MONTANA STATE UNIVERSITY

Biogeography of thermophilic cyanobacteria and the importance of isolation to the evolution of microorganisms

by Robertson Thane Papke

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy m Microbiology

Montana State University

© Copyright by Robertson Thane Papke (2002)

Abstract:
Evolutionary theory predicts the divergence of populations when they become geographically isolated. However, Baas Becking's theory that "everything is everywhere and the environment selects" excludes geographic isolation for microorganisms. In previous diversity and distribution studies, the sequencing of 16S rRNA genes acquired from natural Synechococcus populations residing in hot spring mats from Yellowstone National Park revealed that a single morphology concealed a rich 16S rRNA genotypic diversity. Predominating within that diversity is a group of closely related 16S rRNA genotypes (the A/B cluster) that are uniquely distributed along thermal and light gradients. Curiously, the upper temperature limit for cyanobacterial mat formation is different in globally disparate sites suggesting barriers to dispersal for some populations. I hypothesized that either members of the A/B cluster are distributed globally, but the highest temperature adapted forms (A types) are limited in their dispersal capabilities, or alternatively, globally disparate hot springs are dominated by unrelated Synechococcus genotypes. To test these hypotheses, I performed phylogenetic analysis on PCR-amplified, cloned, 16S rDNA genes recovered from Synechococcus populations residing in hot spring mats in Italy, New Zealand, Japan and the northwest U.S.A. The abundance of detected lineages was determined using lineage-specific oligonuleotide probes; low-abundance genotypes were sought using the same probes as PCR primers. I also assessed 20 different hot spring physical/chemical properties to determine whether adaptation was important to the local and global distributions of Synechococcus populations. Results revealed that: (1) A/B cluster 16S rDNA sequences were not detected outside of the U.S., (2) each country had unique dominating Synechococcus genotypes, (3) within the U.S. and Japan there exist local geographic clades for A/B and Cl lineages, respectively, at the 16S rRNA and internal transcribed spacer region loci, (4) Oscillatoria amphigranulata, a filamentous thermophilic cyanobacterial species also demonstrated unique geographical distributions, and (5) genetic variation did not correlate with tested hot spring physical/chemical parameters. The results revealed that all cyanobacterial lineages had a different dispersal capability, but even the most widely dispersed exhibited substantial evidence of geographic isolation. Additional evidence for isolated prokaryotic populations is reviewed and the general importance of isolation in microbial evolution is emphasized.

BIOGEOGRAPHY OF THERMOPHILIC CYANOBACTERIA AND THE IMPORTANCE OF ISOLATION TO THE EVOLUTION OF MICROORGANISMS

by

Robertson Thane Papke

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

February 2002

D378
P1984

APPROVAL

of a thesis submitted by

Robertson Thane Papke

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Dr. David M Ward _____ (Signature) 26 February 2002 Date

Approved for the Department of Microbiology

Dr. Cliff Bond _____ (Signature) 26 February 2002 Date

Approved for the College of Graduate Studies

Dr. Bruce McLeod _____ (Signature) 2-27-02 Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under the rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted " the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Signature _____

Date 02/27/02

ACKNOWLEDGEMENTS

I would like to thank Dr. David Ward for guiding me through the long process of earning a Ph.D. degree and for teaching me the importance of raising the bar of my own expectations and efforts. Thank you Mary Bateson, your kindness and friendship has made my journey through Bozeman a very happy one. Thank you Kenji Kato for sharing your house, hospitality and friendship and for your supreme efforts in helping me arrange my entire my Japanese hot spring collections. I thank the members of my committee, especially Adam Richman, for their time and effort in making this a better thesis. And thanks to all of the students and fellow scientists at MSU who have enriched my life both personally and scientifically with special thanks to Greg Colores, Mike Franklin, Myke Ferris, Uli Nübel and Marcel van der Meer. I could not have done this without any of you and I am eternally grateful to each and every one of you.

This research was funded by grants from the National Science Foundation, NASA, the Summer Institute in Japan and the Thermal Biology Institute.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Evolutionary theory predicts the divergence of populations when they become geographically isolated. However, Baas Becking's theory that "everything is everywhere and the environment selects" excludes geographic isolation for microorganisms. In previous diversity and distribution studies, the sequencing of 16S rRNA genes acquired from natural *Synechococcus* populations residing in hot spring mats from Yellowstone National Park revealed that a single morphology concealed a rich 16S rRNA genotypic diversity. Predominating within that diversity is a group of closely related 16S rRNA genotypes (the A/B cluster) that are uniquely distributed along thermal and light gradients. Curiously, the upper temperature limit for cyanobacterial mat formation is different in globally disparate sites suggesting barriers to dispersal for some populations. I hypothesized that either members of the A/B cluster are distributed globally, but the highest temperature adapted forms (A types) are limited in their dispersal capabilities, or alternatively, globally disparate hot springs are dominated by unrelated *Synechococcus* genotypes. To test these hypotheses, I performed phylogenetic analysis on PCR-amplified, cloned, 16S rDNA genes recovered from *Synechococcus* populations residing in hot spring mats in Italy, New Zealand, Japan and the northwest U.S.A. The abundance of detected lineages was determined using lineage-specific oligonuleotide probes; low-abundance genotypes were sought using the same probes as PCR primers. I also assessed 20 different hot spring physical/chemical properties to determine whether adaptation was important to the local and global distributions of *Synechococcus* populations. Results revealed that: (1) A/B cluster 16S rDNA sequences were not detected outside of the U.S., (2) each country had unique dominating *Synechococcus* genotypes, (3) within the U.S. and Japan there exist local geographic clades for A/B and C1 lineages, respectively, at the 16S rRNA and internal transcribed spacer region loci, (4) *Oscillatoria amphigranulata*, a filamentous thermophilic cyanobacterial species also demonstrated unique geographical distributions, and (5) genetic variation did not correlate with tested hot spring physical/chemical parameters. The results revealed that all cyanobacterial lineages had a different dispersal capability, but even the most widely dispersed exhibited substantial evidence of geographic isolation. Additional evidence for isolated prokaryotic populations is reviewed and the general importance of isolation in microbial evolution is emphasized.

CHAPTER 1

INTRODUCTION

Development of Evolutionary Theory in Microbiology

Great inroads toward comprehending evolution and the formation of species were made after naturalists and scientists visited locations around the globe, collected plants, animals and fossils and charted the organisms' relatedness against local and/or global distributions and ecological gradients. The independent formation by Darwin and Wallace of the theory of descent with modification via natural selection was completely dependent upon their observations that different yet related species lived in different regions of the world or on separate islands within archipelagos. As biologists searched for and catalogued the diversity of organisms on Earth, the disciplines of biogeography, and more recently phylogeography revealed many more corresponding patterns of organismal relatedness with geography[1]. As a mechanism for speciation geographic isolation is fundamentally different from natural selection, since population differences are driven by neutral genetic drift, not adaptation. Rosenzweig[2] expressed the importance of geographic isolation to the development of species when he articulated that "geographical speciation is the most common mode among most taxa in most places at most times." Indeed, the familiar terms used to describe speciation events, allopatric, parapatric and sympatric speciation all refer to the relative distances (distant, near or

together, respectively) that separate two sister species. Today, it is recognized that populations diverge whenever any kind of barriers to mating success are formed (e.g. different habitats, differential mating periods [day, season or year], anatomical incompatibility, different mating rituals, hybrid death or sterility). In the time since Darwin and Wallace published their great contributions to the science of biology, much has been learned about organismal diversity and mechanisms for speciation.

Unfortunately, evolutionary theory did not have a major impact on the field of microbiology. In 1963, nearly 300 years after van Leeuwenhoek first discovered microorganisms in his microscope and more than 100 years after Darwin and Wallace published, Stanier et al.[3] concluded that, "...any systematic attempt to construct a detailed scheme of natural relationships becomes the purest speculation...." The reasons may be obvious. Macroorganisms can be visualized and collected and morphologically, physiologically, ecologically and genetically described with relative ease. Microorganisms on the other hand are invisible to the naked eye, collections involve cultivation methods that allow recovery of only those that can grow under the conditions presented, their morphologies are exceedingly simple and relatively unvaried, and their diverse phenotypic properties are relatively useless for understanding evolutionary relationships. Because of these limitations, microbiology as a discipline was relegated to the applied side of science (i.e. tools to help the human condition) resulting in countless applications for food science, disease and medicine, genetics, physiology and cellular biology.

Years after the Stanier lament, Woese[4] changed the paradigm of microbiology by describing the three-domain "tree of life" based on sequencing the 16S rRNA molecule of prokaryotes (18S rRNA of eukaryotes). For the first time, the full scope of prokaryotic diversity was placed within the confines of phylogenetic relatedness. Classification based upon evolutionary relationship, once thought impossible, is now possible. The new classification scheme inspired Norman Pace and others[5] to recognize that microorganisms could be identified in situ (without cultivation) by comparing "naturally" occurring 16S rRNA molecule sequences (obtained via molecular techniques) to sequences of cultivated strains in the three-domain tree. Free from the confines of cultivation, microbial ecologists began natural history surveys that further demonstrated the great diversity of microorganisms and stimulated interesting questions about the causes of such diversity. For instance, 16S rRNA analysis of cyanobacterial mats residing in Octopus and Mushroom hot springs in Yellowstone National Park demonstrated that the in situ 16S rRNA gene sequences were different from those of cultivated isolates[6] and that closely related *Synechococcus* (unicellular cyanobacteria) were uniquely distributed across temperature and light gradients[7,8]. It was suggested[9] that the evolutionary/ecological theory, adaptive radiation (i.e. differential adaptation to various environments) could explain the observed relationship of the genotypes to their unique niches, a theory modeled after the adaptation of "Darwin's finches" to different niches on the Galapagos Islands. However, without further distribution analysis (e.g. global sampling) it cannot be determined if the Yellowstone *Synechococcus* radiated within Yellowstone's borders or if they have a wider distribution.

Microbial Biogeography

It is interesting to note that with the new microbial paradigm, lots of problems have been solved, but new problems have arisen. Perhaps the biggest obstacle in the field of microbial biogeography is the question of identity (i.e. how do we know if two populations belong to the same species or if two organisms belong to the same population?). This is of extreme importance when trying to determine the geographic range of a specific species or population. To differentiate species or populations, it is critical to have an established set of criteria, which can be applied to and measured on individuals. In some cases this can be relatively easy. While in New Guinea, Ernst Mayr[10] collected and identified 138 species of birds of which the island's indigenous people identified 137, suggesting that species are not arbitrarily defined but universally accepted regardless of who is counting. However, the myriad of species definitions or concepts, contradicts this notion[11]. Furthermore, it is difficult to identify a single species concept that can be applied to all groups of organisms, extant and extinct, haploid, diploid and polyploid, sexual and asexual or macroorganism and microorganism. Perhaps Darwin[12] expressed the problem best when he wrote "there is no possible test but individual opinion to determine which…shall be considered as species and which as varieties." If species are so difficult to define, then perhaps that unit of identity should not be used, especially with respect to microorganisms where separate species have been arbitrarily defined as organisms with less than 70% similarity in DNA-DNA hybridization,[13] which roughly correlates to 97% similarity at the 16S rRNA locus[14].

Lately, molecular markers (e.g. gene sequence variation) have been extremely successful for linking relatedness with the distribution of organisms[1]. Genetic relatedness can thus be used to define identity. This is appropriate as divergence is really the issue, not what species are. In attempts to determine the biogeography of microorganisms, molecular markers, especially the 16S rRNA gene, have been used in addition to more classical methods of identification (e.g. phenotypic properties). However, there has been little conformity in which measurements should be used to determine the geographic ranges of the studied organisms. The use of conserved genes, like 16S rRNA, to identify and/or define populations may be particularly problematic as they may underestimate the actual diversity and thereby artificially expand our impressions of territorial range. The pitfalls in choosing a wrong level of analysis for determining identity (e.g. morphology, gene restriction enzyme fragment patterns or conserved vs. variable gene sequences) will be considered below.

In a study using microscopy to determine the species diversity of ciliated protozoa (large unicellular eukaryotes whose species are morphologically defined), Fenchel et al.[15] reported (from their study plus others) 181 and 146 species recovered from two ecologically different sediments occurring in a pond (Priest Pot, UK) and shallow bay (Nivå Bay, Helsingør, Denmark). They determined that the diversity discovered was approximately 11% of the total number of free-living ciliate species. Furthermore, they reasoned that similar results would have been found if additional nearby ciliate habitats had been sampled (i.e. 10-20 ecologically different sites). The authors were confident that if the more comprehensive sampling regime had been performed "a very substantial

fraction of all known ciliates" would have been recovered from a relatively small geographical range. From their interpretation of the data, they concluded, "everything is (almost) everywhere". However, the conclusion may be oversimplified. Organisms that live in similar habitats can often have similar morphologies via convergent or parallel evolution thereby concealing genetic diversity within a morphotypically-defined species. Indeed, many planktonic foraminifera species (morphotypically-defined) are comprised of more than one genotype and these geontypes have been considered to be cryptic sibling species[16-18]. In prokaryotes, all unicellular coccoid to rod-shaped cyanobacteria fall within the genus *Synechococcus* [19]. However, this genus is not monophyletic, as the morphology has independently evolved many times[20]. As both examples clearly demonstrate, it is risky to make conclusions about the distribution of microorganisms when identity is based solely upon morphological criteria.

As expressed above, diversity and distribution studies of microorganisms are often performed using the 16S rRNA molecule either by restriction enzyme analysis or by direct sequencing of the molecule. In an attempt to survey the archaeal diversity present in the world's oceans (North Atlantic, Cantabrian Sea [Atlantic Ocean], the Mediterranean Sea, the Santa Barbara Channel [Pacific Ocean], and the Drake Passage [Southern Ocean]) Massana et al.[21] generated 16S rRNA gene libraries from natural samples. They used two restriction enzymes to construct restriction fragment length polymorphism (RFLP) patterns from their clone libraries and interpreted any RFLP patterns that were identical as a single operational taxonomic unit (OTU). The analyses of Massana et al.,[21] revealed that 5 of the 36 OTU's (representing 87% of the analyzed

clones) were "cosmopolitan". The RFLP method is insensitive, as restriction enzymes recognize a very small proportion (e.g. 4-8 nucleotides) of the molecule analyzed. In a computer simulation using prokaryotic 16S rRNA gene sequences, Moyer et al.,[22] tested the efficacy of restriction enzymes in determining the diversity of microorganisms. They found that RFLP could only differentiate among sequences that were at least 3.9% different. This clearly leaves a lot of diversity undetected, especially considering the extremely conserved nature of the 16S rRNA locus. In a study using *Pseudomonas* strains isolated from soil samples collected around the world, Cho and Tiedje [23]compared the effectiveness of 16S rRNA RFLP patterns with repetitive extragenic palindromic-PCR (REP-PCR, a very sensitive method that takes advantage of the entire genomic diversity) for detecting endemic genotypes. In the case of 16S rRNA RFLP pattern analysis, only 4 OTU's were found among 248 isolates and all 4 appeared cosmopolitan in distribution. However, when REP-PCR was applied to each of the strains, 85 genotypes were recovered and identical genotypes were only found in samples from the same geographic sites, indicating high levels of endemism among the strains. Mehta et al.,[24] found similar results when they analyzed *Zylella fastidiosa* isolated from citrus trees in Brazil. It would seem that 16S rRNA RFLP patterns completely underestimate the true diversity of microorganisms and any conclusions as to "cosmopolitan phylotypes" should be avoided when using this technique.

