Microbial Ecology

The Hawaiian Archipelago: A Microbial Diversity Hotspot

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Abstract

The Hawaiian Archipelago is a ''biodiversity hotspot'' where significant endemism among eukaryotes has evolved through geographic isolation and local topography. To address the absence of corresponding region-wide data on Hawaii's microbiota, we compiled the first 16S SSU rDNA clone libraries and cultivated bacteria from five Hawaiian lakes, an anchialine pool, and the Lō'ihi submarine volcano. These sites offer diverse niches over ~5000 m elevation and ~1150 nautical miles. Each site hosted a distinct prokaryotic community dominated by *Bacteria*. Cloned sequences fell into 158 groups from 18 *Bacteria* phyla, while seven were unassigned and two belonged in the *Euryarchaeota*. Only seven operational taxonomic units (each OTU comprised sequences that shared ≥97% sequence identity) occurred in more than one site. Pure bacterial cultures from all sites fell into 155 groups (each group comprised pure cultures that shared ≥97% 16S SSU rDNA sequence identity) from 10 *Bacteria* phyla; 15 *Proteobacteria* and *Firmicutes* were cultivated from more than one site. One hundred OTUs (60%) and 52 (33.3%) cultures shared <97% 16S SSU rDNA sequence identity with published sequences. Community structure reflected habitat chemistry; most δ-*Proteobacteria* occurred in anoxic and sulfidic waters of one lake, while β-*Proteobacteria* were cultivated exclusively from fresh or brackish waters. Novel sequences that affiliate with an Antarctic-specific clade of *Deinococci*, and Candidate Divisions TM7 and BRC1, extend the geographic ranges of these phyla. Globally and locally remote, as well as physically and chemically diverse, Hawaiian aquatic habitats provide unique niches for the evolution of novel communities and microorganisms.

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Introduction

Endemism among eukaryotes in Hawai'i' is well documented, but comparable descriptions of microorganisms in the archipelago are rare [6, 45, 48]. Diverse microbial communities occur in Hawaiian soils, yet the first novel cultivated bacteria from the area were only recently described [12, 14, 34, 49]. Physical and chemical pressures as well as geographic isolation are acknowledged promoters of evolution in organisms. Such factors may vary within a single site, or among representatives of a habitat type. For example, Hawaiian lakes range from meromictic to polymictic, freshwater to hypersaline, and occur below sea level to 13,000 ft. elevation [41]. Furthermore, more than 650 anchialine pools in Hawaii contain brackish water but lack surface connections to the ocean. No phylogenetic studies of prokaryotic communities in Hawaiian lakes or anchialine pools have been reported. Hawaii's newest ''island,'' the Lō'ihi submarine volcano, is over ~1000 m below the surface of the Pacific Ocean. Karl et al. [32] detected metabolically active bacteria in hydrothermal fluids at Lō'ihi, and community structure in a microbial mat at the site was described only through partial 16S rRNA gene sequences [45, 46]. Here we describe a molecular and cultivation-based survey of prokaryotes in five of Hawaii's six lakes, an anchialine pool, and at Lō'ihi. The sixth lake was connected decades ago to the ocean by a subsurface tunnel and is largely considered a tidal pool [41]. The sites considered here are globally remote, are locally isolated, and occur throughout the archipelago. Since all but one experience little, if

Table 1. Physical and hydrographic features of each sample site

Type/basin	*LK Crater*	*LA Sand ridge/atoll*	*LW Cinder cone*	*GL Crater*	*HP Crater*	*AP Atoll*	*LV Pit crater*
Grid reference	21°11′ N, 156°58′ W	25°46′ N, 171°44′ W	19°49′ N, 155°29′ W	19°49′ N, 155°29′ W	20°44′ N, 156°6′ W	27°47′ N, 175°49′ W	18°55′ N, 155°16′ W
Island	Moloka'i	Laysan	Hawai'i	Hawai'i	Maui	Southeast	n/a
Island area (km^2)	421	3.75	4028	4028	729	0.14	n/a
Elevation (m)	0	4	3969	0	2040	0	−969
Max. depth (m)	248	7.5	3	6	6.4	0.3	−5000
Surface area (ha)	0.35	88	0.73	8000	2200	0.03	n/a
T (°C)	24	23	0–13 [39]	25	16	24	3.8 to 90
Salinity (ppt)	6–32	76–80	nd	0.31	0.08	26.9	.34
H_2S (μM)	0–130	2.6	†	†	†	0.6	var.
Surface chl*a* (μg/L)	29	<0.9	<3.8 [39]	0.41	0.21	0.4	n/a

Nd: not determined; †: not detected; var: variable; n/a: not applicable.
LK: Lake Kauhako; LA: Lake Laysan; LW: Lake Waiau; GL: Green Lake; HP: Lake Wai'ele'ele (Haleakela Park); AP: anchialine pool; LV: Lō'ihi submarine volcano.

any, anthropogenic activity, they also present pristine habitats for studies of microbial diversity, processes and discovery of novel taxa.

Materials and Methods

Sample Collection and Water Chemistry. The location and bathymetric characteristics of each site are in Table 1. Water was collected from 18 depths (surface to 200 m) in meromictic LK (L. Kauhakō, Moloka'i, August 2000 and June 2002); from four depths (surface to 7.5 m) in LA (L. Laysan, Laysan Atoll, October 2000); water from the surface and immediately above the bottom was collected in GL (Green L., Hawai'i, March 2003), and from HP (L. Wai'ele'ele, Haleakala National Park, Maui, February 2003); surface water only was taken from AP (anchialine pool, Southeast Is., Pearl and Hermes Reef, October 2000). Surface water and sediment were collected from LW (L. Waiau, Hawai'i, June 2000). All surface samples were collected into sterile Nalgene® bottles, while those from other depths were collected in an ethanol rinsed Beta Plus sampler (Wildco) or an Automated Trace Element sampler [5]. Aerobic, heterotrophic bacteria in the stomach of a penaeid shrimp from LK were cultivated (June 2002) [16, 17]. Hydrogen sulfide and chlorophyll a concentrations were determined in surface water samples [15, 56]. Salinities were measured in nonfrozen samples with an AGE model 2100 Minisal salinometer calibrated against IAPSO standard (Wormley) seawater. Seawater, hydrothermal fluids, and mat material at Lō'ihi were collected during dives PV421 (September 1999) and PV465 to PV473 (October 2001) of the *Pisces V* into a "suction bucket," or temperature and pressure controlled sampler [42] operated from the DSRV *Pisces V*.

