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Geographical isolation in hot spring cyanobacteria

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Summary

It has been proposed that free-living microorganisms exhibit ubiquitous dispersal, do not form geographically isolated populations and rarely (if ever) speciate via allopatry. We studied island-like hot spring cyanobacterial communities in which geographical isolation should be prominent and detectable if it influences the evolution of bacteria. The genetic diversity of cyanobacteria indigenous to North American, Japanese, New Zealand and Italian springs was surveyed by (i) amplification and cloning of 16S rRNA and 16S–23S internal transcribed spacer regions; (ii) lineage-specific oligonucleotide probing (used to verify the predominance of cloned sequences); and (iii) lineage-specific polymerase chain reaction (PCR) (used to search for possible rare genotypes). Phylogenetic and distribution patterns were found to be consistent with the occurrence of geographical isolation at both global and local spatial scales, although different cyanobacterial lineages were found to vary in their distribution. A lack of correspondence between biological patterning and the chemical character of springs sampled suggested that the geographical distribution of thermophilic cyanobacteria cannot be explained by the 20 potential niche-determining chemical parameters that we assayed. Thus, geographical isolation (i.e. genetic drift) must in part be responsible for driving the observed evolutionary divergences. Geographical isolation may be an important underestimated aspect of microbial evolution.

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Introduction

It has been commonly assumed since early in the twentieth century that, in the case of microorganisms, 'everything is everywhere and nature selects' (Beijerinck, 1913; Baas-Becking, 1934). This suggests that microorganisms disperse readily and do not become geographically isolated or undergo allopatric speciation. The apparent absence of allopatric speciation in the microbial world has been exploited to explain why there appear to be fewer microbial species than expected from correlations between body size and number of species (Fenchel, 1993; Finlay *et al*., 1996; Finlay and Clark, 1999; Godfray and Lawton, 2001; Finlay, 2002). Support for the ubiquitous dispersal of free-living microorganisms has mainly come from observations of protist diversity in benthic and planktonic environments that suggest a widespread distribution of those morphospecies (Finlay and Clark, 1999; Patterson and Lee, 2000; Finlay, 2002). A recent molecular study of protist diversity in polar oceans demonstrated that identical 18S rRNA genotypes were present at both poles (Darling *et al*., 2000), further supporting the idea of ubiquitous dispersal and the rarity of allopatry in microbial evolution. It was noted, however, that some closely related genetic variants did exhibit unipolar distribution, and concern was raised that higher resolution genetic markers might be needed to discern geographical patterning (Norris and de Vargas, 2000). Studies of bacterial diversity and distribution in marine (Staley and Gosink, 1999; Massana *et al*., 2000) and near-marine (Garcia-Pichel *et al*., 1996) environments also suggest similar mixed patterns (i.e. the presence of identical as well as slightly different 16S rRNA variants in geographically separate sites). Studies of bacterial diversity in globally separate soil environments have revealed evidence of unique geographical distributions, but only when methods offering more genetic resolution than 16S rRNA sequence variation were used (Fulthorpe *et al*., 1998; Cho and Tiedje, 2000). In the face of conflicting reports, it seemed informative to examine environments in which geographical isolation is a prominent feature and thus likely to contribute to diversification. As pointed out by MacArthur and Wilson (1967) '. . . in the science of biogeography, the island is the first unit that the mind can pick out and begin to comprehend'.

Hot springs are well-isolated habitats occurring as clusters in globally distant regions, and the microorganisms that inhabit them are extremophiles adapted to conditions quite different from the ambient milieu (e.g. air, water) through which they would have to disperse. As such, one

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would expect that geographical isolation might be an important component in the diversification of hot spring microorganisms. Castenholz (1978; 1996) observed anomalous distributions of cyanobacterial morphotypes inhabiting mats in hot springs around the world. In well-studied North American hot springs, such mats are formed by rod-shaped unicellular cyanobacteria of the genus *Synechococcus* with an upper temperature limit of 72°C. Ecologically similar strains are apparently absent from cyanobacterial mats in Japanese, New Zealand, Italian and African hot springs, where *Synechococcus* is reported to occur below ≈63°C, the upper temperature limit for cyanobacterial mat development. *Synechococcus* was not observed at all in hot springs in Iceland, Alaska and the Azores, even though a pure culture of *Synechococcus* would grow in water from Icelandic hot springs. (R. W. Castenholz, personal communication).

Molecular analysis of a single Yellowstone hot spring mat has revealed great diversity within the thermophilic *Synechococcus* morphotype (Ward *et al*., 1990; 1998). Three unrelated phylogenetic lineages (separated by >10% 16S rRNA sequence variation) containing organisms of this morphotype, termed A/B, C1 and C9, have been detected (Fig. 1). The dominant *Synechococcus* in Yellowstone hot springs detected by direct molecular analysis are A/B genotypes (Ruff-Roberts *et al*., 1994). On the basis of distribution (Ferris and Ward, 1997; Ward *et al*., 1998) and pure culture studies (Miller and Castenholz, 2000), the A/B lineage appears to have diverged into high and low temperature-adapted A-like and B-like clades respectively (Fig. 1). Furthermore, different genotypes occur at different depths in the mat (Ramsing *et al*., 2000), leading us to suggest that the pattern of diversity in this lineage resulted from an adaptive radiation (Ward *et al*., 1998). *Synechococcus* spp. C1 and C9 genotypes were also detected in the same Yellowstone springs through cultivation and were less abundant and diverse.

The island-like nature of hot springs, the anomalous distributions (as viewed within the microbiological paradigm 'everything is everywhere') of hot spring *Synechococcus* morphotypes, the enormous genetic diversity masked by the simple unicellular morphology and the hypothesized adaptive radiation of the A/B genotypes (a process usually requiring geographical isolation) collectively suggested that a comprehensive direct molecular analysis was needed to understand the biogeography and thus the evolutionary history of these organisms. Hence, we sought hot spring cyanobacterial mats thought or known to contain *Synechococcus* in North America, Japan, New Zealand and Italy. In each country, we sampled a large number microbial mats from springs varying widely in geographical location and physical/chemical properties in order to obtain a robust sampling of the diversity present within the region and subregions (*Supplementary material*, Table S1). We simultaneously measured 20 chemical parameters (*Supplementary material*, Table S2) to determine possible abiotic effects on distribution of genotypes, an alternative that has not been rigorously addressed in previous microbial biogeography studies. We used polymerase chain reaction (PCR) with general primers to amplify 16S rRNA genes of members of the domain Bacteria and cloning in order initially to investigate the genotypes present in each of the communities. By using one primer targeting a site in the 23S rRNA gene, we simultaneously amplified the adjacent internal transcribed spacer (ITS) region, often used in phylogenetic studies (Suzuki *et al*., 2001; Rocap *et al*., 2002) to examine genetic variation at higher resolution. We quantified the importance of cloned genotypes through specific probing of 16S rRNAs of the lineages detected. Because general primers used in molecular cloning could cause a bias towards dominant genotypes, we also developed a lineage-specific PCR approach to amplify the 16S rRNA genes from rare genotypes at high sensitivity.

