COMPOSITION OF HYDROTHERMAL VENT MICROBIAL COMMUNITIES AS REVEALED BY ANALYSES OF SIGNATURE LIPIDS, STABLE CARBON ISOTOPES AND *AQUIFICALES* CULTURES

Linda L. Jahnke[1]*, Wolfgang Eder[3], Robert Huber[3], Kai-Uwe Hinrichs[4], John M. Hayes[4], David J. Des Marais[1], Sherry Cady[5], Janet M. Hope[2], Roger E. Summons[2]

[1] Exobiology Branch, NASA Ames Research Center, Moffett Field. California, 94035, USA

[2] Australian Geological Survey Organisation, GPO Box 378, Canberra, ACT, 2601, Australia

[3] Lehrstuhl für Mikrobiologie and Archaeenzentrum, Universität Regensburg, D-93053 Regensburg, Germany

[4] Woods Hole Oceanographic Institution, Woods Hole MA 02543. USA

[5] Department of Geology. Portland State University, Portland, OR 97201, USA

ABSTRACT

Extremely thermophilic microbial communities associated with the siliceous vent walls and outflow channel of Octopus Spring, Yellowstone National Park, have been examined for lipid biomarkers and carbon isotopic signatures. These data were compared with that obtained from representatives of three *Aquificales* genera. *Thermocrinis ruber*, "*Thermocrinis* sp. HI", *Hydrogenobacter thermophilus* TK-6, *Aquifex pyrophilus* and *Aquifex aeolicus* all contained phospholipids composed not only of the usual ester-linked fatty acids, but also ether-linked alkyls. The fatty acids of all cultured organisms were dominated by a very distinct pattern of *n*-$C_{20:1}$ and *cy*-C_{21} compounds. The alkyl glycerol ethers were present primarily as $C_{18:0}$ monoethers with the expection of the *Aquifex* spp. in which dialkyl glycerol ethers with a boarder carbon-number distribution were also present. These Aquificales biomarker lipids were the major constituents in the lipid extracts of the Octopus Spring microbial samples. Two natural samples, a microbial biofilm growing in association with deposition of amorphous silica on the vent walls at 92°C, and the well-known 'pink-streamers community' (PSC), siliceous filaments of a microbial consortia growing in the upper outflow channel at 87°C were analyzed. Both the biofilm and PSC samples contained mono- and dialkyl glycerol ethers with a prevalence of C_{18} and C_{20} alkyls. Phospholipid fatty acids were comprised of both the characteristic Aquificales *n*-$C_{20:1}$ and *cy*-C_{21}, and in addition, a series of *iso*-branched fatty acids from *i*-$C_{15:0}$ to *i*-$C_{21:0}$, with *i*-$C_{17:0}$ dominant in the PSC and *i*-$C_{19:0}$ in the biofilm, suggesting the presence of two major bacterial groups. Bacteriohopanepolyols were absent and the minute quantities of archaeol detected showed that Archaea were only minor constituents. Carbon

isotopic compositions of the PSC yielded information about community structure and likely physiology. Biomass was ^{13}C-depleted (10.9‰) relative to available CO_2 from the source water inorganic carbon pool with lipids further depleted by 6.3‰ relative to biomass. The C_{20-21} *Aquificales* fatty acids of the PSC were somewhat heavier (average $\Delta\delta^{13}C_{CO2}$= −18.4‰) than the *iso*-branched fatty acids (average $\Delta\delta^{13}C_{CO2}$= −22.6‰). The carbon isotopic signatures of lipid biomarkers were also explored using a pure culture, *T. ruber*, previously isolated from the PSC. Cells grown on CO_2 with O_2 and both H_2 and thiosulfate as electron donors were only slightly depleted (3.3‰) relative to the C-source while cells grown on formate with O_2 showed a major discrimination (19.7‰), possibly the result of a metabolic branch point involving the assimilation of C-formate to biomass and the dissimilation to CO_2 associated with energy production. *T. ruber* lipids were slightly heavier than biomass (+1.3‰) whether cells were grown using CO_2 or formate. Fatty acids from CO_2 grown *T. ruber* cells were also slightly heavier (average = +2.1‰) than biomass. The relatively depleted PSC C_{20-21} fatty acids suggest that any associated *Thermocrinis* biomass would also be similarly depleted and much too light to be explained by growth on CO_2. The C-fractionations determined with the pure culture suggest that growth of *Thermocrinis* in the PSC is more likely to occur on formate, presumably generated by geothermal activity. This study points to the value of the analysis of the structural and isotopic composition of lipid biomarkers both in pure culture studies, and in establishing community structure and physiology, as a complement to genomic profiles of microbial diversity. This is especially so when the members of the microbial community are novel and difficult to cultivate in the laboratory.

INTRODUCTION

Based on phylogenetic analysis of small subunit rRNA sequences, hyperthermophilic organisms proliferate in the deepest branches of the Bacterial and Archaeal domains. The branch lengths of these hyperthermophilic lineages tend to be short which further suggests that such organisms are the closest known extant descendants of the Last Common Ancestor and retain many ancestral phenotypic properties **[63]**. The recent discovery of probable filamentous microfossils preserved in a submarine, volcanogenic massive sulfide deposit that was dated at 3,235 Ma lends considerable weight to the theory that hydrothermal vent organisms have had a very long history on Earth and to proposals for a hyperthermophilic chemolithotrophic origin of life **[54]**. Hyperthermophilic microbes are also attracting astrobiological and biogeochemical interest. This is because of their potential roles in the formation of many kinds of mineral deposits, and the generation of rock textures and mineral assemblages that may be diagnostic for extant or extinct life beyond Earth **[6, 14, 68]**.

A well known example of a chemolithotrophic ecosystem composed of filamentous streamers is found at Octopus Spring, Yellowstone National Park, USA. The 'pink streamers' were first described by Brock in 1965 **[5]**. Similar streamer communities were first described by Setchell in 1903 **[59]** and have been described in neutral to alkaline springs in geothermal areas in Iceland, Japan and Kamtschatka, Russia **[26, 62, 70]**, and more recently as distinct black streamers at Calcite Springs in Yellowstone **[55]**. The Octopus Spring communities were noted for their propensity to rapidly form macroscopically visible pink streamers on strings or biofilms on glass slides immersed in source waters **[4]**. Molecular analysis of the small subunit 16S rRNA sequences of the filamentous pink streamer community (PSC) suggests dominance of the domain Bacteria, and in particular two deeply diverging phylotypes affiliated with the *Aquificales* and *Thermotogales* **[56]**.

The Yellowstone PSC appear as gelatinous, filamentous masses attached to rocky fragments and small twigs in the swift upper outflow channel, closely adjacent to the vent source of Octopus Spring **[4]**. The masses are imbued with amorphous silica depositing from the SiO_2-rich vent fluids **[2, 4]**. From the PCS, the first 'pink streamer' isolate, *Thermocrinis ruber*, has been otained recently in pure culture **[23]**. *T. ruber* forms a separate lineage within the order *Aquificales*, and shares many features with the two previously isolated genera, *Aquifex* and *Hydrogenobacter* **[27, 35, 37]**, including chemolitho-autotrophic growth with hydrogen, sulfur or thiosulfate in the presence of oxygen. However, *T. ruber* also grows chemoorganoheterotrophically, a previously undescribed metabolic property for this group **[27]**. Various molecular studies of microbial streamers have identified a number of other novel sequences of yet uncultivated organisms **[56, 62]**. Genomic and microscopic analysis of Octopus and other siliceous springs indicate that similar bacterial communities readily colonize the vent walls of these thermal pools **[6, 28]**.

The ability of microorganisms to survive adverse temperature changes depends on a thermoadaptative compensation largely determined by the nature of the hydrocarbon chains that fill the hydrophobic core of their cellular membranes. Numerous studies have shown the predominance of *iso*- and *anteiso*-branched fatty acids and the lack of unsaturated fatty acids in most thermophilic bacteria **[39]**. However, some thermophiles are now recognized for a variety of novel lipids which can serve as valuable biomarkers. *Thermomicrobium roseum* contains internally methyl-branched C_{18} fatty acid and long-chain 1,2-diols as major components **[51]**. The core lipids of members of the order *Thermotogales* are composed of unusual dicarboxylic fatty acids and a new ether lipid, 15,16-dimethyl-30-glyceroloxytriacontanoic acid **[25]**. Other novel mono- and dialkyl glycerol ether (GME and GDE, respectively) lipids have been described in *Thermodesulfobacterium commune* **[38]** and *A. pyrophilus* **[27]** which differ only in the nature of the hydrocarbon chain. The predominant ether lipids in *T. commune* are

composed of *iso*- and *anteiso*-branched chains, while in *A. pyrophilus*, they are straight chains. Also, given the rarity of unsaturated fatty acids in thermophiles, the presence of high amounts of a monounsaturated, straight chain C_{20} in the *Hydrogenobacter* genus **[35]** is of particular importance as described below.

Lipid analyses of natural thermophilic streamer mats are quite limited. Bauman and Simmonds **[2]** reported on the fatty acid composition of 'pink streamer' samples from Firehole Pool and Pool 'A' (Octopus Spring) of the lower Geyser Basin in Yellowstone. Their lipid extracts showed a fatty acid distribution comprised of a series of normal C_{14} to C_{20} members and iso-C_{15} to iso-C_{20}. The major fatty acids appeared somewhat influenced by temperature; the Firehole Pool (82°C) sample contained a monounsaturate C_{20} and iso-C_{17} as the major acids, while in Pool 'A'(87°C), a cyclopropyl C_{21} and iso-C_{19} were more abundant.

In addition to carrying distinctive structures, lipid biomarkers also encode the stable isotopic signature that provides information about the physiologies of the source organisms **[15, 32]**. Interpretation of these isotopic signatures requires knowledge of the inherent C-isotopic discriminations associated with the flow of carbon from natural sources through the biochemical pathways involved in carbon fixation and lipid synthesis. Data that constrains the pCO2 and its isotopic composition are accessible for the Octopus Spring vent waters. On the other hand, only a very limited amount of information is available on the bulk isotopic fractionation factors for some cultured thermophiles and, to our knowledge, nothing has been reported on the isotopic composition of lipids.

In this study, we initially set out to examine the microbial composition of the Octopus Spring PSC and nearby vent biofilms through a comprehensive analysis of signature lipids. The resultant data revealed a more complex situation than was indicated by genomic analysis alone and suggested the need for appropriate support data from pure culture studies. A comparison of the lipid profiles of several genera within the *Aquificales*, as well as, measurements of the carbon isotopic fractionation associated with autotrophic and heterotrophic growth of the only available PSC isolate, *T. ruber*, formed a framework for understanding the population structure of the Octopus Spring 'pink streamer' community.

