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; Escartin 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).
hydrothermal system. It is hosted on a 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

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

<table>
<thead>
<tr>
<th>Constituent or property</th>
<th>Experimental/theoretical</th>
<th>LCHF&lt;sup&gt;a&lt;/sup&gt; (30°N)</th>
<th>Rainbow&lt;sup&gt;b&lt;/sup&gt; (36°14′N)</th>
<th>Sea water&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>25–300</td>
<td>40–93</td>
<td>365</td>
<td>7</td>
</tr>
<tr>
<td>pH</td>
<td>8–12</td>
<td>9–11</td>
<td>2.8</td>
<td>8</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt; (mmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>1–100</td>
<td>0.25–0.43&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; (mmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.01–1</td>
<td>0.13–0.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt; + C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt; (mmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>&lt;1000</td>
<td>&gt;100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1145</td>
<td>0</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;S (mmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.1–1</td>
<td>0.064</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>SO&lt;sub&gt;4&lt;/sub&gt; (mmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0</td>
<td>5.9–12.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>28.6</td>
</tr>
<tr>
<td>NO&lt;sub&gt;x&lt;/sub&gt; (μmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt; (mmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0</td>
<td>ND</td>
<td>16</td>
<td>2.30</td>
</tr>
<tr>
<td>Total Fe (μmol kg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>1.0</td>
<td>ND</td>
<td>24 050</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt;/(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt; + C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>1000–10 000</td>
<td>ND</td>
<td>2183</td>
<td>–</td>
</tr>
</tbody>
</table>

b. Proskurowski et al. (2003; includes C2H6); 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<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub>S values are minimum values because of artifacts associated with sample collection and storage. SO<sub>4</sub> 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<sub>x</sub> values of end-member hydrothermal fluids at the LCHF are believed to be well below 20 μmol kg<sup>−1</sup> because of the highly reducing nature of the system and by comparison with magmatically influenced sites.

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 cocoid 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 (F420) (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 F420-fluorescing cells.

Microscopic analyses of cells extracted from the porous rock matrix revealed cell densities of between 2.0 × 10⁶ and 3.1 × 10⁸ cells g⁻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 × 10⁷–2.7 × 10⁸ cells g⁻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 10⁶ to 10⁷ cells g⁻¹.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Structure</th>
<th>Temperature (°C)</th>
<th>Porosity (%)</th>
<th>Cell counts* (cells g⁻¹)</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Archaea</td>
<td>Eubacteria</td>
</tr>
<tr>
<td>Adjacent to active venting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC944</td>
<td>Chimney</td>
<td>&gt;7</td>
<td>40–50</td>
<td>5.6 (0.9) × 10⁶</td>
<td>7.5</td>
</tr>
<tr>
<td>LC1022</td>
<td>Chimney (in)</td>
<td>&gt;40</td>
<td></td>
<td>2.0 (0.4) × 10⁶</td>
<td>31.4</td>
</tr>
<tr>
<td>LC1022</td>
<td>Chimney (out)</td>
<td>&lt;75</td>
<td>&lt;40</td>
<td>8.6 (0.2) × 10⁷</td>
<td>11.2</td>
</tr>
<tr>
<td>LC1149</td>
<td>Flange (in)</td>
<td>55</td>
<td>40–50</td>
<td>3.1 (0.3) × 10⁸</td>
<td>37.2</td>
</tr>
<tr>
<td>LC1149</td>
<td>Flange (out)</td>
<td>&lt;55</td>
<td>35–40</td>
<td>2.7 (0.4) × 10⁸</td>
<td>5.7</td>
</tr>
<tr>
<td>Extinct structures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC908</td>
<td>Talus</td>
<td>7</td>
<td>25–30</td>
<td>1.7 (0.2) × 10⁶</td>
<td>7.4</td>
</tr>
<tr>
<td>LC938</td>
<td>Talus</td>
<td>7</td>
<td>20–30</td>
<td>1.3 (0.1) × 10⁷</td>
<td>2.9</td>
</tr>
<tr>
<td>LC1123</td>
<td>Talus</td>
<td>7</td>
<td>15–25</td>
<td>1.6 (0.1) × 10⁷</td>
<td>4.5</td>
</tr>
<tr>
<td>LC1231</td>
<td>Chimney</td>
<td>7</td>
<td>20–25</td>
<td>1.2 (0.1) × 10⁷</td>
<td>7.0</td>
</tr>
</tbody>
</table>

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 material in contact with venting hydrothermal fluids (in) and mixtures of hydrothermal fluids and sea water (out).
e. ND, not detected.

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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 binding.

**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.

**Fig. 3.** Epifluorescence microscopy of various morphotypes detected in the LCHF carbonates. A. Autofluorescence of an F420-containing cell cluster from sample LC1022. B. LCMS860-hybridized cells captured on the same filter. Note the similar size and morphology of F420-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 (93% similarity) in public sequence databases (by NCBI-BLAST: http://www.ncbi.nlm.nih.gov/blast) related to the genera Methanobrevibacter, Methanobrevibacter, and Methanoculleus.

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 $F_{160}$-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 Methanomicrobiales: Methanobacterium (H$_2$, hydrogen oxidation), Methanobrevibacter (H$_2$, formate). Order Methanosarcinales: Methane (methane oxidation), ANME-1 (methyl group oxidation), ANME-2 (methyl group oxidation), ANME-3 (acetate), ANME-4 (acetate). Order Methanomicrobiales: Methanobacterium (H$_2$, hydrogen oxidation), Methanobrevibacter (H$_2$, formate), Methanoculleus (H$_2$, formate), Methanosarcina (acetate).](image-url)
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 CO₂, 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...
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

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 promote 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).
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 at 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 F420 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; F420: 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 rDNA gene were performed as described previously (Teske et al., 2002) using the universal primers ARC-8f (5′-TCCGCTTGAACCTGCAG-3′) and ARC-1492R (5′-GGCTACCTTGTAGACTT-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 (Schenk 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 phytypes 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 MSX860 (LCMS860) was the result of this process (5′-GGCTGCTTCTCCGCTTCCCT-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.

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**References**


