Molecular analysis of hot spring microbial mats to study bacterial diversity and physiology
by Stephen Charles Nold

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
Molecular studies investigating 16S rRNA sequence diversity in cyanobacterial mat communities inhabiting hot springs in Yellowstone National Park have revealed that these communities contain numerous uncultivated microbial species. Here, attempts were made to cultivate from one of these mat communities the aerobic chemoorganotrophic bacteria whose 16S rRNA nucleotide sequences were previously observed using molecular retrieval techniques. By utilizing serial dilution enrichment culture and a variety of enrichment conditions, a diversity of bacterial isolates was obtained. 16S rRNA sequence analysis revealed seven genotypically distinct isolates, including Thermus, proteobacterial, and Gram positive representatives. However, only one of these isolates, a β-proteobacterium, contained a 16S rRNA sequence previously observed in Octopus Spring mat. These results illustrate the differing views of microbial community composition which cultivation and molecular techniques provide, and demonstrate the problems encountered when using cultivation approaches to associate microbial activity with bacterial populations whose 16S rRNA sequences were detected in natural samples.

One cultivation-independent approach to associate bacterial activity with retrieved 16S rRNA sequence types would be to selectively capture rRNA molecules synthesized by actively growing microorganisms incubated in the presence of a radiolabeled substrate, then quantify the relative extent of radiolabel incorporation into specific 16S rRNA molecules. Initial studies investigating the feasibility of this approach revealed that although logarithmically growing cyanobacterial cells incorporated photosynthetically fixed $14CO_2$ into rRNA, cyanobacteria inhabiting hot spring mats predominately incorporated $14CO_2$ into polyglucose during periods of illumination (between 77% and 85% of total incorporated carbon). Although photosynthetically active, the cyanobacteria of these mat communities do not appear to be rapidly growing, since only limited synthesis of growth-related macromolecules was detected. The fate of polyglucose reserves was investigated by allowing mat cyanobacteria to photoassimilate $14CO_2$ into polyglucose, then transferring samples to the dark, anaerobic conditions which mat communities experience at night. Radiolabel in the polysaccharide fraction decreased 74.7% after 12 hours dark incubation, of which 58.5% was recovered in radiolabeled fermentation products (i.e. [14C]acetate, $14CO_2$, and [14C]propionate). These results indicate tightly coupled carbon fixation and fermentative processes, and the potential for significant carbon transfer from primary producers to heterotrophic members of these cyanobacterial mat communities.

MOLECULAR ANALYSIS OF HOT SPRING MICROBIAL MATS TO STUDY

BACTERIAL DIVERSITY AND PHYSIOLOGY

by

Stephen Charles Nold

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APPROVAL

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Stephen Charles Nold

This thesis has been read by each member of the thesis 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.

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ABSTRACT

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One cultivation-independent approach to associate bacterial activity with retrieved 16S rRNA sequence types would be to selectively capture rRNA molecules synthesized by actively growing microorganisms incubated in the presence of a radiolabeled substrate, then quantify the relative extent of radiolabel incorporation into specific 16S rRNA molecules. Initial studies investigating the feasibility of this approach revealed that although logarithmically growing cyanobacterial cells incorporated photosynthetically fixed $^{14}CO_2$ into rRNA, cyanobacteria inhabiting hot spring mats predominately incorporated $^{14}CO_2$ into polyglucose during periods of illumination (between 77% and 85% of total incorporated carbon). Although photosynthetically active, the cyanobacteria of these mat communities do not appear to be rapidly growing, since only limited synthesis of growth-related macromolecules was detected. The fate of polyglucose reserves was investigated by allowing mat cyanobacteria to photoassimilate $^{14}CO_2$ into polyglucose, then transferring samples to the dark, anaerobic conditions which mat communities experience at night. Radiolabel in the polysaccharide fraction decreased 74.7% after 12 hours dark incubation, of which 58.5% was recovered in radiolabeled fermentation products (i.e. [^{14}C]acetate, $^{14}CO_2$, and [^{14}C]propionate). These results indicate tightly coupled carbon fixation and fermentative processes, and the potential for significant carbon transfer from primary producers to heterotrophic members of these cyanobacterial mat communities.

CHAPTER 1

MOLECULAR ANALYSIS OF HOT SPRING MICROBIAL MATS:

A GENERAL INTRODUCTION

Studies employing the techniques of molecular biology have revolutionized our understanding of the microbial world. The realization that the information contained in nucleic acid and protein sequences can be used to reconstruct molecular evolutionary history (55) has led to extensive comparative studies of molecular evolution. One molecule in particular, the small subunit ribosomal RNA, has been central to our understanding of evolutionary relationships among microbial species (51). Comparison of small subunit ribosomal RNA nucleotide sequences has allowed construction of a universal phylogeny based on genetic relationships among organisms without reliance on phenotypic traits (31,52). This universal "tree of life" divides life on this planet into three distinct primary groups, the domains Bacteria, Archaea, and Eukarya (51,53). Two of these domains, the Bacteria and the Archaea, are microbial, and the differences separating these domains are more significant than those which distinguish the traditional kingdoms (i.e. plants and animals) from one another (53).

Molecular studies have also revealed that natural habitats harbor a great diversity of undescribed microbial species. Studies of DNA-DNA reassociation of nucleic acids directly extracted from soils showed that DNA from this habitat is extremely heterogeneous (41). The authors of this study deduced that soil DNA

exhibits complexity comparable to ca. 4000 completely different bacterial genomes, most of which are from uncultivated microbial community members. Studies investigating the genotypic diversity of small subunit ribosomal RNA (more specifically, 16S rRNA) sequence types retrieved from natural microbial communities lead to a similar conclusion: that microbial diversity is both very great and easily surpasses the diversity of validly described microbial species cultivated from natural habitats (1,14,16,43,46). The diversity of uncultivated community members is both scientifically interesting and potentially economically valuable, since microbial physiological diversity has been a source of new natural products used in pharmaceutical, biotechnology, and industrial microbiology applications. However, the physiological diversity of uncultivated community members is largely unknown, since few pure cultures of these species have been obtained for phenotypic characterization.