METHODS AND MATERIALS

Sample collection and preparation. Biomass consisting of the 'pink streamers' community (PSC) attached to rock surfaces was collected using forceps from an 87°C, pH 8.3 site in the main outflow just below the source pool vent of Octopus Spring in May 1997. The filaments were placed in glass tubes, sealed with teflon lined caps and frozen on dry ice within 3 hrs, and maintained so in transit to Ames. A vent wall geyserite sample approximately 25 cm^2 was

removed from a site closely adjacent to the main Octopus Spring pool source at 92°C. Working in a glove box, the top 1-2 mm of the surface area of the geyserite-biofilm was carefully removed by scrapping with a spatula, transferred to glass vials and frozen. PSC and vent geyserite samples were lyophilized and then ground to a powder in a glass mortar previously cleaned with sequential solvent washes of dichloromethane, methanol and acetone. All glassware and metal implements used in our procedures were baked at 450°C for a minimum of 4 h. Only teflon stoppers and/or teflon-lined screw caps were used in analyses.

Strains and culture conditions. *Thermocrinis ruber* OC 1/4 (DSM 12173), *Aquifex pyrophilus* Kol5a (DSM 6858), *Hydrogenobacter thermophilus* TK-6 (IAM 12695), *Aquifex aeolicus* VF5 [26] and *Thermocrinis* sp. HI 11/12 [23] were obtained from the culture collection of the Lehrstuhl für Mikrobiologie, Universität Regensburg, Germany. Cell masses of the *Aquificales* strains were grown at 85°C (70°C for *H. thermophilus* and 80°C for *T. ruber* with formate) with stirring (up to 400 rpm) in a 300-liter enamel-protected fermentor (Bioengineering, Wald, Switzerland) as described in Table I. For growth of *T. ruber* in experiment 1 (isotope study), the cell titer was monitored and the culture was gassed with increasing flow rates (2, 5, 7.5 and 10 l/min) to maintain growth rate. At harvest, a titer of 1.1×10^8 cells/ml had been reached from which a biomass dry wt equivalent to 2.1×10^{-13} gm/cell was recovered.

Phylogenetic analyses. For the analyses, an alignment of about 11.000 homologous full primary sequences available in public databases (ARB project, [40, 41]) was used. The *Aquificales* 16S rRNA gene sequences were fitted in the 16S rRNA tree by using the respective automated tools of the ARB software package [41]. Distance matrix (Jukes and Cantor correction), maximum parsimony and maximum likelihood (fastDNAml) methods were applied as implemented in the ARB software package [42]

Lipid extraction, separation and analysis. Lipids were extracted from lyophilized ground sinter or *Aquificales* biomass using a single phase modification of the Bligh and Dyer procedure, and water-soluble contaminants removed as previously reported [29]. Elemental sulfur was removed by passing the total lipid extract over activated copper powder. The total lipid extract (TLE) was dried under nitrogen, then maintained in a vacuum dessicator over Drierite until reaching a constant weight.

A portion of the PSC total lipid was used for an oxidation-reduction procedure to convert bacteriohopanepolyol to its hopanol derivative [58] and analysis as previously reported [30].

Fatty acid methyl esters (FAME) and glycerol ethers (GME and GDE) were prepared by two procedures. In procedure I, FAME were prepared by treating a portion of TLE by a mild alkaline methanolysis procedure [47] modified by heating at 37°C for 1 h. FAME were separated from

the remaining polar ether lipids (GME and GDE) by thin-layer chromatography (TLC) using a methylene chloride mobile phase as previously reported [29]. The FAME (Rf 0.80) were recovered by eluting the silica gel with methylene chloride, and the ether-linked components were recovered from the origin of the TLC plate by Bligh and Dyer extraction of the silica gel zone. The polar ether components were then hydrolyzed in 1 ml of chloroform-methanol- conc. HCl (1:10:1) by heating to 100°C for 2 hr [47], and the glycerol ethers were separated by TLC using hexane-diethyl ether-acetic acid (70:30:1) into monoalkylglycerol ethers (Rf 0.04), dialkylglycerol ethers (Rf 0.40), and diphytanylglycerol ether (Rf 0.49) using reference compounds; 1-*O*-hexadecyl-glycerol and 1,2-di-*O*-hexadecyl-glycerol (Sigma, St. Louis, MO), and diphytanylglycerol ether isolated from *Halobacterium* sp. [67].

Procedure II was used in an attempt to analyze small samples such as the OS vent geyserite. In this procedure, TLE was directly hydrolyzed with acid as described above, followed by trimethylsilyl (TMS) derivatization of the resulting free glycerol ethers, and gas chromatography-mass spectrometry (GC-MS) of the treated TLE. Some of TLE samples were also analyzed for free fatty acids and glycerides by preparation of TMS derivatives. Quantitation was based on comparison of peak areas to standards; methyl tricosanoate (C_{23}) for FAME and cholestanol for glycerol ethers.

Some of the PSC and *T. ruber* (exp. 1) total lipid was also preparatively separated into a polar lipid (phospholipids) and a neutral lipid (glycolipids and diglycerides) fraction by precipitation in cold acetone [34]. The components of the polar and neutral fractions were then separated by thin layer chromatography on Silica gel G plates (Merck) using acetone-benzene-water (91:30:8) [50] or, in some cases, chloroform-methanol-water (65:25:4) [34]. Preliminary characterization of TLC zones was made based on migration of standard diacyl compounds (phosphatidylcholine, phosphatidylethanolamine, digalactosyl diglyceride and monogalactosyl diglyceride) and staining with specific detection reagents for phosphate (phosphomolybdic acid), for amino-lipid (ninhydrin), and for glycolipids (α-naphthol) [34]. TLC zones for lipid analysis were detected by UV fluorescence after spraying with rhodamine 6G, and recovered by eluting the silica gel by the Bligh and Dyer procedure above. FAME and glycerol ethers were prepared from each of these fractions as described above.

The double bond positions of the monounsaturated FAME were determined by preparing the dimethyl disulfide adducts [71]. TMS derivatives of the glycerides were prepared using N,O-bis (trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (1:1 in pyridine).

Alkyl moieties were released from the glycerol ether compounds by reaction with BBr_3 as reported previously [65].

Gas chromatographic analyses. FAME were analyzed by using a Perkin-Elmer Sigma 3B gas chromatograph equipped with a flame ionization detector and 30-m megabore columns (J & W Scientific), either a DB-5ms programmed to increase at 4°C/min from 160°C to 280°C, or a DB-27 programmed to increase at 4°C/min from 120°C to 220°C. Compound identification was based on retention times on the non-polar and the polar columns, and on mass spectral analysis (see below).

GC-MS analyses of fatty acids as FAME or TMS, and glycerol ethers as TMS derivatives were performed using a HP 6890 gas chromatograph equipped with a J&W DB-5 (60-m x 0.32 mm, 0.25-μm film) capillary column and a HP 5973 mass-selective detector operated at 60°C for 10 min, then programmed at 2°C/min to 320°C and held for 60min. The bond positions of the monounsatured FAME were determined by analyzing their dimethyl disulfide adducts as previously described [31].

Isotopic measurements. The dissolved inorganic carbon (DIC) was measured by taking approximately 40-ml water samples from the outflow site using a syringe and immediately filtering through Whatman GF/F filters into preevacuated 130-ml serum bottles sealed with silicon stoppers and containing approximately 0.1 ml of saturated $HgCl_2$ to inhibite bacterial growth. The bottles were kept chilled until analysis. At Ames, samples were withdrawn, acidified and the CO_2 gas collected on a vacuum line for isotopic analysis on a Nuclide 6-60RMS mass spectrometer modified for small samples [11, 18]. Analysis of the DIC composition of the *Thermocrinis* culture medium was similar except samples were collected by filling glass tubes with the gassed culture medium prior to inoculation, and immediately stoppering with crimp-seals before shippment by air to Ames from Germany. Biomass and total lipid were determined at Ames using a Carlo Erba CHN EA1108 elemental analyzer interfaced to a Finnigan Delta Plus isotope ratio mass spectrometer.

Compound specific isotope analyses were done at AGSO as previously described using a Finnigan MAT 252 mass spectrometer equipped with a CuO/Pt microvolume combustion furnace and a Varian 1400 gas chromatograph with DB-5 column [31]. Reported δ values have been corrected for the presence of carbon added during derivtization.

RESULTS

Comparison of pink streamer community and vent biofilm lipids. The total organic carbon (TOC) recovered from the vent geyserite surface was only 0.24% as compared with 7.2% for PSC and 36.7% for *T. ruber* biomass (exp. 1). Procedure II, direct acid hydrolysis, was used to minimize loss of material during preparation of the vent sample, and allow

comparison with PSC and *T. ruber* samples (Fig. 1). Although this method does result in the destruction of the cyclopropyl FAME present in these extracts [2, 7], it provides valuable comparative information.

The fatty acids of vent and PSC were predominantly C_{17} to C_{22} chain lengths, distinguished by *iso*- homologues of the C_{16} to C_{21} acids and, although diminished, *cy*-C_{21}. The TMS derivatives of the free fatty acids present in the vent TLE confirmed that high amounts of *cy*-C_{21} (25% of total) were also characteristic of the OS vent biofilm community. The FAME composition of vent and PSC extracts while qualitatively similar differed quantitatively (Fig. 1). Streamers had more abundant branched chain FAME (41% in PSC vs 13% in OS vent) with a somewhat shorter chain distribution: the ratio of *i*-$C_{17:0}$ to *i*-$C_{19:0}$ was 1:2.1 in PSC but 1:3.7 in OS vent. The *anteiso* analogues, *a*-$C_{17:0}$ and *a*-$C_{19:0}$ were present in PSC (1.3%), but almost absent in OS vent. The OS vent TLE also contained small amounts of even carbon chain FAME, *n*-22:0 to *n*-30:0, and a mid-chain branched octadecanoic acid (1%), possibly 10- or 12-methyl-$C_{18:0}$. The mono- and dialkyl glycerol ethers were abundant in vent and PSC lipids, and a significant peak for an Archaeal biomarker, diphytanylglycerol ether (archaeol), was present in the vent biofilm (Fig. 1). Together the GME and GDE were more abundant than FAME in both vent and PSC lipids. BBr_3 cleavage of the ether alkyl chains detected only straight chain compounds with carbon numbers supporting GC-MS characterization of the intact glycerol moieties. C_{18} chains dominated both GME and GDE.

Pink streamer community and *Aquificales* polar lipids. The acid hydrolysate of the *T. ruber* TLE (procedure II) was composed primarily of saturated and monounsaturated C_{20} FAME and a C_{18}-GME (Fig.1). No *iso*-branched FAME or GDE were detected. Additional analyses of the lipid using an alkaline methanolysis procedure (I), confirmed the high proportion of C_{20} FAME with $C_{20:1}$ which together comprised over 49% of the total for *T. ruber* OC1/4 (Table 2, experiment 1). High amounts of *cy*-C_{21} were also recovered in *T. ruber* and PSC by this procedure, and further FAME analyses were carried out with this procedure (Table 2 & 3).

