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Zeta-Proteobacteria Dominate the Colonization and Formation of Microbial Mats in Low-Temperature Hydrothermal Vents at Loihi Seamount, Hawaii

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In situ **colonization experiments were performed to study the pioneer populations of bacteria at Loihi Seamount, Hawaii. Over a ten-year sampling period, 41 microbial growth chambers (MGCs) were deployed and recovered in Pele's Pit and the surrounding area after short-term (4–10 days) and long-term (∼1–6 years) incubations in the flow of hydrothermal effluent. Terminal-restriction fragment length polymorphism (T-RFLP) analysis of the small subunit rRNA gene (SSU rDNA) revealed that the short-term MGC communities exhibited a low number of represented populations when compared to the long-term MGC communities and naturally occurring microbial mats. Cluster analysis of T-RFLP fingerprints showed the short-term MGC communities all had similar richness but were separated into three distinct groups with different arrays of colonizing populations. Clone library analysis showed that cooler vents (T_{ave} = 40°C) were primarily colonized by *Mariprofundus ferrooxydans*, a neutrophilic Fe-oxidizing *ζ-Proteobacteria* while warmer vents (T_{ave}= 71°C) were colonized by *Sulfurimonas* spp. and other sulfur-cycling members of the *ε-Proteobacteria.* Vents with an intermediate temperature (T_{ave}= 51°C) were colonized by representatives of both *ζ-Proteobacteria* and *ε-Proteobacteria*. Long-term MGC communities did not cluster with any of the short-term communities and exhibited higher richness, indicating a greater number of bacterial populations were able to colonize and grow in the long-term growth chambers.**

Keywords community structure, iron-oxidizing bacteria, molecular ecology

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INTRODUCTION

Deep-sea hydrothermal vents are dynamic habitats known to exhibit high biological productivity primarily fueled by chemosynthesis (Jannasch and Mottl 1985; Nakagawa et al. 2007) and can be some of the most productive ecosystems on earth (Tunnicliffe 1991). In the absence of photosynthetically derived nutrients, chemoautotrophic microorganisms are responsible for primary production and form the basis of food webs at hydrothermal vents, often supporting luxuriant macrofaunal populations (Karl et al. 1980). Hydrothermal vent effluent has high concentrations of reduced chemicals and dissolved gases that mix with cold, ambient seawater across restricted spatial scales, resulting in sharp gradients. These conditions support unique microbial communities existing as microbial mats, as macrofaunal symbionts, as free-living populations in the hydrothermal plume or in the subseafloor vent conduits (Karl 1995; Jeanthon 2000). These regions of the ocean floor bear similarity to the hypothesized temperature and chemistry of the early earth and may have been where life originated (Baross and Hoffman 1985; Martin and Russell 2007).

Loihi Seamount is a seismically active submarine hotspot volcano and the youngest in the chain of Hawaiian islands. The summit is currently at a depth of 956 m, rising over 3 km above the sea floor and is located 35 km SE of the big island of Hawaii. Low temperature hydrothermal venting was detected at Loihi by submersible observation in 1987 and prompted numerous expeditions to explore its geology (Garcia et al. 2006), geochemistry (Sedwick et al. 1992, 1994; Wheat et al. 2000) and biology (Karl et al. 1988, 1989; Moyer et al. 1994, 1995, 1998; Emerson and Moyer 1997, 2002). An eruption at Loihi in 1996 led to the formation of Pele's Pit, a 300 m deep pit crater with multiple actively venting sites (Duennebier et al. 1997). The hydrothermal activity at Loihi allows the formation of luxuriant Fe-rich microbial mats along with the conspicuous absence of large benthic macrofauna (Karl et al. 1989).

The hydrothermal vents found across Loihi have been monitored and shown to be dynamically changing over time. The original vent field discovered was Pele's Vents, which were characterized by high concentrations of dissolved CO_2 (300 mM) causing the pH to be as low as 4.2 (Karl et al. 1988; Sedwick

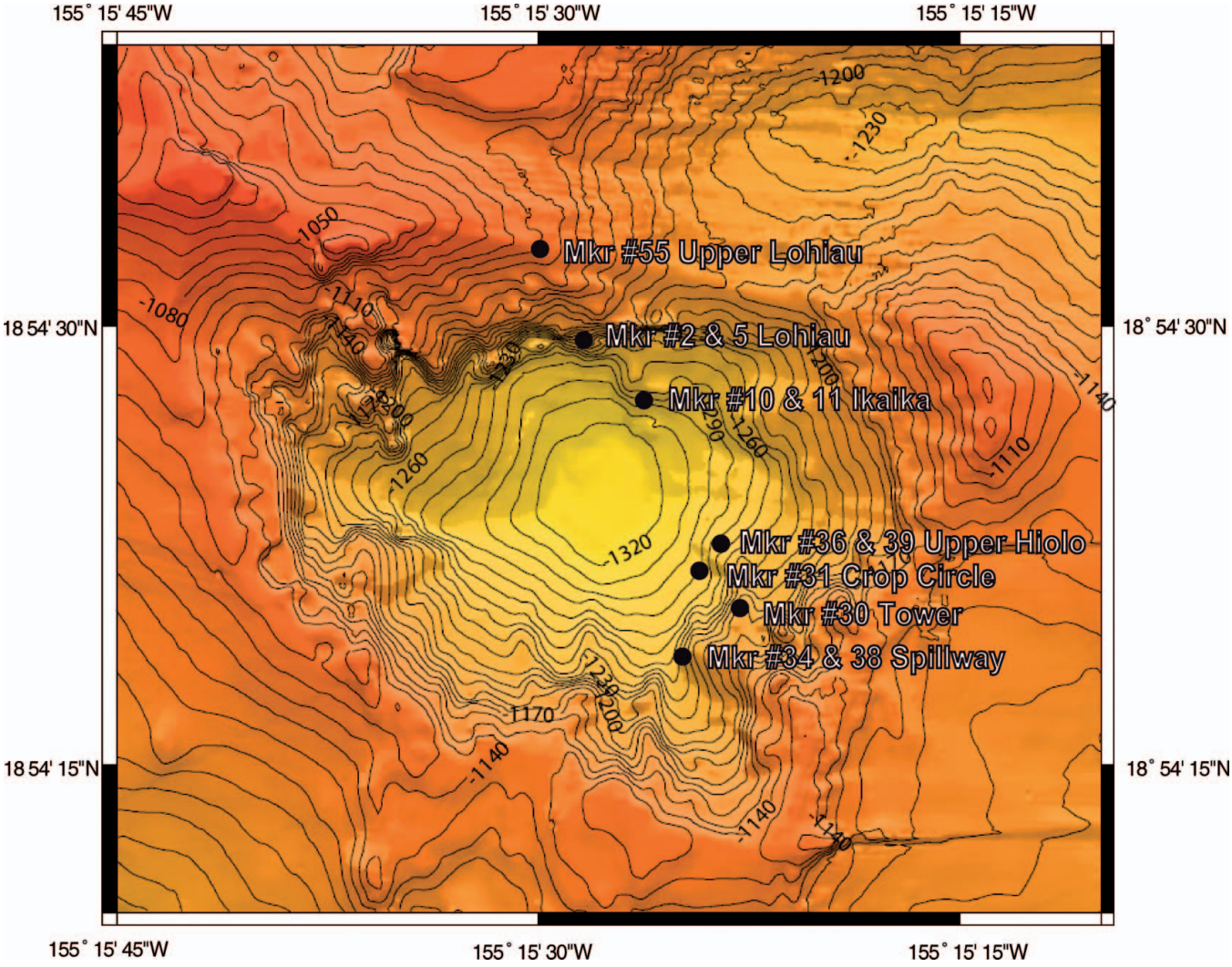

FIG. 1. Bathymetric map of Pele's Pit showing microbial growth chamber deployment sites. (Map courtesy of Brad Bailey, SIO). Naha and Ula Nui Vents are located outside of Pele's Pit, along the southern rift of Loihi at depths of 1325 and 4988 m, respectively.

et al. 1992). Fe(II) concentrations were also enriched at 50 to 750 μM, which is 10^6 times higher than ambient seawater, whereas the levels of dissolved O_2, SO_4^{2-} and Mg^{2+} were considered depleted (Karl et al. 1988; Sedwick et al. 1992). After the 1996 eruption, Pele's Vents ceased to exist and a new caldera, Pele's Pit was formed (Duennebier et al. 1997; Wheat et al. 2000). Vent sites either within the caldera or in close proximity were the focus of this study (Figure 1). Just after the eruption in 1997, effluent from Ikaika Vents (Marker #11) reached 200°C (Wheat et al. 2000), while the highest recorded temperature in 2007 was 55°C at Upper Hiolo Vents (Marker #36). Recently, an extremely deep site (Ula Nui; 4988 m), located near the base of Loihi was discovered and extensive microbial mats representing large-scale iron deposition were observed as a consequence of ultra-diffuse hydrothermal fluid flow coupled with the activities of Fe-oxidizing bacteria (Davis et al. 2007).

