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Estimation of Diversity and Community Structure through Restriction Fragment Length Polymorphism Distribution Analysis of Bacterial 16S rRNA Genes from a Microbial Mat at an Active, Hydrothermal Vent System, Loihi Seamount, Hawaii†

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PCR was used to amplify (eu)bacterial small-subunit (16S) rRNA genes from total-community genomic DNA. The source of total-community genomic DNA used for this culture-independent analysis was the microbial mats from a deep-sea, hydrothermal vent system, Pele's Vents, located at Loihi Seamount, Hawaii. Oligonucleotides complementary to conserved regions in the 16S rRNA-encoding DNA (rDNA) of bacteria were used to direct the synthesis of PCR products, which were then subcloned by blunt-end ligation into phagemid vector pBluescript II. Restriction fragment length polymorphism patterns, created by using tandem tetrameric restriction endonucleases, revealed the presence of 12 groups of 16S rRNA genes representing discrete operational taxonomic units (OTUs). The rank order abundance of these putative OTUs was measured, and the two most abundant OTUs accounted for 72.9% of all of the 16S rDNA clones. Among the remaining 27.1% of the 16S rDNA clones, none of the 10 OTUs was represented by more than three individual clones. The cumulative OTU distribution for 48 bacterial 16S rDNA clones demonstrated that the majority of taxa represented in the clone library were detected, a result which we assume to be an estimate of the diversity of bacteria in the native hydrothermal vent habitat. 16S rDNA fingerprinting of individual clones belonging to particular OTUs by using an oligonucleotide probe that binds to a universally conserved region of the 16S rDNA fragments was conducted to confirm OTU specificity and 16S rDNA identity.

Defining the diversity and structure of natural microbial communities through the quantification of their constituent populations has been a long-standing challenge in microbial ecology. Selective enrichment cultivation as an approach for the description of naturally occurring microbial communities has severe limitations (33, 47). The majority (typically >90 to 99%) of bacteria in nature cannot be cultivated by using traditional techniques (3, 18). Consequently, it is unlikely that collections of bacterial isolates are representative of in situ diversity and community structure. Furthermore, because relatively nutrient-rich media are generally used for isolation, copiotrophic bacteria rather than the bacteria dominant in the natural community may be selected for.

An alternative approach to understanding the composition of natural communities is an approach that uses recently developed techniques of molecular biology and provides a culture-independent analysis of microorganisms. This approach involves examining variations in 16S rRNA or 16S rRNA-encoding DNA (rDNA) within a naturally occurring prokaryotic community (2, 5, 6, 13–15, 40, 47–51). Our objective in this study was to estimate diversity and community structure by performing a restriction fragment length polymorphism (RFLP) analysis of all clones derived from a library of (eu)bacterial 16S rRNA genes following tandem tetrameric site-specific restriction endonuclease treatment. The 16S rRNA gene contains information which makes it an excellent biomarker of microorganisms. For example, each 16S rRNA gene contains both highly conserved regions found in all living organisms and diagnostic variable regions that are unique to particular organisms or closely related groups of organisms. Analysis of the latter regions leads to a specific RFLP pattern, which in turn can be used to define an operational taxonomic unit (OTU). In this study we used tandem tetrameric restriction endonucleases to produce RFLP patterns, which were then analyzed and cataloged. Furthermore, we used 16S rDNA fingerprinting to confirm both the validity of the RFLP analysis results and the 16S rDNA identities.

We define community structure in terms of the number of OTUs present in a community and the abundance of individual clones within each OTU. These values are estimated by a tandem tetrameric RFLP analysis of each 16S rDNA clone. Diversity is a metric for the number of populations in a community and the genetic relatedness among these populations. In this study we focused only on the populations in a bacterial community; genetic relatedness, as assessed by phylogenetic analysis, will be discussed in a subsequent publication. For both community structure and diversity, we estimated the number of populations by the number of OTUs present in the community. In addition, we used an analog of rarefaction to ascertain that the majority of the total diversity in the native habitat was successfully detected in the 48 clones examined.

The microbial community and habitat which we examined

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were the microbial mats at Pele's Vents, which are located on the southwest portion of the summit (980 m below sea level) of Loihi Seamount. The area of active hydrothermal fluid discharge is restricted to the flank of a relatively small volcanic cone approximately 10 to 15 m below the summit. The active field (area, <0.25 km^2) is characterized by numerous individual vents discharging waters heated to temperatures of ≤37°C (compared with the ambient seawater temperature of 4°C). The individual vent orifices are distinguished by a white precipitate, which has been determined to contain high concentrations of elemental sulfur (19, 20). The vent fluid is exceptionally clear and nearly devoid of suspended particulate matter.

Loihi Seamount is an active, submarine, hotspot volcano and potentially the next Hawaiian island. Geochemical evidence and biological evidence suggest that hydrothermal vent systems at midplate sites differ fundamentally from vent systems at plate boundaries (e.g., midocean ridges) (19, 20, 42). Most notably, the vent waters at Pele's Vents contain extremely high concentrations of total dissolved CO_2 (ca. 300 mM) that are more than 100 times greater than the concentration at the Galapagos Rift Vents (9). Consequently, the in situ pH of vent waters can be as low as 4.2 (42). The levels of dissolved iron (ca. 1 mM) are 2×10^6 times greater than the levels in ambient seawater and approximately 40 times greater than the levels at the Galapagos Rift Vents (9). Another compound of potential microbial importance is CH_4; the dissolved CH_4 concentration (ca. 7.2 μM) in the effluent of Pele's Vents is enriched 10^5-fold compared with ambient seawater (19). Loihi Seamount has none of the luxuriant macrobenthos (e.g., tubeworms and giant clams that harbor bacterial endosymbionts) present at other deep-sea hydrothermal systems. This characteristic may be related to the absence or extremely low levels of sulfide in the vent waters (42), to the high levels of dissolved CH_4 and iron (19, 20), or perhaps to the low pH of the effluent waters (42).

There have been previous descriptive and metabolic studies of the microbiological components at Loihi Seamount. Karl et al. (19, 20) described the predominance of iron-depositing sheathed bacteria at the site, demonstrated methanotrophy, utilization of acetate and glutamate, and incorporation of [^{3}H]adenine and [^{3}H]thymidine into nucleic acids, and detected thermophilic bacterial populations. In this study we supplemented this information and estimated the diversity and community structure, as previously defined, of bacteria living at Pele's Vents.

