Environmental Microbiology (2004) doi:10.1111/j.1462-2920.2004.00650.x

Low archaeal diversity linked to subseafloor geochemical processes at the Lost City Hydrothermal Field, Mid-Atlantic Ridge

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Summary

The recently discovered Lost City Hydrothermal Field (LCHF) represents a new type of submarine hydrothermal system driven primarily by exothermic serpentinization reactions in ultramafic oceanic crust. Highly reducing, alkaline hydrothermal environments at the LCHF produce considerable quantities of hydrogen, methane and organic molecules through chemo- and biosynthetic reactions. Here, we report the first analyses of microbial communities inhabiting carbonate chimneys awash in warm, high pH fluids at the LCHF and the predominance of a single group of methane-metabolizing Archaea. The predominant phylotype, related to the Methanosarcinales, formed tens of micrometre-thick biofilms in regions adjacent to hydrothermal flow. Exterior portions of active structures harboured a diverse microbial community composed primarily of filamentous Eubacteria that resembled sulphide-oxidizing species. Inactive samples, away from regions of hydrothermal flow, contained phylotypes related to pelagic microorganisms. The abundance of organisms linked to the volatile chemistry at the LCHF hints that similar metabolic processes may operate in the subseafloor. These results expand the range of known geological settings that support biological activity to include submarine hydrothermal systems that are not dependent upon magmatic heat sources.

Introduction

The most prominent and best-studied hydrothermal systems in deep-sea environments are restricted to sites proximal to mid-ocean ridge axes and volcanic 'hot-spots' (Kelley *et al*., 2002). Most of these systems are located on very young, basaltic oceanic crust and are driven by cooling of magmatic intrusions beneath the seafloor. However, current estimates indicate that at least 20% of exposed seafloor in tectonically active, slow-spreading ridge environments is composed of ultramafic rocks characteristic of the Earth's shallow mantle (Alt and Shanks, 1998; Escartín and Cannat, 1999; Kelley *et al*., 2001; Dick *et al*., 2003; Früh-Green *et al*., 2004). It is likely that an even greater amount of mantle material is exposed away from the ridge axes. Despite the potentially substantial lateral and vertical extent of an ultramafic-hosted subseafloor biosphere, to date, the linkages between biological and geological processes within such systems have been poorly explored.

Peridotite material characteristic of ultramafic seafloor environments consists of >40% of the iron-bearing mineral olivine, which upon contact with sea water is readily altered by serpentinization processes (Alt and Shanks, 1998; Früh-Green *et al*., 2004). These reactions involve the hydration of olivine and its conversion to serpentine minerals, resulting in an increase in pH, the consumption of oxygen and the production of heat (Lowell and Rona, 2002; Früh-Green *et al*., 2004). Serpentinization reactions can also result in a 20–40% gain in rock volume, aiding in the uplift of altered material and exposure of fresh peridotite surfaces through brittle fracturing (Kelley *et al*., 2001; Früh-Green *et al*., 2003; 2004). Chemical reactions coinciding with the alteration of peridotites produce substantial amounts of hydrogen, which, in combination with iron- and nickel-bearing catalysts in the parent materials, can result in the synthesis of methane and other low-molecular-weight organic compounds (Table 1) (Berndt *et al*., 1996; Shock and Schulte, 1998; Horita and Berndt, 1999; McCollom and Seewald, 2001; Charlou *et al*., 2002). The redox active species produced by serpentinization reactions and the organic molecules created as byproducts have the potential to support a rich and metabolically diverse assemblage of autotrophic microorganisms (Shock and Schulte, 1998; McCollom and Seewald, 2001).

The Lost City Hydrothermal Field (LCHF), discovered in December 2000, represents a new class of submarine

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Table 1. Key bioenergetic parameters of fluids associated with serpentinization.

Constituent or property	Experimental/theoretical[a]	LCHF[b] (30°N)	Rainbow[c] (36°14′N)	Sea water[d]
Temperature (°C)	25–300	40–93	365	7
pH	8–12	9–11	2.8	8
H_2 (mmol kg^{-1})	1–100	0.25–0.43[e]	16	0
CH_4 (mmol kg^{-1})	0.01–1	0.13–0.28[e]	2.5	0
$C_2H_4 + C_3H_6$ (nmol kg^{-1})	<1000	>100[e]	1145	0
H_2S (mmol kg^{-1})	0.1–1	0.064	1.2	0
SO_4 (mmol kg^{-1})	0	5.9–12.9[e]	0	28.6
NO_3 (μmol kg^{-1})	ND[f]	ND[g]	ND	20
CO_2 (mmol kg^{-1})	0	ND	16	2.30
Total Fe (μmol kg^{-1})	1.0	ND	24 050	< 0.001
$CH_4/(C_2H_4 + C_3H_6)$	1000–10 000	100	2183	–

a. Palandri and Reed (2004): simulations run at 25°C and 300°C in fresh water and seawater solutions respectively. Horita and Berndt (1999): experiments conducted at 200°C and 300°C in fresh water solutions.
b. Proskurowski *et al*. (2003; includes C_4C_8); Kelley *et al*. (2001).
c. Charlou *et al*. (2002).
d. D. Butterfield, personal communication (measured for 30°N in the Atlantic Ocean at 700 m depth).
e. H_2, CH_4 and H_2S values are minimum values because of artifacts associated with sample collection and storage. SO_4 values are maxima as a result of the partial oxidation of sulphide during storage and the lack of correction for zero Mg end-member hydrothermal fluids.
f. ND, not determined.
g. Although not measured, NO_3 values of end-member hydrothermal fluids at the LCHF are believed to be well below 20 μmol kg^{-1} because of the highly reducing nature of the system and by comparison with magmatically influenced sites.

hydrothermal system. It is hosted on ≈ 1.5-Myr-old ultramafic oceanic crust, nearly 15 km from the axis of the Mid-Atlantic Ridge (Kelley *et al*., 2001). Owing to the geological setting of the LCHF, its distance from the ridge axis and the distinct geochemical signatures of the venting fluids, exothermic heating derived from subseafloor serpentinization reactions is thought to drive hydrothermal flow within this system (Kelley *et al*., 2001; Früh-Green *et al*., 2003). The LCHF hosts >30 active and inactive edifices (Fig. 1A–C) composed primarily of calcium carbonate (aragonite, calcite) and magnesium hydroxide (brucite) minerals (Kelley *et al*., 2001; Früh-Green *et al*., 2003). The towers are associated with fluids venting from a tectonically uplifted peridotite massif that has been highly altered locally to serpentinite, indicating intensive water–rock interaction in the subseafloor. Isotopic age dating of carbonate minerals from the LCHF indicates that this site has been hydrothermally active for at least