Enrichment of Bacteria. Subsamples of lake and pool water or sediment were inoculated to solid and liquid media and incubated at 25 to 50°C (Table 2). LV samples collected into the "suction bucket" were inoculated to the same media but incubated at 4 to 50°C. LV water collected into the pressure and temperature-controlled device was transferred to liquid media at 25 to 90°C and 130 atm. in reactors prepressurized under helium and preheated to sample temperature [42]; pressure and temperature were maintained during transfer. Subsamples drawn from reactors after 6 months' incu-

Table 2. Enrichment media (NaCl concentrations were varied in all but Kushner's media to reflect those of the habitat in question) and target groups

Medium—Target Group
2216E Marine Agar (Difco) — heterotrophs, and obligate halophiles
TSA/TSB, R2A, Nutrient Agar — nonhalophilic heterotrophs
Allen medium and TA medium — thermophilic/acidophilic *Archaea* [1, 36]
Sabouraud Agar — Yeast/fungi
'A', 'A-N', Castenholz D — cyanobacteria [7, 35]
Medium 1 — green and purple sulfur bacteria, pH 6.8, 7.2 [21]
1% Peptone — oligotrophs
ASY — nonsulfur bacteria [44]
Succinate salts — facultative anaerobes [55]
Halophilic *Archaea* [37]
Beggiatoa/Thiothrix — iron oxidizers [3]
Bacto sulfate API broth (lactate or acetate), pH 6.8 — sulfate reducers
Wilkins-Chalgren Agar — anaerobes

Table 3. Oligonucleotides used for amplification of partial 16S rRNA genes

Primer name	*Primer sequence*	*Ref.*
Bacteria		
27F	5′-AGAGTTTGATCCTGGCTCAG-3′	[38]
533F	5′-GTGCCAGCMGCCGCGGTAA-3′	[38]
981R	5′-GGGTTGCGCTCGTTGCGGG-3′	[38]
1492R	5′-GGTTACCTTGTTACGACTT-3′	[38]
Archaea		
S-D-ARCH-0025-a-S-17	5′-CTGGTTGATCCTGCCAG-3′	[61]
S-*-UNIV-1517-a-A-21	5′-ACGGCTACCTTGTTACGACTT-3′	
S-D-ARCH-0344-a-S-20	5′-ACGGGGCGCAGCAGGCGCGA-3′	[61]
S-*-UNIV-1517-a-A-21	5′- ACGGCTACCTTGTTACGACTT-3′	
S-D-ARCH-0025-a-S-17	5′-CTGGTTGATCCTGCCAG-3′	[61]
S-*-UNIV-0907-a-A-20	5′-CCGTCAATTCMTTTRAGTTT-3′	
21F	5′-TCCGGTTGATCCYGCCGG-3′	[52]
1492R	5′- GGTTACCTTGTTACGACTT-3′	
23FPL	5′-TGCA GAYCTGGTYGATYCTGCC-3′	[4]
1391R	5′-GACGGGCGGTGWGTRCA-3′	
ARCH16S1	5′-TTAAAGGAATTGGCGGGGGAGCAC-3′	[a]
ARCH16S2	5′-GGTGAATACGTCCCTGCTCCTTGC-3′	
ARCH16S3	5′-GCAAGGAGCAGGGACGTATTCACC-3′	[a]
ARCH23S5	5′-TTATCGCAGCTTGGCACATCCTTC-3′	

[a]Scott Rogers, Bowling Green State University, OH (pers. comm.).

bation were depressurized and cooled to room temperature over several hours for observation by microscopy [13]. Subsamples from reactors were transferred to enrichment media and incubated at the same temperature and 1 atm. Colonies that arose from all samples on solid media were selected on the basis of colony features for purification; subsamples of turbid liquid media were transferred to plates of the same medium in order to isolate colonies. Strains from all samples and sites were purified through repeated transfers on nutrient media.

DNA Extraction. Water, sediment, and mat subsamples for DNA extraction were transferred upon collection to sterile 1 L Nalgene bottles or sterile 50 mL polypropylene tubes and stored frozen (−20°C for 1 to 8 days depending on the collection site, and thereafter at −80°C) until extraction of community genomic DNA within 1 month of collection. Cells in 1–5 L of water were concentrated in a refrigerated centrifuge (Sorvall Instruments, DuPont, DE) at 4°C and 7000 g for 20 min. Community genomic DNA was isolated from the pellets, or from 1 g (wet wt) of sediment according to Marmur [43]. The G NOME kit was used to isolate genomic DNA from Gram-negative bacterial cultures (Qbiogene, Carlsbad, CA). Genomic DNA was isolated from Gram-positive bacterial cultures after Marmur [43].

Construction of 16S rRNA Libraries, Pure Culture 16S rRNA Gene Amplification, and DNA Sequencing. Clone libraries containing partial *Bacteria* or *Archaea* SSU 16S rDNA genes were prepared by amplification of 16S rDNA from community genomic DNA with universal and domain-specific primers in polymerase chain reactions (PCR) with *Pfu* DNA polymerase (Table 3). Each 50 μL reaction comprised initial denaturation (94°C) for 3 min, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C. Final extension was 7 min at 72°C. PCR products were cleaned with Geneclean (Qbiogene). PCR amplicons of ~1.5 kb were cloned into the pCR4 Blunt-TOPO vector and transformed into *Escherichia coli* Top 10 competent cells (Invitrogen, Carlsbad, CA). Plasmid DNA extracted by alkaline lysis and purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was sequenced in both directions with T3, T7, and internal primers in a Beckman CEQ 2000 Genetic Analysis System. A fragment of the SSU 16S rDNA gene in each pure culture was amplified and sequenced as described above. Sequences for each clone and culture were aligned, assembled and edited in SeqMan (Lasergene).