Similar or identical 16S rRNA gene sequences have been used to declare that some organisms have a worldwide distribution. Indeed, Garcia-Pichel et al.,[25] found identical or nearly identical 16S rRNA gene sequences from hypersaline-adapted

cyanobacteria living in microbial mats from Europe, the Middle East and Baha, Mexico. They concluded that the cyanobacterial species *Microcoleus chthonoplastes* is cosmopolitan. Zwart et al., [26] also found nearly identical 16S rRNA genes from lakes located in North America and Europe, and conjectured that the same species has a global distribution. Although 16S rRNA sequence variation is more sensitive than 16S rRNA RFLP pattern analysis for determining identity, 16S rRNA sequence variation may also unnaturally expand our view of population ranges since the 16S rRNA locus is evolutionarily conserved. For instance Ferris and Ward[7] found that two 16S rRNA genes differing by a single nucleotide had unique distributions along a thermal gradient. Because the 16S rRNA genes were found in different habitats, it was argued that genes were retrieved from different species[27]. Since this locus is barely able to detect differentially adapted populations, it may also be too conserved to detect differences in geographic populations. It is also likely that small changes in the 16S rRNA actually reflect major changes in the organism. The average rate of substitution for 16S and 18S rRNA molecules has been calculated to be 1% per 50 million years[28-30]. This translates to one nucleotide substitution per 3.3 million years, suggesting that two organisms with nearly identical 16S rRNA genes have been divergent for a very long time. The evidence suggests that spatially separated organisms should not be interpreted as having a cosmopolitan distribution when slight differences are detected at the 16S rRNA locus. Indeed, the opposite interpretation may be more likely.

It is difficult to cast blame on researchers for using conserved loci to establish identity, because such genes are commonly assayed and there are often databases to

which results can be compared. However, researchers should recognize the limits of the methods before drawing conclusions. If progress is to be made in microbial biogeography, it is likely that more informative molecular markers with greater resolving power will have to be used. For instance, the DNA-dependent RNA polymerase gene (*rpo*C1) evolves much faster than the 16S rRNA gene. *Synechococcus* sp. strains WH7805 and WH8103 differ by 1.4% at the 16S rRNA locus, but differ by 17% at the *rpo*C1 locus[31]. The internal/intervening/intergenic transcribed spacer (ITS) region located on the rRNA operon between the 16S and 23S rRNA genes also has a much higher resolving power [32-34]. However, for in situ analysis, the ITS region has additional benefits. Because the ITS is adjacent to the 16S rRNA gene, it is possible to PCR amplify both loci simultaneously using the 16S rRNA gene to relate the sequence phylogenetically to other known organisms while using the ITS to discriminate between closely related genetic variants with identical 16S rRNA sequences.

Goals of the Thesis

If Rosenzweig and other evolutionary biologists[2,10,35-37] are correct in thinking that geographic isolation is one of the major causes of speciation, then perhaps it is time for microbiologists to understand this biological paradigm and apply it to investigations concerning microbial diversification and distribution, especially since most of the putative evidence (and dogma) that supports the "cosmopolitan" hypothesis is based on observations that can easily be challenged. With this admonishment in mind, it is the

goal of this thesis to provide convincing evidence that microorganisms can become geographically isolated, that isolation can lead to diverging populations and consequently that genetic drift may play an active role in the evolution of microorganism independently of adaptation (via mutation and lateral gene transfer) and natural selection.

My approach to microbial biogeography was to take advantage of the island-like nature of hot springs and previous observations concerning the diversity and distribution of thermophilic cyanobacterial populations from around the globe. Anomalous distributions such as the lack of high-temperature adapted cyanobacteria in regions outside of the U.S.A.[38,39] led to the main hypotheses:

Synechococcus mats in globally separated hot springs are dominated by A/B genotypes, but there is a barrier to the dispersal of higher temperature-adapted A-like genotypes.

Or, alternatively, mats in globally separated hot springs are dominated by *Synechococcus* unrelated to A/B genotypes.

The first hypothesis supports the idea that everything is everywhere, but nature selects. The hypothesis predicts that both B and A-type *Synechococcus* are ubiquitously dispersed; the inability of type-A *Synechococcus* to live above 63C in some hot springs is explained by environmental selection (e.g., sulfide in combination with high temperature is known to prevent the growth of cyanobacteria[40-41]). The alternative hypothesis is consistent with geographic isolation. A test of either hypothesis must also

address the possibility that distribution is patterned according to adaptation to specific physical/chemical parameters.

To test these hypotheses, I made extensive collections from hot springs in Italy, New Zealand, Japan and the northwest United States and analyzed samples by molecular methods of suitable resolution. I developed a 16S rRNA method that allows genetic comparisons to previous 16S rRNA studies while simultaneously sampling a higher resolution genetic marker (ITS region) for detecting sequence variation between identical or nearly identical 16S-rRNA defined genotypes. This is important because 16S rRNA gene sequences are likely to conceal geographical isolation given their conserved nature. I also generated group-specific 16S rRNA probes to quantify populations in their various locations and, using PCR, to detect rare genotypes that may be present but difficult to detect given the limitations of detection methods. Furthermore, to convincingly demonstrate the role of adaptation or niche specialization in determining the distribution of the thermophilic cyanobacteria, in-depth analysis of the physical/chemical parameters of sampled hot springs was performed. Because the results of this work could potentially shift theoretical paradigms in microbiology, chapter 2 was prepared as a research article for the journal *Nature* and the experimental results are thus presented in a condensed style. Furthermore, much additional literature detail is placed intentionally in a minireview (chapter 3) designed to add my results to a growing body of evidence on physical isolation in microbial evolution, an issue that needs to be emphasized to microbiologists.

REFERENCES CITED

1 Avise, J. 2000. *Phylogeography: The History and Formation of Species.* Harvard University Press, Cambridge, MA.

2 Rosenzweig, M. 1996. *Species Diversity in Space and Time.* Cambridge University Press, UK.

3 Stanier, R.Y., Doudoroff, M. and Adelberg, E.A., 1963. *The Microbial World,* 2nd ed., Prentice-Hall, Inc., Englewood Cliffs, NJ.

4 Woese C. 1987. Bacterial evolution. *Microbiol. Rev.* 51:221-271.

5 Olsen, G.L., Lane, D.J., Giovannoni, S.J., and Pace, N.R. 1986. Microbial ecology and evolution: a ribosomal RNA approach. *Ann. Rev. Microbiol.* 40:337-365.

6 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.

7 Ferris, M., and Ward, D. 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.

8 Ramsing, N., Ferris, M., and Ward, D. 2000. Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobactrial mat. *Appl. Environ. Microbiol.* 66:1038-1049.

9 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.

10 Mayr, E. 1991. *One Long Argument: Charles Darwin and the Genesis of Modern Evolutionary Thought.* Harvard University Press, Cambridge, MA.

11 Claridge, M., Dawah, H., and Wilson, M. (eds.) 1997. *Species: the Units of Biodiversity.* Chapman & Hall, London.

12 Darwin, C. 1859. *Origin of Species, by Means of Natural Selection of the Preservation of Favoured Races in the Struggle for Life.* Mentor ed., Penguin Books Ltd., Harmondsworth, UK.

13 Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H. Moore, W.E.C. Murray, R.G.E., Stackebrandt, E., Starr, M.P., and Trüper, H.G. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37:463-464.

14 Stackebrandt, E., and Goebel B.M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. System Bacteriol.* 44:846-849.

15 Fenchel, T., Esteban, G. and Finlay, F. 1997. Local versus global diversity of microorganisms: cryptic diversity of ciliated protozoa. *OIKOS* 80:220-225.

16 Huber, B., Bijma, J. and Darling K. 1997. Cryptic speciation in the living planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Paleobiol.* 23:33-62.

17 de Vargas, C., Norris, R., Zaninetti, L., Gibb, S., and Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA* 96:2864-2868.

18 Darling, K., Wade, C., Kroon, D., Leigh Brown, A., and Bijma, J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulations. *Paleoceanogr.* 14:3-12.

19 Waterbury, J., and Rippka, R. 1989. Subsection I. Order *Chroococcales* Wettstein 1924, emend. Rippka et al., 1979. in *Bergey's Manual of Systematic Bacteriology*. Staley, J., Bryant, M., Pfennig, N., and Holt, J. (eds.) Williams & Williams. Baltimore.

20 Turner, S., Pryer, K. Miao, M. and Palmer, J. 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J. Eukaryot. Microbiol.* 46:327-338.

21 Massana, R., DeLong, E., and Pedros-Alio, C. 2000. A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. *Appl. Environ. Microbiol.* 66:177-187.

22 Moyer, C., Tiedje, J., Dobbs, F., and Karl, D. 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl. Environ. Microbiol.* 62:2501-2507.

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

24 Mehta, A., Leite, R. Jr., Rosato, Y. 2001. Assessment of the genetic diversity of *Xylella fastidiosa* isolated from citrus in Brazil by PCR-RFLP of the 16S rDNA and 16S-23S intergenic spacer and rep-PCR fingerprinting. *Antonie Van Leeuwenhoek* 79:53-59.

25 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.

26 Zwart, G., Hiorns, W., Methe, B., van Agterveld, M., Huismans, R., Nold, S., Zehr, J., and Laanbroek, H. 1998. Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existance of clades of globally distributed freshwater bacteria. *System. Appl. Microbiol.* 21:546-556.

27 Ward, D. 1998. A natural species concept for prokaryotes. *Curr. Opin. Microbiol.* 1:271-277.

28 Ochman, H., and Wilson, A. 1987. Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J. Mol. Evol.* 26:74-86.

29 Darling, K., Wade, C., Stewart, I., Kroon, D., Dingle, R., and Leigh-Brown, A. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar poulations of planktonic foraminifers. *Nature* 405:43-47.

30 Baumann, P., Baumann, L., Lai, C., Rouhbakhsh, D., Moran, N. and Clark, M. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annu. Rev. Microbiol.* 49:55-94.

31 Toledo, G. and Palenik, B. 1997. *Synechococcus* diversity in the California current as seen by RNA polymerase (*rpo*C1) gene sequences of isolated strains. *Appl. Environ. Microbiol.* 63:4298-4303.

32 Frothingham, R., and Wilson, K. 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *J. Bacteriol.* 175:2818-2825.

33 Scheinert, P., Krausse, R., Ullmann, U., Soller, R., and Krupp, G. 1996. Molecular differentiation of bacteria by PCR amplification of the 16S-23S rRNA spacer. *J. Microbiol. Meth.* 26:103-117.

34 Chun, J., Rivera, I., Colwell R. 2002. Analysis of 16S-23S rRNA intergenic spacer of *Vibrio cholerae* and *Vibrio mimicus* for detection of these species. *Methods Mol. Biol.* 179:171-178.

35 Futuyma, D., 1986. *Evolutionary Biology* 2nd ed. Sinauer Associates, Inc. Sunderland, MA.

36 Wilson, E. 1992. *The Diversity of Life.* W.W. Norton & Co. New York.

37 Mayr, E. 1942. *Systematics and the Origin of Species.* Columbia University Press, New York.

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

39 Castenholz, R.W. 1978. The biogeography of hot spring algae through enrichment cultures. *Mitt. Internat. Verein. Limnol.* 21: 296-315.

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

41 Ward, D.M. and Castenholz, R.W. 2000. in *The Ecology of Cyanobacteria.* Whitton, B.A. & Potts, M. (eds.) Kluwer Academic Publishers, The Netherlands.

CHAPTER 2

GEOGRAPHIC ISOLATION AND THE EVOLUTION OF HOT SPRING CYANOBACTERIA*

Introduction

Genomics and comparative molecular phylogeny have fueled intense consideration of molecular mechanisms for the generation of genetic variation in bacteria, especially lateral gene transfer, and of the role these mechanisms may play in microbial evolution[1,2]. However, less attention has been given to environmental factors that act upon such variation to cause divergence and speciation. Ecological and geographic isolation are recognized as major causes of adaptive and allopatric speciation[3,4]. Microbial ecologists have begun to discover evidence suggesting adaptive radiations as they have used molecular methods to assay microbial diversity[5,6] and distribution patterns along well-defined ecological gradients within natural communities[7-13]. There is, however, considerable debate over the importance of geographic isolation in bacterial speciation.

It has been commonly assumed since early in the 20th century that in the case of microorganisms "everything is everywhere and nature selects"[14,15]. This suggests that microorganisms readily disperse and do not become geographically isolated. Support for the ubiquitous dispersal of microorganisms has come from observations of diversity

*This study has been submitted to *Nature* as: Papke, R.T., N.B. Ramsing, M.M. Bateson and D.M. Ward. Geographic isolation and the evolution of hot spring cyanobacteria.

of protists in sediments, suggesting widespread distribution of morphospecies[16]. One important implication is that the absence of allopatric speciation explains why there appear to be fewer microbial species than expected from correlations between body size and number of species[17]. A recent molecular study of protist diversity in polar oceans demonstrated that the same genetic variants, defined by 18S rRNA sequence variation, were present at both poles[18], 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 geographic patterning[19]. Studies of bacterial diversity and distribution in marine[20,21] and near-marine[22] 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 and distribution in globally separate soil environments have revealed evidence of unique geographic distributions, but only when methods offering more genetic resolution than 16S rRNA sequence variation were employed[23,24]. In the face of conflicting reports it seemed informative to examine environments where geographic isolation is a prominent feature and thus likely to contribute to diversification. As pointed out by MacArthur and Wilson[25] "...in the science of biogeography, the island is the first unit that the mind can pick out and begin to comprehend".

Hot Spring Mats as Island-like Communities

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 through which they would have to disperse. As such, one would expect that geographic isolation might be an important component to the diversification of hot spring microorganisms. Castenholz[26,27] observed anomalous distributions of cyanobacterial morphotypes inhabiting 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 ca. 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 Iceland (Castenholz, personal communication).

Molecular analysis has revealed great diversity within the thermophilic *Synechococcus*[7,28] morphotype. Three unrelated phylogenetic lineages (separated by >10% 16S rRNA sequence variation) containing organisms of this morphotype, termed A/B, C1and C9, have been detected (Figure 1). The predominant *Synechococcus* in Yellowstone hot springs detected by direct molecular analysis is the A/B type[29]. On the basis of distribution[7,10] and pure culture studies[30], the A/B lineage appears to have

diverged into high- and low-temperature adapted A-like and B-like clades, respectively (Figure 1). Furthermore, different genotypes occurred at different depths in the mat[8], leading us to suggest that the pattern of diversity in this lineage resulted from an adaptive radiation[7]. *Synechococcus* spp. C1 and C9 genotypes were also detected in the same Yellowstone spring through cultivation and were less abundant and diverse.

