Enrichment Culture and Microscopy Conceal Diverse Thermophilic *Synechococcus* Populations in a Single Hot Spring Microbial Mat Habitat

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Recent molecular studies have shown a great disparity between naturally occurring and cultivated microorganisms. We investigated the basis for disparity by studying thermophilic unicellular cyanobacteria whose morphologic simplicity suggested that a single cosmopolitan species exists in hot spring microbial mats worldwide. We found that partial 16S rRNA sequences for all thermophilic *Synechococcus* culture collection strains from diverse habitats are identical. Through oligonucleotide probe analysis and cultivation, we provide evidence that this species is strongly selected for in laboratory culture to the exclusion of many more-predominant cyanobacterial species coexisting in the Octopus Spring mat in Yellowstone National Park. The phylogenetic diversity among Octopus Spring cyanobacteria is of similar magnitude to that exhibited by all cyanobacteria so far investigated. We obtained axenic isolates of two predominant cyanobacterial species by diluting inocula prior to enrichment. One isolate has a 16S rRNA sequence we have not yet detected by cloning. The other has a 16S rRNA sequence identical to a new cloned sequence we report herein. This is the first cultivated species whose 16S rRNA sequence has been detected in this mat system by cloning. We infer that biodiversity within this community is linked to guild structure.

A detailed knowledge of metabolically diverse cultivated bacteria dates to the development about a century ago of the enrichment culture approach by Beijerinck and Winogradsky (31). However, studies that employ genetic markers to describe native species have repeatedly demonstrated that there is little correspondence between cultivated and naturally occurring species (1, 7, 8, 12, 13, 19, 26, 32, 34). Therefore, cultivated bacteria provide a weak basis by which to understand the ecology of bacterial communities. We investigated both the basis for this discrepancy and the influence enrichment culture may have in limiting our appreciation of microbial diversity by studying the unicellular cyanobacteria that inhabit a microbial mat community in Octopus Spring, a thermal pool in the Lower Geyser Basin of Yellowstone National Park. Our study focused on a portion of the mat where the temperature range is approximately 50 to 55°C. Microscopic analyses of this region of the mat revealed the presence of cyanobacterial cells having *Synechococcus*-like (rod-shaped) morphologies. Observations such as this from Octopus Spring and many similar but geographically distinct hot springs, combined with the similar moles percent G+C ratios of thermophilic *Synechococcus* strains in culture collections (6), led to the hypothesis that a single *Synechococcus* species, *Synechococcus lividus*, inhabits Octopus Spring (3, 33) and similar springs worldwide (5). In contrast, our initial culture-independent characterization of the Octopus Spring mat revealed four different 16S rRNA sequences which could be recognized as cyanobacterial (34, 35, 37, 38) (types A, B, I, and J). None of these sequences matched that of *S. lividus*.

In a previous study, we used hybridization probes specific for each of the cloned Octopus Spring cyanobacterial 16S rRNA sequence types and for *S. lividus* to investigate the distributions

of these populations in hot spring microbial mats (25). *S. lividus* was detected only in the mat of a slightly acid spring, Clearwater Spring. In this study, we used the same probes to examine the behavior of the type A, B, J, and *S. lividus* Y-7c-s populations during enrichment culture. Information gained from the probe study led to the hypothesis that *S. lividus* was strongly selected in laboratory culture relative to more-predominant cyanobacteria, even from mats in alkaline springs, such as Octopus Spring, where the *S. lividus* population was not detected by probing. We tested this hypothesis by comparing the 16S rRNA sequences of *Synechococcus* isolates obtained from low- and high-dilution enrichment cultures.

