarya contain polymethylenic hydrocarbon chains in fatty acids that are, in the main, linked to glycerol via ester bonds. Notable exceptions to this are membranes of the *Thermotogales*, *Aquificales* and some sulfate-reducing bacteria; they possess ether lipids or mixed ether/ester lipids that contain exclusively polymethylenic (i.e., non-isoprenoid) hydrocarbon chains (*Aquificales* and sulfate-reducing bacteria) (Huber et al. 1992; Rütters et al. 2001) or 15,16-dimethyl-30-glyceroloxytriacontanoic acids (*Thermotogales*) (De Rosa et al. 1988; Jahnke et al. 2001). The diether lipids of Archaea have chains that derive from isoprenoid precursors with 15, 20, 25 or 30 carbon atoms and, ultimately, from acetyl-CoA via the mevalonate pathway (Langworthy 1985; De Rosa and Gambacorta 1986; Lange et al. 2000). C_{40} (biphytane) chains also occur but these form by linking opposed geranylgeranyl (C_{20}) chains in preformed diethers yielding tetraethers. In the case of methanogens and possibly other Archaea, saturation of the geranylgeranyl chains is sometimes incomplete or there are other modifications resulting in isoprenoids with hydroxyl groups or combinations of pentacyclic or hexacyclic rings (Koga et al. 1993). In addition, all glycerol ethers of Archaea contain 2,3-*sn*-glycerol, whereas the glycerol in lipids of Eukarya and Bacteria exhibits a 1,2-*sn* stereochemistry (Kates 1978; De Rosa et al. 1991).

Since no lipid biosynthesis genes have been identified in *N. equitans* (Waters et al. 2003), the analysis of its membrane in comparison to its host should give insights into the origin of *N. equitans* lipids and whether or not either organism contains specific biomarkers. In this study, we report the lipid analysis from purified *N. equitans* cells, the co-culture of *N. equitans* and *Ignicoccus* sp. strain KIN4/I, as well as from a pure culture of *Ignicoccus* sp. strain KIN4/I, cultivated at its minimal, optimal and maximal growth temperatures. Differences between the inner (cytoplasmic) membrane and the outer membrane of *Ignicoccus* sp. strain KIN4/I were examined (Rachel et al. 2002).

Material and methods

Culture conditions and purification of *N. equitans* cells

Pure cultures of *Ignicoccus* sp. strain KIN4/I and the co-culture of *N. equitans* and *Ignicoccus* sp. strain KIN4/I were grown in 1/2 SME medium, pH 5.5 (Huber et al. 2003b), with elemental sulfur as electron acceptor and a gas phase of H_2/CO_2 (80/20 v/v) at 90°C in a 300-l enamel-protected fermenter. In addition, *Ignicoccus* sp. strain KIN4/I was cultivated at 75°C and 95°C. To obtain high amounts of free *N. equitans* cells, it was necessary for the co-culture to be purged with H_2/CO_2 at very high flow rates (15–20 l min^{-1}) after the *Ignicoccus* sp. strain KIN4/I cells reached a cell density of about 10^6 cells ml^{-1} (Huber et al. 2002). *N. equitans* cells were separated from their host cells by differential centrifugation (Huber et al. 2002). All cell masses were freeze-dried for transportation and lipid extraction.

Samples and lipid extraction

Lipids were extracted from the purified *N. equitans* fraction, the co-culture of *N. equitans* and its host, and the pure *Ignicoccus* sp. strain KIN4/I culture, all cultivated at 90°C. In addition *Ignicoccus* sp. strain KIN4/I cells grown at 77°C and 95°C were analyzed. About 0.1 g freeze-dried cells (corresponding to about 0.5 g wet weight) were used for lipid extraction by applying a single-phase modification of the Bligh and Dyer procedure (Bligh and Dyer 1959, modified by Kates 1986). One unavoidable consequence of the culture and harvesting methods was a variable, and sometimes large, content of free elemental sulfur in the samples. This sulfur was removed by one, or more, passages of the total lipid extract over a bed of activated copper (Jahnke et al. 2001) but its presence reduced the accuracy of gravimetric analysis of the total lipid extract. Finally, the total lipid extract was dried under nitrogen, weighed and stored at 4°C.

Following total lipid extract isolation, the insoluble residues were re-extracted with 10:1:1 MeOH/$CHCl_3$/HCl 37% at 95°C overnight. This procedure, which hydrolyzes polar head groups or chains in acid-sensitive cores such as hydroxyarchaeols (Koga et al. 1998), nevertheless allows the presence of lipids that are strongly bound to membranes to be determined, although they cannot be identified precisely (Nishihara and Koga 1987). This resulted in release of a further 10–20% of material, the acid-extractable lipids (AEL), which were analyzed separately for hydrocarbon chain composition.

Preparation of the hydrocarbon chains for GC, GC–MS and GC–IRMS analyses

This was accomplished using two methods. Initially, about 1 mg of the total lipid extract was treated with an excess of BBr_3 in dry dichloromethane for 2 h at 90°C under an atmosphere of dry nitrogen. This afforded a mixture of alkyl bromides (Summons et al. 1998) which were subsequently converted to the corresponding hydrocarbons by reaction with a slight excess of "Superhydride" for 2 h at 70°C in dioxan, also under dry nitrogen. After quenching the reaction, the products were extracted into dichloromethane and the hydrocarbons were purified by passing the mixture through a silica gel column and eluting with hexane. The hexane eluate was carefully reduced under a nitrogen stream to a final volume of 150 µl for analysis by gas

chromatography (GC), GC–mass spectrometry (GC–MS) and GC–combustion-isotope ratio mass spectrometry (GC–C-IRMS). Hydrocarbon samples prepared using this method contained small, but variable, amounts of phytenes and biphytenes as artifacts formed by HBr elimination. Accordingly, ether cleavage products were then obtained on a further aliquot of the total lipid extract and on aliquots of the AEL using hydriodic acid (57%) followed by reduction with superhydride. To these latter samples, an aliquot of 3-methylhenicosane (ai-C_{22}) was added as an internal standard for semi-quantitative analysis of the abundances of C_{20} and C_{40} hydrocarbon chains by GC-FID.

Preparation of the carbohydrates of the total lipid extract

Acetal linkages between the sugars and the lipid residues were cleaved by mild acid hydrolysis. Approximately 0.5 mg of the dried total lipid extract was incubated overnight with 1 M hydrochloric acid, and the sample dried under a stream of nitrogen. Sugar derivatives were prepared using *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (1:1 in pyridine) for 3 h at 80°C. The samples were then analyzed by GC–MS.

