

Methane- and Sulfur-Metabolizing Microbial Communities Dominate the Lost City Hydrothermal Field Ecosystem

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Hydrothermal venting and the formation of carbonate chimneys in the Lost City hydrothermal field (LCHF) are driven predominantly by serpentinization reactions and cooling of mantle rocks, resulting in a highly reducing, high-pH environment with abundant dissolved hydrogen and methane. Phylogenetic and terminal restriction fragment length polymorphism analyses of 16S rRNA genes in fluids and carbonate material from this site indicate the presence of organisms similar to sulfur-oxidizing, sulfate-reducing, and methane-oxidizing *Bacteria* as well as methanogenic and anaerobic methane-oxidizing *Archaea*. The presence of these metabolic groups indicates that microbial cycling of sulfur and methane may be the dominant biogeochemical processes active within this ultramafic rock-hosted environment. 16S rRNA gene sequences grouping within the *Methylobacter* and *Thiomicrospira* clades were recovered from a chemically diverse suite of carbonate chimney and fluid samples. In contrast, 16S rRNA genes corresponding to the Lost City *Methanosarcinales* phylotype were found exclusively in high-temperature chimneys, while a phylotype of anaerobic methanotrophic *Archaea* (ANME-1) was restricted to lower-temperature, less vigorously venting sites. A hyperthermophilic habitat beneath the LCHF may be reflected by 16S rRNA gene sequences belonging to *Thermococcales* and uncultured *Crenarchaeota* identified in vent fluids. The finding of a diverse microbial ecosystem supported by the interaction of high-temperature, high-pH fluids resulting from serpentinization reactions in the subsurface provides insight into the biogeochemistry of what may be a pervasive process in ultramafic seafloor environments.

The Lost City hydrothermal field (LCHF) is located near the summit of the Atlantis Massif at a water depth of ~750 m (37, 38). Long-lived faulting and extensive uplift at the massif have resulted in the exposure of magnesium-rich, variably altered ultramafic rocks with lesser gabbroic material that is 1.5 to 2 million years of age. Fluid circulation within the massif is driven by serpentinization reactions and the cooling of the underlying mantle rocks. These reactions result in a combination of extreme conditions never before seen in the marine environment, which include venting of high-pH (from pH 9 to 11), 40 to 91°C hydrothermal fluids with high concentrations of dissolved hydrogen (H₂), methane (CH₄), and other low-molecular-weight hydrocarbons (38). Mixing of the warm, high-pH fluids with seawater results in carbonate precipitation and growth of chimneys, which tower up to 60 m above the surrounding seafloor (38). Carbon-14 radioisotopic dating indicates that hydrothermal activity has been ongoing for at least 30,000 years (17). A large percentage of exposed seafloor on and near slow- and ultraslow-spreading ridges is likely to contain ultramafic rocks similar to those that host the LCHF (4, 12, 14). Therefore, this system offers a unique opportunity to study an ultramafic-rock-hosted submarine ecosystem that may be both widespread and stable over thousands of years.

The concentration of H₂ in LCHF fluids varies widely by

vent site, with values of 1 to 15 mM (38, 60). This variability may be due in part to biological hydrogen oxidation, as indicated by measurements of the deuterium content of dissolved H₂ in LCHF fluids (60). In contrast to black smoker environments, the high-pH LCHF fluids contain CO₂ concentrations that are small to below detection limits (38). Methane, by contrast, is present at a relatively constant concentration of 1 to 2 mM throughout the LCHF (38, 60). Both H₂ and CH₄ are products of serpentinization (7, 36, 44), so the highly variable H₂ concentrations and consistent values of CH₄ are intriguing. The present study examines the possibility that biological communities are acting as a sink for H₂ and possibly both a source of and sink for CH₄. The CH₄ concentrations of the fluids are similar to those of many environments that support anaerobic methane oxidation (AMO) (55, 75, 76). Therefore, the possibility that AMO exists at the LCHF is specifically addressed.

Previous reports have shown that the most actively venting chimneys at the LCHF are dominated by a single phylotype of *Methanosarcinales* (Lost City *Methanosarcinales* [LCMS]) (65). In areas bathed in >80°C hydrothermal fluid, these organisms form thick biofilms, and they comprise nearly 100% of the archaeal community. Recently, methyl coenzyme M reductase (*mcrA*) gene sequences corresponding to both LCMS and ANME-1, a group of anaerobic methane-oxidizing *Archaea*, have also been recovered from LCHF carbonate chimneys (38). ANME-1 has been identified in many environments, including CH₄ seeps in anoxic marine sediments, CH₄ hydrates, carbonate reefs in the Black Sea, and mud volcanoes (3, 8, 46, 55, 72, 75). Genomic evidence indicates that anaerobic methane-oxidizing *Archaea* harbor nearly all of the genes necessary for methanogenesis, including *mcrA* (21, 45), so it is unclear

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TABLE 1. Description of carbonate samples used for DNA extraction and phylogenetic analysis^a

Marker	Carbonate sample(s)	Fluid sample	Description	Temp (°C)	Mg level (range) (mM/kg)	H ₂ level (mmol/kg)	CH ₄ level (mmol/kg)	No. of cells/g carbonate	No. of cells/ml fluid
2	3864-1524, 3864-1537, LC1149	None	Venting flange, young looking	62	2–39	3.77	1.28	1.4 × 10 ⁹	4.7 × 10 ⁴
3	3881-1408, LC1022	FS242	Peak of chimney complex	81	3–39	13.26	1.55	2.0 × 10 ⁸	3.9 × 10 ⁵
H	3881-1228	FS243	Large chimney with flange	46	28–46	2.21	1.44	3.6 × 10 ⁸	1.4 × 10 ⁵
C	3869-1404, 3869-1443, 3869-1446	FS221	Venting flange, more lithified	70	12–28	14.38	1.98	1.2 × 10 ⁹	1.4 × 10 ⁵
X1	3876-1133	None	Lithified carbonate chimney in serpentinite	ND	ND	ND	ND	1.6 × 10 ⁸	ND
X2	3880-1557	None	Lithified carbonate chimney in serpentinite	ND	ND	ND	ND	3.4 × 10 ⁶	ND
6	None	FS222	Venting chimney	68	45	14.19	1.61	ND	2.0 × 10 ⁵
7	None	FS197	Venting chimney	51	35–52	5.54	1.31	ND	2.8 × 10 ⁵
8	None	FS209	Venting chimney	53	7–53	4.20	1.84	ND	1.7 × 10 ⁵
None	LC1231	None	Inactive chimney	7	ND	ND	ND	1.2 × 10 ⁷	ND

^a Coregistered hydrothermal fluids and carbonate deposits were sampled at markers 3, C, and H. Markers 2, X1, and X2 were sampled for carbonate only, and markers 6, 7, and 8 were sampled for fluid only. Mg concentrations are shown as a measure of the seawater component of the sample. ND, not determined.

whether LCMS and ANME-1 are sources of or sinks for CH₄ within the Lost City system. The limited number of LCHF samples studied to date has not allowed firm conclusions regarding the distribution of LCMS and ANME-1 with respect to temperature. The temperatures of most other sites with high rates of AMO are not significantly different from those of background seawater (earning them the name “cold seeps”). However, Schouten et al. (64) identified lipids that are indicative of methanotrophic *Archaea* throughout a sediment core from hydrothermally influenced sediments of the Guaymas Basin reaching 95°C. Those authors claimed evidence for active microbes involved in AMO at temperatures of >30°C. Kallmeyer and Boetius (34) measured low rates of AMO at up to 85°C at the same site. Therefore, determining whether the ANME-1 phylotype discovered at the LCHF lives at high temperatures and whether the LCMS biofilms known to exist at high temperatures are methanogens or methanotrophs has biogeochemical and ecological importance.

In all known environments where AMO occurs, anaerobic methanotrophic *Archaea* co-occur with sulfate-reducing *Bacteria* (SRB), commonly in tightly coupled consortia (8), although not always in direct physical contact (56). Incubation experiments have shown that this consortium represents a syntrophic metabolic relationship between the methane-oxidizing *Archaea* and SRB (19, 24, 52, 53). The role of *Bacteria* in CH₄ cycling at the LCHF, however, has not been thoroughly explored in previous studies, and it is not known whether SRB are present (38, 65). To further characterize the microbial communities of the LCHF, we have examined the phylogenetic diversity of both archaeal and bacterial 16S rRNA genes in geochemically distinct carbonate and fluid samples. Utilizing terminal restriction fragment length polymorphism (T-RFLP) analyses, the distribution of specific phylogenetic groups of *Archaea* and *Bacteria* was determined. The distribution of microbial groups within the LCHF shows important biogeochemical linkages between serpentinization reactions and microbial metabolism.

