



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 $^{14}\text{CO}_2$ into rRNA, cyanobacteria inhabiting hot spring mats predominately incorporated $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ 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. $[^{14}\text{C}]$ acetate, $^{14}\text{CO}_2$, and $[^{14}\text{C}]$ 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.

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BACTERIAL DIVERSITY AND PHYSIOLOGY

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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

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 $^{14}\text{CO}_2$ into rRNA, cyanobacteria inhabiting hot spring mats predominately incorporated $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ 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, $^{14}\text{CO}_2$, 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

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	
<i>Chloroflexus aurantiacus</i> Y-400-fl	
<i>Thermomicrobium roseum</i>	
	type C OS-V-L-20
<i>Thermus/Deinococcus</i> Group	
<i>Thermus</i> sp. OS-Ramaley-4	
<i>Thermus aquaticus</i> YT-1	
Planctomyces	
<i>Isosphaera pallida</i> 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

^aAdapted 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, $^{14}\text{CO}_2$ was provided as a substrate to predominately radiolabel the photosynthetically active *Synechococcus* cyanobacterial species. Although $^{14}\text{CO}_2$ was readily incorporated into cellular material, no incorporation of $^{14}\text{CO}_2$ 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 $\text{CO}_2 \text{ m}^{-2} \text{ hr}^{-1}$ (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 $\text{m}^{-2} \text{day}^{-1}$. 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 $45 \mu\text{m day}^{-1}$ (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 $\text{m}^{-2} \text{hr}^{-1}$ (calculated from (2)). Acetate and propionate accumulate in mats overnight and are photoheterotrophically 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 $\text{CH}_4 \text{m}^{-2} \text{hr}^{-1}$, (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 $^{14}\text{CO}_2$ 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 MAT¹Introduction

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

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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 x 10 ⁸
ac-14	29 Oct 1992	Glycolate	50	Red	10 ⁻⁵	5.3 x 10 ⁸
ac-2	30 Sept 1992	TYE ^c	50	Red	10 ⁻³	1.8 x 10 ⁹
ac-7	29 Oct 1992	Mat Agar ^d	50	Yellow	10 ⁻³	5.3 x 10 ⁸
ac-1	30 Sept 1992	TYE	70	Yellow	10 ⁻³	1.8 x 10 ⁹
ac-6	29 Oct 1992	Mat Agar	50	Orange	10 ⁻²	5.3 x 10 ⁸

^a*Synechococcus* cells/ml mat homogenate

^b0.1% w/v glycolic acid in Castenholz medium D amended with 1/3 v/v Octopus Spring water[†]

^c0.1% w/v tryptone + 0.1% w/v yeast extract in Castenholz medium D[†]

^d10% 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. <i>Thermus</i> sp. ac-1	—	65	216	220	1	60	60	72	88	212	292	382
2. <i>Thermus</i> sp. ac-7	95.4	—	220	224	64	14	19	68	94	214	315	390
3. <i>Thermus</i> sp. ac-2	84.9	84.6	—	21	218	216	212	218	225	23	284	396
4. <i>Thermus</i> sp. ac-17	84.7	84.4	98.8	—	222	222	218	221	229	35	290	405
5. <i>Thermus</i> sp. ZHGI ^b	99.9	95.5	84.8	84.6	—	57	57	73	89	214	293	386
6. <i>Thermus</i> sp. YSPID ^b	95.8	98.8	84.9	84.6	94.0	—	14	58	81	206	308	379
7. <i>T. aquaticus</i> YT-1 ^b	95.8	98.7	85.2	84.9	94.0	99.0	—	57	86	204	308	384
8. <i>T. thermophilus</i> HB8 ^b	94.9	95.2	84.8	84.7	94.9	95.9	96.0	—	81	214	307	388
9. <i>T. filiformis</i> ^b	93.8	93.4	84.3	84.1	93.8	94.3	94.0	94.3	—	217	295	387
10. <i>T. ruber</i> ^b	85.2	85.0	98.2	97.9	85.1	85.6	85.7	85.1	84.9	—	284	398
11. <i>Deinococcus radiodurans</i> ^c	79.6	78.0	80.0	79.7	79.6	78.5	78.5	78.6	79.5	80.0	—	408
12. <i>Escherichia coli</i> ^c	74.1	73.6	73.2	72.8	73.9	74.4	74.1	73.8	73.9	73.1	72.4	—

^aValues 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.

^bStrains and nucleotide sequences as found in (10)

^cNucleotide sequences were derived from the RDP (20).

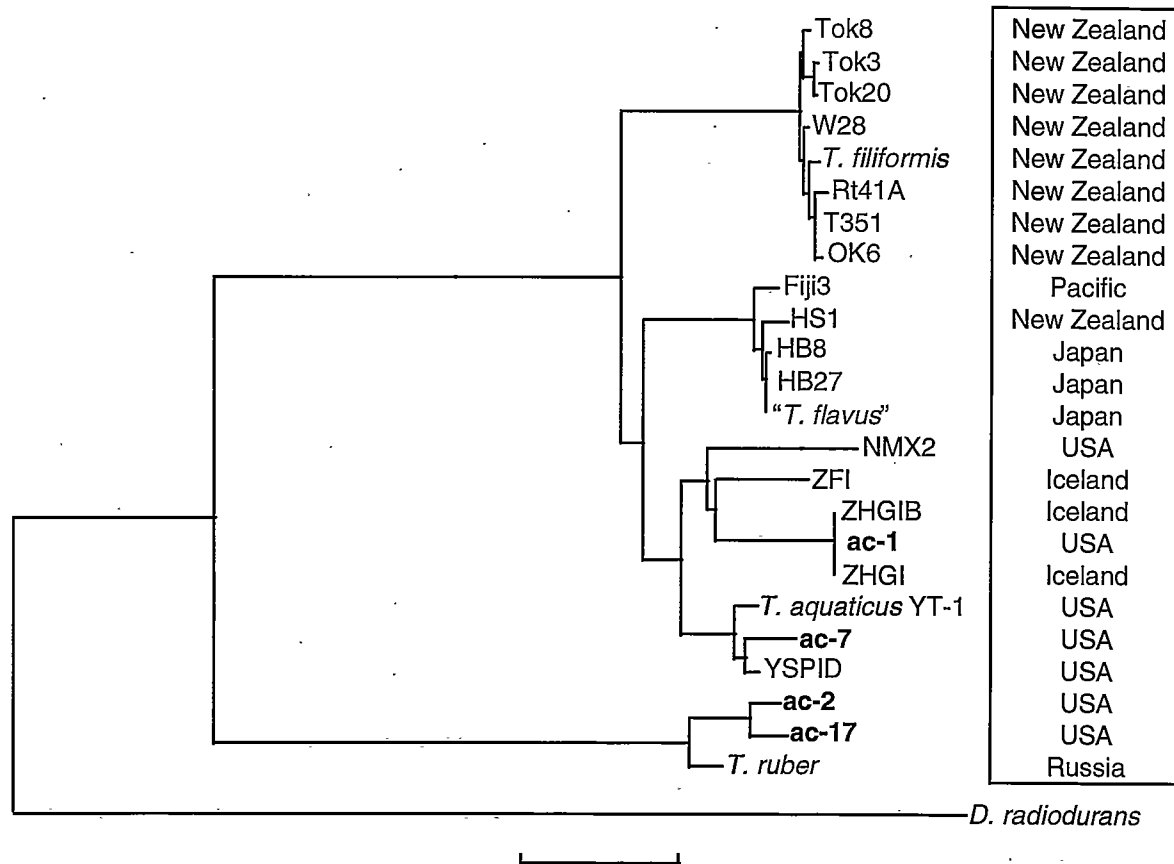


Figure 1. Distance matrix phylogenetic tree of the *Thermus* and *Deinococcus* lines of descent inferred from full 16S rRNA sequence data. Boldface denotes Octopus Spring aerobic chemoorganotrophic isolates. Remaining sequences and geographic origins are as described by Saul et al. (20). The tree was 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

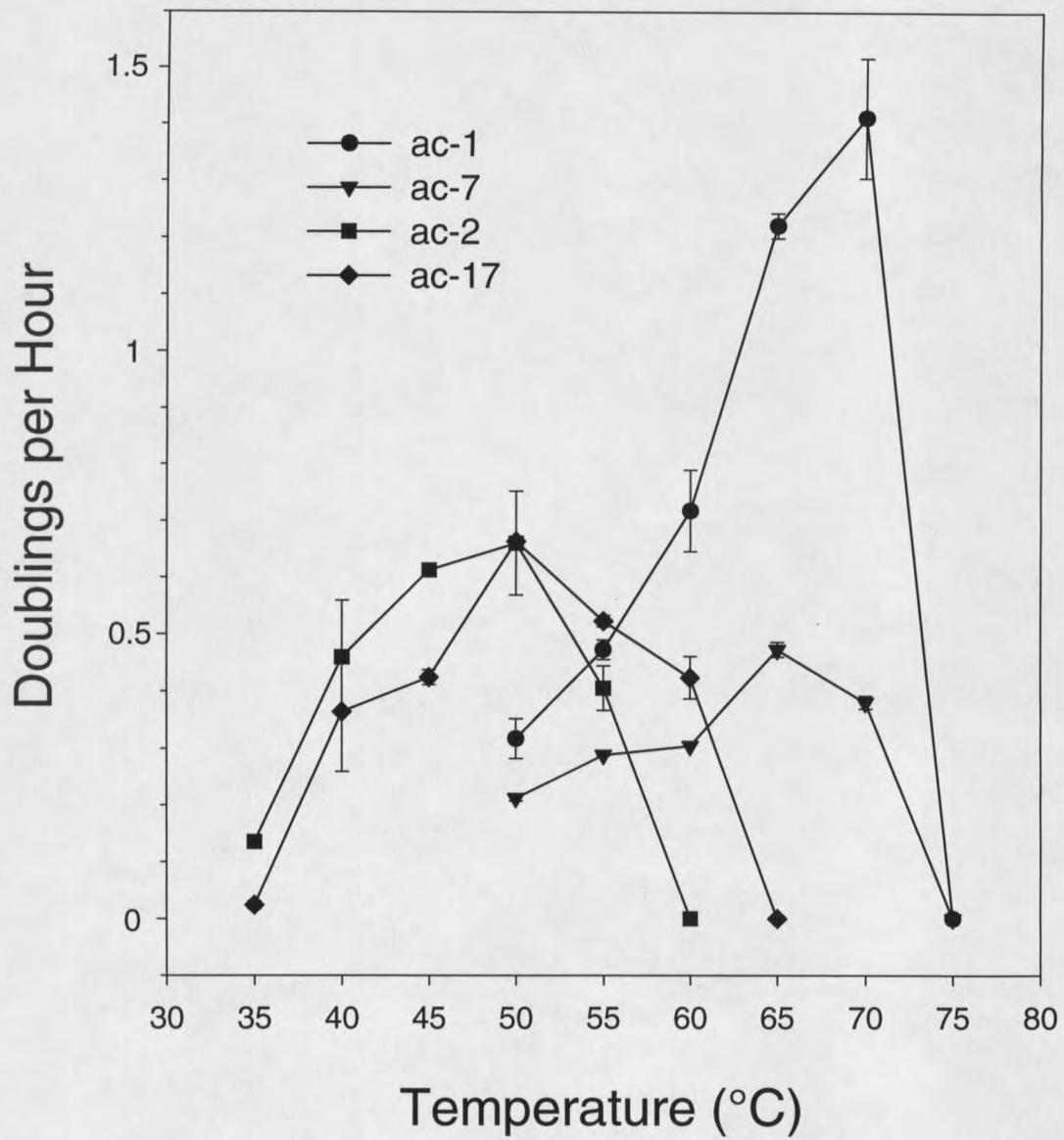


Figure 2. Effect of temperature on growth rates of Octopus Spring *Thermus* isolates. Error bars indicate standard deviation, n=2.

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 MAT¹

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.

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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.

Harvested cells were lysed according to an established enzymatic protocol (36). Nucleic acids were extracted and the 16S rRNA gene was amplified using the polymerase chain reaction, cloned, and full-length (*Escherichia coli* positions 28-1483) (7) 16S rRNA sequence data were generated for isolates ac-15, ac-16, and ac-18 (GenBank accession numbers U46749, U46748, and U46747, respectively) as described by Kopczynski et al. (20). Partial 16S rRNA sequence data were generated for the remaining isolates by directly sequencing PCR products using the Sequenase PCR Product Sequencing Kit (United States Biochemical, Cleveland, OH) according to the manufacturer's directions. The 16S rRNA sequence we report here which was retrieved directly from the mat (Octopus Spring type R, GenBank accession number U46750) was obtained from Octopus Spring library V-L using previously described methods (39). This sequence was previously reported as clone OS-V-L-28 (35), but was not characterized. Sequences were aligned and similarity values were calculated using the SeqEdit program version 3.0.4 provided by the Ribosomal Database Project (RDP) at the University of Illinois (21). All available sequence data, including ambiguous bases and alignment gaps, were included in similarity calculations. Sequences were analyzed for potential chimeric structures using the Check_Chimera program available through the RDP. Phylogenetic trees were constructed using the programs DNADIST, SEQBOOT, FITCH, and CONSENSE from the Phylogenetic Inference Package (PHYLIP) version 3.57c (11) with representative sequences derived from the RDP.

Results

The outcome of enrichment culture experiments conducted on samples collected in September and October 1992 is summarized in Table 4. While phenotypically diverse bacterial isolates were obtained from TYD, MTD, and GLD enrichments, the enrichment containing casein as a carbon source yielded only one isolate (ac-12). Growth occurred to different dilutions depending upon medium type. Enrichments containing glycolate as a carbon source yielded an isolate (ac-21) which was cultivated from a 10^8 -fold dilution, which initially contained 9.0×10^2 *Synechococcus* cells. TYD, CND, and MTD enrichments all yielded less abundant isolates cultivated from 10^3 - to 10^5 -fold dilutions, initially containing 2.7×10^7 to 9.0×10^5 *Synechococcus* cells, respectively. The extent to which the inoculum was diluted before incubation affected the type of growth observed in liquid enrichment cultures. Less abundant populations (10^2 - to 10^5 -fold dilutions) exhibited turbid growth, while more abundant populations growing in GLD enrichments (10^6 - to 10^8 -fold dilutions) were characterized by filamentous organisms growing as faint orange pellicles detected at the interface between air and water in the shaken enrichment flask. Isolation of these sheathed filamentous organisms (isolates ac-19, ac-20, and ac-21) was achieved by streaking pellicle samples onto solidified medium D pre-inoculated with *Synechococcus* isolate C1 (14). Subsequent transfer of colonies to liquid enrichments failed, thereby precluding 16S rRNA sequence characterization of these isolates.

Table 4. Bacterial isolates cultivated from the 50 to 55°C Octopus Spring cyanobacterial mat community sampled during October and November 1992

Isolate	Medium ^b	Highest Dilution	Distinguishing Characteristics	16S rRNA Sequence Type Identical to:
ac-3	TYD ^c	10 ⁻²	Motile coccobacillus	ac-15
ac-4	TYD	10 ⁻³	Brown coccobacillus	ac-18
ac-5	TYD	10 ⁻³	Yellow sporulating rod	ac-18
ac-8	MTD ^d	10 ⁻⁴	Motile coccobacillus	ac-15
ac-9	MTD	10 ⁻⁴	White sporulating rod	ac-18
ac-10	MTD	10 ⁻⁵	Motile rod	ac-16
ac-11	MTD	10 ⁻⁵	Sheathed orange rod, 0.5 × 5 µm	N.D.
ac-12	CND ^e	10 ⁻⁵	Motile rod	ac-16
ac-13	GLD ^f	10 ⁻⁴	Yellow sporulating rod	ac-18
ac-15 ^a	GLD	10 ⁻⁶	Motile coccobacillus	ac-15
ac-16 ^a	GLD	10 ⁻⁶	Motile rod	ac-16
ac-18 ^a	GLD	10 ⁻⁷	Colorless coccobacillus	ac-18
ac-19	GLD	10 ⁻⁷	Sheathed orange trichome, 0.5 × 200µm	N.D.
ac-20	GLD	10 ⁻⁷	Sheathed orange trichome, 1 × 10-50 µm	N.D.
ac-21	GLD	10 ⁻⁸	Sheathed orange trichome, 0.6 × 10-50 µm	N.D.

^aDenotes isolate from which full-length 16S rDNA sequence data are available.

^bpH of all media adjusted to 8.2 before autoclaving.