GC–MS analysis

Qualitative analyses of the hydrocarbons, derivatized total lipid extract, and sugars were carried out using a HP 6890 gas chromatograph interfaced to an HP 5973 mass-selective detector. The GC was equipped with a temperature-programmable, septum-less injector system and a Chrompack CP-Sil5CB column (60 m×0.32 mm, 0.25-μm film) with the samples injected while the oven was held constant at 40°C and the injector programmed to 320°C over 2 min. The oven then followed a temperature program of 10°C min^{-1} to 120°C and 4°C min^{-1} to 320°C where it was held constant for 50 min (Jahnke et al. 2001, modified). The sugars were identified by comparing their mass spectra and retention times to those of the following sugar standards: mannose, glucose, galactose, arabinose, rhamnose, and xylose. Each sugar showed two peaks with different retention times, which represent their α-conformation and *β*-conformation isomers. The ratio of the two conformations does not reflect the state of the sugars in the sample, but is a consequence of the derivatization conditions.

LC–MS analysis

Complex lipids were analyzed as described in Sturt et al. (2004). Briefly, a LiChrospher Diol column (125×2 mm^2, 5 μm (Alltech Associates, Deerfield, Ill., USA) was used at 30°C in a column oven of a ThermoFinnigan Surveyor HPLC system (ThermoFinnigan, San Jose, Calif., USA) . The following linear gradient was applied with a flow rate of 0.2 ml min^{-1}: 100% A to 35% A:65% B over 45 min, hold for 20 min, then back to 100% A for 1 h to re-equilibrate the column for the next run, where A = 79:20:0.12:0.04 of hexane/2-propanol/formic acid/14.8 M NH_{3aq} and B = 88:10:0.12:0.04 of 2-propanol/water/formic acid/14.8 MN H_{3aq}. Typical amounts injected were 100 pg for pure standards and up to 60 μg for microbial total lipid extracts.

MS^n experiments were performed using a ThermoFinnigan LCQ Deca XP ion-trap mass spectrometer with an electrospray interface (ESI). ESI settings were as follows: capillary temperature 200°C, capillary voltage ±11 V, sheath gas flow 40 (arbitrary units), spray voltage was set at ±5 kV. During routine analyses, the mass spectrometer was configured to run "data dependent ion tree" experiments in which the base peak from each full scan (typically 500–2,000 *m*/*z*) was fragmented up to MS^3. Separate runs for positive and negative ion modes yielded complimentary structural information. Direct infusion of microbial total lipid extracts into the electrospray source via a syringe pump allowed for a greater depth of MS^n analysis, while the collision energy was controlled manually to optimize fragmentation.

Some high-resolution LC–MS analyses were conducted using a Waters Q-TOF Micro instrument in positive ion electrospray mode and with the LC conditions specified above. In particular, the molecular species of a putative phospholipid, glycosylphosphoarchaeol, was analyzed in *Ignicoccus* sp. strain KIN4/I (90°C) giving *m*/*z* 895.7001 ($C_{49}H_{100}O_{11}P$ requires 895.7003 vs $C_{49}H_{99}O_{11}S$ requires 895.6908). In addition, modeling of the isotope cluster of the same molecular species in four analyses of *Ignicoccus* sp. strain KIN4/I and two analyses of *N. equitans* was consistent with the presence of a phosphorous atom and inconsistent with the presence of a sulfur atom in the relative abundance of M + 2 ions.

GC–C-IRMS

Compound-specific isotope analysis was conducted using a Thermo-Finnigan Trace GC interfaced to a Thermo-Finnigan MAT Delta XP isotope ratio mass spectrometer via a combustion interface. The GC was equipped with a programmable split/splitless injector system operated in the splitless mode. The column was a J&W DB-5MS (30 m×0.25 mm, 0.25-μm film) and operated at constant flow of 2 ml min^{-1}. The oven conditions for analysis of alkanes were 60–150°C programmed at 10°C min^{-1}, then at 4°C min^{-1} to 310°C, held isothermal for 30 min. Isotopic compositions were determined by comparison with co-injected standards comprising perdeuterated C_{24} and C_{32} *n*-alkanes. The isotopic compositions of all peaks, including the ai-C_{22} internal standard were reproducible to ±0.1‰.

Table 1 Amounts (%/μg per mg total lipid extract/μg per mg biomass) and isotopic compositions of hydrocarbons obtained from the total lipid extracts of the different samples. The weight of biomass was estimated by subtracting the weight of elemental sulfur removed from the Bligh–Dyer total lipid extract from the weight of freeze-dried cells used for lipid extraction. Hydrocarbon data represent the sum of products from the Bligh–Dyer and acid-modified extracts. $\delta^{13}C$ values are reported relative to VPDB. Purified *Nanoarchaeum equitans* cells were derived from the co-culture experiment

Sample	Phytane	$\delta^{13}C$	Biphytane	$\delta^{13}C$
Purified *N. equitans* cells, cultivated at 90°C	89.4/79.6/12.79	−16.0	10.6/9.5/1.52	−16.3
Co-culture of *N. equitans* and *Ignicoccus* sp. strain KIN4/I, cultivated at 90°C	80.3/17.6/1.83	−16.1	19.7/4.3/0.45	−16.0
Ignicoccus sp. strain KIN4/I, cultivated at 77°C	89.3/9.9/0.31	−15.2	10.7/1.2/0.04	−15.2
Ignicoccus sp. strain KIN4/I, cultivated at 90°C	80.7/25.9/0.48	−42.1	19.3/6.2/0.12	−43.6
Ignicoccus sp. strain KIN4/I, cultivated at 95°C	90.5/2.1/0.04	−18.6	9.5/0.2/0.005	−19.8

Results

Yields of lipid from *N. equitans* and *Ignicoccus* sp. strain KIN4/I

Lipid contents of the different cultures were calculated by difference in mass of lipid extracts before and after removal of elemental sulfur. As expected, due to the small cell size, the highest lipid yield was in the cells of *N. equitans*, which contained approximately 16%. Of this, approximately 3% was recovered from the insoluble residues by extraction under strong acids conditions. The *Ignicoccus* sp. strain KIN4/I–*N. equitans* co-culture contained approximately 6.3% lipids, with about 2.3% of this recovered using acid extraction. Cultures of *Ignicoccus* sp. strain KIN4/I grown at 77, 90 and 95°C all contained very low lipid contents, with 3.2, 1.9 and 2.4% lipid, respectively, after correction for the elemental sulfur contents. Of this lipid, an average of 0.5% was recovered from the acid extracts.