The morphological observations of biogeographical anomaly and our molecular observations in Yellowstone hot springs led us to the following alternative hypotheses regarding biogeographical influences on the distribution and evolution of hot spring *Synechococcus*:

Synechococcus mats in globally separate hot springs are dominated by A/B genotypes, but there is a barrier to the dispersal of higher temperature-adapted A-like genotypes.

Or, alternatively, mats in globally separate hot springs are dominated by *Synechococcus* unrelated to A/B genotypes.

The first hypothesis supports the idea that everything is everywhere, but nature selects. The hypothesis predicts that both B and A-type *Synechococcus* are ubiquitously dispersed; the inability of type-A *Synechococcus* to live above 63C in some hot springs

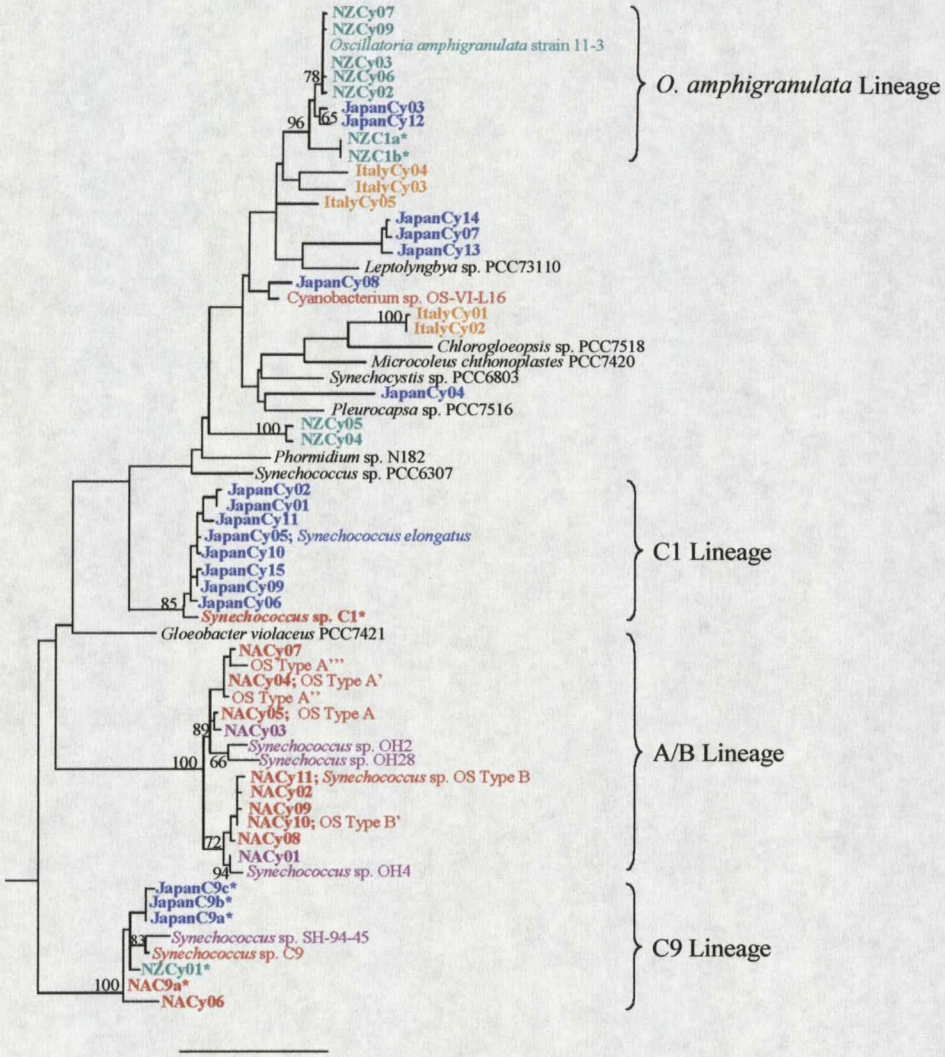

Figure 1. 16S rRNA gene tree demonstrating the relationships of clones retrieved from all countries (those from this study in bold) to other cyanobacterial 16S rRNA sequences including hot spring *Synechococcus* spp. and *Oscillatoria amphigranulata* isolates. The tree was rooted with *E. coli* and *Bacillus subtilus* 16SrRNA sequences. Values at nodes indicate bootstrap percentages for 1000 replicates. Values less than 50% are not reported. Scalebar indicates 0.10 substitutions per site. *, cloned genotypes from linage-specific PCR study. Color highlighting: Green, New Zealand; blue, Japan; yellow, Italy; red, Greater Yellowstone Ecosystem; purple, Oregon.

is explained by environmental selection (e.g., sulfide in combination with high temperature is known to prevent the growth of cyanobacteria[31-33]). The alternative hypothesis is consistent with geographic isolation. A test of either hypothesis must also address the possibility that distribution is patterned according to adaptation to specific physical/chemical parameters.

To test these hypotheses 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 of springs varying widely in geographic location and physical/chemical properties in order to obtain a robust sampling of the diversity present within the region and subregions (Appendix, Table 3). We simultaneously examined a wide range of physical/chemical parameters (Appendix, Table 4) to determine possible abiotic effects on distribution of genotypes, an alternative that has not been rigorously addressed in previous microbial biogeography studies. We used PCR with general primers to amplify 16S rRNA genes of Domain Bacteria and cloning in order to initially investigate the genotypes present. By using one primer targeting a site in the 23S rRNA gene, we simultaneously amplified the adjacent intervening transcribed spacer (ITS) region, often used in phylogenetic studies[34] 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 toward dominant genotypes, we also developed a lineage-specific PCR approach to amplify the 16S rRNA genes from rare genotypes at high sensitivity.

Geographic Patterning of Diversity

From North America, Japan and New Zealand we obtained clones from 17-37 samples collected from 9-14 hot springs located in 3-6 distinct subregions, (Table 1). 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, approximately 6000 partial 16S rDNA and ITS clones (450bp at each locus) were sequenced. Because artifacts are known to occur during PCR, cloning and sequencing[35,36] we report only those sequences that were found in replicate and in more than one mat sample. This approach underestimates the discovered clone sequence diversity, but gives us high confidence that the sequences we report are real (see Methods). Most of the clones exhibited close phylogenetic relatedness (>96%) to 16S rDNA sequences of cultivated *Synechococcus* isolates (Figure 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[37]. 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.

Table 1. Physical, chemical and biological data for hot springs sampled in different geographic regions of all countries.

					Cyanobacteria					
		Range					Relative Abundance (%)[c]			
Region [a]	# Springs/ # Samples	Temp (C)	pH	S^{2-} (uM)	Morpho-type Diversity [b]	Cloned Genotypes	A/B	C-1	C-9	*Synecho-coccus*[d]
North America 1	3/4	49.7-61.8	8.1-8.3	0.7-4.6	R	NACy01, 03	69.5±3.9	nd[e]	3.2±0.6	72.7
North America 2	1/1	57.2-57.5	8.8	7.7	R/f	NACy06, 08, 10	72.4± 10.8	nd	22.3± 6.07	94.7
North America 3	4/4	56.4-60.2	6.6-6.9	nd-39.3	R	NACy09, 10, 11	87.7± 8.8	nd	7.4± 3.9	95.1
North America 4	3/3	54.0-61.9	5.2-7.5	nd-1.3	R	NACy05, 10, OS type-C1*[f]	90.3± 7.7	5.1±2.3	1.5± 0.2	96.9
North America 5	3/6	54.0-64.0	8.2-8.4	nd-0.6	R	NACy02, 04, 05, 06, 07, 10, 11, NAC9a*	83.6± 4.0	nd	2.3± 0.3	85.9
North America 6	3/3	56.6-61.8	8.0-9.2	nd-19.5	R	NACy04, 05, 09, 10, 11	83.6± 4.0	nd	2.3± 0.3	85.9
Japan 1	5/13	48.2-62.2	6.3-8.4	0.1-36.7	R, R/F, F, a	JapanCy01, 02, 03, 05, 07, 12, 14	nd	68.2± 11.3	0.5± 0.2	68.7
Japan 2	1/3	56.6-59.1	7.3	7.5	R	JapanCy05, 06				
Japan 3	3/10	47.2-66.3	6.1-8.5	0.2-34.5	R	JapanC9a*, b*,c*, JapanCy05, 09, 10, 15	nd	105± 13.1	nd	105
Japan 4	2/3	50.6-60.5	7.0-7.3	7.0-7.5	R	JapanCy05, 06				
Japan 5	2/4	49.4-61.9	7.8-9.1	0.9-34.3	R, F, R/O	JapanCy08, 09, 10, 11, 15	nd	111± 10.	nd	111
Japan 6	2/4	51.9-59.2	7.6-8.6	0.4-0.8	R, F, F/r, F/o	JapanCy04, 05, 10, 13	nd	73.4± 6.9	1.6± 0.06	75.0
New Zealand 1	6/14	44.1-60.9	6.4-8.9	1.1-48.6	R/f, F/r, R/F/O, r, f	NZCy01*, 02, 03, 06, 07, 08, NZC1a*, b*	nd	nd	14.2± 3.9	14.2
New Zealand 2	1/4	49.8-59.1	8.5-8.6	nd-0.9	R/f, F,	NZCy01, 03, 04, 05, 08	nd	nd	16.3± 4.5	16.3
New Zealand 3	2/4	47.6-57.3	6.6-7.9	nd-1.3	R/F, r/F	NZCy02, 03, 05, 06	nd	nd	4.7± 2.1	4.7
New Zealand 4	3/3	50.0-55.5	5.6-7.3	0.9-1.5	R/F, F		nd	nd	5.7± 2.9	5.7
Italy 1	1/1	54.4-55.4	7.7	nd	F	ItalyCy04, 05				
Italy 2	2/3	46.9-57.8	7.0-7.9	nd	F	ItalyCy02, 03				
Italy 3	1/2	50.0-54.9	7.1-7.3	nd	F	ItalyCy01, 02				

a, Regions in Japan and most of North America are shown in Figure 2b,d; region 1 and 2 in North America are near Lakeview, OR and Bozeman, MT, respectively. Italian region 1 is near Padua;

regions 2 and 3 are in Naples and Ischia, respectively. New Zealand regions are all on the North Island between Rotorua and the southern shore of Lake Taupo.

b. R or r, unicellular rod; F or f, filamentous; O or o, unicellular, ovoid; a, aggregate (upper and lower cases reflect predominance and presence of the morphotype, respectively. Entry/entry indicates different morphologies in same spring, whereas commas separate morphologies found in different springs.

c, Mean of per-sample lineage-specific probe response relative to cyanobacterial lineage probe response.

d, Sum of means for all three lineages

e, nd, not detected.

f, *, genotype discovered using lineage-specific PCR technique.

Our clone survey revealed evidence for the restricted distribution of cyanobacterial genotypes to specific geographic locations both among and within countries, as emphasized by unique color coding in Figure 1. Members of the A/B *Synechococcus* clade were detected only in North America. 16S rRNA genotypes found in Oregon hot springs are different from those found in Yellowstone and Montana hot springs. Separate clades for Oregon and Yellowstone/Montana B-like genotypes are 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 geographic distribution patterns within the Greater Yellowstone Ecosystem (e.g., clone NACy08 was detected only in Bozeman Hot Springs, located ca. 200 km north of Yellowstone). ITS analysis provided further evidence of localized geographic patterning. Figure 2a shows a phylogenetic tree exhibiting 9 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, comprised of 5 ITS variants retrieved almost exclusively from springs in the northern region of Yellowstone or just north of the park (regions 3 and 4). All sequence variants outside this lineage were retrieved from more

southerly 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 southeasterly 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[38] (Figure 1). Eight distinct Japanese clone sequences formed a clade that included one genotype that is identical to the 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 (Figure 2b). ITS analysis also demonstrate the existence of clades supported by 96-98% bootstrap values separating variants recovered only from the most northerly (region 1) or more southerly (regions 2-5) springs.

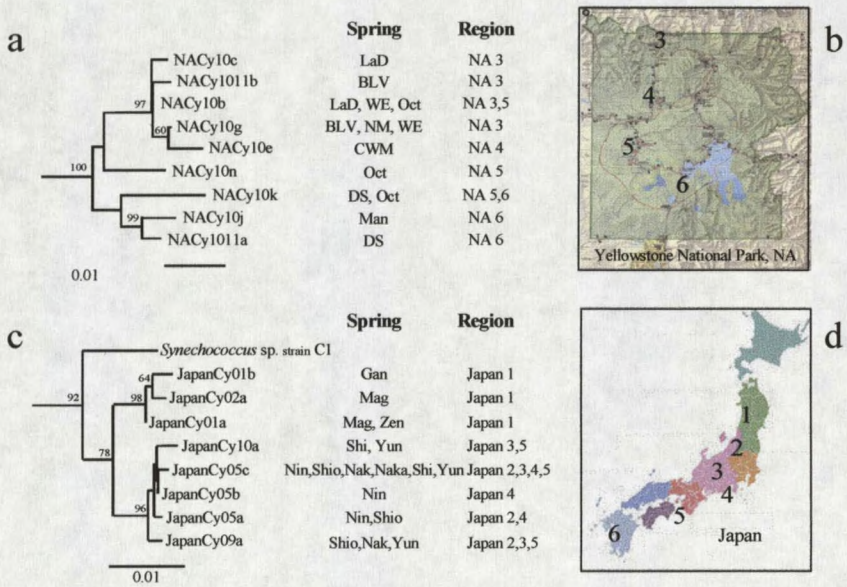

Figure 2. Phylogenies for ITS variants detected in Yellowstone or Japan relative to springs and subregions from which they were retrieved. (a) ITS genotypes linked to 16S rRNA genotype NACy10 (corresponding to the type B' 16S rRNA sequence from previous work[7]) (b) Map of Yellowstone National Park indicating regions sampled (c) Combined 16S rRNA and ITS sequence data for members of the C1 lineage. (d) Map of Japan indicating regions sampled. Values at nodes indicate bootstrap percentages for 1000 replicates. Values less than 50% are not reported. Scalebar indicates 0.01 substitutions per site. Differences between Figure 1 and Figure 2c reflect insufficient replication at the ITS locus for certain 16S rRNA genotypes (see methods for replication criteria). Maps of Yellowstone and Japan were found at URL's www.yellowstone-natl-park.com/ywstone.htm and http://jin.jcic.or.jp/region/index.html, respectively.

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. A new C9-like genotype was also detected in North America. Evidence of geographic patterning of diversity was also found among representatives of the *O. amphigranula* 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% (Figure 1)).