Neutrophilic iron-oxidizing bacteria have been shown to be abundant at Loihi Seamount (Karl et al. 1989; Moyer et al. 1995; Emerson and Moyer 2002) as well as other hydrothermal vent systems (Kennedy et al. 2003; Edwards et al. 2003, 2004) and in similar steep gradient freshwater environments (Emerson and Revsbech 1994; Emerson and Weiss 2004; Neubauer et al. 2002). These chemoautotrophs have not been comprehensively studied in the marine environment because the oceans are considered well-oxygenated and relatively depleted in reduced iron (Emerson 2000; Emerson and Moyer 2002). Additionally, iron-oxidation as a metabolic pathway yields less energy when compared to sulfur-oxidation, which is considered a common metabolic pathway used by bacteria at hydrothermal vents (Jannasch and Mottl 1985; Campbell et al. 2006). Recently, an iron-oxidizing bacterium from Loihi has been described (e.g., *Mariprofundus ferrooxydans*) and shown to represent a novel class, the *ζ (zeta)-Proteobacteria* using both cultivation and molecular techniques (Emerson et al. 2007).

The microbial populations at hydrothermal vents that form microbial mats have the potential to be rich in diversity (Davis

and Moyer 2008), but colonization is often limited to only a few populations (Moyer and Engebretson 2002). Few studies have attempted to characterize the colonizing populations of microorganisms at hydrothermal vent associated microbial mats. An *in situ* growth chamber deployed at a 9°N vent site showed the rapid formation of filamentous microbial mats by sulfur-oxidizing bacteria (Taylor et al. 1999), later hypothesized to be affiliated with the genus *Arcobacter* (Wirsen et al. 2002). In another study, colonization occurred after a single 5-day vent cap incubation by populations of *ε-Proteobacteria, Aquificales* and *Desulfurobacteria* at the Mid-Atlantic Ridge (Reysenbach et al. 2000; Corre et al. 2001). A study involving 4 microbial colonization devices deployed at East-Pacific Rise vents targeting *Alvinella* worm habitats showed a dominance of *ε-Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* as pioneer populations (Alain et al. 2004). Another *in situ* growth chamber was incubated for 3 to 10 days in hot vent fluids (156–305°C) at various sites on Suiyo Seamount, and was frequently colonized by populations of *ε-Proteobacteria* (Higashi et al. 2004). Novel members of *ε-Proteobacteria* have also been discovered using an *in situ* colonization system at hydrothermal systems at the Okinawa Trough, the Western Pacific Ocean and in Kairei Field in the Indian Ocean (Takai et al. 2003). This is the first study to examine the microbial colonization of microbial mats in an iron-dominated hydrothermal vent system.

In this study, we have identified and characterized colonizing populations of bacteria using microbial growth chambers (MGCs) at Loihi Seamount, Hawaii. Several colonizing populations of bacteria were identified from multiple vent sites using 28 short-term (4 to 10-day) and 13 long-term (∼1 to 6-year) deployments. MGCs were deployed and recovered from 1997 to 2007. MGCs were designed to provide an enhanced surface area within hydrothermal vent effluent for the growth of bacterial colonizers. Bacterial community structure was assessed using both terminal-restriction fragment length polymorphism (T-RFLP) community fingerprinting (Marsh 2005) and traditional clone library analysis (Moyer 2001). T-RFLP is a high-throughput, cultivation-independent and sensitive genotyping tool that can accurately resolve populations in microbial communities of low to intermediate richness (Engebretson and Moyer 2003) and has been shown to be reliable for detecting changes in synthetic community compositions (Hartmann and Widmer 2008). Throughout all colonization experiments in this study, archaea were either not detected or not found to dominate above 5% of any of the pioneer mat communities by the same Q-PCR assay used by Davis and Moyer (2008; data not shown).

MATERIALS AND METHODS

Construction of Microbial Growth Chambers. Microbial growth chambers (MGCs) were constructed of three 3″ sections of 4″ o.d. plexiglass cylinders enclosed top and bottom by 202 μm Nytex mesh to restrict the entry of macrofauna. The Nytex was sealed to the edges of the plexiglass using all-purpose glue (Arrow Fastener Co., Saddlebrook, NJ). The interior of the chambers contained ∼300 g of hand-woven, 8 μm-diameter silica wool as a substrate for microbial colonization, which yields a surface area of ∼33 m^2. The structural integrity was tested and maintained in 100°C water at 1 atm pressure. A stainless steel eye-bolt was fastened in the center of the three chambers to achieve negative buoyancy and to function as a point of attachment for polyurethane line to facilitate deployment and recovery operations.

Deployment and Recovery of Microbial Growth Chambers. This was achieved using the submersible *PISCES V* and the remotely operated vehicle (ROV) *JASON II* during oceanographic research cruises in 1997, 1998, 2004, 2006 and 2007. The majority of the MGCs were deployed and recovered in both 2006 and 2007 as part of the Iron Microbial Observatory project (FeMO) at Loihi Seamount. The MGCs were recovered after being directly placed in the hydrothermal vent effluent for a period of 4 to 2206 days. Upon recovery, each chamber was aseptically penetrated and silica wool was removed, placed into sample bags and immediately frozen at −80°C until further processed.

Isolation of Microbial Biomass. Silica wool (inside sample bags) was thawed on ice and then placed in a sterile wide-mouth 1 quart mason jar containing ∼300 ml 1X phosphate-buffered saline. The mason jar was then placed on a rotating platform at 4°C for 30 to 60 min. The cellular biomass was allowed to settle and was then decanted and centrifuged for 5 min (10 K × *g*) to pellet cells. Approximately 0.5 g (wet weight) was used for the subsequent DNA extraction. This step was optimized for biomass recovery and one freeze-thaw cycle was not shown to affect gDNA extraction results (data not shown).

Genomic DNA extraction. Genomic DNA (gDNA) was extracted from samples using the Fast DNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA) according to the manufacturer's protocol with the modification that gDNA was eluted into 10 mM Tris with 0.1 mM EDTA at pH 8 (TE). To optimize the cell lysis step, a FastPrep Instrument (Qbiogene) was used at an indexed speed of 5.5 for 30 sec. gDNA purity and concentration were determined with a NanoDrop ND-1000 spectrophotometer. Shearing of gDNA was minimized by limiting freeze-thawing, pipetting with wide-bore tips and by storage at −80°C.

PCR Amplification of SSU rDNA. gDNA was diluted to ∼10 ng/μl using TE buffer and the bacterial SSU rDNAs were amplified using the forward primer (5′ TdNA dNAC ATG CAA GTC GdKdK CG 3′) corresponding to positions 49 to 68 using the *E. coli* numbering system and the reverse primer (5′ dKGdP TAC CTT GTT ACG ACT T 3′) corresponding to positions 1510 to 1492, where purine analogs are indicated as dK, pyrimidine analogs are indicated as dP and an equal mixture of dK and dP is indicated by dN (Glen Research, Sterling, VA). The forward primer was 5′ end-labeled with 6-FAM when used for T-RFLP analysis. PCRs were performed using 25–50 ng of gDNA template, 5 U of *Taq* polymerase, 1X PCR Buffer (100 mM Tris-HCl at pH 9.0, 500 mM, 1% Triton X-100; Promega, Madison, WI), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 10 μg

bovine serum albumin (BSA), 1 μM each of forward and reverse primers and molecular grade water to a total volume of 50 μl. Reaction mixtures were heated for 2 min at 95°C then chilled on ice before the addition of BSA and *Taq*. The following conditions were used for the amplification process: 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 90 sec) and elongation (72°C for 3 min). A final elongation step at 72°C for 7 min was also used. Amplicons were sized by 1% gel electrophoresis against a 1-kb ladder (Invitrogen, Carlsbad, CA). Negative controls were maintained throughout. Amplicons were desalted with a Montáge PCR centrifugal filtration device (Millipore, Bedford, MA).