MATERIALS AND METHODS

Sample collection. Carbonate chimney samples were collected from the LCHF with DSV *Alvin* (dives 3651 and 3862 to 3881) during cruises AT03-6 and

AT07-34 aboard the R/V *Atlantis* in December 2000 and April to May 2003 (38). Shipboard, subsamples of chimney material were frozen immediately at –80°C and remained frozen until onshore analysis. Filtered fluid samples were obtained using a hydrothermal fluid and particle sampler (10). Approximately 1 liter of fluid was pumped through a Sterivex-GP (0.22-μm) filter while the pump rate and temperature of the fluid were measured. Once on deck, the filters were placed into 50-ml sterile Falcon tubes (BD Sciences Labware) and stored at –20°C until DNA extraction. Fluid samples were also preserved in a 2% formalin solution and stored at 4°C. Cell enumeration of DAPI (4',6'-diamidino-2-phenylindole)-stained cells (59) was performed using a Nikon Eclipse E600 POL epifluorescence microscope.

Sample descriptions. Samples were collected from carbonate structures with a range of venting temperatures and chemistries (Table 1), representing actively venting, hot chimneys (markers 2, 3, C, H, and 7) and less-active, cooler chimneys (markers X1 and X2). Active chimneys tended to cluster around the Poseidon complex (Fig. 1), while carbonate deposits at markers X1 and X2 were found at the periphery of the field. The chimney at marker 3 represents one of four pinnacles at the summit of the Poseidon complex, and the fluid sample from this chimney had the highest temperature (81°C) of any samples in this study and approached the maximum temperature of any vent at the LCHF (91°C). Two carbonate samples from near marker 2 were collected from the interior of a very large flange after the cooler, outer portions of the flange were cleared away by DSV *Alvin*. The carbonate material collected from markers 2 and 3 appeared to be the youngest samples in this study; i.e., the carbonate was very friable and not well lithified. These samples also had the largest quantity of a mucilaginous biofilm-like material, deposited throughout the carbonate matrix. Marker C is at a large flange composed of aragonite, calcite, and brucite [Mg(OH)₂]. The top of the 70°C venting flange is comprised of well-lithified carbonate bathed in seawater. Markers H, 7, and 8 are located on somewhat cooler (46 to 53°C) chimneys and are located at the perimeter of the Poseidon complex on the east side of the field. Carbonate material from marker H appeared to be similar to the young carbonate deposits collected at markers 2 and 3. Marker 6 represents an actively venting chimney (70°C) located between the summit of Poseidon and markers H and 7. The lithified chimneys exhibiting little or no venting near markers X1 and X2 were discovered during exploratory DSV *Alvin* dives and are located several hundred meters downslope from the Poseidon complex. The marker X2 chimney was very highly lithified, having a dark gray and brownish color, while the chimney at marker X1 appeared to have recently precipitated carbonate in its interior. Sample characteristics are summarized in Tables 1 and 2, and Fig. 2 contains photographs of chimneys from which carbonate samples were analyzed.

Clone library construction and sequencing. DNA was extracted from carbonate chimney material as previously described (6, 66), with the following modifications: 80 μl of 5 M NaCl and 80 μl of 10% cetyltrimethylammonium bromide in 0.7 M NaCl were added to 1 g of carbonate chimney sample and incubated at 50°C for 30 min after proteinase K treatment. To prevent shearing of genomic DNA, samples were not subjected to freeze-thaw treatment. Crude DNA extracts were purified with QiaQuick columns (QIAGEN) according to the man-

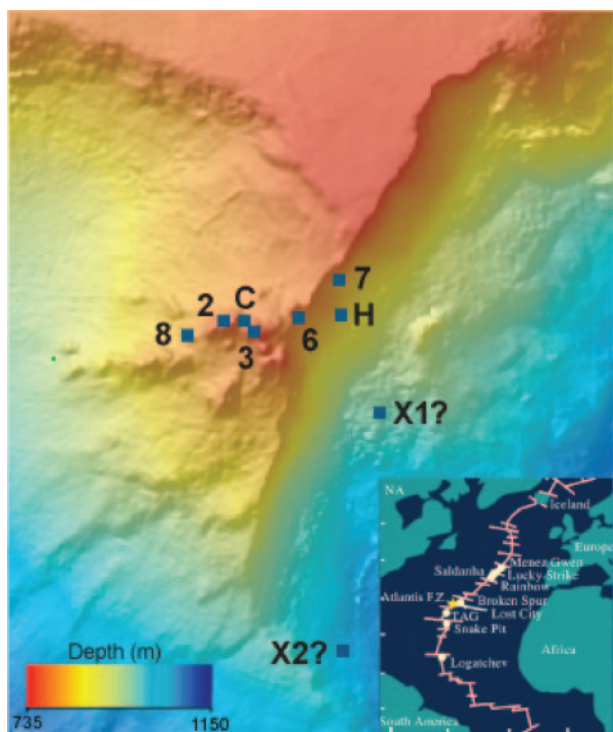


FIG. 1. Sample locations in the Lost City hydrothermal field analyzed in this study. Markers 2, 3, 6, 8, and C are within the central portion of the Poseidon complex of carbonate chimneys. Markers X1 and X2 are isolated chimneys exhibiting greatly reduced or no apparent hydrothermal activity.

ufacturer's instructions for PCR purification. Sterivex filters were extracted and purified as described previously (27). Amplification of the 16S rRNA gene via PCR was performed using the universal primers ARC-21F (5'-TTC CGG TTG ATC CYG CCG GA-3') and ARC-958R (5'-YCC GGC GTT GAM TCC AAT T-3') for *Archaea* and BAC-8F (5'-AGR GTT TGA TCC TGG CTC AG-3') and BAC-1492R (5'-CGG CTA CCT TGT TAC GAC TT-3') for *Bacteria*. The PCR-amplified DNA was reconditioned using the protocol described previously

by Thompson et al. (74) and cloned using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. Nearly full-length sequences of cloned inserts were obtained using a MegaBACE 1000 apparatus (Molecular Dynamics) at the Marine Molecular Biotechnology Laboratory, University of Washington (UW), according to the methods described previously by Huber et al. (27) using the sequencing primers described previously by Huber et al. (29).

T-RFLP analysis. T-RFLP analyses of carbonate chimney and fluid samples were performed using universal primers for archaeal and bacterial 16S rRNA genes. T-RFLP primer sequences for the 16S rRNA gene are as follows (51): ARC21F (5'-6-carboxyfluorescein-TTC [dP]GG TTG ATC C[dP]G CC[dK] GA-3'), ARC922R (5'-[dP]CCG GCG TTG A[dN]T CCA ATT-3'), BAC68F (5'-6-carboxyfluorescein-T[dN]A [dN]AC ATG CAA GTC G[dK][dK] CG-3'), and BAC1492R (5'-[dK]G[dP] TAC CTT GTT ACG ACT T-3'), where [dP] indicates the pyrimidine analog P, [dK] indicates the purine analog K, and [dN] is an equal mixture of the two analogs (Glen Research, Sterling, VA).

Aliquots of approximately 5 µl of purified PCR products (QiaQuick columns; QIAGEN) were digested overnight with 0.25 U of the restriction endonucleases HaeIII, MspI, and BstUI for 16S rRNA genes. Digests were immediately ethanol precipitated according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc.) and analyzed using a MegaBACE 1000 apparatus (Molecular Dynamics). Electrophoretic profiles were visualized with DAX software (Van Mierlo Software Consultancy, The Netherlands) at the UW Marine Molecular Biotechnology Laboratory.