MATERIALS AND METHODS

Collection of bacterial mat samples. Material was collected from Pele's Vents (depth, 980 m) on dives 193–196 (12 October 1991 to 18 October 1991) and 208–215 (18 September 1992 to 1 October 1992) of DSRV *Pisces V* (Fig. 1). Two independent methods were used to sample the microbial mats. Mat-covered rocks were placed into polyvinylchloride "coffins" to prevent winnowing of the mat material en route to the surface. Hydrothermal fluids containing bacterial mat material were collected by using the vacuum produced by opening 2-liter Niskin baggie samplers adapted with a 2-m Tygon tube for directional sampling. Aboard ship, samples were collected either by aseptically scraping mat-covered rocks or by allowing mat particulates to settle inside suspended Niskin baggies. Samples were quick frozen and maintained on dry ice until they were returned to the laboratory, and then they were kept at −85°C until they were processed.

Genomic DNA extraction and purification. Both French press lysis and enzymatic cellular lysis were used to maximize the recovery of genomic DNA. Approximately 10 g (wet weight) of mat material was thawed on ice, ground with a cold (4°C) sterile mortar and pestle, and resuspended in cold (4°C) sucrose lysis buffer (0.75 M sucrose, 700 mM NaCl, 40 mM Na_2EDTA, 50 mM Tris-HCl; pH 8.3) to a total volume of 20 ml; the high salt concentration helped prevent DNA shearing. The mat slurry was passed through a cold (4°C) French pressure cell twice at 20,000 lb/in^2, 1 mg of lysozyme per ml was added, and then the preparation was incubated at 37°C for 30 min. After the addition of 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 100 μg of proteinase K (United States Biochemical Co., Cleveland, Ohio) per ml, 250 μg of achromopeptidase (Sigma Chemical Co., St. Louis, Mo.) per ml, and 50 μg of RNase A (United States Biochemical Co.) per ml, the mixture was incubated at 55°C for 30 min. The cells in the mat slurry were monitored by microscopic examination to determine that complete lysis had occurred. The polysaccharides and residual proteins were aggregated by adding 1% (wt/vol) hexadecyltrimethyl ammonium bromide and incubating the preparation at 65°C for 30 min. Protein and polysaccharide complexes were removed by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (50:49:1) (phenol was first prepared fresh by water saturation, 0.1% [wt/vol] *p*-hydroxyquinoline addition, and buffer saturation with STE buffer [100 mM NaCl, 10 mM Na_2EDTA, 50 mM Tris-HCl; pH 7.4]). The residual phenol was removed by extracting the aqueous phase with an equal volume of chloroform-isoamyl alcohol (24:1). The genomic DNA was allowed to precipitate at −20°C for 8 h after the addition of 0.1 volume of 3 M sodium acetate (pH 4.6) and 2.5 volumes of 100% ethanol and was collected by centrifugation at 10,000 × *g* for 30 min by using 30-ml Corex glass centrifuge tubes (Corning Inc., Horseheads, N.Y.). The genomic DNA pellet was resuspended to a concentration of 100 ng/μl, as measured by A_{260}, and the yield was between 10 and 20 μg/10 g (wet weight) of bacterial mat sample. Approximately 5 to 10 μg of this crude DNA preparation was purified by treatment with Qiaex (Qiagen, Inc., Chatsworth, Calif.), a uniform 3-μm silica gel matrix which selectively binds DNA in the presence of high salt concentrations.

Oligonucleotide synthesis and purification. All oligonucleotides used in this study were synthesized with a model 380B automated DNA synthesizer (Applied Biosystems, Foster City, Calif.) and were purified by using the Surepure thin-layer chromatography system (United States Biochemical Co.) at the University of Hawaii Biotechnology-Molecular Biology Instrument and Training Facility.

Amplification of 16S rRNA genes. The 16S rDNA was selectively amplified from purified genomic DNA by using PCR (10, 36, 45) with oligonucleotide primers designed to anneal to conserved positions in the 3′ and 5′ regions of bacterial 16S rRNA genes. The forward primer (5′-TNANA CATGCAAGTCGAICG) corresponded to positions 49 to 68 of *Escherichia coli* 16S rRNA (4), and the reverse primer (5′-GGYTACCTTGTTACGACTT) corresponded to the complement of positions 1510 to 1492 (22). The reaction conditions were as follows: 100 ng of template DNA, 10 μl of 10× PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0 at 25°C], 15 mM $MgCl_2$, 1% [wt/vol] Triton X-100), 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 1.5 μg of T4 gene 32 protein (United States Biochemical Co.), 1 μM forward primer, 1 μM reverse primer, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 200 μM dTTP were combined in a total volume of 100 μl. The reaction mixtures lacking template DNA, T4 gene 32 protein, and *Taq* DNA polymerase were UV irradiated for 10 min to eliminate potentially contaminating template DNA (38) and then heated at 94°C for 2 min. The