T-RFLP Preparation. Three replicate SSU rDNA PCRs were performed as described above. These pooled amplicons were subsequently divided among 8 treatments with tetrameric restriction endonucleases as described by Davis and Moyer (2008). All reactions were desalted using Sephadex superfine G-75 (Amersham Biosciences, Uppsala, Sweden) and dehydrated. Reactions were resuspended in 15 μl formamide with 0.33 μl ROX-500 internal size standard, denatured at 95°C for 5 min and separated by capillary electrophoresis using an ABI 3100 genetic analyzer with a 50-cm capillary array and POP-6 (Applied Biosystems, Foster City, CA). Each reaction was separated and visualized at least twice to ensure reproducibility.

T-RFLP Normalization and Analysis. Terminal-restriction fragments (T-RFs) were sized against the ROX-500 internal size standard using GeneMapper v.3.7 (Applied Biosystems). Electropherograms were imported into the program BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Community fingerprints were compared in BioNumerics using average Pearson product moment correlation (Häne et al. 1993) and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of all eight restriction digests using the relative fluorescent proportions of each electropherogram. The cophenetic correlation coefficient was calculated to assess the robustness of the cluster analysis groupings. Peak detection was limited to peaks between 50 and 500 base pairs in size and with height at least 3% of the maximum value of the fingerprint (Davis and Moyer 2008).

SSU rDNA Clone Library Construction and Screening. Five replicate PCRs were performed on gDNA extractions from MGCs LoBT_24 and LoBT_L10 as described here (without a 5′ fluorescent tag). PCR reactions were performed as described above, but with AmpliTaq Gold (Applied Biosystems) and AmpliTaq Gold PCR buffer and with a 8-min hot-start at 95°C. For each of the two clone libraries, five replicate PCRs were generated and reactions were concentrated and desalted with a Montáge PCR centrifugal filtration device (Millipore). The PCR amplicons were then cloned with a TA cloning kit following manufacturer's instructions (Invitrogen, Carlsbad, CA). All putative clones were streaked for isolation and the inserts assayed for correct size using PCR with M13F and M13R primers (Moyer 2001). Again, amplicons were sized against a 1-kb ladder using 1% agarose gel electrophoresis.

OTU Determination and Rarefaction. Partial sequence data from all clones were quality controlled and aligned using the program BioEdit (Hall 1999). Sequences were trimmed to exclude the bacterial 68F primer and thereafter include ~350 bp of the 5′-end of the SSU rRNA gene. The program DOTUR was used to determine the number of unique operational taxonomic units (OTUs) for each clone library using a 97% similarity cutoff (Schloss and Handelsman 2005).

SSU rDNA Sequencing. Representative SSU rDNA clones from OTUs containing more than one clone were chosen at random and fully sequenced for phylogenetic analysis. Internal sequencing primers were the same as those of Lane (1991). SSU rDNA gene sequences were contiguously assembled using BioNumerics (Applied Maths) and checked for chimeras using both the Bellerophon server (Huber et al. 2004) and Mallard, which allows comparisons to any potential parent sequence (Ashelford et al. 2006).

Phylogenetic Analysis. All full-length sequences were imported into ARB (Ludwig et al. 2004) and aligned to the ARB-SILVA database using the SINA Webaligner function (Pruesse et al. 2007). Phylogenetic analyses were restricted to regions of moderately to highly conserved nucleotide positions that were unambiguously aligned for all sequences. Phylogenetic placements were calculated using fastDNAml version 1.2.2 (Olsen et al. 1994) using the general two-parameter model of evolution (Kishino and Hasegawa 1989) and allowing for the global swapping of branches. The search for an optimal tree was repeated within these parameters until the best log likelihood tree was calculated in at least 3 independent tree calculations. Each phylogenetic tree was bootstrapped 500 times allowing for the global swapping of branches. The search for each bootstrap was repeated until the best log likelihood score was calculated for at least two independent bootstrap calculations.

Nucleotide Sequence Accession Numbers. The SSU rDNA sequences representing the OTUs used in this analysis have been submitted to GenBank and assigned accession numbers FJ001795 through FJ001807.

RESULTS

Twenty-eight short-term microbial growth chambers (MGCs) were deployed and recovered from seven sample sites on the eastern ridge of Pele's Pit at Loihi Seamount (Table 1, Figure 1). Thirteen long-term MGCs were deployed and recovered from Pele's Pit, Naha and Ula Nui Vents (Table 2, Figure 1). Short-term MGCs were left in the vent effluent for 4 to 10 days and long-term MGCs were left in the vent effluent for 342 to 2206 days. Vent depths ranged between 1116 and 4988 m and temperatures of sample sites ranged between 2 and 165°C (Tables 1 and 2). All vent sites had luxuriant Fe-rich mats covering the area surrounding the vent fissures (Figure 2). No macrofauna were observed at any of the sample sites during the time of deployment.

The short-term MGC communities ranged between 2.5 and 12.3 average terminal-restriction fragments (T-RFs), used as a

TABLE 1
Sample site locations and descriptions, estimated bacterial richness and biomass for each short-term microbial growth chamber

Year	Sample	Vent Site	Recovery Dive No.	Depth (mbsl)[1]	Temp (°C)[2]	Duration (days)	Average T-RFs Bacteria[3]	ng DNA/g sample (wet weight)	Short-term group
2006	LoBT_35	Upper Lohiau (Mkr #55)	J2-245	1116	22	6	7.9 ± 2.9	887	Cluster 1
2006	LoBT_37	Upper Lohiau (Mkr #55)	J2-245	1116	22	7	3.1 ± 1.9	1376	Cluster 1
2007	LoBT_68	Upper Lohiau (Mkr #55)	J2-316	1116	22	6	4.4 ± 1.4	7754	Cluster 1
2006	LoBT_24	Lohiau (Mkr #2-5)	J2-245	1174	22	7	6.7 ± 2.6	2354	Cluster 1
2006	LoBT_26	Lohiau (Mkr #2-5)	J2-245	1174	22	7	9.8 ± 2.9	1224	Cluster 1
2007	LoBT_56	Lohiau (Mkr #2-5)	J2-316	1174	22	10	6.3 ± 2.7	5561	Cluster 1
2004	LoBT_1	Tower Vents (Mkr #30)	PV600	1262	60	4	9.0 ± 3.1	907	Cluster 1
2004	LoBT_4	Tower Vents (Mkr #30)	PV600	1262	60	4	8.0 ± 3.9	1066	Cluster 1
2006	LoBT_20	Spillway (Mkr #34)	J2-245	1273	52	8	7.1 ± 3.6	3504	Cluster 1
2006	LoBT_30	Spillway (Mkr #34)	J2-245	1273	52	8	4.5 ± 2.1	1781	Cluster 1
2007	LoBT_58	Spillway (Mkr #34)	J2-315	1273	50	9	2.5 ± 1.1	5207	Cluster 1
2004	LoBT_5	Spillway (Mkr #38)	PV601	1276	44	4	7.1 ± 2.5	1357	Cluster 1
2004	LoBT_7	Spillway (Mkr #38)	PV601	1276	44	4	6.6 ± 2.4	1040	Cluster 1
2006	LoBT_28	Spillway (Mkr #38)	J2-245	1276	45	8	5.3 ± 1.2	590	Cluster 1
2006	LoBT_29	Spillway (Mkr #38)	J2-245	1276	45	8	4.3 ± 1.6	886	Cluster 1
2006	LoBT_32	Spillway (Mkr #38)	J2-245	1276	45	8	3.3 ± 1.3	1248	Cluster 1
2004	LoBT_8	Upper Hiolo (Mkr #39)	PV601	1302	46	4	7.3 ± 2.3	953	Cluster 1
2006	LoBT_33	Upper Hiolo (Mkr #39)	J2-245	1302	52	8	8.3 ± 2.3	2939	Cluster 1
2006	LoBT_31	Upper Hiolo (Mkr #39)	J2-245	1302	52	8	8.8 ± 1.7	1528	Cluster 2
2006	LoBT_36	Upper Hiolo (Mkr #39)	J2-245	1302	52	5	12.3 ± 4.1	2869	Cluster 2
2006	LoBT_40	Upper Hiolo (Mkr #39)	J2-245	1302	52	6	9.4 ± 3.1	1408	Cluster 2
2007	LoBT_62	Upper Hiolo (Mkr #39)	J2-315	1302	53	9	8.1 ± 3.2	2724	Cluster 2
2006	LoBT_25	Upper Hiolo (Mkr #36)	J2-245	1302	51	8	11.1 ± 4.7	2419	Cluster 2
2006	LoBT_27	Upper Hiolo (Mkr #36)	J2-245	1302	51	8	6.8 ± 2.9	1232	Cluster 2
2007	LoBT_60	Upper Hiolo (Mkr #36)	J2-315	1302	43	9	4.5 ± 1.3	1616	Cluster 2
2007	LoBT_66	Crop Circle (Mkr #31)	J2-315	1311	50	5	4.5 ± 1.3	2763	Cluster 2
1998	LoBT_L9A	Ikaika Vents (Mkr #11)	PV397	1298	77	4	7.1 ± 3.2	3015	Cluster 3
1998	LoBT_L10	Ikaika Vents (Mkr #11)	PV397	1302	64	4	8.8 ± 1.6	8062	Cluster 3

[1]Meters below sea level.
[2]As measured by miniature temperature recorder.
[3]± Standard deviation.

metric for richness, for each treatment with an average of 6.9 ± 2.4 across all treatments (Table 1). Cluster analysis of the short-term MGC communities showed three distinct clusters and these were designated short-term Clusters 1, 2 and 3 (Figure 3). Cluster 1 contained 18 MGC communities which were dominated by a single *ζ-Proteobacteria* population in all eight treatments (data shown for *Mbo*I only). Short-term Cluster 2 contained 8 MGC communities and showed two dominant populations, each from the *ε-Proteobacteria* or *ζ-Proteobacteria*. Short-term Cluster 3 contained two MGC communities from Ikaika Vents, which were dominated by a single *ε-Proteobacteria* population. The average temperature of the vent effluent for Cluster 1, 2 and 3 was 40, 51 and 71°C, respectively.