T. ruber biomass from a variety of growth conditions were analyzed (Table 1). In experiment 1, *T. ruber* was grown using thiosulfate with a constant gassing of H_2-CO_2-O_2 to maintain high substrate levels for measurement of the carbon isotopic discrimination associated with CO_2-fixation (results below). This growth condition resulted in accumulation of relatively large amounts of intracellular sulfur (~15% of dry wt). S° accumulation was not apparent in *T. ruber* grown with thiosulfate, but without H_2 (experiment 2) or in PSC extracts. The lipid composition of additional *Aquificales* cultures (Table 1) was also analyzed to assess the potential use of *Thermocrinis*-like lipids as group biomarkers and aid in characteriztion of the pink

streamer community (Table 2). All *Aquificales* cultures contained GME and no *iso*-branched FAME were detected (Table 2). GDE were only present in the lipids of the PSC and the two marine *Aquifex* cultures, *A. aeolicus* VF5 and *A. pyrophilus* Kol 5a. BBr_3 cleavage of the *Aquificales* glycerol ethers showed only straight chain alkyls with chain length distributions similar to those characterized for the intact molecules.

Using the methods of Rohmer et al.[58] no hopanoids were detected in the PSC total lipid extract, either as the polar bacteriohopanepolyol, or as the free lipids, diplopterol or diploptene.

A preliminary attempt was made to separate the polar lipids of the PSC extract to aid in biomarker identification and as a preparative step to carbon isotope analysis. Isolation of individual polar lipids was not possible using one-dimensional thin layer chromatography, because of the complex lipid composition which resulted from both the diversity of polar head groups and the occurrence of both diacyl and dialkyl moieties. Five zones were isolated that accounted for most of the FAME and dialkyl ethers. With the chloroform-methanol-water system, three phospholipid zones, PL-2 (Rf 0.35 to 0.25), PL-3 (Rf 0.40 to 0.48) and PL-4 (Rf 0.60 to 0.56) which roughly co-migrated with diacylphosphatidylcholine (Rf 0.33), diacylphosphatidylglycerol (Rf 0.49) and diacylphosphatidylethanolamine (Rf 0.66), accounted for 39%, 4% and 12% of the total FAME, respectively. All three PL zones showed the presence of multiple components and PL-4 was dominated by an aminolipid similar to *A. pyrophilus* [27]. TLC of the neutral lipid with the acetone-benzene-water system separated a glycolipid zone that migrated closely with a digalactosyl diglyceride standard (Rf 0.43) and contained an additional 6% of the FAME, and a fast migrating component (Rf 0.95), a probable free glyceride fraction designated NL-2, that accounted for an additional 36% of total FAME. The GDE were recovered in PL-3, PL-4 and NL-2 (15, 60 and 25%, respectively). While recovery of FAME and GDE were in good agreement with analyses made using the TLE, recovery of GME were poor and gave no indication whether these compounds were present as unsubstituted 1-*O*-alkylglycerols or substituted at C-2 with an ester-linked fatty acid.

Lipid fractions as described above were also prepared from the total lipid of *T. ruber* (experiment 1). In this case, most of the FAME were recovered from two fractions equivalent to PL-2 and PL-4 with 24% and 70%, respectively. A small amount of FAME was also recovered from the glycolipid and glyceride zones as described above. No GDE was detected in any of the isolated fractions, and although most of the recovered GME (88%) was also recovered in PL-2 and PL-4, the recovery was not significant (195 nmol/gm dry wt) relative to the amount measured by the direct acid hydrolysis procedure (Table 2). This discrepancy appears to be associated with the use of TLC to separate the polar compounds and not procedure I. as relatively large amounts of GME were recovered during analysis of the additional *Aquificales* cultures using this procedure.

Carbon isotopic composition. The water of the Octopus Spring outflow was relatively rich in dissolved inorganic carbon (DIC) measuring 5.3 mM with a $\delta^{13}C$ of –1.5‰. At the temperature and pH of the 'pink streamer' site, a dissolved CO_2 of 71 μM and $\delta^{13}C$ of –4.7‰ can be calculated based on a pKa for bicarbonate-CO_2 at 90°C of –6.42 and the equation of Mook et al [46] where the discrimination between dissolved CO_2 (d) and dissolved bicarbonate (b) can be expressed: $\varepsilon_{d/b}$ = 24.12‰ - 9866/T with T as the absolute temperature. The streamer biomass and TLE from this site exhibited ^{13}C-depletions relative to the dissolved CO_2 with values for $\Delta\delta^{13}C_{CO2}$ of –10.9‰ and –17.2‰ for biomass and lipid, respectively (Table 3).

For the C-isotope experiment, *T. ruber* was grown by continous flow of a gas mixture containing 20% CO_2 through the fermenter vessel. The culture media sample removed prior to inoculation measured 14.1 mM DIC with a $\delta^{13}C$ of –25.5‰. At the temperature and pH of the culture fluid, a value for the dissolved CO_2 of 6.2 mM and a $\delta^{13}C$ of –27.4‰ was calculated as above. The carbon available from the high flow rates far exceeded the carbon recovered in biomass assuring maintenance of an open system. The *T. ruber* culture was much less depleted than the PSC (Table 3) with $\Delta\delta^{13}C_{CO2}$ for biomass and lipid extract of –3.3‰ and –2.1‰, respectively.

The apparent ^{13}C-discrimination was much greater when *T. ruber* was grown with 0.1% formate with a 19.7‰ depletion for biomass relative to carbon source (Table 3). The biomass yield from 250-l of medium was 2.5 g dry wt (39.2% carbon) which accounted for 0.18% of available carbon.

Growth on either carbon substrate resulted in a similar depletion pattern for the total lipid relative to biomass, a slight enriched of 1.2‰ for CO_2 and 1.7‰ for formate growth.

Carbon isotopic composition of several individual lipid biomarkers was also determined (Table 4). The FAME in the PSC fractions appeared to be composed of two isotopically distinct groups. Among the peaks with sufficient material for isotopic measurement, the *iso*-branched FAME (*i*-$C_{17:0}$, *i*-$C_{18:0}$, *i*-$C_{19:0}$) in PL-2, PL-4 and NL-2 were distinctly more depleted in ^{13}C than the longer chain C_{20} and *cy*-C_{21} FAME with $\Delta\delta^{13}C_{FA\text{-}CO2}$ averaging –22.6‰ ± 0.4 (n=8) for *iso*-branched vs. –18.4 ‰ ± 1.4 (n=8) for $C_{20\text{-}21}$. Likewise, those fractions having higher *iso*-branched content (PL-2 and NL-2) were more depleted than PL-4 which had a high *cy*-C_{21} content.

DISCUSSION

Microbial communities are composed of organisms living in close association, dominated by a few microorganisms well adapted to their environment, with physiologies compatible with

available energy and carbon sources. Since the chemolithoautotrophic origins and early diversification of microorganisms in a hot environment [33, 63], the evolution of microbial life on Earth has been intimately linked to these ecosystems. Numerous molecular studies that depend on small subunit rRNA sequences attest to the microbial diversity that persists in globally dispersed thermal ecosystems today, and provide a window on our past. These studies, together with physiological information gained from culture isolates, are often used to infer ecosystem function [13]; however, molecular techniques that rely on organic and stable isotope composition can also aid in establishing structural and functional relationships.

A previous study of the 'pink streamers' of Octopus Spring using molecular techniques based on 16S rRNA sequences, identified three phylogenetic types, EM3, EM17 and EM19, from amplification of the mixed population DNA [56]. All three sequences diverge deeply within the domain Bacteria. A phylogenetic tree constructed taking advantage of a current, more extensive sequence data base (Fig. 2) confirms that the EM 17 gene sequence clusters among the *Aquificales* and is closely related (99% sequence identity) to the pink streamer isolate, *Thermocrinis ruber* [23], while the EM3 sequence is related to the *Thermotogales*. EM19, however, forms a separate more deeply diverging lineage, well outside the *Aquificales* and *Thermotogales*. In Reysenbach's study [56], the EM17 sequence represented the majority of examined clones (26 out of 35), and a fluorescently labeled oligonucleotide probe complementary to EM17 hybridized *in situ* to the pink filaments. However, no hybridization was noted for EM3 or EM19 probes.

Lipid analysis of the pink streamers of the Octopus Spring outflow channel were characterized by high levels of *iso*-branched and cyclopropane ester-linked fatty acids, and straight chain ether-linked alkyl lipids (Table 2). Similar lipids were associated with the biofilm growing on the siliceous sinter walls of the vent pool (Fig.1). The isolation of one polar fraction enriched in *iso*-branched and another enriched in *cy*-C_{21} fatty acids (Table 3), together with the analysis of *T. ruber* lipids (Table 2) suggested the presence of at least two distinct bacterial populations.

To date, *T. ruber* OC 1/4 is the only cultivated isolate from the PSC [23, 26]. The fatty acid composition of *T. ruber* is similar to that reported previously for a number of *Hydrogenobacter thermophilus* strains [35], and to the additional *Aquificales* cultures analyzed here (Table 2). The fatty acids of these *Aquificales* are dominated by *n*-$C_{18:0}$, *n*-$C_{20:1}$ and *cy*-C_{21} (Table 2). Two sets of monounsaturated isomers were detected, $C_{18:1\Delta9}$ and $C_{18:1\Delta11}$ and their chain elongation products, $C_{20:1\Delta11}$ and $C_{20:1\Delta13}$, with either a *cis* or *trans* configuration. As cyclopropane fatty acids are formed by the addition of a methylene group from *S*-adenosylmethionine across the double bond of a monounsaturated fatty acid, the two *cy*-C_{21} isomers are probable homologues of the *n*-$C_{20:1}$ isomers. Cyclopropane and *trans* fatty acids

are synthesized by a wide variety of bacteria as an postsynthetic in vivo modifications of membrane phospholipids in response to growth condition and/or as a strategy for adaptation to environmental stress [12, 36, 43]. Direct conversion of highly fluid *cis* unsaturated fatty acids to a more rigid cyclopropane ring or *trans* configuration are important short-term adaptive mechisms to maintain membrane integrity particularly for microorganisms living in a thermal environment. C_{20} fatty acids are rare in bacteria, and the presence of large amounts of the *n*-$C_{20:1}$ and cy-C_{21}, with lesser amounts to *n*-$C_{20:0}$, *n*-$C_{21:0}$, *n*-$C_{22:0}$ and *n*-$C_{22:1}$ in respresentatives of four distinct subclusters within the *Aquificales* (Fig. 2, Table 2) suggest that these fatty acids can serve as a taxonomic signature for this order. Notably, the 'pink streamers' also contained similar C_{20}, C_{21} and C_{22} FAME. Based on a value for *T. ruber* of 2.1 x 10^{-13} gm dry wt cell^{-1} and an average value of 57 µmol C_{20-22} FAME gm^{-1} dry wt in *T. ruber* cultures (Table 2), the amount of the C_{20-22} FAME present in the PSC suggest a population of ~1.6 x 10^{13} *T. ruber*-like cells gm^{-1} dry wt. No similar calculation is possible for the population(s) represented by the *iso*-branched FAME, although the fatty acid abundance suggests a biomass similar to that of the *Aquificales* component in this community.