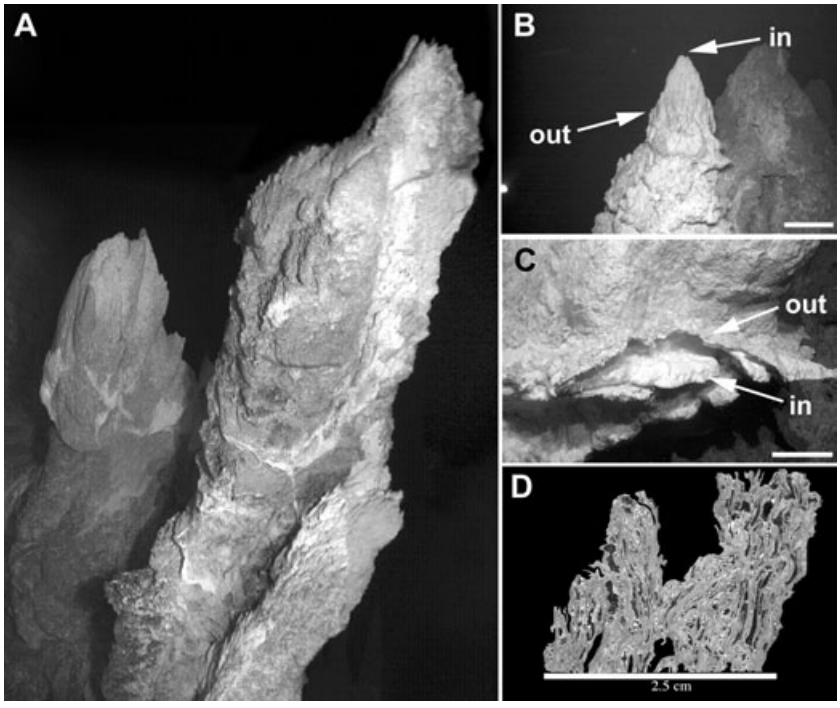

Fig. 1. Geological setting of the carbonate-hosted microbial ecosystem at the LCHF.
A. Portion of a large, ≈ 30-m-tall carbonate structure from the LCHF.
B. A pinnacle near the summit of the ≈ 60-m-tall carbonate chimney 'Poseidon'. Sample LC1022 was obtained from this site and was in contact with 75°C fluids. Pinnacles on Poseidon were venting warm (40–93°C), high pH (9–11) hydrothermal fluids from multiple sites along the complex structure. Scale bar is ≈ 50 cm. Arrows indicate mineralogical zonation associated with regions of active fluid venting (*in*: brucite and aragonite rich) and exterior to the structure (*out*: aragonite and calcite rich).
C. Porous flanges adorned the exterior surfaces of Poseidon and were venting up to 55°C fluids. The fracture 'scar' from where flange sample LC1149 was obtained is shown percolating 'shimmering' hydrothermal fluids. Scale bar is ≈ 40 cm.
D. Digital photograph of a petrographic thin section of chimney material that was in contact with hydrothermal fluids. The image highlights the extremely porous nature of young, active carbonate chimneys. Scale bar is 2.5 cm.

30 000 years (Früh-Green *et al.*, 2003). The largest of these structures, 'Poseidon', is 60 m in height and vents moderate temperature (40–93°C), high pH (9–11), metal-poor, volatile-rich fluids from its summit and from large, parasitic flanges along the sides of the chimney (Table 1; Fig. 1B and C). These fluid compositions are in striking contrast to the extremely high temperature, low pH, metal-rich fluids typical of sulphide-dominated hydrothermal systems that are driven by magmatic heat (Kelley *et al.*, 2001).

Here, we report the first analyses of microbial communities inhabiting porous carbonate environments at the LCHF. Microscopic analyses were used to examine the local distributions of cells within the carbonate structures and to enumerate rock-hosted populations. Phylogenetic surveys were used to characterize the microbial diversity within selected active and extinct chimney structures. An oligonucleotide probe designed for a specific phylotype detected at this site was used to confirm its occurrence *in situ*. These data provide an important initial microbiological description of a novel ecosystem in the deep sea.

Results

Seven carbonate samples were recovered from the chimneys and were frozen or chemically fixed for shore-based analyses. The youngest structures, in contact with diffusely venting fluids, were composed of aragonite and were extremely porous (Fig. 1D; Table 2). The mineralogy and internal porosity of the chimney samples changed progressively with age; older structures were less porous (Table 2) and more calcite rich (Früh-Green *et al.*, 2003). In two samples (LC1022 and LC1149), mineralogical zonation between the interior (in contact with vent fluids) and exterior (in contact with sea water) portions of the structures (Fig. 1C) was evident. The zonations were used as a basis for subsampling the carbonate structures.

Microscopic examination of samples from the LCHF revealed microbial communities inhabiting pore spaces within the carbonate towers. Portions of the carbonate samples in contact with hydrothermal fluids, where pore space was >40% of the rock volume, contained tens of micrometre-thick microbial biofilms composed of irregular coccoid cells, ≈ 1–3 μm in diameter (Fig. 2A and B). The cells from actively venting portions of the chimney were bound to mineral surfaces in a web-like matrix resembling microbial exopolysaccharides (Fig. 2C). Most of the cell populations in these regions autofluoresced when illuminated with ultraviolet light (Fig. 3A), a characteristic of Archaea containing factor 420 (F_{420}) (Doddema and Vogels, 1978), a key coenzyme involved in anaerobic methane cycling (Thauer, 1998). In contrast, microbial communities from exterior portions of the carbonate structures and from inactive, 'extinct' samples were composed of multiple morphotypes including filaments, rods and cocci (Fig. 3C and D) and contained few F_{420}-fluorescing cells.

Microscopic analyses of cells extracted from the porous rock matrix revealed cell densities of between 2.0×10^6 and 3.1×10^8 cells gdw^{-1} in regions awash in hydrothermal fluids (Table 2). Nearly all (≈ 90%) of the cells detected in this region were small cocci (≈ 1–3 μm), commonly aggregated into clusters (Fig. 3A and B). In exterior portions of the active structures, population densities were consistently high (8.6×10^7–2.7×10^8 cells gdw^{-1}) and were composed primarily of long (10–100 μm) filamentous cells (Figs 2D and 3C). In extinct samples, away from active hydrothermal flow, a mixed community of microorganisms was detected (Fig. 3D) with cell densities ranging from 10^6 to 10^7 cells gdw^{-1}.

Table 2. Geo-microbiological characteristics of hydrothermal carbonate samples from the LCHF.