A consensus sequence for each clone and culture was checked for chimeric features in CHIMERA_CHECK at the Ribosomal Database Project [8] and compared with DNA sequences in the public domain through gapped BLASTn searches [2]. Sequences considered chimeric were not included in subsequent analyses. Cloned sequences sharing 97% nucleotide identity were grouped in the same operational taxonomic unit (OTU). Pure cultures were also grouped in this way. Representative sequences from each OTU and culture group were aligned with 16S rDNA sequences of their nearest neighbors and other taxa in the public domain by the neighbor-joining algorithm in CLUSTALX [33]. Sequences were bootstrapped and replicated 10,000 times within CLUSTALX, with bootstrap values over 7000 placed at the respective nodes. Outgroups were fixed in NJPLOT (distributed with CLUSTALX) [60]. Images of the resulting unrooted

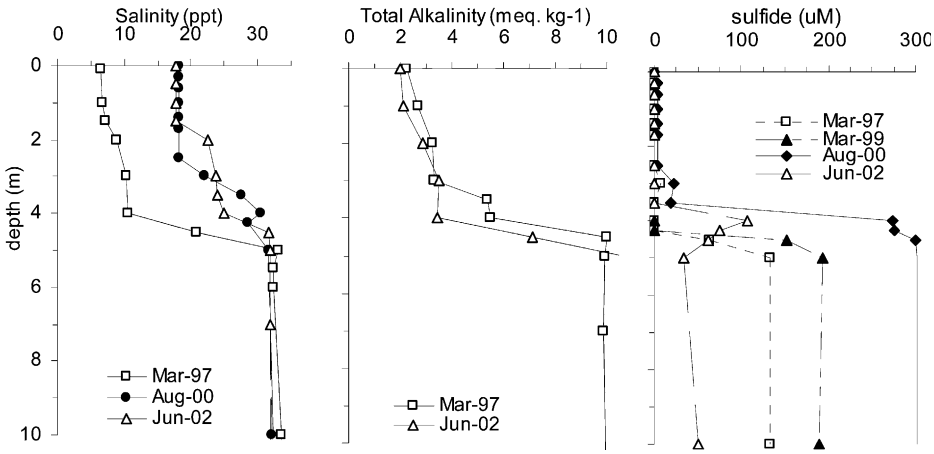

Figure 1. Salinity, alkalinity, and sulfide concentrations in L. Kauhako. Data for 1997 from [15]; March 1999 from Donachie & Zaborsky (unpubl.); August 2000 and June 2002, this work.

trees were exported from TREEVIEW [50] and refined in Adobe Illustrator for publication.

Nucleotide Sequence Accession Numbers. The nucleotide sequences of all 16S rDNA sequences (except those considered chimeras) have been deposited into GenBank at the NCBI and assigned accession numbers AF513927 to AF513968, AY344367 to AY344443, AY345357 to AY345583, AY353700 to AY353702, AY371182 to AY371186, and AY372114 to AY372116. (Accession numbers for each OTU and culture are listed in supporting information at http://www.hawaii.edu/microbiology/MO/supplement_table.htm.)

Results

Sampled Sites Were Physically and Chemically Diverse. Each lake was characterized by unique bathymetry or chemistry (Table 1). Meromixis in L. Kauhakō, the fourth deepest lake in the United States and that with the highest ratio of depth to surface area of any lake in the world, was characterized by a persistent chemocline at 4 to 4.5 m between 1997 and 2002. During this period, salinity at the surface of the lake increased from 6 to 19 ppt, and by ~10 ppt elsewhere above the chemocline [cf. 15]. Alkalinity over the same water column was stable (Fig. 1). L. Laysan is Hawaii's only hypersaline lake, with surface salinity of 80 ppt. L. Waiau, Wai'ele'ele, and Green L. each contained freshwater. L. Kauhakō was the only lake in which surface Chl *a* exceeded 4 μg/L (Table 1). Salinity in the anchialine pool was ~25% lower than that of seawater, and both Chl *a* and hydrogen sulfide concentrations were low. The chemical properties of seawater and hydrothermal vent discharges at Lō'ihi have been described elsewhere [53], but sample temperatures here were 3.4°C to 90°C.

Diverse Uncultivated Bacteria Were Detected. Uncultivated *Bacteria* from all sites grouped into 158 OTUs from 18 phyla and subphyla (Table 4). Seven OTUs were not assigned to existing phyla, while single *Euryarchaeota* OTUs from L. Laysan and L. Waiau belonged in the *Halobacteriaceae* and *Methanosetaceae*, respectively. Diverse uncultivated bacteria were determined in each lake and at Lō'ihi, but only *Proteobacteria* and *Firmicute* OTUs were detected in the anchialine pool (http://www.hawaii.edu/microbiology/MO/supplement_table.htm). Only seven *Bacteria* OTUs (4.4%) occurred in more than one site, including two (from L. Kauhakō and Lō'ihi) that affiliated with the marine green sulfur bacteria *Chlorobium vibrioforme* and *Prosthecochloris aestuarii*. Five other 'shared' OTUs belonged in the *Proteobacteria*, only one of which occurred in more than two sites (Table 5). Three Lō'ihi OTUs shared >99.6% sequence identity with the iron-oxidizing bacteria *Leptothrix cholodnii*, *Marinobacter* sp., and a 'deep-sea iron-oxidizer' [20]. Relatives of the latter two were brought into pure culture; a third culture produces sheaths typical of *Leptothrix/Gallionella* but is not yet axenic (Fig. 2). We also cultivated a spore-forming *Bacillus* sp. (culture R5B) from a 70°C, 130 atm. bioreactor inoculated with material from Lō'ihi (Fig. 2).