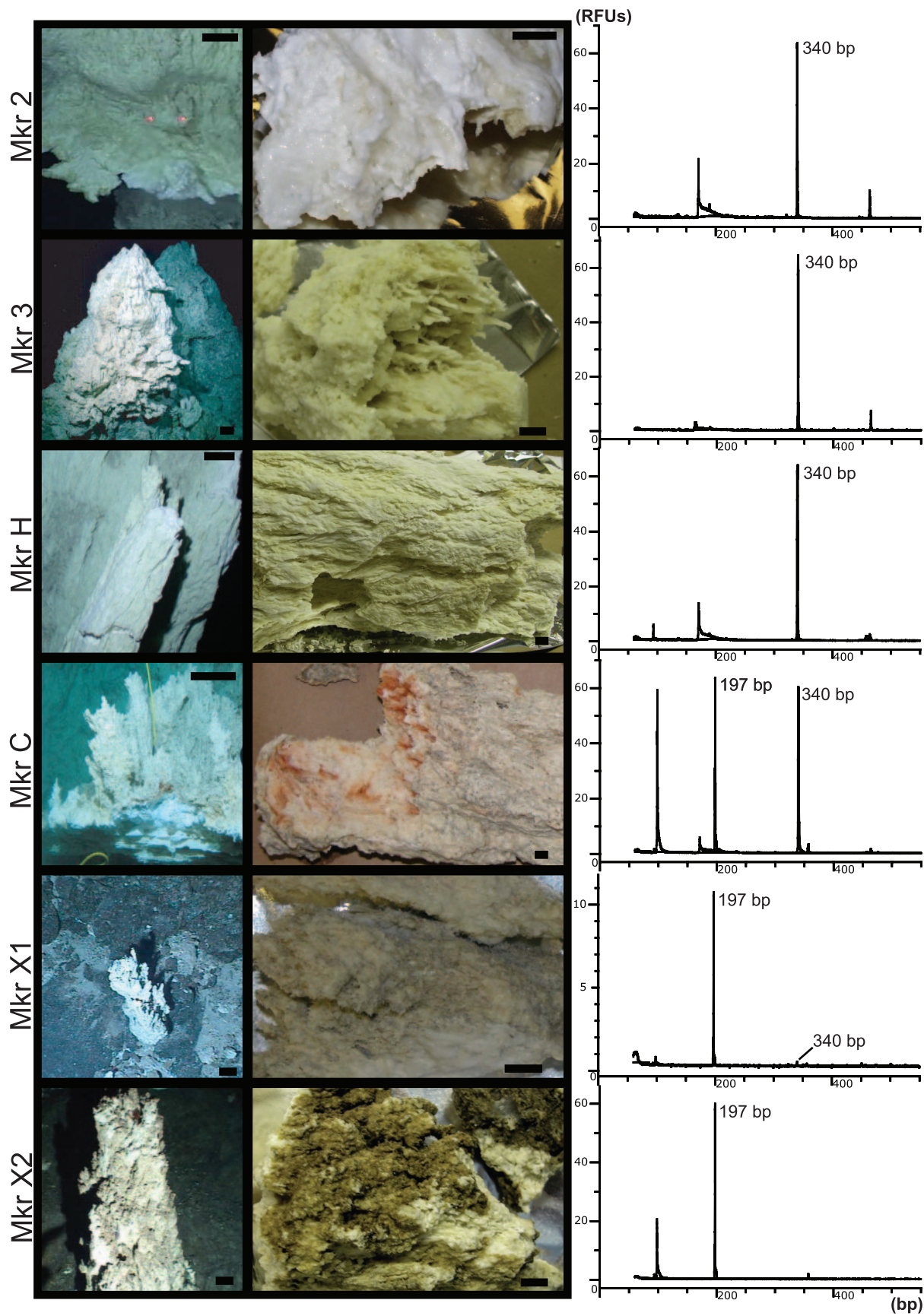
Predicted terminal restriction fragments (TRFs) of clones were obtained using Sequencher (version 4.1.2; Gene Codes Corp.). Actual TRF profiles of environmental samples and some clones were compared to the predicted TRFs. The presence or absence of phylogenetic groups (Table 2 and see Fig. 6) was determined by the positive identification of a predicted TRF in the actual TRF profile of an environmental sample. To score a phylogenetic group as "present," two criteria were required: (i) the predicted TRF size matched the actual TRF size within ±2 bp, and (ii) at least two of the three restriction enzyme digests yielded a positive identification of a predicted TRF of a clone belonging to that group. Clones that had fewer than two predicted TRFs within the 50- to 550-bp size window were not included in the analysis. The counting of operational taxonomic units (OTUs) can yield various results depending on the threshold criteria and variation due to PCR errors (15, 41, 49, 62). For this study, an OTU in a TRF profile was defined as one bin of peaks within ±2 bp having a magnitude of relative fluorescent units greater than that of the signal bleed-through from the standard DNA marker channel. These criteria were validated by replicate PCRs and replicate digests of environmental samples as well as cloned DNA.

Phylogenetic analysis. Archaeal and bacterial 16S rRNA gene nucleotide sequences were aligned using ClustalX, version 1.81 (73). PCR primers and hypervariable regions were omitted from the alignments. Clones were checked for chimeric sequences using the CHIMERA_CHECK program of RDP II and the Bellerophon server (30). The Phylip version 3.62 software package (obtained

TABLE 2. Archaeal and bacterial phylotypes recovered in 16S rRNA gene clone libraries and OTUs resolved by T-RFLP analysis of carbonate chimney and fluid samples^a

Sample	Archaeal phylotype (no. of clones)	Archaeal OTUs (no. identified) (HaeIII-MspI-BstUI)	Bacterial phylotype (no. of clones)	Bacterial OTUs (no. identified) (HaeIII-MspI-BstUI)
3864-1524	ND	ND	7 (27)	12 (4)-15 (5)-15 (4)
3864-1537	ND	5 (1)-7 (2)-4 (1)	15 (46)	12 (8)-16 (11)-15 (6)
3881-1408	1 (29)	5 (1)-6 (2)-4 (2)	37 (55)	13 (6)-ND-13 (7)
FS242	ND	10 (4)-9 (1)-13 (2)	ND	20 (14)-24 (13)-19 (9)
3881-1228	2 (3)	7 (2)-4 (3)-4 (3)	24 (32)	ND
FS243	16 (57)	9 (3)-8 (1)-11 (3)	ND	24 (10)-22 (11)-23 (10)
3869-1404	1 (3)	4 (1)-3 (1)-4 (1)	3 (3)	23 (12)-26 (10)-18 (7)
3869-1443	ND	7 (2)-7 (3)-5 (4)	8 (9)	27 (14)-37 (15)-20 (9)
3869-1446	ND	10 (3)-7 (4)-9 (2)	40 (72)	ND
FS221	ND	ND	ND	8 (6)-9 (4)-7 (2)
3876-1133	1 (2)	4 (2)-4 (2)-4 (2)	30 (54)	23 (12)-ND-22 (6)
3880-1557	1 (2)	2 (1)-3 (2)-4 (1)	ND	21 (10)-24 (7)-20 (8)
FS222	ND	9 (2)-8 (1)-11 (1)	ND	15 (7)-24 (13)-17 (5)
FS197	2 (3)	12 (3)-12 (2)-15 (2)	ND	24 (9)-16 (3)-17 (8)
FS209	12 (50)	ND	ND	11 (4)-7 (4)-11 (5)

^a Archaeal and bacterial phylotypes are defined as 97% DNA sequence similarity. The total number of clones in the library and the number of OTUs that were identified as corresponding to a terminal restriction fragment of a clone in Table 3 are shown in parentheses. Samples for which a clone library or T-RFLP analysis was not attained are indicated by "ND."



from J. Felsenstein, UW) was used to construct maximum-likelihood (DNAML) trees. Confidence estimates for tree topology were calculated by bootstrap analysis (SEQBOOT) using 100 replicates. Trees were drawn with TreeViewPPC (version 1.6.6) and edited with Adobe Illustrator (version 10.0.3).

Nucleotide sequence accession numbers. GenBank accession numbers for the clones reported here are listed in Table 3 and include accession numbers DQ228555 to DQ228583, DQ270590 to DQ270662, and DQ272585.

RESULTS

Archaeal 16S rRNA. As reported previously (38, 65), samples of carbonate chimneys from the LCHF exhibited remarkably low archaeal diversity (Table 2). Phylogenetic analyses of 16S rRNA genes revealed a single phylotype each of Lost City *Methanosarcinales* (LCMS) and ANME-1 (clones LC1022A-1, LC1149A-56, LC1133A-9, LC1133A-17 in Fig. 3a and Table 3). The latter clones are most similar to the ANME-1a subgroup, which includes clones collected from a variety of CH₄ seeps, hydrothermal sediments, and Black Sea carbonate reefs (23, 39, 40, 48, 55, 71, 72). LCMS has 93% similarity to a cluster of sequences that are derived from CH₄ seep sediments and a mud volcano in the Barents Sea (20, 39, 47, 55).

The entire suite of samples (Table 1) was assayed for TRFs corresponding to the 16S rRNA gene clones (Table 3). Actively venting, high-temperature chimneys such as those at markers 2, 3, and H were dominated by LCMS, while less-active carbonate veins hosted in serpentinite (markers X1 and X2) contained ANME-1 but not LCMS. A TRF of very low relative peak height corresponding to LCMS, however, was identified in the marker X1 sample. Peaks with a low relative height corresponding to LCMS were also identified in all fluid samples, likely due to the entrainment of portions of the densely populated LCMS biofilms into venting fluid. T-RFLP profiles for all three samples collected from marker C, which appears to consist of both young and old carbonate minerals (see Discussion), displayed peaks for both LCMS and ANME-1 TRFs (Fig. 2). Digests with alternative restriction enzymes (HaeIII and MspI) also yielded additional peaks but none with relative peak heights comparable to those of the peaks corresponding to LCMS or ANME-1. Although T-RFLP analysis is not a quantitative measurement and is susceptible to PCR biases, the order of magnitude difference in peak heights between the LCMS and ANME-1 peaks and those of all other peaks is consistent with the high percentage of LCMS previously found in phylogenetic and fluorescence in situ hybridization studies at this site (38, 65).

A somewhat wider diversity of archaeal 16S rRNA gene sequences was recovered from hydrothermal fluid samples (Fig. 3b). Many of the clones exhibited significant similarity to a variety of uncultured organisms belonging to the marine

groups I, II, and III of the *Archaea*; these clones were not identified in any chimney samples by T-RFLP analysis, indicating that they are probably derived from the ambient seawater. Clones FS209A-1, FS243A-60, and FS209A-10, however, clustered with a novel group of uncharacterized *Crenarchaeota* that were identified within the walls of a high-temperature sulfide chimney (66). In addition, three sequences belonging to the *Thermococcales* (clones FS197A-23, FS197A-30, and FS209A-41) were identified in three fluid samples (from markers H, 7, and 8). These clones have extremely high sequence similarity (>99%) to an isolate from the Axial Volcano (28).