^cTYD, 0.1% (w/v) tryptone + 0.1% (w/v) yeast extract in Castenholz medium D.

^dMTD, 10% (v/v) cyanobacterial mat homogenate with 3% (w/v) agar in Castenholz medium D amended with 1/3 (v/v) Octopus Spring water.

^eCND, 4% (w/v) casein in Castenholz medium D amended with 1/3 (v/v) Octopus Spring water.

^fGLD, 0.1% (w/v) glycolic acid in Castenholz medium D amended with 1/3 (v/v) Octopus Spring water.

N.D. no data

In enrichment culture experiments conducted on samples collected in November 1993, carbon source concentration did not influence the extent of growth (all TYD concentrations exhibited growth to a 10^8 -fold dilution), but evidence of growth was observed sooner in the more dilute enrichments (0.001% and 0.01% TYD, 6 days) than in 0.1% TYD (44 days). Growth at the highest dilutions resembled the orange filamentous pellicle described above, and characterization attempts similarly failed.

Isolates clustered into three phylogenetically distinct groups: proteobacteria, Gram positive bacteria, and *Thermus* (previously reported in (22)) (Figure 3). Isolate ac-15 contains a 16S rRNA nucleotide sequence which is identical to the previously retrieved β -proteobacterial Octopus Spring type N sequence (34) over the 277 nucleotides available for comparison (Table 5). Isolate ac-16 and the retrieved but previously uncharacterized Octopus Spring type R sequence also displayed similarity to proteobacterial sequences (Table 5). Isolates ac-15 and ac-16, and the Octopus Spring type R sequence are compared to other mat proteobacterial sequence types and representatives of the major proteobacterial lines of descent in Figure 4. Analysis of diagnostic secondary structure (*E. coli* positions 140 to 223) and diagnostic nucleotide signatures (*E. coli* positions 50, 108, 124, 640, 690, 722, 760, 812, 871, 929, 947, and 1234) (42), as well as sequence similarity (Table 5) and tree results (Figure 4) support the inference that isolates ac-15, ac-16, and the retrieved Octopus Spring type R sequence belong to the β subdivision of the proteobacteria. Isolate ac-18 exhibited a 16S rRNA nucleotide sequence nearly identical to the sequence from *Bacillus*

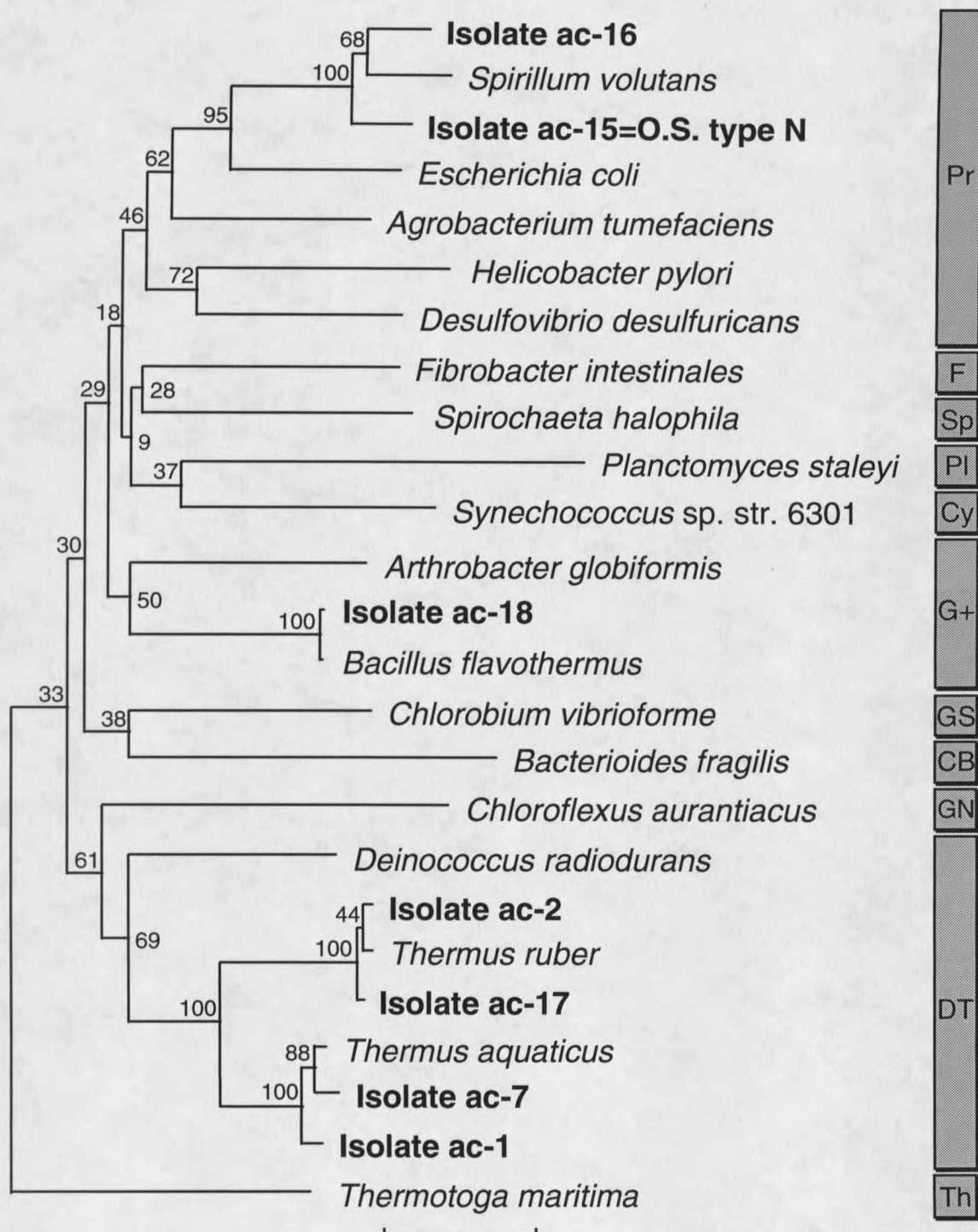


Figure 3. Distance matrix phylogenetic tree showing the placement of 16S rRNA sequences of aerobic chemoorganotrophic isolates cultivated from the Octopus Spring mat community relative to those of representatives of the major Bacterial lines of descent. Figure legend continued on page 43.

Table 5. 16S rRNA sequence similarities of Octopus Spring isolates and selected proteobacterial and Gram positive sequence types^a

Sequence	% similarity\number of unambiguous differences with sequence									
	1	2	3	4	5	6	7	8	9	10
Proteobacteria										
1. Isolate ac-15 (1392)	-	0	172	142	66	152	177	365	363	385
2. O.S. type N (277)	99.8	-	29	22	27	28	44	65	65	79
3. Isolate ac-16 (1456)	87.7	89.7	-	161	60	87	185	357	358	391
4. O.S. type R (1346)	88.9	91.9	88.0	-	56	148	181	333	334	349
5. O.S. type G (588)	88.8	90.2	89.8	90.6	-	70	93	120	120	145
6. <i>Azoarcus denitrificans</i> (1458)	89.1	90.0	93.9	89.0	88.2	-	193	367	365	402
7. O.S. type O (728)	76.0	77.6	75.0	75.0	79.8	73.8	-	170	169	211
Gram positive bacteria										
8. Isolate ac-18 (1478)	74.6	76.8	76.1	75.9	79.9	75.5	77.1	-	6	341
9. <i>Bacillus flavothermus</i> (1477)	74.7	76.8	76.0	75.8	79.9	75.6	77.2	99.6	-	340
10. <i>Thermotoga maritima</i> (1481)	73.4	72.6	74.0	74.9	75.9	73.3	71.7	77.2	77.2	-

^aValues in the lower left are percent sequence similarities based on all available sequence data (number of nucleotides available for comparison in parentheses); values in the upper right are the absolute number of unambiguous nucleotide substitutions.

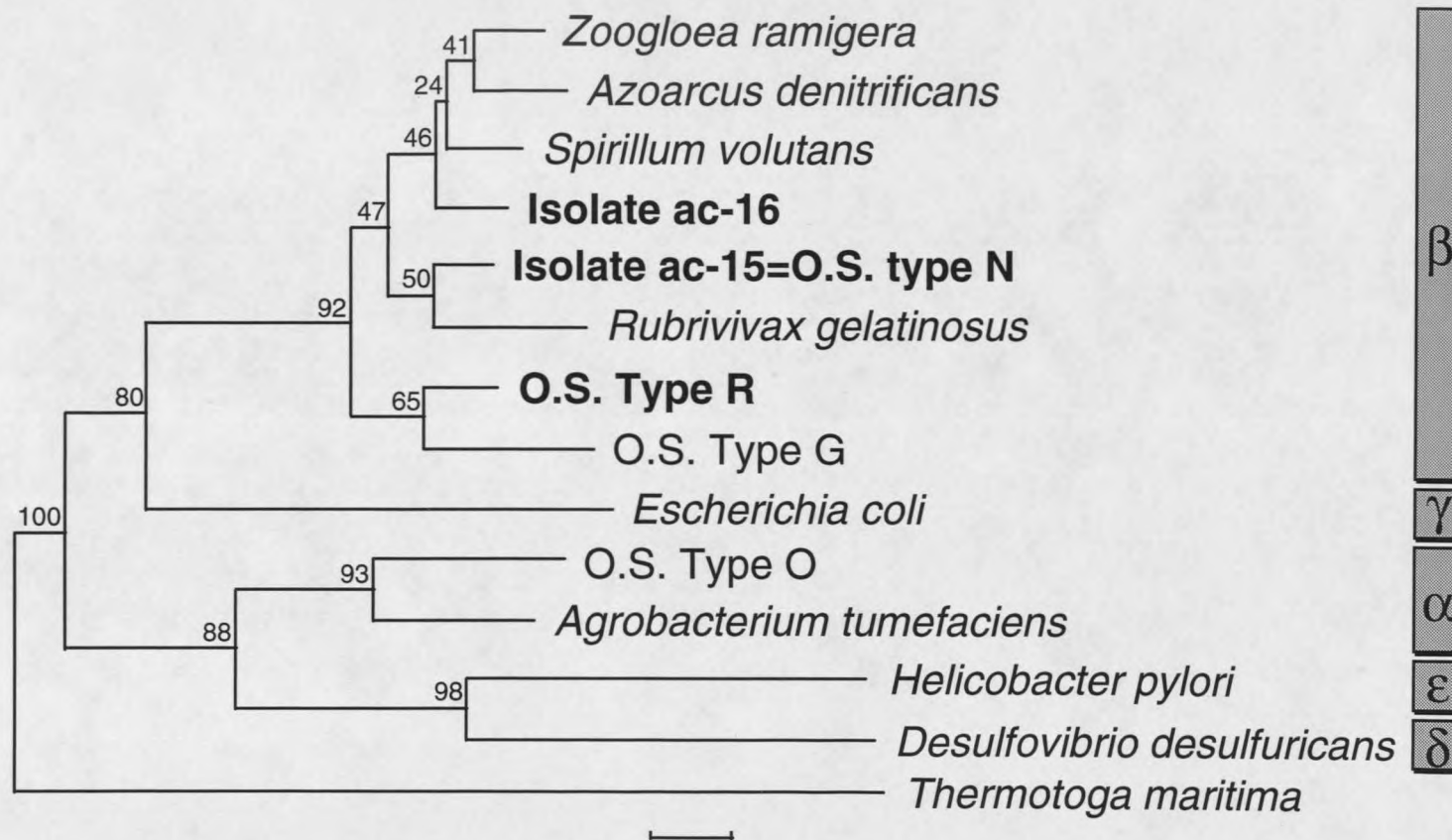


Figure 4. Distance matrix phylogenetic tree showing the placement of 16S rRNA sequences of cultivated and cloned proteobacterial Octopus Spring cyanobacterial mat populations relative to those of representatives of the major proteobacterial lines of descent (α , β , γ , δ , ϵ). Boldface denotes proteobacterial sequences reported in this paper. The consensus values at the nodes indicate the number of times the group consisting of the species to the right of the node occurred among 100 trees inferred from the bootstrapped data set sampled by analysis of nucleotides which align with *Escherichia coli* 16S rRNA positions 802-825, 875-886, 1046-1114, 1157-1250, 1287-1392. Scale bar represents 0.01 fixed point mutations per sequence position.

Figure 3 legend (continued). Representatives were chosen from the following lines: Pr, proteobacteria; F, *Fibrobacter*; Sp, spirochetes and relatives; Pl, *Planctomyces* and relatives; Cy, cyanobacteria; G+, Gram positive; GS, green sulfur bacteria; CB, *Flexibacter-Cytophaga-Bacteriodes*; GN, green non-sulfur bacteria and relatives; DT, *Deinococcus-Thermus* subdivision of the green non-sulfur bacteria; Th, Thermotogales. *Thermus* sequences reported by Nold and Ward (22) are included to exhibit the full phylogenetic range of the aerobic chemoorganotrophic bacteria cultivated during these studies (isolates denoted by boldface type). The consensus values at the nodes indicate the number of times the group consisting of the species to the right of the node occurred among 100 trees inferred from the bootstrapped data set sampled by analysis of restricted nucleotide positions (39) which were common to all sequences (at least 898 nucleotides). This tree was rooted using the 16S rRNA sequence of *Methanobacterium thermoautotrophicum*. Scale bar represents 0.05 fixed point mutations per sequence position.

flavothermus, a group 5 *Bacillus* in the low G+C subdivision of the Gram positive bacteria (25) cultivated from a hot spring habitat (16) (Figure 3, Table 5). We found no evidence of chimera formation in the 16S rRNA sequences of any of the isolates using Check_Chimera (low maximum improvement scores and lack of peakedness) and secondary structural analyses, but detecting chimeration can be problematic (20,28).

Stable phenotypic differences were displayed by isolates ac-4, ac-5 (which was phenotypically similar to ac-13), ac-9, and ac-18 (Table 4), yet all exhibit Gram positive 16S rRNA nucleotide sequences identical to isolate ac-18 through the region 1086-1295, which includes the V9 variable region of the molecule (nucleotides 1110-1276). Only two of these isolates (isolates ac-5 and ac-13) exhibited phenotypic similarity to *B. flavothermus* (i.e. rod-shaped morphology, spore formation, and yellow colony color) (16). Other isolates displayed similar morphology and identical β proteobacterial 16S rRNA sequence types. A motile rod (isolates ac-10, ac-12, and ac-

16) and a motile coccobacillus (isolates ac-3, ac-8, and ac-15) were isolated from different dilutions and substrate types and displayed identical 16S rRNA nucleotide sequences through nucleotides 1087-1289 and 1085-1280, respectively.

Discussion

Here we present the cultivation and full-length 16S rRNA sequence characterization of an organism whose sequence was previously observed in the Octopus Spring mat using molecular retrieval techniques. We detect no unambiguous 16S rRNA nucleotide differences between isolate ac-15 and the previously retrieved sequence fragment Octopus Spring type-N (Table 5). It appears that the type-N population corresponds to an aerobic chemoorganotrophic motile coccobacillus of β -proteobacterial descent which is able to utilize glycolic acid, tryptone and yeast extract, and mat homogenate as carbon sources. Other examples of bacterial populations detected by both cultivation and 16S rRNA sequence retrieval are rare; Ferris et al. (14) successfully cultivated a relevant thermophilic cyanobacterium from this habitat, and Huber et al. (17) obtained an archaeal isolate whose 16S rRNA was previously detected.

Isolates ac-15=O.S. type N and ac-16 are the first proteobacterial isolates to be cultivated from Octopus Spring mat community (35). These isolates are phylogenetically related to members of the β subdivision of the proteobacteria, a physiologically diverse clustering of organisms which display nitrogen fixation

(18,26,40), aerobic and anaerobic respiration (32), and photoautotrophic and photoheterotrophic capabilities (40). While isolates ac-15=O.S. type N and ac-16 clearly exhibit aerobic chemoorganotrophic metabolic capabilities, they may, like other β -proteobacteria, display physiological versatility. Thus, their metabolic functions exhibited in Octopus Spring mat community are currently unknown. Four of the five known proteobacterial sequences detected in the Octopus Spring mat are most similar to members of the β subdivision. We have observed a similar pattern of multiple representatives within one phylogenetic type in the cyanobacterial, green sulfur, green non-sulfur, and *Thermus/Deinococcus* lines of descent (12,22,30). One possible explanation for this recurring pattern could be that progenitor bacteria within a phylogenetic group became specialized to conditions which vary in the habitat, resulting in subsequent evolutionary radiation and the observed diversity of modern 16S rRNA types (37,43).