Results

Geographical patterning of diversity

From North America, Japan and New Zealand, we obtained clones from 17 to 37 samples collected from nine to 14 hot springs located in three to six distinct subregions (*Supplementary material*, Table S1). In Italy, sampling was less rigorous because of the difficulty of locating hot spring cyanobacterial mats; no mats with *Synechococcus* were observed. From the sample collection, ≈ 6000 partial 16S rDNA and ITS sequences (450 bp at each locus) were examined. Because artifacts are known to occur during PCR cloning and sequencing (Qiu *et al*., 2001; Speksnijder *et al*., 2001) we report only those sequences that were found in replicate or in more than one mat sample. This approach underestimates the discovered clone sequence diversity (and endemism), but gives us confidence that the sequences we report are real (see *Experimental procedures*). Most of the clones exhibited close phylogenetic relatedness (>96%) to 16S rDNA sequences of cultivated *Synechococcus* isolates (Fig. 1) representative of the three identified thermophilic *Synechococcus* lineages (A/B, C1 and C9). Some of the clones from New Zealand and Japan were closely associated phylogenetically (>98%) with *Oscillatoria amphigranulata*, a filamentous hot spring cyanobacterium isolated from New Zealand (Garcia-Pichel and Castenholz, 1990). Only a few clones (e.g. NZCy04, 05) are novel in the sense that they are phylogenetically unrelated to any known isolates but form clades within the cyanobacterial kingdom.

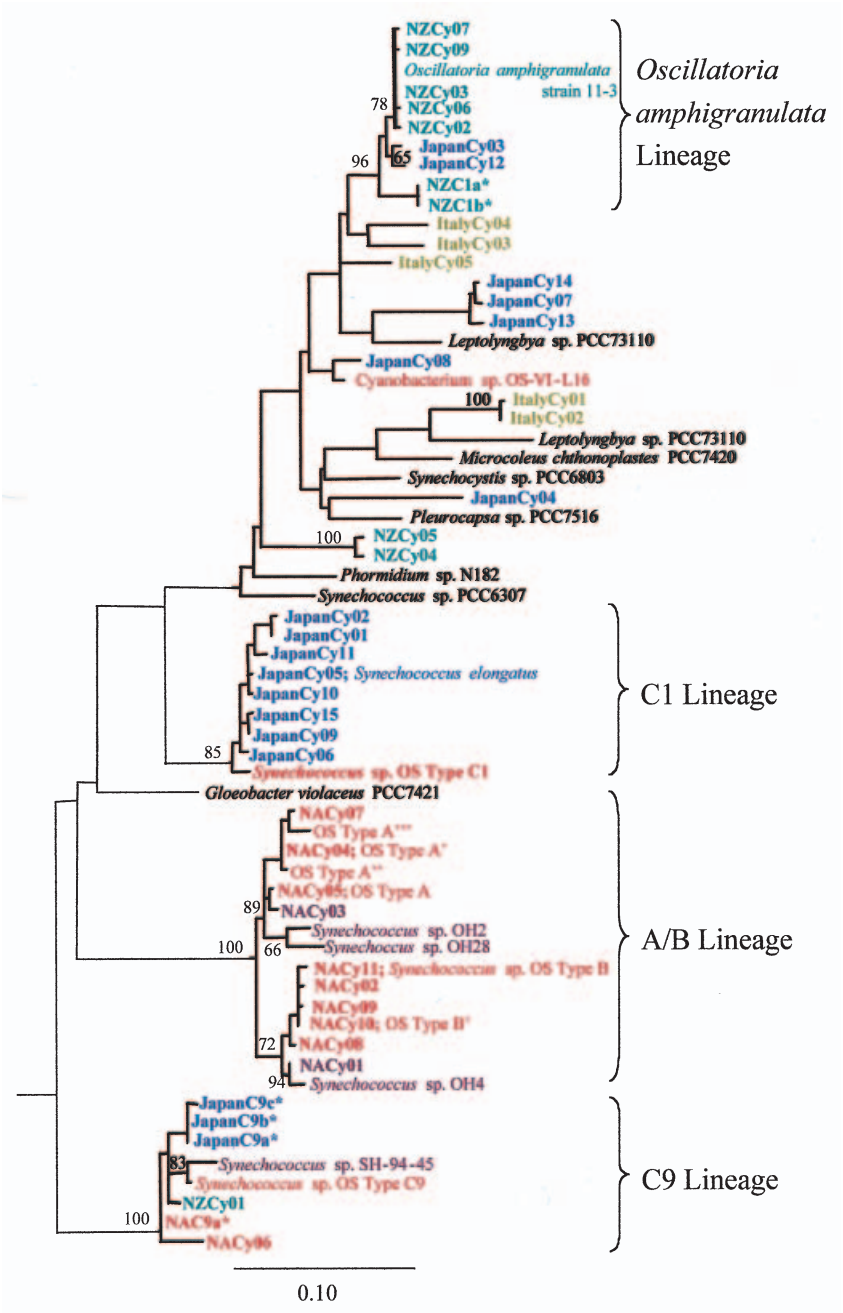

Fig. 1. 16S rRNA gene tree demonstrating the relationships of clones retrieved from all countries to other cyanobacterial 16S rRNA sequences including hot spring *Synechococcus* spp. and *Oscillatoria amphigranulata* isolates. The tree was rooted with *E. coli* and *Bacillus subtilis* 16S rRNA sequences. Values at nodes indicate bootstrap percentages for 1000 replicates. Values less than 50% are not reported. Scale bar indicates 0.10 substitutions per site. *Genotypes recovered exclusively from lineage-specific PCR study. Colour highlighting: green, New Zealand; blue, Japan; yellow, Italy; red, Greater Yellowstone Ecosystem; purple, Oregon.