Sixty percent of OTUs shared <97% sequence identity with their nearest neighbor in GenBank (additional data at: http://www.hawaii.edu/microbiology/MO/supplement_table.htm). OTUs from frequently cultivated subphyla were common, e.g., γ-*Proteobacteria* (46 OTUs) and α-*Proteobacteria* (25 OTUs), but 37 and 80% of these OTUs, respectively, were also novel sequences. Most γ-*Proteobacteria* and α-*Proteobacteria* OTUs were from saline lakes or Lō'ihi. Low sequence identities (<95%) were common for OTUs that affiliated with phyla with few sequences in the public domain, e.g., *Deinococci*, *Planctomycetes*, *Fibrobacter/Acidobacter*, *Verrucomicrobia*, and Candidate Divisions TM7 and BRC1. An OTU from L. Laysan grouped with *Deinococcus*-related sequences in an "Antarctic-only" clade (Fig. 3), and two OTUs from L. Waiau affiliated with Candidate Division TM7 se-

Table 4. Number of OTUs and cultures (in parentheses) per taxon determined in each site

Taxon	*LK*[a]	*LA*	*LW*	*GL*	*HP*	*AP*	*LV*	*Totals*[b]	*Unique*[c]
Actinobacteria	6(8)	1(0)	5(3)		1(0)		1(2)	14(13)	14(13)
α-*Proteobacteria*	9(15)	5(10)	1(0)	1(2)	2(0)	1(3)	7(5)	26(35)	25(33)
β-*Proteobacteria*	2(1)	1(0)	3(0)	2(4)	3(5)		3(0)	14(10)	10(9)
γ-*Proteobacteria*	17(26)	6(8)	8(2)	5(3)	4(3)	2(9)	7(23)	49(74)	46(60)
δ-*Proteobacteria*	12(1)	1(0)						13(1)	13(1)
ε-*Proteobacteria*		2(1)	1(0)					3(1)	3(1)
Bacteroidetes	3(3)	3(3)	3(0)	1(1)	0(2)			10(9)	10(9)
Chlorobi	4(0)	0(1)					2(0)	6(1)	4(1)
Chlamydiae			1(0)					1(0)	1(0)
Chloroflexi		1(0)						1(0)	1(0)
Cyanobacteria	2(0)					0(1)		2(1)	2(1)
Deinococci/Thermus		1(0)						1(0)	1(0)
Fibrobacter/Acidobacter	1(0)							1(0)	1(0)
Firmicutes	1(18)	0(3)	4(1)	0(2)	2(3)	1(3)	0(2)	8(32)	8(27)
Candidate Division BRC1	1(0)							1(0)	1(0)
Candidate Division TM7			2(0)					2(0)	2(0)
Planctomycetes	5(0)	3(0)	3(0)					11(0)	11(0)
Verrucomicrobia	2(0)	1(0)	2(0)					5(0)	5(0)
Unassigned	5(0)		2(0)					7(0)	7(0)
Euryarchaeota		1(0)	1(0)					2(0)	2(0)
Totals[b]	70(72)	26(26)	36(6)	9(12)	12(13)	4(16)	20(32)	**177(177)**	**167(155)**[e]
OTUs/cultures ≥97% ID[d]	0	4	0	0	0	1	3	8	8

[a]See text for abbreviations.
[b]These totals are sums of OTUs and cultures from each site and do not take into account those that occurred in more than one.
[c]Seven OTUs and 15 cultures were isolated from more than one site; the number of distinct OTUs and cultures was 167 and 155, respectively. All sequences in an OTU and all cultures in the same group shared ≥97% 16S SSU rDNA sequence identity.
[d]Refers to OTUs and cultures from the same sample and which shared ≥97% sequence identity.
[e]Number of unique OTUs (and cultures) from all sites.

quences (Fig. 4). An OTU from anoxic water in L. Kauhakō affiliated with Candidate Division BRC1 sequences, while another (GenBank accession number AF424445) from L. Kauhakō shared 85% identity over 977 bases with an Antarctic Candidate Division OP11 clone. In light of this low identity, we did not assign the OTU here.

Ten Bacteria Phyla and Subphyla Were Represented by Cultivated Strains. Phylogenetically diverse pure bacterial cultures were prepared, with the initial 499 cultures from all samples (L. Kauhakō, 276; L. Laysan, 53; L. Waiau, 20; Green L., 27; L. Wai'ele'ele, 35; anchialine pool, 35; Lō'ihi, 53) falling into 155 groups from ten phyla and sub-phyla (http://www.hawaii.edu/microbiology/MO/supplement_table.htm). Fifty-two culture groups (33.3%) shared <97% 16S rDNA sequence identity with valid strains in seven phyla or subphyla (Table 6). No *Archaea* were cultivated. As with OTUs, most cultivated strains occurred in single sites. Fifteen of the 155 culture groups were cultivated from more than one site (Table 6).

Community Structure Reflected Habitat Chemistry. Obligately aerobic *Bacteroidetes* were cultivated only from oxic waters, while eight of nine β-*Proteobacteria* came from freshwater Green L. and Wai'ele'ele. *Planctomycetes* OTUs were prepared from DNA extracted from L. Kauhakō's anoxic waters (Table 7). Of 13 δ-*Proteobacteria* OTUs detected throughout this work, 12 were from H_2S-containing water below the L. Kauhakō chemocline. A single δ-*Proteobacterium* (K2-52) was cultivated from the surface of L. Kauhakō, *Bacteria* were cultivated from the gut of the penaeid taken from L. Kauhakō, but none were detected as OTUs or enriched from contemporaneously collected water samples; a strain cultivated from the gut sample is being described as a new *Actinobacterium*.

A Polyphasic Approach Revealed Considerable Microbial Diversity. The numbers of *Bacteria* OTUs and distinct pure cultures (158 and 155, respectively) were remarkably similar (Table 4). Phylogenetically, however, the data sets had little in common; only eight OTUs and cultures from the same samples shared >97% sequence identity (Fig. 5). Five other OTUs and cultures that shared >97% identity were from different samples at the same site, while OTUs and cultures in four other such pairs were from different sites. The highest number of 'identical' OTUs and cultures from one site was 4 from L. Laysan.