Sample	Structure	Temperature (°C)	Porosity (%)	Cell counts[a] (cells g^{-1})	Proportion[b] (%)		
					Archaea	Eubacteria	LCMS[c]
Adjacent to active venting							
LC944	Chimney	>7	40–50	$5.6\ (0.9) \times 10^6$	7.5	14.8	6.3
LC1022	Chimney (*in*[d])	75	>40	$2.0\ (0.4) \times 10^6$	31.4	3.5	29.1
LC1022	Chimney (*out*)	<75	<40	$8.6\ (0.2) \times 10^7$	11.2	26.4	8.3
LC1149	Flange (*in*)	55	40–50	$3.1\ (0.3) \times 10^8$	37.2	4.2	32.5
LC1149	Flange (*out*)	<55	35–40	$2.7\ (0.4) \times 10^8$	5.7	23.1	3.1
Extinct structures							
LC908	Talus	7	25–30	$1.7\ (0.2) \times 10^6$	7.4	9.1	1.1
LC938	Talus	7	20–30	$1.3\ (0.1) \times 10^7$	2.9	19.6	ND[e]
LC1123	Talus	7	15–25	$1.6\ (0.1) \times 10^7$	4.5	16.5	ND
LC1231	Chimney	7	20–25	$1.2\ (0.1) \times 10^7$	7.0	15.8	ND

a. Microbial abundances are reported as mean cells per gram dry weight (±SD) calculated from three independent extractions.
b. Determined by FISH; data are mean percentage of the total cell populations.
c. LCMS indicates cells that hybridized with probe LCMS860, targeting the phylotype found in this study.
d. Indicates materials in contact with venting hydrothermal fluids (*in*) and mixtures of hydrothermal fluids and sea water (*out*).
e. ND, not detected.

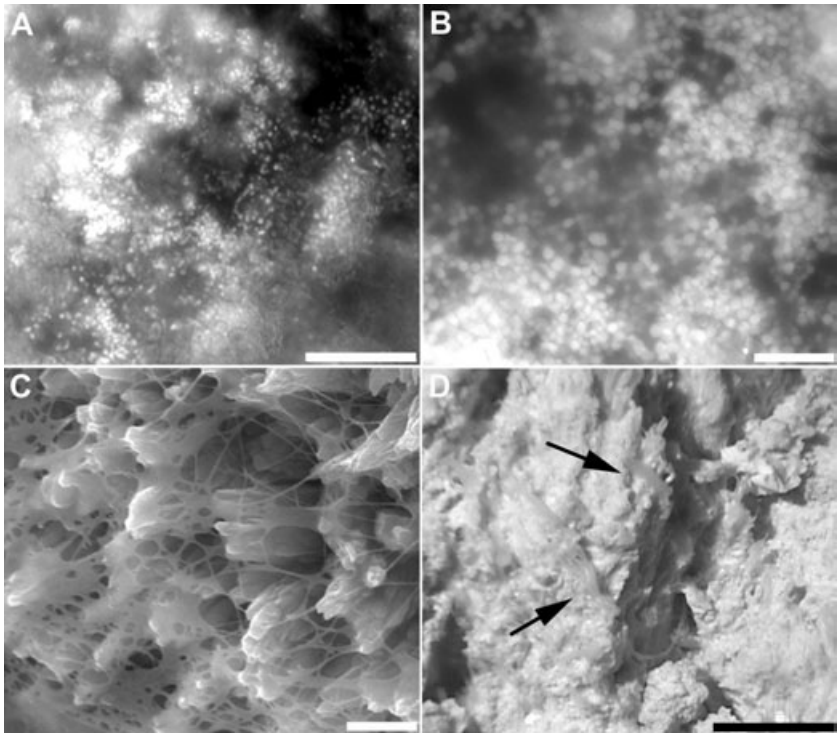

Fig. 2. Microbial biofilms associated with active hydrothermal edifices at the LCHF.
A. Planar view of a DAPI-stained biofilm within a porous carbonate sample in contact with 75°C fluids (LC1022).
B. Sagittal image of the same sample demonstrates the vertical continuity of the biofilm structures.
C. Scanning electron micrograph of fixed carbonate, showing the fibrous nature of polysaccharide-like material associated with microorganisms on mineral surfaces.
D. Filamentous microbial communities (arrows) were visually evident along exterior surfaces of actively venting carbonate structures. Scale bar is 20 μm in A and B, 1 μm in C and 5 cm in D.

Both Archaea and Eubacteria were detected by fluorescence *in situ* hybridization (FISH) in each of the seven samples analysed (Table 2). Overall, a relatively low percentage (16.5–41.4%) of the total cell populations fluoresced with the oligonucleotide probes used in this study. The relatively low percentage of FISH-hybridizing cells is consistent with other analyses of natural samples (Glöckner *et al*., 1999; Bouvier and del Giorgio, 2003) and may result from a number of factors including low ribosome content, cell impermeability or the lack of probe target

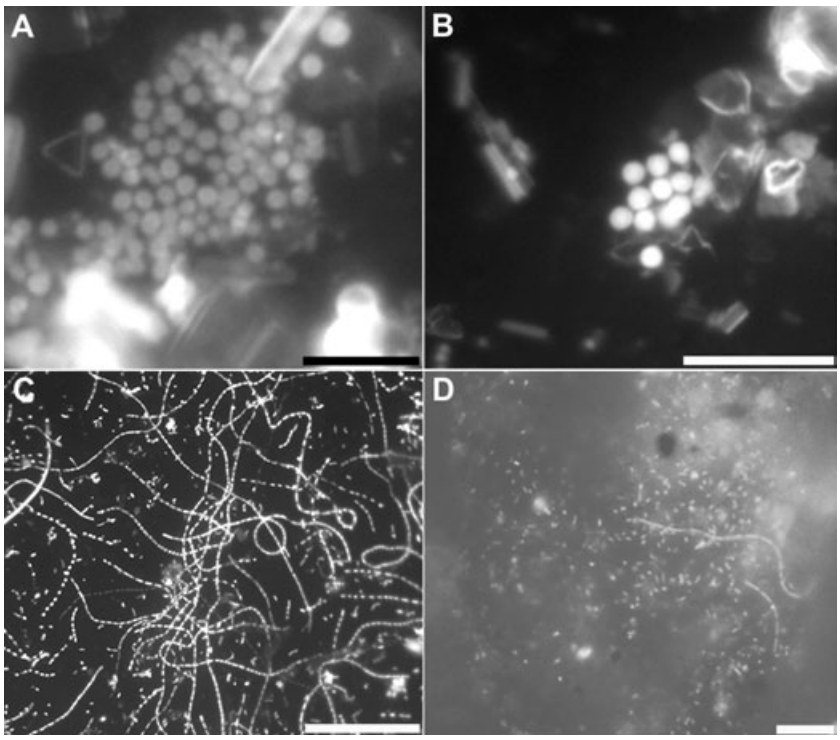

Fig. 3. Epifluorescence microscopy of various morphotypes detected in the LCHF carbonates.
A. Autofluorescence of an F_{420}-containing cell cluster from sample LC1022.
B. LCMS860-hybridized cells captured on the same filter. Note the similar size and morphology of F_{420}-containing and hybridized cells.
C. DAPI-stained photomicrograph of bacterial filaments detected in sample LC1022 (*out*).
D. DAPI-stained attached microbial community from an inactive sample (LC1231). Scale bar is 20 μm in A–C and 10 μm in D.

sequence in the populations analysed (Amann *et al*., 1995; Bouvier and del Giorgio, 2003). Despite these potential biases, the FISH analyses of LCHF carbonates yielded a higher percentage of Archaea (7.5–37.2%) in samples in contact with hydrothermal fluids relative to the exterior of the carbonate chimneys (5.7–11.2%) and extinct samples (2.9–7.4%) (Table 2).