Our clone survey revealed evidence for the restricted distribution of cyanobacterial genotypes to specific geographical locations both among and within countries, as emphasized by unique colour coding in Fig. 1. Members of the A/B *Synechococcus* clade were detected only in North America. A/B 16S rRNA genotypes found in Oregon hot springs are different from those found in Yellowstone and Montana hot springs. A clade separating Oregon from Yellowstone/Montana B-like genotypes is supported by a bootstrap value of 94%. Two of three Oregon A-like sequences also form a clade separate (66% bootstrap support) from all Yellowstone/Montana A-like sequences. Some variation within the A/B lineage may also reflect unique geographical distribution patterns within the Greater Yellowstone Ecosystem (GYE; e.g. clone NACy08 was detected only in Bozeman Hot Springs, located ≈ 200 km north of Yellowstone). ITS analysis provided further evidence of localized geographical patterning. Figure 2A shows a phylogenetic tree exhibiting nine ITS variants found within one B-like 16S rRNA genotype (NACy10). A main feature of the tree is a clade, supported by a 97% bootstrap value,

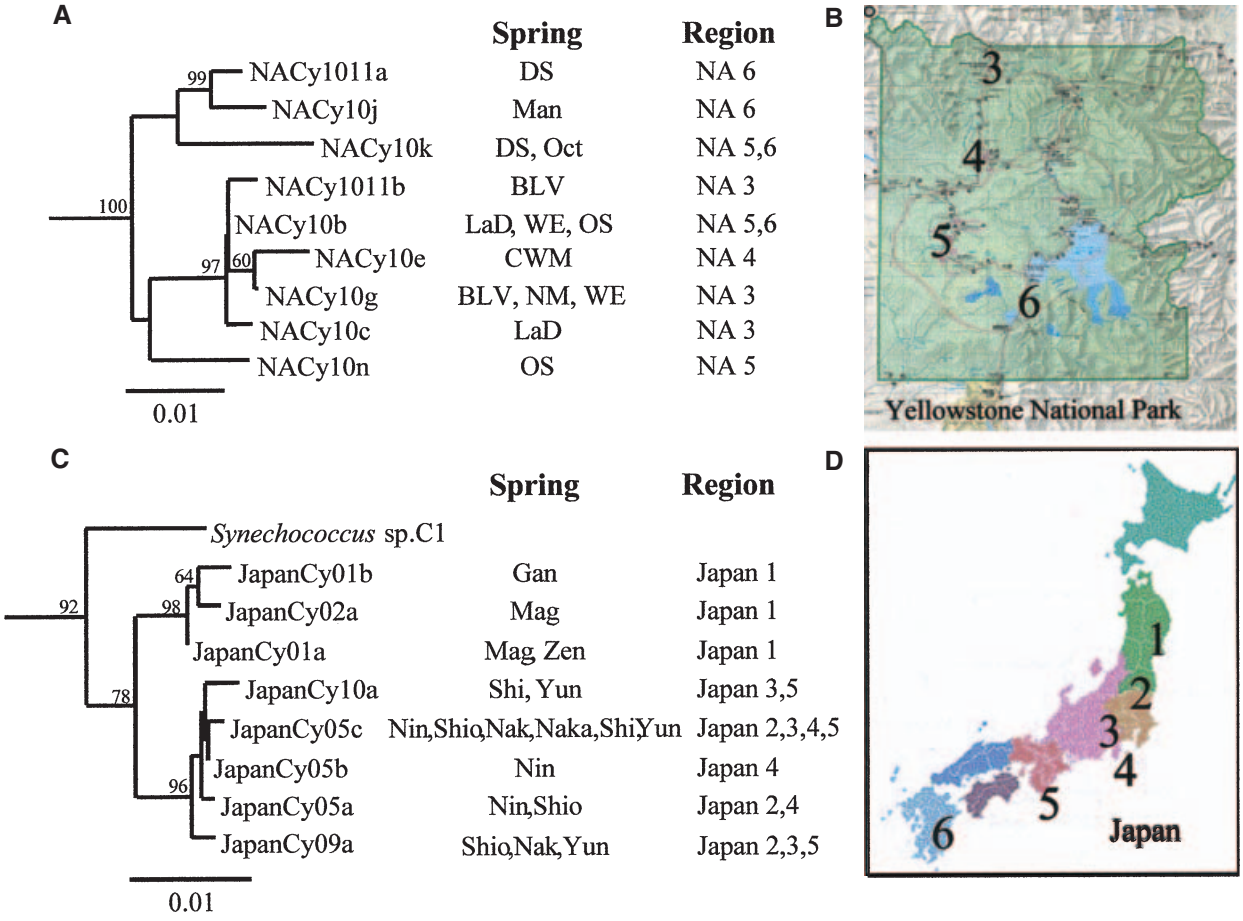

Fig. 2. Phylogenies for ITS variants detected in Yellowstone or Japan relative to springs and subregions from which they were retrieved.
A. ITS genotypes with an identical 16S rRNA genotype [NACy10 corresponding to the type B′ 16S rRNA sequence from previous work (Ward *et al.*, 1998)].
B. Map of Yellowstone National Park (http://www.yellowstone_natl_park.com/ywstone.htm) indicating regions sampled.
C. Concatenated 16S rRNA and ITS sequence data for members of the C1 lineage.
D. Map of Japan (http://jin.jcic.or.jp/region/index.html) indicating regions sampled. Values at nodes indicate bootstrap percentages for 1000 replicates. Values less than 50% are not reported. Scale bar indicates 0.01 substitutions per site. Abbreviated spring names defined in *Supplementary material*, Table S1. Differences between Figs 1 and 2C reflect insufficient replication at the ITS locus for certain 16S rRNA genotypes.

composed of five ITS variants retrieved exclusively from springs in the western regions (regions 3–5) (and almost exclusively from the most northerly regions) of Yellowstone. All sequence variants outside this lineage were retrieved from more southerly or south-easterly springs in the Lower Geyser Basin and West Thumb area (regions 5 and 6). A second clade, supported by a 99% bootstrap value, contains two of these genotypes, which were obtained only from the most south-easterly sites (region 6).