The importance of non-isoprenoid, alkyl glycerol ethers as membrane lipids among members of the domain Bacteria is becoming increasingly appreciated. In many respects, these non-isoprenoid alkyl ethers are similar to the glycerol diphytanyl ethers found in the domain Archaea. In addition to the GME and GDE with the *n*-C_{16-18} alkyl chains previously described in *A. pyrophilus* [27], GME and GDE with *iso*- and *anteiso*-branched chains have also been identified as major membrane lipids in two anaerobic thermophiles, a sulfate reducer, *Thermodesulfotobacterium commune* [38], and a novel nitrate reducer, *Ammonifex degensii* [24]. An unusal glycerol monoether with a dimethyltriacontanoic acid chain has been identified in another thermophile, *Thermotoga maritima* [8]. Additionally, small amounts of glycerol monoethers having normal- or methylbranched-chains have been detected in mesophilic and thermophilic clostridia where they are most probably found as 1-*O*-alkyls substituted at carbon 2 with an ester-linked fatty acid [39]. Environmental analyses have identified small amounts of *n*-C_{18} and *br*-C_{17} 1-*O*-alkyl ethers and a C_{15}:C_{15} 1,2-*O*-alkyl ether in hot spring cyanobacterial mats [72, 73], and more recently, relatively abundant *n*- and *br*-C_{15-18} 1-*O*-alkyls in association with anaerobic methane-oxidizing consortia in marine sediments [20].

All of the *Aquificales* cultures in our study (Table 2) synthesize alkyl ether lipids to some degree although with the exception of *A. pyrophilus* and *A. aeolicus*, these alkyls were present at much lower abundances than the ester-linked fatty acyls. Only small amounts of the GME was detected in *H. thermophilus* and the "*Thermocrinis* sp.HI" which was isolated from a greyish-white streamer mat in Iceland. While the *T. ruber* cultures contained somewhat higher amounts of the monoethers, there was only a suggestion of the presence of the diether

C_{18} in two of the cultures (Table 2). Both *A. pyrophilus* and *A. aeolicus* contained GME and GDE. The *A. pyrophilus* ether lipids most closely approximated the distribution of the PSC and vent ether alkyl lipids. To date, however, the only identified *Aquifex* spp. are marine bacteria. The presence of a similar diether alkyl chain distribution in the PSC, and the apparent lack of diethers in *T. ruber* suggest that the synthesis of these diethers is under some environmental control not readily expressed in the laboratory grown cultures, or that an additional *Aquifex*-like organism was present in the streamer and vent biofilm communities.

Although our information about the OS vent community is somewhat limited by the biomass available in this study, our analysis suggests that those organisms represented by the *iso*-branched FAME are less abundant than the *Aquificales* in the biofilm population, a possible result of a lower thermal growth range for the *iso*-group. *Iso*-$C_{17:0}$, *iso*-$C_{18:0}$ and *iso*-$C_{19:0}$ are abundant in the PSC, and while present in the vent biofilm, the iso-$C_{19:0}$ is now the only major branched acid and is present in much lower amounts relative to the C_{20-22} FAME and the glycerol ethers representing the *Aquificales*-like community members.

An increase in growth temperature can result in a higher proportion of *iso*-branched fatty acids and/or acyl chains with greater length. Most of the known bacterial groups with thermophilic representatives contain methylbranched fatty acids [39]. Growth of moderate thermophiles such as *Thermus aquaticus* at higher temperatures has been shown to result in a shift from synthesis of *iso*-C_{15} to the longer chain *iso*-C_{17} [19], and in the more extremely thermophilic *Thermus thermophilum* grown, the branched-C_{17} and C_{19} account for 82% of the fatty acid [49]. Similar results have been recorded for the extremely thermophilic *Bacillus* spp. [69], suggesting that the relatively high levels of *iso*-$C_{19:0}$ detected in the PSC and vent biofilm may be a consequence of higher environmental growth temperature and an attempt by the organisms to maintain membrane integrity.

Little is know about the magnitude of the carbon isotopic fractionation associated with growth of thermophiles in laboratory pure cultures or natural environments. Most of the information available is limited to characterization of the enzymes of the various CO_2 fixation mechanisms in thermophiles and analogy to C-isotopic studies with their mesophilic counterparts. Four CO_2 fixation pathways have been described. Three of these, the reductive pentose phosphate or Calvin cycle, the reductive citric acid cycle, and the reductive acetyl coenzyme A (CoA) pathway, have been reviewed by Fuchs [16]. The fourth, the 3-hydroxypropionate cycle, has only recently been elaborated [21, 64].

Carbon isotopic discrimination varies widely depending on the mechanism responsible for CO_2 assimilation. Generally, the enzymes of the reductive acetyl CoA pathway express the strongest ^{13}C discriminations with $\Delta\delta^{13}C$ values for biomass relative to CO_2 ranging from –20

to –36‰ [16, 52]. Organisms using the Calvin cycle and ribulose-1,5-bisphosphate carboxylase (Ribisco) for CO_2 incorporation display somewhat less discrimination and depending on whether the bacterium expresses Form I or II Ribisco [57], $\Delta\delta^{13}C_{CELL}$ will range from –11 to –26‰ [16, 17, 52, 53, 61]. A broad range of values has also been reported for the diverse metabolic group of organisms known to use the reverse citric acid cycle having $\Delta\delta^{13}C_{CELL}$ of –3.5 to –12.2‰ [16, 52, 53, 61]. For the 3-hydroxypropionate cycle, only one value of –13.7‰ is available for a phototroph, *Chloroflexus aurantiacus*, grown autotrophically at 55°C [22].

The presence of the enzymes of the reductive citric acid cycle have been identified in three *Aquificales*, *A. pyrophilus*, *A. aeolicus* and *H. thermophilus* [3, 9, 60], however, no information has been available about the C-isotopic discrimination associated with growth of these autotrophic bacteria. Our results suggest that the ^{13}C-discrimination expressed by *T. ruber* cells grown autotrophically at 85°C is –3.3‰. Although this discrimination seems low, the carbon available during growth does not suggest a ^{12}C-limiting or closed-system effect (ref.), and it does approach some values previously reported for *Chlorobium* spp. using the reverse citric acid cycle [53].

The carbon isotopic composition of the PSC with a $\Delta^{13}C_{BIOMass}$ of –10.8‰ relative to CO_2 is much more depleted than suggested by the depletion measured for growth of *T. ruber* (-3.3‰), even though the available CO_2 level in the culture vessel was almost two orders of magnitude higher than in the alkaline outflow waters. This conclusion is supported by the highly depleted PSC lipids. In particular, the PSC $\delta^{13}C$ values for the *Aquificales* biomarker, the C_{20-21} fatty acids ($\Delta^{13}C_{FA\text{-}CO2}$ of –18.4‰), suggest a relatively depleted *Thermocrinis* biomass (perhaps $\Delta^{13}C_{BIOM\text{-}CO2}$ ~ -25‰), which based on the results of our pure culture work, is more likely a consequence of growth on formate (Table 3, Fig 3).

The geothermal outgassing of Octopus Spring vent provides a relatively nutrient-limited environment. Hydrogen is thought to be a primary electron source and to support this ecosystem [56]. Oxygen would be limited by its solubility at these high temperatures, and indeed, *T. ruber* is a microaerophile, growing optimally with 3% O_2 [23]. Small amounts of sulfide (9μM), sulfate (240μM) and nitrate (5μM) are also present [4]. Formic acid has been identified in many hydrothermal and geological fluids, where it may form at high temperatures from CO_2 and H_2 [44], and could provide an alternate carbon source in the Octopus Spring vent waters. *T. ruber* is the only member of the *Aquificales* to grow either autotrophically with H_2 and O_2, or as a chemoorganoheterotroph with O_2 [23]. It is of interest that initial PSC enrichment attempts with hydrogen were unsuccessful, and that *T. ruber* was isolated from a mixed organic acid enrichment [23]. Growth with organic substrates is, however, limited to formate or formamide suggesting a relatively specific metabolic potential. The nature of this metabolic capacity is unknown [23].

The PSC is characterized by high amounts of phospholipids with relatively ^{13}C-depleted *iso*-branched fatty acids which presumably represent the non-*Thermocrinis* PSC population (Fig. 3 *iso*-biomass). No hyperthermophiles have been described with such a lipid composition. *Thermus* spp., which have been identified in amorphous silica deposits in geothermal water in Japanese power stations [28], are known to contain principally *iso*-branched fatty acids [49]; however, these bacteria are heterotrophs. Some of the more extremely thermophilic *Bacillus* spp. also contain appropriate *iso*-branched fatty acids and at least some of these may be chemolithoautotrophs [1, 69]. Other thermophiles which are chemolithoautotrophs, such as *Thermothrix* spp., contain only straight chain fatty acids [48].

This putative *iso*-group would also be somewhat unusual in the isotopic relationship between its biomass and fatty acids. An alternate heavy source of organic carbon must be present in the PSC in order to isotopically balance the 'light' *Thermocrinis*(Fig. 3). From the lipid composition, an estimate of '*iso*-biomass' abundance would be from 40 to 50% with *Thermocrinis* representing the remaining carbon. If *Thermocrinis* has a $\delta^{13}C$ of ~ -25‰, an *iso*-biomass with a $\delta^{13}C$ of –1 to –6‰ results (Fig. 3). The *iso*-fatty acids, however, were much more depleted with a $\delta^{13}C$ of –27.3‰ (Fig 3). Fatty acid synthesis in bacteria which use pyruvate dehydrogenase for synthesis of acetyl CoA generally results in fatty acids depleted relative to biomass by about 3‰ [10, 45]. Examples of larger relative depletions associated with fatty acid synthesis have been reported for bacteria using the serine pathway and a lyase mechanism for synthesis of acetyl CoA. In *Shewanella putrefaciens* grown anaerobically a $\Delta\delta^{13}C_{CELL\text{-}FA}$ of as much as –10‰ can result, while in the type II methanotroph, *Methylosinus trichosporium*, as much as –13‰ has been reported [32, 66]. The difference between the *iso*-fatty acids and the calculated $\delta^{13}C$ for associated biomass appears too great, and suggests the presence of a novel heavy component associated with the PSC and/or the *iso*-biomass.

Unfortunately our knowledge of the consequences of isotope discrimination associated with the biosynthesis of metabolic pools and lipid biomarkers is limited. Such information, when available, is normally acquired under optimal laboratory growth conditions and may not be appropriate for extrapolation to growth in natural environments. Explanations of the isotopic profile associated with the components of the 'pink streamer community' based on our pure culture work with *T. ruber* are not necessarily compeling. Information is needed about the nature of a formate source in Octopus Spring water, the potential nature of a 'heavy component' associated with the PSC filaments, the apparent lack of dialkyl glycerol ethers in *T. ruber*, and the physiological mechanisms leading to the novel fractionations associated with its growth on CO_2 and formate in order to fully assess the PSC on physiological, structural and ecosystem levels. What is apparent from our study is that the combination of lipid biomarker and stable carbon isotopic analysis can provide semi-quantitative information on the

composition of microbial ecosystems. Gene probes are not quantitative and do not reveal everything about the structure of microbial communities. A comprehensive lipid biomarker profile can contribute enormously to identifying the presence and nature of uncultivated organisms. Therefore, lipid data, including structural and isotopic features, is an extremely valuable tool for establishing community structure and physiology, and a useful supplement and complement to genomic profiles of microbial diversity.