Hydrocarbon chains prepared from the lipid fractions

The purified *N. equitans* cells, the co-culture of *N. equitans* and its host, as well as the *Ignicoccus* sp. strain KIN4/I cells exhibited no difference in the qualitative composition of hydrocarbons. All cultures contained phytane as the main hydrocarbon, with a retention time of 29.18 min, and a smaller amount of biphytane, with a retention time of 60.66 min, and with mass spectra identical to the same hydrocarbons released from other Archaea. Traces of phytenes and biphytenes (monounsaturated phytane/biphytane, retention times ∽29.6 min and ∽61.3 min) were detectable in the hydrocarbons released by the boron tribromide procedure but absent from those released by hydriodic acid, suggesting these were artifacts.

By comparing the relative amounts of the hydrocarbons, major differences in the quantitative composition became evident, as shown in Table 1. The co-culture of *N. equitans* with *Ignicoccus* sp. strain KIN4/I contained approximately 20% biphytane, while the purified *N. equitans* cell fraction contained only 10%. *Ignicoccus* sp. strain KIN4/I grown at its optimal temperature of 90°C showed a higher proportion of biphytane (20%) than when cultivated at its minimal and maximal growth temperatures (77°C and 95°C). These samples contained only about 10% biphytane.

Results of this analysis conducted on the fractions of the lipid that were rendered extractable in the presence of hot hydrochloric acid yielded hydrocarbon mixtures dominated by biphytane in all samples. The ratio of phytane to biphytane varied from 2:3 to 1:3 (individual data not shown); Table 1 reflects the results of the combined Bligh–Dyer lipids and AEL. An analysis of a small aliquot of the total lipid extract which had been derivatized with BSFTA/pyridine showed only archaeol and that there were no hydroxyarchaeols present.

In addition, the ^{13}C contents of the hydrocarbons in the total lipid extracts of the co-culture and of purified *N. equitans* cells from the same co-culture were determined. As shown in Table 1, essentially the same $\delta^{13}C$ values for phytane and biphytane were measured in each sample analyzed. $\delta^{13}C$ values for each cultivation experiment were different as a consequence of using different and multiple CO_2 tanks for the various incubations.

Intact polar lipids

The LC–MS analyses of the intact polar lipids (IPL) in the total lipid extracts of all samples revealed 2,3-di-*O*-phytanyl glycerol diether (archaeol) and glycerol dibiphytanyl glycerol tetraether (GDGT or caldarchaeol) core lipids (structures shown in Fig. 1). Figure 2 shows ion chromatograms constructed from the most intense

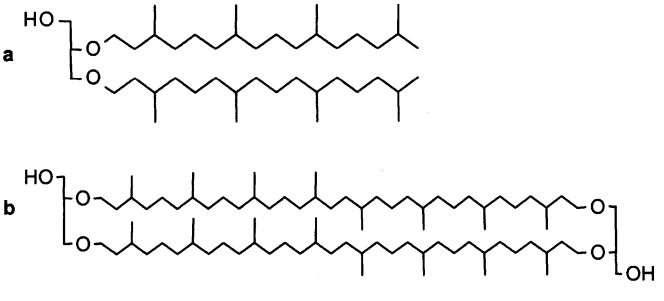

Fig. 1 Structures of archaeal core lipids. **a** archaeol; **b** glycerol dibiphytanyl glycerol tetraether or caldarchaeol