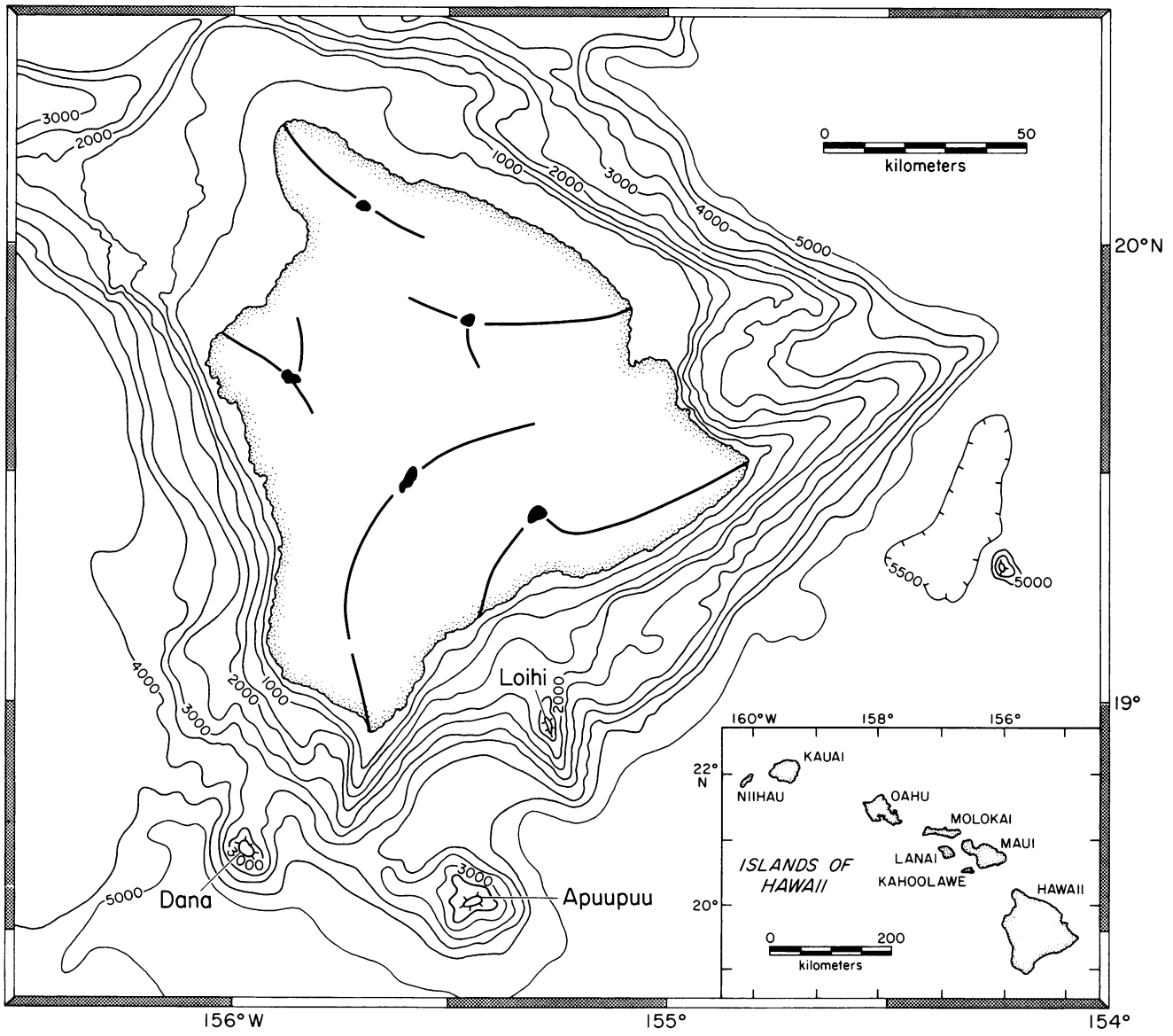


FIG. 1. Map showing the location of Loihi Seamount in relation to the island of Hawaii. (Inset) Larger-scale view of the major islands which make up the state of Hawaii. Redrawn from reference 12. Isobaths are in meters.

complete reaction mixture was overlaid with mineral oil and incubated in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) as follows: denaturation at 94°C for 1 min, primer annealing at 60°C for 1.5 min, and chain extension at 72°C for 3 min with an additional extension time of 7 min on the final cycle, for a total of 33 cycles. Each lot of T4 gene 32 protein and *Taq* DNA polymerase was tested for potential contamination of 16S rDNA templates as described by Schmidt et al. (41). PCR-amplified gene products were separated chromatographically in 1.5% agarose (0.75% NuSieve and 0.75% SeaKem; FMC Bioproducts, Rockland, Maine) electrophoresis gels stained with 0.5 μg of ethidium bromide per ml and visualized by UV excitation.

Construction of bacterial 16S rDNA clone library. Amplified 16S rDNA gene products from four to six individual PCRs were pooled, purified by treatment with Qiaex, and made blunt ended by treatment with 10 U of the large (Klenow) fragment of DNA polymerase I and 10 U of T4 polynucleotide kinase (Promega). The reaction mixture also included 10 μl of 10× Klenow buffer (0.5 M Tris-HCl [pH 7.5 at 25°C], 100 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mg of bovine serum albumin per ml), 1 mM ATP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 200 μM dTTP; the total volume was 100 μl, and the preparation was incubated at 37°C for 1.0 h. The blunt-ended PCR-amplified 16S rDNA gene products were again purified by treatment with Qiaex and ligated into the *Sma*I restriction site of pBluescript II KS$^-$ (Stratagene, La Jolla, Calif.). The ligation reaction mixture contained 270 ng of insert and 15 ng of vector (i.e., the molar ratio was 9:1), as well as 1 μl of 10× ligase buffer (300 mM Tris-HCl [pH 7.8 at 25°C], 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM ATP) and 2 U of T4 DNA ligase (Promega) in a final reaction volume of 10 μl and

was incubated at 16°C for 12 to 14 h. The resulting ligation products from four independent ligation reactions were pooled, diluted 2:3 with TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA [pH 8.0 at 25°C]), and used to transform competent Epicurian Coli XL1-Blue MRF′ cells (Stratagene) according to the manufacturer's protocol. Clones were screened for α-complementation by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as the substrate (37) on YT agar supplemented with ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml).

16S rDNA RFLP analysis. Plasmid DNA was prepared from clones by using the Magic Miniprep DNA purification system (Promega). Insert 16S rDNA gene fragments were cut from the plasmid vector with restriction enzymes *Bam*HI plus *Pst*I (United States Biochemical Co.), concentrated by ethanol precipitation with ammonium acetate (37), separated by gel electrophoresis in 1.5% agarose (0.75% NuSieve and 0.75% SeaKem; FMC Bioproducts) gel stained with 0.5 μg of ethidium bromide per ml, and visualized by UV excitation. Insert 16S rDNA gene fragments were excised from the agarose gel, extracted by using Qiaex, restricted by using the tetrameric endonuclease pairs *Hae*III plus *Msp*I or *Alu*I plus *Rsa*I (New England Biolabs Inc., Beverly, Mass.), and concentrated by ethanol precipitation with ammonium acetate (37). The resulting RFLP products were separated by gel electrophoresis in 4.0% agarose (3.0% NuSieve and 1.0% SeaKem; FMC Bioproducts) gels stained with 0.5 μg of ethidium bromide per ml and visualized by UV excitation.