Molecular analyses have also aided our understanding of the physiological activity exhibited by microorganisms as they occur in nature. Studies investigating elemental composition, patterns of photosynthetically fixed carbon allocation among molecular classes, and synthesis of individual macromolecules have shown that phytoplankton communities are both photosynthetically active and exhibit growth at or near maximal rates (18,19,22,25,26). Not all microbial communities are rapidly growing, however. Novitsky (30) observed a high ATP content combined with a low rate of nucleic acid synthesis in a marine sediment microbial community, indicating that the microbial cells were active, but not rapidly dividing. In other studies, the effects of disturbance and environmental perturbation on molecular synthesis have been investigated by analyzing phospholipid and sterol biomarkers in marine sediment

communities (12,13,24). These studies illustrate the utility of molecular analyses to investigate microbial response to environmental change.

These advances in our knowledge of microbial evolution, diversity, and activity have led to the questions addressed by this thesis. I have chosen the laminated cyanobacterial mat communities inhabiting mildly alkaline silicious hot springs in Yellowstone National Park as units of study. The microbial mat community inhabiting one hot spring in particular, Octopus Spring, has been intensively studied in an effort to make fundamental observations of microbial species composition and physiological activity (5,43-45). This existing information base allows the construction of testable hypotheses concerning the activities which occur in microbial communities and the identity of the microorganisms which perform those activities. The theme which unifies the investigations presented in this thesis is the goal of associating microbial activity with microbial diversity. More specifically, the unifying goal of this thesis is to associate microbial activities which occur in hot spring cyanobacterial mat communities with microbial populations which share identical 16S rRNA sequences. The remainder of this chapter introduces the experiments which were performed to accomplish this goal, and highlights the hypotheses which were tested by those experiments.

Cultivation to Associate Microbial Activity with Microbial Diversity

Selective enrichment culture techniques have been used for more than a century to obtain naturally occurring microorganisms for study in pure culture. The

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microbiologists who originally developed these techniques cautioned that the methods may select for organisms which are best adapted to the enrichment culture environment, but which may not be the dominant organisms in nature (42,50). Comparison of the 16S rRNA sequences of organisms cultivated from Octopus Spring mat to 16S rRNA sequences detected in the mat using molecular retrieval techniques reveals that enrichment culture does indeed fail to cultivate the dominant microorganisms whose rRNAs are detected in natural microbial communities. To illustrate this point, bacteria which were cultivated from Octopus Spring mat and which exhibit an aerobic chemoorganotrophic type of metabolism appear in Table 1, column 1. These five species include representatives from the green non-sulfur bacteria, *Thermus*, and planctomyces lines of descent. Molecular retrieval approaches have revealed many 16S rRNA sequence types representing microorganisms which might exhibit aerobic chemoorganotrophic metabolisms (Table 1, column 2). Some of the 13 unique 16S rRNA sequence types retrieved from this community are related to members of the green non-sulfur, green sulfur, and proteobacterial lines of descent, while others do not readily cluster into known phylogenetic groups. However, Table 1 clearly shows that the species detected by molecular retrieval and cultivation approaches are completely different, illustrating the conflicting views of microbial community composition provided by these two techniques. Similar observations are repeated in other physiologically related groups in the Octopus Spring mat (11,43,44,47,48), as well as other microbial communities (16,37,39). Clearly, our knowledge of microbial diversity based on organisms cultivated from natural environments is incomplete.

Table 1. Bacteria known to inhabit Octopus Spring which have or could have aerobic chemoorganotrophic metabolisms based on cultivation and molecular studies[a].

Previously Cultivated	16S rRNA Sequence Retrieved
Green non-sulfur bacteria	
Chloroflexus aurantiacus Y-400-fl	
Thermomicrobium roseum	
	type C
	OS-V-L-20
***Thermus/Deinococcus* Group**	
Thermus sp. OS-Ramaley-4	
Thermus aquaticus YT-1	
Planctomyces	
Isosphaera pallida IS1B	
Proteobacteria	
Alpha subdivision	type O
Beta subdivision	type G
	type N
	type R
Green sulfur-like bacteria	
	type E
	type M
	OS-III-9
Uncertain Affiliation	
	type L
	type D
	type F
	OP-I-2

[a]Adapted from reference (44).

Careful assessment of the enrichment culture techniques used by previous investigators to cultivate aerobic chemoorganotrophic bacteria from hot spring cyanobacterial mats may help to explain the failure of these methods to recover the numerically abundant microbial populations (i.e those microbial species whose 16S rRNA sequences were detected using molecular retrieval approaches). In general, isolates were obtained by directly streaking mat material onto solidified media (17,21,32) or by the direct addition of undiluted inoculum to enrichment flasks (6) containing relatively high levels of carbon substrates (0.1% to 3% tryptone and yeast extract) (6,21,32) and incubating at high temperatures (70°C) regardless of the temperature of the collection site (6,21). There are several potential problems with these methods. Incubating cultures at temperatures which are different than the sample collection site may select against microorganisms adapted for optimal growth at collection site temperatures. Strain purification by picking isolated colonies requires growth on solidified media; if a bacterial strain is incapable of colonial growth, this species will not appear in culture collections. Directly plating mat inoculum onto solidified media precludes attempts to measure the relative abundance of the cultivated organisms, and directly adding undiluted inoculum to enrichment flasks may promote culture overgrowth by numerically insignificant species. Recently, researchers attempting to cultivate microorganisms from seawater have successfully obtained isolates of oligotrophic ultramicrobacteria (i.e. bacteria smaller than 2 μm which are adapted to low organic carbon concentrations) using serial dilution enrichment culture techniques (7,38). These researchers inoculated a series of flasks containing unamended sterile seawater with a serially diluted inoculum source, initially resulting

in 10-fold fewer microorganisms in each enrichment flask. The isolates obtained from very high (10^6-fold) dilutions resembled the majority of the microorganisms in the original seawater. These experiments illustrate two important points. First, employment of serial dilution enrichment culture techniques may lead to successful cultivation of the more numerically abundant microorganisms from natural samples, and second, not all bacteria are adapted to copiotrophic conditions (i.e. high organic carbon concentrations). Thus, enrichments containing relatively high concentrations of organic carbon may select against microorganisms adapted to oligotrophic conditions. Finally, although microbial mat communities are characterized by extreme environmental gradients (33,34), conditions in culture media are remarkably homogenous. The environmental homogeneity which characterizes most culture media may limit the diversity of cultivated species by failing to provide a range of conditions from which the numerically abundant microorganisms may select for growth (8,54).