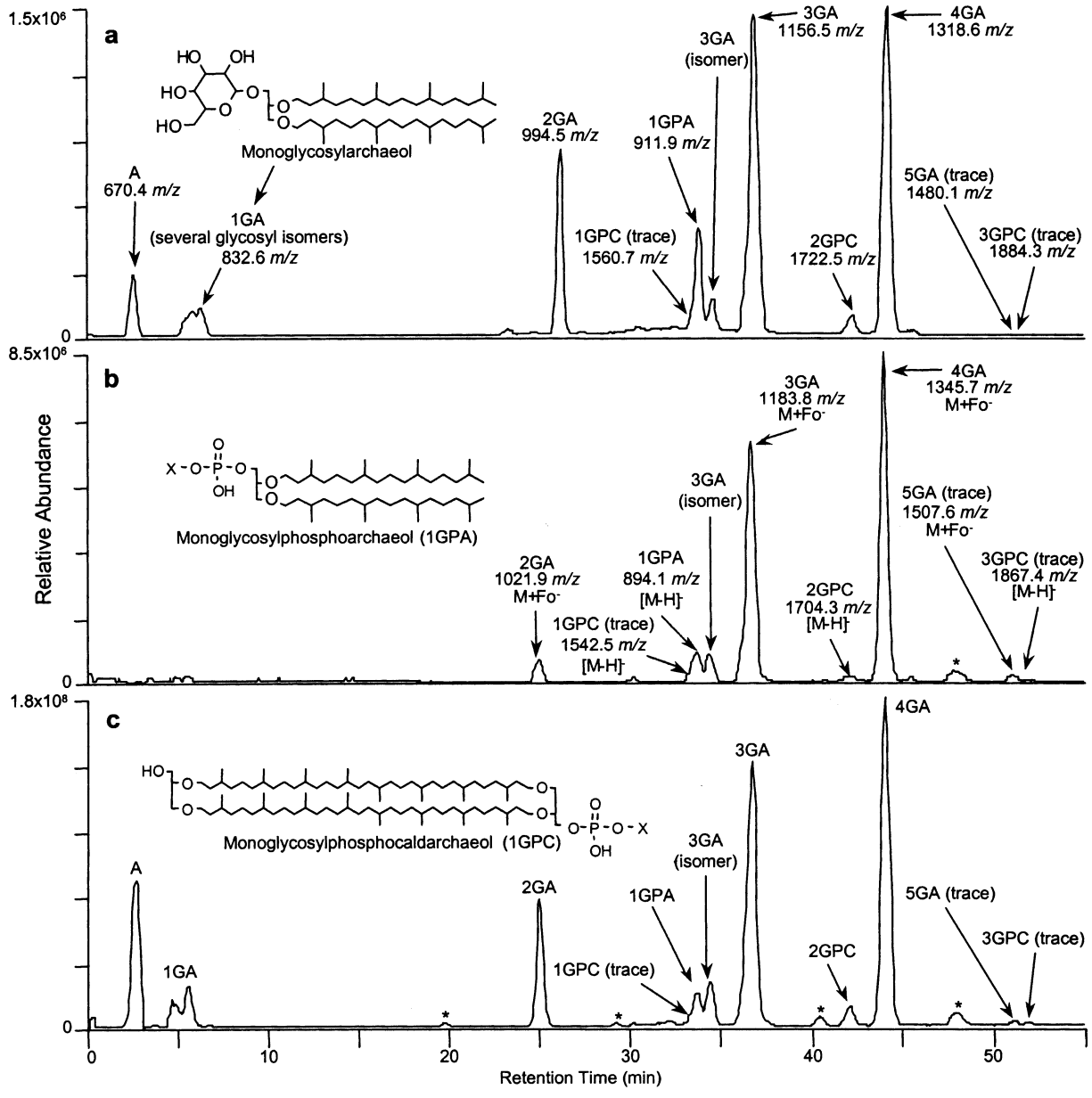

Fig. 2 Base peak chromatograms of full-scan (650–2,000 *m/z*) mass spectra of *N. equitans* (**a** positive ion; **b** negative ion) and a *N. equitans*/*Ignicoccus* sp. strain KIN4/I co-culture (**c** positive ion). Identifications were made on the basis of MS^n spectra in both positive and negative ion modes from LC–MS and from direct infusion of total lipid extracts. Peaks are labeled as follows: *1–5* number of glycosyl units, *G* glycolipid, *GP* phospholipid, *A* archaeol lipid core, *C* caldarchaeol lipid core and * unidentified lipid. The dominant lipids are glycolipids with an archaeol core, with smaller amounts of phospholipids which consist of a phosphate group with glycosyl headgroup and either an archaeol or caldarchaeol lipid core

ions (i.e., base peak) in both positive ion and negative ion modes for *N. equitans* and positive ion mode for a *N. equitans*/*Ignicoccus* sp. strain KIN4/I co-culture. Differences in relative responses for the IPLs can be observed for positive and negative ion modes and, while the overall response in negative ion mode is two orders of magnitude lower, there is less background interference so that small peaks tend to be more obvious. The predominant IPLs are glycolipids (1–5 sugar residues) with an archaeol core (Fig. 2). Much smaller amounts of phospholipids with caldarchaeol and archaeol cores were observed with one or more sugar residues attached to the phosphate or glycerol moiety of the caldarchaeol. Isomers of these complex lipids were also evident in the LC–MS analysis and may represent the presence of different hexoses linked to the core lipids (see below) or some other form of isomerism.

The comparison of the LC–MS results from the purified *N. equitans* cells, the co-culture of *N. equitans* and *Ignicoccus* sp. strain KIN4/I, and of the pure host *Ignicoccus* sp. strain KIN4/I (Table 2) showed that there are no qualitative differences in the lipid compositions of their membranes. *Ignicoccus* sp. strain KIN4/I cells

Table 2 Summary of the distributions of intact polar lipids (IPL) of all samples as determined by LC–MS analysis. – Not detectable, + less than 20%, + + between 20 and 50%, + + + more than 50%, *GDGT* Gycerol dibiphytanyl glycerol tetraether or caldarchaeol

	Purified *N. equitans* cells, cultivated at 90°C	Co-culture of *N. equitans* and *Ignicoccus* sp. strain KIN4/I, cultivated at 90°C	*Ignicoccus* sp. strain KIN4/I, cultivated at 77°C	*Ignicoccus* sp. strain KIN4/I, cultivated at 90°C	*Ignicoccus* sp. strain KIN4/I, cultivated at 95°C	*Ignicoccus* sp. strain KIN4/I, outer membrane
Archaeol	+	+	+	+	+	+
Monoglycosyl-archaeol						
isomer a	+	+	+	+	+	+
isomer b	+	+	+	+	+	+
isomer c	+	+	+	+	+	–
Diglycosyl-archaeol						
isomer a	+	+	+	+	+	+
isomer b	+	+	+	+	+	+
isomer c	+ +	+ +	+	+	+	–
Monoglycosyl-phosphoarchaeol	+	+	+	+	+	+
Triglycosyl-archaeol						
isomer a	+	+	+	+	+	+
isomer b	+	+	+	+	+	+
isomer c	+	+	+	+	+	–
Tetraglycosyl-archaeol						
isomer a	+ +	+ +	+ + +	+ + +	+ + +	+ +
isomer b	+	+	+	+	+	–
Monoglycosyl-phospho-GDGT	+	+	+	+	+	–
Diglycosyl-phospho-GDGT	+	+	+	+	+	–

grown at 77, 90, and 95°C exhibited similar lipid patterns. The only deviation from this was found in the purified outer membrane of *Ignicoccus* sp. strain KIN4/I, which was devoid of biphytane in the hydrocarbons released by ether cleavage or polar lipids with caldarchaeol cores. This indicated that the two membranes of *Ignicoccus* sp. strain KIN4/I (the cytoplasmic and the outer membrane) must exhibit a significant difference in their compositions.

The identification of glycolipids is based upon LC retention times, mass spectra and MS^n spectra such as those shown in Figs. 3, 4. These spectra were compared with those of authentic standards and with lipids known to be present in reference organisms. The identification of glycolipids is based upon our own analyses of total lipid extracts of Archaea, namely, *Methanocaldococcus jannaschii*, *Methanopyrus kandleri*, *Methanothermus fervidus* and *Sulfolobus shibatae* (Ferrante et al. 1990; Hafenbradl et al. 1996; Sprott et al. 1997; Sugai et al. 1995), whereas the identification of the glycosylphospho-lipids (Fig. 4) is more tentatively based on our own analyses of phosphoarchaeol standards with other headgroups and diacylglycerophosphoinositol standards together with well established fragmentation patterns for diacylglycerophosphoinositols.

The presence of a phosphate moiety in the putative glycophosphoarchaeol in Fig. 4 was demonstrated by high-resolution LC–MS analysis. Accurate mass measurement of the 895.7-Da molecular ion gave results that were more consistent with a phospholipid than with a sulfur-containing species. Sulfur (i.e., sulfate) was excluded on the basis of the isotope patterns of the molecular species being incompatible with the presence of an element with such a high M + 2 isotope. The caldarchaeol lipid structures shown in Fig. 4 are one possible arrangement of glycosyl units, although it is reasonable to assume that there is only one glycosyl unit attached to the phosphate, and subsequent glycosyl units are attached to the glycerol end of the caldarchaeol core, since no diglycosylphosphoarchaeol was observed. The ion at 1,381 Da corresponds to the loss of one glycosyl unit (−162) to leave the caldarchaeol core with the phosphate group attached. The ion at 1,543 Da retains one of the two original sugar moieties. The 241-Da ions are not observed in these MS^n spectra since the scan range of the ion trap is limited by the mass of the parent. Note that the molecular ions shown as 1,543 Da and 1,705 Da correspond to the species containing one ^{13}C atom, this being the most common for molecules of this size (e.g., $[M-H]^-$ for diglycosylphospho caldarchaeol = $C_{98}H_{192}O_{19}P^-$, monoisotopic mass 1,704.38 (91.1%), with one $^{13}C = 1{,}705.38$ (100.0%).