The long-term MGC communities ranged between 6.5 and 21.9 average T-RFs with an average of 13.7 ± 4.9 across all treatments (Table 2). Cluster analysis of the long-term MGCs placed a group of five communities (long-term Cluster 1) between short-term Clusters 1 and 2 with the remaining eight communities clustering as an outgroup (long-term Cluster 2) to all other communities (Figure 4). Long-term Cluster 1 communities had an average of 11.9 ± 4.4 T-RFs, while long-term Cluster 2 communities had an average of 20.5 ± 5.2 T-RFs (Table 2).

Two communities were chosen for clone library analysis to identify and describe the bacterial phylotypes representative of the short-term colonizing populations. Sample LoBT_24 from short-term Cluster 1 and sample LoBT_L10 from short-term Cluster 3 were selected as each was dominated by an *ζ-Proteobacteria* or *ε-Proteobacteria*, respectively (Figure 3). Sample LoBT_24 was from Lohiau Vents, which had a temperature of 22°C (Table 1, Figure 1). Sample LoBT_L10 was

TABLE 2
Sample site locations and descriptions, estimated bacterial richness and biomass for each long-term microbial growth chamber

Year	Sample	Vent Site	Recovery Dive No.	Depth (mbsl)[1]	Temp (°C)[2]	Duration (days)	Ave. T-RFs Bacteria[3]	ng DNA/g sample (wet weight)	Long-term group
2007	LoBT_42	Upper Hiolo (Mkr #39)	J2-308	1302	53	344	14.6 ± 3.3	4844	Cluster 1
2007	LoBT_43	Upper Hiolo (Mkr #39)	J2-308	1302	53	344	8.0 ± 4.0	3603	Cluster 1
2007	LoBT_47	Upper Lohiau (Mkr #55)	J2-308	1116	22	347	6.5 ± 1.7	3574	Cluster 1
2004	LoBT_L11	Naha (Mkr #1)	PV599	1325	6-10	2206	16.4 ± 6.7	3098	Cluster 1
2004	LoBT_L15	Naha (Mkr #1)	PV599	1325	6-10	2206	14.1 ± 6.4	2712	Cluster 1
2007	LoBT_50	Lohiau (Mkr #2-5)	J2-308	1174	22	344	19.1 ± 3.0	3675	Cluster 2
2007	LoBT_48	Lohiau (Mkr #2-5)	J2-308	1174	22	344	15.4 ± 4.2	3148	Cluster 2
2006	LoBT_21	Spillway (Mkr #38)	J2-308	1273	52	352	7.9 ± 1.1	3505	Cluster 2
2007	LoBT_46	Spillway (Mkr #38)	J2-308	1276	51	344	10.1 ± 3.4	2333	Cluster 2
1998	LoBT_L4	Ikaika Vents (Mkr #10)	PV393	1305	87	393	14.1 ± 3.8	3796	Cluster 2
1998	LoBT_L6	Ikaika Vents (Mkr #11)	PV393	1298	64	393	10.1 ± 3.7	5595	Cluster 2
2007	LoBT_51	FeMO Deep (Ula Nui)	J2-307	4988	2	342	20.1 ± 5.6	4631	Cluster 2
2007	LoBT_52	FeMO Deep (Ula Nui)	J2-307	4988	2	342	21.9 ± 3.9	3509	Cluster 2
1998	PV397_b1-4	Ikaika Vents (Mkr #11)	PV397	1298	77-165	n.a.	12.2 ± 4.1	841	Microbial Mat
2006	J2-241_black	Lohiau (Mkr #2-5)	J2-241	1174	22	n.a.	8.3 ± 1.4	1284	Microbial Mat

[1]Meters below sea level.
[2]As measured by miniature temperature recorder.
[3]± Standard deviation.

from Ikaika Vents, which had a temperature of 64°C (Table 1, Figure 1).

Seven operational taxonomic units (OTUs) were detected in the clone library from the Lohiau Vents and five OTUs were detected in the library from Ikaika Vents (Table 3). Rarefaction analysis of the two clone libraries showed that the colonizing populations from both sites are not significantly different in terms of OTU richness (Figure 5). The communities were significantly different in terms of OTU composition when calculated using the integral form of the Cramér-von Mises statistic (p-values of 0.001) using the program Libshuff (Schloss et al. 2004).

The clone library from Lohiau Vents, designated LOH, contained OTUs 1, 2, 5 and 7, which were most closely related to the *ζ-Proteobacteria.* Of the four OTUs clustering in this group, LOH OTU 7 was the least abundant OTU detected, however, it was phylogenetically the most closely related to *Mariprofundus ferrooxydans* while LOH OTU 1 was the most abundant as well as the most dissimilar to the type strain (Table 3, Figure 6).

IKA (Ikaika Vents) OTU 1 and LOH OTU 3 were affiliated with the Sulfurimonas group of the *ε-Proteobacteria* (Figure 6). All *Sulfurimonas* isolates thus far are mesophilic sulfur- and thiosulfate-oxidizing bacteria (Inagaki et al. 2003) and grow in anaerobic, microaerophilic or aerobic conditions (Takai et al. 2006). Culture-independent studies have shown that *ε-Proteobacteria* can be abundant in vent microbial mats (Moyer et al. 1995; Nakagawa et al. 2005a) as well as in hydrothermal fluids (Corre et al. 2001). All *ε-Proteobacteria* isolates from deep-sea hydrothermal vents are either sulfur-oxidizing or sulfur-reducing and grow at varied oxygen levels (Campbell et al. 2006).

LOH OTU 4 was contained in the Sulfurovum group of the *ε-Proteobacteria* (Figure 6). The closest cultured representative is *Sulfurovum lithotrophica*, a mesophilic anaerobic or microaerophilic sulfur- and thiosulfate-oxidizer, which can reduce oxygen and nitrate (Inagaki et al. 2004).

IKA OTU 5 was contained in the Nitratiruptor group of the *ε-Proteobacteria* (Figure 6). The closest cultured representative is *Nitratiruptor tergarcus*, a mesophilic chemoautotrophic hydrogen-oxidizing bacterium (Nakagawa et al. 2005b). This OTU contained two clones, both of which underwent full SSU gene sequencing and phylogenetic analysis. Secondary structure analysis of the SSU rDNA of these clones revealed an extension of the variable H198 loop (*E. coli* numbering system) in the cruciform region (Lane et al. 1992; Cannone et al. 2002).