The first research objective of this thesis was to attempt to overcome some of the existing problems with enrichment culture techniques in order to cultivate the numerically abundant aerobic chemoorganotrophic bacteria from Octopus Spring mat (see hypothesis I, page 15). Serial dilution enrichment culture was used to provide a measure of the relative abundance of the isolates obtained, and to avoid culture overgrowth by numerically insignificant species (11). Enrichments were also performed under more natural conditions (e.g. incubating at the temperature of sample collection, and using more relevant carbon substrates and concentrations). By successfully cultivating the microorganisms whose 16S rRNA sequences were retrieved from Octopus Spring mat, and associating those sequence types with the

aerobic chemoorganotrophic metabolism, the major goal of this thesis would be addressed. Namely, a microbial activity (aerobic chemoorganotrophy) would be associated with microbial populations whose 16S rRNA sequence types are detected in natural mat samples.

During these investigations, a diversity of bacterial isolates was identified and characterized. Analysis of 16S rRNA sequence types revealed seven genotypically distinct isolates, including representatives belonging to the *Thermus*, proteobacterial, and Gram positive lines of descent. Characterization of the relative abundance and growth characteristics of the cultivated *Thermus* isolates led to the conclusion that *Thermus* distribution may be controlled by specialization to temperature, a condition which varies in hot spring habitats (Chapter 2). Phenotypically distinct Gram positive isolates exhibited identical 16S rRNA nucleotide sequence through a variable region of the molecule, indicating the conserved nature of bacterial diversity estimates based on 16S rRNA sequence information (Chapter 3). However, only one of the seven isolates, a β-proteobacterium, contained a 16S rRNA sequence identical to a sequence type previously detected in Octopus Spring mat using molecular retrieval techniques (Chapter 3). By combining these results with a closely related study of species composition in similar enrichment cultures before strain purification (36), it becomes possible to speculate about the causes of the failure of enrichment culture techniques to recover the numerically abundant microorganisms from natural microbial communities (Chapter 6).

Clearly, there exists a need to cultivate the microorganisms which actually occur in natural habitats. However, even if abundant populations were successfully

cultivated, it would be difficult to extrapolate the phenotypes observed in pure culture

to the activities the cultivated microorganisms exhibit in nature. For example,

photoheterotrophic carbon uptake (the light-induced incorporation of organic carbon)

by the filamentous *Chloroflexus*-like hot spring cyanobacterial mat inhabitants is

considered to be an important mechanism of carbon transfer from the primary

producers (*Synechococcus* spp.) to the primary consumers (the *Chloroflexus*-like cells)

in these communities (2,35). However, cultivation conditions may be sufficiently

different that an ecologically important phenotype such as photoheterotrophy may not

be expressed in pure culture. When *Chloroflexus* cells were first cultivated under

dark, aerobic conditions, the photoheterotrophic metabolic capability of this bacterium

was not fully appreciated (4,5). Likewise, demonstration of a phenotype in pure

culture does not necessarily demonstrate an ecologically important activity.

There appears a need for cultivation-independent methods to assess the activity

exhibited by genetically related microbial populations. Ideally, such a method would

accurately measure microbial activity in situ, and successfully associate microbial

activity with a 16S rRNA sequence type. One possible solution to this challenge may

come from the techniques of molecular biology.

rRNA Synthesis to Monitor Activity of Microbial Populations

Ribosomal RNA exhibits several properties that make it uniquely suited as a

species-specific indicator of bacterial activity. The nucleotide sequence of rRNA

contains conserved and variable regions, allowing design of oligodeoxynucleotide

hybridization probes specific to phylogenetic groups and individual species (23).

Synthesis of ribosomes and ribosomal RNA is also proportional to growth rate.

Studies using *Escherichia coli* as a model organism showed remarkable growth rate-dependent regulation of ribosomal component synthesis (15,29). Using fluorescently

labelled rRNA-targeted oligodeoxynucleotide probes, DeLong et al. (9) showed a

similar result; as growth rate of *E. coli* increased, probe response increased

proportionally. I hypothesized that by providing a microbial community with a

specific radiolabeled substrate, only those populations which are actively growing and

utilizing that substrate would incorporate radiolabel into rRNA. By assaying the

relative amounts of radiolabel incorporation into group- or species-specific rRNA

sequence types, it should be possible to address the major goal of this thesis: to

monitor microbial activity (by measuring radiolabel incorporation into rRNA) and

associate that activity with microbial populations whose 16S rRNA sequence types are

detected in natural mat samples. Obtaining radiolabeled rRNA should not be difficult

if microbial populations are growing. However, separating specific 16S rRNA

sequence types from a mixture containing diverse rRNA sequence types (such as

would be found in a microbial community) requires specialized molecular techniques.

Selectively retrieving sequence-specific rRNA types from a mixture of

community rRNAs has been accomplished using oligodeoxynucleotide hybridization-

based capture probe technology (20,27,28,40). This method requires the specific

hybridization of a biotinylated oligodeoxynucleotide probe to a target 16S rRNA

nucleotide sequence. The probe/target rRNA hybrid is then "captured" onto a

magnetic bead particle by high affinity binding which occurs between the biotin

moiety on the probe and strepavidin molecules which are covalently attached to the magnetic bead. Separation of target rRNA from non-target community rRNAs is accomplished by washing the uncaptured rRNA from the magnetic bead/capture probe/target RNA complex. Thus, capture probes can be used to separate individual 16S rRNA sequence types to assay the extent of radiolabel incorporation into specific rRNAs.