Sugar analysis

Figure 5 shows the gas chromatographic separation of the sugars prepared by hydrolysis of the total lipid extracts and analyzed as their TMS derivatives. The samples were compared to the TMS derivatives of reference sugars mannose and glucose. The two peaks for each standard reflect co-occurrence of these sugars as their α-conformations and β-conformations. Qualitatively identical and quantitatively similar sugar compositions were obtained for the purified *N. equitans* cells, the co-culture and the *Ignicoccus* sp. strain KIN4/I cells grown

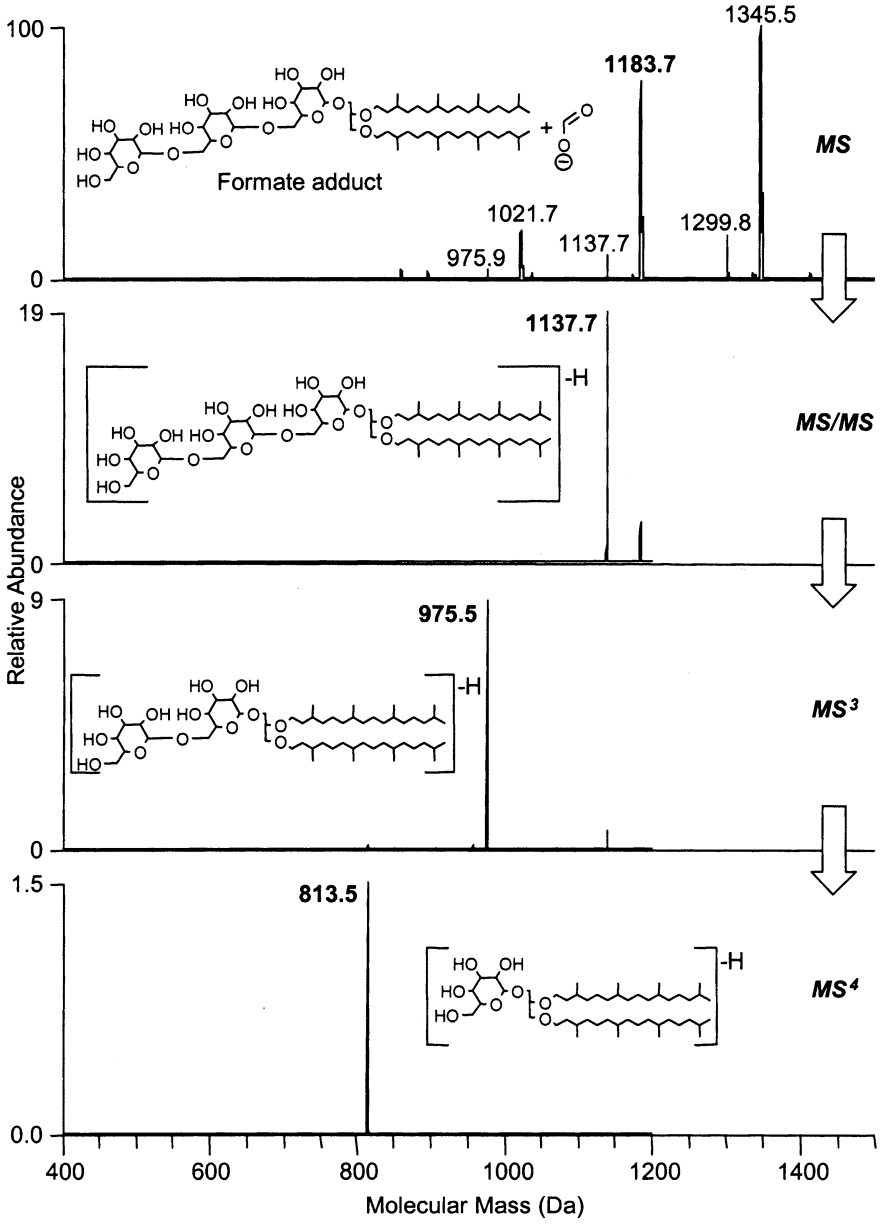

Fig. 3 Negative ion MS^n spectra of the lipid identified as triglycosylarchaeol. The data were collected by infusion of the total lipid extract dissolved in the starting eluent used for LC–MS analyses. In the MS spectrum (*top*), a mixture of related glycolipids is observed with the molecular species at 1,021, 1,183 and 1,345 Da, corresponding to the monoisotopic masses of formate-adducted glycolipids (i.e., $[M+45]^-$) containing 2, 3 and 4 glycosyl groups, respectively. The smaller ions at 975, 1,137, 1,299 Da correspond to the equivalent, but non-adducted glycolipids i.e., $[M-H]^-$. The MS/MS spectrum of the formate adduct of 1,183 Da gives a major ion at 1,137 Da, corresponding to loss of the formate. Subsequent loss of a glycosyl group is observed in each of the MS^3 and MS^4 spectra. A similar analysis of the diglycosyl archaeol in positive ion mode (994 Da, data not shown) yielded fragments of 653 Da (archaeol) and 373 Da (archaeol–phytanyl) as verified by MS^n analysis of an authentic archaeol standard

at different temperatures, each of which showed approximately 95.5% mannose and 4.5% glucose. Neither inositol nor other polyols were positively identified in the hydrolysates. However, they may have been present in trace amounts but overwhelmed by the other sugars.