Isolate ac-18 contains the first thermophilic *Bacillus*-like 16S rRNA sequence observed in the Octopus Spring mat (35). Isolates ac-4, ac-5 (and ac-13), ac-9, and ac-18 are phenotypically distinct (Table 4), yet share this 16S rRNA nucleotide sequence at least through the V9 variable region (nucleotides 1239-1298). This observation could be due to either the highly conserved nature of the 16S rRNA molecule (42), or to undetected differences in other regions of the molecule. Since only limited data were obtained from those isolates which exhibited identical 16S rRNA sequences (207 to 222 nucleotides), we cannot reject this latter possibility. Sequence similarity within phenotypically diverse thermophilic Gram positive bacteria

has been previously observed (3). Caution should be applied when interpreting bacterial diversity detected by a conservative genetic marker, since populations exhibiting identical 16S rRNA nucleotide sequences may contain highly related yet phenotypically distinct members.

By observing the extent of growth from a serially diluted inoculum, an estimate of the relative abundance of cultivated strains can be obtained (8). The highest dilution in which we observed isolate ac-15=O.S. type N (10^6 -fold) originally contained almost five orders of magnitude more *Synechococcus* cells in the inoculum (9.0×10^4), indicating the relative numerical insignificance of this cultivated species in native mat material. If we can assume that the frequency at which a sequence is observed in cloning libraries reflects the abundance of that sequence in nature, then the low abundance of isolate ac-15 in mat homogenate may explain why the Octopus Spring type N sequence was detected only once using molecular retrieval techniques (34). Isolates ac-16 and ac-18 were cultivated from similar dilutions (10^6 - and 10^7 -fold, respectively), but these populations have not been previously observed in cloning libraries constructed from Octopus Spring mat nucleic acid.

Isolates ac-19, ac-20, and ac-21, which exhibited growth in the highest dilutions (10^7 - and 10^8 -fold), could not be grown to sufficient quantity for sequence analysis. These organisms grew as sheathed trichomes similar to the *Chloroflexus* species described by Pierson and Castenholz (24), and also obtained from aerobic chemoorganotrophic enrichments by Brock (4). Glycolate has previously been identified as a substrate for aerobic chemoorganotrophic metabolism in Octopus Spring

mat. Under illuminated conditions, glycolate is excreted by photosynthetically active *Synechococcus* cells and is readily incorporated by filamentous *Chloroflexus*-like organisms (2). This result confirms the importance of glycolate as a carbon source for aerobic chemoorganotrophy in Octopus Spring mat, and illustrates the importance of using ecologically relevant carbon substrates to cultivate the more numerically abundant bacterial species. Combining cultivation and molecular retrieval approaches has allowed us to confirm the *Chloroflexus*-like nature of the sheathed trichomes which grow to the highest dilutions in GLD medium (30).

The other carbon substrates (i.e. CND, TYD, and MTD) only yielded growth to a 10^5 -fold dilution. Further growth in these enrichments may have been inhibited by the inappropriateness of the carbon source provided or the relatively high substrate concentrations provided in these enrichments. To test the hypothesis that substrate concentration influences the extent of growth in serial dilution enrichment culture, 0.1%, 0.01%, and 0.001% tryptone and yeast extract were provided in separate dilution series. The observation that substrate concentration did not influence the extent of growth (all substrate concentrations yielded growth to a 10^8 -fold dilution) was unexpected, since the levels of soluble organic substrates in densely populated cyanobacterial mats may be quite low, favoring organisms adapted to low substrate concentrations. The aerobic mat heterotroph *Isosphaera pallida* was successfully cultivated using oligotrophic substrate concentrations (i.e. unamended mineral salts medium) (15). The relatively high substrate concentrations provided in the enrichments reported in this paper may have selectively recovered only those

populations adapted to high organic carbon concentrations.

These results allow the comparison of cloning and cultivation techniques as methods to describe microbial diversity in natural environments. Although attempts were made to cultivate the more numerically abundant aerobic chemoorganotrophic bacteria from Octopus Spring mat by utilizing serial dilution enrichment culture and possibly more natural incubation conditions, we still failed to cultivate most organisms whose 16S rRNA sequences were previously retrieved. Since samples for cultivation and molecular cloning experiments were not collected simultaneously, this failure may have been due to seasonal bacterial population variation. However, recent studies of 16S rRNA sequence type variation in Octopus Spring mat have shown remarkable seasonal stability of bacterial populations (13). Alternatively, our failure to cultivate organisms with retrieved 16S rRNA sequences may indicate either that the retrieved sequences do not correspond to an aerobic chemoorganotrophic metabolism or that we did not cultivate the relevant organisms. The inability of enrichment culture to recover predominant populations is well documented (1), and isolation techniques which require growth as a colony on solid media may further limit retrieval of relevant organisms (29,37), so simplified species diversity using cultivation methods is not unexpected. However, 16S rRNA sequence retrieval methods may also underestimate species diversity by only detecting those species whose nucleic acids are readily cloned or PCR amplified (10,27). Although we expect the 16S rRNA of numerically abundant organisms to appear in cloning libraries, these techniques may suffer from a lack of sensitivity, resulting in the inability to detect less abundant populations such as

those presented in this paper.

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CHAPTER 4

PHOTOSYNTHATE PARTITIONING AND FERMENTATION IN HOT SPRING
MICROBIAL MAT COMMUNITIES¹.Introduction

The laminated cyanobacterial mat communities inhabiting alkaline silicious hot springs in Yellowstone National Park have been intensively investigated in an effort to make fundamental observations about bacterial species diversity (16,18,45,46,55,63) and microbial community ecology (6,62,65). Previous studies investigating the activity and growth of cells in these well-characterized communities suggested that hot spring cyanobacterial mats display high rates of metabolic activity and growth. Using oxygen and pH microelectrodes, Revsbech and Ward (49) measured oxygen production and pH variation corresponding to very high rates of gross primary productivity by oxygenic photosynthetic cyanobacteria (*Synechococcus* spp.). After darkening a mat and observing the decrease of *Synechococcus* cells over time, Brock (6) calculated washout rates for cells of this morphology. By assuming that washout rate equals

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replacement, or growth rate, cyanobacterial productivity was calculated to be 5.7×10^{11} to 1.6×10^{12} cells $\text{m}^{-2} \text{day}^{-1}$, corresponding to 0.098 to 0.263 doublings day^{-1} . In other experiments, accretion rates of mat material above a silicon carbide layer sifted onto the mat surface were used to measure growth rates of 18 to 45 $\mu\text{m day}^{-1}$ (13). Microautoradiographic studies of hot spring cyanobacterial mat samples demonstrated light-stimulated $^{14}\text{CO}_2$ uptake and glycolate excretion (up to 7% of total photosynthate) by cells conforming to the *Synechococcus* morphology (3). Clearly, cyanobacterial cells in Octopus Spring mat are photosynthetically active and appear to be rapidly growing.

Analysis of molecular synthesis from carbon substrates can provide information about the physiological state of microbial cells. Synthesis of ribosomes and ribosomal RNA is closely associated with cellular growth regulation, since modulation of ribosome content is a primary mechanism by which growth rates are controlled (38). Ribosomal RNA content is proportional to cellular growth rate in logarithmically growing cultures of *Escherichia coli* (21), and RNA content was found to increase exponentially with growth rate of the freshwater cyanobacterium *Anacystis nidulans* (39) and a marine *Synechococcus* strain (32). Synthesis of lipids can provide information about cellular activity (61), and protein synthesis is closely related to cellular growth rate (12). The relative incorporation of photosynthetically fixed carbon into protein, polysaccharide, lipid, and low molecular weight metabolites has been used to determine which environmental factors affect the physiological state of cyanobacterial cultures (28,30,31), and naturally occurring phytoplankton communities

(e.g. (19,22,42,47)). These studies show that nutrient availability, light conditions, and temperature can influence how photosynthate is partitioned into growth-related molecules (i.e. protein, low molecular weight metabolites, and membrane lipids) or storage polymers (polysaccharide and storage lipids) (19,42).

A study of photosynthate partitioning in a hot spring cyanobacterial mat community revealed that mat cyanobacteria primarily synthesize polysaccharide under illuminated conditions (29). Other studies of cyanobacterial pure cultures have shown that carbon and energy is stored in the form of polyglucose during photoautotrophic growth (57). This polymer is accumulated when carbon assimilation exceeds nutritional requirements for the synthesis of cellular components, especially under conditions of nutrient limitation (1,10,36,60). Fermentation of endogenous polyglucose reserves under dark anaerobic conditions has been shown in a variety of cyanobacterial species (58), including unicellular (41) and thermophilic (50) types. Although microbial mat communities undergo marked diel fluctuations in oxygen concentration (48,49), and dark cyanobacterial fermentation of stored polyglucose has been considered an important mechanism of carbon flux through microbial mat communities (58), to our knowledge, no studies have investigated this phenomenon in situ.

In this study, we investigated the qualitative and quantitative incorporation of photosynthetically fixed carbon into molecular components of a logarithmically growing *Synechococcus* isolate C1 culture and hot spring cyanobacterial mat communities. We showed that photosynthate is primarily incorporated into

polyglucose during periods of illumination instead of other cellular components, including protein and rRNA. We also demonstrated the fermentation of these stored polyglucose reserves under the dark, anaerobic conditions cyanobacterial mats experience at night.

Materials and Methods

Mat Samples and Cultures

Cyanobacterial mat samples were collected between June and November of 1994 and 1995 from similar temperature regions of mildly alkaline silicious hot springs in Yellowstone National Park, Wyoming. The locations of Octopus Spring (pH 8.7, 48 to 65°C) and Twin Butte Vista Spring (pH 9.1, 60 to 62°C) are described by Brock (6). Clearwater Springs site D (pH 7.8, 52 to 59°C) is located north of Norris Geyser Basin as described by Ruff-Roberts et al. (52). Cores of mat material were collected using a no. 4 cork borer and the top 2-3 mm of photosynthetically active mat was separated from lower layers using a razor blade. The top green layer was transferred to screw-capped glass vials containing 3 ml water from the collection site before addition of radiolabel.

The Octopus Spring cyanobacterial mat isolate *Synechococcus* sp. C1 (C1) (18) was generously provided by M. J. Ferris. Cultures were grown to late log phase in 50 ml medium D (8) in a 250 ml culture flask with shaking (150 rpm) at 50°C under constant illumination provided by three 50 W fluorescent bulbs. Irradiance measured

with a Lutron light meter (Coopersburg, PA) was 1150 to 1200 Lux. For comparison, ambient irradiance measured above the mat surface in Yellowstone National Park during field incubations was 40 to 55 kLux, but light is rapidly attenuated in the top 1 to 2 mm of the mat (34). Klett absorbance readings were compared to a standard curve relating absorbance to cell number determined by direct microscopy to detect logarithmic growth (1.49 ± 0.53 doublings day⁻¹, mean $\pm$ SD, n=5). Cell densities in the culture flasks were 3.2×10^7 to 5.2×10^7 cells ml⁻¹ before radiolabel administration. The pH of cultures was adjusted to 8.5 before addition of radiolabel.

Radiolabeling

Each cyanobacterial mat core received either 2 μ Ci [¹⁴C]sodium bicarbonate (54 mCi/mmol), 2 μ Ci [³²P]sodium phosphate dibasic (1 Ci/mmol), or 1 μ Ci [2-¹⁴C]sodium acetate (3.1 mCi/mmol). Logarithmically growing C1 cultures received 2 μ Ci [¹⁴C]sodium bicarbonate. Radiolabel was added from a concentrated stock solution and dispersed by gentle agitation. All isotopes were procured from New England Nuclear (Boston, MA).

Triplicate samples were incubated in the presence of radiolabeled substrate for 3 hours unless otherwise noted. Cyanobacterial mat cores were incubated at ambient hot spring temperatures and light intensities by placing vials in the effluent channel at the site of sample collection during time periods proximate to solar noon. Dark conditions were achieved by wrapping vials with aluminum foil and black tape.

During nutrient addition experiments, nitrogen and phosphorous were added to 10% of

the concentrations present in medium D (which contains 922 μM NO_3^- and 77 μM PO_4^{2-}) from a concentrated stock solution before addition of the mat core. During cell density reduction manipulations, individual mat cores were homogenized in 3 ml spring water. Following incubation, field samples were frozen on dry ice to stop biological activity, and cultures were harvested immediately. Since frozen cells may leak organic carbon, measured amounts of incorporated radiolabel may underestimate actual radiolabel incorporation, especially into low molecular weight cellular components. Cells were concentrated by centrifugation (20 minutes, $15,000 \times g$) and washed once with sterile water to reduce unincorporated $^{14}\text{CO}_2$ and excreted photosynthate. We measured $98.5\% \pm 1.7\%$ (mean $\pm$ SD, $n=21$) removal of extracellular ^{14}C in the first cell wash. $^{14}\text{CO}_2$ uptake into cellular material during nutrient addition and homogenization experiments was compared to unmanipulated controls using a two sample t-test.

During fermentation experiments, Clearwater Springs site D mat cores were pre-incubated in the presence of 2 μCi $^{14}\text{CO}_2$ for three hours in the light, then shifted to dark anaerobic conditions. Radiolabeled water was replaced with spring water rendered anoxic by bubbling with nitrogen. In some cases, 2-bromoethanesulfonic acid (to 50 mM) was added to inhibit methanogenesis (BES^+) (2) or formalin (to 4%) was added to terminate biotic activity. In a separate experiment, we determined that cells removed during the replacement procedure contained $4.7\% \pm 4.5\%$ (mean $\pm$ SD, $n=6$) of the radiolabel associated with cells remaining in the vial. After replacement, vials were darkened as described above and the gas headspace of each sealed vial was

flushed with nitrogen. Samples were then incubated for 0, 3, 6, 12, and 24 hours at temperatures within 4°C of the ambient hot spring temperature.

Nucleic Acid Analysis

Cell lysates were prepared from radiolabeled Octopus Spring mat and C1 cultures in a FastPrep bead beater (Bio 101, Vista, CA) using sterile 0.1 mm diameter zirconium beads and a high salt lysis buffer containing 1 M NaCl, 5 mM MgCl₂, and 10 mM Tris base, pH 8.0 to minimize nucleic acid degradation (4). Nucleic acids were obtained from the lysate using standard phenol-chloroform extraction and ethanol precipitation procedures modified to minimize nuclease degradation of RNA (53). Nucleic acids were separated on a 3.5% to 10% linear polyacrylamide gradient denaturing gel or a 10% denaturing polyacrylamide gel overlaid with a 3.5% denaturing polyacrylamide gel (53). Equivalent amounts of radioactivity were loaded onto the gel without regard for nucleic acid concentration. Nucleic acids were stained with ethidium bromide and photographed using standard procedures (53). The gel was dried using a Drygel Sr. vacuum drying apparatus (Hoeffer Sci. Instr., San Francisco, CA). Autoradiography was performed on the dried gel with a PhosphorImager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Polysaccharide Identification

An aliquot of radiolabeled Octopus Spring mat nucleic acid extract or 1.5 mg glycogen (Sigma Chemical Co., St. Louis, MO) was transferred to 4 ml teflon-lined

screw-capped glass test tubes. Inositol was added to each tube as an internal standard (20 μ l of 10 mM inositol in glass-distilled water) and samples were lyophilized overnight. Polysaccharides were hydrolyzed and the resulting monosaccharides were methylated in a single step by adding 1 M methanolic hydrochloric acid (500 μ l) under a stream of nitrogen and allowing the reaction to proceed in the sealed tubes overnight at 80°C. Samples were dried under a stream of nitrogen at room temperature and a trimethylsilyl (TMS) derivitizing reagent (Sigma Sil-A, Sigma Chemical Co.) was added (200 μ l) to replace hydroxyl groups with ester-linked TMS moieties. The derivitization reaction was allowed to proceed for 20 minutes at 80°C. Samples were again dried under a stream of nitrogen and dissolved in 100 μ l hexane for coupled gas chromatography-mass spectrometry analysis.

TMS-derivitized monosaccharides were separated in a Varian (model 3700) gas chromatograph equipped with a 30 m DB-1 glass capillary column (0.25 mm i.d., J & W Scientific, Folsom, CA). A splitless injector operating 30 seconds after injection (injector temperature 260°C) was used. A temperature program was begun at injection with an initial column temperature of 160°C for 3 minutes followed by a 3°C min⁻¹ rise to 260°C, which was then held for 15 minutes. Helium carrier gas flow rate was 30 cm sec⁻¹. The gas chromatograph was directly coupled to a VG 70E-HF double focussing magnetic mass spectrometer operating at a mass resolution of 1500. Ion source temperature was 200°C, the electron energy was 70 eV, and the ions were accelerated with 6 KeV of energy. Data were acquired on a VG 11-250 data system based upon a DEC 11-73 computer.