Successful capture probe retrieval of radiolabeled rRNA sequence types requires that rRNA is synthesized in detectable quantities. Before investing the time to develop capture probe methods, the extent of radiolabel incorporation into rRNA in Octopus Spring mat was investigated. Since rates of cyanobacterial oxygenic photosynthesis are very high (34), and previous studies have suggested that cyanobacterial populations are growing (5,10) (see below), photoautotrophic carbon incorporation by $Synechococcus$ species was chosen as a test case to maximize the amount of radiolabel incorporation into rRNA. Therefore, $^{14}CO_2$ was provided as a substrate to predominately radiolabel the photosynthetically active $Synechococcus$ cyanobacterial species. Although $^{14}CO_2$ was readily incorporated into cellular material, no incorporation of $^{14}CO_2$ into rRNA could be detected in Octopus Spring mat (Chapter 4). This observation was somewhat unexpected, given what is known about the growth, activity, and interactions of hot spring cyanobacterial mat inhabitants.

Hot spring cyanobacterial mats are active and dynamic microbial communities. Cyanobacterial gross primary productivity calculated from measured oxygen production rates is 20 to 40 mmol CO_2 m^{-2} hr^{-1} (34). For comparison, these values are greater than the carbon incorporated by the entire photic zone of the world's most

productive lakes (calculated from data provided in Table 15-9 of reference (49)). Other experiments indicate that hot spring cyanobacterial mats are growing. By measuring the decrease of *Synechococcus* cells over time after darkening a mat, Brock (5) estimated cyanobacterial productivity to be 5.7×10^{11} to 1.6×10^{12} cells m^{-2} day^{-1}. Accretion rates of mat material above a silicon carbide layer sifted onto the mat surface provide further evidence for mat growth. Long-term (1 year) measurements of organic material accretion above silicon carbide layers indicated growth rates of 18 to 45 μm day^{-1} (10). There is also evidence for carbon transfer between primary producers and primary consumers in hot spring mat communities. Photosynthetically active *Synechococcus* species photoexcrete glycolic acid (up to 7% of total photosynthate) which is readily incorporated by the *Chloroflexus*-like primary consumers in the mats (3). Under dark, anaerobic conditions, fermentation products are produced at a rate of ca. 10 mmol acetate and propionate m^{-2} hr^{-1} (calculated from (2)). Acetate and propionate accumulate in mats overnight and are photohetero-trophically incorporated by *Chloroflexus*-like cells the following day (2,35). Terminal anaerobic processes occurring in the mats include methanogenesis (methane production rates are ca. 0.8 mmol CH_4 m^{-2} hr^{-1}, (2,14)) and possibly acetogenesis, the anaerobic conversion of hydrogen and carbon dioxide to acetate (2,3). Clearly, cyanobacterial primary production fuels active carbon transfer within microbial mat communities. Mat communities also appear to be growing, which is why the observation that $^{14}CO_2$ is not readily incorporated into rRNA was unexpected.

The second research objective of this thesis was to investigate the fate of photosynthetically fixed carbon in hot spring cyanobacterial mat communities (see

hypotheses II to IV, page 15). This research objective represents a significant divergence from the major goal of this thesis. However, the observation that rRNA was not synthesized by photosynthetically active mat cyanobacteria leads to the possibility that these populations may not be rapidly growing (hypothesis II). The physiological ecology of a microbial community which is very active, but may not be rapidly growing was sufficiently unique to justify this change in research direction. It was hoped that investigations into the fate of photosynthetically fixed carbon would change our perceptions about the activity of cyanobacterial mat inhabitants. Further, it was hoped that conditions under which rRNA synthesis did occur would be discovered, thus permitting use of the capture probe approach.

These investigations revealed that mat cyanobacteria do not allocate significant amounts of photosynthetically fixed carbon into growth-related molecules such as rRNA, protein, and lipid. Instead, photosynthate is stored in the form of polyglucose during periods of illumination (Chapter 4). These results lead to the conclusion that although mat cyanobacteria are photosynthetically active, they do not appear to be rapidly growing. Mat cyanobacteria were shown to ferment polyglucose reserves under the dark, anaerobic conditions which mats experience at night (Chapter 4). Although unanticipated, this finding helped to achieve the unifying goal of this thesis by associating fermentative activities with the genotypically related *Synechococcus* cyanobacterial populations. Cyanobacterial fermentation has been demonstrated in pure cultures, but has not until now been demonstrated in situ. These results indicate the potential for massive carbon transfer between the *Synechococcus* primary producers and the *Chloroflexus*-like primary consumers in these mat communities.

There remain significant questions regarding the basis for this symbiotic relationship (Chapter 6). Although mat communities normally synthesize only limited amounts of rRNA, disturbance to the mat community resulted in patterns of molecular synthesis which more closely resembled logarithmically growing cyanobacterial cells (Chapter 5). These results indicate the potential for successful application of capture probe technologies when mat communities are undergoing post-disturbance recolonization. The experiments described in chapters 4 and 5 have changed our perception of the physiological ecology of hot spring cyanobacterial mat inhabitants, and have allowed the construction of a conceptual model which describes the fate of photosynthetically fixed carbon in these mat communities (Chapter 6).

Hypotheses

The introductory material presented in this chapter allows the construction of testable hypotheses concerning the activity of hot spring cyanobacterial mat inhabitants and the identity of the microorganisms which perform those activities. Hypothesis I was tested by experiments described in Chapters 2 and 3, hypotheses II and III were tested by experiments described in Chapter 4, and hypothesis IV was tested by experiments described in Chapter 5.

I. Application of cultivation techniques which employ more rational enrichment culture approaches will lead to the cultivation of aerobic chemoorganotrophic bacteria whose 16S rRNA sequences have been previously observed in Octopus Spring mat.

II. Since cyanobacterial cells inhabiting hot spring mat communities are photosynthetically active and appear to be rapidly growing, it should be possible to detect synthesis of growth-related molecules such as ribosomal RNA and protein.

III. Cyanobacteria ferment photoautotrophically fixed polyglucose under the dark, anaerobic conditions hot spring cyanobacterial mats experience at night.

IV. Cyanobacterial biomass lost from mat communities during disturbance events is replaced by growth and cellular division.