ACKNOWLEDGEMENTS

Tsege Embaye (ARC), Kendra Turk (ARC), Sean Sylva (WHOI) and Thomas Hader (Regensburg) provided technical assistance. We thank Mitch Schulte for useful discussions on the geothermal chemical mechanisms. We are gratful to Karl O. Stetter for stimulating and critical discussions. The work of Linda Jahnke and David Des Marais was supported by grants from NASA's Exobiology Program. The work of Wolfgang Eber was financially supported by the Fonds der Chemischen Industrie (to KOS). Roger Summons and Janet Hope publish with the permission of the CEO, AGSO.

1. **Aragno, M.** 1992. Aerobic, chemolithoautotrophic, thermophilic bacteria, p. 77-103. *In* J.K. Kristjansson, (ed.). Thermophilic Bacteria. CRC Press, Inc., Boca Raton.

2. **Bauman, A.J. , and P.G. Simmonds.** 1969. Fatty acids and polar lipids of extremely thermophilic filamentous bacterial masses from two Yellowstone hot springs. J. Bacteriol. **98**:528-531.

3. **Beh, M., G. Strauss, R. Huber, K.-O. Stetter, and G. Fuchs.** 1993. Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and the archaebacterium *Themoproteus neutrophilus*. Arch. Microbiol. **160**:306-311.

4. **Brock, T.D.** 1978. Thermophilic microorganisms and life at high temperatures. p. Pages. Springer-Verlag, New York, N.Y.

5. **Brock, T.D.** 1995. The road to Yellowstone–and beyond. Annu. Rev. Microbiol. **49**:1-28.

6. **Cady, S.L. , and J.D. Farmer.** 1996. Fossilization processes in siliceous thermal springs: trends in preservation along thermal gradients, p. 150-173. *In* Evolution of hydrothermal ecosystems on Earth (and Mars?). Wiley, Chichester.

7. **Christie, W.W.** 1989. Gas Chromatography and Lipids: a practical guide, The Oily Press, Alloway, Scotland.

8. **De Rosa, M., A. Gambacorta, R. Huber, V. Lanzotti, B. Nicolaus, K.O. Stetter, and A. Trincone.** 1988. A new 15,16-dimethyl-30-glyceryloxytriacontanoic acid from lipids of *Thermotoga maritima*. J. Chem. Soc., Chem. Comm. **19**:1300-1301.

9. **Deckert, G., P.V. Warren, T. Gaasterland, W.G. Young, A.L. Lenox, D.E. Graham, R. Overbeek, M.A. Snead, M. Keller, M. Aujay.** 1998. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature **392**:353-358.

10. **DeNiro, M.J. , and S. Epstein.** 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Science **197**:261-263.

11. **Des Marais, D.J., Y. Cohen, H. Nguyen, M. Cheatham, T. Cheatham, and E. Munoz.** 1989. Carbon isotopic trends in the hypersaline ponds and microbial mats at Guerrerro Negro, Baja California Sur, Mexico: Implications for Precambrian stromatolites, p. 191-203. *In* Y. Cohen and E. Rosenberg, (ed.). Microbial Mats: Physiological Ecology of Benthic Microbial Communities. American Society for Microbiology, Washington, D.C.

12. **Diefenbach, R. , and H. Keweloh.** 1994. Synthesis of trans unsaturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids. Arch. Microbiol. **162**:120-125.

13. **Eber, W., L.L. Jahnke, M. Schmidt, and R. Huber.** 2001. Microbial diversity of the brine-seawater interface of the Kebrit Deep, Red Sea, studied via 16S rRNA gene sequences and cultivation methods. Appl. Environ. Microbiol. **submitted**:.

14. **Farmer, J.D. , and D.J. Des Marais.** 1999. Exploring for a record of ancient Martian life. J. Geophys. Res. **104**:26,977-26,995.

15. **Freeman, K.H., J.M. Hayes, J.-M. Trendel, and P. Albrecht.** 1990. Evidence from carbon isotope measurements for diverse origins of sedimentary hydrocarbons. Nature **343**:254-256.

16. **Fuchs, J.** 1989. Alternative pathways of autotrophic CO_2 fixation, p. 365-382. *In* H.G. Schlegel and B. Bowien, (ed.). Autotrophic Bacteria. Science Tech, Madison, Wis.

17. **Guy, R.D., M.L. Fogel, and J.A. Berry.** 1993. Photosynthetic fractionation of the stable isotopes of oxygen and carbon. Plant Physiol. **101**:37-47.

18. **Hayes, J.M., D.J. Des Marais, D.W. Peterson, D.A. Schoeller, and S.P. Taylor.** 1977. High precision stable isotope ratios from microgram samples. Adv. Mass Spectrom. **7**:475-480.

19. **Heinen, W., H.P. Klein, and C.M. Volkmann.** 1970. Fatty acid composition of *Thermus aquaticus* at different growth temperatures. Arch. Mikrobiol. **72**:199-2022.

20. **Hinrichs, K.-U., R.E. Summons, V. Orphan, S.P. Sylva, and J.M. Hayes.** 2000. Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments. Org. Geochem. **31**:1685-1701.

21. **Holo, H.** 1989. *Chloroflexus aurantiacus* secretes 3-hydroxypropionate, a possible intermediate in the assimilation of CO_2 and acetate. Arch. Microbiol. **151**:252-256.

22. **Holo, H. , and R. Sirevåg.** 1986. Autotrophic growth and CO_2 fixation of *Chloroflexus aurantiacus.* Arch. Microbiol. **145**:173-180.

23. **Huber, R., W. Eder, S. Heldwein, G. Wanner, H. Huber, R. Rachel, and K.O. Stetter.** 1998. *Thermocrinis ruber* gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. Appl. Environ. Microbiol. **64**:3576-3583.

24. **Huber, R., P. Rossnagel, C.R. Woese, R. Rachel, T.A. Langworthy, and K.O. Stetter.** 1996. Formation of ammonium from nitrate during chemolithoautotrophic growth of the extremely thermophilic bacterium *Ammonifex degensii* gen. nov. sp. nov. represents a novel

group of marine hyperthermophilic hydrogen-oxidizing bacteria. System. Appl. Microbiol. **19**:40-49.

25. **Huber, R. , and K.O. Stetter.** 1992. The order *Thermotogales*. p. 3809-3815. *In* A. Balows, H.G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer. (ed.). The Prokaryotes. Springer-Verlag. Berlin.

26. **Huber, R. , and K.O. Stetter.** 1999. *Aquificales*, http://www.els.net, *In* Embryonic encyclopedia of life sciences. Nature Publishing Group. London, England.

27. **Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. Koenig, R. Rachel, I. Rockinger, H. Fricke, and K.O. Stetter.** 1992. *Aquifex-pyrophilus* new-genus new-species represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. System. Appl. Microbiol. **15**:340-351.

28. **Inagaki, F., S. Hayashi, K. Doi, Y. Motomura, E. Izawa, and S. Ogata.** 1997. Microbial participation in the formation of siliceous deposits from geothermal water and analysis of the extremely thermophilic bacterial community. FEMS Microbiol. Ecol. **24**:41-48.

29. **Jahnke, L.L. , and P.D. Nichols.** 1986. Methyl sterol and cyclopropane fatty acid composition of *Methylococcus capsulatus* grown at low oxygen tensions. J. Bacteriol. **167**:238-242.

30. **Jahnke, L.L., H. Stan-Lotter, K. Kato, and L.I. Hochstein.** 1992. Presence of methyl sterol and bacteriohopanepolyol in an outer-membrane preparation from *Methylococcus capsulatus* (Bath). J. Gen. Microbiol. **138**:1759-1766.

31. **Jahnke, L.L., R.E. Summons, L.M. Dowling, and K.D. Zahiralis.** 1995. Identification of methanotrophic lipid biomarkers in cold-seep mussel gills: Chemical and isotopic analysis. Appl. Environ. Microbiol. **61**:576-582.

32. **Jahnke, L.L., R.E. Summons, J.M. Hope, and D.J. Des Marais.** 1999. Carbon isotopic fractionation in lipids from methanotrophic bacteria II: The effects of physiology and environmental parameters on the biosynthesis and isotopic signatures of biomarkers. Geochim. Cosmochim. Acta **63**:79-93.

33. **Kandler, O.** 1994. The early diversification of life. p. 152-160. *In* S. Bengtson, (ed.). Early Life on Earth. Nobel Symposium No. 84. Columbia U. P., New York.

34. **Kates, M.** 1986. Techniques of lipidology: isolation, analysis and identification of lipids, *In* R.H. Bubon and P.H. van Knippenberg, (ed.). Laboratory techniques in biochemistry and molecular biology. Elsevier, Amsterdam.

35. **Kawasumi, T., Y. Igarashi, T. Kodama, and Y. Minoda.** 1984. *Hydrogenobacter thermophilus* gen. nov., sp. nov., an extremely thermophilic, aerobic, hydrogen-oxidizing bacterium. Int. J. Syst. Bacteriol. **34**:5-10.

36. **Keweloh, H. , and H.J. Heipieper.** 1996. *Trans* unsaturated fatty acids in bacteria. Lipids **31**:129-137.

37. **Kristjansson, J.K., A. Ingason, and G.A. Alfredsson.** 1985. Isolation of thermophilic obligately autotrophic hydrogen-oxidizing bacteria, similar to *Hydrogenobacter thermophilus*, from Icelandic hot springs. Arch. Microbiol. **140**:321-325.

38. **Langworthy, T.A., G. Holzer, J.G. Zeikus, and T.G. Tornabene.** 1983. *Iso*- and *anteiso*-branched glycerol diethers of the thermophilic anaerobe *Thermodesulfotobacterium commune*. System. Appl. Microbiol. **4**:1-17.

39. **Langworthy, T.A. , and J.L. Pond.** 1986. Membranes and lipids of thermophiles, p. 107-135. *In* T.D. Brock, (ed.). Thermophiles: general, molcular and applied microbiology. John Wiley and Sons, New York.

40. **Ludwig, W.** 1995. Sequence databases (3.3.5), p. 1-22. *In* A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn, (ed.). Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, Niederlande.

41. **Ludwig, W. , and O. Strunk.** 1997. ARB:a software environment for sequence data. http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps.

42. **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner, and K.-H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19**:554-568.

43. **Magnuson, K., S. Jackowski, C.O. Rock, and J. Cronan, J.E.** 1993. Regulation of fatty acid biosynthesis in *Escherichia coli.* Microbiol. Rev. **57**:522-542.

44. **McCollom, T.M., G. Ritter, and B.R.T. Simoneit.** 1999. Lipid synthesis under hydrothermal conditions by Fischer-Tropsch-type reactions. Origins of Life **29**:153-166.

45. **Monson, K.D. , and J.M. Hayes.** 1982. Carbon isotopic fractionation in the biosynthesis of bacterial fatty acids. Ozonolysis of unsaturated fatty acids as a means of determining the intramolecular distribution of carbon isotopes. Geochim. Cosmochim. Acta **46**:139-149.

46. **Mook, W.G., J.C. Bommerson, and W.H. Staberman.** 1974. Carbon isotope fractionation between dissolved bicarbonate and gaseous carbon dioxide. Earth Planetary Science Letters **22**:169-176.