Discussion

Lipid analyses of *Ignicoccus* sp. strain KIN4/I and *N. equitans* revealed qualitatively identical compositions. The hydrocarbons, derived from the ether lipids by chemical degradation, comprised just phytane derived from lipids with an archaeol core and biphytane derived from caldarchaeol (i.e., GDGT-based lipids). A significant quantitative difference was obtained for the relative amount of biphytane, which was half as abundant in purified *N. equitans* cells as in *Ignicoccus* sp. strain KIN4/I. Biphytanyl ether lipids tended to be more tightly bound and difficult to extract than archaeol-based core lipids. Re-extraction of the Bligh–Dyer residue in the presence of strong acid liberated more caldarchaeol than archaeol from all samples, although the amounts of extra lipid released in this way were only 20% of the total. Carbon isotopic analyses of the hydrocarbons derived from *N. equitans* and its host yielded essentially identical results. All cultures showed the same isotopic composition for phytane and biphytane. However, there was a wide dispersion in the $\delta^{13}C$ values for various experiments due to the necessity of using multiple CO_2 tanks to provide the high gassing rates required by the cultures. Accordingly, the variable isotopic composition of the carbon source precluded

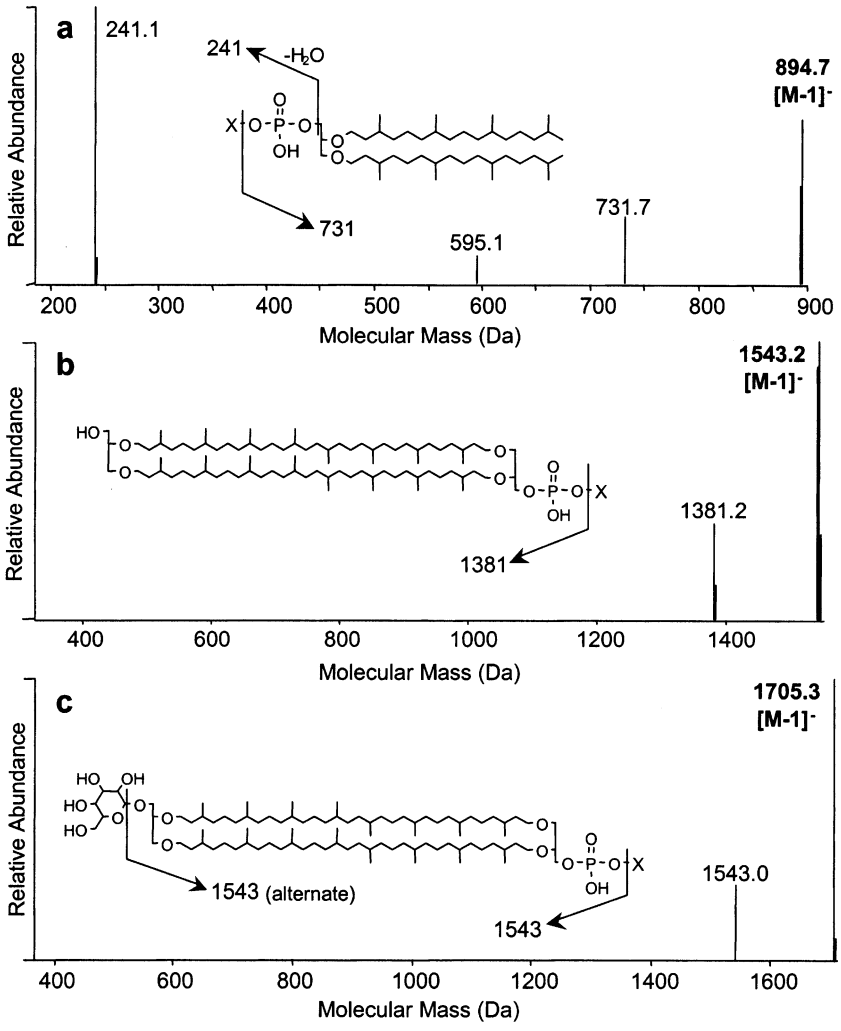

Fig. 4 Negative ion mode MS/MS spectra of three glycosyl-bearing phospholipid species observed in the total lipid extracts of both *N. equitans* (shown above) and *Ignicoccus* sp. strain KIN4/I. Unlike glycolipids, these phospholipids do not form formate adducts and are directly observed as [M−H]-ions. Spectrum **a** is identified as a glycosylphosphoarchaeol (at 33 min in Fig. 2), since the 241-Da ion is diagnostic for phosphoinositol lipids and corresponds to the dehydrated glycophosphate headgroup. The 731-Da ion corresponds to an archaeol phosphate ion and is observed in phosphoarchaeol standards with different headgroups. The exact structure of the glycosyl unit attached to the phosphate is unknown since these MS^n analyses do not give detailed structural information on the nature of the glycosyl units. Spectra **b**, **c** are identified as phosphoglycosyl caldarchaeols with one phosphate and one (at 32.6 min in Fig. 2) or two (at 41.1 min in Fig. 2) glycosyl groups, respectively, attached either via the phosphate group or the glycerol moiety

measurement of the isotopic fractionation associated with carbon assimilation in the experiments reported here.

LC–MS analyses of the IPL of both organisms confirmed the results of the degradative experiments and showed that they contained only archaeol and caldarchaeol cores. These are the basic lipid elements of most Archaea (Kates 1993). Glycolipids with an archaeol core made up the vast majority of the IPL while small amounts of glycosylphospholipids were observed with archaeol and caldarchaeol cores. Although the differential ionization efficiencies means that different compound classes may be detected with quite different sensitivities, the data from LC–MS were quite consistent with the GC and GC–MS data showing that phytanyl ether lipids were in excess of those of biphytane. In Fig. 3, it is also important to note that all of the molecular ions in the positive ion mode are ammonium adducts (i.e., $M+NH_4^+$). In negative ion mode, only the glycolipids form an adduct with formate ion, while the phospholipids form $[M-H]^-$ molecular ions. Ammonium formate is present in the LC solvent system to promote ionization under electrospray conditions. The propensity for different headgroups to form adducts, or not, is diagnostic and is observed in glycolipid and phospholipid standards.

Our structural assignments for the major polar lipids of *Ignicoccus* sp. strain KIN4/I and *N. equitans* are fully supported by additional LC–MS and MS^n analyses we conducted on other thermophilic Archaea known to contain abundant glycosyl archaeols and caldarchaeols (Sturt et al. 2004 and other unpublished results). The IPLs of *M. jannaschii* comprise a number of core lipids, including archaeol, a macrocyclic version of archaeol and caldarchaeol. The polar headgroups encompass a diverse array of glycosyl and phosphate-based headgroups, with phosphatidic acid derivatives being the most abundant. Other polar headgroups include phosphatidylethanolamine and phosphatidylserine (Ferrante et al. 1990; Sprott et al. 1991). The IPL distribution in *S. shibatae* is much simpler than that in *M. jannaschii*: glycolipids with tetraether core lipids. The lipids of the order Sulfolobales have been studied in detail (Sugai et al. 1995) since they were found to contain a novel derivative of caldarchaeol unique to the order. *M. kandleri* IPLs were exclusively archaeol core lipids