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CHAPTER 2

DIVERSE *THERMUS* SPECIES INHABIT

A SINGLE HOT SPRING MICROBIAL MAT[1]

Introduction

Recent studies of the laminated cyanobacterial mat community located in

Octopus Spring (Yellowstone National Park, Wyoming) have focussed on species

diversity and evolution (24,26) and microbial community ecology (18,23). To further

understand the diversity of aerobic chemoorganotrophic bacteria in the Octopus Spring

mat we attempted to cultivate numerically abundant species using a variety of different

strategies. Enrichment conditions were designed to favor recovery of species adapted

to substrates and temperatures which reflect prevailing environmental resources and

conditions. Inoculum was serially diluted to extinction to provide an estimate of

relative abundance of the species cultivated (21) and to eliminate rapid overgrowth by

numerically insignificant species (5). In the course of this work we isolated and

characterized several *Thermus* strains. Our results reveal a diversity of *Thermus*

isolates within one hot spring microbial community whose distribution may be

controlled by specialization to environmental features such as temperature that vary in the habitat.

Materials and Methods

Cultivation of *Thermus* Isolates

Samples were collected from the top 1 cm of the cyanobacterial mat community located in the shoulder region (50-55°C, pH 8.5) of Octopus Spring (2) on 30 September 1992 and 29 October 1992. Samples were kept between 46°C and 50°C for 3 hours in transit to the laboratory, then homogenized with a Dounce tissue homogenizer and serially diluted (1:10) in sterile enrichment medium (30 September 1992) or medium D (29 October 1992) (17) before inoculation. Enumeration of *Synechococcus* cells by direct microscopic count was performed on appropriate dilutions using a Petroff-Hausser counting chamber. A standard procedure to cultivate *Thermus* species (0.1% w/v tryptone, 0.1% w/v yeast extract (TYE) in Castenholz medium D, incubation temperature 70°C) (17) was compared to the use of the same basal medium with substrates that seemed more rational replacing TYE and incubating at lower temperatures (details provided in Table 2). Substrates included glycolate, a compound shown to be excreted by thermophilic cyanobacteria in Octopus Spring mat (1), and solidified autoclaved mat homogenate as carbon sources (Table 2). Liquid media (TYE, glycolate) were incubated with shaking (150 rpm) until turbid, then transferred to solid media containing 3% agar for isolation. Mat agar was spread inoculated with 100 μL homogenized and diluted sample and placed in a static

incubator at 50°C.

Growth rates were measured at different temperatures in duplicate, using sidearm flasks containing 50 mL of medium optimized for each isolate (3.0% TYE, pH 8.2 for isolates ac-1 and ac-7; 1.0% TYE, pH 7.5 for isolates ac-2 and ac-17). Klett absorbance readings were taken during logarithmic growth, compared to a standard curve, and doublings per hour were calculated.

Characterization of 16S rRNA Sequences

Harvested cells were lysed according to an established enzymatic protocol (25). Nucleic acids were extracted and the 16S rDNA gene was amplified using the polymerase chain reaction, cloned, and sequenced as described by Kopczynski et al. (9). Full-length 16S rRNA sequences (*Escherichia coli* positions 28-1483) for isolates ac-1, ac-2, ac-7, and ac-17 (GenBank accession numbers L37520, L37521, L37522, and L37523, respectively) were aligned relative to existing *Thermus* sequences (see Saul et al. (20) for a full description of strains and GenBank accession numbers), *Deinococcus radiodurans*, and *E. coli*, using the sequence editor provided by the Ribosomal Database Project (RDP) (10). *D. radiodurans* and *E. coli* sequences were derived from the Ribosomal Database Project on the anonymous ftp server at the University of Illinois in Urbana, Illinois updated on August 1, 1993. Similarity values were calculated and phylogenetic trees constructed from evolutionary distances using the evolutionary distance matrix algorithm as described by Olsen (14) and available through the RDP.

Results and Discussion

The most numerically abundant *Thermus* isolates were ac-17 and ac-14, which survived dilution to 10^{-6} and 10^{-5}, respectively (Table 2). These were enriched on glycolate medium at 50°C. All other isolates were obtained from 10^{-2} to 10^{-3} dilutions in TYE media or on mat agar incubated at 50°C or 70°C.

Characterization of the 16S rRNAs of Octopus Spring *Thermus* isolates revealed four unique sequence types (sequences 1-4, Table 3). Isolates ac-6 and ac-14 were found to be identical to isolate ac-17 over 469 and 1369 bases through several variable regions, respectively, so no further sequence analysis of these isolates was performed. Although isolates ac-17 and ac-14 show identical 16S rRNA sequences, they exhibit stable differences in pigmentation (Table 2). Pigment variation in *T. ruber* has been previously described (6,13). Isolate ac-1 is nearly identical to *Thermus* strains ZHGI and ZHGIB cultivated from hot springs in Iceland (7). Isolate ac-7 is similar to *T. aquaticus* YT-1 and *Thermus* sp. YSPID, two isolates obtained from hot springs in Yellowstone National Park (3,7). Strain YSPID was previously cultivated from Octopus Spring. Isolates ac-2 and ac-17 are very similar but not identical, and most similar to *T. ruber*, which was originally cultivated from hot springs in Russia (12). A distance matrix phylogenetic tree including these strains (Figure 1) was quite similar to that produced by Saul et al. (20). The Octopus Spring isolates clustered within existing clades.

Geographic isolation has been suggested to be important in determining *Thermus* distribution (8,19) using numerical classifications which assign organisms to

Table 2. *Thermus* strains cultivated from the 50-55°C Octopus Spring cyanobacterial mat community

Strain	Sampling Date	Medium	Incubation Temperature (°C)	Pigmentation	Highest Dilution	Total Cell Counts[a]
ac-17	29 Oct 1992	Glycolate[b]	50	Orange	10^{-6}	5.3×10^8
ac-14	29 Oct 1992	Glycolate	50	Red	10^{-5}	5.3×10^8
ac-2	30 Sept 1992	TYE[c]	50	Red	10^{-3}	1.8×10^9
ac-7	29 Oct 1992	Mat Agar[d]	50	Yellow	10^{-3}	5.3×10^8
ac-1	30 Sept 1992	TYE	70	Yellow	10^{-3}	1.8×10^9
ac-6	29 Oct 1992	Mat Agar	50	Orange	10^{-2}	5.3×10^8

[a]*Synechococcus* cells/ml mat homogenate
[b]0.1% w/v glycolic acid in Castenholz medium D amended with 1/3 v/v Octopus Spring water[†]
[c]0.1% w/v tryptone + 0.1% w/v yeast extract in Castenholz medium D[†]
[d]10% v/v mat homogenate with 3% w/v agar in Castenholz medium D amended with 1/3 v/v Octopus Spring water[†]
[†]pH of all media adjusted to 8.2 before autoclaving