47. **Nichols, P.D., C.A. Mancuso, and D.C. White.** 1987. Measurement of methanotroph and methanogen signature phospholipids for use in assessment of biomass and community structure in model systems. Org. Geochem. **11**:451-461.

48. **Odintsova, E.V., H.W. Jannasch, A. Mamone, and T.A. Langworthy.** 1996. *Thermothrix azorensis* sp. nov., an obligate chemolithoautotrophic sulfur-oxidizing. thermophilic bacterium. Int. J. Syst. Bacteriol. **46**:422-428.

49. **Oshima, M. , and A. Miyagawa.** 1974. Comparative studies on the fatty acid composition of moderately and extremely thermophilic bacteria. Lipids **9**:476-480.

50. **Pohl, P., H. Glasl, and H. Wagner.** 1970. Zur analytik pflanzlicher glyko- und phospholipoide und ihrer fettsäure-zusammensetzung. J. Chromatography **49**:488-492.

51. **Pond, J.L., T.A. Langworthy, and G. Holzer.** 1986. Long-chain diols: A new class of membrane lipids from a thermophilic bacterium. Science **231**:1134-1136.

52. **Preuß, A., R. Schauder, and G. Fuchs.** 1989. Carbon isotope fractionation by autotrophic bacteria with three different CO2 fixation pathways. Z. Naturforsch. **44c**:397-402.

53. **Quandt, L., G. Gottschalk, H. Ziegler, and W. Stichler.** 1977. Isotope discrimination by photosynthetic bacteria. FEMS Microbiol. Lett. **1**:125-128.

54. **Rasmussen, B.** 2000. Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit. Nature (London) **405**:676-679.

55. **Reysenbach, A.-L., M. Ehringer, and K. Hershberger.** 2000. Microbial diversity at 83°C in Calcite Springs, Yellowstone National Park: another environment where the *Aquificales* and "*Korarchaeota*" coexist. Extremophiles **4**:61-67.

56. **Reysenbach, A.-L., G.S. Wickham, and N.R. Pace.** 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. **60**:2113-2119.

57. **Robinson, J.J. , and C.M. Cavanaugh.** 1995. Expression of form I and form II Rubisco in chemoautotrophic symbioses: Implications for the interpretation of stable carbon isotope values. Limnol. Oceanogr. **40**:1496-1502.

58. **Rohmer, M., P. Bouvier-Nave, and G. Ourisson.** 1984. Distribution of hopanoid triterpenes in prokaryotes. J. Gen. Microbiol. **130**:1137-1150.

59. **Setchell, W.A.** 1903. The upper temperature limits of life. Science **17**:.

60. **Shiba, H., T. Kawasumi, Y. Igarashi, T. Kodama, and Y. Minoda.** 1985. The CO_2 assimilation via the reductive tricarboxylic acid cycle in an obligately autotrophic, aerobic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus*. Arch. Microbiol. **141**:198-203.

61. **Sirevåg, R., B.B. Buchanan, J.A. Berry, and J.H. Troughton.** 1977. Mechanisms of CO_2 fixation in bacterial photosynthesis studied by the carbon isotope fractionation technique. Arch. Microbiol. **112**:35-38.

62. **Skirnisdottir, S., G.O. Hreggvidsson, S. Hjörleifsdottir, V.T. Marteinsson, S.K. Petursdottir, O. Holst, and J.K. Kristjansson.** 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. Appl. Environ. Microbiol. **66**:2835-2841.

63. **Stetter, K.O.** 1994. The lesson of Archaebacteria. p. 143-151. *In* S. Bengtson, (ed.). Early Life On Earth. Columbia U. P., New York.

64. **Strauß, G. , and G. Fuchs.** 1993. Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxyprionate cycle. Eur. J. Biochem. **215**:633-643.

65. **Summons, R.E., P.D. Franzmann, and P.D. Nichols.** 1998. Carbon isotopic fractionation associated with methylotrophic methanogenesis. Org. Geochem. **28**:465-475.

66. **Teece, M.A., M.L. Fogel, M.E. Dollhopf, and K.H. Nealson.** 1999. Isotopic fractionation associated with biosynthesis of fatty acids by a marine bacterium under oxic and anoxic conditions. Org. Geochem. **30**:1571-1579.

67. **Tomilinson, G.A., L.L. Jahnke, and L.I. Hochstein.** 1986. *Halobacterium denitrificans*, sp. nov., an extremely halophilic denitrifying bacterium. Int. J. Syst. Bacteriol. **36**:66-70.

68. **Walter, M.R. , and D.J. Des Marais.** 1993. Preservation of biological information in thermal spring deposits: developing a strategy for the search for fossil life on Mars. ICARUS **101**:129-143.

69. **Weerkamp, A. , and W. Heinen.** 1972. Effect of temperature on the fatty acid composition of the extreme thermophiles *Bacillus caldolyticus* and *Bacillus calotenax*. J. Bacteriol. **109**:443-446.

70. **Yamamoto, H., A. Hiraishi, K. Kato, H.X. Chiura, Y. Maki, and A. Shimizu.** 1998. Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. Appl. Environ. Microbiol. **64**:1680-1687.

71. **Yamamoto, K., A. Shibahara, T. Nakayama, and G. Kajimoto.** 1991. Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts. Chem. Phys. Lipids **60**:39-50.

72. **Zeng, Y.B., D.M. Ward, S.C. Brassell, and G. Eglinton.** 1992. Biogeochemistry of hot spring environments 3. Apolar and polar lipids in the biologically active layers of a cyanobacterial mat. Chem. Geol. **95**:347-360.

73. **Zeng, Y.B., D.M. Ward, S.C. Brassell, and G. Eglinton.** 1992. Biogeochemistry of hot spring evironments 2. Lipid compositions of Yellowstone (Wyoming, U.S.A.) cyanobacterial and *Chloroflexus* mats. Chem. Geol. **95**:327-345.

TABLE 1. Growth conditions for *Aquificales* cultures.

Organism	Medium	Gasphase (300 kPa, by volume)	pH	Reference
Aquifex aeolicus VF5	SME*-medium + 0.2% thiosulfate + 0.1% $NaHCO_3$	H_2-CO_2-O_2 = 79:20:1 (gassing)	6.5	[Huber, 1992 #888]
Aquifex pyrophilus Kol5a	SME*-medium + 0.2% thiosulfate + 0.1% $NaHCO_3$	N_2-CO_2-O_2 = 79:20:1 (gassing)	6.5	[Huber, 1992 #888]
Hydrogenobacter thermophilus TK-6	TK-6-medium + 0.1% thiosulfate	H_2-CO_2-O_2 = 79:20:1	7	[Ishii, 1987 #1294]
Thermocrinis ruber OC 1/4 (Exp. 1)	OS-medium + 0.1% thiosulfate	H_2-CO_2-O_2 = 79:20:1 (gassing)	6.5	[Huber, 1998 #1073]
Thermocrinis ruber OC 1/4 (Exp. 2)	OS-medium + 0.1% $NaHCO_3$ + 0.1% thiosulfate	N_2-O_2 = 97:3	6.5	[Huber, 1998 #1073]
Thermocrinis ruber OC 1/4 (Exp. 3)	OS-medium + 0.1% $NaHCO_3$	N_2-O_2-H_2 = 94:3:3	6.5	[Huber, 1998 #1073]
Thermocrinis ruber OC 1/4 (Exp. 4)	OS-medium + 0.1% Na formate	N_2-O_2 = 99:1	6.5	[Huber, 1998 #1073]
Thermocrinis sp. HI 11/12	OS-medium + 0.1% thiosulfate	H_2-CO_2-O_2 = 78:19:3	6.5	[Huber, 1998 #1073]

TABLE 2. Comparison of ester-linked fatty acid and glycerol ether composition of Octopus Spring Pink Streamer Community (PSC) and *Aquificales* cultures[a].

Compound	Distribution (%)							
	PSC	OC 1/4 (Exp 1)	OC 1/4 (Exp 2)	OC 1/4 (Exp 3)	HI 11/12	Kol5a	VF5	TK-6
Fatty Acid[b]								
14:0	<0.1	0.1	nd	0.1	<0.1	<0.1	0.4	<0.1
i15:0	0.2	nd	nd	nd	nd	nd	nd	nd
15:0	<0.1	nd	nd	nd	<0.1	0.1	0.1	<0.1
i16:0	1.1	nd	nd	nd	nd	nd	<0.1	nd
16:1c Δ7	0.1	0.2	0.4	0.4	0.4	0.6	0.5	0.3
16:1c Δ9	0.1	nd	nd	nd	0.1	0.1	0.1	<0.1
16:0	1.9	0.4	1.0	1.2	0.6	2.7	1.2	0.5
i17:0	20.9	nd	nd	nd	nd	nd	nd	nd
a17:0	0.8	nd	nd	nd	nd	nd	nd	nd
17:0	0.8	<0.1	<0.1	<0.1	<0.1	0.2	nd	<0.1
cy17	0.1	nd	nd	nd	nd	nd	nd	nd
i18:0	4.7	nd	nd	nd	nd	nd	nd	nd
18:1 c Δ9	0.6	2.4	4.3	2.3	2.3	4.1	3.7	3.7
18:1c Δ11	0.1	2.6	1.9	0.1	1.1	1.8	2.4	2.6
18:1t Δ9	0.1	0.4	0.3	0.2	0.6	0.7	nd	0.2
18:0	13.6	12.9	27.2	33.4	15.3	27.3	15.2	17.7
i19:0	16.6	nd	nd	nd	nd	nd	nd	nd
a19:0	0.6	nd	<0.1	<0.1	<0.1	0.1	nd	nd
cy19 (2 isomers)	1.8	2.2	0.7	3.9	4.8	2.6	1.8	0.6
19:0	1.3	<0.1	<0.1	0.1	0.1	0.3	nd	<0.1
i20:0	0.6	nd	nd	nd	nd	nd	nd	nd
20:1cΔ11	3.5	22.6	41.9	8.8	10.6	18.0	17.3	48.7
20:1cΔ13	0.3	14.0	1.5	1.1	8.2	1.2	3.7	11.1
20:1*t*Δ11	0.3	9.2	nd	1.1	5.4	5.4	11.1	nd
20:1*t*Δ13	nd	3.7	nd	nd	3.5	nd	nd	nd
20:0	6.6	3.8	5.1	6.9	4.5	5.5	3.4	3.1
i21:0	0.7	nd	nd	nd	nd	nd	nd	nd
cy21 (2 isomers)	21.4	23.1	14.6	39.8	40.4	27.8	38.4	10.9
21:0	0.8	<0.1	<0.1	0.1	0.1	0.1	0.1	nd
22:1c Δ13	0.4	0.6	0.3	0.4	0.8	0.4	0.3	0.3
22:0	0.2	0.3	<0.1	<0.1	<0.1	0.1	<0.1	<0.1
µmol FA/g dwt	5.5	92.2	123.7	92.6	103.5	13.8	35.3	38.8
Glycerol Ether[c]								
GME-18:1	nd	1.8	nd	1.9	trace	3.9	2.2	2.7
GME-18:0	62.4	75.2	90.6	86.7	78.5	57.3	73.0	82.7
GME-19:0	11.5	nd	nd	0.9*	trace	2.5*	0.2*	nd
GME-20:1	nd	13.2	7.0	2.9	17.6	14.4	13.9	11.1
GME-20:0	24.1	4.1	2.5	5.1	2.0	13.8	10.3	3.5
GME-21:1	nd	3.8	nd	2.6	1.9	8.1	0.5	nd
µmol GME/g dwt	3.0	5.9	1.7	4.5	0.9	3.1	19.3	1.2
GDE-17:0,17:0	0.7	nd	nd	nd	nd	8.9	nd	nd
GDE-17:0,18:0	0.8	nd	nd	nd	nd	2.1	nd	nd
GDE-18:0,18:0	33.0	nd	trace	trace	nd	34.5*	32.2	nd
GDE-18:0,19:0	7.7	nd	nd	nd	nd	10.0	2.5	nd
GDE-18:0,20:0	16.2	nd	nd	nd	nd	13.5	9.1	nd
GDE-18:0,21:1	33.1	nd	nd	nd	nd	23.4	44.9	nd
GDE-20:0,20:0	3.1	nd	nd	nd	nd	7.7	11.2	nd
GDE-19:0,21:1	3.5	nd	nd	nd	nd	nd	nd	nd
µmol GDE/g dwt	1.2	nd	trace	trace	nd	1.1	0.6	nd

[a] For culture identification and growth condition see Table 1.