From Japanese springs, the only *Synechococcus* 16S rRNA genotypes recovered were members of the C1 clade, originally defined by isolates from Yellowstone and Oregon hot springs (Ferris *et al.*, 1996a) (Fig. 1). Eight distinct Japanese clone sequences formed a clade that included one genotype that is identical to a Japanese thermophilic *Synechococcus elongatus* isolate. The clade is a sister group of the sequence from North American isolates, as suggested by a bootstrap value of 78% for the Japanese clade based on analysis of combined 16S rRNA and ITS sequence data (Fig. 2C). ITS analysis also demonstrated the existence of clades supported by 96–98% bootstrap values separating variants recovered only from springs in the most northerly (region 1) or more southerly (regions 2–5) regions.

From New Zealand, we only cloned a single *Synechococcus* genotype that is closely related (96.1–98.1% similar) to Yellowstone and Oregon C9-like *Synechococcus* isolates (Fig. 1). A new C9-like genotype (NACy06) was also detected in North America. Evidence of geographical

Table 1. Relative abundance of each 16S rRNA lineage in mats in each country containing *Synechococcus*.

	% ± SE of cyanobacterial 16S rRNA in:			
Lineage	North America	Japan	New Zealand	Italy
A/B	77.8 ± 3.87	ND	ND	ND
C1	0.60 ± 0.36	87.5 ± 6.46	ND	ND
C9	4.4 ± 1.2	0.46 ± 0.13	14.0 ± 3.03	ND

SE, standard error; ND, not detected.

patterning of diversity was also found among representatives of the *O. amphigranulata* clade. Five distinct New Zealand clones formed a clade together with an isolate of this species, whereas two Japanese clones formed a separate clade (supported by bootstrap values of 65–78%; Fig. 1).

All the Italian samples contained cyanobacterial clones with sequences that were phylogenetically distinct from those of any cultivated isolates or clones, further indicating that different genotypes were found in different geographical sites.

Lineage-specific 16S rRNA probing

Because PCR and cloning might bias against some cyanobacterial 16S rRNA sequences (Reysenbach *et al*., 1992), we developed specific 16S rRNA oligonucleotide probes for the three known thermophilic *Synechococcus* lineages in order to test the significance of cloned genotypes and to seek autoecological evidence of the distribution of the members of each lineage. We probed samples from all 42 hot springs containing *Synechococcus* cells (*Supplementary material*, Table S1). Members of the A/B lineage were detected only in North America, where they accounted for most of the cyanobacterial 16S rRNA (Table 1). Members of the C1 lineage accounted for most of the cyanobacterial 16S rRNA in Japanese samples and were detected in one North American spring at 10.3% of cyanobacterial 16S rRNA, consistent with previous results (Ruff-Roberts *et al*., 1994). Members of the C9 lineage were the only *Synechococcus* type in New Zealand, constituting a small fraction of the cyanobacterial 16S rRNA, as filamentous cyanobacteria dominated these hot springs. Members of the C9 lineage were also detected in several North American and Japanese springs in low abundance compared with the total cyanobacterial 16S rRNA.

We also developed a lineage-specific PCR (LS-PCR) approach (Fig. 3A) to confirm and extend probing results, in particular by increasing the sensitivity of detection. We analysed a subset of probed samples to evaluate further the presence of members of the A/B lineage in Japan and New Zealand. Again, the A/B lineage was not detected outside North America (Fig. 3B), even though we were able to detect type B 16S rDNA at a sensitivity of the order of 4–100 genomes (Fig. 3C; see *Experimental procedures*).

We analysed further the PCR products obtained from samples that were positive in lineage-specific PCR for members of the C1 and C9 lineages by cloning and sequencing in order to detect additional diversity if present. We detected the previously observed C9-like genotype NZCy01 in several New Zealand springs and also recovered one new C9-like genotype from North America and three new C9-like genotypes from Japan, the latter forming a separate clade (sequences marked with an asterisk in Fig. 1 and *Supplementary material*, Table S1). We detected the previously observed C1 genotype in several North American springs, but no new C1-like genotypes were recovered from New Zealand, and no additional C1 diversity was discovered in North America.

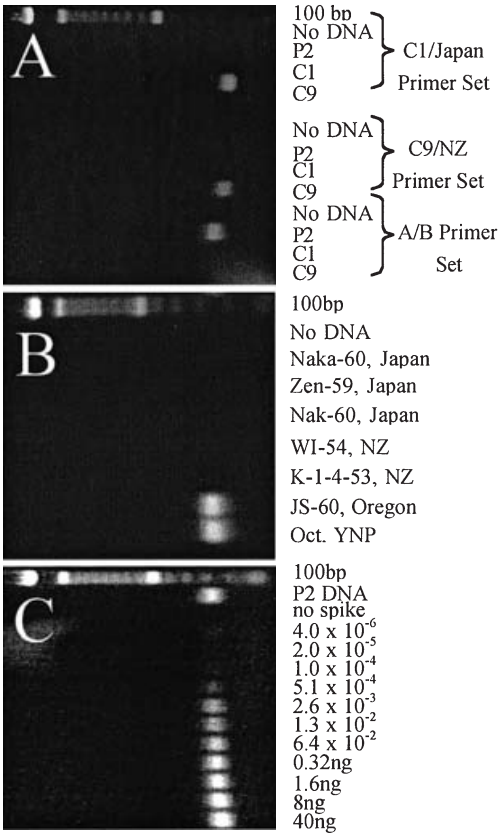

Fig. 3. Lineage-specific PCR of *Synechococcus* in different geographical regions.
A. Specificity of PCRs for A/B (strain P2), C9 and C1 *Synechococcus* lineages, and reactivity of A/B lineage PCR with (B) samples from all countries and (C) a Japanese sample containing various amounts of added DNA from a type B *Synechococcus* isolate. Abbreviated spring names defined in *Supplementary material*, Table S1. Direction of electrophoresis is left to right.