Table 3. 16S rRNA sequence similarities of selected *Thermus* species[a]

	1	2	3	4	5	6	7	8	9	10	11	12
1. *Thermus sp.* ac-1	—	65	216	220	**1**	.60	60	72	88	212	292	382
2. *Thermus sp.* ac-7	95.4	-	220	224	64	**14**	**19**	68	94	214	315	390
3. *Thermus sp.* ac-2	84.9	84.6	-	**21**	218	216	212	218	225	**23**	284	396
4. *Thermus sp.* ac-17	84.7	84.4	**98.8**	-	222	222	218	221	229	**35**	290	405
5. *Thermus sp.* ZHGI[b]	**99.9**	95.5	84.8	84.6	-	57	57	73	89	214	293	386
6. *Thermus sp.* YSPID[b]	95.8	**98.8**	84.9	84.6	94.0	-	14	58	81	206	308	379
7. *T. aquaticus* YT-1[b]	95.8	**98.7**	85.2	84.9	94.0	99.0	-	57	86	204	308	384
8. *T. thermophilus* HB8[b]	94.9	95.2	84.8	84.7	94.9	95.9	96.0	-	81	214	307	388
9. *T. filiformis*[b]	93.8	93.4	84.3	84.1	93.8	94.3	94.0	94.3	-	217	295	387
10. *T. ruber*[b]	85.2	85.0	**98.2**	**97.9**	85.1	85.6	85.7	85.1	84.9	-	284	398
11. *Deinococcus radiodurans*[c]	79.6	78.0	80.0	79.7	79.6	78.5	78.5	78.6	79.5	80.0	—	408
12. *Escherichia coli*[c]	74.1	73.6	73.2	72.8	73.9	74.4	74.1	73.8	73.9	73.1	72.4	—

[a]Values on the lower left are percent sequence similarities based on all available sequence data, values on the upper right are the absolute number of unambiguous nucleotide differences, many of which occurred as complimentary nucleotide substitutions in double-stranded regions.

[b]Strains and nucleotide sequences as found in (10)

[c]Nucleotide sequences were derived from the RDP (20).

Tok8	New Zealand
Tok3	New Zealand
Tok20	New Zealand
W28	New Zealand
T. filiformis	New Zealand
Rt41A	New Zealand
T351	New Zealand
OK6	New Zealand
Fiji3	Pacific
HS1	New Zealand
HB8	Japan
HB27	Japan
"*T. flavus*"	Japan
NMX2	USA
ZFI	Iceland
ZHGIB	Iceland
ac-1	USA
ZHGI	Iceland
T. aquaticus YT-1	USA
ac-7	USA
YSPID	USA
ac-2	USA
ac-17	USA
T. ruber	Russia
D. radiodurans	

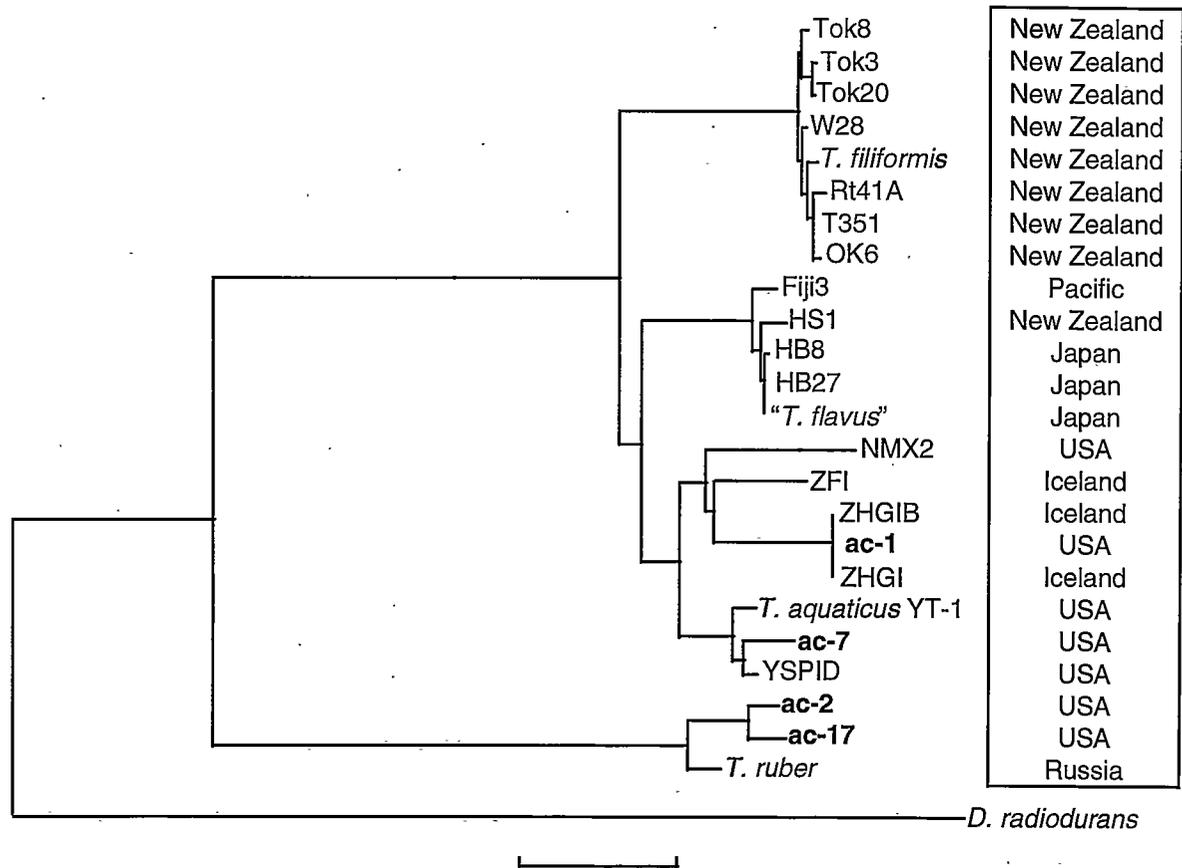Figure 1. Distance matrix phylogenetic tree of the *Thermus* and *Deinococcus* lines of descent inferred from full 16S rRNA sequence data. Boldface denotes Octopus Spring aerobic chemoorganotrophic isolates. Remaining sequences and geographic origins are as described by Saul et al. (20). The tree was rooted to the 16S rRNA sequence of *Escherichia coli*. Scale bar represents 0.02 fixed point mutations per sequence position.