To characterize further the identity of the microbial communities, clone libraries were constructed of archaeal 16S rRNA genes (16S rDNA) from selected samples. A single phylotype within the order Methanosarcinales comprised the entire archaeal clone libraries of two spatially distinct (>15 m apart) samples in contact with venting fluids (LC1022 and LC1149; Fig. 4). These results are in striking contrast to the microbial populations of sulphide-hosted hydrothermal systems, which commonly show a relatively high degree of 16S rDNA sequence diversity (Takai *et al*., 2001; Schrenk *et al*., 2003). Nearly full-length 16S rDNA sequences from LC1022 and LC1149 were >97% similar to one another and phylogenetically related to the genera *Methanococcoides* and *Methanosarcinales* (Table 3). The closest match to this phylotype (≈ 93% similarity) in public sequence databases (by NCBI-BLAST: http://www.ncbi.nlm.nih.gov/blast) is a clone that was a component of anaerobic methane-oxidizing microbial communities found near gas hydrates at the Eel River Basin, California (Orphan *et al*., 2001a). Screening of the clone libraries by restriction fragment length polymorphism (RFLP) analysis indicated subpopulations of different ribotypes (Table 3), with variations occurring as a result of mutations at the restriction sites rather than large-scale variations in gene sizes or base compositions.

The archaeal clone library from an extinct sample (LC1231) also contained limited phylogenetic diversity based upon screening of 16S rDNA by RFLP and sequencing of representative ribotypes (Table 3). However, in contrast to the clone libraries from the active samples, an extinct sample (LC1231) contained sequences solely related to the Marine Group I Crenarchaeota, a common component of pelagic environments (Karner *et al*., 2001).

An oligonucleotide probe targeting the Methanosarcinales phylotype (MSLC860) was applied to the seven carbonate samples to document the occurrence of this group of organisms *in situ*. Cells hybridizing with the probe were detected in four of the seven samples, including all the samples in contact with hydrothermal fluids (Table 2). MSLC-hybridizing cells comprised up to 32.5% of the total cell populations in the carbonate samples and corresponded to the F_{420}-fluorescing morphotypes (Fig. 3B).

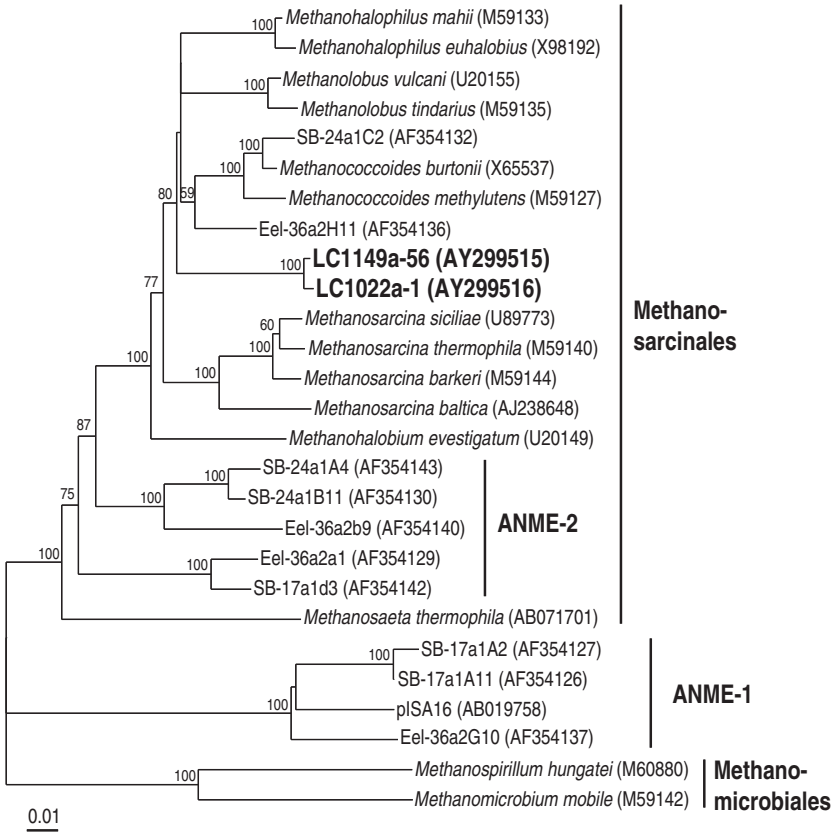

Fig. 4. Neighbour-joining tree depicting the phylogenetic affiliation of archaeal clone sequences from carbonate chimney samples in contact with vent fluids (LC1149 and LC1022) relative to the Methanosarcinales. Scale bar represents one nucleotide change per 100 bases. Bootstrap values for tree topology are adjacent to corresponding nodes. GenBank accession numbers for reference sequences are provided in parentheses. Principal energy sources of phylogenetic groups used in the tree. Order Methanosarcinales: *Methanolobus* (CH_3-), *Methanohalophilus* (CH_3-), *Methanococcoides* (CH_3-), Eel 36a2H11 (unknown), *Methanosarcina* (acetate/CH_3-/H_2), *Methanohalobium* (CH_3-), *Methanosaeta* (acetate), AMNE-2 (methane oxidation), ANME-1 (methane oxidation). Order Methanomicrobiales (H_2, formate).

Table 3. Archaeal phylogenetic groups detected in carbonate chimneys from the LCHF.

Phylogenetic affiliation	Clone	n_{ribo}[a]	Closest 16S rDNA match[b] (accession number)	Identity (%)
Euryarchaeota				
Methanosarcinales	LC1022-a1	9	Gas hydrate clone Eel-36a2H11 (AF354136)	93
	LC1149-a56	18	Gas hydrate clone Eel-36a2H11 (AF354136)	93
Crenarchaeota				
Marine Group I	LC1231-a51	1	Pacific Ocean clone MBMPA68 (AJ567666)	96
	LC1231-a68	7	Pacific Ocean clone (AJ567632)	98
	LC1231-a76	1	Pacific Ocean clone (AJ567622)	98
	LC1231-a78	5	Deep-sea sediment clone APA4-0cm (AF119138)	98

a. n_{ribo} indicates the number of distinct patterns per sequence type determined by RFLP.
b. Determined by NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast).