MATERIALS AND METHODS

Hybridization probe analysis. Samples for hybridization probe analysis were collected from a 54°C site at Clearwater Spring site A (pH 6.2) on 31 July 1991 and from a 50°C site in the shoulder region of Octopus Spring on 8 August 1991 (25). A no. 15 cork borer (415.5 mm²) was used to remove the top 5 mm of mat. Triplicate cores were frozen in liquid N₂ for later extraction of RNA. Two cores for enrichment cultures were placed into a sterile polypropylene tube, and the volume was brought to 15 ml with water from the collection site. The cores were transported to the laboratory in the dark at approximately 50°C and processed the same day. Samples were homogenized in a sterile 40-ml Dounce tissue grinder (Corning). A 5-ml portion of the homogenate was used to inoculate a screw-cap culture tube containing 5 ml of medium D (5); this tube was termed the low-dilution enrichment. For some Clearwater Spring enrichments, the pH of the medium was adjusted to 6.5, the lowest pH permitting growth of *S. lividus* from this spring (17). In all other cases, the pH of the medium was 8.2, resembling the pH of Octopus Spring (pH 8.4). From each low-dilution enrichment, a 10-fold dilution series was prepared in tubes of sterile medium D. The tubes were incubated at 54°C under fluorescent lighting for 2 months. These enrichments were maintained by periodically subculturing 10% of the total volume into tubes containing 5-ml portions of medium D. For each enrichment series, rRNA was extracted from cells in both the low-dilution enrichment and the highest dilution enrichment in which growth occurred, hereafter referred to as high-dilution enrichments. To ensure sufficient biomass for RNA extraction, cells from 8 to 10 replicate subcultures were pooled and harvested by centrifugation. Procedures for RNA extraction of mat samples and enrichment culture pellets and radiolabeled oligonucleotide probe analyses were described previously (25). The probe sequences with the corresponding *Escherichia coli* 16S rRNA position numbers in parentheses were as follows: type A (1254 to 1275), 5'TCCCGCTCGCGC GTTCGCTGCC3'; type B (1254 to 1275), 5'TCAGCTCGCGCGCTCGC

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GACC3'; type J (1240 to 1281), 5'ATTATCCCTCTGTCCACGCCA3'; and *S. lividus* Y-7c-s (1273 to 1294), 5'CGTGGTTTAAAGAGATTAGTCG3'. Probe specificity was reported previously (25), but we have since discovered additional populations with which probe cross-reactivity was not empirically determined. Two of these populations, Octopus Spring isolates C9 and B10 reported herein, contain sequences which exhibit 4 to 10 single-base-pair mismatches with the probe sequences and would not cross-react with any of the probes. However, type A- and B-like populations discovered in denaturing gradient gel electrophoresis analyses have sequences at the probe site which are identical to the type A and B sequences, respectively, and thus will cross-react with the type A and B probes (11). The relative probe response is the response of a probe specific to one 16S rRNA sequence divided by the response of a universal probe designed to measure total 16S rRNA in the sample (25).

Culture isolation. Two samples for enrichment and subsequent culture isolation were collected from different 50°C sites in the shoulder region of Octopus Spring on 8 June 1992. Fifteen milliliters of spring water from the collection site was added to each sterile 50-ml polypropylene tube. The volume of each tube was then brought to 30 ml by adding viscous mat material collected, using a small weighing spatula, from the top green surface layer (ca. 1 mm) of the mat. Each sample was transported as described above, and then the entire 30 ml was homogenized in a sterile 40-ml tissue grinder. Direct counts of *Synechococcus*-like cells in the homogenized material were made with a Petroff-Hauser counting chamber according to the manufacturer's directions. Each homogenate was used to create a separate 10-fold dilution series by inoculating 5.0 ml of homogenate into 45 ml of sterile medium D contained in 250-ml DeLong shake flasks with metal closures (Bellco). The flasks were incubated at 50°C in an orbital shaker (New Brunswick) at 150 rpm under constant 3,000-lux fluorescent illumination for several months. During the incubation, sterile distilled water was periodically added to the flasks to maintain medium volume. For each dilution series, attempts were made to obtain axenic cyanobacterial cultures from the lowest and highest dilutions that exhibited cyanobacterial growth, termed the low- and high-dilution enrichments, respectively. Culture isolation attempts employed standard dilution and spread plate techniques, using medium DG (5) solidified with 0.8% Gelrite (Kelco), since it has been our experience that some isolates grow poorly or not at all on media solidified with agar. All plates were incubated at 45°C under constant fluorescent illumination and were kept in transparent plastic freezer bags to reduce the rate of drying. Isolated cyanobacterial colonies were picked with the aid of a dissecting microscope and repeatedly restreaked to plates containing fresh medium DG to obtain pure isolates.