16S rDNA fingerprinting by Southern blotting and oligonucleotide probe hybridization. RFLP gels were denatured in a 1.5 M NaCl–0.5 N NaOH solution and Southern blotted (43) onto Nytran membranes (Schleicher & Schuell, Keene, N.H.) with a vacuum blotter (model 785; Bio-Rad Laboratories, Richmond, Calif.) by using a transfer buffer (2.16 M NaCl, 12 mM Na_2EDTA, 120 mM $NaPO_4$ buffer [pH 7.6]) (16). The following DNA oligonucleotides were used as hybridization probes with RFLP blots: oligonucleotide 1406F (5′-GYACA CACCGCCCGT), corresponding to *E. coli* 16S rRNA positions 1392 to 1406; oligonucleotide 926F (5′-CAGCMGC CGCGGTAATIC), corresponding to positions 907 to 926; and oligonucleotide 536F (5′-AAACTYAAAKGAATTGACGG), corresponding to positions 519 to 536 (23). These oligonucleotides correspond to highly conserved or "universal" regions found in all known small-subunit ribosomal genes (23), and each was 5′ end labeled with [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) by using T4 polynucleotide kinase (Promega) to a specific activity of approximately 2×10^8 to 4×10^8 dpm/μg (44). The labeled probes were purified on C_{18} reverse-phase Sep-Pak columns (Millipore Corp., Milford, Mass.) (23). Southern-blotted RFLP membranes were dried at 80°C for 30 min under a vacuum and UV cross-linked for 2 min (0.1 J/cm^2). The membranes were prehybridized for 15 min at 25°C in 10 ml of hybridization buffer (1× Denhardt's reagent, 0.1% SDS, 1.08 M NaCl, 6 mM Na_2EDTA, 60 mM $NaPO_4$ [pH 7.6]) in a hybridizer oven (model HB-1D; Techne Inc., Princeton, N.J.). The solution was replaced with 10 ml of fresh hybridization buffer containing 20 μCi of γ-^{32}P-labeled oligonucleotide probe, and the preparation was incubated at 25°C for 4 to 12 h. Following hybridization, the membranes were washed three times (15 min each) in 25 ml of washing buffer (0.1% SDS, 1.08 M NaCl, 6 mM Na_2EDTA, 60 mM $NaPO_4$ [pH 7.6]) at 28°C. The final 15-min wash at 37°C was done in washing buffer. Hybridized membranes were kept moist for the purpose of reprobing by placing them on blotting paper saturated with TE buffer and sealed inside Micro-Seal bags (Dazey Corp., Kansas City, Kans.). The Micro-Seal bags were placed directly into autoradiography cassettes with intensifying screens for 6 to 8 h of exposure to X-ray film. Oligonucleotide probes were stripped from membranes by washing the membranes twice (15 min each) with washing buffer at 65°C prior to probing with a different oligonucleotide.

RESULTS

A total of 76 colonies were chosen after they tested positive for α-complementation of β-galactosidase, and 51 clones contained an insertion detectable by primary restriction with *Bam*HI plus *Pst*I. A total of 48 of these clones contained the entire 1.5-kb 16S rDNA insert, including three discrete, universally conserved oligonucleotide hybridization sites. These universally conserved sites were assessed by 16S rDNA fingerprinting analyses (data not shown). The RFLP patterns of the 48 intact 16S rDNA clones indicated that only three specific patterns were obtained after primary restriction with *Bam*HI

TABLE 1. Data matrix for OTU polymorphisms as detected by restriction digestion

Restriction digestion	Estimated size of rDNA fragment (bp)	Presence in:											
		OTU 1 (n = 12)[a]	OTU 2 (n = 23)	OTU 3 (n = 3)	OTU 4 (n = 1)	OTU 5 (n = 1)	OTU 6 (n = 2)	OTU 7 (n = 1)	OTU 8 (n = 1)	OTU 9 (n = 1)	OTU 10 (n = 1)	OTU 11 (n = 1)	OTU 12 (n = 1)
Primary[b]	1,500	+	+	−	+	+	+	−	+	−	−	+	+
	1,250	−	−	−	−	−	−	+	−	+	−	−	−
	800	−	−	+	−	−	−	−	−	−	+	−	−
	700	−	−	+	−	−	−	−	−	−	+	−	−
	250	−	−	−	−	−	−	+	−	+	−	−	−
Secondary[c]	550	−	−	−	−	−	+	−	−	−	−	−	+
	350	+	−	−	+	+	−	+	−	−	+	−	−
	300	−	+	+	−	−	−	+	+	+	−	−	−
	250	−	−	−	+	−	+[d]	−	−	−	−	+	+[d]
	175	+[d]	+	+	+[d]	+	+	+[d]	+	+	+[d]	+	+
	150	−	−	−	+	+	−	−	−	−	+	−	+
	125	−	+[d]	+[d]	−	−	+	+	+[d]	+[d]	+	−	+
	100	−	−	−	−	+	−	−	−	−	−	−	−
	75	−	+	+	−	−	+	−	−	+	−	+[d]	−
	50	+	+	−	−	+[d]	−	+	+	−	+	+	+

[a] n is the number of clones examined.
[b] Restriction with *Bam*HI plus *Pst*I.
[c] Restriction with *Alu*I plus *Rsa*I.
[d] After hybridization with universal oligonucleotide probe 536F