IKA OTU 2 was affiliated with the Thiomicrospira group of the *γ-Proteobacteria* (Figure 6). *Thiomicrospira* isolates are usually chemolithoautotrophic bacteria that oxidize sulfide, thiosulfate and sulfur (Takai et al. 2004). These bacteria have been cultured from a variety of marine environments and hydrothermal vents (Ruby et al. 1981; Brinkhoff and Muyzer 1997; Brinkhoff et al. 1999; Takai et al. 2004). LOH OTU 6 also clustered within the *γ-Proteobacteria* and is closely related to two

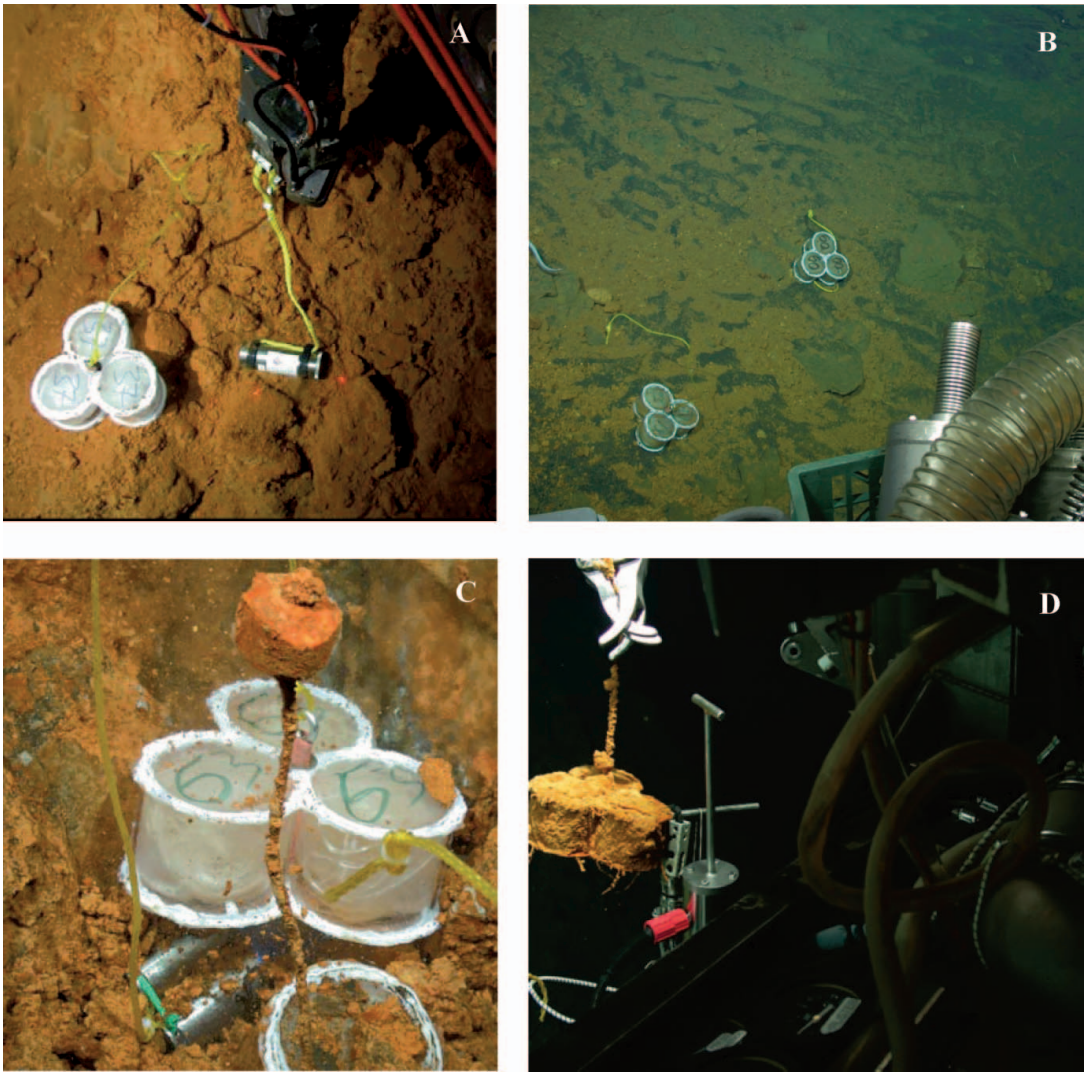

FIG. 2. Photographs of microbial growth chambers (MGCs) deployed at (A) Upper Lohiau Vents (Marker #55) next to a miniature temperature recorder (MTR). (B) Crop Circle Vents (Marker #31). (C) Upper Hiolo Vents (Marker #39) with a pair of MTRs beneath. (D) Recovery of an MGC from Upper Hiolo Vents (Marker #39) after a 344-day deployment.

bacterial clones found to colonize mineral surfaces within a sulfide-microbial incubator (Kelly et al. 2004).

IKA OTU 3 was affiliated with the *Rhodobacteraceae* of the *α-Proteobacteria* (Figure 6). This includes *Rhodovulum robiginosum*, an isolate that uses Fe^{2+} as an electron donor for anoxygenic photosynthesis (Straub et al. 1999). *Rhodobacter* spp. are also in this family and are early biofilm colonizers of submerged surfaces in coastal marine waters (Dang and Lovell 2000, 2002). Iron-oxidizing bacteria affiliated with the *α-Proteobacteria* have also been isolated from weathered basalts near hydrothermal vents and have been shown to be chemoautotrophic (Edwards et al. 2003).

IKA OTU 4 clustered within a deeply rooted, monophyletic lineage in the *Proteobacteria*. This group lacks any cultured representatives, but contains many environmental clones detected from hydrothermal vents at the Mid-Atlantic Ridge (Corre et al. 2001). The robustness of this unique lineage is supported by a 100% bootstrap value (Figure 6).

Representative T-RFLP electropherograms were examined from both Lohiau (Figure 7) and Ikaika Vents (Figure 8) using *Mbo*I digests. Predicted T-RFs were generated *in silico* using full-length sequence data (data not shown) so that phylotypes could be identified (Figure 7C, Figure 8C). At both vent locations, the trend was for the dominant colonizing populations to be maintained as community complexity increased in both the long-term MGCs and the naturally occurring microbial mats (Figures 7 and 8).

DISCUSSION

The majority of short-term MGCs (18 out of 28; Table 1) were colonized primarily by populations contained within the

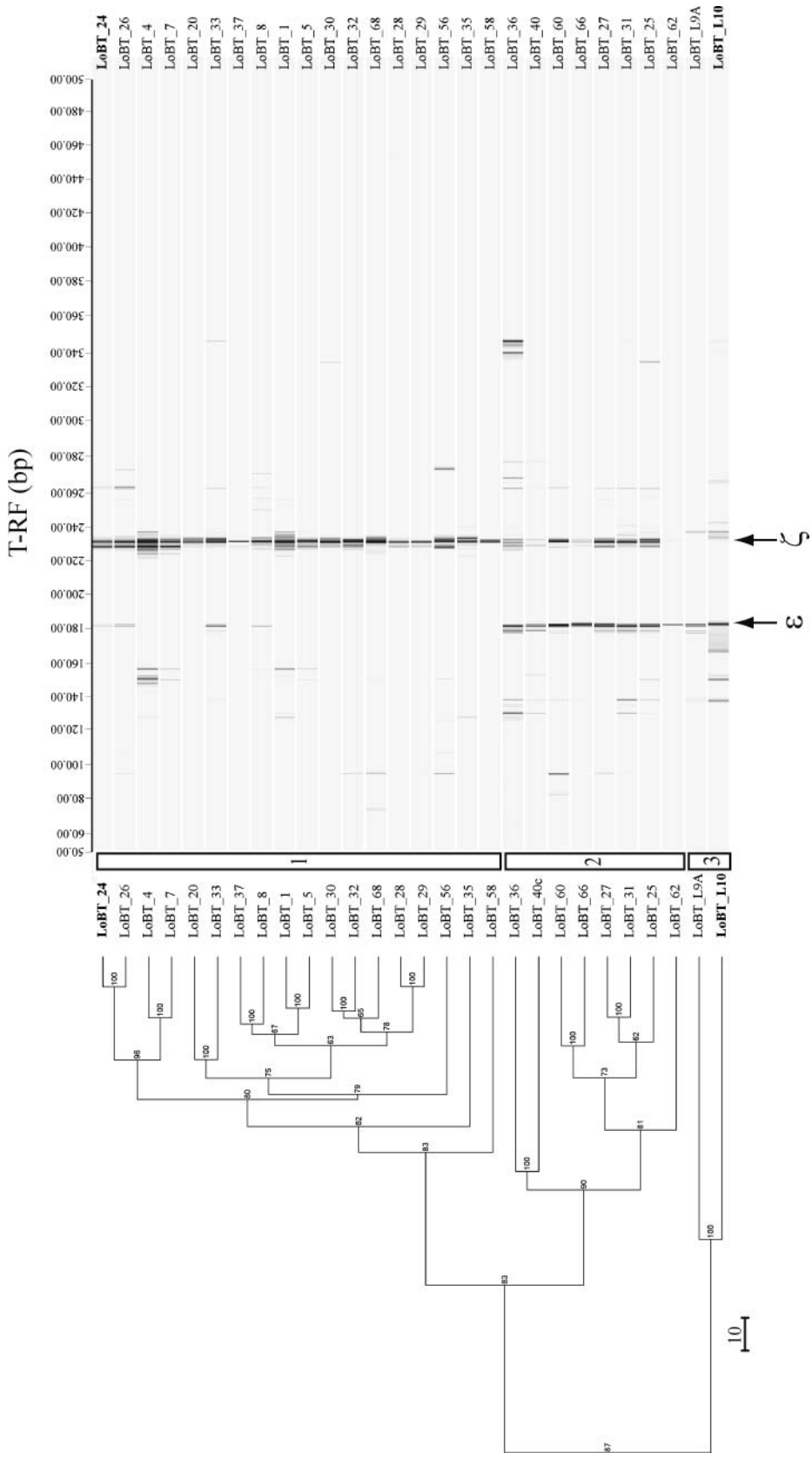

FIG. 3. UPGMA/Pearson product moment correlation cluster analysis of bacterial TRFLP fingerprints from 28 short-term MGCs deployed within Pele's Pit (Loihi Seamount) showing three separate clusters. The center panel is an image of the *Mbo*I digest showing the presence of two prominent populations. The ε arrow shows the *Sulfurimonas* spp. fragment and the ζ arrow shows the *Mariprofundus* spp. fragment. Scale bar is Pearson product moment correlation r-value X 100. Numbers at nodes are cophenetic correlation coefficients. Samples in bold were further examined by SSU rDNA clone library analysis.