[b] FAME prepared by procedure I as described in methods. Acyl-alkyl chain nomenclature designates carbon number as saturated (:0) or monounsatured (:1) with double bond of *cis* (*c*) or *trans* (*t*) configuration and position relative to carboxyl end (Δ); or cyclopropyl ring (cy), with *iso*- (i) and *anteiso*- (a) methyl branching.

[c] GME, GDE results for PSF and Thermocrinis (1) from procedure II, remaining samples from procedure I.

* Designates inclusion of a minor, unresolved peak with unsaturated mass spectrum (:1 or cy).

Table 3. The C-isotopic composition of isolated components from the 'Pink Streamer Community' (PSC) and *Thermocrinis ruber* grown as a lithoautotroph with thiosulfate-H_2-O_2-CO_2 and as a chemoorganotroph with formate and O_2.

Sample	Carbon Source	$\delta^{13}C$ (‰)	Component	$\Delta\delta^{13}C_{component-source}$ (‰)
PSC	CO_2	-4.7	Biomass	-10.9
			Extracted Residue	-10.5
			Total Lipid	-17.2
T. ruber	CO_2	-27.4	Biomass	-3.3
			Extracted Residue	na
			Total Lipid	-2.1
T. ruber	Formate	-23.3	Biomass	-19.7
			Extracted Residue	-21.0
			Total Lipid	-17.9

TABLE 4. Stable carbon isotopic composition ($\Delta\delta^{13}C$ relative to CO_2, ‰)[a] and the distribution (%) of fatty acids in the major lipid fractions isolated from 'Pink Streamer Community' (PSC) and *Thermocrinis ruber*.

Fatty Acid	PSC						*T. ruber*	
	PL-2		PL-4		NL-1		PL-4	
	$\Delta\delta^{13}C$ (‰)	Distribution %	$\Delta\delta^{13}C$ (‰)	Distribution %	$\Delta\delta^{13}C$ (‰)	Distribution %	$\Delta\delta^{13}C$ (‰)	Distribution %
i-17	-22.9	24.9	-22.5	3.2	-22.7	28.6	-	nd[b]
i-18	-23.2	6.4%	nm[b]	0.2	-22.5	5.5	-	nd
18:1	nm	0.5	nm	1.5	nm	0.8	-1.9	3.8
18:1	-	nd	-	nd	-	nd	-2.5	2.9
18:0	-20.0	12.9	-18.5	20.9	-20.2	10.4	-2.0	12.5
i-19	-22.5	18.9	-22.5	2.7	-21.9	26.2	-	nd
cy19	nm	1.5	-18.5	2.7	nm	1.0	+3.5	2.3
19:0	-19.0	2.0	-17.8	4.2	nm	1.1	nm	<0.1
20:1	-16.9	3.0	-15.7	7.6	nm	2.1	-3.2	51.2
20:0	-18.9	5.5	-18.6	8.9	-19.0	6.6	-2.9	3.1
cy21	-19.8	19.1	-19.1	40.6	-19.5	8.4	+2.2	22.5
22:1	nm	0.2	nm	0.7	nm	0.4	-2.5	0.6
Average Composition[c]	-20.4‰		-18.7‰		-21.5‰		-1.2‰	

[a] Pink Streamer site at main vent outflow of Octopus Spring was 87°C, pH 8.3 with a total dissolved inorganic carbon (DIC) of 5.4 mM and $\delta^{13}C$ of –1.5‰ (CO_2 = –4.7‰). *Thermocrinis* culture was grown at 85°C, pH 6.5, constantly gassed with 20% CO_2 under a 300 KPa headspace pressure; DIC measured 14.3 mM with a $\delta^{13}C$ of –25.5‰ (CO_2 = –27.4‰).

[b] Abbreviations: nd, not detected; nm, insufficient material for measurement.

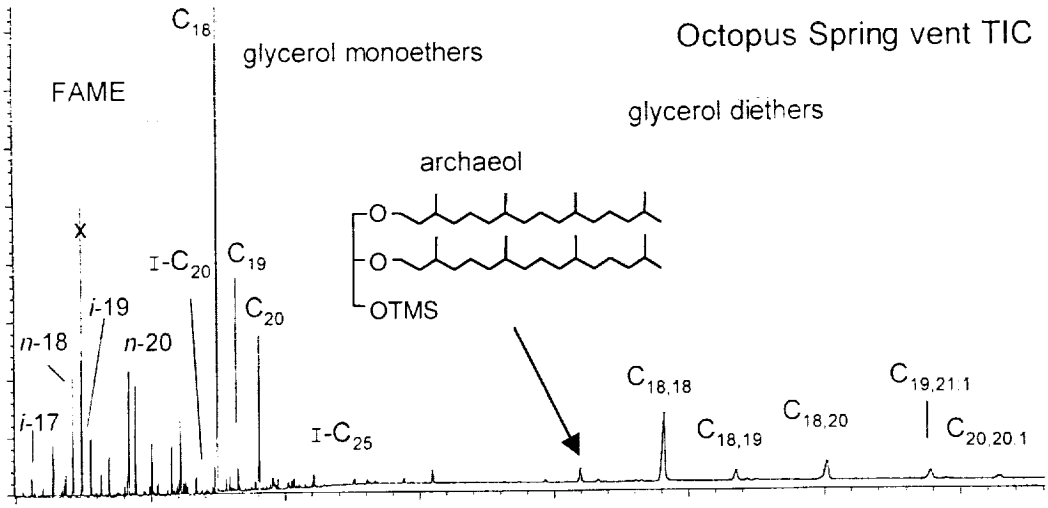
[c] $\Delta\delta^{13}C$ calculated by mass balance for each fraction based on relative abundance and $^{13}C/^{12}C$ ratio for individual fatty acids where $\delta^{13}C$ FAME = (Ratio FAME - Ratio PDB) / Ratio PDB x 1000 = per mil.

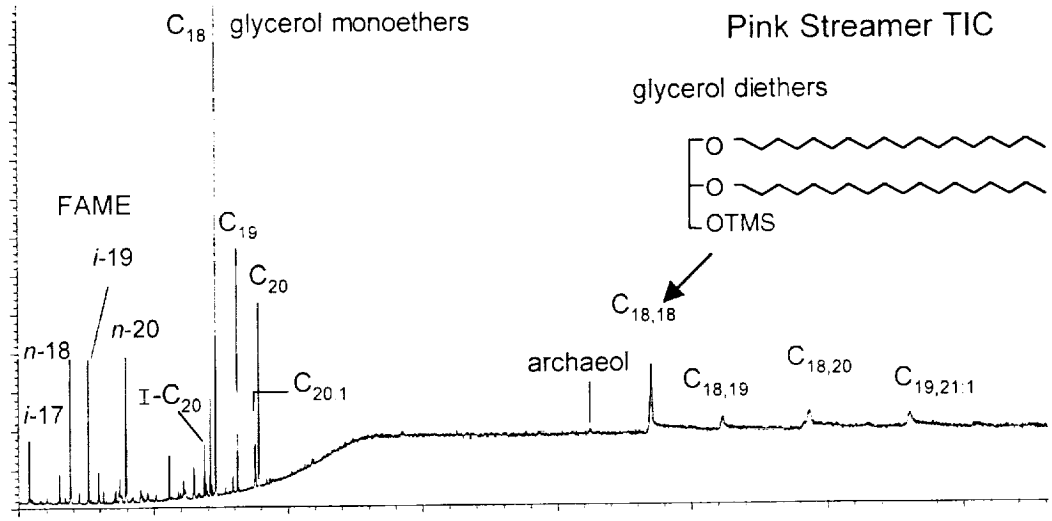
Figure legend

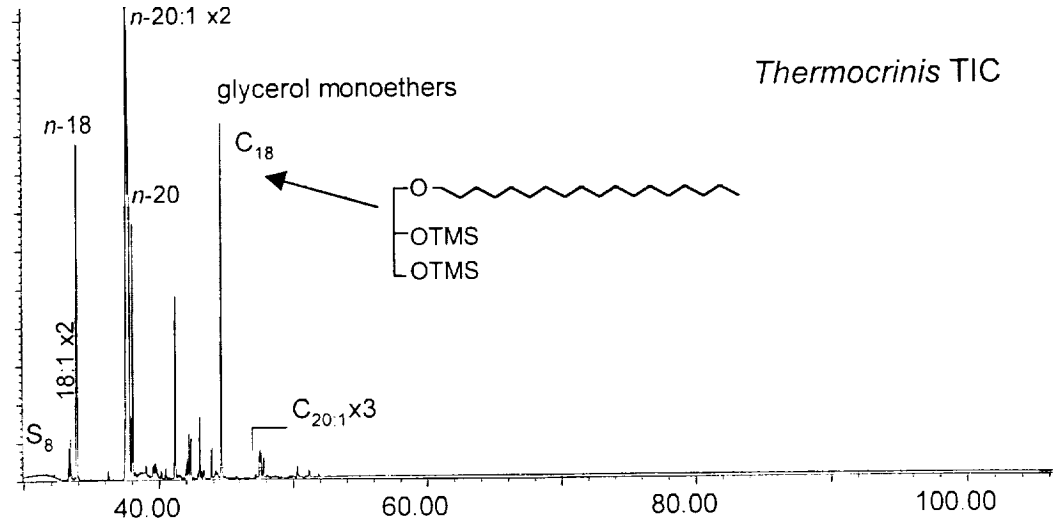
Figure 1. GC-MS analyses of derivatized total lipid extracts of the Octopus Spring PSC, a biofilm on siliceous sinter from within the vent and *T. ruber*. The data illustrate the major compositional differences between the natural and cultivated biomass samples with respect to the presence of dialkyl glycerol diethers and more complex distributions of FAME in the Octopus Spring samples. Symbols designate FAME (*n*- or *i*-18); isoprenoid lipid (I-); monoalkyl glycerol (C_{18}); dialkyl glycerol ($C_{18,18}$).