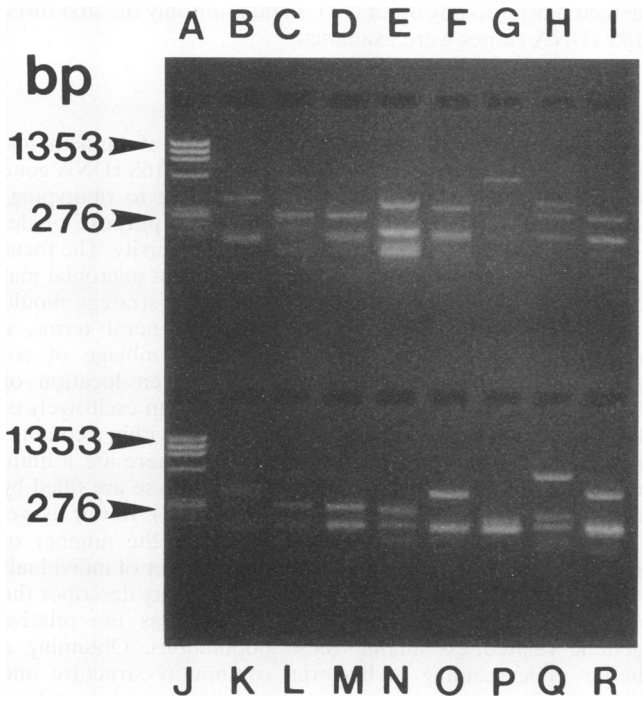


FIG. 2. OTUs determined from RFLP analysis of representative bacterial 16S rDNA cloned inserts restricted with *Alu*I plus *Rsa*I. Lanes A and J contained *Hae*III-restricted ϕX174 DNA size markers. Lanes B, K, and R, OTU 1 clones; lanes C and L, OTU 2 clones; lanes D and M, OTU 3 clones; lane E, OTU 4 clone; lane F, OTU 5 clone; lane G, OTU 6 clone; lane H, OTU 7 clone; lane I, OTU 8 clone; lane N, OTU 9 clone; lane O, OTU 10 clone; lane P, OTU 11 clone; lane Q, OTU 12 clone. Two or three lanes containing members of the same OTU demonstrate that there was reproducibility among different 16S rDNA clones in that OTU.

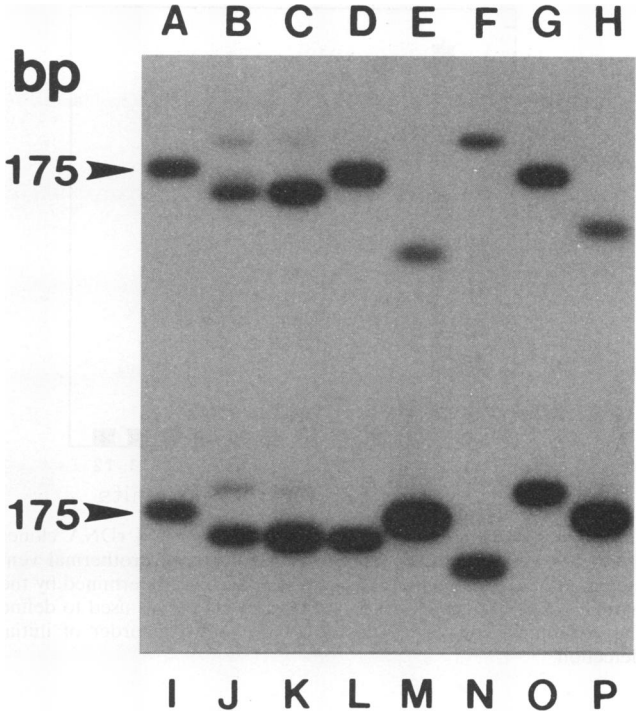


FIG. 3. 16S rDNA fingerprinting of RFLPs from clones representing OTUs with oligonucleotide probe 536F. Lanes A, I, and P, OTU 1 clones; lanes B and J, OTU 2 clones; lanes C and K, OTU 3 clones; lane D, OTU 4 clone; lane E, OTU 5 clone; lane F, OTU 6 clone; lane G, OTU 7 clone; lane H, OTU 8 clone; lane L, OTU 9 clone; lane M, OTU 10 clone; lane N, OTU 11 clone; lane O, OTU 12 clone. Two or three lanes containing members of the same OTU demonstrate that there was reproducibility among different 16S rDNA clones in that OTU.

plus *Pst*I (Table 1). These RFLP patterns resulted from no more than one internal cut site within the 16S rDNA insert of any individual clone. Two of the three clones with inserts not used in the OTU analysis contained incomplete inserts that were approximately 600 bp long, and the other clone had an approximately 1.8-kb insert containing a 16S rDNA chimeric structure. The latter was detected after the cloned 16S rDNA insert was digested after secondary restriction with tetrameric endonuclease pairs, which was followed by the hybridization of two discrete sites to a single universally conserved oligonucleotide probe (data not shown).

Secondary restriction of complete 16S rDNA inserts was performed with tetrameric endonuclease pairs (either *Hae*III plus *Msp*I or *Alu*I plus *Rsa*I) to identify discrete OTUs. In evaluating the RFLP patterns that emerged, we classified each discrete pattern, which was either unique for a single clone or similar for two or more clones, as an OTU. After secondary restriction of the entire 16S rDNA clone library with *Hae*III plus *Msp*I, 11 OTUs, which included all the clones contained in the two dominant OTUs (data not shown), were detected. Secondary restriction of all 16S rDNA clones with *Alu*I plus *Rsa*I was slightly more discriminating in that one additional OTU, which contained a single clone, was detected; OTU 11 was separated from OTU 1 in this manner. Overall, when *Alu*I plus *Rsa*I were used after excision with *Bam*HI plus *Pst*I, a total of 12 OTUs were detected (Table 1; Fig. 2).

The validity of the 12 OTUs was confirmed by the results of 16S rDNA fingerprinting of representative 16S rDNA clones that underwent secondary restriction with *Alu*I plus *Rsa*I by using oligonucleotide probe 536F (Table 1; Fig. 3). In addition, 16S rDNA fingerprinting confirmed that cloned inserts were intact 16S rDNA gene fragments; each of the three oligonucleotide probes used hybridized to the universally conserved regions in the 16S rRNA gene (data not shown for probes 926F and 1406F). Intact 16S rDNA inserts were determined both from the overall sizes of cloned inserts after primary restriction with *Bam*HI plus *Pst*I from the phagemid vector and from the occurrence of a single hybridization site among the RFLP bands for each of the three universally conserved oligonucleotide probes.