Geochemical patterns

To assay the general chemical character of the sampled springs, we measured 20 chemical parameters, including several that are known to affect cyanobacterial distribution within geographical regions (Castenholz, 1976; Brock, 1978; Ward *and* Castenholz, 2000). The North American, Japanese and New Zealand collections were from springs exhibiting a broad array of temperature, pH and sulphide concentrations (46–66°C, pH 5.2–9.1, 0–48.6 μM sulphide; *Supplementary material*, Table S1). Figure 4 shows the results of cluster analysis based on chemical parameters (*Supplementary material*, Table S2) with springs from different countries highlighted in different colours. Although there is evidence of small-scale clustering of springs within the same geographical region, clearly, the springs do not all group according to geography. In each country, there are at least two chemically distinct groups of springs. For instance, two large-scale chemical clusters of Yellowstone springs reflect a major difference between calcium magnesium carbonate (BLV, NM,WE, LaD) and alkaline siliceous (HP, ClwE, ClwN, ClwS, DS, Man, Oct and TBV) water chemistries that distinguish the carbonate-depositing Mammoth hot springs from the siliceous sinter-depositing pools of Yellowstone's Upper, Midway, Lower and West Thumb area geyser basins. In New Zealand, there are two widely separated clusters (one includes springs OK-MA, OK-HC, K, WI, WHN, OHS-1; the other includes springs T1, 2, 4 and 5); one spring (OHS-2) appears to have unique chemistry. In Japan, there is a small cluster of four springs (Tsu, Naka, Shi, Zen), but otherwise no other clusters, indicating that a diversity of chemistries occurs among the remaining eight springs (Miz, Geto, Nak, Tan, Yun, Gan, Tou and Mag).

As shown in Fig. 4, no strong association between distribution of genotypes and the chemical character of hot springs was observed. Members of all lineages are found in chemically distinct hot springs both within and among countries. Specifically, members of the A/B lineage are found in both major North American chemical groups,

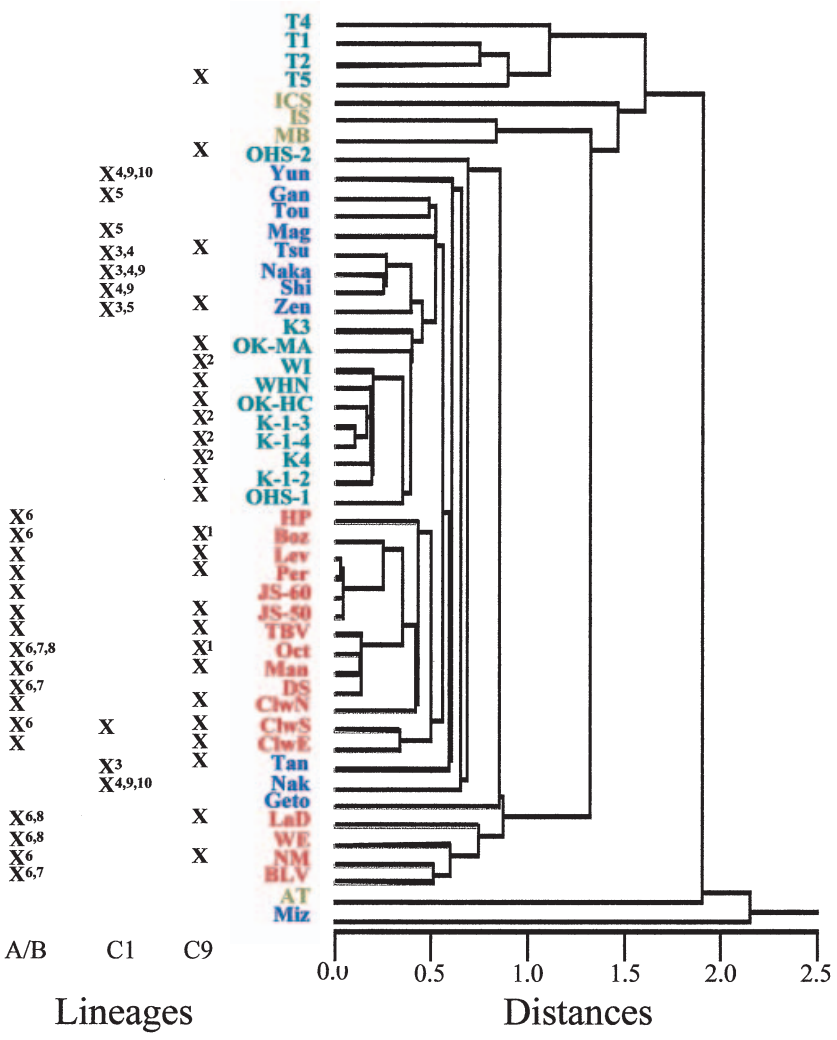

Fig. 4. Hierarchical cluster analysis of hot spring chemical parameters compared with 16S rRNA lineages and specific genotypes found in each hot spring. Hot springs are colour coded by country: green, New Zealand; blue, Japan; yellow, Italy; and red, North America. An X indicates that a 16S rRNA genotype from the A/B, C9 or C1 lineage was detected by either cloning or probing. Xs with numbers identify springs in which identical genotypes from that lineage have been recovered by cloning 16S rRNA genotypes: 1, NACy06; 2, NZCy01; 3, JapanCy05; 4, JapanCy09; 5, JapanCy01; 6, NACy10; 7, NACy11; and ITS genotypes: 8, NACy10a; 9, JapanCy05c; 10, JapanCy09a. JS, Jack Stream; Per, Perpetual; Lev, Levee; Boz, Bozman; LaD, LaDuke; NM, New Mound; BLV, Bath Lake Vista; WE, White Elephant; ClwE, Clearwater East; ClwS, Clearwater South; ClwN, Clearwater North; Oct, Octopus; TBV, Twin Butte Vista; Mush, Mushroom; Man, Mantrap; DS, Double Spring; HP, Heart Pool; Zen, Zenikawa; Mag, Magaroku; Gan, Ganiba; Miz, Mizusawa; Naka, Nakafusa; Shi, Shinhokata; Nak, Nakanoyu; Yun, Yunomine; Tou, Tousenji; Tsu, Tsuetate; K, Kuirau Reserve; OHS, Ohinemutu; WHN, Whakarewatewa-Ngararatuatara; WI, Wiamongu-Iodine; OK-HC, Orakei Korako-Hochstetter Cauldron; OK-MA, Orakei Korako-Map of Africa; T, Tokaanu Thermal Reserve; MB, Montegrotto Therme Buena Vista Hotel; ICS, Island of Ischia–Cave Scurra; IS, Island of Ischia–Sorgenta; AT, Agnano Terme.

members of the C1 lineage are found in chemically dissimilar springs in Japan and North America, and members of the C9 lineage are found in springs of diverse chemistries in all countries. This is even true for some identical 16S rRNA and ITS genotypes (numbered in Fig. 4). Conversely, geographically separated springs that are nearly identical in chemistry (e.g. Oct, Man, DS) do not always contain the same genotypes (Fig. 2A).