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

Lineage-Specific 16S rRNA Probing

Because PCR and cloning might bias against some cyanobacterial 16S rRNA sequences[39], we developed specific 16S rRNA oligonucoeotide probes for the three known thermophilic *Synechococcus* lineages in order to test the significance of cloned genotypes and to seek autecological evidence of the distribution of the members of each lineage. We probed samples from all 42 hot springs containing *Synechococcus* cells (Table 1 and Appendix, Supplemental Table 1). Members of the A/B lineage were detected only in North America, where they accounted for most of the cyanobacterial 16S rRNA (Table 2). 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[29]. Members of the C9 lineage were the most prominent *Synechococcus* type in New Zealand but only constituted 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 to the total cyanobacterial 16S rRNA.

We also developed a lineage-specific PCR (LS-PCR) approach to confirm and extend probing results, in particular by increasing the sensitivity of detection. We analyzed a subset of probed samples to further evaluate the presence of members of the

Table 2. Relative abundance of *Synechocooccus* 16S rRNA lineages in mats from each country.

Lineage	% ±SE[a] of cyanobacterial 16S rRNA in			
	North America	Japan	New Zealand	Italy
A/B	77.8±3.87	nd[b]	nd	nd
C1	0.60±0.36	87.5±6.46	nd	nd
C9	4.41±1.2	0.46±0.13	14.0±3.03	nd

a, standard error
b, not detected

A/B lineage in Japan and New Zealand. Again, the A/B lineage was not detected outside of North America (Figure 3).To test our sensitivity limits, we titrated genomic DNA from a type-B *Synechococcus* isolate into purified DNA from a Japanese hot spring sample that was previously found in probe and lineage-specific PCR studies to be negative for the A/B lineage. We were able to detect type-B 16S rDNA at a

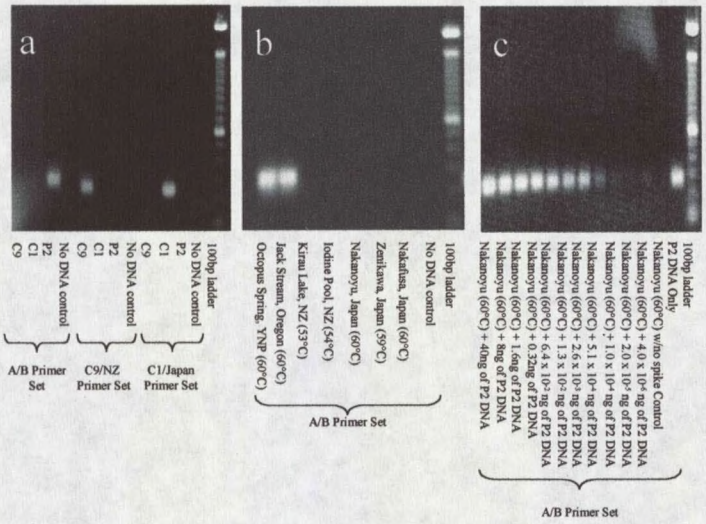

Figure 3. Lineage-specific PCR of *Synechococcus* in different geographic regions. (a) Specificity of PCR reactions for A/B, C9 and C1 *Synechococcus* lineages, and reactivity of A/B lineage PCR with (b) samples from all countries and (c) a Japanese sample containing various amounts of added DNA from a type-B *Synechococcus* isolate.

sensitivity of on the order of 4-100 genomes (see Methods). We further analyzed 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 verify that primers amplified 16S rRNA molecules belonging to those lineages and to detect additional diversity if present. We recovered only 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 Figure 1 and Table 1).

Geochemical Patterns

To assay the general physical/chemical character of the sampled springs we measured 20 parameters, including several that are known to affect cyanobacterial distribution within geographic regions[31-33]. The North American, Japanese and New Zealand collections were from springs exhibiting a broad array of temperature, pH and sulfide concentration (46-66 °C, 5.2-9.1 pH, 0-48.6 μM sulfide; Table 1 and Appendix, Supplemental Table 3). Figure 4 shows the results of cluster analysis based on chemical parameters, with springs from different countries highlighted in different colors. While there is evidence of small-scale clustering of springs within the same geographic 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

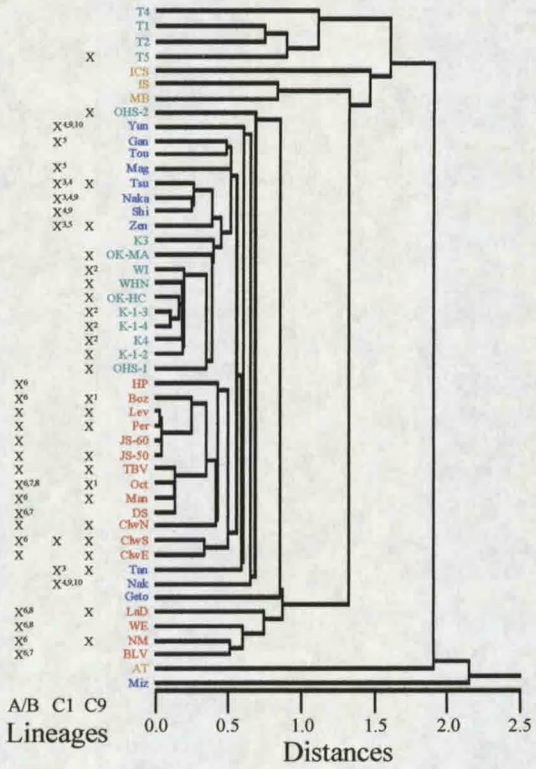

Figure 4. Hierarchical cluster analysis of hot spring chemical parameters compared to 16S rRNA lineages and specific genotypes found in each hot spring. Hot springs are color coded by country: green, New Zealand; blue, Japan; yellow, Italy; and red, North America. An X indicates that a 16S rRNA genotype from the A/B, C9 or C1 lineage was detected by either cloning or probing. X's 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. For spring names, see Appendix, Supplemental Table 3.

(BLV, NM,WE, LaD) and alkaline siliceous (HP, ClwE, ClwN, ClwS, DS, Man, Oct and TBV) water chemistries. Oregon springs (Per, Lev, and JS) and a spring at the northern extreme of the Greater Yellowstone Ecosystem (Boz) cluster among alkaline silicious springs. 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 and5); 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 Figure 4, no strong association between distribution of genotypes and the physical/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, 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 Figure 4). Conversely, geographically separated springs that are nearly identical in chemistry (e.g., Oct, Man, DS) do not always contain the same genotypes (Figure 2a).

Importance of Geographic Isolation

Our first hypothesis reflects the idea that there are no dispersal barriers for bacteria. If this were true we would expect identical genotypes and the same patterns of diversity in all geographic sites. This was certainly not found (Figures 1 and 2 and Table 1 and 2). A/B type *Synechococccus* were detected only in North America where they predominate, even though extremely sensitive lineage-specific PCR could detect numerically rare members of this lineage if present (Figure 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. The C9 lineage was the dominant *Synechococcus* lineage detected in New Zealand and was the only lineage detected in all three countries. The patterns observed instead support our alternative hypothesis that each site is dominated by different *Synechococcus* linages, as would be expected if dispersal barriers exist. The pattern is made more complex by the occurrence of some lineages in more than one country. If dispersal and invasion involving members of such lineages were frequent, we would expect that the specific genotypes detected in different geographic 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 and in some cases separate clades were observed for genotypes detected in different countries. There was even some evidence to suggest that genotypes within lineages differed within geographic subregions in Japan and North America. The differences in distribution of the three *Synechococcus* 16S rRNA-defined lineages and the specific genotypes they

contain suggest that geographic barriers do exist, and that members of different lineages exhibit different abilities to disperse and/or invade.

The distribution of biological variation relative to hot spring physical/chemical variation provides additional evidence of geographic isolation. 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 physical/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. Second, there is evidence that different geographic clades contain genotypes found in springs of similar chemistry. Third, widely varying chemical environments do not a priori restrict *Synechococcus* colonization (or adaptation), as exemplified by lineage C9. Collectively, the data suggest strongly that geographic isolation is involved in divergence of hot spring cyanobacteria.

In the case of the North American A/B lineage we have clear evidence of evolutionary radiation that can be explained by a combination of adaptation and geographic isolation. Evolutionary radiations have apparently also occurred in other *Synechococcus* lineages as well as the *O. amphigranulata* lineage (Figure 1), but we have little evidence at present for the roles that adaptation and/or geographic isolation may have played. It is interesting to note the difference in patterning of diversity within a lineage at different geographic 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 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.

Conclusion

Our results demonstrate that geographic isolation does influence the distribution and evolution of bacterial species. This raises the interesting possibility that divergence and speciation of bacteria does not necessarily depend 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 therefore an oversimplification, since 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 geographic effects at local spatial scales or in less island-like habitats it might be necessary to use highly sensitive approaches, especially in the case of organisms with a propensity to disperse. Our results also suggest that both adaptation and geographic

isolation must be considered as factors acting upon variation within 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 like Yellowstone National Park, especially in times when there is increasing sampling pressure, and especially since Yellowstone contains the greatest lineage diversity and the most unique genotypes among all countries we investigated.

Methods

Sample Collection and Microscopy.

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) prior to cell lysis and DNA extraction and purification[40]. PCR was performed[41] with Taq polymerase (Fisher) with primers 1070F (5'-ATGGCTGTCGTCAGCT)[41] and 23R (5'-TGCCTAGGTATCCACC) (*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, San

Diego, Ca). Either 32 or 48 colonies per sample were picked for sequence analysis. Cloned plasmids were purified using the QIAprep 96 Turbo Miniprep Kit (Qiagen, Valencia, CA). Sequencing was performed on an Applied Biosystems 310 genetic analyzer using primers 1070F and 1505F (5'-GTGAAGTCGTAACAAGG). Sequences have been submitted to GenBank. 16S rRNA sequences were edited using Sequencher 3.0 (Gene Codes Corp. Inc., Ann Arbor, MI) 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.[42]. ITS trees were constructed using the neighbor joining distance algorithm within the PAUP* phylogenetic software package[43]. 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[35,36]. Speksnijder et al.[35] 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 4-fold for 16S rRNA and 3-fold for ITS) and additionally 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 nontarget 16S rRNA molecules in the Ribosomal Database Project[44]: A/B lineage probe, *E. coli* position 1282 (5'-CTGAGACGCGGTTTTTGG); C-9 lineage

probe, *E. coli* position 1250 (5'-CGCTGGCTGGCTACCCTT); C-1 lineage probe, *E.coli* position 1253 (5'GCCCTCGCGGGTTGGCAACT); cyanobacterial lineage probe CYA359F[45]. Total RNA was extracted, purified and quantified[46]. 400 ng of community cyanobacterial 16S rRNA from triplicate mat samples was fixed to a nylon membrane using a Bio-Rad 96 well dot blot apparatus. Oligonucleotide probes were end-radiolabeled 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 analyzer (Molecular Dynamics) and images were analyzed using Scion Image (Scion Corporation, Worman's Mill, CT). Specific lineages were quantified as a percentage of the total cyanobacterial community 16S rRNA from standard curves (R^2 values: ranged 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 ca. 3ng 16S rRNA, which is 0.75% of the spotted RNA.

Lineage-SpecificPCR.

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 into 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 $4 \times 10^{-6} - 10^{-4}$ 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, sulfate, arsenic, boron, chromium, copper, iron, manganese and zinc were analyzed by Peak Analytical Services, Inc. (Bozeman, MT) using standard analytical methods[47]. We analyzed sulfide, nitrate, nitrite, ammonia and phosphate colorimetrically[48-50]. All water samples were subjected to 0.2 micron filtration prior to analysis. Hierarchical clustering analysis of the above 19 parameters plus pH (Appendix, Tables 3 and 4) was done with Systat 9 software (SPSS Inc, Chicago) using single linkage and Euclidian distance algorithms. Prior to analysis all concentration values were standardized to alleviate artifacts possibly arising from large scale differences.

REFERENCES CITED

1 Ochman, H., Lawrence, J.G. and Groisman, E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405: 299-304.

2 Doolittle, W.F. 2000. Uprooting the tree of life.. *Sci. Am.* 282: 90-95.

3 Mayr, E. 1999. Understanding evolution. *Trends Ecol. Evol.* 14: 372-373.

4 Rozenzweig, M.L. 1995. *Species Diversity in Space in Time*. Cambridge University Press, Cambridge.

5 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.

6 Giovannoni, S.J., Britschgi, T.B., Moyer, C.L. and Field, K.G. 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* 345: 60-63.

7 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.

8 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.

9 Ferris, M.J. and Palenik, B. 1998. Niche adaptation in ocean cyanobacteria. *Nature* 396: 226-228.

10 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.

11 Moore, L.R., Rocap, G. and Chisholm, S.W. 1998. Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. *Nature* 393: 464-467.

12 West, N.J. and Scanlan, D.J. 1999. Niche-partitioning of *Prochlorococcus* populations in a stratified water column in the eastern North Atlantic Ocean. *Appl. Environ. Microbiol.* 65: 2585-2591.

13 Field, K., Gordon, D., Wright, T., Rappé, M., Urbach, E., Vergin, K., and Giovannoni, S.J. 1997. Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. *Appl. Environ. Microbiol.* 63:63-70.

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

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

16 Finlay, B.J. and Clark, K.J. 1999. Ubiquitous dispersal of microbial species. *Nature* 400: 828.

17 Fenchel, T. 1993. There are more small than large species. *Oikos* 68: 375-378.

18 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.

19 Norris, R.D. and de Vargas, C. 2000. Evolution all at sea. *Nature.* 405: 23-24.

20 Staley, J.T. and Gosink, J.J. 1999. Poles apart: biodiversity and biogeography of sea ice bacteria. *Annu. Rev. Microbiol.* 53: 189-215.

21 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.

22 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.

23 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.

24 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.

25 MacArthur, R.H. and Wilson, E.O. 1967. *The Theory of Island Biogeography* Princeton University Press, Princeton.

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

27 Castenholz, R.W. 1978. The biogeography of hot spring algae through enrichment cultures. *Mitt. Internat. Verein. Limnol.* 21: 296-315.

28 Weller, R., Bateson, M.M., Heimbuch, B.K., Kopczynski, E.D., and Ward, D.M. 1992. Uncultivated cyanobacteria, *Chloroflexus*-like inhabitants, and *Spirochete*-like inhabitants of a hot spring microbial mat. *Appl. Environ. Microbiol.* 58: 3964-3969.

29 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.

30 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.

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

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

33 Ward, D.M. and Castenholz,. R.W. 2000. in *The Ecology of Cyanobacteria.* Whitton, B.A. & Potts, M. (eds.) Kluwer Academic Publishers, The Netherlands.

34 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.

35 Speksnijder, A.G., Kowalchuk, G.A., DeJong, 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.

36 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.

37 Garcia-Pichel, F. and Castenholz, R.W. 1990. Comparative anoxygenic photosynthetic capacity in 7 strains of a thermophilic cyanobacterium. *Arch. Microbiol.* 153: 344-351.

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

39 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.