Having identified 12 putative OTUs, we determined the distribution of the 48 16S rDNA clones among these OTUs (Fig. 4). The OTUs were numbered solely in the order of detection, which was assumed to be stochastic, as determined by the first 16S rDNA clone found in each OTU. OTUs 1 and 2 together accounted for 72.9% of all of 16S rDNA clones. Of the two dominant OTUs, OTU 2 clones were nearly twice as prevalent as OTU 1 clones; OTUs 1 and 2 accounted for 25.0 and 47.9%, of the 16S rDNA clones examined, respectively. The remaining 13 bacterial 16S rDNA clones were distributed among 10 OTUs. Only 2 of the remaining 11 OTUs contained more than one clone. Thus, 8 of the 12 OTUs were represented by a single 16S rDNA clone.

To determine whether in situ bacterial diversity was well described by the 16S rDNA clones examined, the cumulative number of OTUs was plotted as a function of clone number

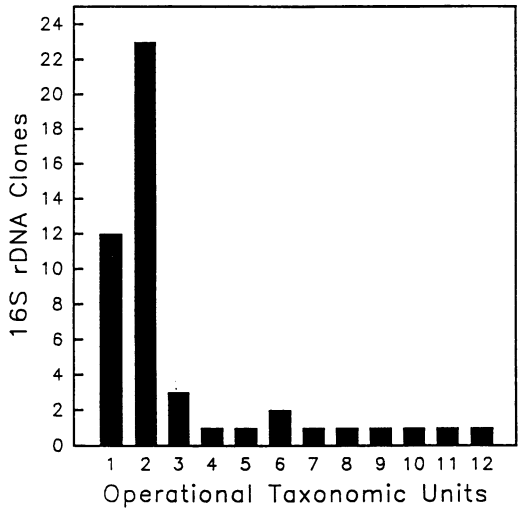


FIG. 4. Distribution in OTUs of 48 bacterial 16S rDNA clones from the bacterial mat community at an active, hydrothermal vent system at Loihi Seamount, Hawaii. Abundance, as determined by the number of 16S rDNA clones found in each OTU, was used to define the community structure. The OTUs are shown in order of initial detection.

(Fig. 5). The bacterial 16S rDNA clones were numbered solely on the basis of initial detection, which was assumed to be stochastic. This technique is analogous to generating a rarefaction curve to estimate species richness from a deterministic transform of species abundance data (46). After the first 27 bacterial 16S rDNA clones were examined, 11 of the 12 OTUs had been detected. Only one additional OTU was detected among the remaining 21 clones. The two dominant OTUs were detected prior to any other OTUs and after only the first three 16S rDNA clones were examined.

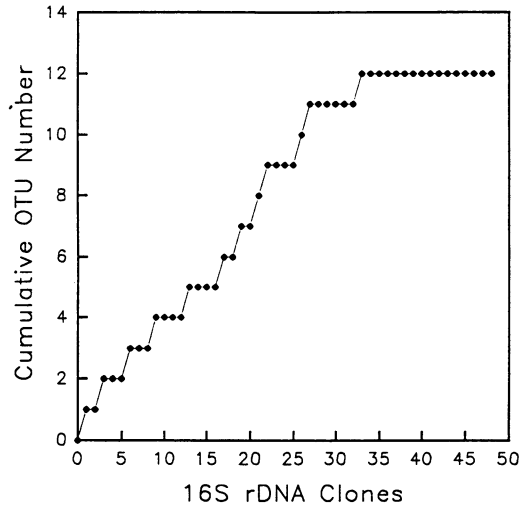


FIG. 5. Estimation of diversity in the bacterial mat community at an active, hydrothermal vent system at Loihi Seamount, Hawaii. The sequential detection of cumulative OTUs following RFLP analysis of a 48-clone bacterial 16S rDNA clone library is represented. For example, after 27 (and 32) clones were examined, 11 (and 12) OTUs were detected. The 16S rDNA clone numbers reflect the order of initial detection, which was assumed to be stochastic relative to the distribution of clones generated in the library.

DISCUSSION

In this paper, we describe the novel use of tandem tetrameric restriction endonucleases with cloned 16S rDNA gene fragments to generate RFLP data (equivalent to ribotyping) combined with rDNA fingerprinting for the purpose of describing bacterial community structure and diversity. The focus of our study was a deep-sea, hydrothermal vent microbial mat community, and we believe that our analytical strategy should apply equally well in other habitats. In general terms, a microbial community is defined as an assemblage of cooccurring microorganisms interacting at given location or habitat; it is the highest biological unit made up exclusively of individuals and populations. Each population within a community has a distinct functional role or niche. There are a finite number of niches within a community, and these are filled by the intrinsic populations of that community (1). In our usage, the term community structure encompasses the number of populations within a community and the number of individuals within each population. A community's diversity describes the number of individual populations, as well as the relative genetic relatedness among these populations. Obtaining a better understanding of bacterial community structure and diversity is crucial to aspects of microbial ecology where bacteria interact with one another and with their environment (e.g., global biogeochemical cycling of matter, risk assessment related to the release of genetically engineered microorganisms, predator-prey relationships, and trophic-level interactions).

A keystone of our approach was the use of tandem tetrameric restriction enzymes to establish OTUs. While any one of the four tetrameric restriction enzymes by itself yielded too few recognition sites for OTU analysis (data not shown), either pair of enzymes (*Hae*III plus *Msp*I or *Alu*I plus *Rsa*I) would have been sufficient for the detection of the majority of the OTUs present in the hydrothermal bacterial community examined. The 1.5-kb cloned 16S rDNA inserts yielded restriction fragments in the size range from 50 bp (lower limit of detection) to 800 bp. In a perfectly random DNA sequence that contained 50% G+C, a tetrameric recognition site would occur every 256 bases. Therefore, the use of two tetrameric restriction enzymes theoretically would yield 11 or 12 recognition sites within any given 1.5-kb gene fragment. Because of the moderately higher G+C contents of 16S rRNA genes (most bacterial 16S rDNAs have G+C contents between 55 and 65% [52]), we initially used restriction enzymes *Hae*III plus *Msp*I. However, *Alu*I plus *Rsa*I yielded more restriction fragments within the required size range and were slightly more discriminating for the bacterial 16S rDNA clones examined in this study (Table 1; Fig. 2), which resulted in detection of an additional OTU.