Discussion

The prevalence of a single phylotype related to the archaeal order Methanosarcinales in samples bathed in 55–75°C, alkaline fluids is intriguing in that such low diversity has rarely been observed in natural ecosystems. The closest relative to the phylotype detected in this study did not originate from similarly extreme environments, but rather from marine gas hydrates (Orphan *et al*., 2001a). Other hydrogen-rich, anaerobic ecosystems (e.g. marine sediments) harbour different methanogenic and sulphidogenic species from the group found at the LCHF (Boone *et al*., 2001). Furthermore, the temperature maxima for cultured isolates of the Methanosarcinales is ≈ 55°C, and most species do not grow above pH 10 (Boone *et al*., 2001). In concert, these data indicate that a highly selective set of environmental conditions exists within actively venting carbonate structures at the LCHF, which may include a combination of factors such as warm temperatures, high pH, distinct redox gradients and the availability of specific energetic and nutritional resources (Fig. 5).

Unique interpretation of the phylogenetic data is frustrated by the fact that the Methanosarcinales are the most metabolically diverse group of all of the methanogens. They can catalyse the production of methane from acetate, hydrogen/carbon dioxide or by dismutating methyl compounds (e.g. methanol, methyl amines or methyl sulphides) (Boone *et al*., 2001). In addition, close relatives of the Methanosarcinales have been implicated in anaerobic methane oxidation (Orphan *et al*., 2001b). The most abundant electron donors present within the hydrothermal fluids at the LCHF include hydrogen and methane (Table 1) (Kelley *et al*., 2001; Proskurowski *et al*., 2003). However, these environments also appear to be limited in potential electron acceptors because of the highly reducing nature of the subseafloor source region (Früh-Green *et al*., 2004) and the rapid kinetics of calcium carbonate precipitation (Früh-Green *et al*., 2003). In fact, the restricted availability of CO_2, as both a carbon and an energy source (Table 1) may explain the apparent absence of methanogenic species commonly found in deep-sea vent ecosystems (i.e. *Methanococcus* and *Methanopyrus* spp.).

Carbonate-hosted microbial communities at the LCHF may benefit from gradients in temperature and chemistry within the chimney walls that allow the perfusion of volatile-rich fluids throughout the pore network. Methanosarcinales may be the dominant populations in more restrictive habitats of the carbonate structures, while organisms with different metabolisms (such as sulphur oxidation/reduction and heterotrophy) probably prevail at other locations (Fig. 5). Eubacterial 16S rDNA clone libraries from parallel samples (data not shown) indicate the presence of sequences related to the ε-proteobacteria (sulphur- and hydrogen-using species), γ-proteobacteria (*Thiomicrospira* spp.) and firmicutes within the active samples, which supports this hypothesis. In addition, preliminary results from a follow-on cruise to the LCHF in 2003 indicate heterogeneous microbial lipid signatures within the carbonate structures when sampled comprehensively at high resolution (Bradley *et al*., 2003).

The aggregation and localization of methane-metabolizing cells in thick biofilms within the porous carbonate structures in contact with warm, alkaline fluids has several important implications. In particular, the biofilms may play a role in limiting the diffusion of carbon and energy sources present within hydrothermal fluids and electron acceptors present in sea water, and maintain anaerobic conditions within porous interior regions of the carbonate chimneys (Stewart, 2003). Additionally, biofilms have been shown recently to support significant phenotypic diversity even among genetically similar organisms (Stoodley *et al*., 2002; Rainey and Rainey, 2003). In the carbonate chimney ecosystem, the single Methanosarcinales phylotype may carry out several cooperative roles.

The identity and isotopic signature of microbial membrane lipids have been extremely informative in investigating carbon and energy flow in natural methane-influenced ecosystems (Orphan *et al*., 2001a,b; Teske *et al*., 2002). Unfortunately, the serendipitous discovery of the LCHF