Discussion

We observed clear patterns of phylogeny and distribution consistent with geographical isolation. Among countries, different distribution patterns were observed for each of the *Synechococcus* and *O. amphigranulata* 16S rRNA lineages. A/B type *Synechococcus* were detected only in North America where they dominate, even though extremely sensitive lineage-specific PCR could detect numerically rare members of this lineage if present elsewhere (Fig. 3). C1-type *Synechococcus* were dominant in Japan and were found at low abundance in some North American springs, but were not detected in New Zealand. *O. amphigranulata*-like genotypes were also found in just two countries, New Zealand and Japan. The C9 lineage was the dominant *Synechococcus* lineage detected in New Zealand and was the only lineage detected in all three countries. If dispersal and invasion involving members of such lineages were frequent, as predicted by the 'everything is everywhere' hypothesis, we would expect that the specific genotypes detected at different geographical sites would be identical, especially at such a highly conserved genetic locus as the 16S rRNA gene. Yet, we found no evidence for this, as all genotypes within a lineage were different in different countries. In many cases, separate clades were observed for genotypes detected in different countries. There was even evidence to suggest that genotypes within lineages differed within geographical subregions in Japan and North America (Fig. 2). The distribution of biological variation relative to hot spring chemical variation strongly suggests that geographical isolation must be involved in the observed biological patterning. If 'everything is everywhere and nature selects', chemically different springs within and between countries should have genotypically different organisms, but that was not observed. The distribution of lineages relative to chemical differences is, in fact, what would be expected for geographically isolated populations. First, members of lineages tolerate the variety of chemistries encountered in the places to which they have dispersed (Fig. 4). Secondly, different genotypic clades (e.g. Fig. 2A) were observed for springs of similar chemistry (Fig. 4) that occur in different regions. Thirdly, widely varying chemical environments do not a priori restrict *Synechococcus* colonization, as exemplified by lineage C9. Collectively, the data strongly suggest that geographical isolation is involved in the divergence of hot spring cyanobacteria.

In the case of the North American A/B lineage, we have clear evidence of an evolutionary radiation that can be explained by a combination of adaptation and geographical isolation. Although the effect of geographical isolation must be on contemporary evolving populations, geographical isolation appears to have left a record of its historical importance throughout the evolution of the A/B lineage (Fig. 1). As far as can be determined, all lineage members are endemic to North American springs, suggesting that the A/B lineage common ancestor exhibited a more restricted distribution than is found in other lineages. More recent geographical effects are suggested by separate 16S rRNA-defined clades within the A/B lineage, the members of which are endemic to specific regions (e.g. Oregon versus GYE), and ITS-defined clades within a single 16S rRNA-defined genotype, the members of which are endemic to very localized subregions (e.g. within GYE; Fig. 2A and B). The amount of genetic difference associated with each geographical isolation event is presumably a function of the evolutionary divergence (i.e. time) since the event occurred. Evolutionary radiations have apparently also occurred in other *Synechococcus* lineages as well as the *O. amphigranulata* lineage (Fig. 1), but we have little evidence at present for the roles that adaptation and/or geographical isolation may have played. In order to unravel the ordering of geographical and adaptive events in the evolutionary history of a lineage, it may be necessary to use very high-resolution genetic markers (e.g. protein-encoding genes) as our current observations are based on relatively small differences at relatively conserved loci.

It is interesting to note differences in the patterning of diversity at different geographical sites. For instance, eight distinct C1-like variants were detected in Japan, whereas only a single C1-like variant was detected in North America. Similarly, the C9 lineage shows restricted diversity relative to *O. amphigranulata* in New Zealand and to type A/B *Synechococcus* in North America. We hypothesize that such patterning indicates either insufficient time to diverge at the sequenced loci or that evolutionary radiation is restricted by the presence of competing cyanobacterial species, which have already radiated and established themselves in various local niches. It has been noted that, where *Synechococcus* is absent from hot springs, there may have been insufficient time for its dispersal to geologically young thermal areas (Castenholz, 1978; 1996). Although it could be argued that *Synechococcus* does disperse to such sites and is outcompeted by predominant cyanobacteria, this argument would not hold for sites above 63°C, the upper temperature limit for cyanobacteria in such locations, which is well below the upper temperature tolerances of *Synechococcus* belong-

ing to the A clade found in North America (Ferris and Ward, 1997; Miller and Castenholz, 2000). The geological ages of hot springs undoubtedly influence microbiota distribution patterns, although one must be careful with such reasoning as it is extremely difficult to estimate the spatiotemporal history of geothermal activity associated with modern sites (R. Smith, personal communication).

Our results demonstrate that ubiquitous dispersal is not a universal trait of free-living microorganisms, and that geographical isolation does impact on the distribution and evolution of bacterial species. This raises the interesting possibility that divergence and speciation of bacteria do not depend solely on selection or molecular mechanisms that confer adaptive value, such as lateral gene transfer. Rather, isolation (perhaps in combination with associated limitations in population size, i.e. founder effects) may lead to divergence through genetic drift. Another important observation is that bacteria that have different evolutionary histories may have different dispersal and/or invasiveness capabilities. The idea that everything is everywhere is incorrect, because its tenet is that all microorganisms have no dispersal barriers. By studying island-like sites, it was possible to make these observations at a global scale using a conserved genetic marker and at local scales using a less conserved genetic marker. Thus, our results reinforce earlier results and suggestions that, to witness geographical effects at local spatial scales or in less island-like habitats, it is necessary to use highly sensitive approaches. Our results also suggest that both adaptation and geographical isolation must be considered as factors acting upon variation within and among microbial populations and influencing their speciation. Endemism should be of interest to biotechnology companies who seek unique resources from microorganisms. Local endemism might also have important implications for the identification and management of microbial resources in reserves such as Yellowstone National Park, especially in times when there is increasing sampling pressure, and as Yellowstone contains the greatest *Synechococcus* lineage diversity and the most unique genotypes among all countries that we investigated.

Experimental procedures

Sample collection and microscopy

We made collections at 48 hot springs ranging in geographical separation from tens to hundreds of metres (within basin) to tens of kilometres (between basins of major geothermal regions, e.g. Yellowstone National Park geyser basins) to hundreds of kilometres (within country, e.g. Yellowstone versus Oregon) to thousands of kilometres (between countries). Additionally, the sampled springs exhibited a broad range of physical/chemical parameters (see *Supplementary material*, Tables S1 and S2). At each spring, replicate biomass samples (6–8) were taken with a cork borer or forceps and immediately preserved on dry ice, then stored at −80°C. Water samples were preserved for chemical analysis. Cyanobacterial morphologies were observed using autofluorescence microscopy.