16S rRNA characterization of cyanobacterial isolates. Culture collection *Synechococcus* strains were obtained from the American Type Culture Collection, the Paris Culture Collection, and the Castenholz Culture Collection (R. W. Castenholz, University of Oregon). Strain Y-7c-s was originally isolated in 1976 from material collected within a Clearwater Spring pH 5.5 pool in Yellowstone Park (17) and is the only *S. lividus* isolate whose 16S rRNA sequence is available (14, 35). DNA extraction, PCR amplification, and cloning and sequencing methods were described previously (18). Percent similarities were determined after aligning sequences to others in our Octopus Spring database and the Ribosomal Database Project database (22). The sequence of each Octopus Spring isolate was evaluated using Check Chimera of the Ribosomal Database Project (20). Initially all cultures were screened by obtaining partial 16S rRNA sequences through hypervariable region V9 (16) which includes the sites targeted by specific oligonucleotide probes (25). The emphasis on a hypervariable region maximized the probability of determining identity between isolates. The percent similarity values in such comparisons are lower than if the entire 16S rRNA sequences are compared. Additional sequence data were obtained for each unique *Synechococcus* isolate obtained in this study.

Characterization of 16S rRNA sequences cloned from the Octopus Spring mat. The new 16S rRNA sequence we report here was obtained from Octopus Spring library V-L by methods described previously (37).

Phylogenetic analyses. Phylogenetic analyses were performed by using the programs DNADIST, FITCH, DNAPARS, DNAML, SEQBOOT, and CONSENSE from the Phylogenetic Inference Package (PHYLIP) version 3.57c (10). Cyanobacterial phylogenetic trees were inferred by analysis of nucleotides which align with *E. coli* 16S rRNA positions 332 to 452, 480 to 507, 712 to 892, and 1140 to 1364, comparing mainly conserved and semiconserved regions. The type A cyanobacterial sequence was too short to include but is closely related to the type B sequence (see Table 1).

Nucleotide sequence accession numbers. Nucleotide sequences have been submitted to GenBank under the accession numbers: Octopus Spring type P, L35331; isolate C1, L35345, L35479, and L35480; and isolate C9, L35481, L35482, and L35483.

RESULTS

Oligonucleotide probe analyses. Figure 1 illustrates the results of the oligonucleotide probe analyses of Clearwater Spring mat and enrichments. Here, *S. lividus* was detected directly in the mat and in the low- and high-dilution enrichments at both pH 6.5 and 8.2. The same was true for type

FIG. 1. Relative responses of oligonucleotide probes targeting 16S rRNA of *S. lividus* and uncultivated cyanobacteria with RNA extracted from a homogenized mat sample (MAT) from a 54°C site in Clearwater Spring or from enrichments at pH 6.5 or 8.2 inoculated with low- or high-dilution samples of this mat.

B-like sequences. Type A-like sequences were not probed directly in the mat or in the low- or high-dilution enrichment at pH 8.2. They were detected in both the low- and high-dilution enrichments at pH 6.5.

The results of the probe analyses of Octopus Spring mat and enrichments are presented in Fig. 2. *S. lividus* was not detected in the mat but was readily detected in the low-dilution enrichment. However, *S. lividus* was not detected in the high-dilution enrichment. In contrast, two of the Octopus Spring cyanobacterial populations previously detected as clones, types A and B, were readily detected in the high-dilution enrichment. The other clone population, type J, was detected in the mat samples but not in the low- or high-dilution enrichment.

The results of the probe analyses suggested that *S. lividus* was present at low population density in the Octopus Spring mat but was particularly competitive in enrichment medium environments. It appeared as though diluting mat samples prior to inoculation might eliminate *S. lividus* from enrichments, thus permitting the growth and isolation of some of the Octopus Spring cyanobacterial populations previously detected as 16S rRNA clones.

Dilution cultures. Direct cell counts of *Synechococcus*-shaped cells in the Octopus Spring mat samples indicated a concentration of approximately 10^{10} *Synechococcus* cells per ml in the cyanobacterial layer. Similar concentrations of *Synechococcus* cells in the surface layer of this microbial mat have

FIG. 2. Relative responses of oligonucleotide probes targeting 16S rRNA of *S. lividus* and uncultivated cyanobacteria with RNA extracted from a homogenized mat sample (MAT) from a 50 to 55°C region of Octopus Spring or from enrichment cultures inoculated with low- or high-dilution samples of this mat.

been reported previously (2). For the Octopus Spring enrichment studies, this allowed us to determine the number of *Synechococcus* cells inoculated into each culture vessel. The high-dilution enrichment in the probe study received approximately 1,330 *Synechococcus* cells. The two high-dilution enrichments prepared for culture isolation attempts received approximately 25 and 250 *Synechococcus* cells and yielded cyanobacterial isolates B10 and C9, respectively.