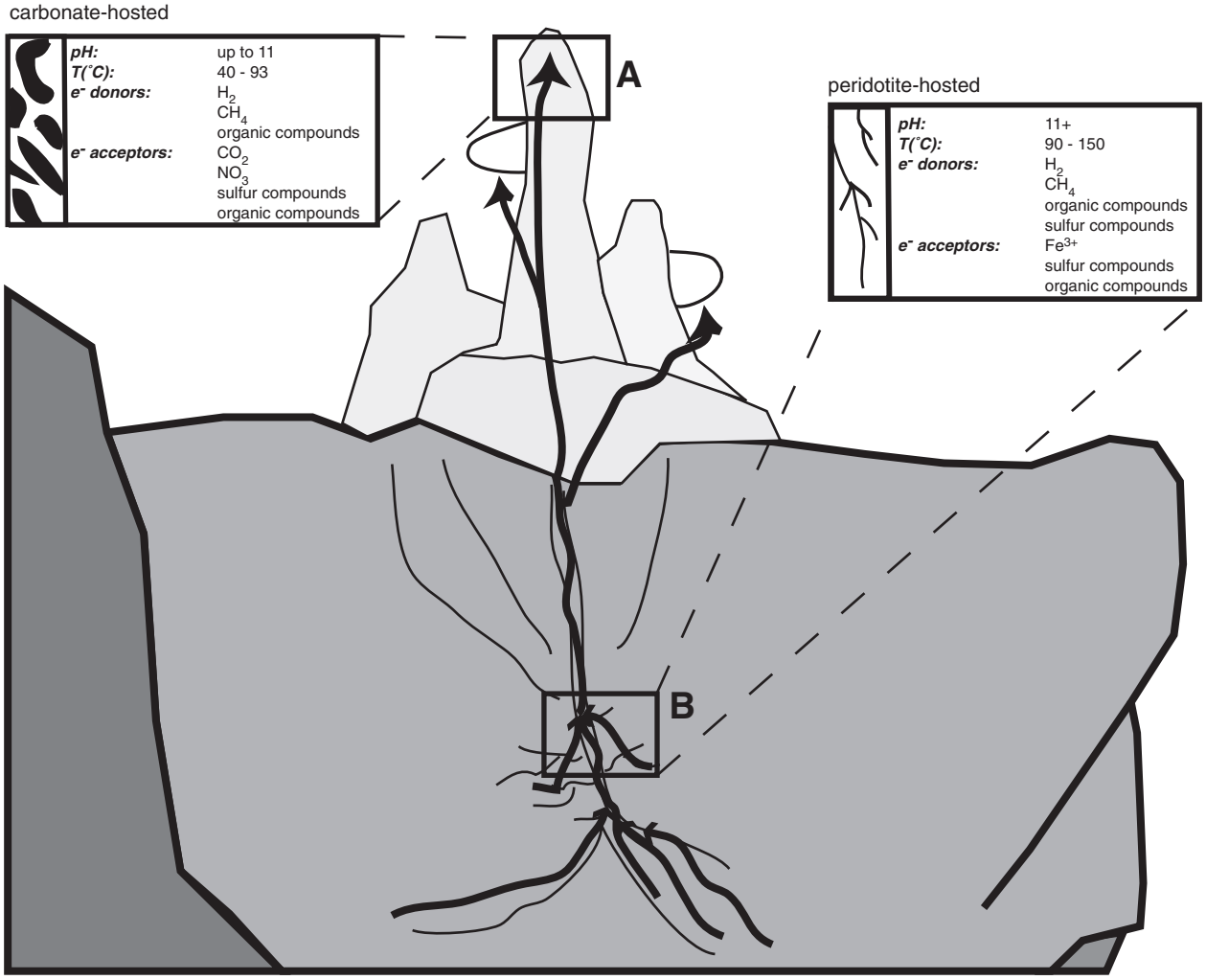

Fig. 5. Schematic of rock-hosted hydrothermal environments at the LCHF. Circulation of sea water through fractures in the subseafloor facilitates exothermic serpentinization of peridotites. Volume changes associated with serpentinization promotes additional fracturing and exposure of fresh peridotite surfaces. Serpentinization reactions result in a net increase in pH, the production of heat and the generation of hydrogen. Byproducts of serpentinization include methane and low-molecular-weight organic molecules. The warm, high pH fluids created by serpentinization vent on to the seafloor. Mixing with sea water results in the precipitation of calcium carbonate and chimney formation.
A. Habitats within the porous chimney structures of the LCHF are likely to span the gradients between warm (>90°C), high pH (11), highly reduced hydrothermal fluids and cold, oxygenated sea water.
B. Microenvironments within fractures near the sites of active serpentinization are likely to be highly reducing, contain abundant volatile compounds and reach high temperatures (90–150°C) and pHs (>11).

prohibited collection of sufficient material to extract lipids from these samples. However, in future studies, the stable isotopic signatures of lipids (Orphan *et al*., 2001a; Teske *et al*., 2002), fluids (Horita and Berndt, 1999) and rock samples (Alt and Shanks, 1998; Kelley *et al*., 2001) will be key to identifying important metabolic processes within this novel ecosystem. Such studies should incorporate the potential for heterogeneity among closely related organisms within the gradients of the carbonate chimney walls and the possibility of diverse metabolisms and carbon assimilation pathways into their sampling schemes and subsequent data interpretation. The predominance of Methanosarcinales-related cells is a unique aspect of the LCHF system and is apparent only at sites associated with active hydrothermal venting. These data are consistent with anaerobic microbial metabolisms linked to subseafloor serpentinization processes that generate substantial amounts of hydrogen, methane and low-molecular-weight organic compounds (Berndt *et al*., 1996; Shock and Schulte, 1998; Horita and Berndt, 1999; Kelley and Früh-Green, 2000; McCollom and Seewald, 2001; Charlou *et al*., 2002; Proskurowski *et al*., 2003; Früh-Green *et al*., 2004).

Although comparable deep-sea ecosystems have not been investigated in detail from a microbiological perspective, rock alteration processes have been described as a

source of metabolic energy in terrestrial subsurface ecosystems hosted in basaltic rock (Stevens and McKinley, 1995; 2000; Chapelle *et al*., 2002). In particular, the study by Chapelle *et al*. (2002) found an extremely low diversity of hydrogenotrophic, methanogenic Archaea in an aquifer venting ≈ 58.5°C, pH 6.77 fluids. Ultramafic rocks within mid-ocean ridge environments are considerably more reactive than basalts and, consequently, are capable of generating significant quantities of hydrogen through water–rock reactions (Janecky and Seyfried., 1986; Abrajano *et al*., 1988; Wetzel and Shock, 2000). Given the adaptation of ultramafic-hosted communities to extreme environmental conditions (temperature, pH) and sufficient nutrient and carbon sources, serpentinization processes have the potential to support an even greater degree of lithoautotrophic microbial activity than terrestrial basalt-hosted ecosystems.

The LCHF represents the first ecosystem in the deep sea where hydrothermal flow appears to be driven by exothermic serpentinization reactions in the subsurface (Kelley *et al*., 2001; Früh-Green *et al*., 2003). The majority of spreading at mid-ocean ridges is slow to ultraslow, which favours attenuation, fracturing and faulting of brittle oceanic crust. In concert, these tectonic processes result in significant exposures of mantle material at the seafloor (Dick *et al*., 2003; Früh-Green *et al*., 2004). Serpentinization of shallow mantle material and associated generation of microbial energy sources may be an extremely common process and persist away from mid-ocean ridge axes (Kelley *et al*., 2001; Früh-Green *et al*., 2004). Yet, serpentinized ultramafic rock is a geo-biological setting virtually unexplored in the oceans. Microbial niches at depth within these systems, near sites of active serpentinization fronts, may be isolated from biological sources of carbon and retain primitive and unusual metabolisms and physiologies that have not yet been considered (Fig. 5). Peridotite-hosted hydrothermal vents may resemble those present on the early Earth and, indeed, alkaline hydrothermal systems have been postulated to have played a key role in the origin and early evolution of life (Russell and Hall, 1996; Kelley *et al*., 2001).

Our data show that active magmatic heating is not a prerequisite for warm, life-supporting ecosystems. These results expand our understanding of the distribution of life on Earth to include submarine hydrothermal environments independent of volcanic sources of heat. An exciting implication of this discovery is that it provides a novel mechanism by which geological processes can support microbial ecosystems that should be incorporated into our search for extraterrestrial life. In particular, the early history of Mars may also have included liquid water and exposures of ultramafic rocks that would have favoured geochemical processes similar to those at Lost City (Shock and Schulte, 1998).

Experimental procedures

Carbonate samples

Samples were collected from the LCHF during cruise AT03-6 aboard the *R/V Atlantis* using the *DSV Alvin* (Dive 3651). Parallel subsamples of rock material were frozen immediately at −80°C in the shipboard laboratory or partitioned for petrological and geochemical analyses (Kelley *et al*., 2001; Früh-Green *et al*., 2003). Pieces of frozen rock were subsequently fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored in 70% ethanol at −20°C until examination (Glöckner *et al*., 1999).