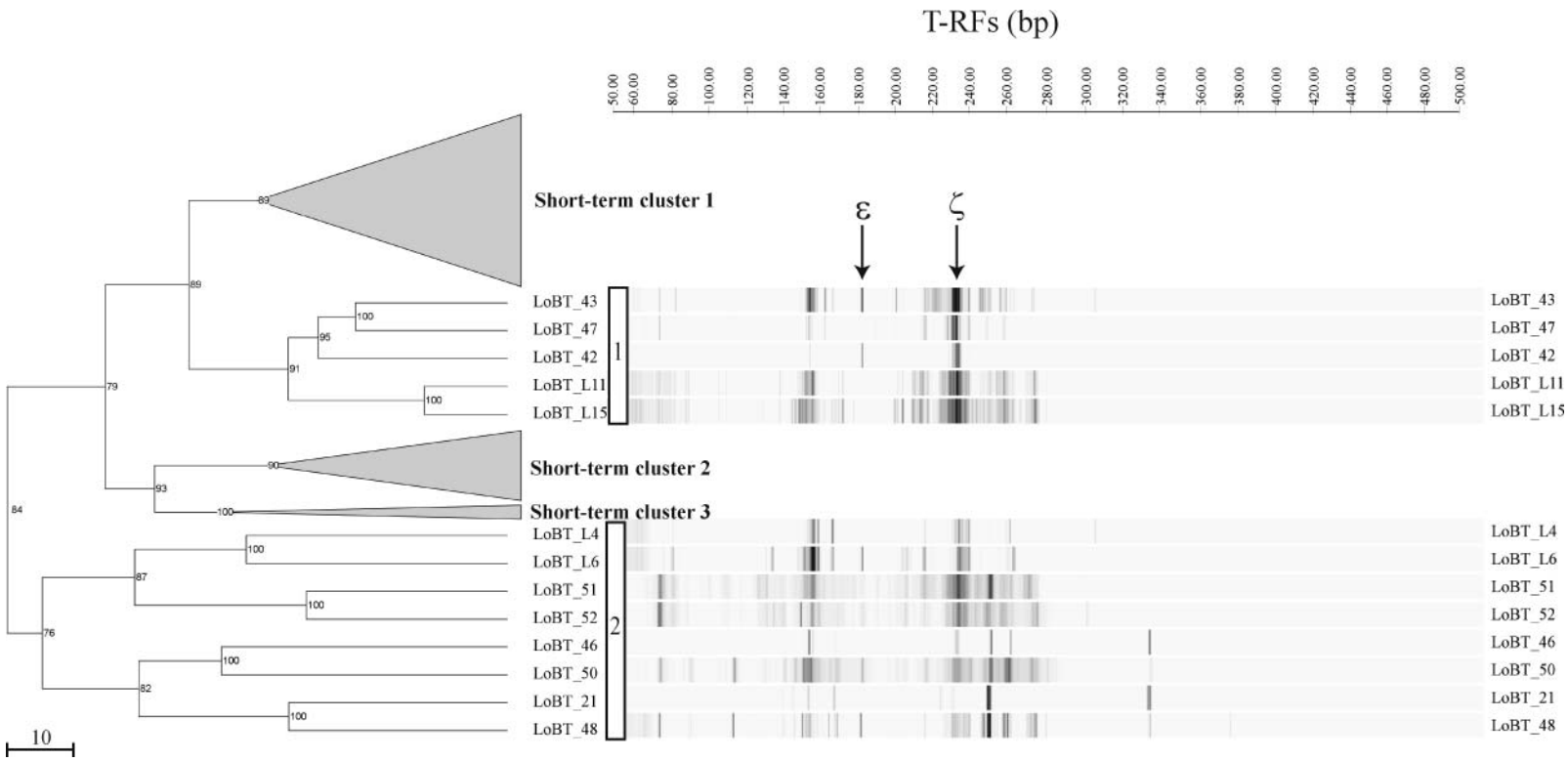

FIG. 4. UPGMA/Pearson product moment correlation cluster analysis of bacterial TRFLP fingerprints from both short-term (encapsulated) and long-term MGCs. The center panel is an image of the *Mbo*I digest for the long-term microbial growth chambers. Long-term clusters 1 and 2 are indicated. The ε arrow shows the *Sulfurimonas* spp. fragment and the ζ arrow shows the *Mariprofundus* spp. fragment. Scale bar is Pearson product moment correlation r-value X 100. Numbers at nodes are cophenetic correlation coefficients.

TABLE 3
Operational Taxonomic Unit (OTU) determination, representative clone number, percent recovery, phylogenetic affiliation and similarity comparisons of short-term MGC clone libraries from Lohiau and Ikaika

OTU	Sequenced clone no.	Clones recovered (%)[1]	Phylogenetic Grouping	Closest Cultured Relative[2]	S_{ab} score[2]	Similarity (%)[3]
LOH OTU 1	67	24	*ζ-Proteobacteria*	*Mariprofundus ferrooxydans*	0.624	94
LOH OTU 2	60	24	*ζ-Proteobacteria*	*Mariprofundus ferrooxydans*	0.828	96
LOH OTU 3	50	12	*ε-Proteobacteria*	*Sulfurimonas* sp.	0.945	98
LOH OTU 4	30	12	*ε-Proteobacteria*	*Sulfurovum* sp.	0.869	95
LOH OTU 5	26	10	*ζ-Proteobacteria*	*Mariprofundus ferrooxydans*	0.735	94
LOH OTU 6	47	4	*γ-Proteobacteria*	n.a.	n.a.	n.a.
LOH OTU 7	5	4	*ζ-Proteobacteria*	*Mariprofundus ferrooxydans*	0.873	97
IKA OTU 1	8	55	*ε-Proteobacteria*	*Sulfurimonas* sp.	0.946	96
IKA OTU 2	48	20	*γ-Proteobacteria*	*Thiomicrospira* sp.	0.730	93
IKA OTU 3	24	8	*α-Proteobacteria*	*Roseobacter* sp.	0.747	93
IKA OTU 4	46	4	Unclassified *Proteobacteria*	n.a.	n.a.	n.a.
IKA OTU 5	37 & 59	4	*ε-Proteobacteria*	*Nitratiruptor* sp.	0.587	94

[1]Calculated by dividing the number of group-specific clones by the total number of clones analyzed (n = 51 for both libraries).
[2]As determined by RDP Release 9.0.
[3]As determined by NCBI BLAST.

ζ-Proteobacteria (Cluster 1), which includes the neutrophilic Fe-oxidizing bacterium *Mariprofundus ferrooxydans* as the closest cultured relative (Emerson et al. 2007). These colonizing communities where shown to cluster together by T-RFLP fingerprinting (Figure 3) comprising a single group that correlated with lower vent effluent temperatures (T_{ave} = 40°C). Cluster 2 vent sites had an average temperature of 51°C and Cluster 3 had an average of 71°C. The remaining short-term MGCs were colonized primarily by populations from both the *ζ-Proteobacteria* and the Sulfurimonas group (Inagaki et al. 2003) of the *ε-Proteobacteria* (Cluster 2), with the exception of two MGCs from Ikaika Vents that were exclusively colonized by *ε-Proteobacteria* (Cluster 3).