Bacterial 16S rRNA genes. A high diversity of bacterial 16S rRNA gene sequences was recovered from LCHF carbonate chimney samples spanning many phyla (Fig. 4 and 5). The most diverse group was the gamma-proteobacteria (Fig. 4), which contained clones belonging to several methylophilic genera including *Methylobacter*, *Methylomonas*, and *Methylophaga*. A number of clones formed a new clade within the genus *Thiomicrospira* (94% similar to *Thiomicrospira* sp. strain Milos-T2), a group of sulfur-oxidizing organisms commonly associated with hydrothermal vents. TRFs corresponding to clones from the *Methylobacter/Methylomonas*, *Methylophaga*, and *Thiomicrospira* clades (Table 3) were found in all carbonate chimney and fluid samples analyzed in this study (Table 1 and Fig. 5). The two clones representing the CH₄ seep/hydrate group (clones LC1133B-18 and LC1228B-91) (Table 3 and Fig. 4) were present in four of the six fluid samples analyzed but in only one chimney sample.

There was an unusually large diversity of *Firmicutes* in the carbonate chimneys (Fig. 5). Clones LC1537B-22 and LC1228B-98 have 88 to 92% sequence similarity to *Desulfotomaculum halophilus* and *Desulfotomaculum alkaliphilus*; many members of the *Desulfotomaculum* genus are known to be sulfate reducers (31) and are found in high-temperature, high-pH environments (61). In contrast to other AMO environments, there is a notable lack of delta-proteobacterial sequences in our Lost City clone libraries. The LCHF *Desulfotomaculum* clones, the only candidate sulfate-reducing *Bacteria* identified, were not detected in the ANME-1-dominated sample from marker X1 (Fig. 6), casting doubt on its ability to be the syntrophic partner in anaerobic methane oxidation.

Clones LC1133B-75, LC1446B-28, and LC1133B-108 consistently grouped apart from other sequences from *Firmicutes* spp. with both neighbor-joining (data not shown) and maximum-likelihood phylogenetic trees (Fig. 5), even though the three previously published sequences with which they group (88 to 89% sequence similarity among all six clones) have been described as low-GC-content, gram-positive *Bacteria* (43).

FIG. 2. T-RFLP peaks generated by BstUI digestion of archaeal 16S rRNA genes amplified from carbonate chimney samples. Photographs of the chimneys at each marker are shown (first column), as are the samples of each chimney used in the analysis (second column). The 340-bp LCMS fragment is present in the active chimneys at markers 2, 3, H, and C. The 196-bp ANME-1 fragment is also detected at marker C as well as a weakly venting chimney at marker X1 and an extinct chimney at marker X2. A very short peak corresponding to LCMS is also barely noticeable in the marker X1 profile. The carbonate material recovered from the active chimneys (at markers 2, 3, and H) was whiter and softer than the gray, lithified minerals of the extinct chimneys from markers X1 and X2. A milky, biofilm-like substance pervaded the sample from marker 2, as can be seen in the photo. The sample from the marker C flange exhibited both types of mineralogies; in the photo, bright white brucite deposits are evident in the actively venting portion underneath a gray, more-lithified zone that is bathed in background-temperature seawater (see the text for details). RFUs, relative fluorescent units.

TABLE 3. Archaeal and bacterial 16S rRNA gene clones generated in this study, in addition to previously published LCMS and marine group I clones^a

Domain	Clone	GenBank accession no.	Phylogenetic group	Fragment size (bp)			
				HaeIII	MspI	BstUI	
<i>Archaea</i>	LC1022A-1	AY299516	LCMS	219	130	340	
	LC1149A-56	AY299515	LCMS	219*	130*	340*	
	LC1133A-9	AY760632	ANME-1	236*	96*	196*	
	LC1133A-17	DQ270605	ANME-1	235	96	197	
	FS197A-23	DQ270592	<i>Thermococcales</i>	154*	78	73	
	FS197A-30	DQ270593	<i>Thermococcales</i>	154	78	73	
	FS209A-41	DQ270596	<i>Thermococcales</i>	154	78	73	
	FS243A-6	DQ270598	Unc. Eury.	22	81	93	
	FS243A-60	DQ270602	Unc. Cren.	152	124	331	
	FS209A-10	DQ270595	Unc. Cren.	165	68	82	
	<i>Bacteria</i>	LC1228B-35	DQ270650	<i>Actinobacteria</i>	187	426	184
		LC1022B-39	DQ228573	<i>Actinobacteria</i>	191	105	472
		LC1022B-1	DQ228574	<i>Actinobacteria</i>	309	455	96
LC1022B-27		DQ228572	<i>Actinobacteria</i>	ND	ND	ND	
LC1133B-64		DQ270644	Alpha-proteobacteria	188	399	58	
LC1133B-90		DQ270648	Alpha-proteobacteria	188	400	58	
LC1133B-8		DQ270640	Alpha-proteobacteria	188	400	58	
LC1133B-74		DQ270646	Alpha-proteobacteria	183	429	72	
LC1133B-45		DQ270642	Alpha-proteobacteria	163	410	167	
LC1231B-170		DQ228567	Alpha-proteobacteria	188	400	58	
LC1022B-48		DQ228566	Alpha-proteobacteria	188	399	58	
LC1133B-23		DQ270615	Animal	152	288	26	
LC1228B-46		DQ270625	Animal	151	286	26	
LC1133B-24		DQ270616	Animal	155	107	358	
LC1524B-50		DQ270636	Beta-proteobacteria	165	395	354	
LC1537B-16		DQ270638	Beta-proteobacteria	165	440	354	
LC1022B-30		DQ228559	Beta-proteobacteria	165	455	354	
LC1537B-77		DQ272585	<i>Chloroflexus</i>	181	174	361	
LC1231B-173		DQ228575	<i>Chloroflexus</i>	179	256	202	
LC1408B-77		DQ270634	CFB	361	442	65	
LC1446B-1		DQ270658	CFB	362	161	68	
LC1524B-89		DQ270637	CFB	376	458	351	
LC1228B-138		DQ270654	CFB	243	50	70	
LC1133B-32		DQ270641	CFB	368	50	70	
LC1022B-31		DQ228583	CFB	235	441	57	
LC1228B-98		DQ270651	<i>Desulfotomaculum</i>	270	131*	77*	
LC1537B-22		DQ270639	<i>Desulfotomaculum</i>	225	130	26	
LC1404B-6		DQ270655	Epsilon-proteobacteria	769	431	355	
LC1133B-68		DQ270645	Epsilon-proteobacteria	767	431	355	
LC1228B-116		DQ270653	Epsilon-proteobacteria	767	431	355	
LC1133B-58		DQ270643	Epsilon-proteobacteria	246	431	455	
LC1408B-26		DQ270633	Epsilon-proteobacteria	855	431	355	
LC1149B-14		DQ228557	Epsilon-proteobacteria	270	127	340	
LC1149B-70		DQ228579	Epsilon-proteobacteria	1,133	162	349	
LC1149B-104		DQ228555	Epsilon-proteobacteria	1,419	125	335	
LC1231B-176		DQ228582	Epsilon-proteobacteria	ND	436	360	
LC1149B-9		DQ228581	Epsilon-proteobacteria	ND	ND	353	
LC1149B-130		DQ228580	Epsilon-proteobacteria	ND	162	349	
LC1149B-115		DQ228556	Epsilon-proteobacteria	ND	124	264	
LC1408B-88		DQ270635	<i>Firmicutes</i>	267*	435^	76*	
LC1022B-45		DQ228570	<i>Firmicutes</i>	270	515	196	
FS209A-1		DQ270594	Unc. Cren.	192	124	332	
FS197A-14		DQ270591	Marine group I	214*	284*	460	
FS243A-39		DQ270601	Marine group I	214	284	460	
FS243A-7		DQ270590	Marine group I	214*	285*	461^	
FS243A-90		DQ270604	Marine group I	214	284	460	
LC1231A-51		AY505046	Marine group I	214	284	460	
FS243A-20		DQ270599	Marine group II	208	191	260	
FS243A-33		DQ270600	Marine group II	208	191	59	
FS243A-89		DQ270603	Marine group II	315	191	130	
FS243A-3	DQ270597	Marine group III	216	75	131		
LC1022B-35	DQ228568	<i>Firmicutes</i>	236	28	196		
LC1149B-139	DQ228571	<i>Firmicutes</i>	268	434	76		
LC1022B-12	DQ228569	<i>Firmicutes</i>	269	114	195		