Radiolabeled Octopus Spring mat nucleic acid extract was incubated for 3 hours at 37°C with 1 U α -amylase and/or 1 U amyloglucosidase (Boehringer Mannheim Corp., Indianapolis, IN) in an aqueous reaction buffer containing 5 mM NaCl and 2 mM phosphate buffer, pH 5.8. Samples were precipitated with 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol, then centrifuged for 20 minutes at 16,000 $\times g$. The supernatant was removed and the remaining pellet was dissolved in sterile water. Radioactivity in the supernatant and the dissolved pellet was determined as described below. Radiolabeled nucleic acid extracts from Octopus Spring mat and logarithmically growing C1 cultures were also incubated with 40 $\mu\text{g ml}^{-1}$ proteinase K (Sigma Chemical Co.) in water for 10 minutes at 45°C, or RNase A (1 μg , Sigma Chemical Co.) or RNase-free DNase I (10 U, Boehringer Mannheim Corp.) in a buffer containing 3 mM MgCl_2 for 1 hour at 37°C. Protease and nuclease digestions were phenol-chloroform extracted and ethanol precipitated as described above. All samples were separated on a 10% denaturing polyacrylamide gel overlaid with a 3.5% polyacrylamide gel. Gels were dried and autoradiography was performed as described above.

Protein Analysis

Radiolabeled cell lysates from Octopus Spring mat and C1 cultures were prepared as described for nucleic acid extractions but with sterile water instead of high salt lysis buffer. Proteins were separated by denaturing polyacrylamide gel electrophoresis using the discontinuous system of Laemmli (35). The separating gel

was 1.5 mm thick and contained a 7% to 20% linear polyacrylamide gradient. Equivalent amounts of radioactivity were loaded onto the gel without regard for protein concentration. The gel was dried and autoradiography was performed as described above. Silver staining was performed according to the procedure of Blum et al. (5).

Lipid Analysis

Lipids were extracted from radiolabeled Octopus Spring mat and C1 cultures using a chloroform/methanol/phosphate buffer extraction solvent as described by Guckert et al. (25). Total lipid was separated by 2-dimensional thin layer chromatography according to the procedure of Evans et al. (15) using the following solvent systems: chloroform:methanol:acetic acid:diethylamine:water (120:35:37.5:6:4.5, by vol.) in the first dimension, and chloroform:methanol:ammonium hydroxide (65:25:5, by vol.) in the second dimension. Lipids were detected by charring with sulfuric acid (27). Equivalent amounts of radioactivity were loaded onto the plates without regard for lipid concentration. Autoradiography was performed as described above. Diagnostic stains for phospholipids (molybdenum blue), aminolipids (ninhydrin), and glycolipids (α -naphthol) were applied to the developed plates according to the directions supplied by the manufacturer (Alltech Assoc. Inc., Deerfield, IL).

Photosynthate Partitioning

The major end products of photosynthesis were determined in mat samples and C1 cultures by partitioning incorporated carbon into chloroform-soluble (lipid), methanol-water-soluble (low molecular weight metabolites), hot trichloroacetic acid (TCA)-soluble (polysaccharide) and hot TCA-insoluble (protein) fractions by the method of Morris et al. (44) as modified by Fitzsimons et al. (20) to maximize removal of polysaccharide from the TCA-insoluble pellet. Nucleic acids partition into the polysaccharide fraction in this fractionation scheme (43). An aliquot of radiolabeled mat nucleic acid extract was lyophilized overnight and subjected to the fractionation scheme described above. To validate cell fractionation procedures, the amount of anthrone-reactive polysaccharide in each of the four fractions was determined as described by Herbert et al. (26). Of the original anthrone-reactive material present in unfractionated cells, 80.8% was recovered in the polysaccharide fraction, 4.8% was recovered in both the protein and lipid fractions, and 1.0% was recovered in the low molecular weight metabolite fraction. Radioactivity in each of these fractions was determined as described below. Total incorporated radiolabel was calculated by summing the radioactivity detected in each fraction. By comparison to whole-cell incorporation, recovery of radiolabel by this method was determined to be $94.6\% \pm 13.2\%$ (mean $\pm$ SD, $n=7$).

Headspace Gas Analysis

Headspace gasses (carbon dioxide and methane) in the unopened vials after

dark anaerobic incubations were measured with a gas chromatograph as previously described (64). Radioactivity in $^{14}\text{CO}_2$ and $^{14}\text{CH}_4$ was determined by coupling the gas chromatograph to a gas proportional counter (64).

Volatile Fatty Acid Detection

The supernatants of samples incubated under dark anaerobic conditions were analyzed for volatile fatty acids (VFAs) after centrifugation (20 minutes, $15,000 \times g$) to remove cells. VFAs were separated by high performance liquid chromatography using a DX300 ion chromatography system and detected with a conductivity detector (Dionex, Sunnyvale, CA). VFAs were separated isocratically using a HPICE-AS10 ion exchange column, an eluent consisting of 3.5 mM $\text{K}_2\text{B}_4\text{O}_7$, and a self-regenerating suppressor. A column regeneration step consisting of an increase in eluent strength to 100 mM $\text{K}_2\text{B}_4\text{O}_7$ was performed after each run. Calibration was achieved by comparison of retention times and peak heights to external standards. The detection limits for acetate, propionate, and butyrate were approximately $0.5 \mu\text{g ml}^{-1}$, whereas the detection limits for n-butyrate and i-butyrate were approximately $1.0 \mu\text{g ml}^{-1}$. The lowest reported acetate concentration was above $3 \mu\text{g ml}^{-1}$. Column eluent fractions corresponding to the retention time of each VFA, as well as fractions prior to and after VFA elution were collected manually. Radiolabeled carbon was determined as described below. VFA specific activity was calculated by dividing the total radioactivity detected in the collected fraction by the measured concentration of the VFA.

Radioassays

Water samples and molecular fractions were radioassayed for ^{32}P and ^{14}C content using a Packard Tri-Carb liquid scintillation analyzer (Model 1900 TR). Aliquots of aqueous samples were added to Optima Gold (Packard Instr. Co., Meriden, CT) scintillation cocktail. Lipid samples were dried under a stream of nitrogen, dissolved in chloroform, and 10% was removed to a scintillation vial and dried prior to scintillant addition. The entire protein fraction was reacted with tissue solublizer (Scintigest, Fisher Scientific, Pittsburg, PA, or Soluene 350, Packard Instr. Co.) for 1 hour prior to scintillant addition. Background counts were subtracted from sample counts before counts per minute were converted to disintegrations per minute (DPM) using an external standards ratio method of quench correction. Counting continued until a constant coefficient of variation was achieved ($\text{cv}=0.5\%$) or until 10 minutes had elapsed.

Results

Logarithmically growing C1 cultures and mat cores from three different hot springs actively incorporated $^{14}\text{CO}_2$ into cellular material under illuminated conditions (Table 6). When normalized to the number of cells present in the incubations, C1 cultures and intact mat cores incorporated approximately equivalent amounts of radioactivity. $^{14}\text{CO}_2$ incorporation did not deplete available radiolabel under both light and dark conditions during the three hour incubation time. Dark incorporation of

Table 6. $^{14}\text{CO}_2$ uptake in various hot spring cyanobacterial mat samples and logarithmically growing *Synechococcus* isolate C1 cells^a.

Sample	Light		Dark	
	$^{14}\text{CO}_2$ incorporation (DPM cell ⁻¹ × 10 ⁻⁶)	% label incorporated of total added	$^{14}\text{CO}_2$ incorporation (DPM cell ⁻¹ × 10 ⁻⁶)	% label incorporated of total added
Octopus Spring ^b	1404 ± 409	26.8 ± 5.7	97 ± 7	2.0 ± 0.2
Twin Butte Vista Spring ^b	2181 ± 310	39.1 ± 3.1	65 ± 53	1.3 ± 1.1
Clearwater Springs site D ^b	1673 ± 88	70.5 ± 4.4	38 ± 6	1.9 ± 0.4
<i>Synechococcus</i> isolate C1	1250 ± 487	54.0 ± 9.2	-	-

^aMean ± 95% confidence interval (Twin Butte Vista Spring and Clearwater Springs site D, n=3; Octopus Spring, n=4; *Synechococcus* isolate C1, n=5).

^bIncorporation values assume 1×10^{10} cells ml⁻¹ in mat material (13).

$^{14}\text{CO}_2$ was 6.9%, 3.0%, and 2.3% of photoautotrophic carbon fixation in Octopus Spring, Twin Butte Vista Spring, and Clearwater Springs mats, respectively.

Nucleic Acid Synthesis

Radiolabeling with $^{14}\text{CO}_2$. We detected nucleic acids in both logarithmically growing C1 cultures and cyanobacterial mat samples (Figure 5a) which corresponded to known sizes of standard *Escherichia coli* ribosomal RNA. Although we detected incorporation of $^{14}\text{CO}_2$ into 23S, 16S, and 5S ribosomal RNAs in logarithmically growing C1 cultures, we failed to detect incorporation of $^{14}\text{CO}_2$ into rRNAs in cyanobacterial mat samples (Figure 5b). Instead, radiolabel was detected in material which only slightly entered a 3.5% polyacrylamide gel. This material accumulated in mat samples incubated in the light, but not in dark incubated samples, and was also detected to a lesser extent in C1 cultures. We estimate the lower detection limit of radiolabeled rRNA by this method to be ca. 1.5×10^3 DPM, 60 times lower than the ca. 9.0×10^4 DPM associated with rRNAs in the C1 culture.

Radiolabeling with $^{32}\text{PO}_4^{2-}$. When $^{32}\text{PO}_4^{2-}$ was provided to the mat as a radiolabeled substrate, synthesis of rRNAs was detected (Figure 6). Of the added $^{32}\text{PO}_4^{2-}$, $51.0\% \pm 2.9\%$ was incorporated by the mat cells after 3.25 hours of light incubation (mean $\pm$ 95% confidence interval, $n=3$).

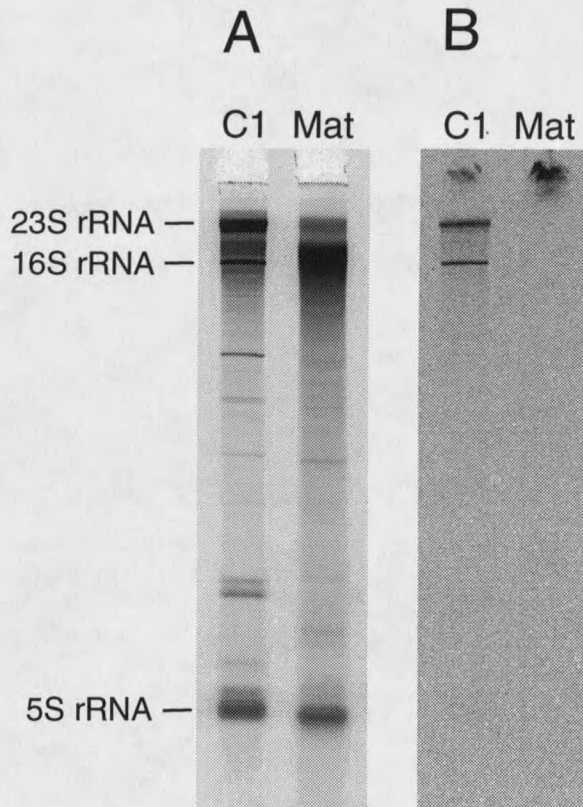


Figure 5. Composition of nucleic acid extracts from a logarithmically growing *Synechococcus* culture (C1) and Octopus Spring cyanobacterial mat community (Mat) after radiolabeling with $^{14}\text{CO}_2$ in the light. (A), negative photographic image of ethidium bromide-stained nucleic acids separated on a 3.5-10% linear polyacrylamide gradient denaturing gel; (B), autoradiogram of the same gel.

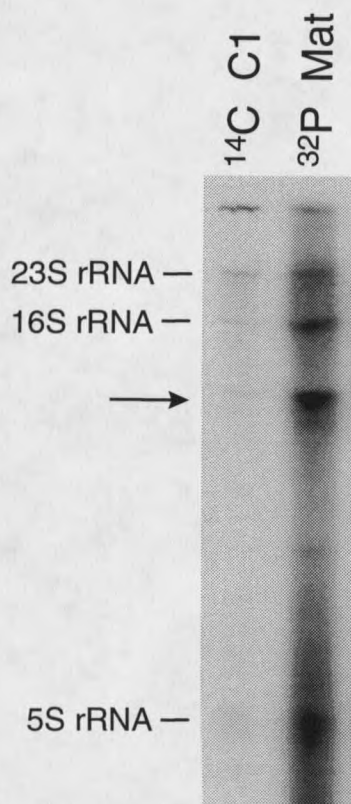


Figure 6. Autoradiogram of polyacrylamide gel containing nucleic acid extract from Octopus Spring cyanobacterial mat community after radiolabeling with $^{32}\text{PO}_4^{2-}$ in the light (^{32}P Mat) and ^{14}C -labeled *Synechococcus* isolate C1 nucleic acid extract (^{14}C C1). Arrow indicates interface between 3.5% and 10% polyacrylamide gel concentration.

Effect of Environmental Manipulations on rRNA Synthesis. Neither addition of nitrogen and phosphorous to the incubation water to alleviate possible nutrient imbalance nor homogenization and dilution of mat material to alleviate possible density-dependent regulation of cell physiology resulted in increased $^{14}\text{CO}_2$ uptake into cellular material when compared to unmanipulated controls ($p=0.5481$ and $p=0.8228$, respectively). rRNA synthesis from $^{14}\text{CO}_2$ could not be demonstrated for treatments including nutrient addition, homogenization and dilution, extended light and dark incubations (5 hours each), or shifting incubations from 2.5 hours light to 2.5 hours dark conditions (data not shown).

Identification of Radiolabeled Material in the Nucleic Acid Extract.

Radiolabeled material in mat nucleic acid extract was resistant to protease (proteinase K) and nuclease (RNase A, DNase I) treatments (data not shown). To test the hypothesis that mat nucleic acid extract contained radiolabeled polysaccharide, the extract was hydrolyzed, TMS-derivitized, and analyzed by combined gas chromatography-mass spectrometry. Gas chromatographic analysis showed that mat nucleic acid extract contained a compound which exhibited monomers with retention times identical to glucose derived from glycogen (Figure 7a). The two peaks observed in the glycogen standard correspond to the α - and β -anomeric forms of TMS-derivitized D-glucose (11). The major peaks in the Octopus Spring mat nucleic acid extract detected by gas chromatography exhibit mass spectra similar to TMS-derivitized hexose sugars (Figure 7b) by comparison to standard library spectra.