Figure 2. 16S rRNA gene based phylogenetic tree of the *Aquificales* based on the results of a maximum-likelihood analysis. Reference sequences were chosen to represent the broadest diversity of *Bacteria*. Only sequence positions that share identical residues of 50% or more of all available bacterial 16S rRNA sequences were included for tree reconstruction. Accession numbers for the sequences are indicated. The scale bar represents 0.10 fixed mutations per nucleotide position.

Fig. 3 Proposed carbon isotopic profile for 'Pink Streamer Community' showing measured $\delta^{13}C$ values for isolated PSC components on the left (open boxes). On the right, an assumed value of ~25‰ for *Thermocrinis* biomass is based on the PSC biomarker C_{20} fatty acids and the established relationship between *T. ruber* biomass and fatty acids (solid box). Growth of *Thermocrinis* on formate would require dissimilation of ~80% of formate-C for energy production resulting in release of heavy CO_2. Isotopic value of formate and CO_2 (thatched boxes) and associated fractionations are unknown. In this scenario, mass balance between PSC, *Thermocrinis* and *Iso*-biomasses requires a relatively heavy composition for *Iso*-biomass (thatched box) and leaves unresolved relationship of PSC *iso*-fatty acids to *Iso*-biomass.


Octopus Spring vent TIC
FAME
glycerol monoethers
glycerol diethers
archaeol
OTMS
x
i-17
n-18
i-19
n-20
I-C20
C19
C20
I-C25
C18,18
C18,19
C18,20
C19,21:1
C20,20:1


Pink Streamer TIC
C18 glycerol monoethers
glycerol diethers
OTMS
FAME
i-17
n-18
i-19
n-20
I-C20
C19
C20
C20:1
C18,18
archaeol
C18,19
C18,20
C19,21:1


Thermocrinis TIC
n-20:1 x2
glycerol monoethers
n-18
C18
n-20
OTMS
OTMS
18:1 x2
S8
C20:1x3
40.00
60.00
80.00
100.00

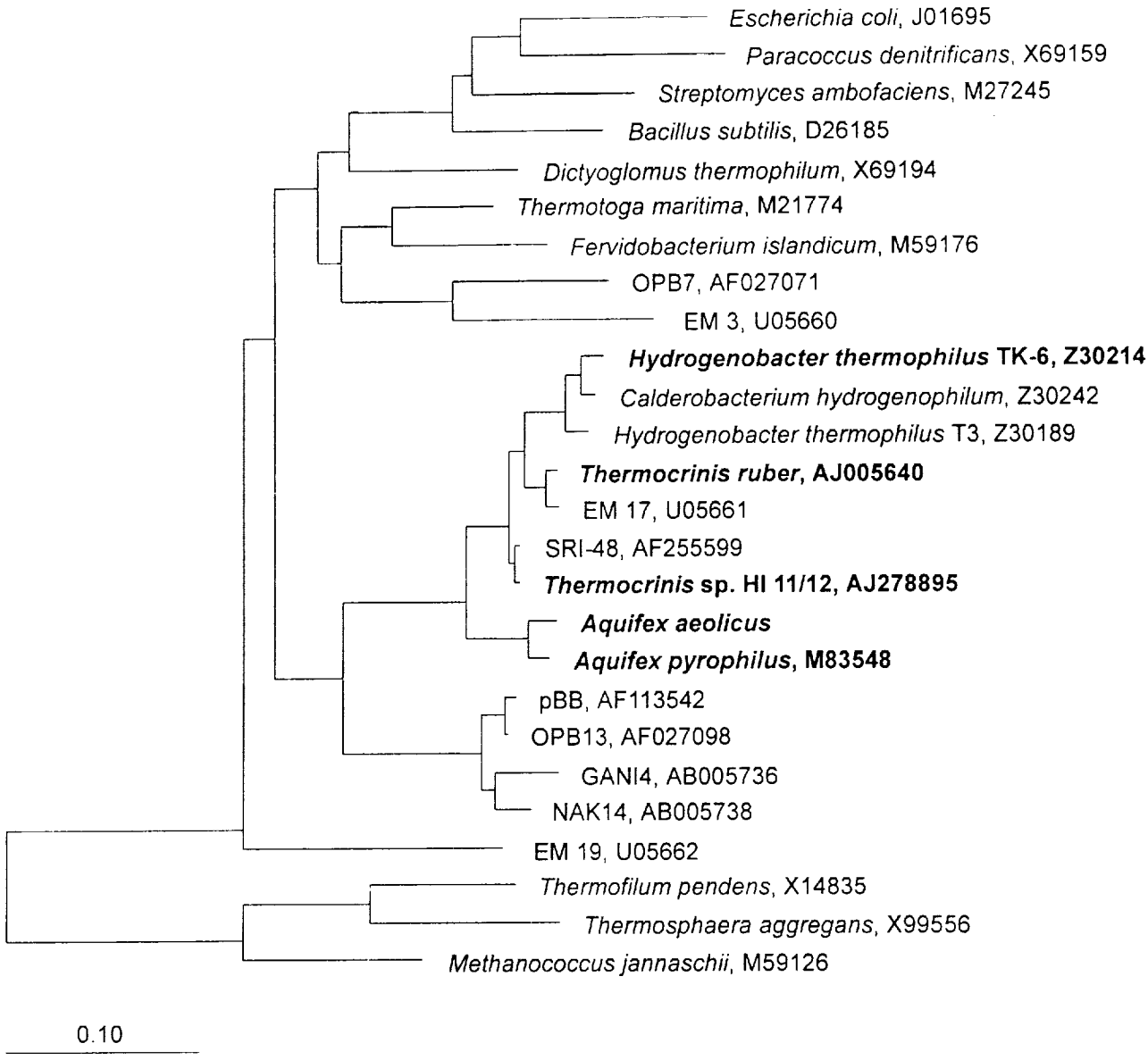

Escherichia coli, J01695
Paracoccus denitrificans, X69159
Streptomyces ambofaciens, M27245
Bacillus subtilis, D26185
Dictyoglomus thermophilum, X69194
Thermotoga maritima, M21774
Fervidobacterium islandicum, M59176
OPB7, AF027071
EM 3, U05660
***Hydrogenobacter thermophilus* TK-6, Z30214**
Calderobacterium hydrogenophilum, Z30242
Hydrogenobacter thermophilus T3, Z30189
***Thermocrinis ruber*, AJ005640**
EM 17, U05661
SRI-48, AF255599
***Thermocrinis* sp. HI 11/12, AJ278895**
Aquifex aeolicus
***Aquifex pyrophilus*, M83548**
pBB, AF113542
OPB13, AF027098
GANI4, AB005736
NAK14, AB005738
EM 19, U05662
Thermofilum pendens, X14835
Thermosphaera aggregans, X99556
Methanococcus jannaschii, M59126
0.10

Fig. 2

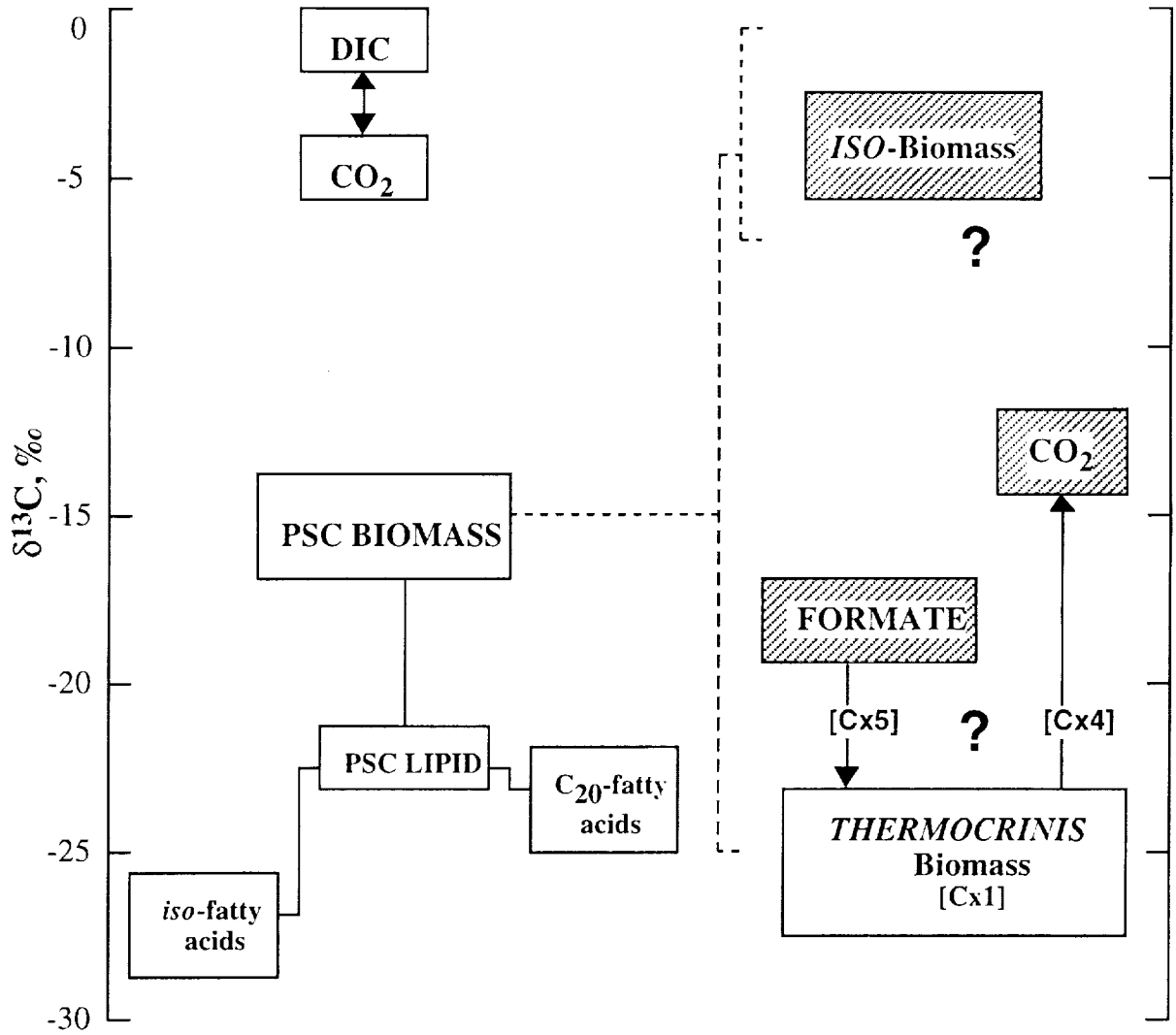

0
-5
-10
-15
-20
-25
-30
δ13C, ‰
DIC
CO2
PSC BIOMASS
PSC LIPID
C20-fatty acids
iso-fatty acids
ISO-Biomass
?
CO2
FORMATE
[Cx5]
?
[Cx4]
THERMOCRINIS Biomass [Cx1]

Fig. 3