16S rRNA characterization of Octopus Spring *Synechococcus* isolates. Table 1 summarizes the results of the culture isolation experiment. Visible cyanobacterial growth was apparent within a few days in low-dilution enrichments. These enrichments yielded axenic cyanobacterial isolates B1 and C1

which formed relatively large colonies on isolation plates within a few days. Both isolates have the same 16S rRNA sequence as *S. lividus* Y-7c-s through the V9 region. More-extensive sequence data for isolate C1 showed that it exhibits a similarity of 99.6% to strain Y-7c-s through 682 nucleotide positions including two additional variable regions, V6 and V8. In contrast, high-dilution enrichments required several weeks for visible growth to occur, eventually yielding *Synechococcus* isolates C9 and B10. These isolates formed much smaller colonies on isolation plates and were difficult to discern without the aid of a dissecting microscope. The 16S rRNA sequences of isolates C9 and B10 exhibit similarities of only 71.1 and 85.5% to that of *S. lividus* Y-7c-s, respectively. Isolate C9 has a novel 16S rRNA sequence on the basis of 1,385 nucleotides through nine variable regions, which has not been previously detected among those cloned from the mat. The sequence of isolate B10 is identical to a new cyanobacterial sequence (type P) we have recently cloned from the mat and report herein. This similarity is based on 643 nucleotide positions which include variable regions V1, V2, V3, V6, and V9. This is the first example we have observed of a cultivated Octopus Spring isolate having a 16S rRNA sequence matching that of a sequence retrieved by cloning. No indication of the formation of chimeras was found for the 16S rRNA sequences of any isolate using Check Chimera (low maximum improvement scores and lack of peakedness), but this is difficult to prove with such methods (18, 24). Furthermore, denaturing gradient gel electrophoresis analysis of genomic DNA of each isolate gave only a single band, indicating a single rRNA operon (11). The best evidence against the formation of chimeras is provided by detection of a sequence using multiple methods that recover large amounts of sequence, as in the case of the clone type P and cultivated isolate B10 sequences above. All the isolates have a common *Synechococcus*-like morphology.

16S rRNA sequences of thermophilic *Synechococcus* culture collection strains. To investigate further the tendency for enrichment culture to favor the isolation of *S. lividus*, we screened the three other thermophilic *Synechococcus* strains available in culture collections. These isolates were originally obtained by independent investigators from various Yellowstone and Oregon hot spring mats (9, 27, 29) (Table 1). All

TABLE 1. Pairwise similarities between 16S rRNA sequences of thermophilic *Synechococcus* strains and cyanobacterial sequence types^a

SSU rRNA sequence ^b	Source ^c	% Similarity with Octopus Spring mat 16S rRNA sequence:											
		1	2	3	4	5	6	7	8	9	10	11	12
1 (ATCC 27179)	Hunter Sp. Oreg.												
2 (ATCC 27180)	Fairy Sp. YNP	100											
3 (PCC 6715)	Mixed YNP Sps	100	100										
4 (isolate B1)	Octopus Sp. YNP	100	100	100									
5 (isolate C1)	Octopus Sp. YNP	100	100	100	100								
6 (<i>S. lividus</i> Y-7c-s)	Clearwater Sp. YNP	100	100	100	100	100							
7 (isolate C9)	Octopus Sp. YNP	71.8	71.8	71.8	71.8	71.8	71.1						
8 (isolate B10 [type P])	Octopus Sp. YNP	85.9	85.9	85.9	85.9	85.9	85.5	76.5					
9 (type A)	Octopus Sp. YNP	78.7	78.7	78.7	78.7	78.7	79.4	78.7	81.1				
10 (type B)	Octopus Sp. YNP	78.8	78.8	78.8	78.8	78.8	79.5	80.0	82.4	95.3			
11 (type I)	Octopus Sp. YNP	83.5	83.5	83.5	83.5	83.5	84.3	70.6	82.4	82.3	83.5		
12 (type J)	Octopus Sp. YNP	75.3	75.3	75.3	75.3	75.3	75.0	67.1	72.6	75.2	74.0	72.6	
13 (<i>Oscillatoria</i> sp. strain 7515)		84.1	84.1	84.1	84.1	84.1	84.9	76.7	78.0	78.0	78.0	75.5	70.7

^a Pairwise similarities between 16S rRNA sequences (*E. coli* nucleotide positions 1223 to 1307) of culture collection strains of thermophilic *S. lividus*, *Synechococcus* strains cultivated from the 50 to 55°C region of the Octopus Spring mat, and uncultivated cyanobacterial sequence types cloned from the mat.