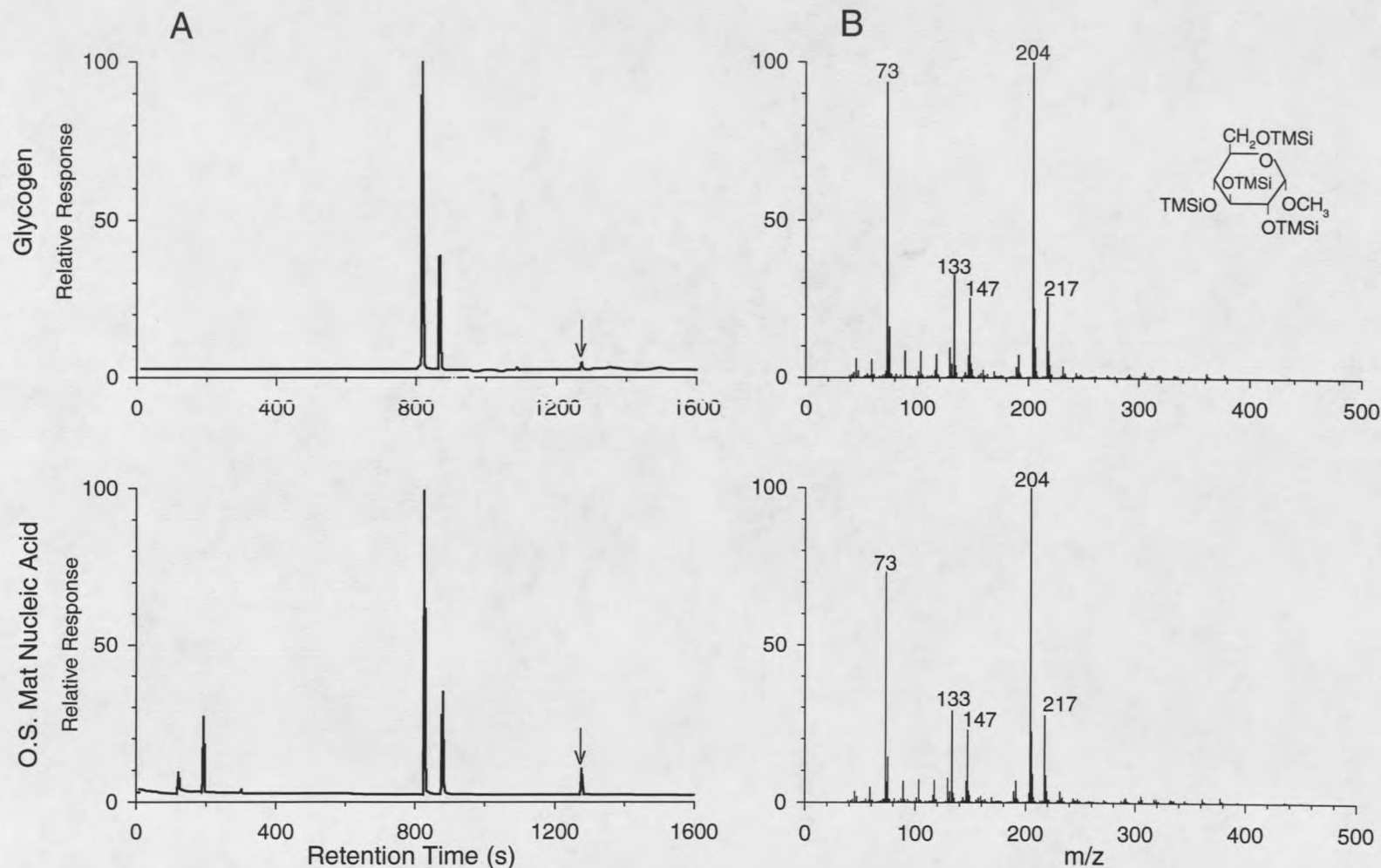


Figure 7. (A), Hydrolyzed and TMS-derivitized glycogen (top panel) and Octopus Spring mat nucleic acid extract (bottom panel) samples analyzed by gas chromatography. (B), Mass spectra of peaks with retention times of 838 and 833 seconds in glycogen and mat samples, respectively. O.S., Octopus Spring; TMSi, trimethylsilyl moiety. Arrows indicate inositol internal standard.

Minor peaks were not identified.

Radiolabeled material in the mat nucleic acid extract was labile in the presence of α -amylase and amyloglucosidase, two polyglucose-specific enzymes (Figure 8).

White precipitate observed in untreated controls could not be observed in the enzyme-treated samples after ethanol precipitation, and radiolabel could not be detected in the pellet after centrifugation. Of the original radiolabel added to the enzymatic digest, 87.3% was recovered in the ethanol wash removed after centrifugation.

Protein Synthesis

Although diverse proteins were detected in logarithmically growing C1 cultures and cyanobacterial mat material, there is little correspondence between observed protein sizes (Figure 9a). While C1 cells actively incorporated $^{14}\text{CO}_2$ into protein, we detected only limited protein synthesis in the cyanobacterial mat (Figure 9b). Instead, radiolabel was detected primarily in the well of the polyacrylamide gel.

Lipid Synthesis

Logarithmically growing C1 cultures and cyanobacterial mat material displayed similar lipids (Figure 10a) and similar patterns of $^{14}\text{CO}_2$ incorporation into lipids (Figure 10b). Aminolipids which were detected in the mat were not detected in C1 cultures (Figure 10a). Nearly all lipids which appear in Figure 10a received radiolabel, including pigments (Figure 10b).

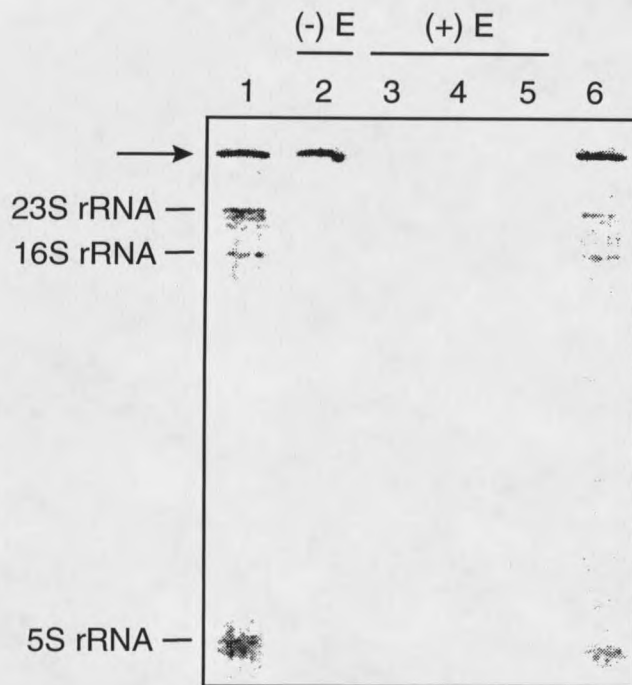


Figure 8. Autoradiogram of polyacrylamide gel containing enzymatically treated Octopus Spring mat nucleic acid extract after radiolabeling with $^{14}\text{CO}_2$ in the light. (-) E, no enzymatic treatment; (+) E, samples treated with polyglucose-specific enzymes as follows: lane 3, α -amylase and amyloglucosidase treatment; lane 4, α -amylase only; lane 5, amyloglucosidase only; lanes 1 and 6, radiolabeled *Synechococcus* isolate C1 nucleic acid (no enzymatic treatment). Arrow denotes location of well of polyacrylamide gel.

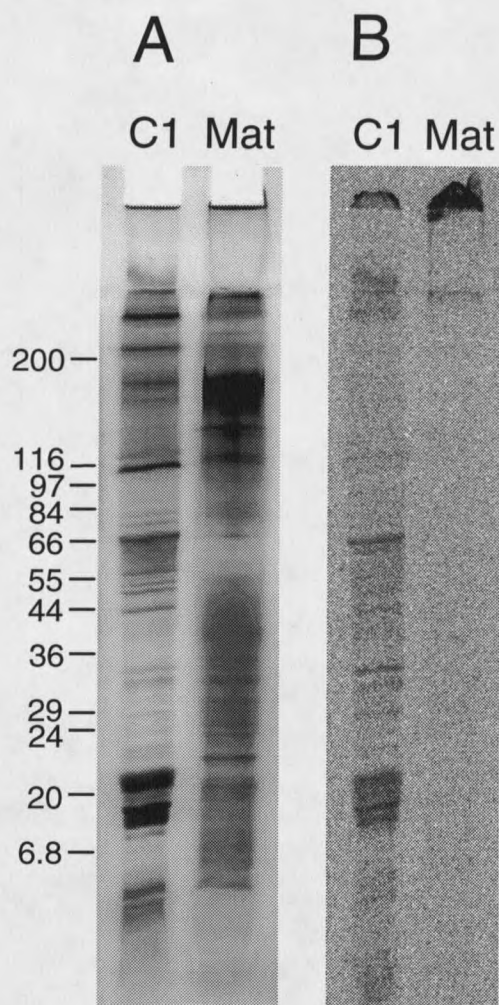


Figure 9. Composition of proteins solubilized from whole-cell extracts of a logarithmically growing *Synechococcus* isolate C1 culture (C1) and Octopus Spring cyanobacterial mat community (Mat) after radiolabeling with $^{14}\text{CO}_2$ in the light. (A), photographic image of silver-stained proteins separated by denaturing polyacrylamide gel electrophoresis; (B), autoradiogram of the same gel.

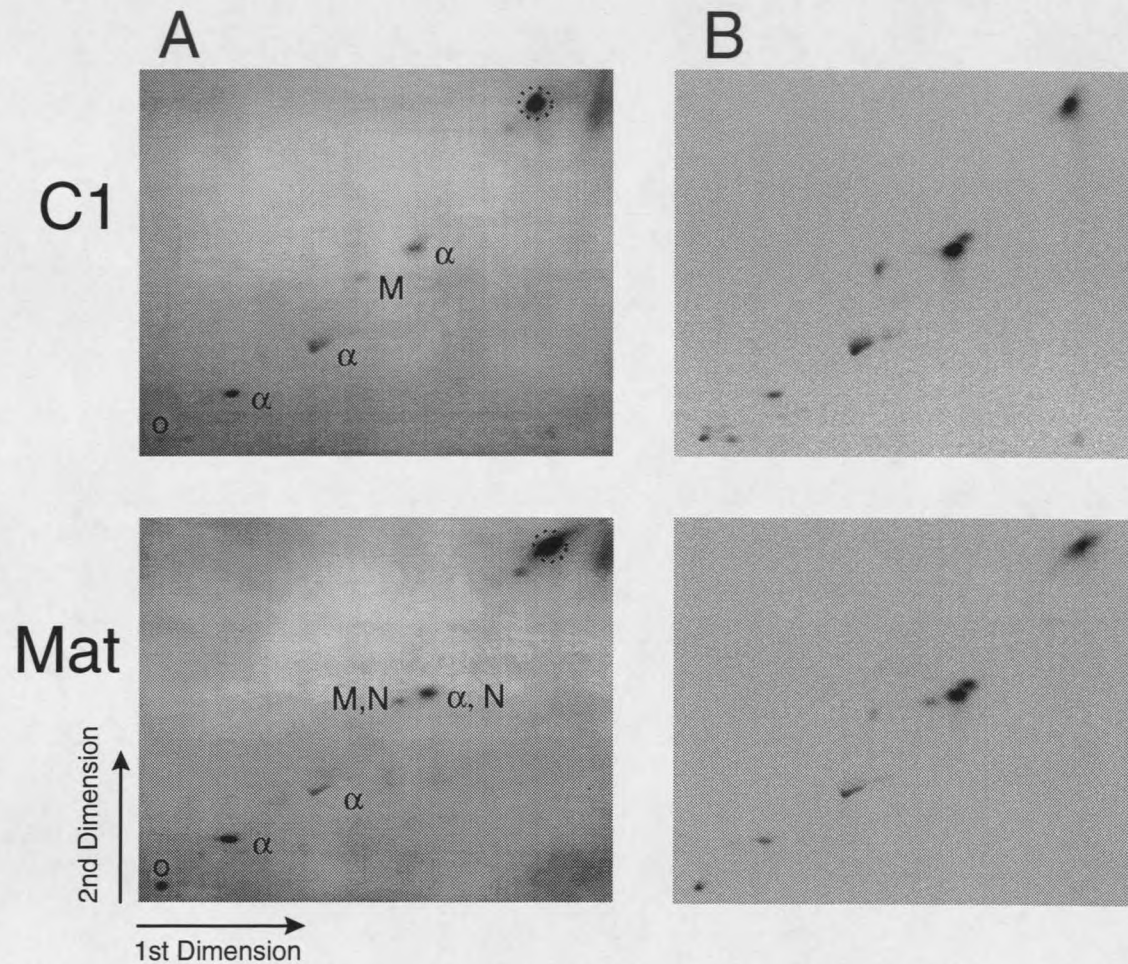


Figure 10. Composition of lipids extracted from a logarithmically growing *Synechococcus* isolate C1 culture (C1) and Octopus Spring cyanobacterial mat community (Mat) after radiolabeling with $^{14}\text{CO}_2$ in the light. (A), photographic image of sulfuric acid-charred 2-dimensional thin layer chromatography plates; (B), autoradiogram of the same plates. M, molybdenum blue reactive; N, ninhydrin reactive; α , α -naphthol reactive; O, origin. Encircled spots were pigmented green.

$^{14}\text{CO}_2$ Partitioning into Cellular Components

Various hot spring cyanobacterial mat samples and a logarithmically growing C1 culture were fractionated to quantify patterns of photosynthate partitioning into major molecular classes. Different hot spring cyanobacterial mat communities displayed remarkably similar patterns of photosynthate partitioning (Table 7). When incubated in the light, mat samples partitioned the majority of photosynthetically fixed carbon into the polysaccharide-containing fraction, whereas mat samples incubated in the dark primarily synthesized protein. In contrast, logarithmically growing C1 cultures partitioned photosynthate primarily into protein, and the remaining carbon was approximately equally divided between polysaccharide, lipid, and low molecular weight metabolites (Table 7). In an experiment to determine which cellular fraction contained the radiolabeled polyglucose detected in cyanobacterial mat nucleic acid extract, $98.1\% \pm 1.1\%$ (mean $\pm$ SD, $n=2$) was recovered in the polysaccharide fraction.

Polysaccharide Fermentation

Cyanobacterial mat samples were pre-incubated with $^{14}\text{CO}_2$ in the light to accumulate radiolabeled polysaccharide, then shifted to dark anaerobic conditions for up to 24 hours. Following incubation, radiolabel in cellular fractions and extracellular fermentation products was then determined. Upon transfer of photoautotrophically grown cells to dark anaerobic conditions, we observed a decrease in ^{14}C -labeled polysaccharide. Simultaneously, [^{14}C]acetate, [^{14}C]propionate, and $^{14}\text{CO}_2$ were produced (Figure 11a). Overall, radiolabel in the polysaccharide fraction decreased

Table 7. $^{14}\text{CO}_2$ partitioning among molecular fractions in logarithmically growing *Synechococcus* isolate C1 cultures and hot spring cyanobacterial mat samples incubated under light and dark conditions.

Fraction	% label in fraction ^a						
	Light				Dark		
	O.S.	C.W.S.	T.B.V.	C1	O.S.	C.W.S.	T.B.V.
Polysaccharide	84.6 ± 3.6	77.0 ± 3.8	84.0 ± 2.8	14.9 ± 2.5	5.7 ± 2.8	5.2 ± 4.9	11.3 ± 3.2
Protein	9.3 ± 3.0	11.3 ± 2.7	9.9 ± 3.3	44.8 ± 2.7	88.4 ± 3.2	88.8 ± 9.3	78.8 ± 4.4
Lipid	1.9 ± 0.3	8.3 ± 1.6	2.1 ± 0.3	18.5 ± 3.2	3.1 ± 1.4	2.5 ± 1.8	6.2 ± 1.4
LMW	4.0 ± 0.5	3.5 ± 0.9	4.5 ± 0.3	21.8 ± 1.9	2.0 ± 0.3	3.4 ± 3.0	3.8 ± 2.5

^aPercent of total incorporated radiolabel detected in molecular fraction. Mean ± 95% confidence interval (n=3, T.B.V. and C.W.S.; n=4, O.S. and C1)

O.S., Octopus Spring; C.W.S., Clearwater Springs site D; T.B.V., Twin Butte Vista Spring; C1, *Synechococcus* isolate C1