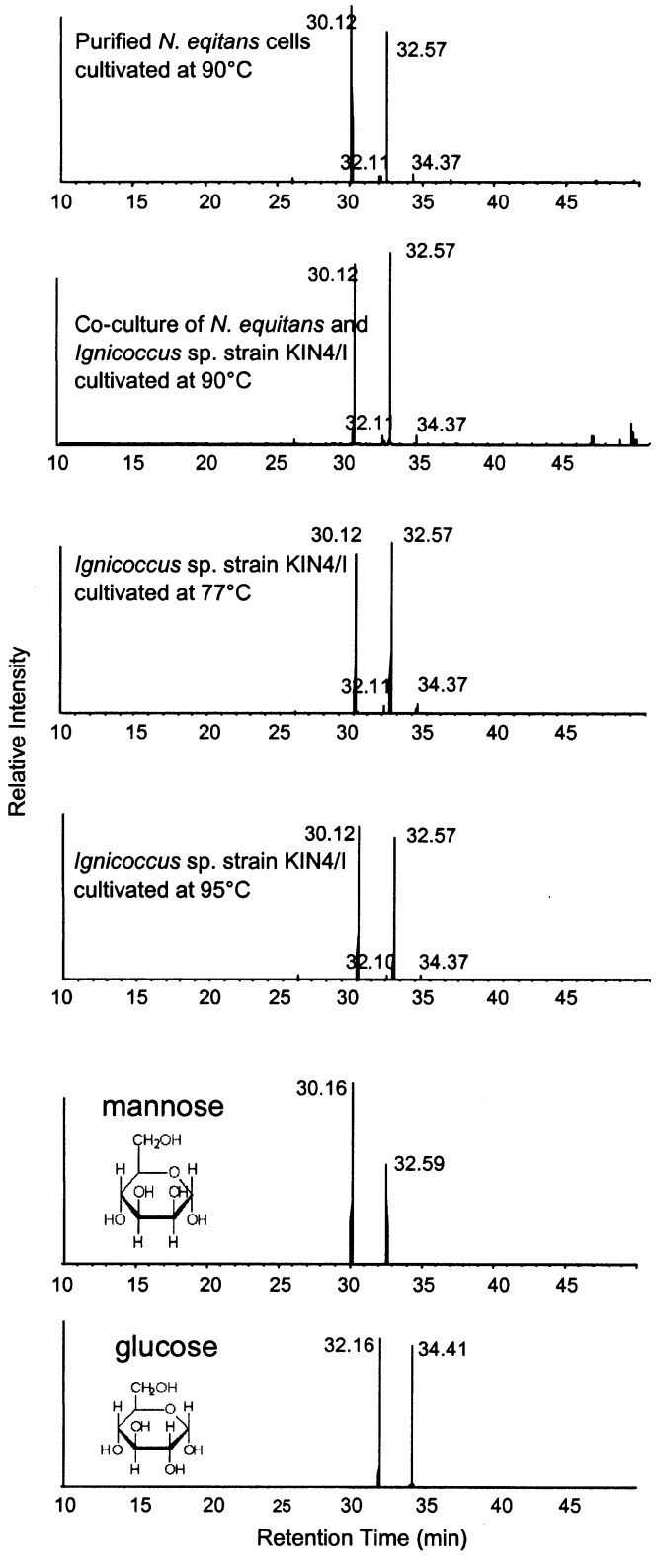

Fig. 5 Gas chromatographic separation of the sugars residues in the total lipid extracts (including sugar standards)

with mainly glycosyl headgroups and some phospholipids with phosphoethanolamine, phosphoglycerol and phosphocholine headgroups (Sprott et al. 1997).

Since multiple isomers of some structures were evident in the LC–MS of total lipids, the sugar residues were released by hydrolysis and analyzed separately by GC–MS. Mannose was the dominant sugar residue in all samples, at about 95.5% with glucose subordinate at around 4.5%.

The results demonstrate that there is a remarkable similarity in the compositions of the membrane lipids of *N. equitans* and its host *Ignicoccus* sp. strain KIN4/I, both qualitatively and quantitatively.

Considering the great phylogenetic distance between *N. equitans* and *Ignicoccus* sp. strain KIN4/I (Huber et al. 2002) and particularly the result of the whole-genome analysis of *N. equitans*, in which no genes for the biosynthesis of lipids could be identified (Waters et al. 2003), we conclude that *N. equitans* does not synthesize its own membrane lipids. Instead, it must obtain them from its host. While the mechanism of the lipid uptake is unknown, the differences in abundances of certain compounds may be explained by a selective uptake of the components by *N. equitans*. As the genome analysis of *N. equitans* shows the presence of genes for modifications such as glycosylation (Waters et al. 2003) it remains an open question as to whether or not this capacity is expressed.

Growth of *Ignicoccus* sp. strain KIN4/I at different temperatures also resulted in the same qualitative lipid pattern. LC–MS analysis showed that the patterns of glycosylation were essentially identical at all temperatures. However, there was a marked decrease in the relative abundance of biphytane in *Ignicoccus* sp. strain KIN4/I, from about 20% at the optimum growth temperature of 90°C to both its minimal growth temperature (77°C) and maximal (95°C) growth temperatures. Other Archaea, such as *M. jannaschii* (Sprott et al. 1991), have been shown to increase the amount of tetraether as a function of increasing temperature. However, several hyperthermophilic Archaea, such as *M. kandleri* and *Thermococcus celer*, contain no tetraether lipids in the cytoplasmic membrane (Kurr et al. 1991; De Rosa et al. 1987). Therefore, an increase of the amount of tetraethers in the cytoplasmic membrane is neither characteristic nor essential for preserving membrane stability at very high temperatures. More likely, for *Ignicoccus* sp. strain KIN4/I, growth at sub-optimal temperatures disrupts the biosynthetic processes leading to tetraether formation.