40 More, M.I., Herrick, J.B., Silva, W.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.

41 Ferris, M.J., Muyzer, G. and Ward, D.M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defmed populations inhabiting a hot springs microbial mat community. *Appl. Environ. Microbiol.* 62: 340-346.

42 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.

43 Swofford, D.L. 2001. *Phylogenetic analysis using parsimony (*and other methods)* Sinauer Associates, Sunderland, MA.

44 Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T. Jr., Saxman, P.R., Rarris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., and Tiedje, J.M. 2001. The RDP-II (Ribosomal Database Project). *Nucleic Acids. Res.* 29: 173-174.

45 Nübel, U., Garcia-Pichel, F. and Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.* 63: 3327-3332.

46 Kingston, R.E., Chomczynski, P. and Sacchi, N. 1998. Unit 4.2. in *Current Protocols in Molecular Biology*. Ausubel, F.M. *et al.* (eds) John Wiley & Sons, Inc., U.S.A.

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

and American Water Works Association and Water Environment Federation, Baltimore.

48 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.

49 Wetzel, R. and Likens, G. 1991. *Limnological Analyses*. Springer-Verlag, New York.

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

CHAPTER 3

THE IMPORTANCE OF ISOLATION IN MICROBIAL EVOLUTION*

Introduction

With respect to the diversification or speciation of prokaryotes, most studies focus on the importance of variation created by mutation and/or lateral gene transfer (LGT) and subsequent Darwinian selection through competition for resources (i.e. adaptation[1-11]). However, in sexual populations, adaptation and selection are not the sole mechanisms of evolution; other mechanisms such as neutral evolution and/or genetic drift also impact population divergences. Curiously, genetic drift has not been seriously considered as a mechanism for prokaryotic evolution. This probably stems from two assumptions about bacterial populations in nature: (1) they are extremely large and (2) they can freely disperse to all parts of the globe. In recent years, with the advent of molecular techniques for the identification of prokaryotes, many studies have revealed evidence of population isolation occurring in many types of habitats (ranging from insect hosts to marine and soil environments). Our objective is to review studies that demonstrate microbial population isolation and to consider their findings within well-established general evolutionary theory to illustrate that adaptation and selection

*After chapter 2 is published, this manuscript will be submitted to *Environmental Microbiology* as a minireview as: Papke, R.T. and D.M. Ward. The importance of isolation in microbial evolution.

may not account for all microbial diversification or speciation. We first review evolutionary theory, proceed to the evidence for physical isolation, and then consider the ramifications of isolation in microbial evolution.

Isolation in Sexual Species

In modeling the processes involved in the speciation of plants and animals, evolutionary theorists proposed that the isolation of organisms generates barriers to gene flow and that isolation events are considered "extrinsic impediments ... envisioned as prerequisite for genetic divergence...."[12]. For sexual organisms, gene flow within a population tends to act as a cohesive force preventing genetic divergences by homogenizing the gene pool. When organisms become isolated from their parental population (for whatever reason) this cohesive force is escaped. Subsequent to an isolation event, the restriction of gene flow between the two resulting populations allows a nascent population to independently accumulate mutations and/or lose allelic diversity through genetic drift, eventually culminating in the formation of a new species or extinction. Indeed, it has been suggested that "the evolution of new species is equivalent to the evolution of genetic barriers to gene flow between populations[13].

Allopatric speciation (resulting from physically separated populations) is the best documented and the easiest isolation process to contemplate. As a result of discrete geographical ranges, interpopulation reproduction is prevented because individuals from the different populations rarely or never co-occur. Isolation can also

be viewed as a pre-mating mechanism for the prevention of gene flow. Other premating mechanisms include evolved barriers to genetic exchange, such as mate recognition or behavioral displays. Additionally, post-mating mechanisms can isolate populations (i.e. chromosomal inversions or different numbers of chromosomes), but these mechanisms only tend to reduce reproductive success, not eliminate it[13]. While pre- and post-mating mechanisms are not usually thought to apply to bacterial evolution, they certainly can in the case of acquisition of laterally transferred DNA/genes.

Isolation in Prokaryotes

Despite the clonal reproductive mode of prokaryotes, they do have a "sexual" component to their survival strategy, but unlike truly sexual organisms, genetic exchange is not required for their life cycle or reproductive success. Prokaryotes have the ability to integrate horizontally transferred genes or gene segments into their genomes from virtually any donor using homologous recombination (acquisition of a different allele(s) for an existing gene(s)), nonhomologous recombination (acquisition of a novel gene(s)) or through the acquisition of genetic elements (i.e. plasmids). The creation of metabolic pathways[14] and operons[15] are prime examples of the importance of LGT in the generation of vast physiologic and genetic diversity both within a single organism and among all of the prokaryotes. Like sexual organisms, prokaryotes have also evolved mechanisms for "sexually" isolating their genomes that restrict or exclude extraneous or "non-self" DNA. For a successful sexual event (i.e. recombination) to

take place close physical proximity of the recipient to the donor cells or their DNA is required. The absence of proximity acts as a pre-mating barrier. Furthermore, a series of post-mating barriers must be overcome that include: uptake of DNA into the cell, escape from restriction enzymes that destroy inappropriately modified donor DNA inside the cell, DNA sequence divergence, recombination joint formation, mismatch repair (MMR) and SOS systems and functional compatibility of the gene in its new host (For review see[16,17]). The frequency of homologous recombination can be predicted by a mathematical log-linear relationship for *Bacillus*[18], enterobacteria[19] and yeast[20] and is determined by the DNA sequence divergence between the two strands; the more differences that exist between the two strands, the less likely they are to recombine in a transformation assay. However, the frequency of homologous recombination can be skewed away from that predicted by the mathematical models through the regulation of MMR and SOS systems, thus permitting recombination between unrelated organisms or prohibiting recombination between even the closest relatives[19,21]. This is especially true for microorganisms experiencing stress in their environment[22].

The role of recombination as a cohesive force for homogenizing prokaryotic population diversity is different from that observed in sexually reproducing organisms. In an asexual population, acquisition of a new allele via LGT may allow an individual cell that has gained such variation to out-compete its conspecific relatives for resources and systematically replace the entire genetic structure of the population with a genome descending from that single organism[2,23,24]. In evolutionary terms, the selection coefficient of this variant is greater than that of other members of the population.

Mutations that increase the selection coefficient can have the same effect. This purging effectually homogenizes the diversity within the entire population and acts as a cohesive force that prevents the population from diverging. It is only when individual organisms within a population escape this cohesive force, which places boundaries on the population diversity, that individuals can begin to diverge along separate evolutionary pathways. One way an individual can escape genetic diversity purges would be to avoid direct competition for nutrients via previously acquired ecological novelty; in this regard, lateral gene flow, especially nonhomologous gene flow, may be the cornerstone of variation acquisition and speciation. However, in the light of studies from plant and animal evolution, another way of enabling microorganisms to escape the cohesive forces restraining their population diversity would be physical isolation from the parental population.

Physical Isolation of Bacterial Populations

Host-symbiont Population Isolation

If a symbiotic association between a microbe and a host is permanent, and if the period of interaction has extended back in time through a series of host speciation events, it is possible for the symbiont to evolve in parallel with its host. When this occurs it is because each bacterial symbiont forms an allopatric population. As such, each is physically removed from periodic purges of sister populations in other hosts and each is thus free to diverge at a mechanistic level. For instance, the two populations are likely to

be physically prevented from acquiring DNA from each other via LGT events (i.e. sexual isolation). This type of relationship effectively breaks the cohesive forces that would ordinarily keep closely related bacterial populations from diverging, thus resulting in the formation of allopatric bacterial species whenever the host speciates. Probably the simplest method for detecting co-speciation events between a host and its symbiont is when their two evolutionary histories are congruent (i.e. phylogenetic trees have identical topologies for hosts and symbionts) (Figure 5). Microorganisms associated in symbiotic relationships provide excellent models enabling microbiologists to observe the formation of bacterial allopatric species and this is perhaps the simplest and best way to understand microbial isolation and its ramifications.

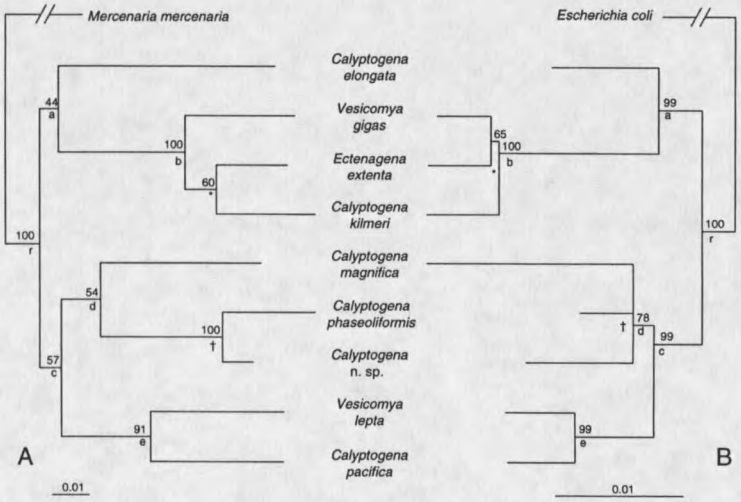

Figure 5. Maximum likelihood phylogenies for nine species of vesicomyid clams and their associated endosymbionts. (A) The vesicomyid tree was based on combined data from portions of the mitochondrial cytochrome oxidase subunit I and 16S rDNA genes (B) The bacterial tree was based on a portion of small subunit 16S rDNA. Host and symbiont trees were not drawn to the same rate scale (scale below each tree) After Peek et al.[25].

We would assert that any time there is phylogenetic congruence between host species and their symbionts there is a high likelihood of population isolation. We do not exclude the possibility that other evolutionary mechanisms (e.g. selection) have also played a part in the association. However, we emphasize that once the initial symbiosis is formed, subsequent allopatric speciation in concert with a host need not be the result of different populations having acquired adaptations and becoming differentially selected in the Darwinian sense.

In the past decade, several microbe-host associations have been discovered to be co-speciating. We have categorized these parallel evolutionary paths according to the relationship between the host and the symbiont. Host-endosymbiotic relationships occur when the symbionts live in specialized cells of the host (bacteriocytes) and are transmitted vertically from generation to generation through eggs of the host, and when the host requires a nutritional supplement(s) from the symbiont. Host-pathogen relationships are those in which the symbiont causes a disease exclusively in its host despite the symbiont's intermediate "free-living" life stage. An example of the first type of relationship is deep-sea hydrothermal vent vesicomyid clams and their proteobacterial sulfur-oxidizing endosymbionts. To ascertain and compare the phylogeny of the host with its symbiont, Peek et al.[25] sequenced two genes of the hosts' mitochondrial genomes (cytochrome oxidase subunit I and 16S rRNA) and the 16S rRNA gene of the symbionts' genomes (Figure 5). After rigorous statistical analysis of the phylogenetic trees produced from the sequenced genes, congruence between the hosts' and symbionts' evolutionary histories was strongly established. This, in combination with factors such as the vertical

transmission of the symbiont via host eggs, the dependence of the host on the symbiont for nutrients, and lack of evidence for a "free-living" version of the symbiont in surrounding waters, strongly suggested that these organisms have maintained a long term relationship and have co-speciated as a result of their dependence upon each other. Other well-studied co-speciating animal-bacterial host-endosymbiotic relationships have been observed in the following hosts: aphids[26-28], whiteflies[29-30], carpenter ants[31], cockroaches and termites[32], tsetse flies[33] and gutless worms[34].

The host-pathogen type of symbiotic relationship can be illustrated by the marine red algal genus *Prionitis* (Rhodoyphyta, Halymeniaceae, Gigartinales) and its gall-forming (tumorigenesis) pathogen from the *Roseobacter* group within the α-*Proteobacteria*. In research presented by Ashen and Goff[35], the internal transcribed spacer regions (ITS) from different *Prionitis* species were sequenced and compared phylogenetically to the 16S rRNA gene sequences of the pathogens found in galls of each host (Figure 6). The results are complicated by the fact that the named species *P. filiformis* is not monophyletic. However, the co-evolution pattern is still "consistent and parsimonious" if we consider only the gall + phenotypes: gall + *P. decipiens* is more closely related to gall + *P. lanceolata,* with the gall + *P. filifomis* forming a sister group to the other two species. Cross inoculations of the pathogens to the hosts showed that the host-pathogen relationships were specific, thus increasing confidence that the different gall-forming pathogens were co-speciating with their specific *Prionitis* host species. Other excellent examples of infectious agents co-evolving with their hosts include *Silene* spp. and its anther smut fungus *Microbotryum violceum*[36], hantaviruses with *Peromyscus*

leucopus mice in North America[37], and herpesviruses with their mammalian and avian hosts[38].

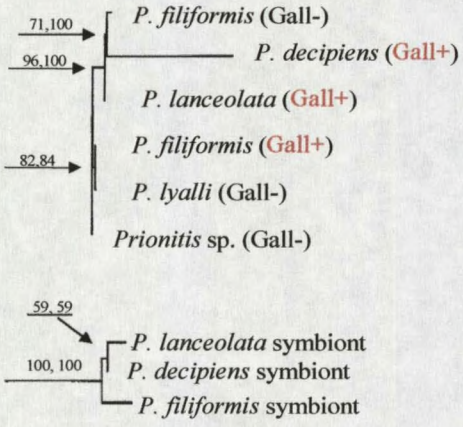

Figure 6. Red algal species (*Prionitis*) phylogenetically compared to the pathogens found in galls of each host. (A) Phylogeny of different *Prionitis* species using the internal transcribed spacer regions. Values at nodes are from bootstrap analysis performed using maximum likelihood. The second number is a bootstrap value after the most diverged sequence was removed from the analysis. (B) Phylogeny of different pathogens using 16S rRNA gene sequences. Bootstrap analysis was performed using parsimony (1st number) and maximum likelihood (2nd number). Modified after Ashen and Goff[35].

Geographic Isolation

The concept that "free-living" bacteria are incapable of forming isolated populations appears to date back to the beginning of the 20th century when it was hypothesized that "everything is everywhere and nature selects"[39,40]. This hypothesis suggests that population genetic cohesiveness can never be broken by isolation. Support for this notion generally relies upon the assumptions that bacteria are small, can form resistant physiologically inactive stages and have extremely large population sizes. There

are many examples of studies in which researchers claim evidence in support of this hypothesis[41-51]. However, the data can often be inconclusive with respect to what constitutes identity between organisms found in different places. For instance, similar morphology and/or physiology, identical restriction patterns of highly conserved genes (e.g. 16S rRNA), or nearly identical 16S rRNA genotypes may be too insensitive to detect important differences in genetic variation among organisms investigated (see below).