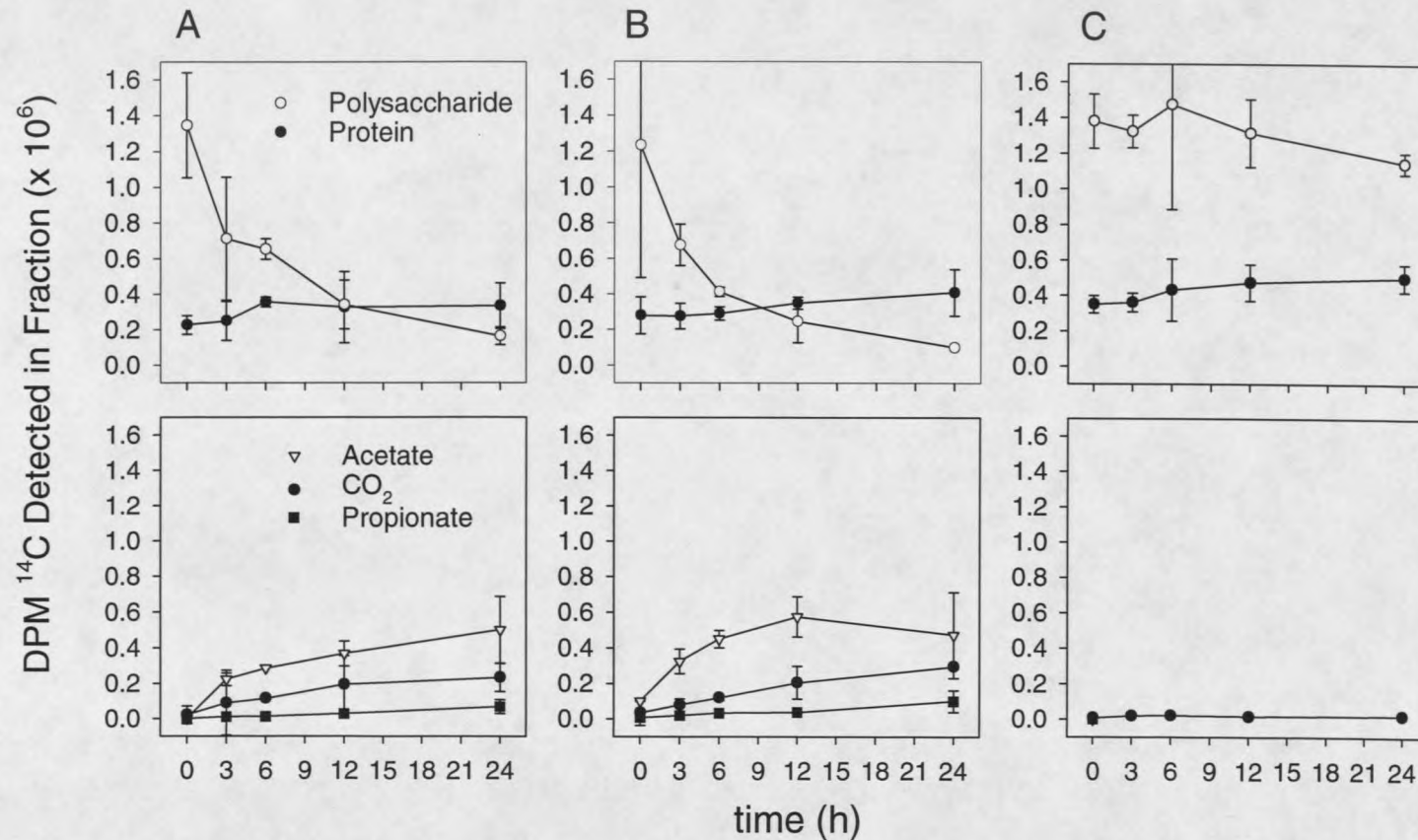


Figure 11. (A), Changes in ¹⁴C detected in polysaccharide and protein cellular fractions, and acetate, CO₂, and propionate fermentation products in Clearwater Springs site D mat cores shifted from a 3 hour light incubation in the presence of ¹⁴CO₂ to a 0, 3, 6, 12, and 24 hour dark anaerobic incubation. (B), with bromoethanesulfonic acid added to inhibit methanogenesis; (C), formalin killed control. Error bars indicate 95% (top panel) and 90% (bottom panel) confidence intervals about the mean (n=3, except for 0 hours BES⁺ samples n=2, and 6 hours unamended fermentation product values n=1, so only this value is reported).

74.7% after 12 hours of dark incubation, of which 58.5% was recovered in ^{14}C -labeled fermentation products. Acetate was the most abundant ^{14}C -labeled fermentation product. Radiolabel in the protein fraction remained constant or increased only slightly during dark anaerobic incubation. Addition of BES to inhibit methanogenic hydrogen and CO_2 consumption (2) resulted in a more rapid accumulation of radiolabeled acetate (compare Figure 11b to Figure 11a). Radiolabeled polysaccharide in BES^+ samples decreased 80.0% after 12 hours, and recovery of radiolabeled fermentation products was higher in BES^+ treatments (82.9%) than in unamended samples. Small amounts of $^{14}\text{CH}_4$ were detected in unamended samples after 24 hours dark incubation, but none was detected in equivalent BES^+ samples. We observed a linear decrease in the specific activity of acetate from 3 to 24 hours dark incubation (43.9% and 43.1% decrease in unamended and BES^+ treatments after 24 hours, respectively). Specific activity of propionate did not significantly change over time. Formalin-killed controls displayed no readily identifiable trend (Figure 11c).

^{14}C Acetate Partitioning into Cellular Components

The fate of excreted acetate was investigated by fractionating mat material incubated under light and dark conditions in the presence of ^{14}C acetate into molecular classes. Dark incorporation of ^{14}C acetate was 29.9% and 60.5% of light incorporation in Octopus Spring and Clearwater Springs site D mats, respectively. Incorporated ^{14}C acetate was primarily partitioned into polysaccharide and protein fractions in the light, and into protein in the dark (Table 8). rRNA synthesis could not

be detected when [^{14}C]acetate was added as a radiolabeled substrate. Instead, labeled material was detected in the well of a 3.5% polyacrylamide gel which was resistant to α -amylase and amyloglucosidase enzymatic digestion (data not shown).

Table 8. [^{14}C]Acetate partitioning among molecular fractions in Octopus Spring and Clearwater Springs site D cyanobacterial mat samples incubated under light and dark conditions.

Fraction	% label in fraction ^a			
	Light		Dark	
	O.S.	C.W.S.	O.S.	C.W.S.
Polysaccharide	56.3 $\pm$ 2.3	37.2 $\pm$ 6.5	9.9 $\pm$ 3.5	7.6 $\pm$ 1.9
Protein	27.7 $\pm$ 3.5	52.2 $\pm$ 7.0	61.7 $\pm$ 23.1	76.2 $\pm$ 3.9
Lipid	12.3 $\pm$ 2.8	8.5 $\pm$ 2.3	25.7 $\pm$ 17.0	12.1 $\pm$ 4.4
LMW	3.7 $\pm$ 0.7	2.1 $\pm$ 0.4	2.6 $\pm$ 2.8	4.1 $\pm$ 1.1

^aPercent of total incorporated radiolabel detected in molecular fraction. Mean $\pm$ 95% confidence interval (n=3, O.S.; n=4, C.W.S.)

O.S., Octopus Spring; C.W.S., Clearwater Springs site D

Discussion

Here we present several lines of evidence which suggest that although the oxygenic photosynthetic cyanobacteria inhabiting hot spring mat communities are very active (49), they do not appear to be rapidly dividing. First, we failed to detect incorporation of $^{14}\text{CO}_2$ into ribosomal RNA, a sensitive indicator of the rate of cyanobacterial cell division (32,39). Manipulation of nutrient levels, cell density, and light conditions failed to induce detectable rRNA synthesis from $^{14}\text{CO}_2$. Since rRNA

synthesis from $^{14}\text{CO}_2$ was below detection limits, a high specific activity preparation of $^{32}\text{PO}_4^{2-}$ was provided as a radiolabeled substrate to increase the sensitivity of detecting synthesis of phosphorous-containing molecules such as rRNA. Although the observed incorporation of $^{32}\text{PO}_4^{2-}$ indicates limited rRNA synthesis, we cannot necessarily associate this synthesis with cyanobacterial growth, since both photoautotrophic and heterotrophic organisms assimilate inorganic phosphate. Second, we detected only limited synthesis of proteins and lipids in the mat. Instead, mat samples primarily accumulated polyglucose reserves in the light (77.0% to 84.6% of total incorporated carbon). In contrast to mat samples, logarithmically growing C1 cultures readily incorporated $^{14}\text{CO}_2$ into rRNA, protein, lipid, and low molecular weight metabolites.

These results are consistent with the hypothesis that hot spring cyanobacterial mats are active, but contradict earlier studies which suggested that mats are rapidly growing. Calculation of cyanobacterial productivity from measured washout rates following darkening (6) requires the assumption that washout rate equals replacement rate. Washout rates may more accurately measure cyanobacterial death rate after elimination of light as the energy source for oxygenic photosynthesis. Accretion rates above silicon carbide layers added to hot spring mat surfaces provide compelling evidence for rapid mat growth (13), but these experiments may merely reflect a disturbance artifact. The community above the silicon carbide layer might be in a post-disturbance successional phase which would exhibit different species composition and different growth rates than the original community below the layer (see below).

Studies of elemental composition, carbon partitioning, and molecular synthesis

in natural phytoplankton populations have shown that phytoplankton are photosynthetically active and often exhibit growth at or near maximal rates (22,23,33,42,44). Phytoplankton populations are also subject to disturbance (i.e. removal of biomass (24)) by zooplankton grazing which may force phytoplankton populations to grow and divide to replace lost biomass (e.g. (7,40,56)). In contrast, hot spring cyanobacterial mats inhabit high temperature environments that are devoid of eukaryotic grazers and which allow microbial mats to accumulate without grazing disturbance (6). The low "disturbance potential" (59) which characterizes hot spring environments may be one reason we observe limited synthesis of growth-related molecules in mature mat communities. In support of this hypothesis, we observed changes in cyanobacterial populations and patterns of carbon partitioning after disturbing the photosynthetically active layer of the Octopus Spring cyanobacterial mat. Five days after removing the green surface layer, we detected a pattern of photosynthate partitioning similar to logarithmically growing C1 cultures (i.e. protein, lipid, and low molecular weight metabolite synthesis). After 21 days, the pattern of photosynthate partitioning had changed to more closely resemble undisturbed regions of the mat (i.e. primarily polysaccharide synthesis) (17) [See Chapter 5].

The physiological state of hot spring cyanobacteria characterized by high rates of activity combined with relatively slow growth may occur in other microbial communities. For example, bacteroid-forming root nodule symbionts (i.e. *Rhizobium* spp.) are also found at high cell densities and actively fix elemental nitrogen, but do not rapidly grow (67). The bioluminescent fish and squid light organ symbiont *Vibrio*

fischeri actively produces light but does not rapidly divide under the high cell density conditions which characterize these habitats (14,51). Perhaps other microbial communities characterized by high activity and high cell densities (e.g. temperate cyanobacterial mats and biofilms) may also exhibit slow growth.

The decrease in photosynthetically fixed radiolabeled polyglucose and corresponding increase in radiolabeled fermentation products suggests that *Synechococcus* spp. switch to a fermentative metabolism under dark anoxic conditions. Interestingly, we detected no significant increase of radiolabel in the protein fraction during dark incubation. Other studies investigating photosynthate partitioning in phytoplankton and microbial mat communities have shown movement of radiolabel from storage products to protein at night (9,22,29,37), but these experiments were performed on low cell density cell suspensions incubated under aerobic conditions. Our observation that protein synthesis does not readily occur even after an extended dark incubation supports the conclusion that native mat *Synechococcus* populations are not rapidly growing.

The measured accumulation of fermentation products under dark anaerobic conditions closely parallels results obtained by Anderson et al. (2) who showed that acetate and propionate were the major fermentation products to accumulate overnight. The populations which perform fermentative metabolisms have, until now, not been identified, although numerous fermentative bacteria have been isolated from hot spring mats (e.g. (66,68,69)). By detecting the accumulation of radiolabeled fermentation products from radiolabeled polyglucose, we have identified cyanobacterial populations

as contributors to the pool of fermentation products.

Cyanobacterial fermentation may not be the only anaerobic process contributing to acetate pools, since other acetate producing processes such as acetogenesis and non-cyanobacterial fermentation may be occurring simultaneously. Acetogenic bacteria (2) and the previously discussed fermentative bacteria have been cultivated from hot spring cyanobacterial mats, and acetogenic conversion of CO_2 and H_2 to acetate was suspected to occur in these habitats under dark anaerobic conditions (3). Here we present evidence in support of the hypothesis that acetate production occurs by both cyanobacterial fermentation and other acetate-producing processes. If cyanobacterial fermentation was the sole source of anaerobic acetate production, acetate specific activity would remain constant over time. However, acetate specific activity decreased over time, indicating that unlabeled acetate accumulated concurrently with radiolabeled acetate. In addition, acetate accumulated more quickly in BES^+ treatments than in unamended samples, possibly due to removal of competition between methanogens and acetogens for hydrogen, leading to increased acetate production. Finally, we detected greater recovery of fermentation products in BES^+ treatments (82.9% recovery versus 58.5% in unamended samples), possibly due to $^{14}\text{CO}_2$ conversion to ^{14}C acetate rather than to $^{14}\text{CH}_4$, a product which was not quantified. The lower recovery of fermentation products in unamended samples may result in an underestimation of the extent of cyanobacterial fermentation. Thus, both cyanobacterial fermentation and other forms of acetate production must occur simultaneously. However, cyanobacteria must play an important role in acetate production as acetate specific activity was only

diluted ca. 43% during a 24 hour dark incubation. Combined with the observation that acetoclastic methanogenesis does not occur in Octopus Spring mat (54), these mat communities appear to be physiologically poised to produce and accumulate acetate under dark anaerobic conditions (2).

By combining the results from this paper with previous physiological studies, it is possible to construct a plausible description of carbon flow through hot spring cyanobacterial mat communities. During the day, carbon is photoautotrophically incorporated by *Synechococcus* spp. and is mainly stored as polyglucose. Fixed carbon is also photoexcreted as glycolate, which is readily incorporated by *Chloroflexus*-like filaments (3). At night, *Synechococcus* spp. respond to anaerobic conditions by fermenting the majority of stored polyglucose, thereby producing acetate and propionate, most of which accumulates overnight (2). CO₂ produced from polyglucose may be further converted to acetate by acetogenic bacteria (2) or lost from the system as methane (54). CO₂ incorporated in the dark is primarily synthesized into protein, which could either be the product of dark cyanobacterial incorporation (57) or the product of carbon assimilation by acetogenic or methanogenic bacteria. The next day, *Chloroflexus*-like filamentous organisms photoheterotrophically incorporate accumulated fermentation products (2,54), and primarily synthesize protein and polysaccharide, but not rRNA. This scenario describes significant transfer of carbon from the primary producers (*Synechococcus* spp.) to the filamentous heterotrophic *Chloroflexus*-like primary consumers throughout the diel cycle. The extent of carbon transfer is nearly complete, leaving little carbon for *Synechococcus* cell replication. The basis for this symbiotic association is not understood.

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CHAPTER 5

PHYSIOLOGICAL SUCCESSION AFTER DISTURBANCE
OF A HOT SPRING CYANOBACTERIAL MAT¹Introduction

The preceding chapter of this thesis argues that although the thermophilic cyanobacteria (*Synechococcus* spp.) inhabiting hot spring microbial mat communities are photosynthetically active, they do not appear to be rapidly growing (5). There are several lines of evidence which lead us to this conclusion. First, we failed to detect incorporation of $^{14}\text{CO}_2$ into ribosomal RNA, a sensitive indicator of cell division (Figure 5). Manipulation of light conditions and nutrient levels, as well as homogenization of mat samples to reduce cell densities failed to induce rRNA synthesis. We also detected only limited synthesis of growth-related molecules such as protein, lipid, and low molecular weight metabolites when $^{14}\text{CO}_2$ was provided as a radiolabeled substrate. Instead, when incubated in the light, mat samples primarily synthesized polyglucose, a carbon and energy storage molecule (77 to 85% of total

¹This study has been submitted for publication as part of a larger study in Applied and Environmental Microbiology as: Ferris, M. J., S. C. Nold, N. P. Revsbech, and D. M. Ward. Bacterial population succession following a disturbance of a hot spring microbial mat community.

incorporated carbon, Table 7). This pattern of polyglucose synthesis in the light was demonstrated in three different hot spring cyanobacterial mats and appears to be a stable physiological response of mat cyanobacteria to conditions of illumination. In contrast, logarithmically growing cyanobacterial cells do incorporate $^{14}\text{CO}_2$ into ribosomal RNA (Figure 5). We also detected extensive synthesis of protein, lipid, and low molecular weight metabolites (cumulatively accounting for 85% of total incorporated carbon, Table 7) in growing *Synechococcus* cultures.

The major goal of this thesis is to associate microbial activity with microbial populations whose 16S rRNA sequence types were detected in natural mat samples. One method which could potentially accomplish this goal is the use of capture probes to investigate the extent of radiolabel incorporation into the 16S rRNA sequence types synthesized by growing microbial populations (this concept is more fully developed in Chapter 1). Successful use of capture probes, however, requires that sufficient amounts of radiolabel are incorporated into ribosomal RNA. Since we failed to detect incorporation of $^{14}\text{CO}_2$ into rRNA in active cyanobacterial mats, it would appear that the use of capture probes to associate microbial activity with individual 16S rRNA sequence types would similarly fail.