taxa according to phenotypic characteristics. Based on the geographic location of *Thermus* isolates used to construct a 16S rRNA-based phylogenetic tree, Saul et al. (20) also hypothesized a geographic basis for *Thermus* distribution. In contrast, within one hot spring we find a diversity of *Thermus* species that segregate into several major clades of the phylogenetic tree (Figure 1). Our results are consistent with the observation that *T. ruber* has been cultivated from diverse geographic sources when incubation temperatures were lowered to 50-60°C (4,6,11,22).

Octopus Spring *Thermus* isolates belonging to distinct clades displayed optimal growth rates at different temperatures (Figure 2). *T. aquaticus*-like isolates ac-7 and ac-1 had temperature optima of 65°C and 70°C, respectively, while *T. ruber*-like isolates ac-17 and ac-2 displayed optimal growth rates at 50°C. Differences in temperature adaptations between *T. ruber* and *T. aquaticus* have been previously reported (3,11).

Abundance of *Thermus* spp. within the 50-55°C Octopus Spring mat appears to be related to temperature adaptation. Low temperature adapted isolate ac-17 was more numerically abundant in the 50-55°C mat (surviving a 10^{-6} dilution) than were high temperature adapted isolates ac-7 and ac-1 (surviving 10^{-3} dilutions). Ramaley and Bitzinger (16) also observed differential dominance of differently pigmented *Thermus* strains in a man-made thermal gradient. Specialization to temperature has been shown in other thermophilic genera (15), and may represent an evolutionary strategy driving diversity and community structure in thermal environments (24). Perhaps other *Thermus* populations exist in Octopus Spring mat that are specialized to different
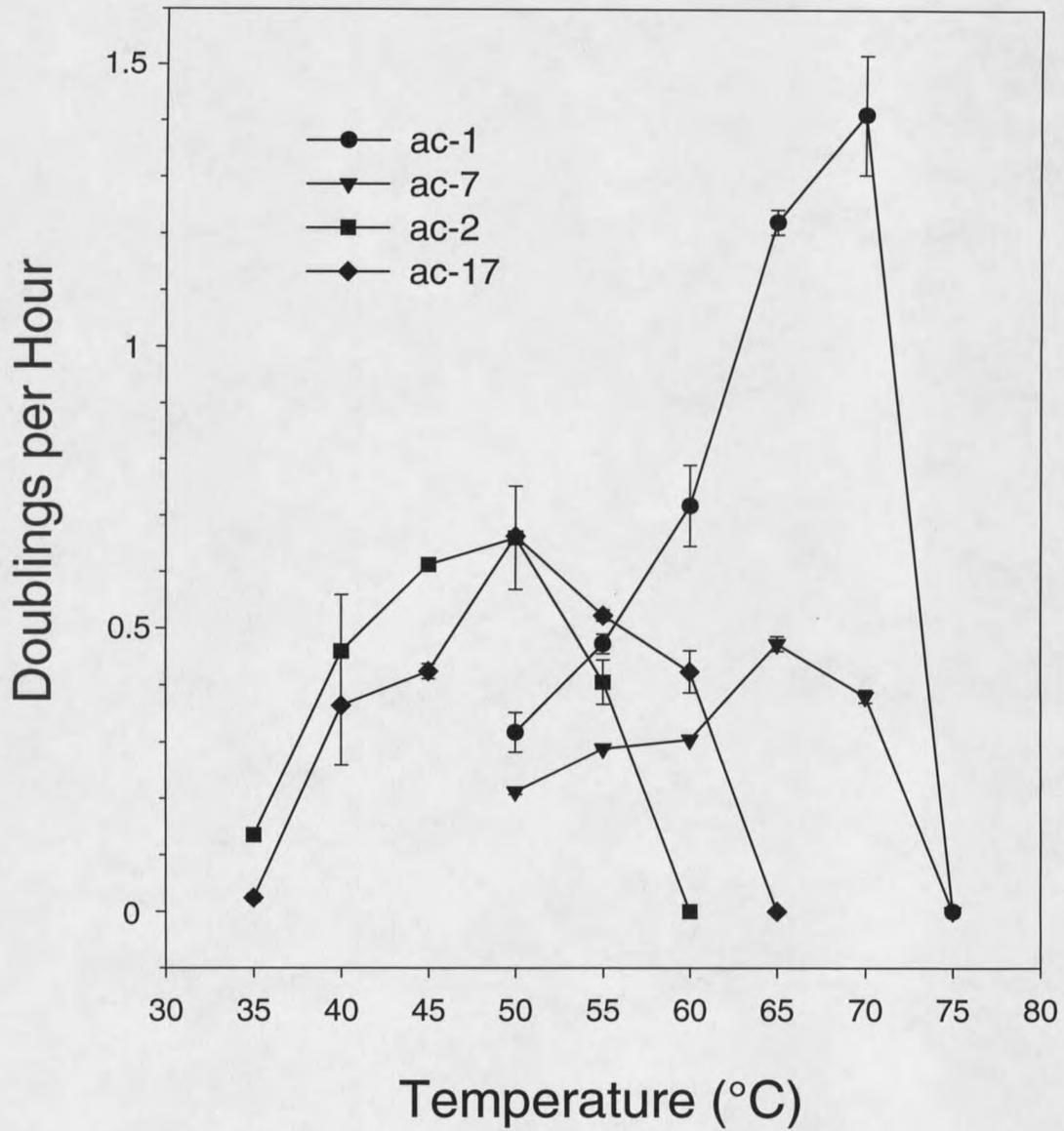
Figure 2. Effect of temperature on growth rates of Octopus Spring *Thermus* isolates. Error bars indicate standard deviation, n=2.

parameters such as substrate and pH.