Table 5. OTUs and cultivated *Bacteria* detected in samples from more than one site

OTU affiliation	*LK*	*LA*	*LW*	*GL*	*HP*	*AP*	*LV*
Chlorobi							
Chlorobium vibrioforme	X						X
Prosthecochloris aestuarii	X						X
α-*Proteobacteria*							
Ochrobacter sp.			X				X
β-*Proteobacteria*							
Achromobacter/Alcaligenes sp.	X	X	X		X		X
γ-*Proteobacteria*							
Aeromonas bestiarum			X			X	
Pantoea sp.	X			X			
Stenotrophomonas maltophilia			X		X		
Culture Affiliation	**LK**	**LA**	**LW**	**GL**	**HP**	**AP**	**LV**
α-*Proteobacteria*							
α-*Proteobacterium*	X						X
Rhodovulum sp.	X					X	
β-*Proteobacteria*							
Matsuebacter sp.				X	X		
γ-*Proteaobacteria*							
Aeromonas sp.				X	X		
'Alteromonas alvinellae'	X					X	X
Enterobacter sp.				X	X		
Marinobacter sp.	X	X					
Pseudomonas stutzeri	X						X
Salinivibrio costicola	X	X				X	
Serratia marcesens	X					X	
Shewanella alga	X					X	
Vibrio sp.	X	X				X	
Firmicutes							
Bacillus pumilis	X	X					
Bacillus sp.	X						X
Bacillus sp.	X			X	X	X	

X: Detected at this site.

Discussion

Physical and Chemical Diversity among Sites. The aquatic systems considered in this work offer physically and chemically diverse niches. The range over which parameters such as salinity, depth, morphometry, and even elevation varied between each site was quite remarkable, more so considering the lakes alone are the only five natural lakes in the Hawaiian Archipelago, i.e., they have not been converted to reservoirs, dredged, or connected to the ocean. Although the same suite of enrichment media (with changes only in salinity) was applied to samples from each site, and clone libraries were prepared in the same manner, there was no evidence to support the maxim that 'everything is everywhere'. Indeed, only a handful of OTUs and cultivated bacteria occurred in more than one site, and they all belonged in phyla commonly cultivated or detected in clone libraries.

An increase in salinity in surface waters of L. Kauhakō during a persistent local drought accompanied a decrease with time in the depth at which we detected H_2S. During the same period, we detected obligately halophilic sulfate-reducers in shallower (saline) water. If the drought persists and salinity in the lake's surface waters continues to increase, biogenic hydrogen sulfide from bacterial sulfate reduction may well vent to the atmosphere. A biogeochemical manifestation of the same drought is evident elsewhere in Hawai'i, with salinity increases in the Subtropical North Pacific Gyre having weakened the strength of the annual CO_2 sink [18]. With respect to other sites we sampled, L. Laysan is the archipelago's only hypersaline lake. It was not the only one in this work, however, in which a low concentration of Chl *a* was detected. Small standing stocks of photoautotrophs were also indicated for freshwater Green L. and L. Wai'ele'ele. Such observations are consistent with the only other report of chl *a* concentrations in these lakes [41]. Chemistry in the anchialine pool on the Pearl and Hermes Atoll was typical of such features in Hawai'i, with salinity less than that of the nearby ocean. Lō'ihi water chemistry has been discussed elsewhere [53].

Uncultivated Bacterial Diversity. That most OTUs from γ-*Proteobacteria* and α-*Proteobacteria* were from

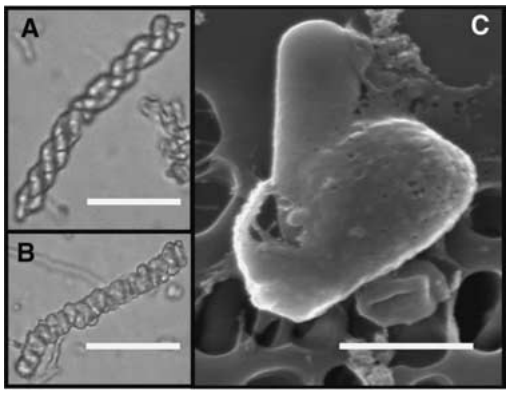

Figure 2. (A, B). A mixed bacterial culture from Lō'ihi containing sheaths characteristically produced by iron-oxidizing *Bacteria*. Scale bars are 5 μm. (C). SEM of a spore emerging from a vegetative cell in a Lō'ihi vent water sample incubated in Marine Broth at 70°C and 130 atm. in a pressure and temperature controlled reactor. A *Bacillus* sp. was subsequently isolated from this reactor. Scale bar is 1 μm.

saline lakes or Lō'ihi is consistent with reports of their strong representation in (marine) clone libraries [22, 26]. The fact that fluorescence *in situ* hybridization suggests

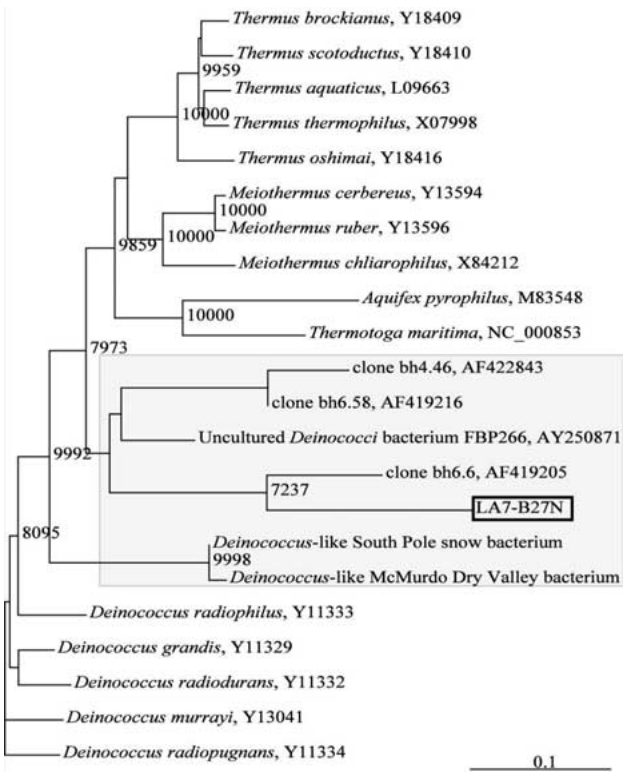

Figure 3. The closest neighbors of a "*Deinococcus*-like" sequence from hypersaline L. Laysan form a clade of "Antarctic-only" *Deinococci*-like sequences (shaded); Laysan clone is boxed. See text for details of the alignment procedure. Scale bar represents the number of nucleotide substitutions per site.