Continued on facing page

TABLE 3—Continued

Domain	Clone	GenBank accession no.	Phylogenetic group	Fragment size (bp)		
				HaeIII	MspI	Bstul
	LC1022B-45	DQ228570	<i>Firmicutes</i>	270	515	196
	LC1537B-86	DQ270611	<i>Marinomonas</i>	212	449	69
	LC1228B-136	DQ270627	<i>Methylobacter</i>	365	451	350
	LC1133B-145	DQ270623	<i>Methylobacter</i>	367	453	477
	LC1133B-37	DQ270617	<i>Methylobacter</i>	163	452	168
	LC1446B-16	DQ270628	<i>Methylobacter</i>	289	459	176
	LC1133B-99	DQ270621	<i>Methylobacter</i>	230	465	70
	LC1446B-37	DQ270629	<i>Methylobacter</i>	279	449	70
	LC1133B-5	DQ270612	<i>Methylobacter</i>	282	453	168
	LC1133B-61	DQ270619	<i>Methylobacter</i>	214	451	70
	LC1133B-86	DQ270620	<i>Methylobacter</i>	161	451	70
	LC1231B-184	DQ228565	<i>Methylobacter</i>	215	452	70
	LC1408B-19	DQ270606	<i>Methylophaga</i>	182	451	352
	LC1446B-88	DQ270631	<i>Methylophaga</i>	282	453	352
	LC1231B-11	DQ228564	<i>Methylophaga</i>	208	426	26
	LC1443B-4	DQ270657	<i>Nitrospira</i>	357*	408*	520 [^]
	LC1228B-113	DQ270652	<i>Nitrospira</i>	355	406	518
	LC1404B-28	DQ270656	<i>Nitrospira</i>	357*	408*	520 [^]
	LC1446B-146	DQ270662	<i>Planctomycetes</i>	247	88	74
	LC1446B-77	DQ270660	<i>Planctomycetes</i>	217	150	84
	LC1231B-20	DQ228576	<i>Planctomycetes</i>	173	103	20
	LC1231B-166	DQ228578	<i>Planctomycetes</i>	220	438	217
	LC1231B-5	DQ228577	<i>Planctomycetes</i>	245	134	112
	LC1228B-91	DQ270626	Seep/hydrate	152	455	26
	LC1133B-18	DQ270614	Seep/hydrate	216	104	72
	LC1446B-28	DQ270659	Subsurface firm	297	157	40
	LC1133B-75	DQ270647	Subsurface firm	297	157	40
	LC1133B-108	DQ270649	Subsurface firm	171	151	211
	LC1446B-39	DQ270630	<i>Thiomicrospira</i>	85	355	89
	LC1537B-12	DQ270608	<i>Thiomicrospira</i>	184	455	72
	LC1408B-58	DQ270607	<i>Thiomicrospira</i>	220	457	481
	LC1537B-49	DQ270609	<i>Thiomicrospira</i>	184	454	170
	LC1133B-148	DQ270624	<i>Thiomicrospira</i>	186	432	74
	LC1133B-127	DQ270622	<i>Thiomicrospira</i>	185	455	73
	LC1149B-99	DQ228561	<i>Thiomicrospira</i>	184	455	72
	LC1022B-40	DQ228560	<i>Thiomicrospira</i>	297	255	86
	LC1537B-81	DQ270610	<i>Vibrio</i>	174	464	181

^a See reference 65. The predicted terminal restriction fragment size for each restriction enzyme is generated by identifying the first restriction site for each enzyme 3' to the fluorescently tagged forward primer. Fragment sizes were experimentally verified within ± 2 bp (*) or ± 4 bp (°). The absence of a restriction site present in the portion of the clone that was sequenced is indicated by "ND." Unc. Eury., uncultured *Euryarchaeota*; Unc. Cren, uncultured *Crenarchaeota*; CFB, *Cytophaga-Flavobacterium-Bacteroides*.

Since all three previously published clones have been recovered from sediment or subsurface sources (43), these clones are tentatively identified as a "subsurface" clade in Fig. 3b. TRFs corresponding to clones from the "subsurface" clade were positively identified in three fluid samples (markers 3, H, and C) using the restriction enzyme HaeIII, but the predicted TRFs of these clones for MspI and Bstul either were outside the 50- to 500-bp analysis window of this study or did not match any environmental TRFs within 2 bp. Therefore, the uncertain presence of the "subsurface" clade in these fluid samples is indicated by the question marks in Fig. 6.

A number of clones group within the epsilon-proteobacteria (Fig. 5) and were identified in samples of carbonate chimneys and fluids of various temperatures and chemical characteristics (Fig. 5). The only cultured isolate with significant similarity to these clones is *Sulfurovum lithotrophicum* (94% sequence similarity to clones LC1133B-58 and LC1133B-68), a sulfur-oxidizing chemolithoautroph from hydrothermal sediments in a black smoker environment (33). Clone LC1537B-77 groups with environmental clones from three different mud volcanoes

in the eastern Mediterranean (90 to 92% sequence similarity) (Fig. 5) (S. K. Heijs et al., unpublished data).

DISCUSSION

Archaea associated with active and inactive carbonate structures. Phylogenetic and T-RFLP analyses of the LCHF show that the diversity of archaeal 16S rRNA genes is dominated by LCMS in sites of active venting and by ANME-1 in sites that are only weakly venting or inactive (Fig. 2 and 6). A temperature-controlled zonation of ANME communities at the Lost City is consistent with in vitro studies (53), which found that the temperature optimum for AMO was the biggest physiological difference between ANME-1 and ANME-2 communities.

The only exceptions to the segregation of LCMS and ANME-1 are data from the marker C samples, where both phylotypes were present. The underside of the flange at marker C hosts a shallow pool of 70°C hydrothermal fluid. Analyses of material from the flange interior showed that it is porous and comprised of carbonate minerals and brucite. Since brucite

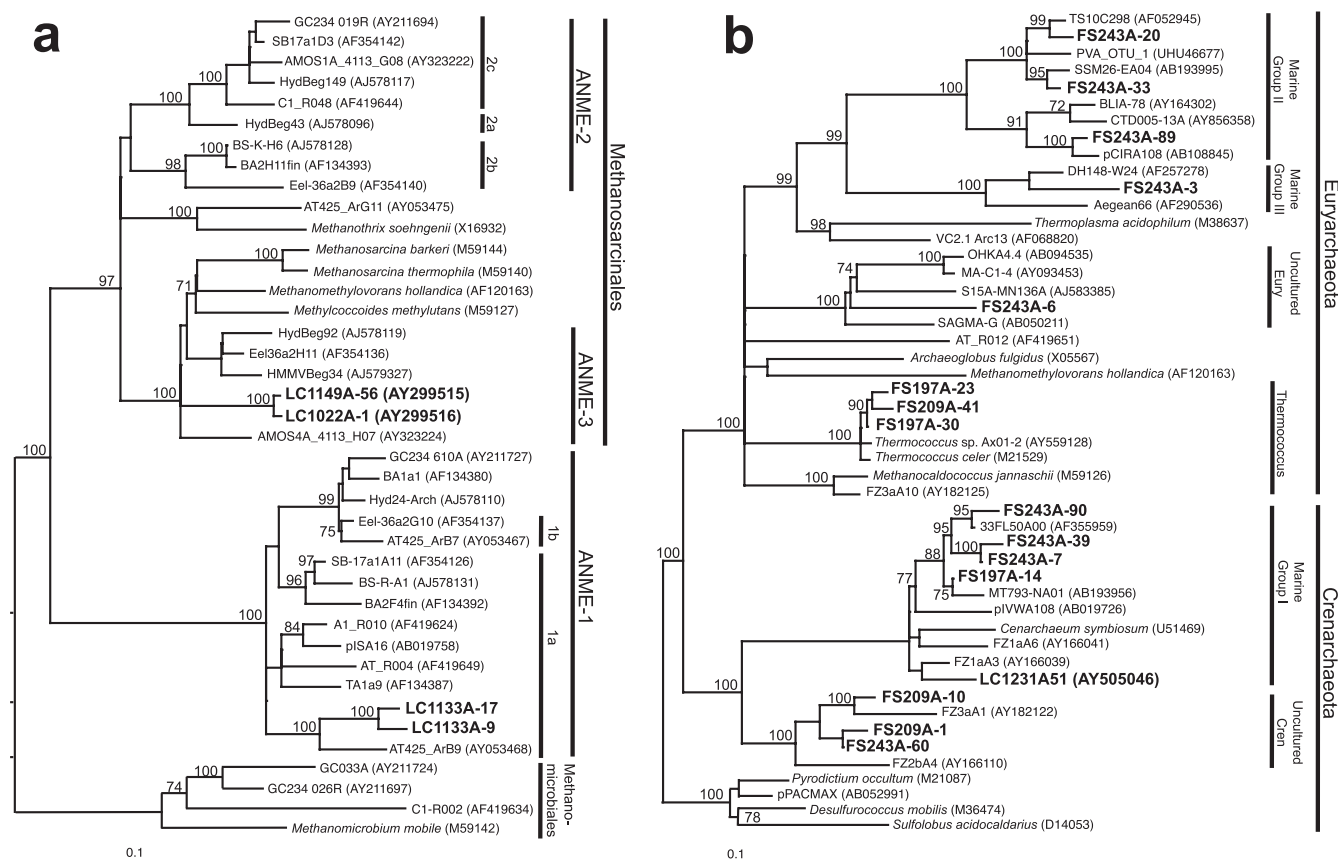


FIG. 3. Maximum-likelihood phylogenetic trees depicting the relationships of archaeal 16S rRNA gene sequences from LCHF carbonate chimneys to other ANME-related sequences. LC1022A-1 and LC1149A-56 (65) were cloned from carbonate mineral samples from active chimneys (a). LC1133A-9 and LC1133A-17 were cloned from sample X1, a chimney with very little venting fluid. Fluid samples (b) represent a mixture of hydrothermal fluid and ambient seawater (see Table 1 and the text for details). *Methanomethylivorans hollandica* is included in b as a representative of the *Methanosarcinales*. Lost City hydrothermal field environmental clones are in boldface type. Phylogenetic groups used in the T-RFLP analysis are labeled. Trees are unrooted, and scale bars represent 1 nucleotide change per 10 bases. GenBank accession numbers are in parentheses.