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References

Barns SM, Delwiche CF, Palmers JD, Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci USA 93:9188–9193

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

De Rosa M, Gambacorta A (1986) Lipid biogenesis in archaebacteria. In: Kandler O, Zillig W (eds) Archaebacteria'85. Fischer, Stuttgart, pp 278–285

De Rosa M, Basso A, Gambacorta A, Holz I, Trincone A, Zillig W (1987) Lipid of *Thermococcus celer*, a sulfur-reducing Archaebacterium—structure and biosynthesis. Syst Appl Microbiol 9(1–2):1–5

De Rosa M, Gambacorta A, Huber R, Lanzotti V, Nicolaus B, Stetter KO, Trincone A (1988) A new 15,16-dimethyl-30-glyceroloxytriacontanoic acid from lipids of *Thermotoga maritima*. J Chem Soc Chem Commun 19:1300–1301

De Rosa M, Trincone A, Nicolaus B, Gambacorta A (1991) Archaebacteria: lipids, membrane structures, and adaptation to environmental stresses. In: DiPrisco G (ed) Life under extreme conditions. Springer, Berlin Heidelberg New York, pp 61–86

Ferrante G, Richards JC, Sprott GD (1990) Structures of polar lipids from the thermophilic, deep-sea archaeobacterium *Methanococcus jannaschii*. Biochem Cell Biol 68(1):174–283

Hafenbradl D, Keller M, Stetter KO (1996) Lipid analysis of *Methanopyrus kandleri*. FEMS Microbiol Lett 136(2):199–202

Hohn MJ, Hedlund BP, Huber H (2002) Detection of 16S-rDNA sequences representing the novel phylum "*Nanoarchaeota*": indication for a wide distribution in high temperature biotopes. Syst Appl Microbiol 25:551–554

Huber R, Wilharm T, Huber D, Trincone A, Burggraf S, König H, Rachel R, Rockinger I, Fricke H, Stetter KO (1992) *Aquifex pyrophilus* gen. nov., sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst Appl Microbiol 15:340–351

Huber H, Hohn MJ, Rachel R, Fuchs T, Wimmer VC, Stetter KO (2002) A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature 417:63–67

Huber H, Hohn MJ, Stetter KO, Rachel R (2003a) The phylum Nanoarchaeota: present knowledge and future perspectives of a unique form of life. Res Microbiol 154:165–171

Huber H, Hohn MJ, Rachel R, Stetter KO (2003b) "*Nanoarchaeota*". In: Dworkin M, Schleifer KH, Stackebrandt E (eds) The prokaryotes: an evolving electronic resource for the microbiological community, 3rd edn. Springer, Berlin Heidelberg New York www.prokaryotes.com(release 3.13, 12th May 2003)

Jahn U (2003) Physiologische, molekularbiologische und biochemische Untersuchungen am Organismensystem *Nanoarchaeum equitans* und *Ignicoccus* sp. KIN4/I. Diplomarbeit, Universität Regensburg

Jahnke LL, Eder W, Huber R, Hope JM, Hinrichs K, Hayes JM, Des Marais DJ, Cady S, Summons R (2001) Signature lipids and stable carbon isotope analysis of octopus spring hyperthermophilic communities compared with those of *Aquificales* representatives. Appl Environ Microbiol 67:5179–5189

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

Kates M (1986) Techniques of lipidology: isolation, analysis and identification of lipids. In: Bubon RH, van Knippenberg PH (eds) Laboratory techniques in biochemistry and molecular biology. Elsevier, Amsterdam

Kates M (1993) Membrane lipids of archaea. In: Kates M, Kushner DJ, Matheson AT (eds) The biochemistry of archaea (Archaebacteria). Elsevier, Amsterdam, pp 261–295

Koga Y, Nishihara M, Morii H, Akagawa-Matsushita M (1993) Ether polar lipids of methanogenic bacteria: structures, comparative aspects, and biosynthesis. Microbiol Rev 57:164–182

Koga Y, Kyuragi T, Nishihara M, Sone N (1998) Did archaeal and bacterial cells arise independently from non-cellular precursors? A hypothesis stating that the advent of membrane phospholipid with enantiomeric glycerophosphate backbones caused the separation of the two lines of descent. J Mol Evol 46(1):54–63

Kurr M, Huber R, König H, Jannasch HW, Fricke H, Trincone A, Kristjansson JK Stetter KO (1991) *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. Arch Microbiol 156:239–247

Lange BM, Rujan T, Martin W, Croteau R (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci USA 97:13172–13177

Langworthy TA (1985) Archaebacteria. In: Woese CR, Wolfe RS (eds) The bacteria, vol 8. Academic, New York, pp 459–497

Nishihara M, Koga Y (1987) Extraction and composition of polar lipids from the archaebacterium, Methanobacterium thermoautotrophicum: effective extraction of tetraether lipids by an acidified solvent. J Biochem 101:997–1005

Rachel R, Wyschkony I, Riehl S, Huber H (2002) The ultrastructure of *Ignicoccus*: evidence for a novel outer membrane and for intracellular vesicle budding in an archaeon. Archaea 1:9–18

Rütters H, Sass H, Cypionka H, Rullkötter J (2001) Monoalkylether phospholipid in the sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*. Arch Microbiol 176:435–442

Sprott GD, Meloche M, Richards JC (1991) Proportions of diether, macrocyclic diether, and tetraether lipids in *Methanococcus jannaschii* grown at different temperatures. J Bacteriol 173:3907–3910

Sprott GD, Agnew BJ, Girshchandra BP (1997) Structural features of ether lipids in the archaeobacterial thermophiles *Pyrococcus furiosus*, *Methanopyrus kandleri*, *Methanothermus fervidus*, and *Sulfolobus acidocaldarius*. Can J Microbiol 43:467–476

Stetter KO (1989) Extremely thermophilic chemolithoautotrophic archaebacteria. In: Schlegel HG, Bowien B (eds) Autotrophic bacteria. Science Tech/Springer, Madison/Berlin Heidelberg New York London Paris Tokyo, pp 167–175

Stetter KO (1995) Microbial life in hyperthermal environments. Am Soc Microbiol News 61:285–290

Sturt HF, Summons RE, Smith K, Elvert M, Hinrichs K-U (2004) Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry—new biomarkers for biogeochemistry and microbial ecology. Rapid Commun Mass Spectrom 18(6):617–628

Sugai A, Sakuma R, Fukuda I, Itoh YH, Kon K, Ando S, Itoh (1995) The structure of the core polyol of the ether lipids from *Sulfolobus acidocaldarius*. Lipids 30:339–344

Summons RE, Franzman PD, Nichols PD (1998) Carbon isotopic fractionation associated with methylotrophic methanogenesis. Org Geochem 28:465–475

Waters E, Hohn MJ, Ahel I, Graham DE, Adams MD, Barnstead M, Beeson KY, Bibbs L, Bolanos R, Keller M, Kretz K, Lin X, Mathur E, Ni J, Podar M, Richardson T, Sutton GG, Simon M, Söll D, Stetter KO, Short JM, Noordewier M (2003) The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. Proc Natl Acad Sci USA 100:12984–12988

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87:4576–4579