Recently, the "everything is everywhere" hypothesis has been invoked to explain why the relationship between body size and species numbers breaks down for organisms smaller than 1mm[52-54]. Among animals there is a general trend of more species per taxon when the average body size is small, (e.g. 751,000 insect species vs. 281,000 for all other animals[55]). Despite the small size of bacteria, there is a paucity of named bacterial species (ca. 5000). The researchers cited above explain the lack of microbial species as a consequence of ubiquitous dispersal; therefore, allopatric speciation (i.e. population isolation) is considered an extremely rare event in the evolution of "free-living" microbes. However, the "everything is everywhere" hypothesis has recently been challenged by a number of studies suggesting that isolation, even in "free-living" microorganisms may be more common than thought[56-73].

An excellent example of the dependence of observing geographic isolation on suitably sensitive methods comes from work on soil bacteria. From soils collected at 10 sites on four continents (North America, South America, Australia, and Africa), Cho and Tiedje[61] cultivated 248 fluorescent pseudomonads, organisms containing a

chromosomally derived selectable trait. Three different levels of identity analysis were examined: (i) restriction patterns of the conserved 16S rRNA gene, (ii) restriction patterns of the more highly variable 16S-23S rRNA ITS region, and (iii) an extremely sensitive method that takes advantage of the entire genome's complexity, repetitive extragenic palindromic-PCR (REP-PCR). Restriction pattern analysis of the 16S rRNA gene revealed very few differences among all of the strains and no endemism. Restriction analysis of the ITS region revealed some endemism. However, with REP-PCR no identical genotypes were found among any of the different regions sampled (Figure 7) leading the authors to conclude that "geographic isolation plays an important

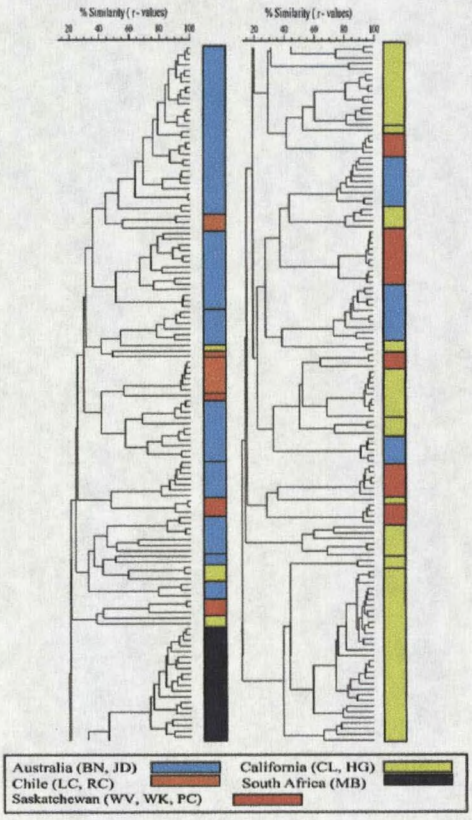

Figure 7. REP-PCR band pattern similarity dendrogram demonstrating the relationship between the genetic diversity of fluorescent *Pseudomonas* cultivated from soils and the geographical origins of the isolates. Modified after Cho and Tiedje[61].

role in bacterial diversification". Fulthorpe *et al.*[67] found similar results with 3-chlorobenzoate-utilizing strains using similar analyses and soil samples.

A second example of geographical isolation comes from our own observations of hot spring cyanobacterial mats[56]. From 42 hot springs located in four countries (United States, Japan, New Zealand and Italy), the biogeographical patterns of thermophilic cyanobacteria were determined using PCR amplification of the 16S rRNA (Figure 1) and 16S-23S rRNA ITS region (Figure 2). Lineage-specific probing was used to verify that the cloning approach detected dominant populations and lineage-specific PCR was used to search for rare genotypes (Figure 3). Each of the four main cyanobacterial lineages appeared to vary with respect to dispersal capabilities: the A/B *Synechococcus* lineage was detected only in North America, the C1 *Synechococcus* lineage was detected in North America and Japan, the *Oscillatoria amphigranulata* lineage was detected in Japan and New Zealand; and, the C9 *Synechococcus* lineage was detected in all of these countries. Each country's hot springs were dominated by a different *Synechococcus* lineage (Tables 1,2 and 3). We never found identical cyanobacterial 16S rRNA genotypes in the different countries. Sequences found in different countries and subregions often exhibited distinct clades at the 16S rRNA or ITS loci (Figure 2), providing evidence that endemism exists within as well as between countries. Distribution patterns did not correlate with hot spring physical/chemical parameters.

The Ramifications of Population Isolation

From the examples above and numerous other studies, it is evident that physical isolation can be an integral element in the biology of microbial populations (symbiotic and free-living), though its importance may vary among different bacteria. Therefore, genetic drift, founder effects and/or neutral evolution should be considered when studying microbial diversity, evolution and ecology. Population isolation may be more common than is currently appreciated, as the ability to detect isolation depends upon the resolution of the genetic marker(s) used. The role of isolation should be also be considered when contemplating LGT, as physical proximity and barriers to gene flow are realities that must affect the LGT process. In a general theory of microbial evolutionary ecology, isolation should be thought of as one mechanism of speciation, not the only mechanism of speciation. Adding isolation to a developing theoretical framework for microbial evolution does not displace other mechanisms, such as adaptation arising from mutation, LGT, or both. All of these mechanisms must be considered to gain complete insight into how microbes evolve.

REFERENCES CITED

1 Cohan, F.M. 1996. The role of genetic exchange in bacterial evolution. *Am. Soc. Microbiol. News.* 62:631-636.

2 Cohan, F.M. 2001. Bacterial species and speciation. *Syst. Biol.* 50:513-524.

3 Hacker, J and Carniel E. 2000. Ecological fitness, genomic islands and bacterial pathogenicity: a Darwinian view of the evolution of microbes. *EMBO Reports* 2:376-381.

4 Rainey, P.B., Buckling, A., Kassen, R., and Travisano, M. 2000. The emergence and maintenance of diversity: insights from experimental bacterial populations. *Trends Ecol. Evol.* 15:243-247.

5 Palys, T., Nakamura, L.K., and Cohan, F.M. 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. *Int. J. System. Bacteriol.* 47:1145-1156.

6 de la Cruz, F. and Davies, J. 2000. Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol.*. 8:128-133.

7 Ochman, H., Lawrence J.G., and Groisman, E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405: 299-304.

8 Lawrence, J.G. 1997. Selfish operons and speciation by gene transfer. *Trends Microbiol.* 5:(9)355-359.

9 Lawrence, J.G. 1999. Gene transfer, speciation, and the evolution of bacterial genomes. *Cur. Opin. Microbiol.* 2:519-523.

10 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.

11 Ward, D. 1998. A natural species concept for prokaryotes. *Curr. Opin. Microbiol.* 1:271-277.

12 Avise, J. 2000. *Phylogeography: The History and Formation of Species.* Harvard University Press. Cambridge, MA.

13 Futuyma, D. 1986. *Evolutionary Biology*, 2nd ed. Sinauer Associates, Inc. Sunderland, MA.

14 Boucher, Y. and Doolittle, W.F. 2000. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol. Microbiol.* 37: 703-718.

15 Lawrence, J.G., and Roth, J.R. 1996. Selfish operons: horizontal transfer may drive the evolution of gene clusters. *Genetics* 143:1843-1860.

16 Majewski, J. 2001. Sexual isolation in bacteria. *FEMS Microbiol Lett.* 199:161-169.

17 Matic, I., Taddei, F., and Radman, M. 1996. Genetic barriers among bacteria. *Trends Microbiol.* 4:69-72.

18 Roberts, M. and Cohan, F. 1993. The effect of DNA seqence divergence on sexual isolation in *Bacillus*. *Genetics* 134:401-408.

19 Vulic, M., Dionisio, F., Taddei, F., and Radman, M. 1997. Molecular keys to speciation: DNA polymorphism and the control of genetic exchange in enterobacteria. *Proc. Natl. Acad. Sci. USA* 94:9763-9767.

20 Datta, A., Hendrix, M., Lipsitch, M., and Jinks-Robertson, S. 1997. Duel roles for DNA sequence identity and the mismatch repair system in the regulation of mitotic crossing-over in Yeast. *Proc. Natl. Acad. Sci. USA* 94:9757-9762.

21 Rayssiguier, C., Thaler, D.S., and Radman, M. 1989. The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature* 342:396-401.

22 Taddei, F., Vulic, M., Radman, M. and Matic, I. 1997. Genetic variability and adaptation to stress. *EXS* 83:271-290.

23 Muller, H.J. 1932. Some genetic aspects of sex. *Am. Nat.* 8:118-138.

24 Atwood, K.C., Schneider, L.K. and Ryan, F.J. 1951. Periodic selection in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 37:146-155.

25 Peek, A.S., Feldman, R.A., Lutz, R.A., and Vrijenhoek, R.C. 1998. Cospeciation of chemoautotrophic bacteria and deep sea clams. *Proc. Natl. Acad. Sci. USA* 95:9962-9966.

26 Bauman, P., Lai, C., Bauman, L., Rouhbakhsh, D., Moran, N.A., and Clark, M.A. 1995. Mutalistic associations of aphid and prokaryotes: Biology of the genus *Buchnera*. *Appl. Envirnon. Microbiol.* 61:1-7.

27 Clark, M.A., Moran, N., Baumann, P., and Wernegreen, J. 2000. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution* 54:517-525.

28 Martinez-Torres, D., Bauades, C., Latorre, A., and Moya, A. 2001. Molecular systematics of aphids and the primary endosymbionts. *Mol. Phylogen. Evol.* 20:437-449.

29 Spaulding, A.W. and von Dohlen, C.D. 2001. *Psyllid* endosymbionst exhibit patterns of co-speciation with hosts and desapilizing substitutions in ribosomal RNA. *Insect Mol. Biol.* 10:57-67.

30 Thao, M.L., Moran, N.A., Abbot, P., Brennan, E.B., Burckhardt, D.H and Bauman, P. 2000. Cospeciation of *Psyllids* and their primary prokaryotic endosymbionts. *Appl. Envirnon. Microbiol.* 66:2898-2905.

31 Sauer, C., Stackebrandt, E., Gadau, J., Holldobler, B., and Gross, R. 2000. Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus Blochmannia* gen. nov. *Int. J. Syst. Evol. Microbiol.* 50:1877-1886.

32 Bandi, C., Sironi, M., Damiani, G., Magrassi, L., Nalepa, C.A., Laudani, U., and Sacchi, L. 1995. The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. *Proc. R. Soc. Lond. B.* 259:293-299.

33 Chen, X., Song, L., and Aksoy, S. 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *J. Mol. Evol.* 48:49-58.

34 Dubilier, N., Giere, O., Distel, D.L., and Cavanaugh C.M. 1995. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. *Appl. Envirnon. Microbiol.* 61:2346-2350.

35 Ashen, J.B. and Goff, L.J. 2000. Molecular and ecological evidence for species specificity and coevolution in a group of marine algal-bacterial symbioses. *Appl. Environ. Microbiol.* 66:3024-3030.

36 Bucheli, E., Gautschi, B., and Shykoff, J.A. 2001. Differences in population structure of the anther smut fungus *Microbotryum violceum* on two closely related host species, *Silene latifolia* and *S. dioica*. *Mol. Ecol.* 10:285-294.

37 Morzunov, S.P., Rowe, J.E., Ksiazek, T.G., Peters, C.J., St. Jeor, S.C., and Nichol, S.T. 1998. Genetic analysis of the diversity and origin of hantaviruses in *Peromyscus leucopus* mice in North America. *J. Virol.* 72:57-64.

38 McGeoch, D.J., Dolan, A., and Ralph, A.C. 2000. Toward a comprehensive phylogeny for mammalian and avian herpesviruses. *J. Virol.* 74:10401-10406.

39 Beijerinck, M.W. 1913. De infusies en de ontdekking der backteriën. In *Jaarboek van de Koninklijke Akademie v. Wetenschoppen*. Müller. Amsterdam.

40 Baas-Becking L.G.M. 1934. *Geobiologie of Inleiding Tot de Milieukunde*. The Hague: Van Stockum & Zon.

41 Estrada-de los Santos, P., Bustillos-Cristales, R., and Caballero-Mellado, J. 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl. Environ. Microbiol.* 67:2790-2798.

42 Massana, R., DeLong E.F., and Pedros-Alio, C. 2000. A few cosmopolitan phylotypes dominate planktonic assemblages in widely different oceanic provinces. *Appl. Environ. Microbiol.* 66:1777-1787.

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

44 Zwart, G., Hiorns, W.D., Methe, B.A., van Agterveld, M.P., Huismans, R., Nold, S.C., Zehr, J.P., and Laanbroek, H.J. 1998. Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. *Syst. Appl. Microbiol.* 21:546-556.

45 Finlay, B.J. and Clarke, K.J. 1999. Ubiquitious dispersal of microbial species. *Nature*. 400, 828-828.

46 Kuske, C., Barns, S.M., and Busch, J.D. 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. *Appl. Environ. Microbiol.* 63:3614-3621.

47 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.

48 Castenholz, R.W. 1978. The biogeography of hot spring algae through enrichment cultures. *Mitt. Internat. Verein. Limnol.* 21:296-315.

49 Castenholz, R.W. 1996. Endemism and biodiversity of thermophilic cyanobacteria. *Nova Hedwigia* 112:33-47.

50 Beeder, J., Nielsen, R.K., Rosnes, J.T., Torsvik T., and Lien T. 1994. *Archaelglobus fulgidus* isolated from hot North Sea oil field waters. *Appl. Environ. Microbiol.* 60:1227-1234.

51 Stetter, K.O., Huber, R., Blochl, E., Kurr, M., Eden R.D. 1993. Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature* 365:743-745.

52 Godfray, H.C. and Lawton, J.H. 2001. Scale and species numbers. *Trends Ecol. Evol.* 16:400-404.

53 Finlay, B.J., Esteban, G.F. and Fenchel, T. 1999. Global diversity and body size. *Nature* 383:132-133.

54 Fenchel, T 1993. There are more small than large species. *Oikos* 68, 375-378.

55 Wilson, E. O. 1992. *The Diversity of life.* W.W. Norton & Co., New York.

56 Papke, R.T., Ramsing, N.B.,Bateson, M.M., and Ward D.M. Geographic isolation and the evolution of hot spring cyanobacteria. Submitted.

57 Pride, D. T., Meinersmann, R.J., and Blaser, M.J. 2001. Allelic variation within *Helicobacter pylori babA* and *babB*. *Infect. Immun.* 69:1160-1171.

58 Edmands, S. 2001. Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. *Mol. Ecol.* 10: 1743-1750.

59 Bertout, S., F. Renaud, R.B., Symoens, F., Burnod, J., Piens, M.-A., Lebeau, B., Viviani, M., Chapuis, F., Bastide, J.-M., Grillot, R., Mallie, M., and The European research group on biotype and genotype of *Aspergillus* 2001. Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. *J. Clin. Microbiol.* 39:1731-1737.

60 Petursdottir S.K., Hreggvidsson, G.O., Da Costa, M.S., and Kristjansson, J.K. 2000. Genetic diversity analysis of *Rhodothermus* reflects geographical origin of the isolates. *Extremophiles*. 4:267-274.