Sequence acquisition and analysis

Samples were thawed on ice and washed with Na-phosphate buffer (pH 8) before cell lysis and DNA extraction and purification (More *et al*., 1994). PCR was performed with *Taq* polymerase (Fisher) with primers 1070F(Ferris *et al*., 1996b) (5′-ATGGCTGTCGTCAGCT) and 23R (5′-TGCCTAGGTATC CACC) (*Escherichia coli* numbering system) to amplify the last third of the 16S rRNA gene, the ITS region and the beginning of the 23S rRNA gene. PCR products were cloned and transformed using the TOPO TA cloning kit (Invitrogen). Either 32 or 48 colonies per sample were picked for sequence analysis. Cloned plasmids were purified using the QIAprep 96 Turbo miniprep kit (Qiagen). Sequencing was performed on an Applied Biosystems 310 genetic analyser using primers 1070F and 1505F (5′-GTGAAGTCGTAACAAGG). Sequences have been submitted to GenBank under accession numbers AF505882–AF506001. 16S rRNA sequences were edited using SEQUENCHER 3.0 (Gene Codes), and the 16S rRNA gene alignment and tree was made within the ARB software package (http://www.mikro.biologie.tu-muenchen.de/) by adding short sequences to a backbone tree established with full-length sequences (Nübel *et al*., 2001). ITS trees were constructed using the neighbour-joining distance algorithm within the PAUP* phylogenetic software package (Swofford, 2001). Clades with high bootstrap support were also evident in trees constructed by parsimony and maximum likelihood methods.

Minimizing PCR and cloning artifacts

The potential for generating artifactual sequences in PCR and cloning approaches is significant and cannot be ignored (Qiu *et al*., 2001; Speksnijder *et al*., 2001). Speksnijder *et al*. (2001) found that such artifacts were observed as singletons, whereas real sequences, representing the majority of clones, were always recovered in replicate. Hence, we minimized the chances of including artifacts by reporting only genotypes that were replicated (at least fourfold for 16S rRNA and threefold for ITS) or were found in more than one spring.

rRNA dot blot hybridization

Probes for lineage-specific 16S rRNA membrane hybridization were designed using the Probe Design subroutine within the ARB software package to have at least one mismatch with all non-target 16S rRNA molecules in the Ribosomal Database Project (Maidak *et al*., 2001): A/B lineage probe, *E. coli* position 1282 (5′-CTGAGACGCGGTTTTTGG); C9 lineage probe, *E. coli* position 1250 (5′-CGCTGGCTGGCTACCCTT); C1 lineage probe, *E. coli* position 1253 (5′-GCCCTCGCGGGTTGGCAACT); cyanobacterial lineage probe CYA359F (Nübel *et al*., 1997) (5′-CCCATTGCG GAAAATTCCCC). Total RNA was extracted, purified and

quantified (Kingston *et al*., 1998). Community cyanobacterial 16S rRNA (400 ng) from each of triplicate mat samples was fixed to a nylon membrane using a Bio-Rad 96-well dot blot apparatus. Oligonucleotide probes were end-radiolabelled with kinase and [^{32}P]-ATP. Probe hybridization conditions were optimized by varying concentrations of formamide (A/B, 40%; C1, 50%; C9, 60%; and CYA359, 40%). Probed membranes were visualized using a phosphorimage analyser (Molecular Dynamics), and images were analysed using SCION IMAGE (Scion Corporation). Specific lineages were quantified as a percentage of the total cyanobacterial community 16S rRNA from standard curves (R^2 values of 0.57–0.99, mean 0.83) obtained from serial dilutions of RNA purified from *Synechococcus* sp. strains P2, C9 and C1 that represent the A/B, C9 and C1 lineages respectively. Our detection limit for each of the probes was ≈3 ng of 16S rRNA, which is 0.75% of the spotted RNA.

Lineage-specific PCR

PCR conditions were the same as above except for the use of lineage-specific probes as primers in conjunction with primer 1070F. To determine detection limits, DNA from type B *Synechococcus* sp. strain P2 was added to purified DNA from Nakanoyou hot spring, which had probed negative for the A/B lineage. The isolate's 16S rRNA gene was amplified from as little as 4×10^{-6}–10^{-4} ng of added DNA. We estimate that this is roughly equivalent to the weight of 4–100 genomes of the cyanobacterium *Synechocystis* PCC6803.

Chemical analysis

Alkalinity, calcium, chloride, magnesium, silica, sodium, sulphate, arsenic, boron, chromium, copper, iron, manganese and zinc were analysed by Peak Analytical Services using standard analytical methods (Clesceri *et al*., 1998). We analysed sulphide, nitrate, nitrite, ammonia and phosphate colorimetrically (Cline, 1969; Johnson and Pilson, 1972; Wetzel and Likens, 1991). All water samples were subjected to 0.2 μm filtration before analysis. Hierarchical clustering analysis of the above 19 parameters plus pH (*Supplementary material*, Tables S1 and S2) was done with SYSTAT 9 software (SPSS) using a single linkage and Euclidean distance algorithm. Before analysis, all concentration values were standardized to alleviate artifacts possibly arising from large-scale differences.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/emi/emi460/emi460sm.htm

Table S1. Physical, chemical and biological data for all hot springs sampled.

Table S2. Chemical measurements for each hot spring.

References

Baas-Becking, L.G.M. (1934) *Geobiologie of Inleiding tot de Milieukunde.* The Hague: Van Stockum & Zon.

Beijerinck, M.W. (1913) *Jaarboek van de Koninklijke Akademie v. Wetenschoppen.* Amsterdam: Muller.

Brock, T.D. (1978) *Thermophilic Microorganisms and Life at High Temperatures*. New York: Springer-Verlag.

Castenholz, R.W. (1976) The effect of sulfide on the blue-green algae of hot springs. I. New Zealand and Iceland. *J Phycol* **12:** 54–68.

Castenholz, R.W. (1978) The biogeography of hot spring algae through enrichment cultures. *Mitt Int Verein Limnol* **21:** 296–315.

Castenholz, R.W. (1996) Endemism and biodiversity of thermophilic cyanobacteria. *Nova Hedwigia Beiheft* **112:** 33–47.

Cho, J.C., and Tiedje, J.M. (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. *Appl Environ Microbiol* **66:** 5448–5456.