The *ε-Proteobacteria* responsible for colonization at the warmer Ikaika Vents were closely related to known *Sulfurimonas* spp., which are sulfur- and thiosulfate-oxidizing bacteria (Inagaki et al. 2003). Previous studies have shown phylotypes most closely related to *Sulfurimonas* spp. as the dominant colonizers at a number of hydrothermal vent sites (Corre et al. 2001; Higashi et al. 2004), whereas some have found the dominant phylotypes to be related to *Sulfurovum* spp. (López-Garcia et al. 2003; Alain et al. 2004). Other colonizing populations found at Ikaika Vents include relatives of *Thiomicrospira* (*γ-Proteobacteria*), *Roseobacter* (*α-Proteobacteria*), a lineage of unclassified *Proteobacteria* (formerly known as Group D; Corre et al. 2001) and *Nitratiruptor* (*ε-Proteobacteria*). No *ζ-Proteobacteria* were detected in the short-term MGCs from Ikaika Vents, suggesting that higher temperatures may inhibit colonization by these mesophilic iron oxidizers (Table 3; Figure 6).

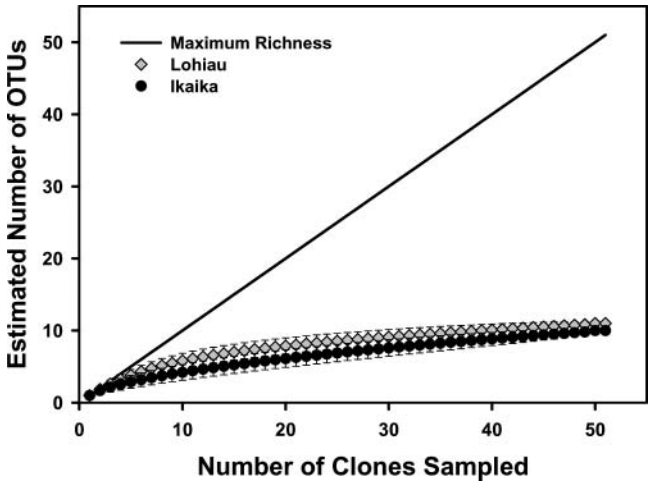

FIG. 5. Rarefaction curves comparing the estimated population richness from clone libraries of colonizers detected in short-term MGCs from Lohiau (LoBT_24) and Ikaika (LoBT_L10) Vents. OTU richness is not significantly different between these two communities as represented by their clone libraries. Error bars represent standard deviation.

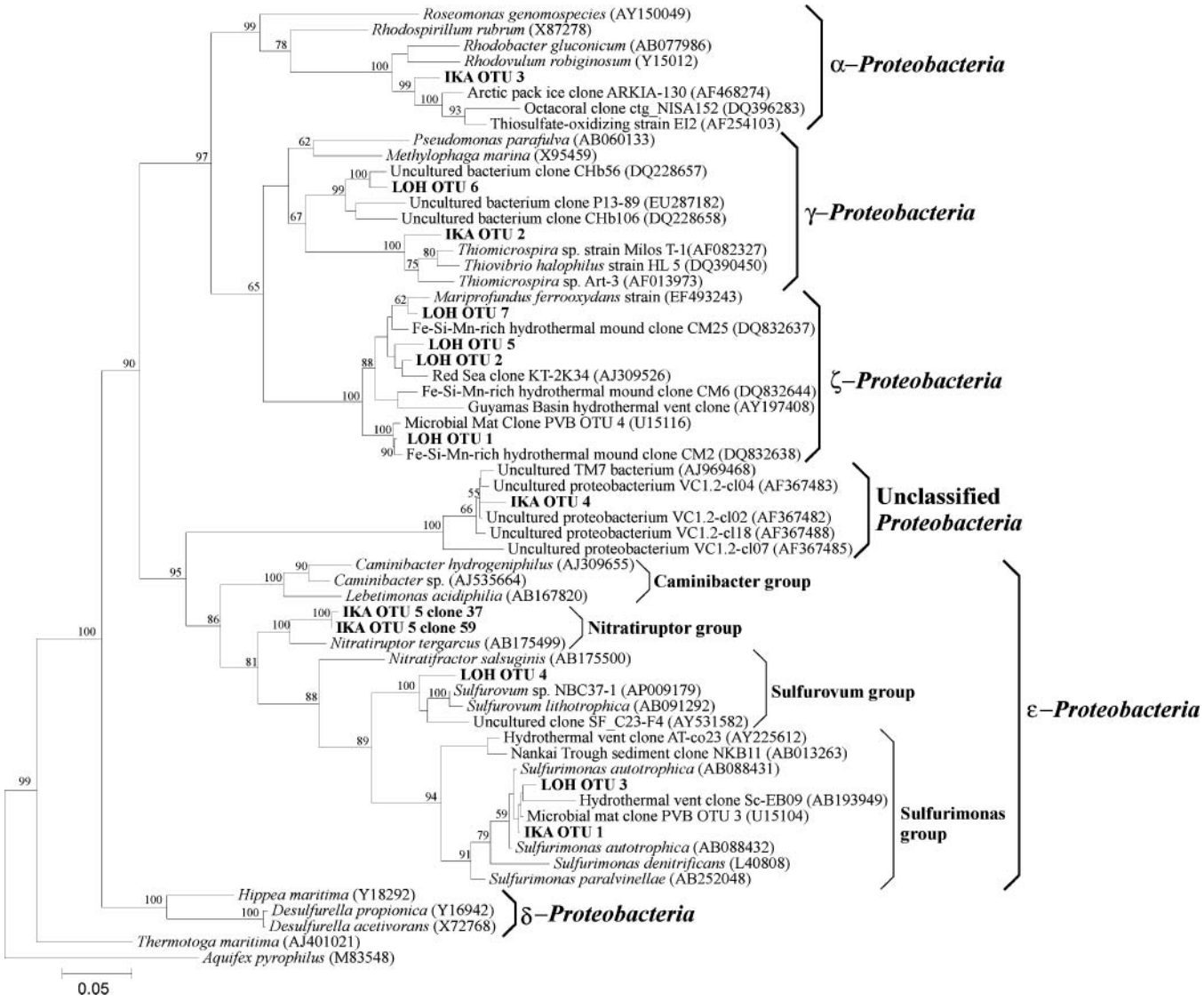

FIG. 6. Maximum-likelihood phylogenetic tree showing the evolutionary placement of OTUs belonging to the *Proteobacteria*. Only bootstrap values above 50 are shown. Scale bar represents 5 nucleotide substitutions per 100 positions.

In addition to the *ζ-Proteobacteria* phylotypes that comprised OTUs 1, 2, 5 and 7, the other colonizing populations detected at Lohiau Vents include relatives of both *Sulfurimonas* spp. and *Sulfurovum* spp. as well as an unclassified *γ-Proteobacterium* (Table 3; Figure 6). Further examination of representative clone libraries showed that microbial mat colonizers were not significantly different in terms of OTU richness (Figure 5), but were significantly different in terms of OTU composition when calculated using the program Libshuff (Schloss et al. 2004).

Five long-term MGCs (from ∼1 to 6 years; Table 2) were shown to maintain populations of *ζ-Proteobacteria* (due to the presence of representative T-RFs; Figure 4) that acted to unify this cluster (long-term Cluster 1). This cluster demonstrated enhanced community complexity (11.9 ± 4.4 $T\text{-}RF_{ave}$) relative to the short-term MGC communities (6.9 ± 2.4 $T\text{-}RF_{ave}$). Long-term Cluster 2 had consistently less representative T-RFs from both the *ζ-Proteobacteria* and *ε-Proteobacteria* and had significantly more complexity in terms of richness for each of the eight communities surveyed (20.5 ± 5.2 $T\text{-}RF_{ave}$) with respect to any of the short-term communities (Table 2; Figure 4). It was this enhanced community complexity that lead to this group clustering well outside of all other MGC-derived communities that we examined. We hypothesize that the observed increase in complexity for all long-term MGC communities can be attributed to populations with a wider array of chemoautotrophic metabolic potential as well as the establishment of mixotrophic and heterotrophic populations in addition to the original