dissolves in seawater, it is likely that the porous interior of the flange was dominated by hydrothermal fluid. In contrast, the outer portion of the flange is well lithified, gray, and bathed in seawater (Fig. 2). Carbonate minerals sampled for T-RFLP analyses likely contained material from both the exterior and interior of the flange. Based on known environmental conditions at other sampling sites (markers 2, 3, X1, and X2), we infer that LCMS sequences were derived from the interior and that ANME-1 sequences were derived from the exterior of the flange at marker C.

The presence of LCMS in warm, actively venting chimneys and the presence ANME-1 in cold, inactive or very weakly venting structures suggest that ecological succession occurs in the microbial community as carbonate chimneys cool down, become less active, and are progressively bathed in seawater. Succession is also consistent with the increased number of bacterial OTUs (Table 2) identified in markers C, X1, and X2 compared to those at presumably younger chimneys. Preliminary age dating of a few carbonate samples, coupled with modeling, shows that hydrothermal activity at the Lost City may be remarkably stable and long lived, in contrast to the transient environments that typify most black smoker environments (35). Carbon isotopic analyses showed that venting has

been ongoing for at least 30,000 years, with localized sites active for at least 50 to 300 years, and modeling results indicate that venting may last for 100,000 to up to 1 million years (17).

An important, unresolved question regarding LCMS and ANME-1 organisms at the LCHF is whether they are methanogens or methanotrophs. Knittel et al. (39) designated a cluster of sequences containing LCMS as "ANME-3," which could indicate that these organisms are also involved in AMO. However, this seems unlikely at the LCHF, because the very high H_2 concentrations (1 to 15 mM) should strongly favor methanogenesis. In addition, archaeal lipids collected from carbonate chimney samples at this site have a much higher $\delta^{13}C$ level (-8.5‰ to 4.8‰ versus Pee Dee Belemnite) (A. S. Bradley, J. M. Hayes, and R. E. Summons, Eos Trans. AGU Fall Meet. Suppl. 85[47], abstr. B33B-0263, 2004) than would be predicted for organisms involved in AMO, whose lipids can have $\delta^{13}C$ values of -85‰ or lower (38, 57). The abundant H_2 and enriched ^{13}C content of lipids must be reconciled, however, with the lack of dissolved inorganic carbon (DIC) available to putative methanogens living in an environment dominated by serpentinization-derived fluids. Unlike other H_2 - and CH_4 -rich subsurface environments where the $\delta^{13}C$ values of CH_4 are significantly more depleted than those of the source

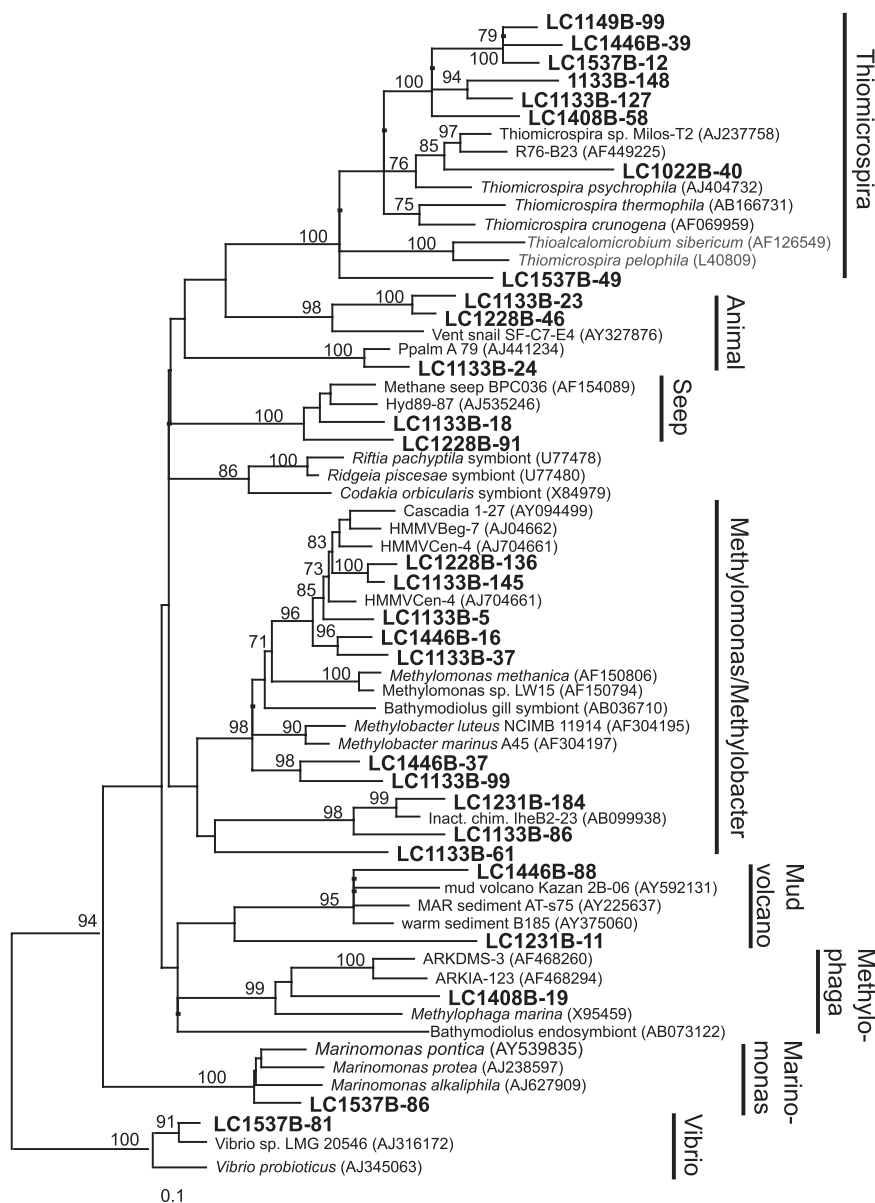


FIG. 4. Maximum-likelihood phylogenetic tree of gamma-proteobacterial 16S rRNA genes cloned from LCHF carbonate chimneys. Lost City hydrothermal field environmental clones are in boldface type. Labeled phylogenetic groups correspond to the groups used in the T-RFLP analysis (Fig. 6). Trees are unrooted, and scale bars represent 1 nucleotide change per 100 bases. GenBank accession numbers are in parentheses.

carbon and thus indicate a strong biological source (50, 70), the $\delta^{13}\text{C}$ content of CH_4 (-8.8‰ to -13.6‰) at the LCHF is similar to that of fluid DIC (-2‰ to -8‰), chimney carbonate minerals ($+13\text{‰}$ to -7‰), and chimney total organic carbon (-3.1‰ to -18.1‰) (38). The absence of ^{13}C -depleted CH_4 and archaeal lipids could be a result of carbon limitation due to high pH and an apparent absence of magmatic CO_2 effectively eliminating available DIC. Because DIC is limiting and because CH_4 has multiple possible sources (methanogens and serpentinization-associated reactions) and sinks (anaerobic and aerobic methanotrophy), stable isotope ratio measurements in this system are difficult to interpret. Mixing models of abiotic and biologically derived CH_4 (42) may allow a more intricate understanding of the carbon budget

of the LCHF. Nevertheless, it is interesting that the most ^{13}C -depleted organic carbon from the LCHF is derived from archaeal lipids recovered from a sample at marker X2, which is dominated by ANME-1 (A. Bradley, personal communication). Therefore, while the stable isotope data are enigmatic, it is broadly consistent with a temperature-associated zonation of methanogenesis and AMO.

Archaea in hydrothermal fluid samples. The microbial populations present in the fluid samples reflect the widespread mixing of seawater with serpentinization-derived fluids that occurs at the LCHF: 16S rRNA gene sequences likely originating from seawater (marine groups I, II, and III) as well as likely hydrothermal activity-associated organisms (novel *Crenarchaeota* and *Thermococcus* species) were recovered (Fig. 3).