^b The emphasis on a hypervariable region lowers the percent similarity from the value when sequences are compared in general. For example, the percent similarity between isolates C1 and C9 is 71.8% over 85 nucleotide positions through the V9 region but 92.6% overall (1,127 nucleotides).

^c Abbreviations: Sp, Spring; YNP, Yellowstone National Park.

FIG. 3. Distance matrix phylogenetic tree illustrating the relationships of Octopus Spring cyanobacterial 16S rRNA sequences to those of representatives of the major cyanobacterial lineages described by the Ribosomal Database Project. The tree was rooted by using the 16S rRNA sequences of *Thermotoga maritima*, *Chlorobium vibrioforme*, and *E. coli*. The consensus values at the forks indicate the number of times the group consisting of the species to the right of the fork occurred among 100 trees inferred from the bootstrapped data set of 606 nucleotides. Evolutionary distance is indicated by horizontal lines; bar corresponds to 0.01 fixed point mutation per sequence position.

have 16S rRNA sequences through the V9 region identical to that of *S. lividus* Y-7c-s.

Phylogenetic diversity among Octopus Spring cyanobacteria. Figure 3 is a least-squares distance matrix phylogenetic tree of 16S rRNA sequences of Octopus Spring cyanobacteria and representatives of the major lines of cyanobacterial descent, including those which are most deeply divergent (*Gleobacter violaceus* and *Pseudoanabaena galeata*). Consensus values derived from bootstrapped data are low for all of the branches, except those linking very closely related organisms. Octopus Spring cyanobacterial populations C9, B, and J are the most deeply branching lineages, with consensus values of 41 and 35%. This same deep branching of populations C9, B, and J was observed in trees inferred by DNA parsimony (consensus values, 45.5 and 23.9%) and DNA maximum likelihood (data not evaluated by bootstrap analysis) and when this set of sequences was tested for possible thermophilic convergence (36).

DISCUSSION

It is now clear that the diversity of *Synechococcus* populations inhabiting thermal mats must be greater than previously suspected. While it has been known for some time that members of the genus *Synechococcus* are genetically diverse and that the genus is in need of revision (6), such diversity was not suspected among thermophilic *Synechococcus* populations. On the basis of the similar moles percent G+C ratios of thermophilic *Synechococcus* isolates from different hot spring habitats, this group was thought to consist of a single species, *S. lividus* (5). Our sequencing results confirm the genetic similarity among these thermophilic *Synechococcus* culture collection

strains but at a higher level of resolution than moles percent G+C ratios; i.e., the strains have identical sequences within a 16S rRNA hypervariable region. In contrast, our molecular cloning and cultivation efforts have revealed seven cyanobacterial populations within the Octopus Spring microbial mat community. Two additional Octopus Spring cyanobacterial populations have been discovered by denaturing gradient gel electrophoresis analyses (11). Three of these populations have now been cultivated and are known to have *Synechococcus*-like morphologies. Thus, while *S. lividus* appears to be widely distributed, it exists in the Octopus Spring mat as one of at least nine cyanobacterial populations defined by our molecular and cultivation efforts. The selective enrichment of *S. lividus*, combined with the single rod-shaped cell morphology of the cyanobacteria inhabiting the mat, concealed striking genetic diversity among the Octopus Spring mat cyanobacteria, the magnitude of which is similar to that observed among all cyanobacteria. Several Octopus Spring cyanobacteria may represent the most primordial lines of cyanobacterial descent yet discovered. This possibility is supported by the results of three different phylogenetic inference methods. However, as indicated by the low bootstrap values for both parsimony and distance matrix methods, the explosive radiation in cyanobacterial evolution makes documentation of the branching orders of major lineages difficult. To answer this question more definitively, additional sequence data must be obtained (15).