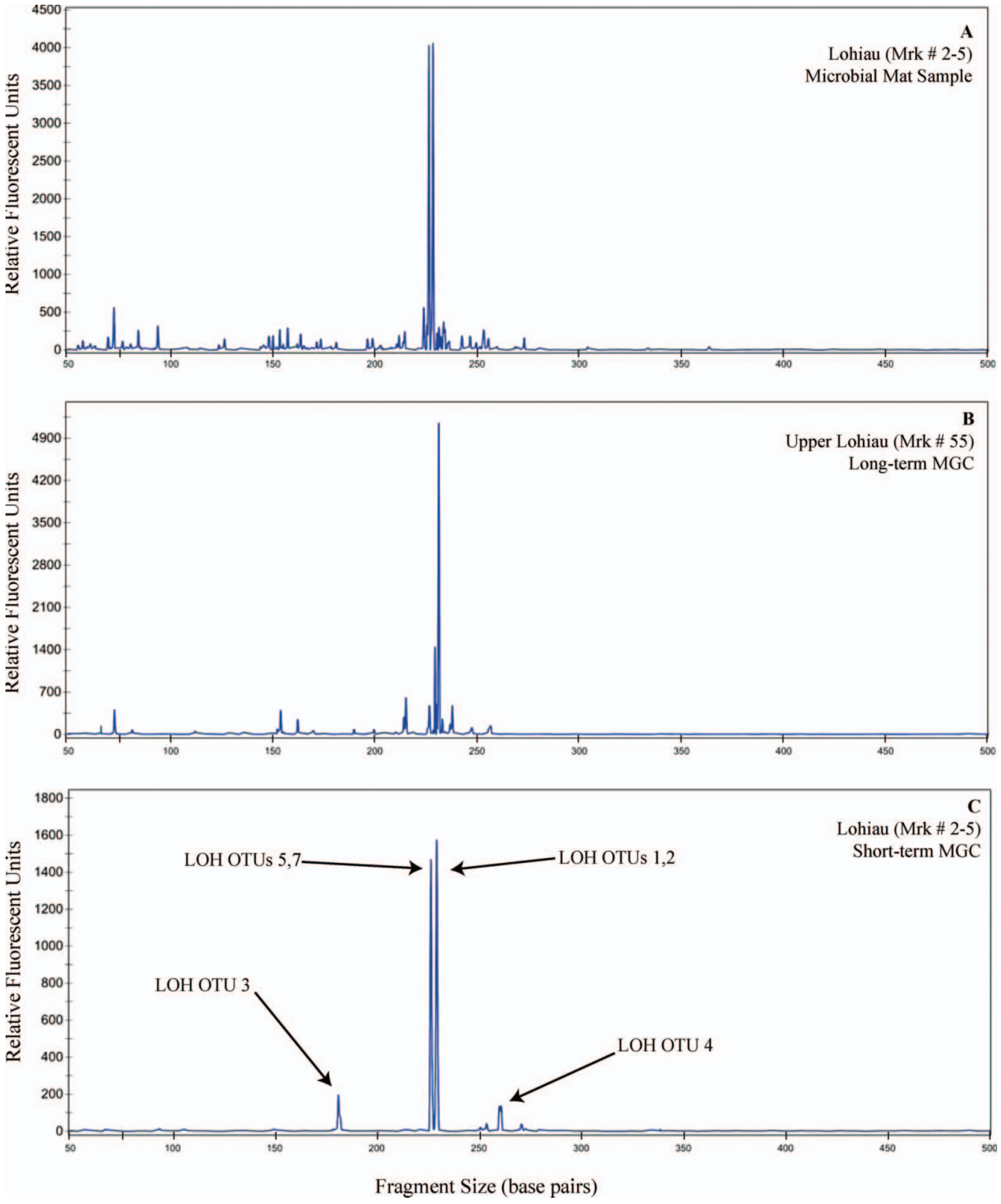

FIG. 7. T-RFLP electropherogram traces showing the *Mbo*I digests of three samples from Lohiau Vents. (A) Microbial mat (J2-241_black), (B) long-term MGC (LoBT_47) and (C) short-term MGC (LoBT_24). Arrows indicate fragments corresponding to OTUs detected in LoBT_24 clone library.

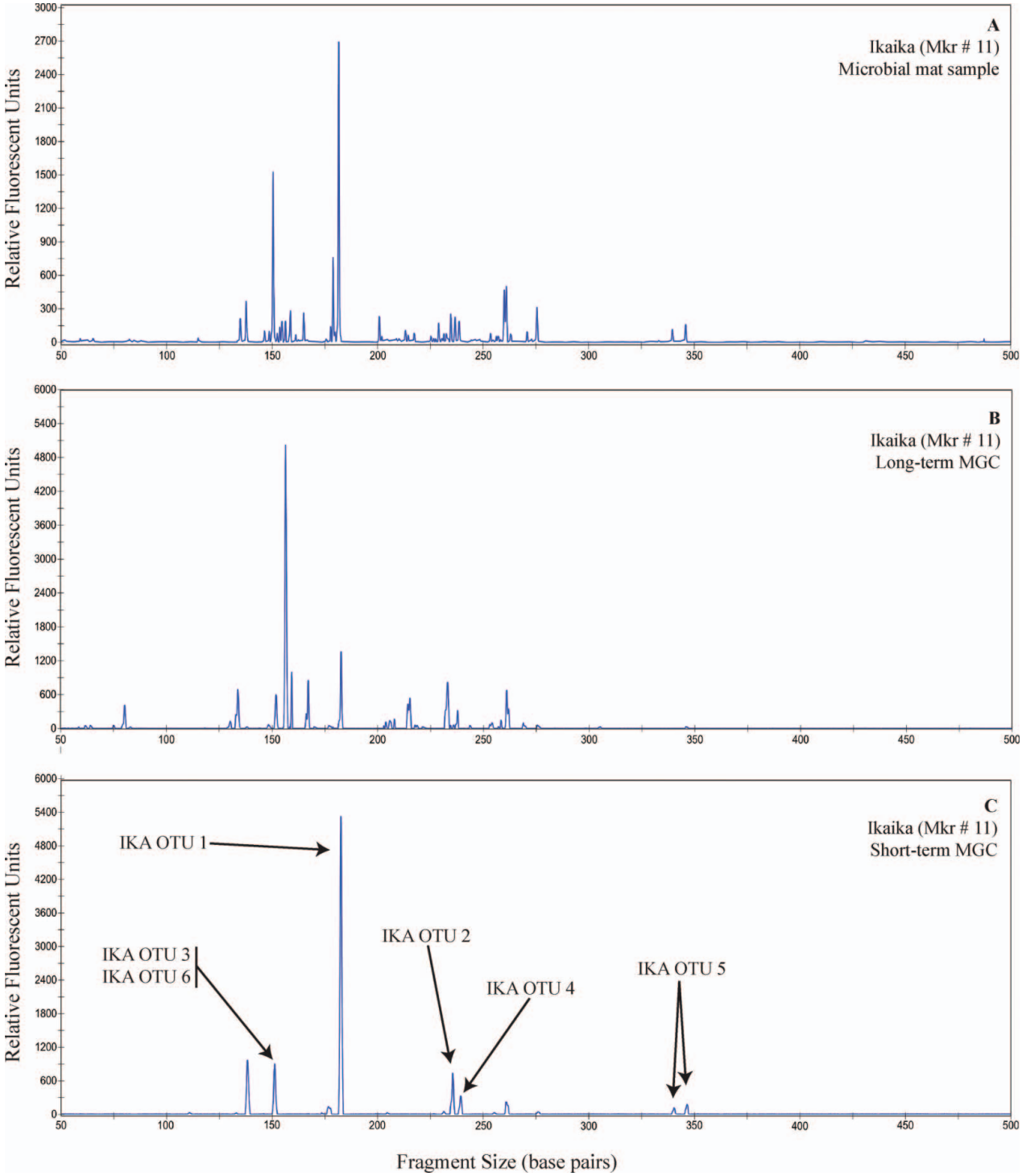

FIG. 8. T-RFLP electropherogram traces showing the *Mbo*I digests of three samples from Ikaika Vents. (A) Microbial mat (PV397_b1-4), (B) long-term MGC (LoBT_L6) and (C) short-term MGC (LoBT_L10). Arrows indicate peaks corresponding to OTUs detected in LoBT_L10 clone library.

bacterial colonizers. This hypothesis is also supported by the trend observed at Lohiau Vents (Figure 7) and Ikaika Vents (Figure 8), where enhanced community complexity was seen in both the long-term MGCs and the naturally occurring microbial mats. Nearly all phylotypes detected in the short-term MGCs were chemoautotrophic, primarily iron- and to a lesser degree sulfur-oxidizers. The long-term MGC communities probably contain additional populations operating at multiple trophic levels.

This is the first study to identify the colonizing microbial populations at Loihi Seamount and the first study to show *ζ-Proteobacteria* as the dominant colonizers in a hydrothermal vent system. Iron-oxidizing bacteria in hydrothermal vent systems are probably underestimated, since they are able to thrive in lower temperature habitats at the boundary layer between oxygenated seawater and the reducing subsurface (Emerson et al. 2007). These types of habitats are more common than is often appreciated since both divergent and convergent plate boundaries as well as areas of hot spot volcanism are common throughout the world's oceans and all manifest varying degrees of fluid flow (Delaney et al. 1998).

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