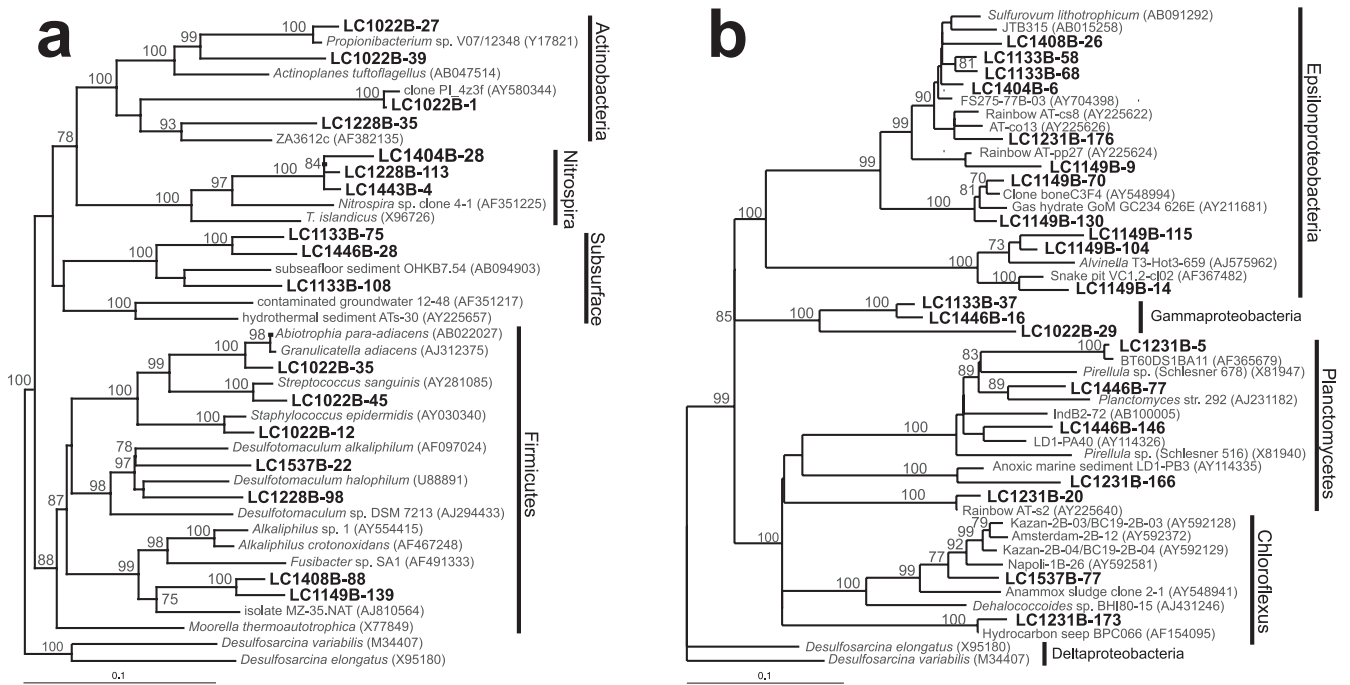


FIG. 5. Maximum-likelihood phylogenetic tree of bacterial 16S rRNA genes cloned from LCHF carbonate chimneys similar to (a) *Firmicutes* and (b) epsilon-proteobacteria and their relatives. Lost City hydrothermal field environmental clones are in boldface type. Labeled phylogenetic groups correspond to the groups used in T-RFLP analysis (Fig. 6). Trees are unrooted, and scale bars represent 1 nucleotide change per 10 bases. GenBank accession numbers are in parentheses.

Three *Crenarchaeota* clones (Fig. 3b) have high sequence similarity (90 to 94%) to sequences collected from within the walls of a 300°C sulfide chimney from a hydrothermal vent on the Juan de Fuca Ridge (66). All LCHF clones similar to high-

temperature *Crenarchaeota* species were recovered only from hydrothermal fluid samples (Fig. 6); none were detected in carbonate chimney samples. These relationships may reflect the entrainment of *Crenarchaeota* into upflowing fluids within

	Phylogenetic Group	X1	X2	2	3	H	F	C	F	6	Z	8
		C	C	C	C	F	C	F	C	F	F	F
Archaea	ANME-1											
	LCMS											
	Thermoc.											
	Unc. Eury.											
	Unc. Cren											
	MG I											
	MG II											
Bacteria	MG III											
	Methylobacter							nd				
	Animal							nd				
	Seep							nd				
	Thiomicrospira							nd				
	Methylophaga							nd				
	Marinomonas							nd				
	Vibrio							nd				
	Desulfotom.							nd				
	Other Firmicutes							nd				
	Subsurface Firm.						?	nd	?		?	
	Nitrospira							nd				
	Chloroflexus							nd				
CFB							nd					
Unc. Epsilon							nd					

FIG. 6. Presence or absence of phylogenetic groups in LCHF carbonate chimney and fluid samples. Colored and empty boxes indicate the presence and absence, respectively, of specific phylogenetic groups identified in Fig. 3 to 5. The gray box indicates the presence of a peak corresponding to LCMS of very low relative peak height, and the question marks indicate that TRFs corresponding to the “subsurface” clade were identified in the samples but did not match the sample profile within ± 2 bp (see the text for details). “C” and “F” in the top row indicate carbonate chimney or fluid samples, respectively. “nd,” no data are available. Abbreviations: ANME-1, anaerobic methanotrophic *Archaea*; LCMS, Lost City *Methanosarcinales*; Thermoc., *Thermococcus*; Unc. Eury., uncultured *Euryarchaeota*; Unc. Cren, uncultured *Crenarchaeota*; MG I, MG II, and MG III, marine groups I, II, and III, respectively; CFB, *Cytophaga-Flavobacterium-Bacteroides*. Desulfotom., *Desulfotomaculum*. Unc. Epsilon, uncultured epsilon-proteobacteria.

large, open channels that did not come into direct contact with the carbonate chimneys. The presence of *Thermococcales* sequences has been used as a bioindicator of hot seafloor habitats (25, 26, 28, 69). Their presence in moderate-temperature fluids (~50°C) within the LCHF may reflect the presence of a hot seafloor microbial habitat in the shallow mantle rocks beneath the field. This is consistent with modeling calculations that indicate temperatures of 150 to 250°C at depth (2).

Sulfur-cycling Bacteria. Although LCHF fluids contain dissolved H₂ concentrations that are several orders of magnitude higher than those present in other "hydrogen-based" ecosystems (11, 67), none of the H₂-oxidizing *Bacteria* (*Aquificales* and delta-proteobacteria) found at those sites were identified in this study. Instead, the LCHF bacterial community appears to be dominated by aerobic or microaerophilic organisms that use reduced sulfur species as electron donors. All chimney and fluid samples analyzed in this study contained 16S rRNA gene sequences belonging to *Thiomicrospira* species (Fig. 6), known as chemolithoautotrophic sulfur and sulfide oxidizers in hydrothermal vent environments (9, 63). *Thiomicrospira*-like organisms from microbial mats have been shown to mediate the sequential oxidation of sulfide to sulfur and sulfate (54), which could obscure the chemical and isotopic signature of any sulfate-reducing *Bacteria* in the system. The epsilon-proteobacteria identified in this study (Fig. 5) are also putative sulfur-oxidizing chemolithoautotrophs, evidenced by their strong sequence similarity to *Sulfurovum lithotropicum*. Building an integrated view of the LCHF ecosystem will require resolving the relationship, in terms of carbon and energy flow, between these ubiquitous sulfur-cycling *Bacteria* and the microbial mats of CH₄-cycling *Archaea* within the chimneys.

In all known instances of AMO reported to date, *Archaea* partner with SRB to form an electron-shuttling couple that makes this process more metabolically feasible (8, 24, 57, 72, 75). At the LCHF, sequences belonging to *Desulfotomaculum* were recovered, but no clones exhibited any significant sequence similarity to organisms previously reported to be associated with AMO. Some species of *Desulfotomaculum* are known to be alkaliphilic (58) and have the *dsrAB* gene encoding dissimilatory sulfite reductase, the enzyme necessary for sulfate reduction (75, 77). The LCHF *Desulfotomaculum*-like clones (LC1537B-22 and LC1228B-98), however, were identified in high-temperature chimneys lacking ANME-1 and were not identified at marker X1, where ANME-1 dominates. Attempts to amplify the *dsrAB* gene from carbonate chimney samples have been unsuccessful (unpublished observations). However, the *mcrA* gene, encoding methyl coenzyme M reductase, which is present in all known methanogens and anaerobic methanotrophs, has been amplified and sequenced from the same samples (38). These phylogenetic data, therefore, are inconsistent with *Desulfotomaculum* playing a role in AMO. Although sulfate reducers belonging to the delta-proteobacteria have been found in all AMO-associated environments, microscopic observations indicate that a close association between sulfate reducers and methanotrophic *Archaea*, especially the ANME-1 group, is not necessary (56). Therefore, it is possible that sulfate reducers within the LCHF, if they exist, are only loosely associated with ANME-1.