Microscopy and cell enumeration

Petrographic thin sections were prepared from each of the seven samples of LCHF carbonate and used to estimate rock porosity through transmitted light microscopy at 50× magnification (500 μm field of view). Intact associations between microbial cells and rock material were observed in pieces of whole carbonate embedded in 2.5% low-melting-point agarose (GeneMate), which were fractured or cut axially with a sterile scalpel. Embedded rock fragments were either stained with 4′6-diamidino-2-phenylindole (DAPI; Sigma) or the autofluorescence of F_{420} was observed. Two of the active structures (LC1022 and LC1149) were mineralogically zoned and were cut parallel to the zonation and used in independent cell extractions. Microbial cells were extracted from three subsamples per carbonate structure using previously described methods (Harmsen *et al*., 1997; Schrenk *et al*., 2003) and captured on 0.22 μm pore size black polycarbonate filters (Glöckner *et al*., 1999). Individual filters were cut into four, approximately equivalent sections and used for enumeration by epifluorescence microscopy. Total cell populations were quantified using the DNA stain DAPI (Porter and Feig, 1980). Fluorescence *in situ* hybridization (FISH) was performed with the Cy3-labelled oligonucleotides EUB338 and ARC915 (Qiagen-Operon) (Amann *et al*., 1995), according to the method of Glöckner *et al*. (1999). FISH with the newly designed LCMS860 probe (see description below) was performed under similar hybridization conditions using solutions containing 30% formamide. All epifluorescence and light microscopic observations were made using a Nikon Eclipse E600 POL epifluorescence microscope equipped with the appropriate fluorescence filter sets (DAPI: 360/400/460, ex/di/em; F_{420}: 410/440/470; Cy3: 545/575/610). Digital images were obtained using a Roper Scientific digital CCD camera and processed in Adobe PHOTOSHOP version 6.0 (Adobe). Fixed samples were also observed with an ISI DS-130S scanning electron microscope at NOAA-PMEL (Seattle, WA) with the assistance of Geoff LeBon.

DNA extraction

Extraction and purification of nucleic acids (Schrenk *et al*., 2003) and polymerase chain reaction (PCR) amplification of the archaeal 16S rRNA gene were performed as described previously (Teske *et al*., 2002) using the universal primers ARC-8f (5′-TCCGGTTGATCCTGCC-3′) and ARC-1492R (5′-GGCTACCTTGTTACGACTT-3′). The PCR-amplified DNA

was reconditioned according to the protocol of Thompson *et al*. (2002) and cloned using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. Clones were PCR amplified with vector-specific primers and screened by RFLP as described previously (Schrenk *et al*., 2003). One hundred, 300 and 93 clones were screened for libraries LC1022-a, LC1149-a and LC1231-a respectively. Representative ribotypes were selected for partial sequencing and, in several cases, full-length sequences of the clones were obtained.

Phylogenetic analysis

Nearly complete 16S rDNA sequences obtained from this study were generally aligned using the Ribosomal Database Project II (RDP II: http://rdp.cme.msu.edu/html) SEQUENCE ALIGNER program (Maidak *et al*., 2001) and manually aligned to reference sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/GenBank) in BIOEDIT version 5.0.9 (Hall, 1999). Approximately 1390 nucleotide bases were used in phylogenetic analyses, with only homologous positions used in comparisons. Alignments were checked for chimeric sequences using the CHIMERA_CHECK program of RDP II. Sequence similarities were calculated using BIOEDIT. The sequences for *Methanospirillum hungatei* (M60880) and *Methanomicrobium mobile* (M59142) were used as outgroups. The PHYLIP version 3.6 software package (obtained from J. Felsenstein, University of Washington, Seattle, WA, USA) was used to construct distance (NEIGHBOR and FITCH) and maximum likelihood (DNAML) trees, which resulted in congruent topologies. Confidence estimates for tree topology were obtained through bootstrap analysis (SEQBOOT) using 100 replicates.

Oligonucleotide probe design

An alignment of new sequences obtained in this study was compared with nearly full-length sequences of related phylotypes obtained from GenBank in BIOEDIT. Conserved regions within the target sequences were compared with the oligonucleotide probes used in the study by Raskin *et al*. (1994). A modification of the probe MSMX860 (LCMS860) was the result of this process (5′-GGCTCGTTTCACRGCTTCCCT-3′). LCMS860 was synthesized and labelled with Cy3 (Qiagen-Operon) and empirically tested using target and non-target control cultures.

The sequence data reported in the study have been submitted to the GenBank database and are available under the accession numbers AY299515–AY299516 and AY505046–AY505054.

Acknowledgements

We wish to thank K. Ludwig and G. Früh-Green for their thoughtful insight and helpful discussions of the petrogenesis and mineralogy of the LCHF, and P. J. Cimino for assistance with cell enumeration and sample characterization. We thank the crew and the science party of the *R/V Atlantis* (AT03-6) and the *DSV Alvin*. We gratefully acknowledge funding from NSF (OCE9712549), NSF-IGERT (DGE-9870713) and the NASA Astrobiology Institute through the Carnegie Geophysical Institute.

References

Abrajano, T.A., Sturchio, N.C., Bohlke, J.K., Lyon, G.L., Poreda, R.J., and Stevens, C.M. (1988) Methane-hydrogen gas seeps, Zambales ophiolite, Philippines: deep or shallow origin? *Chem Geol* **71:** 211–222.

Alt, J.C., and Shanks, W.C. (1998) Sulfur in serpentinized oceanic peridotites: serpentinization processes and microbial sulfate reduction. *J Geophys Res* **103:** 9917–9929.

Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59:** 143–169.

Berndt, M.E., Allen, D.E., and Seyfried, W.E. (1996) Reduction of CO_2 during serpentinization of olivine at 300°C and 500 bar. *Geology* **24:** 351–354.

Boone, D.R., Whitman, W.B., and Koga, Y. (2001) Order III Methanosarcinales. In *Bergey's Manual of Systematic Bacteriology, The Archaea, Photosynthetic Bacteria, and Deeply Branched Bacteria*, 2nd edn, vol. 1. Boone, D.R., Castenholz, R.W., and Garrity, G. (eds). Berlin: Springer Verlag, pp. 268–293.

Bouvier, T., and del Giorgio, P.A. (2003) Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): a quantitative review of published reports. *FEMS Microbiol Ecol* **44:** 3–15.

Bradley, A.S., Hayes, J.M., and Summons, R.E. (2003) Geolipids produced by methanogens and sulfate-reducing bacteria at the Lost City Hydrothermal Field. *Eos, Trans Am Geophys Union* **84** (46)**:** Fall Meet. Suppl., Abstract B12A-0773.

Chapelle, F.H., O'Neill, K., Bradley, P.M., Methé, B.A., Ciufo, S.A., Knobel, L.L., and Lovley, D.R. (2002) A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* **415:** 312–315.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., and Holm, N. (2002) Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14′N, MAR). *Chem Geol* **191:** 345–359.

Dick, H.J.B., Lin, J., and Schouten, H. (2003) An ultraslow-spreading class of ocean ridge. *Nature* **426:** 405–412.