Dilution of inoculum prior to enrichment permitted the isolation of two previously uncultivated *Synechococcus* populations, C9 and B10. Similar results have been reported for heterotrophic marine bacteria, where dilution of the original populations to near extinction of the ability to grow favored isolation of more abundant rather than more nutrient-tolerant

organisms (4). High-dilution populations B10 and C9 were isolated from enrichments which received approximately 25 and 250 *Synechococcus* cells, respectively. It is likely that they were among the predominant cyanobacterial populations in the original mat samples. However, it cannot be concluded that these are the only predominant *Synechococcus* populations present in the mat. It is possible that multiple populations developed in the high-dilution enrichments, but some were unable to grow on the solidified isolation medium or were missed by not sampling all the colonies that developed on isolation plates. This might explain why type A and B cyanobacterial populations were detected by probing high-dilution enrichments but were not recovered as isolates on plates. Alternatively, if multiple populations of approximately equivalent densities exist in initial mat samples, different populations might predominate in replicate dilutions. Another possibility is that some *Synechococcus* populations are simply unable to grow or compete effectively in the liquid enrichment medium. For example, the type J population was detected in the Octopus Spring mat sample but was not detected in any enrichment.

The recovery of strain C9, whose unique molecular signature had not been previously detected in the mat, raises interesting questions about the correspondence, or lack thereof, between molecular and culture approaches. It must be emphasized that because this approach is labor-intensive, so far only a few samples have been studied by cloning and sequencing methods and these might not have contained the C9 population at high density. Alternatively, molecular methods might select against the C9 sequence (e.g., priming and/or cloning biases). In ongoing studies, we have found that the C9 sequence behaves normally when using other molecular procedures involving the same priming sites used in cloning work. We hope that these studies, involving denaturing gradient gel electrophoresis (11), will enable more rapid profiling of populations within larger numbers of samples so that the question of changing spatial and temporal distributions among populations can be more adequately addressed.

Because cyanobacteria are functionally related, it is appropriate to consider that the many Octopus Spring cyanobacterial populations constitute at least part of a guild of oxygenic phototrophs within the mat community. The existence of multiple 16S rRNA types within other groups of Octopus Spring mat inhabitants which may be both phenotypically and phylogenetically related (32) suggests that this may be a general pattern of community organization. Previous hybridization probe studies indicate that the different cyanobacterial populations have adapted to different environmental parameters such as temperature, light, and pH (25). Temperature-adapted *Synechococcus* isolates, considered at the time to be strains of *S. lividus* (5), have been reported elsewhere (23). We hypothesize that as in plant and animal communities (21, 28, 30), intraguild biodiversity may help stabilize guild function. In the mat habitat where parameters such as temperature, light, and pH vary daily and seasonally, the existence of diverse, specialized populations within a guild would provide a level of flexibility beyond physiological change within individual species to buffer guild function (e.g., oxygenic photosynthesis) against environmental variation. Clusters of phylogenetically related populations are commonly observed in 16S rRNA analyses of natural communities (1, 7, 8, 12, 13, 19, 26, 32, 34). These clusters might also be explained as intraguild specialization to local environmental parameters, if these populations conduct similar functions.

Microbiologists have been aware of the selectivity of culture media since the inception of the enrichment culture technique (31). Molecular techniques now allow us to detect many of the

microorganisms we have been missing by cultivation and, to some extent, to determine their abundance and phenotypes. This permits more-informed attempts at cultivating relevant species.