Our results do not exclude the possibility that geographic barriers may limit dispersal, thereby affecting distribution of some *Thermus* species. Indeed, major clades of the phylogenetic tree are composed of organisms cultivated from geographically distinct locations (e.g. *T. filiformis* and *T. aquaticus* clades are only known to contain organisms cultivated from New Zealand and Yellowstone National Park, respectively). However, our results do suggest that for some *Thermus* species, adaptation to local environmental conditions might help explain population distributions within the environmental gradients found in hot spring habitats.

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CHAPTER 3

CULTIVATION OF AEROBIC CHEMOORGANOTROPHIC PROTEOBACTERIA

AND GRAM POSITIVE BACTERIA FROM A

HOT SPRING MICROBIAL MAT[1]

Introduction

Recent studies investigating microbial species diversity in the Octopus Spring

cyanobacterial mat community have revealed a marked disparity between the native

16S rRNA sequence types observed in the mat using molecular retrieval techniques

and the 16S rRNA sequences of aerobic chemoorganotrophic bacteria cultivated from

this and other geothermal habitats (34,35,38). Sequences retrieved from the Octopus

Spring mat which may belong to organisms exhibiting aerobic chemoorganotrophic

metabolic capabilities include planctomycete, proteobacterial, and Gram positive

bacterial representatives, as well as relatives of green sulfur and green nonsulfur

bacteria (35) **[See Table 1]**. However, characterizing the metabolic capabilities of the

bacteria which contain retrieved 16S rRNA sequence types is difficult without first

cultivating these organisms.

[1]This study has been accepted for publication in Applied and Environmental Microbiology as:
Nold, S. C., E. D. Kopczynski, and D. M. Ward. Cultivation of Aerobic Chemoorganotrophic
Proteobacteria and Gram Positive Bacteria from a Hot Spring Microbial Mat.

Previous efforts to cultivate aerobic chemoorganotrophic bacteria from alkaline silicious hot spring cyanobacterial mats including Octopus Spring mat have yielded *Bacillus* (5), *Thermomicrobium* (19), *Thermus* (6), and *Chloroflexus* (4) isolates. These studies utilized similar cultivation techniques characterized by relatively high organic substrate concentrations (usually 0.1% w/v tryptone and yeast extract in liquid medium). An exception was the cultivation of the oligotrophic bacterium *Isosphaera pallida* using culture media devoid of organic substrates (15). Enrichments were often conducted at high incubation temperatures (70°C) regardless of the temperature of the collection site and the existence of temperature-adapted strains (23). Isolates were obtained by directly streaking mat material onto solidified media or by the direct addition of undiluted inoculum to enrichment flasks. Either of these methods preclude attempts to measure the relative abundance of the organisms cultivated. Since the development of enrichment culture techniques, microbiologists have suspected that these methods may select for the organisms which are best adapted to the enrichment culture environment, but which may not be the dominant organisms in nature (33,41). Our investigations resulted from our suspicion that the selectivity of the enrichment culture environment may explain the discrepancy between cultivated and naturally occurring populations detected by molecular retrieval techniques.

In this study we attempted to cultivate more numerically abundant aerobic chemoorganotrophic bacteria from the 50 to 55°C region of Octopus Spring cyanobacterial mat community located in Yellowstone National Park. We utilized serial dilution enrichment culture (8,31) to provide a relative measure of the

abundance of the isolates obtained and to avoid culture overgrowth by numerically insignificant species (14). We also enriched under seemingly more natural conditions (e.g. incubating at the temperature of sample collection, using more relevant substrates known to be present in the habitat such as mat material and glycolic acid (2), and using lower substrate concentrations). Previously reported results from this study (22) revealed a diversity of *Thermus* isolates cultivated from Octopus Spring mat whose distribution may be controlled by specialization to different temperatures which occur within the habitat. Here we report the cultivation and 16S rRNA sequence characterization of phenotypically and phylogenetically distinct proteobacterial and Gram positive aerobic chemoorganotrophic bacteria from the Octopus Spring mat.

Materials and Methods

Cultivation of Isolates

Procedures for sample collection and enrichment culture conditions were performed as described in Nold and Ward (22). Briefly, cyanobacterial mat samples were collected in September and October 1992 and November 1993 from the shoulder region of Octopus Spring (50 to 55°C, pH 8.5). Samples were kept between 46°C and 50°C for 3 hours in transit to the laboratory, then homogenized with a Dounce tissue homogenizer and serially diluted (1:10) in sterile medium D (9) before inoculation. Carbon sources included glycolic acid (GLD), casein (CND), solidified autoclaved mat homogenate (MTD), and a standard substrate used to cultivate *Thermus* species (6),

tryptone and yeast extract in medium D (TYD); details provided in Table 4. The

sample for TYD enrichments was collected 30 September 1992, and the sample for

MTD, CND, and GLD enrichments was collected 29 October 1992. On a later

sampling date (9 November 1993) 0.1%, 0.01%, and 0.001% (w/v) tryptone and yeast

extract were used as carbon sources. Liquid enrichments (50 ml GLD, TYD, or CND

in 300 ml shake flasks) were inoculated with 5 ml serially diluted mat homogenate

and incubated at 50°C with shaking (150 rpm) until turbid, then transferred to

solidified medium containing 3% agar for isolation. Solidified mat homogenate was

spread inoculated with 100 µl serially diluted inoculum and placed in a static incubator

at 50°C. Individual colonies which exhibited unique and stable phenotypic properties

(colony color, cell morphology, spore formation, motility) were re-streaked for

purification and perpetuated from each medium type. Enumeration of cyanobacterial

(*Synechococcus* spp.) cells by direct microscopic count was performed on appropriate

dilutions of mat homogenate using a Petroff-Hausser counting chamber. Total direct

counts of cyanobacterial cells in undiluted homogenized mat inocula were 5.3×10^8

unicells ml^{-1} (TYD) and 1.8×10^9 unicells ml^{-1} (GLD, CND, MTD). The abundance

of each isolate is reported relative to the number of *Synechococcus* cells present in the

diluted inoculum source used to inoculate the flask in which the isolate was observed.

Characterization of 16S rRNA Sequences

16S rRNA sequence data were generated for each phenotypically unique isolate

which grew from the most highly diluted inoculum in each enrichment type.