61 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.

62 Sanson, G. and Briones, M. 2000. Typing of *Candida glabrata* in clinical isolates by comparative sequence analysis of the cytochrome c oxidase subunit 2 gene distinguishes two clusters of strains associated with geographical sequence polymorphisms. *J. Clin. Microbiol.* 38:227-235.

63 Beltran, E.C. and Neilan, B.A 2000. Geographical segregation of neurotoxin-producing cyanobacterium *Anabaena circinalis*. *Appl. Environ. Microbiol.* 66:4468-4474.

64 Staley, J. and Gosink, J.J. 1999. Poles apart: biodiversity and biogeography of sea ice bacteria. *Annu. Rev. Microbiol.* 53:189-215.

65 Habeshaw, G., Yao, Q., Beli, A.I., Morton, D., and Rickinson, A. 1999. Epstein-Barr virus nuclear antigen 1 sequences in endemic and sporadic Burkist's lymphoma reflect virus strains prevalent in different geographic areas. *J. Virol.* 73: 965-975.

66 Poussier, S., Vandewalle, P., and Luisetti, J. 1999. Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. *Appl. Environ. Microbiol.* 65:2184-2194.

67 Fulthorpe, R., Rhodes, A., and Tiedje, J. 1998. High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. *Appl. Environ. Microbiol.* 64:1620-1627.

68 Wang G., van Dam, A.P., Spanjaard, L., and Dankert, J. 1998. Molecular typing of *Borrelia burgdorferi* sensu lato by randomly amplified polymorphic DNA fingerprinting analysis. *J. Clin. Microbiol.* 36:768-776.

69 Williams, R.A., Smith, K.E., Welch, S.G., and Micallef, J. 1996. *Thermus oshimai* sp. nov., isolated from hot springs in Portugal, Iceland , and the Azores, and comment on the concept of a limited geographical distribution of *Thermus* species. *Int. J. Syst. Bacteriol.* 46:403-408.

70 Yoshino, K., Ramamurthy, T., Nair, G.B., Fukushima, H., Ohtomo, Y., Takeda N., Kaneko, S., and Takeda, T. 1995. Geographical heterogeneity between Far

East and Europe in prevalence of *ypm* gene encoding the novel superantigen among *Yersinia pseudotuberculosis* strains. *J. Clin. Microbiol.* 33:3356-3358.

71 Vilgalys, R. and Sun, B.L. 1994. Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *Proc. Natl. Aca. Sci. USA* 91:4599-4603.

72 Saul, D.J., Rodrigo, A.G., Reeves, R.A., Williams, L.C., Borges, K.M., Morgan, H.W. and Bergquist, P.L. 1993. Phylogeny of twenty *Thermus* isolates constructed from 16S rRNA gene sequence data. *Int. J. Syst. Bacteriol.* 43:754-760.

73 Hudson, J., Morgan, H. and Daniel R. 1989. Numerical classification of *Thermus* isolates from globally distributed hot springs. *System. Appl. Microbiol.* 11:250-256.

CHAPTER 4

SUMMARY

Analyses of the distribution patterns of organisms can lead to valuable insights into the causes of those patterns. For instance, studies using the 16S rRNA molecule as a molecular marker revealed that from a single model hot spring (Octopus Spring, Yellowstone National Park) a group of closely related *Synechococcus* populations, termed the A/B cluster, was surprisingly organized. Analysis of their distribution suggested adaptation to different habitats (e.g. temperatures and light quantities and/or qualities)[1,2]. Though model systems can be extremely helpful for interpreting the natural history of the organisms studied, they provide an over-focused view of just a part of nature and its explanations. Therefore, it was the purpose of this thesis to extend and broaden the knowledge of the distribution of hot spring *Synechococcus* populations and improve our view of the evolutionary forces that were and are acting upon them. To do so, I analyzed global and local biogeographic distributional patterns using the conserved 16S rRNA gene and the adjacent, more variable, ITS region. My major contribution to the study of microbial evolution and ecology is that every recovered *Synechococcus* genotype (16S rRNA and ITS) had a limited geographical range (and therefore restricted dispersal capability) with many genotypes being restricted to single hot springs. Such results are contrary to the microbial paradigm that "everything is everywhere and nature selects". Furthermore, analyses described in chapter 2 revealed that the geographic

distributions (both within and among countries) could not be correlated with hot spring physical/chemical features (i.e. ecological factors that might determine population ranges) suggesting that geographic isolation (possibly involving founder effects and random genetic drift) plays a role in the diversification of thermophilic *Synechococcus* species. By examining the broader distributional patterns, it becomes clear that natural selection and adaptation are not the only evolutionary forces acting upon the A/B radiation.

The implications of my research are unlikely to be limited to just thermophilic *Synechococcus* species. Though there are relatively few examples of microbial population isolation, the phenomenon is now being discovered in many organisms living in many habitats. Such evidence has led me to propose that population isolation and genetic drift may be important to the divergence of microbial populations (chapter 3). This important aspect of evolutionary theory is not generally considered in explanations of microbial diversity and distribution, as microbiologists generally believe that the major forces shaping microbial diversification and speciation are lateral gene transfer, and natural selection[3-6]. That is to say, microbiologists generally believe that adaptation to specific niches largely explains the divergence and distribution patterns of microbes. The complete scope and importance of population isolation to the evolution of microbes has yet to be evaluated. The simple observation of endemism suggests lateral gene transfer and natural selection are not the only forces affecting microbial evolution. Geographic/physical isolation should be integrated within a general theory of bacterial evolution.

REFERENCES CITED

1 Ferris, M., and Ward, D. 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.

2 Ramsing, N., Ferris, M., and Ward, D. 2000. Highly ordered vertical structure of *Synechoccus* populations within the one-millimeter-thick photic zone of a hot spring cyanobactrial mat. *Appl. Environ. Microbiol.* 66:1038-49.

3 Ochman, H., Lawrence, J.G., and Groisman, E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405: 299-304

4 Doolittle, W.F. 2000. Uprooting the tree of life.. *Sci. Am.* 282: 90-95.

5 Hacker, J and Carniel E. 2000. Ecological fitness, genomic islands and bacterial pathogenicity: a Darwinian view of the evolution of microbes. *EMBO Reports* 2:376-381.

6 Cohan, F.M. 2001. Bacterial species and speciation. *Syst. Biol.* 50:513-524.

APPENDIX A

SUPPLEMENTAL TABLES

Table 3. Physical, chemical and biological data for all hot springs sampled.

					Cyanobacteria					
							Relative Abundance (%)[b]			
Region/ Sample	**Latitude Longitude**	**Temp (C)**	**pH**	**S^{2-} (uM)**	**Morphology[a]**	**Cloned genotypes**	**A/B**	**C-1**	**C-9**	***Synechococcus*[c]**
North America 1										
Jack Stream (JS)-60	42°11' 20"N 120° 20' 41"E	59.8-61.2	8.3	2.5	R	NACy03				
JS-50	42°11' 20"N 120° 20' 41"E	49.7-50.2	8.4	0.7	R	NACy01	60.4± 4.16	nd[d]	4.48± 1.39	65.3
Perpetual (Per)	42° 11' 20"N 120° 20' 41"E	60.1-60.9	8.2	4.6	R		83.3± 2.81	nd	2.76± 0.00	86.1
Levee (Lev)	42° 11' 20"N 120° 20' 41"E	59.5-61.8	8.1	5.5	R		64.6± 2.74	nd	2.11± 0.69	66.7
North America 2										
Bozeman (Boz)	45° 39' 39"N 111° 11' 12"E	57.2-57.5	8.8	7.7	R, f	NACy06, NACy08, NACy10	72.4± 10.8	nd	22.3± 6.07	94.7
North America 3										
LaDuke (LaD)	45° 05' 25"N 111° 46' 28"E	59.8-60.2	6.7	nd	R	NACy10	91.9± 39.2	nd	29.0± 4.41	121
New Mound (NM)	44° 57' 90"N 110° 42' 50"E	56.4	6.6	25.4	R	NACy10	88.6± 12.2	nd	0.60± 0.05	89.3
Bath Lake Vista (BLV)	44° 57' 90"N 110° 42' 50"E	57.0-57.3	6.6	39.3	R	NACy10, NACy11	86.1± 1.74	nd	nd	86.1
White Elephant (WE)	44° 57' 90"N 110° 42' 50"E	59.7-60.0	6.9	8.2	R	NACy10, NACy09	84.3± 3.41	nd	nd	84.3
North America 4										
Clearwater East (ClwE)	44° 47' 19"N 110° 44' 21"E	57.3-61.9	5.2	1.3	R		1.92± 0.08	nd	1.51± 0.50	3.43
Clearwater South (ClwS)	44° 47' 19"N 110° 44' 21"E	56.3-56.5	6.1	0.2	R	NACy10, OS type-C1*[e]	93.4± 8.37	10.3± 1.08	1.27± 0.11	105
Clearwater North (ClwN)	44° 47' 19"N 110° 44' 21"E	54.0-56.2	7.5	nd	R	NACy05	87.1± 17.8	nd	1.61± 0.29	88.7

North America 5										
Octopus (Oct)	44° 32' 35"N 110° 46' 06"E	54.0-64.0	8.2	0.1	R	NACy02, NACy04, NACy05, NACy06, NACy07, NACy10, NACy11, NAC9a*	76.2± 10.6	nd	1.28± 0.36	77.5
Twin Butte Vista (TBV)	44° 32' 35"N 110° 46' 06"E	59.3-60.7	8.4	0.6	R		88.8± 1.75	nd	2.62± 0.24	91.4
Mushroom (Mush)	44° 32' 35"N 110° 46' 06"E	58.9-60.6	8.3	nd	R		85.9± 5.71	nd	2.88± 0.24	88.7
North America 6										
Mantrap (Man)	44° 24' 56"N 110° 34' 29"E	58.2-61.8	8.3	nd	R	NACy04, NACy09, NACy10	92.5± 12.5	nd	1.24± 0.12	93.6
Double Spring (DS)	44° 24' 56"N 110° 34' 29"E	59.1-60.2	8.0	0.6	R	NACy10, NACy11	81.5± 7.52	nd	nd	81.5
Heart Pool (HP)	44° 24' 56"N 110° 34' 29"E	56.6-58.3	9.2	19.5	R	NACy05, NACy10	83.7± 4.12	nd	nd	83.7
Japan 1										
Geto-48	39° 12' 54"N 140° 52' 82"E	48.2	6.3	0.1	F	JapanCy03	nd	nd	nd	nd
Zenikawa (Zen)-62	40° 00' 76"N 140° 48' 20"E	62.0-62.4	8.1	0.2	R	JapanCy01				
Zen-59	40° 00' 76"N 140° 48' 20"E	59.3-59.6	8.1	0.3	R	JapanCy01, JapanCy05	nd	89.8± 10.1	1.39± 0.09	91.2
Zen-57a	40° 00' 76"N 140° 48' 20"E	57.1-56.8	8.1	0.3	R	JapanCy01, JapanCy05				
Zen-57b	40° 00' 76"N 140° 48' 20"E	57.5-57.7	8.3	0.3	R	JapanCy01, JapanCy05				
Zen-55	40° 00' 76"N 140° 48' 20"E	56.1-56.3	8.4	0.3	R	JapanCy01				
Magaroku (Mag)-62	39° 48' 26"N 140° 48' 27"E	62.2	7.0	7.0	R	JapanCy01, JapanCy02				
Mag-60	39° 48' 26"N 140° 48' 27"E	59.8-59.9	7.1	19.7	R	JapanCy01, JapanCy02	nd	93.5± 16.6	nd	93.5
Mag-56	39° 48' 26"N 140° 48' 27"E	56.0	7.0	7.0	R	JapanCy01, JapanCy02				
Mag-51	39° 48' 26"N 140° 48' 27"E	51.1	7.9	4.1	R, F	JapanCy02				
Mag-50	39° 48' 26"N 140° 48' 27"E	49.7-50.2	7.2	23.2	F	JapanCy07, JapanCy12				

Ganiba (Gan)-49	39° 48' 90"N 140° 47' 58"E	49.3	8.4	36.6	R, F	JapanCy01, JapanCy12, JapanCy14	nd	21.3± 3.55	nd	21.3
Mizusawa (Miz)-55	39° 45' 92"N 140° 45' 20"E	55.0	7.6	36.7	a	nd	nd	nd	nd	nd
Japan 2										
Shionoyu (Shio)-59	37° 11'N[#f] 139° 46'E	58.5-59.1	7.3	7.5	R	JapanCy05				
Shio-58	37° 11'N[#] 139° 46'E	58.0	7.3	7.5	R	JapanCy05, JapanCy06				
Shio-56	37° 11'N[#] 139° 46'E	56.6	7.3	7.5	R	JpnCy05				
Japan 3										
Nakafusa (Naka)-66	36° 23' 35"N 137° 44' 80"E	65.8-66.3	8.5	34.5	R	JapanCy09				
Naka-60	36° 23' 35"N 137° 44' 80"E	60.1-60.4	8.5	1.9	R	JapanCy09, JapanCy15, JapanC9a*, JapanC9b*, JapanC9c*	nd	141± 28.7	nd	142
Naka-56	36° 23' 35"N 137° 44' 80"E	55.8-56.4	8.7	4.5	R, F	JapanCy05, JapanCy09				
Shinhokata (Shi)-65	36° 15' 33"N 137° 34' 60"E	64.4-65.4	6.1	0.3	R	JapanCy09, JapanCy10				
Shi-57a	36° 15' 33"N 137° 34' 60"E	57.2-57.6	6.9	0.4	R	JapanCy09, JapanCy10	nd	100.26 ± 9.09	nd	100
Shi-57b	36° 15' 33"N 137° 34' 60"E	57.4-57.6	8.2	0.2	R	JapanCy09, JapanCy10				
Nakanoyu (Nak)-64	36° 12' 34"N 137° 36' 27"E	63.3-61.9	7.5	1.0	R, o	JapanCy09				
Nak-60	36° 12' 34"N 137° 36' 27"E	59.4-60.0	7.8	5.8	R	JapanCy09	nd	74.4± 3.60	nd	74.4
Nak-55	36° 12' 34"N 137° 36' 27"E	54.3-55.3	8.1	6.7	R	JapanCy09				
Nak-49	36° 12' 34"N 137° 36' 27"E	47.2-47.5	8.3	0.5	R, F, O					
Japan 4										
Ninotaira (Nin)-52	35° 15'N[#] 139° 09'E	52.0	7.3	7.5	R	JapanCy05				
Nin-50	35° 15'N[#] 139° 09'E	50.6	7.3	7.5	R	JapanCy05,				
Sokokura (Sok)-60	35° 15'N[#] 139° 09'E	59.2-60.5	7.0	7.0	R	JapanCy05, JapanCy06				