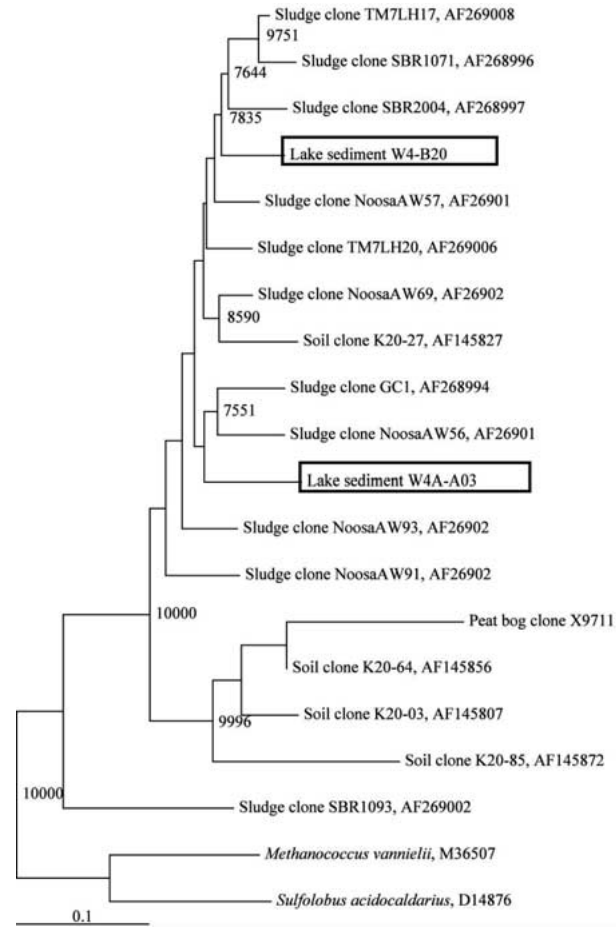

Figure 4. Candidate Division TM7-like sequences from L. Waiau extend the geographic range and habitat type reported for this division. The L. Waiau clones are boxed. See text for details of the alignment procedure. The scale bar represents the number of nucleotide substitutions per site.

they are actually small fractions of freshwater and marine communities, however, is an example of how different methods can give contrasting views of community structure [27]. Our belief that distinct prokaryote communities would occur at each site was supported by the fact that just over 4% of the *Bacteria* OTUs occurred in more than one site. Two such OTUs (from L. Kauhakō and Lō'ihi) affiliated with *Chlorobium vibrioforme* and *Prosthecochloris aestuarii*, strict anaerobes and obligate phototrophs. This is in fact the first report of these bacteria at Lō'ihi. Similarly, we also extended the geographic ranges of the TM7 and BRC1 lineages, as well as that of a group of *Deinococcus*-related sequences described previously as an "Antarctic-only" clade [10]. An OTU from anoxic water in L. Kauhakō affiliated with Candidate Division BRC1 sequences from anoxic rice paddy soil and anoxic marine sediments, both extending this lineage's geographic range and corroborating reports that its members are obligate anaerobes [11, 25]. One

Table 6. Cultivated *Bacteria* sharing <97% 16S SSU rDNA sequence identity with nearest published neighbor

Source		*Culture*	*nt*	*Nearest neighbor*	*Accession no.*	*Identity*	*%ID*
Actinobacteria							
LK	1	K2-78	1497	*Arthrobacter cumminsii*	X93354	1391/1472	94.50
LK	2	KA72	1291	*Arthrobacter sp.*	AB076400	1237/1275	97.02
LK	3	K2-27	1492	*Cellulomonas cartae*	X79456	1410/1467	96.11
LK	4	K2-66	1526	*Nesterenkonia lacusekhoensis*	AJ290397	1410/1464	96.31
LV	5	Ph1	767	*Corynebacterium* cf. *aquaticum*	AJ244681	749/781	95.90
LV	6	Ph10	848	*Kocuria* sp.	AB094467	657/679	96.76
Bacteroidetes							
LK	1	K2-15	1499	*Cellulophaga* sp.	AY035869	1280/1427	89.70
LK	2	K2-1	1506	*Cyclobacterium* sp.	AJ244689	1404/1489	94.09
GL	3	G21	1488	*Flavobacterium johnsoniae*	AB078043	1405/1453	96.70
HP	4	H22	1468	*Flectobacillus speluncae*	AY065625	1428/1447	98.69
LA	5	LA48	1396	*Flexibacter tractuosus*	M58789	1346/1391	96.76
LA	6	[a]LA1^T	1428	*Psychroflexus torquis*	U85881	1350/1433	94.21
α-*Proteobacteria*							
LK	1	K2-11	1455	α-*Proteobacterium*	AJ133762	1363/1412	96.53
LK	2	K2-53B	1454	α-*Proteobacterium*	AJ133762	1359/1412	96.25
LA	3	LA5	1364	α-*Proteobacterium*	AJ133762	1288/1366	94.29
LK	4	K2-75	1376	α-*Proteobacterium*	U70683	1270/1349	94.14
LA	5	LA33B	1384	α-*Proteobacterium*	AB015896	1299/1400	92.79
LA	6	LA52	1330	α-*Proteobacterium*	AB013079	1293/1313	98.48
LV	7	Ph15	514	*Caulobacter* sp.	AJ227773	507/527	96.20
LV	8	Ph12	596	*Erythrobacter citreus*	AF118020	593/615	96.42
LA	9	LA9	1280	Marine bacterium	AJ002565	1194/1255	95.14
LK	10	K2-76	1364	*Rhizobium loti*	U50165	1317/1361	96.77
LK	11	K2-57B	1453	*Rhizobium* sp.	AY056831	1332/1419	93.87
LA	12	LA7	1363	*Roseobacter* sp.	AF098495	1305/1366	95.53
GL	13	G6	1437	*Roseomonas cervicalis*	AY150047	1354/1436	94.29
AP	14	PH30	1396	*Thalassospira lucentensis*	AB024595	1332/1361	97.87
LK	15	KA 77	1136	Uncultured α-*Proteobacterium*	AF445712	1045/1078	96.94
LK	16	K2-53A	1461	Uncultured *Rhodobacter* LA1-B32N	AF513928	1368/1422	96.20
LK	17	K2-19	1449	Uncultured *Rhodobacter* LA4-B3	AF513932	1372/1429	96.01
LK	18	K2-12	1445	Unidentified α-*Proteobacterium*	U70683	1276/1374	92.87
LV	19	L12	1398	Unidentified α-*Proteobacterium*	U70683	1263/1340	94.25
β-*Proteobacteria*							
HP	1	H2	1485	*Iodobacter fluviatilis*	M22511	1418/1466	96.73
γ-*Proteobacteria*							
LK	1	K55	1394	'Alteromonas alvinellae'	AF288360	1153/1194	96.57
LK	2	K2-7	1528	Biphenyl-degrading bacterium	AB086226	1388/1445	96.06
LV	3	JB3	653	*Halomonas aquamarina*	AJ306888	642/662	96.98
LV	4	[b]L2-TR^T	1415	Unculturable Mariana eubacterium	D87345	1413/1415	99.86
AP	4	PH27A	1436	*Oceanospirillum maris*	AB006763	1151/1252	91.93
LV	5	JB10	680	*Pseudoalteromonas* sp.	AF227238	626/651	96.16
LV	6	JB18	1403	*Pseudomonas aeruginosa*	AY162139	1388/1404	95.73
LK	7	K51	1355	*Pseudomonas* sp.	AJ007005	1025/1060	96.70
LV	8	Ph23	673	*Salinivibrio costicola*	X95532	659/694	94.96
AP	9	PH35	1457	Uncultured γ-*Proteobacterium*	AJ310680	1339/1461	91.65
LK	10	K2-14	1520	Uncultured γ-*Proteobacterium*	AJ240916	1431/1493	95.85
LV	11	JB11	1408	Uncultured soda lake γ-*Proteobacterium*	X92128	1257/1386	90.69
LK	12	K10	1357	*Vibrio* sp.	AF246980	1256/1338	93.87
LK	13	K20	1527	*Vibrio* sp.	AB038029	1434/1507	95.16
δ-*Proteobacteria*							
LK	1	K2-52	1595	Sulfate-reducing bacterium	AF216646	1384/1429	96.85
ε-*Proteobacteria*							
LA	1	LA31B	1402	*Arcobacter* sp.	L42994	1345/1402	95.93
Firmicutes							
LK	1	K2-72	1489	*Bacillus aestuarii*	AB062696	1426/1500	95.07
LK	2	K2-24	1536	*Bacillus* sp.	AB055097	1486/1536	96.74
LK	3	K2-37	1508	*Bacillus* sp.	AB043854	1452/1500	96.80
LV	4	R5B	1202	*Bacillus* sp.	AF326369	1155/1197	96.49
LK	5	K2-69	1532	*Planomicrobium* sp.	AF144750	1440/1486	96.90