An integral part of our analysis was rDNA fingerprinting through Southern blotting and 16S rDNA universal oligonucleotide probe hybridizations (Table 1; Fig. 3). In doing this, we confirmed the occurrence of 12 OTUs and the identities of the 16S rDNA inserts for each clone. In addition, we detected a chimeric 16S rDNA clone most likely produced by PCR-mediated amplification. Without either rDNA fingerprinting or a complete secondary-structure analysis of the primary-sequence data from the entire 16S rRNA gene, this PCR-mediated potential risk could produce erroneous results and thereby suggest the presence of organisms that in fact do not exist (24). In our study, the chimeric 16S rDNA clone was

detected after secondary restriction by hybridization with a universal oligonucleotide probe. This probing revealed the presence of dual sites for a universally conserved position that normally occurs only once in a 16S rDNA gene. This result, found during an initial inspection of the bacterial clone library by rDNA fingerprinting, caused us to eliminate this suspect clone from the final OTU analysis.

The bacterial mat community collected at Pele's Vents was dominated by two OTUs, which accounted for 72.9% of the total 16S rDNA bacterial clone library (48 clones), and there were an additional 10 OTUs present at low levels. Our estimate of bacterial community structure, consisting of 12 OTUs detected among the 48 bacterial 16S rDNA clones and their respective levels of abundance, approximates a log-normal distribution (Fig. 4). This distribution of individual 16S rDNA clones in OTUs (community structure) for Pele's Vents is especially interesting because this habitat may be considered an "extreme" environment because of its low pH (≈4.0), strong thermal gradient, high dissolved CO_2 content (≈300 mM), and elevated trace metal concentrations (e.g., an Fe concentration of ≥1 mM). This finding is accentuated by the ephemeral nature of hydrothermal vent water emissions in general, which directly affect the temperature of the habitat.

In examining the cumulative OTU distribution of 48 bacterial 16S rDNA clones (Fig. 5), we interpreted the significant decrease in the rate of OTU detection as evidence that most of the diversity in the clone library was detected by the RFLP analysis. We detected 11 of the 12 OTUs among the first 27 bacterial 16S rDNA clones examined. The remaining 21 bacterial 16S rDNA clones yielded only 1 additional OTU. As there certainly must be rarely occurring bacteria in the mat community, an examination of additional 16S rDNA clones may have detected more OTUs. It is clear, however, that the level of analysis was sufficient to detect the community's predominant OTUs and infer their distribution within the microbial mat community at Pele's Vents.

A crucial factor to address in an analysis of microbial community structure and diversity when 16S rRNA biomarkers are used is whether the focus of study should be on the potential genetic diversity represented at the DNA level or on physiologically active genetic diversity represented at the RNA level. No habitat can optimally support the growth of all of the bacteria that it contains (25, 26), and in marine environments it is likely that starvation-survival processes are a common metabolic strategy for a majority of the bacteria (27–29). When in a starvation survival-state, marine bacteria lose viability as well as cellular DNA and RNA disproportionally, depending on the prestarvation growth rate (29–32). The rRNA content decreases predictably during starvation-survival, with loss rates that depend on the physiological state at the onset of starvation (11, 21). A positive correlation between cellular ribosomal (rRNA) content over a wide range of growth rates for bacteria is a long-standing axiom in microbial physiology (8, 17, 35, 39). Recently, this relationship was demonstrated elegantly by using fluorescent 16S rRNA-targeted hybridization probes for single cells of *E. coli* B/r (7) and for single cells of sulfate reducers found in biofilms (34). Direct analysis of 16S rRNA can potentially bias diversity estimations in favor of rapidly growing populations of cells and can underestimate the genetic diversity present in a given habitat. Therefore, the 16S rRNA of a natural microbial community better estimates the physiologically active microorganisms that are present than the absolute genetic diversity and community structure. Consequently, estimating diversity at the DNA level, rather than at the RNA level, theoretically provides a more accurate measurement of taxonomic group variability by potentially detecting slowly growing or dormant microorganisms present within the community.

Estimating community structure and diversity at the DNA level is an invaluable tool for microbial ecology, but this strategy also has potential problems and limitations. The oligonucleotide primers used to amplify the 16S rRNA genes from the bacterial mat community are complementary to regions conserved over the entire bacterial domain. Therefore, we assumed that the clone library contained an array of 16S rDNAs approximately as diverse as that of the bacterial mat community at Pele's Vents. Furthermore, the distribution of 16S rDNA clones within a library ultimately should approximate the relative distribution of cells in the habitat. However, the possibility of selection during the DNA extraction process exists, and care must be taken to achieve the highest possible level of efficiency in cell extraction and DNA recovery to avoid selection prior to PCR amplification of the cloned 16S rDNA. It is also possible that "cell-free" detrital DNA, which may have been adsorbed onto the mineral-rich microbial mat material found at Pele's Vents was extracted. The potential for bias at the level of the PCR and ligation reactions also exists, which is why care was taken to use multiple PCR and ligation reactions to construct the bacterial clone library. Finally, as shown previously, when conducting community structure and diversity analyses with these techniques, workers must be especially alert to and test for the possibility of PCR-mediated chimeric gene amplification.

In summary, in this study we demonstrated a novel approach for estimating microbial diversity and community structure from environmental samples by using recently developed molecular biological techniques. We used tetrameric restriction endonuclease pairs to detect OTUs by an RFLP analysis of a PCR-amplified 16S rDNA bacterial clone library. Using this technique coupled with rDNA fingerprinting, we estimated the number of OTUs and the abundance of each OTU. We applied this approach to the bacterial mats at Pele's Vents, a deep-sea hydrothermal vent system, and showed that the bacterial community is dominated by 2 OTUs and contains at least 12 OTUs, entities analogous to bacterial species. Phylogenetic analyses of the 16S rRNA genes from each of the bacterial OTUs, as well as archaeal community structure and diversity analyses of the microbial mats at Pele's Vents, are currently under way.