Japan 5										
Yunomine (Yun)-62	33° 49' 75"N 135° 45' 47"E	61.7-61.9	7.8	0.9	R, O	JapanCy09, JapanCy10, JapanCy15				
Yun-55	33° 49' 75"N 135° 45' 47"E	53.8-56.3	8.4	4.1	R	JapanCy09, JapanCy10, JapanCy11	nd	111± 10.8	nd	111
Tousenji (Tou)-55	33° 59' 74"N 135° 48' 36"E	56.8-57.1	9.0	34.3	F	nd	nd	nd	nd	nd
Tou-50	33° 59' 74"N 135° 48' 36"E	49.4-50.9	9.1	8.2	F	JapanCy08				
Japan 6										
Tsuetate (Tsu)-60	33° 10' 95"N 131° 02' 06"E	58.0-59.2	8.6	0.8	R	JapanCy05	nd	87.0± 6.97	1.52± 0.08	88.4
Tsu-55	33° 10' 95"N 131° 02' 06"E	55.8-55.9	8.8	0.4	F	JapanCy05, JapanCy10				
Tsu-50	33° 10' 95"N 131° 02' 06"E	51.9-53.4	8.8	0.5	F, o	JapanCy04, JapanCy05				
Tanoharu (Tan)-53	33° 04' 86"N 131° 07' 54"E	52.4-52.9	7.6	0.6	F,r	JapanCy05, JapanCy13	nd	60.0± 2.93	1.72± 0.07	61.7
New Zealand 1										
Kuirau Reserve (K)-1-4-53	38° 07' 74"S 176° 14' 65"E	48.7-52.9	7.8	1.3	F, r	NZCy03, NZCy01*, NZC1a*, NZC1b*	nd		1.16± 0.19	1.16
K-1-4-50	38° 07' 74"S 176° 14' 65"E	47.1-48.4	8	1.1	R, F	NZCy03				
K-1-3-60	38° 07' 74"S 176° 14' 65"E	58.7-60.9	7.7	1.3	R, f	NZCy03				
K-1-3-50	38° 07' 74"S 176° 14' 65"E	47.4-53.4	7.9	1.3	R, f	NZCy01, NZCy03, NZCy06				
K3	38° 07' 74"S 176° 14' 65"E	50.7-51.7	6.4	1.5	F	NZCy03	nd		nd	nd
K4	38° 07' 74"S 176° 14' 65"E	44.1-48.0	6.9	1.1	R, f	NZCy01	nd		46.8± 6.3	46.8
K-1-2-54	38° 07' 74"S 176° 14' 65"E	55.7-57.7	7.3	1.1	R, f, O	NZCy02				
K-1-2-48	38° 07' 74"S 176° 14' 65"E	46.3-50.6	7.2	1.9	R, f	NZCy03, NZCy07	nd		29.0± 1.60	29
Ohinemutu (OHS)-1	38° 07' 71"S 176° 14' 83"E	50.4-51.7	8.3		R, F	NZCy03	nd		1.13± 0.01	1.13
OHS-2	38° 07' 71"S 176° 14' 83"E	50.4-54.2	8.9	48.6	R, F	NZCy03	nd		20.0± 2.54	20

Whakarewatewa-Ngararatuatara (WHN)-57	38° 09' 94"S 176° 14' 97"E	55.0-59.0	8.2	1.4	F, r	NZCy03, NZCy08	nd		1.33± 0.16	1.33
WHN-52	38° 09' 94"S 176° 14' 97"E	51.3-54.0	8.2	1.1	F, r	NZCy03				
WHN-50	38° 09' 94"S 176° 14' 97"E	46.6-50.1	8.3	1.1	R, f	NZCy03				
New Zealand 2										
Wiamongu-Iodine (WI)-59	38° 16' 65"S 176° 24' 59"E	59.1	8.5	0.8	F	NZCy03	nd		16.3± 4.50	16.3
WI-57	38° 16' 65"S 176° 24' 59"E	57.6	8.5	nd	F	NZCy03				
WI-54	38° 16' 65"S 176° 24' 59"E	53.6-53.7	8.6	0.9	R, f	NZCy01, NZCy03, NZCy08				
WI-50	38° 16' 65"S 176° 24' 59"E	49.8	8.6	0.9	R, f	NZCy04, NZCy05				
New Zealand 3										
Orakei Korako-Hochstetter Cauldron (OK-HC)-57	38° 28' 41"S 176° 08' 82"E	56.6-57.3	7.9	1.3	r, F		nd		9.43± 1.16	9.43
OK-HC-52	38° 28' 41"S 176° 08' 82"E	51.2-53.2	8.2	1.3	r, F	NZCy03, NZCy06				
OK-HC-50	38° 28' 41"S 176° 08' 82"E	47.6-49.7	8.3	nd	R, F	NZCy02, NZCy03, NZCy05				
Orakei Korako-Map of Africa (OK-MA)	38° 28' 41"S 176° 08' 82"E	55.8-56.4	6.6	nd	r, F		nd		0.06± 0.03	0.06
New Zealand 4										
Tokaanu Thermal Reserve (T)1	38° 41'S# 176° 05'E	55	5.6	1.5	F		nd		nd	nd
T3	38° 41'S# 176° 05'E	51	6.3	1.3	F		nd		nd	nd
T5	38° 41'S# 176° 05'E	50.0-51.0	7.3	0.9	R, F		nd		17.2± 1.58	17.2
Italy 1										
Montegrotto Therme Buena Vista Hotel (MB)	45° 31'N# 11° 58'E	54.4-55.4	7.7	nd	F	ItalyCy04, ItalyCy05				

Italy 2										
Island of Ischia-Cave Scurra (ICS)	40° 44'N# 13° 57'E	46.9-49.9	7.9	nd	F	ItalyCy02				
Island of Ischia-Sorgenta (IS)-57	40° 44'N# 13° 57'E	56.2-57.8	7.0	nd	F	ItalyCy03				
IS-52	40° 44'N# 13° 57'E	50.7-51.9	7.0	nd	F	ItalyCy03				
Italy 3										
Agnano Terme (AT)-55	40° 49'N# 14° 07'E	54.2-54.9	7.3	nd	F	ItalyCy02				
AT-50	40° 49'N# 14° 07'E	50	7.1	nd	F	ItalyCy01, ItalyCy02				

a, R/r, unicellular rod; F/f, filamentous; O/o, unicellular, ovoid; a, aggregate (upper and lower cases reflect predominance and presence of the morphotype, respectively.
b, Mean of per-sample lineage-specific probe response relative to cyanobacterial lineage probe response.
c, Sum of means for all three lineages
d, not detected.
e, *, genotype discovered using lineage-specific PCR technique.
f, #, estimated for the general area, but not the spring itself.

Table 4. Chemical measurements for all hot springs sampled

Spring[a]	PO_4[b]	NH_3[b]	NO_2[b]	NO_3[b]	$CaCO_3$[c]	Ca[c]	Cl[c]	Mg[c]	SiO_2[c]	Na[c]	SO_4[c]	As[c]	B[c]	Cr[c]	Cu[c]	Fe[c]	Mn[c]	Zn[c]
JS-60	0.37	2.48	0.57	0.54	64.0	9.00	130	0.50	73.0	190	270	0.15	7.40	0.01	0.01	0.02	0.01	0.03
JS-50	0.04	2.48	0.57	0.54	65.0	9.00	130	0.50	74.0	200	280	0.02	7.50	0.01	0.01	0.02	0.01	0.03
Per	0.37	4.12	0.57	0.54	65.0	10.0	120	0.50	73.0	190	260	0.15	7.40	0.01	0.01	0.02	0.01	0.03
Lev	0.37	10.1	0.57	0.54	72.0	10.0	120	0.50	71.0	190	260	0.13	7.20	0.01	0.01	0.02	0.01	0.03
Boz	0.45	2.48	0.57	0.54	87.0	1.00	53.0	0.50	34.0	130	130	0.11	0.60	0.01	0.01	0.02	0.01	0.03
LaD	0.37	2.48	1.09	0.54	230	340	49.0	59.0	23.0	220	1300	0.02	0.93	0.01	0.01	0.19	0.01	0.03
NM	0.37	24.1	0.57	0.54	720	350	160	65.0	27.0	120	600	0.82	4.30	0.01	0.01	0.70	0.05	0.03
BLV	0.37	26.5	0.57	0.54	720	330	170	63.0	27.0	120	600	0.94	4.70	0.01	0.01	0.02	0.01	0.03
WE	0.37	18.8	0.57	0.54	610	330	170	67.0	30.0	120	670	0.75	4.40	0.01	0.01	0.22	0.01	0.07
ClwE	0.37	14.0	0.57	0.54	8.00	6.00	130	0.50	40.0	74.0	26.0	0.68	2.70	0.01	0.01	0.53	0.04	0.03
ClwS	0.37	6.47	0.57	0.54	31.0	7.00	140	0.50	45.0	94.0	33.0	0.67	2.80	0.01	0.01	0.14	0.10	0.03
ClwN	0.37	4.12	5.65	0.54	84.0	23.0	300	1.00	86.0	190	100	1.30	5.00	0.01	0.01	0.11	0.23	0.03
Oct	0.37	35.3	1.09	6.13	290	0.50	290	0.50	110	300	26.0	1.40	3.30	0.01	0.01	0.02	0.01	0.03
TBV	0.37	2.48	0.57	0.54	270	0.50	320	0.50	110	290	21.0	2.10	3.30	0.01	0.01	0.02	0.01	0.03
Man	0.37	2.48	0.57	1.12	350	0.50	180	0.50	94.0	270	29.0	1.00	2.50	0.01	0.01	0.02	0.01	0.03
DS	0.37	2.48	0.57	0.54	390	0.50	290	0.50	85.0	350	42.0	1.80	3.60	0.01	0.01	0.02	0.01	0.03
HP	0.37	2.48	0.57	2.58	480	0.50	350	0.50	120	410	44.0	2.10	4.30	0.01	0.01	0.02	0.01	0.08
Geto	0.37	12.4	0.20	9.75	314	239	1535	49.0	92.0	574	408	0.01	20.6	0.05	0.05	0.23	0.08	0.03
Mag	0.31	32.5	0.13	1.04	134	34.0	219	2.40	35.0	86.0	130	0.08	2.50	0.05	0.05	0.17	0.14	0.03
Gan	0.31	14.4	0.05	0.73	14.0	121	204	0.50	11.0	45.0	454	0.01	0.12	0.05	0.05	0.12	0.03	0.05
Miz	6.52	476	0.08	1.29	671	186	452	123	71.0	142	1026	0.03	1.90	0.05	0.05	0.05	2.20	0.03
Naka	0.62	3.25	0.08	2.17	67.0	1.60	173	0.50	92.0	68.0	43.0	0.06	0.92	0.05	0.05	0.23	0.03	0.03
Shi	0.19	4.20	0.99	11.2	54.0	3.50	142	0.50	102	48.0	40.0	0.21	1.30	0.05	0.05	0.05	0.03	0.03
Nak	0.64	40.8	0.13	2.80	234	31.0	194	4.00	245	118	91.0	0.04	2.30	0.05	0.05	0.19	0.97	0.03
Yun	0.99	19.7	0.05	1.02	648	8.60	308	1.70	112	374	43.0	0.00	3.00	0.05	0.05	0.17	0.09	0.03
Tou	0.31	7.71	0.08	1.02	94.0	1.50	125	0.50	43.0	55.0	35.0	0.01	2.30	0.05	0.05	0.05	0.09	0.03
Tsu	0.93	23.4	1.24	7.99	35.0	12.0	620	0.50	81.0	287	95.0	0.31	5.40	0.05	0.05	0.05	0.03	0.03
Tan	1.18	21.8	0.43	24.4	231	14.0	259	17.0	245	171	222	0.07	2.60	0.05	0.05	0.12	0.10	0.08
K-1-4	0.62	20.7	0.08	1.37	284	0.50	257	0.50	126	374	132	0.10	6.01	0.05	0.05	0.05	0.05	0.07
K-1-3	0.62	7.94	0.13	1.97	292	0.50	282	0.50	127	385	130	0.10	6.18	0.05	0.05	0.05	0.05	0.06
K3	0.87	51.3	0.13	1.10	78.0	0.50	230	0.50	147	293	262	0.09	5.16	0.05	0.05	0.47	0.05	0.07
K4	0.50	13.6	0.18	1.09	280	0.50	251	0.50	130	343	127	0.09	5.85	0.05	0.05	0.05	0.05	0.07
K-1-2	1.18	15.9	0.15	10.5	318	0.50	277	0.50	127	386	132	0.10	6.27	0.05	0.05	0.05	0.05	0.08
OHS-1	2.24	7.45	0.43	0.27	334	0.50	262	0.50	123	376	77.0	0.04	5.47	0.05	0.05	0.05	0.05	0.07
OHS-2	0.93	8.73	0.08	0.97	378	0.50	275	0.50	146	354	62.0	0.06	5.71	0.05	0.05	0.05	0.05	0.08
WHN	0.62	24.5	0.18	1.57	127	0.50	490	0.50	122	395	86.0	0.74	5.28	0.05	0.05	0.05	0.05	0.07
WI	0.99	19.4	0.50	1.24	202	0.50	667	0.50	133	512	112	0.86	5.92	0.05	0.05	0.05	0.05	0.07
OKHC	0.37	3.09	0.35	2.27	160	0.50	193	0.50	129	239	100	0.38	2.72	0.05	0.05	0.05	0.05	0.07
OKMA	1.18	6.24	0.45	2.21	131	0.50	161	0.50	222	235	102	0.31	2.27	0.05	0.05	0.05	0.05	0.08
T1	0.31	948	0.18	63.8	31.0	5.20	1960	0.50	233	1100	81.0	4.93	55.8	0.05	0.05	0.17	0.05	0.10
T2	0.44	760	0.18	18.9	52.0	7.40	2140	0.50	244	1330	85.0	6.23	61.1	0.05	0.05	0.05	0.05	0.08
T4	0.87	493	17.0	58.2	76.0	7.30	1940	0.50	241	1140	59.0	5.27	57.3	0.05	0.05	0.27	0.05	0.07

T5	0.25	548	0.03	2.19	67.0	6.90	3080	0.50	126	1810	74.0	8.04	89.7	0.05	0.05	0.05	0.05	0.07
MB	0.16	111	13.5	43.8	130	198	1480	43.0	52.0	740	500	0.05	6.50	0.27	0.05	0.14	0.05	0.03
ICS	3.75	16.8	1.45	2.53	940	12.0	1700	1.40	190	1540	680	1.38	3.40	0.26	0.05	0.27	0.51	0.06
IS	0.59	0.24	16.9	56.4	310	14.0	2530	31.0	120	1790	660	0.42	4.20	0.27	0.05	0.14	0.05	0.10
AT	2.02	437	0.16	1.51	1190	230	2870	65.0	160	1800	400	0.45	12.0	0.05	0.05	2.29	0.96	0.10

a, see Appendix Supplemental Table 3 for full spring names
b, μM
c, mg/L

MONTANA STATE UNIVERSITY - BOZEMAN
3 1762 10358950 1