[a]LA1^T: *Psychroflexus tropicus* [12].
[b]L2-TR^T: *Idiomarina loihiensis* [14].

Table 7. Depth distribution of OTUs and cultivated *Bacteria* by phylum in Lake Kauhakō

Depth, m	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.1	❼	▯		▯	❼	⌘	⌘	▯		❼				
0.4	❼	❼	❼	❼		⌘		⌘		❼				
0.7	▯	❼		▯	⌘		⌘	⌘						
1.2	⌘	⌘		❼			⌘	⌘						
1.6	▯	⌘		❼			⌘	⌘		❼				⌘
2	⌘						⌘	⌘		❼				
2.4	⌘			▯	⌘			⌘		❼				
2.7		⌘		▯			⌘	⌘		⌘				⌘
3				❼	⌘				⌘					
3.25		⌘			⌘			⌘	⌘	⌘				
3.5				▯	⌘				⌘				⌘	
4	❼	❼	❼	▯	⌘			❼	⌘	❼				
4.2	❼			❼					⌘					
4.4				❼					⌘	❼				
8				⌘					⌘					
30	⌘	▯	⌘	▯	⌘	⌘	⌘	⌘	⌘	▯	⌘			⌘
60				⌘	⌘			⌘				⌘	⌘	
125		⌘							⌘					
200				⌘					⌘	❼			⌘	

⌘: OTU only.
❼: culture only.
▯: OTU and culture.
1: *Actinobacteria*; 2: α-*Proteobacteria*; 3: β-*Proteobacteria*; 4: γ-*Proteobacteria*; 5: δ-*Proteobacteria*; 6: *Cyanobacteria*; 7: *Planctomycetes*; 8: *Bacteroidetes*; 9: *Chlorobi*; 10: *Firmicutes*; 11: *Acidobacter/Fibrobacter*; 12: *Verrucomicrobia*; 13: Candidate Division BRC1; 14: Unassigned.

sequence (AF424445) from L. Kauhakō shared 85% identity over 977 bases with an Antarctic Candidate Division OP11 clone. Although we did not assign the OTU here, it may constitute a novel lineage within the division. Overall, 60% of OTUs shared <97% sequence identity with their nearest neighbors in GenBank and thus represent novel sequences [cf. 54].

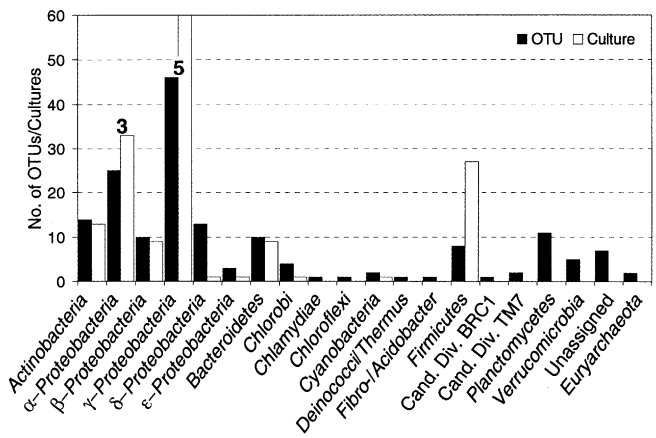

Figure 5. Phylogenetic affiliations of *Bacteria* cultivated from each site. The data labels indicate how many OTUs and cultures from that phylum and the same sample shared 97% 16S rRNA sequence identity.