Doddema, J.J., and Vogels, G.D. (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. *Appl Environ Microbiol* **36:** 752–754.

Escartín, J., and Cannat, M. (1999) Ultramafic exposures and the gravity signature of the lithosphere near the Fifteen-Twenty Fracture Zone (Mid-Atlantic Ridge, 14°–16.5°N). *Earth Planet Sci Lett* **171:** 411–424.

Früh-Green, G.L., Kelley, D.S., Bernasconi, S.M., Karson, J.A., Ludwig, K.A., Butterfield, D.A., *et al.* (2003) 30,000 years of hydrothermal activity at the Lost City Vent Field. *Science* **301:** 495–498.

Früh-Green, G.L., Connolly, J.A.D., Plas, A.P., Kelley, D.S., and Grobéty, B. (2004) Serpentization of oceanic peridotites: Implications for geochemical cycles and biological activity. In *The Subseafloor Biosphere at Mid-Ocean Ridges, Geophysical Monograph Series*. Wilcock, W.D.,

Baross, J.A., Kelley, D.S., DeLong, E.F., and Cary, C. (eds). Washington, DC: AGU, in press.

Glöckner, F.O., Fuchs, B.M., and Amann, R. (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence *in situ* hybridization. *Appl Environ Microbiol* **65:** 3721–3726.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Series* **41:** 95–98.

Harmsen, H.J.M., Prieur, D., and Jeanthon, C. (1997) Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridization and enrichment culture of thermophilic subpopulations. *Appl Environ Microbiol* **63:** 2876–2883.

Horita, J., and Berndt, M.E. (1999) Abiogenic methane formation and isotopic fractionation under hydrothermal conditions. *Science* **285:** 1055–1057.

Janecky, D.R., and Seyfried, W.E., Jr (1986) Hydrothermal serpentinization of peridotite within the oceanic crust; experimental investigations of mineralogy and major element chemistry. *Geochim Cosmochim Acta* **50:** 1357–1378.

Karner, M.B., DeLong, E.F., and Karl, D.M. (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409:** 507–510.

Kelley, D.S., and Früh-Green, G.L. (2000) Volatiles in mid-ocean ridge environments. In *Ophiolites and Oceanic Crust; New Insights from Field Studies and the Ocean Drilling Program*. Dilek, Y., Moores, E.M., Elthon, D., and Nicolas, A. (eds). Boulder, CO, USA: Geological Society of America (GSA), pp. 237–260.

Kelley, D.S., Karson, J.A., Blackman, D.K., Früh-Green, G.L., Butterfield, D.A., Lilley, M.D., *et al.* (2001) An off-axis hydrothermal vent field near the Mid-Atlantic Ridge at 30°N. *Nature* **412:** 145–149.

Kelley, D.S., Baross, J.A., and Delaney, J.R. (2002) Volcanoes, fluids, and life at mid-ocean ridge spreading centers. *Annu Rev Earth Planet Sci* **30:** 385–491.

Lowell, R.P., and Rona, P.A. (2002) Seafloor hydrothermal systems driven by the serpentinization of peridotite. *Geophys Res Lett* **29:** 26–29.

McCollom, T.M., and Seewald, J.S. (2001) A reassessment of the potential for reduction of dissolved CO_2 to hydrocarbons during serpentinization of olivine. *Geochim Cosmochim Acta* **65:** 3769–3778.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Jr, Saxman, P.R., Farris, R.J., *et al.* (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29:** 173–174.

Orphan, V.J., Hinrichs, K.-U., Ussler, W., III, Paull, C.K., Taylor, L.T., Sylva, S.P., *et al.* (2001a) Comparative analysis of methane-oxidizing Archaea and sulfate-reducing bacteria in anoxic marine sediments. *Appl Environ Microbiol* **67:** 1922–1934.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., and DeLong, E.F. (2001b) Methane-consuming Archaea revealed by directly-coupled isotopic and phylogenetic analysis. *Science* **293:** 484–487.

Palandri, J.L., and Reed, M.H. (2004) Geochemical models of metasomatism in ultramafic systems: serpentinization, rodingitization, and seafloor carbonate chimney precipitation. *Geochim Cosmochim Acta* **68:** 1115–1133.

Porter, K.G., and Feig, Y.S. (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* **25:** 943–948.

Proskurowski, G., Lilley, M.D., Olson, E.J., and Larson, B. (2003) Preliminary geochemistry of volatile species from active vents at the Lost City Hydrothermal Field. *Eos, Trans Am Geophys Union* **84** (46)**:** Fall Meet. Suppl., Abstract B12A-0770.

Rainey, P.B., and Rainey, K. (2003) Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425:** 72–74.

Raskin, L., Stromley, J.M., Rittmann, B.E., and Stahl, D.A. (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl Environ Microbiol* **60:** 1232–1240.

Russell, M.J., and Hall, J.A. (1996) The emergence of life from monosulphide bubbles at a submarine hydrothermal redox and pH front. *J Geol Soc (London)* **153:** 1–26.

Schrenk, M.O., Kelley, D.S., Delaney, J.R., and Baross, J.A. (2003) Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Appl Environ Microbiol* **69:** 3580–3592.

Shock, E.L., and Schulte, M.D. (1998) Organic synthesis during fluid mixing in hydrothermal systems. *J Geophys Res* **103:** 28,513–28,528,527.

Stevens, T.O., and McKinley, J.P. (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270:** 450–454.

Stevens, T.O., and McKinley, J.P. (2000) Abiotic controls on H_2 production from basalt-water reactions and implications for aquifer biogeochemistry. *Environ Sci Technol* **34:** 826–831.

Stewart, P.S. (2003) Diffusion in biofilms. *J Bacteriol* **185:** 1485–1491.

Stoodley, P., Sauer, K., Davies, D.G., and Costerton, J.W. (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* **56:** 187–209.

Takai, K., Komatsu, T., Inagaki, F., and Horikoshi, K. (2001) Distribution of Archaea in a black smoker chimney structure. *Appl Environ Microbiol* **67:** 3618–3629.

Teske, A., Hinrichs, K.-U., Edgcomb, V., de Vera Gomez, A., Kysela, D., Sylva, S.P., *et al.* (2002) Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. *Appl Environ Microbiol* **68:** 1994–2007.

Thauer, R.K. (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* **144:** 2377–2406.

Thompson, J.R., Marcelino, L.A., and Polz, M.F. (2002) Heteroduplexes in mixed-template amplification: formation, consequence, and elimination by 'reconditioning PCR'. *Nucleic Acids Res* **30:** 2083–2088.

Wetzel, L.R., and Shock, E.L. (2000) Distinguishing ultramafic- from basalt-hosted submarine hydrothermal systems by comparing calculated vent fluid compositions. *J Geophys Res* **105:** 8319–8340.