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REFERENCES

1. **Atlas, R. M., and R. Bartha.** 1993. Microbial ecology, 3rd ed. Benjamin Cummings, Redwood City, Calif.
2. **Britschgi, T. B., and S. J. Giovannoni.** 1991. Phylogenetic analysis

of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **57:**1707–1713.
3. **Brock, T. D.** 1987. The study of microorganisms *in situ*: progress and problems. Symp. Soc. Gen. Microbiol. **41:**1–17.
4. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.
5. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
6. **DeLong, E. F., D. G. Franks, and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:**924–934.
7. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
8. **Dennis, P. P., and H. Bremer.** 1974. Macromolecular composition during steady-state growth of *Escherichia coli* B/r. J. Bacteriol. **119:**270–281.
9. **Edmond, J. M., A. C. Campbell, M. R. Palmer, K. K. Falkner, and T. S. Bowers.** 1987. Chemistry of low temperature vent fluids from Loihi and Larson's Seamounts. EOS Trans. Am. Geophys. Union **68:**1553–1554.
10. **Erlich, H. A., D. Gelfand, and J. J. Sninsky.** 1991. Recent advances in the polymerase chain reaction. Science **252:**1643–1651.
11. **Flärdh, K., P. S. Cohen, and S. Kjelleberg.** 1992. Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. **174:**6780–6788.
12. **Fornari, D. J., M. O. Garcia, R. C. Tyce, and D. G. Gallo.** 1988. Morphology and structure of Loihi Seamount based on Seabeam sonar mapping. J. Geophys. Res. **93:**15227–15238.
13. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature (London) **356:**148–149.
14. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific oceans. Appl. Environ. Microbiol. **59:**1294–1302.
15. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345:**60–63.
16. **Grimont, F., and P. A. D. Grimont.** 1991. DNA fingerprinting, p. 249–279. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., London.
17. **Ingraham, J. L., O. Maaløe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Associates, Inc., Sunderland, Mass.
18. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater as determined by different methods of enumeration. Limnol. Oceanogr. **4:**128–139.
19. **Karl, D. M., A. M. Brittain, and B. D. Tilbrook.** 1989. Hydrothermal and microbial processes at Loihi Seamount, a mid-plate hot-spot volcano. Deep-Sea Res. **36:**1655–1673.
20. **Karl, D. M., G. M. McMurtry, A. Malahoff, and M. O. Garcia.** 1988. Loihi Seamount, Hawaii: a mid-plate volcano with a distinctive hydrothermal system. Nature (London) **335:**532–535.
21. **Kramer, J. G., and F. L. Singleton.** 1992. Variations in rRNA content of marine *Vibrio* spp. during starvation-survival and recovery. Appl. Environ. Microbiol. **58:**201–207.
22. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., London.
23. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82:**6955–6959.
24. **Liesack, W., H. Weyland, and E. Stackebrandt.** 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed culture of strict barophilic bacteria. Microb. Ecol. **21:**191–198.
25. **Morita, R. Y.** 1982. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. **6:**171–198.
26. **Morita, R. Y.** 1985. Starvation and miniaturisation of heterotrophs, with special emphasis on maintenance of the starved viable state, p. 111–130. *In* M. Fletcher and G. Floodgate (ed.), Bacteria in the natural environments: the effect of nutrient conditions. Academic Press, London.
27. **Morita, R. Y.** 1986. Starvation-survival: the normal mode of most bacteria in the ocean, p. 243–248. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
28. **Morita, R. Y.** 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. **34:**436–441.
29. **Morita, R. Y., and C. L. Moyer.** 1989. Bioavailability of energy and the starvation state, p. 75–79. *In* T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita, and A. Uchida (ed.), Proceedings of the 5th International Symposium on Microbial Ecology. Japan Scientific Societies Press, Tokyo.
30. **Moyer, C. L., C. W. Mordy, D. J. Carlson, and R. Y. Morita.** 1990. Ethidium homodimer used for the sensitive measurement of DNA and RNA of a psychrophilic marine bacterium grown at different growth rates during starvation-survival. J. Microbiol. Methods **12:**75–81.
31. **Moyer, C. L., and R. Y. Morita.** 1989. Effect of growth and starvation-survival on cellular DNA, RNA, and protein of a psychrophilic marine bacterium. Appl. Environ. Microbiol. **55:**2710–2716.
32. **Moyer, C. L., and R. Y. Morita.** 1989. Effect of growth rate and starvation-survival on the viability and stability of a psychrophilic marine bacterium. Appl. Environ. Microbiol. **55:**1122–1127.
33. **Poindexter, J. S., and E. R. Leadbetter.** 1986. Enrichment cultures in bacterial ecology, p. 229–292. *In* J. S. Poindexter and E. R. Leadbetter (ed.), Bacteria in nature, vol. 2. Plenum Press, New York.
34. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
35. **Rosset, R., J. Julien, and R. Monier.** 1966. Ribonucleic acid composition of bacteria as a function of growth rate. J. Mol. Biol. **18:**308–320.
36. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Sarkar, G., and S. S. Sommer.** 1990. Shedding light on PCR contamination. Nature (London) **343:**27.
39. **Schaechter, M., O. Maaløe, and N. O. Kjeldgaard.** 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19:**592–606.
40. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.
41. **Schmidt, T. M., B. Pace, and N. R. Pace.** 1991. Detection of DNA contamination in *Taq* polymerase. BioTechniques **11:**176–177.
42. **Sedwick, P. N., G. M. McMurtry, and J. D. MacDougall.** 1992. Chemistry of hydrothermal solutions from Pele's Vents, Loihi Seamount, Hawaii, Geochim. Cosmochim. Acta **56:**3643–3667.
43. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
44. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., London.
45. **Steffan, R. J., and R. M. Atlas.** 1991. Polymerase chain reaction: applications in environmental microbiology. Annu. Rev. Microbiol. **45:**137–161.
46. **Tipper, J. C.** 1979. Rarefaction and rarefiction—the use and abuse of a method in paleoecology. Paleobiology **5:**423–434.
47. **Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts.**

1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. **12:**219–286.

48. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:**63–65.
49. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. FEMS Microbiol. Rev. **75:**105–116.
50. **Weller, R., M. M. Bateson, B. K. Heimbuch, E. D. Kopczynski, and D. M. Ward.** 1992. Uncultivated cyanobacteria, *Chloroflexus*-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. Appl. Environ. Microbiol. **58:**3964–3969.
51. **Weller, R., J. W. Weller, and D. M. Ward.** 1991. 16S rRNA sequences of uncultivated hot spring cyanobacterial mat inhabitants retrieved as randomly primed cDNA. Appl. Environ. Microbiol. **57:**1146–1151.
52. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.