On the basis of previous studies (5, 32, 43), clones LC1133B-

75, LC1446B-28, and LC1133B-108 were expected to group among the other *Firmicutes* sequences identified in this study. However, the three clones repeatedly formed their own distinct clade, despite attempts to produce one monophyletic clade of *Firmicutes* by editing multiple sequence alignments, jumbling the order of sequence addition to the alignment, adding and subtracting outgroups, and using both neighbor-joining and maximum-likelihood treeing algorithms. The T-RFLP profiles from this study are ambiguous regarding the distribution of these three clones, but the tentative identification of their TRFs in three different fluid samples but in no chimney samples suggests that they are more prevalent in hydrothermal fluids than in carbonate chimneys. This possibility, coupled with the fact that previously described clones with high sequence similarity to these clones were recovered from sediments and subsurface sources, may indicate that these *Firmicutes*-like organisms represent another member of the subsurface community at the LCHF.

Biogeochemical cycling. Clones with high sequence similarity to methanotrophic *Bacteria* were identified in six carbonate chimney samples and six hydrothermal fluid samples included in the T-RFLP survey (Fig. 6). The co-occurrence of CH₄-oxidizing *Bacteria* with obligately anaerobic methanogens and anaerobic methane-oxidizing *Archaea* raises questions about microscale redox and temperature gradients present in this system. The highly porous chimney structures are likely to favor anaerobic niches within areas dominated by hydrothermal fluids but that are in close physical proximity to the seawater-influenced exterior of the chimney. Indeed, Schrenk et al. (65) have previously described distinct microbial communities inhabiting the inner and outer portions of LCHF carbonate chimneys. Figure 7 depicts a biogeochemical model of carbon and sulfur cycling in the carbonate chimneys. The system is characterized by the interaction of H₂- and CH₄-rich hydrothermal fluid with oxygenated seawater. Biofilms of LCMS are restricted to high-temperature anoxic zones within the carbonate matrix that are dominated by high-pH fluids, which lack measurable CO₂. The biofilms may promote the maintenance of microscale anoxic niches by trapping dissolved H₂ and CH₄ for use as metabolic substrates and excluding dissolved O₂ (68). Where highly reducing hydrothermal fluid meets seawater laden with abundant electron acceptors, a diverse bacterial community flourishes. As carbonate chimneys age, cool down, and are progressively bathed in fluids less enriched with H₂, ANME-1 displaces LCMS as the dominant archaeal population, while the aerobic methanotrophic and sulfur-oxidizing bacterial community remains.

Many of the putative CH₄- and sulfur-cycling organisms identified in this study have close phylogenetic relationships to organisms found at CH₄ seeps and mud volcanoes. Although overlain by sediment, many mud volcanoes are also influenced by serpentinization reactions in the subsurface and are significant sources of CH₄ (13). In addition to the CH₄- and sulfur-cycling organisms discussed above, other clones in carbonate chimney and fluid samples represent uncultured lineages with unknown physiologies. Clone LC1537B-77, for example, shares significant sequence similarity with clones recovered from three different mud volcanoes in the eastern Mediterranean (78). Clones LC1231B-173, LC1149B-70, LC1149B-130, LC1446B-88, and LC1231B-11 also have high sequence similarity to clones

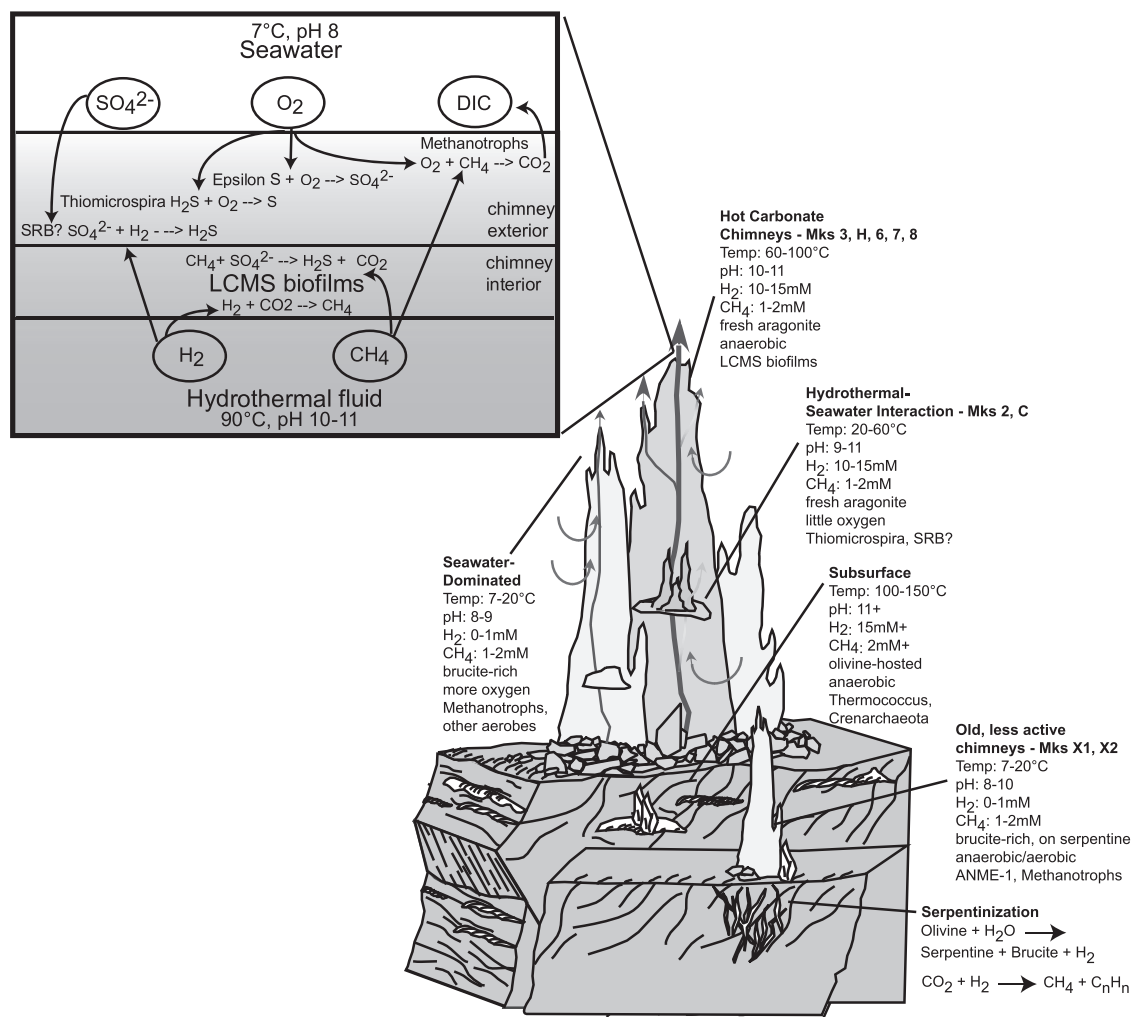


FIG. 7. Model of biogeochemical zonation at the Lost City hydrothermal field. Exothermic reactions in the serpentinization reaction zone produce H_2 , methane, and other hydrocarbons to drive the ecosystem. Hyperthermophilic *Thermococcales* and *Crenarchaeota* inhabit the subsurface where both temperature and pH could be very high. LCMS biofilms, consuming the abundant H_2 and/or methane present in hydrothermal fluids, coat the surface of fresh aragonite precipitated at actively venting carbonate chimneys. Electron acceptors are introduced via seawater circulating through the highly porous chimney structures, allowing aerobic sulfide oxidizers and methanotrophs to inhabit the exterior portions of the chimneys. As chimneys age and cool down, they vent less H_2 -dominated hydrothermal fluid but retain enough methane to support anaerobic methanotrophic *Archaea* (ANME-1), which replace LCMS as the dominant phylotype.

recovered from CH_4 seeps and mud volcanoes, and all represent uncultured lineages. Recently, a low-diversity community of anaerobic methanotrophic *Archaea* and diverse bacterial assemblages in terrestrial mud volcanoes were discovered in the Carpathian Mountains (1). There was also a report of an H_2 - and CH_4 -rich deep borehole dominated by methanogens and sulfate-reducing *Desulfotomaculum* (50). Thus, it appears that CH_4 produced by abiotic processes has selected for similar microbial communities in geologically diverse sites. The LCHF represents the first hydrothermal chimney environment where such an assemblage of CH_4 -cycling organisms has been discovered.

With abundant dissolved H_2 as an energy source and high concentrations of CH_4 as energy and carbon sources, a geochemically complex ecosystem that includes methanogens, aerobic and anaerobic methanotrophs, sulfate reducers, and sulfur oxidizers is thriving at the LCHF. Further biochemical and

genetic investigations are necessary to determine whether LCMS is a methanogen or methanotroph and to resolve the issues of very limited DIC concentrations at the LCHF and the ambiguous evidence for syntrophic SRB associated with LCMS biofilms. Considering the widespread global distribution of ultramafic subsurface environments (16) and the possibility of their existence on other planetary bodies such as Mars (18, 22), exploration of serpentinite-hosted ecosystems has the potential to yield profound discoveries with implications for understanding the linkages between abiotic water-rock reactions and microbial evolution.

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