There must be conditions, however, under which mat communities do synthesize rRNA. Ecologically, disturbance has been defined as the loss of biomass from a community (4). Disturbance to hot spring microbial mat communities occurs during hailstorms or after sudden shifts in environmental conditions such as temperature and pH. After such events, newly exposed surfaces become covered with

microbial mat material. This replacement of lost biomass can occur either by species immigration into the disturbed area, or by growth and cellular division of immigrant or native species (1). If biomass replacement after disturbance occurs at least in part by growth and cellular division, then it should be possible to detect synthesis of growth-related molecules such as protein, lipid, low molecular weight metabolites, and rRNA. The following experiments were performed (i) to assess our ability to detect synthesis of growth related molecules under conditions when mat cyanobacteria may not be primarily synthesizing polyglucose, and (ii) to test the hypothesis that cyanobacterial biomass lost from mat communities during disturbance events is replaced primarily by growth and cellular division rather than immigration.

Materials and Methods

An area of the top green cyanobacterial layer (ca. 7 by 15 by 0.5 cm deep) was scraped away from the surface of the mat community inhabiting the effluent channel of Octopus Spring (Yellowstone National Park, Wyoming, USA) (2). Previous studies have shown that microbial populations in these environments are essentially homogenous within an area of this size (3). Sampling began on June 9th, 1995 (day 0) and continued at intervals of 5, 12, and 21 days. Triplicate cores of mat material from both the disturbed site and an adjacent undisturbed site were collected with a no. 4 cork borer and the top 2-3 mm of photosynthetically active mat was separated from lower layers using a razor blade. The top green layer was transferred to glass vials

containing water from the collection site, and samples were incubated in situ for 3 hours at ambient temperatures and light intensities in the presence of 1 μCi [^{14}C]sodium bicarbonate (54.6 mCi/mmol; New England Nuclear). Following incubation, samples were frozen on dry ice to stop biological activity. Cells were concentrated by centrifugation (20 minutes, $15,000 \times g$) and washed once with sterile water to reduce unincorporated $^{14}\text{CO}_2$. The major end products of photosynthesis were determined by partitioning cellular carbon into chloroform-soluble (lipid), methanol-water-soluble (low molecular weight metabolites), hot trichloroacetic acid-soluble (polysaccharide) and hot trichloroacetic acid-insoluble (protein) fractions using methods described in Chapter 4. Radioactivity in each of these fractions was determined as described in Chapter 4. Total incorporated radiolabel was calculated by summing the radioactivity detected in each fraction. The extent of radiolabel incorporation into cellular material in disturbed and undisturbed regions of the mat was compared using a two sample t-test.

Results

Within 5 days, an evenly distributed, faintly green layer was observed on the surface of the disturbed mat. Undisturbed mat material incorporated similar amounts of radiolabel (3.96×10^5 and 5.97×10^5 DPM $^{14}\text{CO}_2 \cdot \text{core}^{-1}$) and partitioned the majority of photosynthetically fixed carbon into the polysaccharide fraction (67 to 79% of total incorporated radiolabel) during the experimental time period (Figure 12a). In

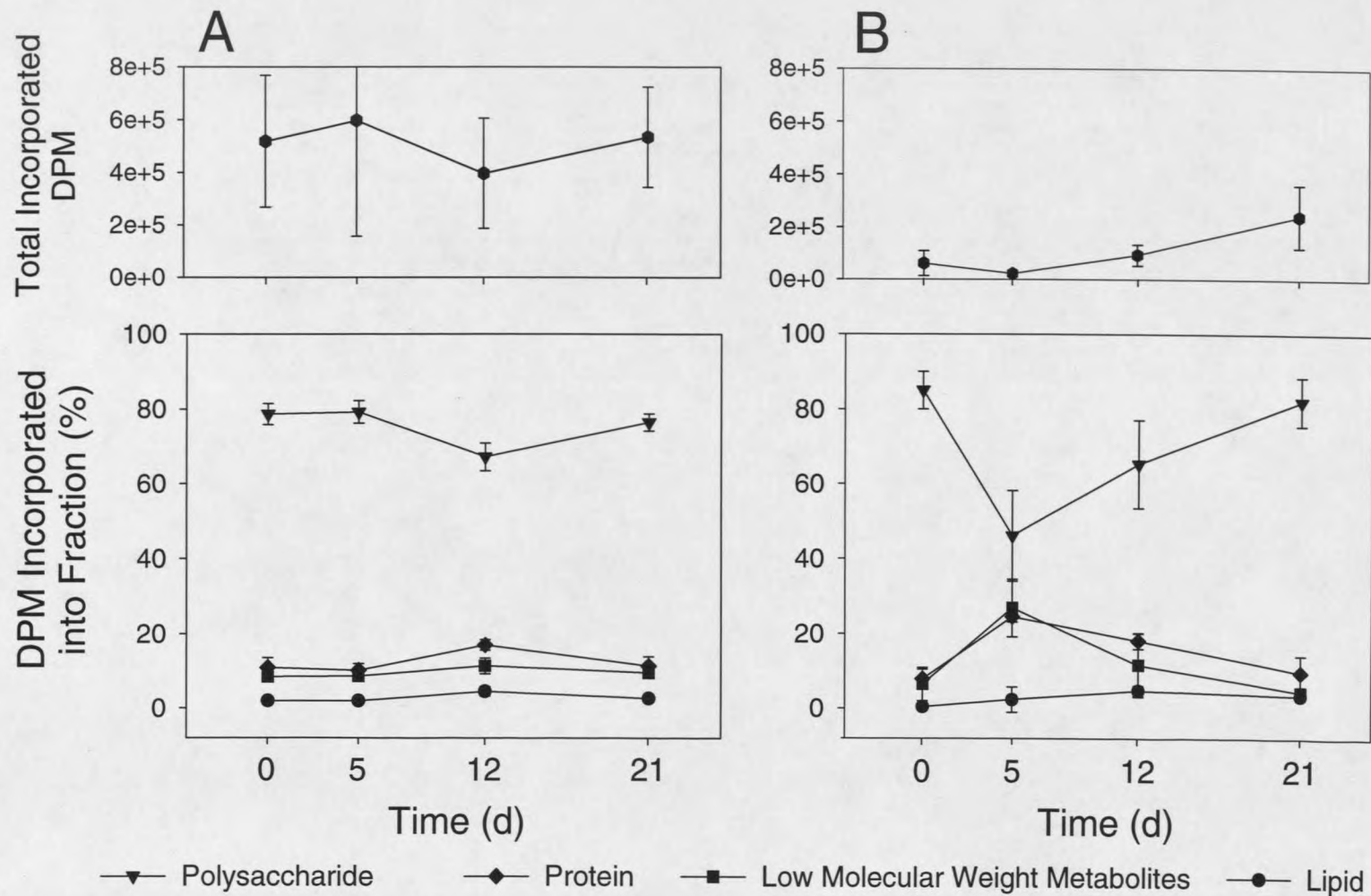


Figure 12. Carbon incorporation (top panel) and carbon partitioning into polysaccharide, protein, lipid, and low molecular weight metabolite cellular fractions (bottom panel). (A), undisturbed mat; (B), scraped mat 0, 5, 12, and 21 days after disturbance. Error bars represent 95% confidence intervals about the mean (n=3).

contrast, cells in the disturbed mat incorporated significantly less carbon than in undisturbed mat on all sampling dates ($p < 0.0305$, $n = 3$), and displayed an increase in carbon incorporation between days 5 and 21 (Figure 12b, top panel). In addition, cells in the disturbed region of the mat displayed variable patterns of carbon partitioning between molecular components (Figure 12b, bottom panel). On day 5, significantly less carbon was incorporated into the polysaccharide fraction, and significantly more carbon was incorporated into the protein and low molecular weight fractions in disturbed mat samples than in undisturbed mat samples. Carbon incorporated into the polysaccharide fraction increased from days 5 to 21, and carbon incorporated into protein and low molecular weight fractions decreased over the same time interval. By day 21, the pattern of carbon partitioning was similar to undisturbed controls. Immediately after removal of the *Synechococcus* layer (day 0), the majority of the radiolabel associated with cellular material was detected in the polysaccharide fraction.

Discussion

These results demonstrate our ability to detect changes in carbon incorporation into molecular classes when the mat cyanobacteria are not primarily synthesizing polyglucose. The proportion of photosynthetically fixed carbon allocated to polysaccharide synthesis increased, and allocation of carbon to protein and low molecular weight metabolite synthesis decreased during the 5 to 21 day time interval. As early as 5 days after the disturbance event, incorporated carbon was synthesized

into growth related molecules such as protein and low molecular weight metabolites in a manner similar to logarithmically growing *Synechococcus* cultures (Table 7). By 21 days, the pattern of carbon partitioning among molecular classes was similar to undisturbed mat communities in this and other hot spring environments (Chapter 4).

These results are consistent with the hypothesis that cyanobacterial biomass lost during disturbance events is replaced by growth and cellular division. We cannot, however, reject the possibility that immigration contributes to biomass replacement, since this process must be occurring simultaneously. No attempt was made to exclude deposition of cells from the water flowing over the mat. Therefore, colonizers growing on the newly exposed substrate may have been immigrants which were deposited onto the disturbed mat from adjacent undisturbed regions of the mat.

These results were obtained as part of a larger study investigating the effects of disturbance on a hot spring microbial mat community which included microelectrode measurements of oxygenic photosynthesis (N. P. Revsbech, unpublished) and molecular characterization of microbial community composition (M. J. Ferris, unpublished). Rates of oxygenic photosynthesis increased from net oxygen consumption on day 0 to measurable oxygen production after 21 days which was approximately 45% of the photosynthetic rate measured in undisturbed mat. Although no cyanobacterial 16S rRNA sequence types were detected on day 0, cyanobacterial sequences were observed during subsequent sampling dates. Oxygen production and community composition results correspond to the physiological results presented in this chapter to describe the successive changes in microbial activity and community

composition which occurred after disturbance to the mat community.

Since microelectrode measurements showed no cyanobacterial oxygen production on day 0, and no cyanobacterial 16S rRNA sequences were detected at this time point, the observed incorporation of $^{14}\text{CO}_2$ into polysaccharide on day 0 (see Figure 12b, bottom panel) is unlikely to be a valid measure of cyanobacterial photoautotrophy.

Previous studies have demonstrated the effect of environmental change on the distribution of 16S rRNA sequence types in hot spring cyanobacterial mat communities. Using ^{32}P -labeled oligodeoxynucleotide hybridization probes designed to target individual 16S rRNA sequence types, Ruff-Roberts et al. (6) demonstrated changes in bacterial populations when mat samples were exposed to different temperatures, pHs, and light intensities. The probe response of some rRNA sequence types increased from below detection limit to readily detectable levels after only 1 week of environmental perturbation, suggesting that ribosomal RNA was actively synthesized. These observations, combined with the conclusion from this study that cyanobacterial cells may grow after a disturbance event, indicate that there may be conditions under which rRNA is synthesized by cyanobacterial mat community members. Although native mat material does not normally synthesize significant amounts of rRNA, disturbance to the mat community or perturbation of environmental conditions may induce synthesis of detectable quantities of radiolabeled rRNA.

The potential for mat communities which are undergoing post-disturbance or post-perturbation physiological changes to synthesize rRNA has implications for the

use of capture probe technologies. Successful capture probe assessment of metabolic activity may be possible when mat communities are undergoing recolonization. If sufficient quantities of radiolabel associated with individual rRNA sequence types can be detected, then this method may become a viable mechanism to address the major goal of this thesis. However, conclusions based on such studies should be approached with caution. As this study shows, the physiological status of microorganisms after a disturbance event are clearly different than in an undisturbed mat. Synthesis of ribosomal RNA after disturbance or perturbation should be considered an artifact of environmental manipulation which does not reflect the prevailing physiological activity of hot spring cyanobacterial mat inhabitants.

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CHAPTER 6

MOLECULAR ANALYSIS OF HOT SPRING MICROBIAL MATS TO STUDY
BACTERIAL DIVERSITY AND PHYSIOLOGY: A SUMMARY

The preceding studies investigating the diversity of aerobic chemoorganotrophic bacteria and the fate of photosynthetically fixed carbon have not only increased our understanding of bacterial diversity in hot spring cyanobacterial mat communities, they have revealed that although mat cyanobacteria are photosynthetically active, they grow unexpectedly slowly. These experiments are also the first to demonstrate cyanobacterial fermentation in situ, a critical step in understanding the nature and extent of carbon flux from mat photoautotrophs to other microbial community members. By combining the results presented in this thesis with other observations, this chapter reviews our present knowledge of (i) the diversity of aerobic chemoorganotrophic bacteria in hot spring cyanobacterial mat communities, and (ii) the physiological activity of mat cyanobacteria as they exist in nature. In addition, the validity of the hypotheses tested by these experiments is discussed.

Aerobic Chemoorganotrophic Bacterial Diversity

The bacterial isolates which were cultivated during these studies (Chapters 2

and 3) (12,13) are compared to aerobic chemoorganotrophic bacterial populations known to inhabit Octopus Spring mat in Table 9. Cultivation attempts yielded seven genotypically distinct isolates which belong to the *Thermus*, proteobacterial, and Gram positive lines of descent (Table 9, column 3). Although attempts were made to use more ecologically relevant incubation conditions, and mat inoculum was diluted to extinction to provide a measure of relative abundance of the cultivated species, only one bacterial isolate whose 16S rRNA sequence had been previously detected in the mat was successfully cultivated (isolate ac-15=O.S. type N). This result is significant because it confirms that the 16S rRNA sequences retrieved from the mat by molecular cloning techniques do correspond to actual bacterial species which inhabit the mat. However, most of the organisms whose sequences were retrieved from the mat remain uncultivated (Table 9, compare columns 2 and 3), underscoring how cultivation and molecular retrieval approaches provide very different views of microbial community composition. The failure of these cultivation attempts to recover microorganisms whose 16S rRNA sequences were observed in mat samples also illustrates the limitations of using cultivation approaches to achieve the major goal of this thesis, to associate microbial activities with 16S rRNA sequence types detected in natural mat samples.

Isolates ac-15=O.S. type N and ac-16 are the first proteobacterial isolates to be cultivated from Octopus Spring mat community. Although these isolates are phylogenetically related to members of the β subdivision of the proteobacteria, the physiological diversity exhibited by bacteria in this subdivision precludes attempts to

Table 9. Bacteria known to inhabit Octopus Spring which have or could have aerobic chemoorganotrophic metabolisms based on cultivation and molecular studies.
Correspondence between methods is indicated by underlining between adjacent columns.

Previously Cultivated	16S rRNA Retrieved	Cultivated During This Study	Populations Detected in Broth Enrichment Cultures ^a
Green non-sulfur bacteria			
			env.OS_ace3
			env.OS_ace4
			env.OS_ace5
<i>Chloroflexus aurantiacus</i> Y-400-fl			
<i>Thermomicrobium roseum</i>			
	O.S. type C		
	OS-V-L-20		
Deinococcus/Thermus Group			
<i>Thermus</i> sp. OS-Ramaley-4			
<i>Thermus aquaticus</i> YT-1			
		<i>Th. aquaticus</i> -like isolate ac-7	
		<i>Th. aquaticus</i> -like isolate ac-1	<i>Th. aqu.</i> -like ac-1
			<i>Th. aqu.</i> -like ac-7'
		<i>Th. ruber</i> -like isolate ac-2	<i>Th. ru.</i> -like ac-2
		<i>Th. ruber</i> -like isolate ac-17	
Planctomyces			
<i>Isosphaera pallida</i> IS1B			
Gram positive bacteria			
Low G + C subdivision		isolate ac-18	
High G + C subdivision			env.OS_ace1
			env.OS_ace6
Proteobacteria			
Alpha subdivision	O.S. type O		
			env.OS_ace2
Beta subdivision	O.S. type G		
	O.S. type N	isolate ac-15=O.S. type N	O.S. type N
			O.S. type N'
	O.S. type R		
		isolate ac-16	
Gamma subdivision			env.OS_ace7
Delta subdivision			env.OS_ace8
Green sulfur-like bacteria			
	O.S. type E		
	O.S. type M		
	OS-III-9		
Uncertain Affiliation			
	O.S. type L		O.S. type L
	O.S. type D		
	O.S. type F		
	OP-I-2		

^aDetected in broth enrichments using denaturing gradient gel electrophoresis separation of PCR-amplified 16S rRNA sequence fragments (20).

infer the physiological activities which these isolates exhibit in nature based on 16S rRNA nucleotide sequence information. However, by cultivating isolate ac-15=O.S. type N using enrichment culture techniques which selected for aerobic chemoorganotrophic bacteria, a metabolic potential (i.e. aerobic chemoorganotrophy) was associated with a 16S rRNA sequence type detected in nature.