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REFERENCES

- Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring. *Proc. Natl. Acad. Sci. USA* **91**:1609–1613.
- Bauld, J., and T. D. Brock. 1974. Algal excretion and bacterial assimilation in hot spring algal mats. *J. Phycol.* **10**:101–106.
- Brock, T. D. 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, Berlin.
- Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Robertson. 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Appl. Environ. Microbiol.* **59**:881–891.
- Castenholz, R. W. 1981. Isolation and cultivation of thermophilic cyanobacteria, p. 236–246. In M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes: a handbook on habitats, isolation, and identification of bacteria*. Springer-Verlag, Berlin.
- Castenholz, R. W., and J. B. Waterbury. 1989. Oxygenic photosynthetic bacteria, p. 1710–1806. In J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 3. Williams & Wilkins, Baltimore.
- Choi, B. K., B. J. Paster, F. E. Dewhirst, and U. B. Gobel. 1994. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. *Infect. Immun.* **62**:1889–1895.
- DeLong, E. F. 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA* **89**:5685–5689.
- Dyer, D. L., and R. D. Gafford. 1961. Some characteristics of a thermophilic blue-green alga. *Science* **134**:616–617.
- Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164–166.
- Ferris, M. J., G. Muyzer, and D. M. Ward. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* **62**:340–346.
- 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.
- 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.
- Giovannoni, S. J., S. Turner, G. J. Olsen, D. J. Lane, and N. R. Pace. 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. *J. Bacteriol.* **170**:3584–3592.
- Gouy, M., and W. Li. 1989. Phylogenetic analysis based on rRNA sequences supports the archaeobacterial rather than the eocyte tree. *Nature (London)* **339**:145–147.
- Gray, M. W., D. Sankoff, and R. J. Cedergren. 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit RNA. *Nucleic Acids Res.* **12**:5837–5852.
- Kallas, T., and R. W. Castenholz. 1982. Initial pH and ATP-ADP pools in the cyanobacterium *Synechococcus* sp. during exposure to growth-inhibiting low pH. *J. Bacteriol.* **149**:229–236.
- Kopczynski, E. D., M. M. Bateson, and D. M. Ward. 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Appl. Environ. Microbiol.* **60**:746–748.
- Liesack, W., and E. Stackebrandt. 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J. Bacteriol.* **174**:5072–5078.
- Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese. 1994. The Ribosomal Database Project. *Nucleic Acids Res.* **22**:3485–3487.
- Naeem, S., L. J. Thompson, S. P. Lawler, J. H. Lawton, and R. M. Woodfin. 1994. Declining biodiversity can alter the performance of ecosystems. *Nature (London)* **368**:734–737.
- Olsen, G. J., R. R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W. M. Kaun, T. J. Macke, Y. Xing, and C. R. Woese. 1992. The Ribosomal Database Project. *Nucleic Acids Res.* **20**:2199–2200.
- Peary, J. A., and R. W. Castenholz. 1964. Temperature strains of a thermophilic blue-green alga. *Nature (London)* **220**:720–721.

24. Robinson-Cox, J. F., M. M. Bateson, and D. M. Ward. 1995. Evaluation of nearest-neighbor methods for detection of chimeric small-subunit rRNA sequences. *Appl. Environ. Microbiol.* **61**:1240–1245.
25. Ruff-Roberts, A. L., G. J. Kuenen, and D. M. Ward. 1994. Distribution of cultivated and uncultivated cyanobacteria and *Chloroflexus*-like bacteria in hot spring microbial mats. *Appl. Environ. Microbiol.* **60**:697–704.
26. Schmidt, T. M., E. F. DeLong, and N. R. Pace. 1991. Analysis of marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* **173**:4371–4378.
27. Schrader, M., G. Drews, and J. Weckesser. 1981. Chemical analysis on cell wall constituents of the thermophilic cyanobacterium *Synechococcus* PCC 6716. *FEMS Microbiol. Lett.* **11**:37–40.
28. Schulze, E., and H. A. Mooney. 1993. Biodiversity and ecosystem function. Springer-Verlag, Heidelberg, Germany.
29. Sheridan, R. P., and R. W. Castenholz. 1968. Production of hydrogen sulfide by a thermophilic blue-green alga. *Nature (London)* **217**:1063.
30. Tilman, D., and J. A. Downing. 1994. Biodiversity and stability in grasslands. *Nature (London)* **367**:363–365.
31. 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.
32. Ward, D. M., M. J. Ferris, S. C. Nold, M. M. Bateson, E. D. Kocczynski, and A. L. Ruff-Roberts. 1994. Species diversity in hot spring microbial mats as revealed by both molecular and enrichment culture approaches—relationship between biodiversity and community structure, p. 33–44. In L. J. Stal and P. Caumette (ed.), *Microbial mats: structure, development and environmental significance*. Springer-Verlag, Heidelberg, Germany.
33. Ward, D. M., T. A. Tayne, K. L. Anderson, and M. M. Bateson. 1987. Community structure and interactions among community members in hot spring cyanobacterial mats. *Symp. Soc. Gen. Microbiol.* **41**:179–210.
34. 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.
35. 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.
36. Weisburg, W. G., S. J. Giovannoni, and C. R. Woese. 1989. The *Deinococcus-Thermus* phylum and the effect of rRNA composition on phylogenetic tree construction. *Syst. Appl. Microbiol.* **11**:128–134.
37. Weller, R., M. M. Bateson, B. K. Heimbuch, E. D. Kocczynski, 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.
38. 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.