Clesceri, L.S., Arnold, G.E., and Andrew, D.E. (eds) (1998) *Standard Methods for the Examination of Water and Wastewater.* Baltimore, MD: American Public Health Association and American Water Works Association and Water Environment Federation.

Cline, J.D. (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol Oceanogr* **14:** 454–458.

Darling, K.F., Wade, C.M., Stewart, I.A., Kroon, D., Dingle, R., and Brown, A.J. (2000) Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature* **405:** 43–47.

Fenchel, T. (1993) There are more small than large species. *Oikos* **68:** 375–378.

Ferris, M.J., and Ward, D.M. (1997) Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **63:** 1375–1381.

Ferris, M.J., Ruff-Roberts, A.L., Kopczynski, E.D., Bateson, M.M., and Ward, D.M. (1996a) Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. *Appl Environ Microbiol* **62:** 1045–1050.

Ferris, M.J., Muyzer, G., and Ward, D.M. (1996b) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined

populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62:** 340–346.
Finlay, B.J. (2002) Global dispersal of free-living microbial eukaryote species. *Science* **296:** 1061–1063.
Finlay, B.J., and Clark, K.J. (1999) Ubiquitous dispersal of microbial species. *Nature* **400:** 828.
Finlay, B.J., Esteban, G.F., and Fenchel, T. (1996) Global diversity and body size. *Nature* **383:** 132–133.
Fulthorpe, R.R., Rhodes, A.N., and Tiedje, J.M. (1998) High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. *Appl Environ Microbiol* **64:** 1620–1627.
Garcia-Pichel, F., and Castenholz, R.W. (1990) Comparative anoxygenic photosynthetic capacity in 7 strains of thermophilic cyanobacterium. *Arch Microbiol* **153:** 344–351.
Garcia-Pichel, F., Prufert-Bebout, L., and Muyzer, G. (1996) Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium. *Appl Environ Microbiol* **62:** 3284–3291.
Godfray, H.C.J., and Lawton, J.H. (2001) Scale and species number. *Trends Ecol Evol* **16:** 400–404.
Johnson, D.L., and Pilson, M.E.Q. (1972) Spectrophotometric determination of arsenite, arsenate and phosphate in natural waters. *Anal Chem Acta* **58:** 289–299.
Kingston, R.E., Chomczynski, P., and Sacchi, N. (1998) Guanidine methods for total RNA preparation. In *Current Protocols in Molecular Biology.* Ausubel, F.M., *et al.* (eds). Unit. 4.2. New York: Wiley & Sons.
MacArthur, R.H., and Wilson, E.O. (1967) *The Theory of Island Biogeography.* Princeton: Princeton University Press.
Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Jr, Saxman, P.R., Rarris, R.J., *et al.* (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29:** 173–174.
Massana, R., DeLong, E.F., and Pedros-Alio, C. (2000) A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. *Appl Environ Microbiol* **66:** 1777–1787.
Miller, S.R., and Castenholz, R.W. (2000) Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcus*. *Appl Environ Microbiol* **66:** 4222–4229.
More, M.I., Herrick, J.B., Silva, M.C., Ghiorse, W.C., and Madsen, E.L. (1994) Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl Environ Microbiol* **60:** 1572–1580.
Norris, R.D., and de Vargas, C. (2000) Evolution all at sea. *Nature* **405:** 23–24.
Nübel, U., Garcia-Pichel, F., and Muyzer, G. (1997) PCR primers to amplify rRNA genes from cyanobacteria. *Appl Environ Microbiol* **63:** 3327–3332.
Nübel, U., Bateson, M.M., Madigan, M.T., Kühl, M., and Ward, D.M. (2001) Diversity and distribution in hypersaline microbial mats of bacteria related to *Chloroflexus* spp. *Appl Environ Microbiol* **67:** 4365–4371.
Patterson, D.J., and Lee, W.J. (2000) Geographic distribution and diversity of free living heterotrophic flagellates. In *The Flagellates. Unity, Diversity and Evolution.* Leadbetter, B.S.C., and Green, J.C. (eds). London: Taylor & Francis, pp. 269–287.
Qiu, X., Wu, L., Huang, H., McDonel, P.E., Palumbo, A.V., Tiedje, J.M., and Zhou, J. (2001) Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Appl Environ Microbiol* **67:** 880–887.
Ramsing, N.B., Ferris, M.J., and Ward, D.M. (2000) Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. *Appl Environ Microbiol* **66:** 1038–1049.
Reysenbach, A.L., Giver, L.J., Wickham, G.S., and Pace, N.R. (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Appl Environ Microbiol* **58:** 3417–3418.
Rocap, G., Distel, D.L., Waterbury, J.B., and Chisholm, S.W. (2002) Resolution of *Prochlorococcus and Synechococcus* ecotypes by using 16S–23S ribosomal DNA internal transcribed spacer sequences. *Appl Environ Microbiol* **68:** 1180–1191.
Ruff-Roberts, A.L., Kuenen, J.G., and Ward, D.M. (1994) Distribution of cultivated and uncultivated cyanobacteria and *Chloroflexus*-like bacteria in hot spring microbial mats. *Appl Environ Microbiol* **60:** 697–704.
Speksnijder, A.G., Kowalchuk, G.A., De Jong, S., Kline, E., Stephen, J.R., and Laanbroek, H.J. (2001) Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. *Appl Environ Microbiol* **67:** 469–472.
Staley, J.T., and Gosink, J.J. (1999) Poles apart: biodiversity and biogeography of sea ice bacteria. *Annu Rev Microbiol* **53:** 189–215.
Suzuki, M.T., Beja, O., Taylor, L.T., and DeLong, E.F. (2001) Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. *Environ Microbiol* **3:** 323–331.
Swofford, D.L. (2001) *paup*: Phylogenetic Analysis Using Parsimony (*and Other Methods).* Sunderland, MA: Sinauer Associates.
Ward, D.M., and Castenholz, R.W. (2000) Cyanobacteria in geothermal habitats. In *The Ecology of Cyanobacteria.* Whitton, B.A., and Potts, M. (eds). The Netherlands: Kluwer Academic Publishers, pp. 39–57.
Ward, D.M., Weller, R., and Bateson, M.M. (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345:** 63–65.
Ward, D.M., Ferris, M.J., Nold, S.C., and Bateson, M.M. (1998) A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol Mol Biol Rev* **62:** 1353–1370.
Wetzel, R., and Likens, G. (1991). *Limnological Analyses.* New York: Springer-Verlag.