A diversity of *Thermus* isolates were cultivated which displayed 16S rRNA nucleotide sequence similarity to *Thermus aquaticus* and *Thermus ruber*, two thermophilic bacterial species which were previously cultivated from hot spring habitats. The genus *Thermus* has recently been emended to reflect the differences in temperature adaptation and 16S rRNA nucleotide sequence which distinguish *Thermus ruber* from other *Thermus* species. *Thermus ruber* and closely related species (22) have been transferred to a new genus, *Meiothermus* (11). The *Thermus* isolates cultivated during these investigations displayed optimal growth rates at different temperatures, which corresponded to their observed abundance in the mat (e.g. the low temperature-adapted isolate ac-17 was more abundant in the 50-55°C region of the mat than were isolates ac-7 and ac-1, both of which were adapted for optimal growth at higher temperatures). These results indicate that *Thermus* species distribution in hot spring cyanobacterial mats may be controlled at least in part by adaptation to temperature.

Four Gram positive bacterial isolates were cultivated which displayed stable phenotypic differences, yet they all exhibited 16S rRNA nucleotide sequences identical to isolate ac-18 through a variable region of the molecule. These results illustrate the

conserved nature of estimates of bacterial diversity based on 16S rRNA molecular sequence comparisons.

The most numerically abundant bacterial isolates (ac-19, ac-20, and ac-21, which survived 10^7 - and 10^8 -fold dilutions) were cultivated on media containing glycolate as a carbon source. Unfortunately, these isolates were difficult to perpetuate in pure culture, and could not be grown to sufficient quantity for 16S rRNA sequence characterization. They did, however, resemble the *Chloroflexus* cells suspected to incorporate glycolate excreted by photosynthetically active cyanobacterial cells (2). This result confirms the importance of glycolate as a carbon source for aerobic chemoorganotrophy in Octopus Spring mat, and illustrates the importance of using ecologically relevant carbon substrates to cultivate the more numerically abundant bacterial species.

However, even the most abundant bacterial species cultivated from highly diluted inocula (10^7 - to 10^8 -fold) still contained 2-3 orders of magnitude more *Synechococcus* cells in the inoculum source, indicating the relative numerical insignificance of the species cultivated. Isolate ac-15=O.S. type N was estimated to be present at even lower population densities, containing almost 5 orders of magnitude (9.0×10^4) more *Synechococcus* cells in the inoculum source. These observations indicate either that most populations containing retrieved 16S rRNA sequences types are incapable of aerobic chemoorganotrophy, or that we did not cultivate the numerically abundant populations. Although seemingly more ecologically relevant culture conditions were provided, the microenvironments which characterize microbial

mat habitats are probably poorly reproduced in enrichment culture environments. The results presented in Chapter 4 indicate that acetate and propionate are important carbon substrates for mat heterotrophy and would be logical choices as carbon substrates in future attempts to cultivate the bacteria whose 16S rRNA sequences have been previously detected in Octopus Spring mat.

These experiments demonstrated the ability of selective enrichment culture techniques to cultivate physiologically related microbial populations from natural habitats. However, the aerobic chemoorganotrophic isolates obtained using enrichment culture techniques were different from the bacterial populations detected using molecular retrieval techniques. To further examine how enrichment culture conditions may influence culture composition, a separate study was initiated by a visiting Dutch student, Sjila Santegoeds, with whom I collaborated. This study employed similar enrichment culture conditions to those utilized in Chapters 2 and 3, but used denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA fragments to monitor the species composition of broth enrichment cultures (20). In this study, we detected 14 different 16S rRNA sequence types in broth enrichment cultures. These sequence types are compared to bacterial populations known to inhabit Octopus Spring mat in Table 9. Only three of the bacterial species contributing sequence types detected in broth enrichments were recovered on solidified media after similar enrichment (*Thermus* isolates ac-1 and ac-2, and β -proteobacterial isolate ac-15=O.S. type N; Table 9, compare columns 3 and 4). Only two of the sequences detected in broth enrichment cultures were previously detected in the mat using

molecular retrieval techniques (O.S. type N and O.S. type L, Table 9, compare columns 2 and 4). These results reveal an even greater diversity of aerobic chemoorganotrophic bacterial populations inhabiting Octopus Spring mat than was previously detected.

Combining the results presented in Chapters 2 and 3 with the study which monitored liquid enrichment culture composition illustrates the difficulty encountered when attempting to cultivate the numerically abundant microbial species from natural habitats. With two exceptions, O.S. type N and O.S. type L, these cultivation attempts failed to recover those microbial species whose 16S rRNA sequences were retrieved from Octopus Spring mat. There appear to be two levels of selection during enrichment culture that change community composition. First, enrichment in liquid media whose elemental composition and physical conditions may be very different from the natural hot spring mat environment appears to change species composition so that only those organisms which can divide and grow under the provided conditions are enriched (23,25). Second, the requirement for organisms to grow as a colony on solidified media appears to reduce the number of species recovered from liquid enrichments. This result is demonstrated by comparing populations detected in broth enrichments to those recovered on solidified media (Table 9, compare columns 3 and 4). Clearly, the species recovered in liquid media are very different than those organisms which are observed using molecular retrieval techniques, and the species recovered on solidified media are different than those recovered in liquid media. Molecular retrieval methods may also bias our view of bacterial species diversity by

only detecting those species which are readily cloned or PCR amplified (7,18).

Molecular retrieval techniques may also suffer from a lack of sensitivity, resulting in the inability to detect the less abundant populations in natural microbial communities.

Two potential solutions to the problem of our failure to recover numerically abundant microbial species from natural habitats have recently been demonstrated. Advances in enrichment culture technology have increased our ability to cultivate relevant microbial populations. By serially diluting inoculum before enrichment (8,21), providing a range of conditions (i.e. pH and salt concentration) from which the relevant microorganisms can choose (6,26), and using optical micromanipulator techniques to isolate single microbial cells for use as an inoculum source (24), researchers have begun to cultivate more numerically abundant microbial species from natural environments. The other solution might be to avoid studying pure cultures altogether. Dr. D. E. Caldwell has recently argued that the era of the study of pure cultures is over, and that we should concentrate on microbial assemblages as units of study. By studying intact microbial communities, the possibility of disrupting potentially interesting interactions is avoided, and the full complexity of microbial community activities can be appreciated (5). More likely, the solution to this problem will require careful cultivation and phenotypic characterization of the numerically abundant microorganisms followed by assessment of their distribution, abundance, and activity in the microbial community from which they were cultivated.

Fate of Photosynthetically Fixed Carbon

The preceding section of this chapter summarizes the difficulties encountered when employing cultivation techniques to address the major goal of this thesis. One cultivation-independent approach to associate the activity of microbial populations whose 16S rRNA types are detected in mat communities is the use of capture probes to investigate synthesis of 16S rRNA by growing microbial populations (this concept is more fully developed in Chapter 1). To investigate the feasibility of using capture probe techniques, the extent of $^{14}\text{CO}_2$ incorporation into rRNA molecules in Octopus Spring mat was measured. Although the cyanobacterial cells inhabiting hot spring cyanobacterial mat communities are photosynthetically active (17), we could not detect incorporation of $^{14}\text{CO}_2$ into ribosomal RNA (14). We also detected only limited synthesis of molecules associated with microbial growth (i.e. lipid, protein, and low molecular weight metabolites). Instead, mat samples incubated in the light primarily synthesized polyglucose, a polymer used for carbon and energy storage. In contrast, we detected incorporation of $^{14}\text{CO}_2$ into rRNA, lipid, protein, and low molecular weight metabolites in logarithmically growing cyanobacterial cells (Chapter 4). These results suggest that hot spring mat cyanobacterial populations are not rapidly growing. We also demonstrated cyanobacterial fermentation of polyglucose reserves under the dark, anaerobic conditions which mats experience at night (Chapter 4).

These results allow the construction of a conceptual model which describes the flux of carbon through hot spring cyanobacterial mat communities (Figure 13). During

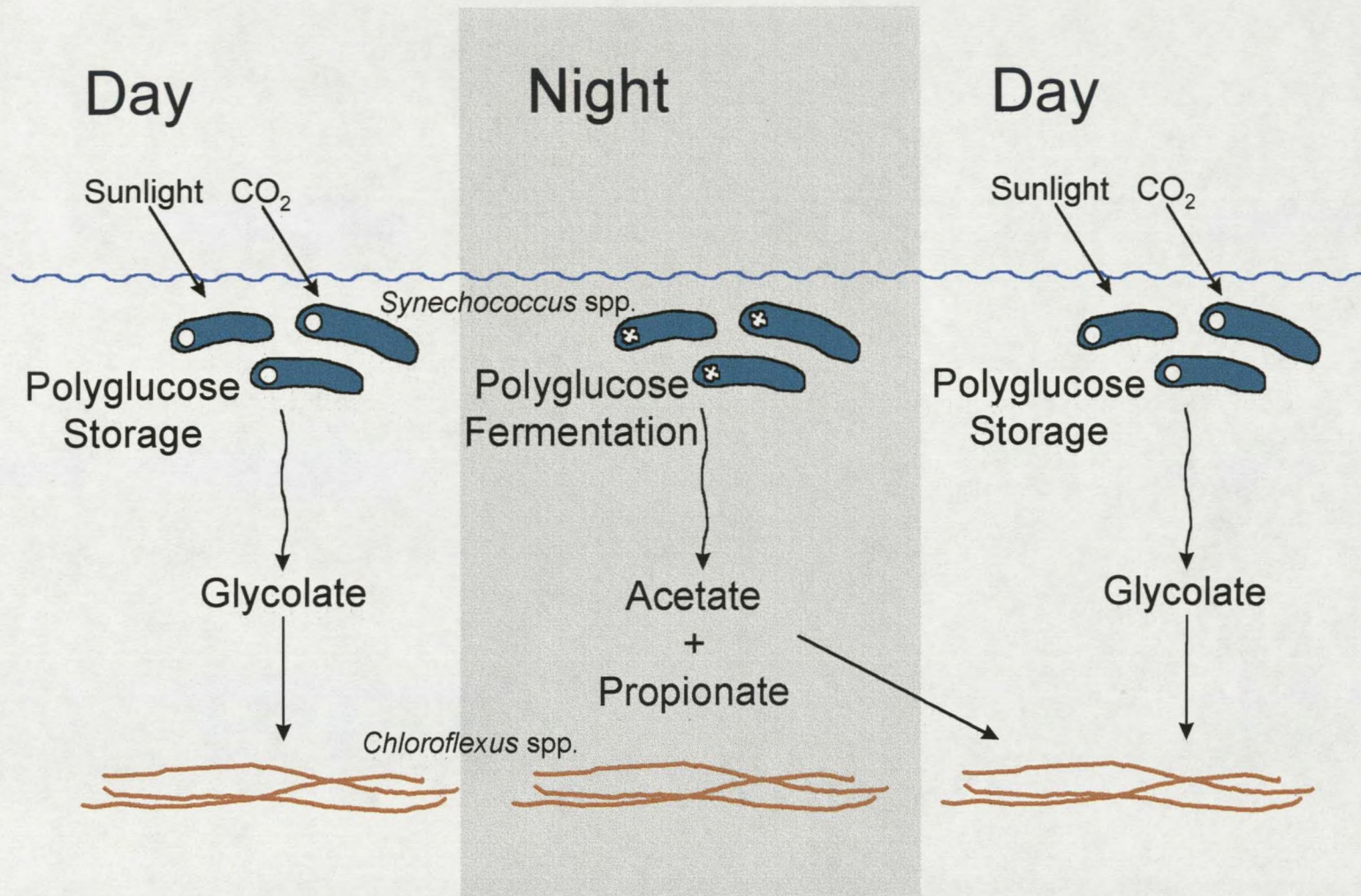


Figure 13. Conceptual model of carbon flux through primary producers (*Synechococcus* spp.) to heterotrophs (*Chloroflexus* spp.) in hot spring cyanobacterial mat communities

the day, mat cyanobacteria photosynthetically reduce carbon dioxide and synthesize the fixed carbon into molecules which are either excreted or incorporated into cellular material. Mat cyanobacteria incorporate 77 to 85% of the photosynthetically fixed carbon into polyglucose storage molecules, and photoexcrete up to 7% of the fixed carbon as glycolate (2). Photoexcreted carbon is readily photoassimilated by *Chloroflexus*-like filamentous mat inhabitants (2,3). At night, cyanobacteria ferment the majority (>58.5%) of the polyglucose reserves, excreting acetate and propionate which accumulate overnight. The following day, *Chloroflexus*-like filamentous organisms photoheterotrophically incorporate accumulated fermentation products (1,19). This model describes massive carbon transfer from the *Synechococcus* primary producers to the *Chloroflexus*-like primary consumers in these mat communities. Transfer of photosynthetically fixed carbon is nearly complete, leaving little carbon for *Synechococcus* cell replication.

Since only limited synthesis of growth-related molecules (i.e. protein, lipid, low molecular weight metabolites, and especially ribosomal RNA) was detected, using capture probes to assay activity associated with 16S rRNA sequence types is not likely to succeed, at least in undisturbed microbial mat communities. After disturbance, however, the mat cyanobacteria allocate proportionally more carbon into proteins and low molecular weight metabolites than before disturbance, indicating that mat cyanobacteria may be growing (Chapter 5). If cyanobacteria also synthesize detectable quantities of rRNA after disturbance, then capture probes may be feasible tools for associating microbial activity with bacterial populations in mats undergoing post-

disturbance recolonization.

It is yet unclear why mat cyanobacteria do not appear to be rapidly growing while exporting the vast majority of their photosynthetically fixed carbon to the filamentous *Chloroflexus*-like heterotrophs. Cyanobacteria have been shown to enter into symbiotic relationships with other organisms, including fungi to form lichens (4), and primordial eukaryotic cells to form the chloroplasts of modern green plant cells (10). Likewise, hot spring cyanobacteria may be symbiotically associated with the *Chloroflexus*-like heterotrophs. Mat heterotrophs clearly benefit from their association with cyanobacteria, but whether *Synechococcus* cells benefit from their massive carbon export is not well understood. Hopefully, future studies will investigate whether mat cyanobacteria benefit from metabolic products of *Chloroflexus*-like populations such as exopolysaccharide (9) or fixed nitrogen (15,16), or from the fabric these organisms create, which may aid *Synechococcus* cell loss by preventing washout in a flowing aquatic environment.

Validity of Hypotheses

Hypothesis 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.

Although one bacterial isolate was cultivated which exhibited a 16S rRNA

nucleotide sequence identical to a sequence previously observed in Octopus Spring mat (isolate ac-15=O.S. type N), most of the numerically abundant aerobic chemoorganotrophic bacteria from the habitat remain uncultivated. This hypothesis forces the researcher to use more ecologically relevant enrichment culture conditions, which I apparently failed to provide.

Hypothesis 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.

The relatively limited incorporation of $^{14}\text{CO}_2$ into growth-related molecules and the observation that hot spring cyanobacteria incorporate most of the photosynthetically fixed carbon into polyglucose during the day and ferment the polyglucose reserves at night are inconsistent with hypothesis II. Since only limited synthesis of protein and lipid was detected, and synthesis of rRNA from $^{14}\text{CO}_2$ was below detection limits, cyanobacterial populations may be dividing, but only at relatively slow rates.

Hypothesis III

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

The decrease in photosynthetically fixed radiolabeled polyglucose and the corresponding increase in radiolabeled fermentation products is consistent with hypothesis III. The only way for cyanobacterial polyglucose to become radiolabeled under the provided conditions would be through autotrophic incorporation of $^{14}\text{CO}_2$. The most plausible explanation for the increase of radiolabeled fermentation products would be cyanobacterial fermentation. If radiolabeled polyglucose did not decrease, and if accumulated fermentation products did not contain radiolabel, hypothesis III would be rejected, since this hypothesis requires the fermentation of the polyglucose synthesized by mat cyanobacteria in the light.

Hypothesis IV

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

The observed increase in cyanobacterial oxygen production measured by oxygen microelectrodes and the appearance of cyanobacterial 16S rRNA sequence types detected by DGGE analysis of PCR fragments do not indicate microbial growth as a mechanism of biomass replacement. Oxygen production and detection of cyanobacterial 16S rRNA sequence types would be expected if the alternative hypothesis that species immigration replaces lost biomass is true. However, the detection of carbon allocation into growth-related molecules such as protein and low molecular weight metabolites allows rejection of immigration as the sole mechanism of biomass replacement. Immigration may contribute to biomass replacement, but the

successive change in physiological activity from days 5 to 21 after disturbance are consistent with growth as a mechanism of biomass replacement.

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