Phylogenetic Diversity among Cultivated Bacteria. Parameters to consider when designating new species are discussed elsewhere, but <97% nucleotide identity between the 16S rDNA genes in two bacterial strains might indicate the strains are phylogenetically distinct [54]. Differentiating bacteria through this degree of identity is not a taxonomic rule, of course. In this respect, however, although one of our Lō'ihi cultures (L2-TR) shared >99% 16S rDNA gene nucleotide identity with its nearest neighbor (*Idiomarina abyssalis*), phenotypic characteristics and DNA–DNA reassociation supported the establishment of L2-TR as the type strain of a new species, *Idiomarina loihiensis* [14]. Had we elected to define phylogenetically distinct species through ≤98% 16S rDNA sequence identity (rather than ≤97%), 20 more of the 155 cultivated strains would be potentially novel. Moreover, a 99% identity limit (cf. *I. loihiensis* L2-TR^T and *I. abyssalis*) would encompass an additional 63 strains, giving a total of 134 potentially novel cultivated *Bacteria*. Regardless of the 16S rDNA nucleotide sequence identity level that may only loosely suggest phylogenetic novelty, our data for other strains support their placement as new species [e.g., 12, 14].

Phylogeny and Habitat Chemistry. Phylum-level characteristics of some OTUs and pure cultures reflected their distribution in terms of habitat chemistry. For example, obligately aerobic *Bacteroidetes* were cultivated only from oxic waters, while β-*Proteobacteria* were cultivated only from the freshwater sites, a typical habitat for the β-*Proteobacteria* [27]. Rare *Bacteroidetes* and *Planctomycetes* OTUs from L. Kauhakō's anoxic waters are likely from sinking cells since sedimentation rates in this lake are prodigious (Kempe, pers. obs.). Recent evidence, however, does support the existence of anaerobic or facultatively anaerobic *Planctomycetes* [57]. The majority of δ-*Proteobacteria* OTUs were derived from samples taken from H_2S-containing water below the L. Kauhakō chemocline, while a single δ-*Proteobacterium* (K2-52) was cultivated from the surface. Low sequence identities of OTUs and K2-52 with nearest neighbors suggest phylogenetic novelty in this subphylum in L. Kauhakō. A single δ-*Proteobacteria* OTU from L. Laysan was related to an SO_4–reducing endosymbiont from an oligochaete [19], but such a relationship could not be determined here. Moreover, an OTU from directly above the chemocline in L. Kauhakō was distantly related to a sulfide-oxidizing symbiont in a bivalve [29]; bivalves are not found in L. Kauhakō, although juveniles of an unidentified ostracod and a small gastropod have been found (Kempe, pers. obs.). A penaeid shrimp and an endemic Hawaiian atyid shrimp, *Halocaridina rubra*, are the lake's only pelagic invertebrates [41].

Phylogeny and Function. The degree to which physiology and function *in situ* might be inferred through

phylogeny is limited. With this *caveat* in mind, three Lō'ihi OTUs shared >99.6% sequence identity with iron-oxidizing bacteria. In this respect, *Leptothrix*-like cells, from Lō'ihi were recently described [23]. Bacteria that oxidize other metals at Lō'ihi have not been reported, but the closest relative of a strain we isolated from a 70°C, 130 atm. bioreactor was a *Bacillus* sp. from marine sediments whose spores oxidize manganese [24]. Our results show that the strain we isolated from Lō'ihi weakly oxidizes manganese (data not shown). The cultivation from L. Laysan and Lō'ihi of α-*Proteobacteria* affiliating with marine and vent strains that degrade organic sulfur compounds, and/or oxidize sulfite and thiosulfate, is indirect evidence of bacterial sulfurbased metabolism in these sites [28, 59], a contention supported by the presence in L. Laysan of H_2S and benthic mats of cyanobacteria and sulfurbacteria.

A Polyphasic Approach Is Required to Describe Microbial Diversity. The similarity between the number of *Bacteria* OTUs and distinct pure cultures was remarkable. It was equally striking, however, that the respective data sets had little in common [cf. 30]. The poor representation of the *Firmicutes* in clone libraries might result from the failure of these cells to lyse during DNA extraction. For example, we cultivated 19 different *Firmicutes* from L. Kauhakō, yet detected just one in L. Kauhakō clone libraries. Lack of primer specificity cannot be invoked since all pure cultures were sequenced with the same primers used in construction of clone libraries. It may be posited, however, that we cultivated a greater fraction of the relatively small "culturable" community than we sequenced of a much larger "nonculturable" community. Rarefaction curves of clones sequenced versus the number of different OTUs often show that diversity in terms of OTUs can be represented with fewer than 100 clones, as previously demonstrated for Lō'ihi [45]. In this respect, we sequenced ~6.5 million base pairs from more than 3000 clones from all sites. Such disparity between OTU and culture phylogeny was demonstrated in L. Laysan, where α-*Proteobacteria* OTUs belonged only in the *Rhodobacteraceae*, while several α-*Proteobacteria* cultures affiliated with three families from which no OTUs were detected. Similarly, *Vibrionaceae* and *Enterobacteriaceae* cultivated from L. Laysan and the anchialine pool were absent from these sites clone libraries. The relatively poor representation of *Archaea* in habitats in which they might be expected was surprising. As control PCRs with different *Archaea* primers and varying PCR conditions consistently yielded a fragment of the 16S rDNA gene from *Halobacterium salinarum* DNA, we might conclude that only an inordinate number of clones, primer combinations, or PCR conditions might determine greater *Archaea* diversity or presence at these sites [11, 31, 51, 58].

Molecular phylogenetic approaches describe parts of the community that cultivation methods overlook, but cultivation methods also appear routinely to determine fractions of the community not reported by molecular approaches [e.g., 47]. Since prokaryote diversity in any sample may never be known *in toto* [9], we can only speculate how much diversity and novel taxa are overlooked by single method approaches [40]. With habitat degradation and loss of biodiversity worldwide encouraging significant investment in compilation of species inventories, it is noteworthy that 47 of our 52 potentially novel strains did not occur in clone libraries from the same sample. Phylogenetic novelty among OTUs and cultivated bacteria reported here demonstrate the Hawaiian Archipelago hosts previously uncultivated bacteria. Moreover, the sizeable fractions of each in their respective libraries might also qualify the archipelago as a microbial diversity hotspot.

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