

TEMPERATURE AND LIGHT ADAPTATIONS OF *SYNECHOCOCCUS* ISOLATES  
FROM A HOT SPRING MICROBIAL COMMUNITY

by

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## ABSTRACT

Previous molecular analysis of a well-studied microbial mat system in Yellowstone National Park revealed numerous genetically distinct 16S rRNA sequences distantly related to the 16S rRNA sequence of the unicellular cyanobacterium *Synechococcus lividus*. These new genotypes were shown to be contributed by the predominant cyanobacterial populations. Patterns in genotype distribution relative to temperature and light conditions suggested that these populations may have evolved through adaptive radiation to fill ecological niches. In order to test this hypothesis, *Synechococcus* isolates were cultivated using a dilution and filtration approach, then shown to be genetically relevant compared to natural mat populations by similarities of 16S rRNA genes and the 16S-23S intervening transcribed spacer (ITS) regions. Several isolates were retrieved that were identical or closely related to predominant mat genotypes at both loci. Other *Synechococcus* isolates were relevant at the 16S rRNA locus, but had ITS sequences not yet found in the mat. The growth rate and photosynthetic response of one representative of each genotype was then measured under various temperature and light conditions. Isolates with predominant mat genotypes had distinct temperature ranges and optima for growth, suggesting that specific temperature adaptations exist for these organisms. Isolates with non-predominant genotypes exhibited different temperature ranges and optima that may not be representative of relevant mat populations. Isolates with non-predominant genotypes also grew more slowly, which may signify a lower fitness *in situ*. Temperature effects on  $^{14}\text{CO}_2$  fixation did not exactly reflect temperature relations for growth, but they were consistent with a higher upper temperature limit of the most thermally stable genotype. Growth rate and photosynthetic responses of the isolates to light did not provide conclusive evidence of light adaptation; however, there was evidence for acclimation to light.

## INTRODUCTION

The theories central to ecology and evolution have long been applied to plant and animal species and their interactions within communities. In the mid-19<sup>th</sup> century Charles Darwin developed the theory of natural selection (Darwin, 1860). A classic example of its value is that it explains the level of species diversity seen in finch populations of the Galapagos Islands. Through detailed observations, Darwin concluded that finch populations had diverged from one another as they became specifically adapted to different ecological niches. Over time, the divergence resulted in different species of finches, each species associated with a particular ecological niche.

Although this theory has been widely used to explain plant and animal species, it is only recently that it and other theories have been used to formulate concepts about species of microorganisms. Microorganisms exist in almost every type of habitat, including some of the most inhospitable and extreme environments on the planet. This creates the potential for an enormous amount of species diversity. However, defining microbial species, especially prokaryotic species, is a difficult task. Prokaryotes are asexual, and therefore, species cannot be delineated using theories based on sexual reproduction such as Mayr's Biological Species Concept (Mayr, 1944). According to this concept, genetic exchange occurring through sexual reproduction is the cohesive force that holds plant and animal species together. Organisms are considered separate species if they cannot sexually reproduce and create viable offspring. Despite being asexual, individuals in microbial populations are subject to the same kinds of evolutionary and ecological forces (e.g., mutation (including recombination) and natural selection) as

sexual species. Divergence must be limited by the presence of some other cohesive force.

It has been suggested that for prokaryotes, the cohesive force is adaptive mutation (Cohan, 2001) and periodic selection (Ward and Cohan, in preparation). If an individual cell in a population acquires a new trait (through mutation) that allows it to be more successful than the other cells, then the newly adapted organism and its descendants will eventually replace the other cells in the population with whom they are competing (Cohan, 2001). This loss in diversity and rise of a new population is known as periodic selection (Atwood et al., 1951). Periodic selection may also give rise to entirely new populations (or lineages) of prokaryotes. For instance, if an adaptive mutant gains a trait that allows it to occupy a different ecological niche than that of the parent population, then the two populations will diverge and the adaptive mutant will create a new population constrained by the characteristics of its new niche (Ward and Cohan, in preparation). Periodic selection events will then act on the two populations independently and they will be free to diverge permanently, creating new lineages (Cohan, 2001). Cohan (2002) defines such populations as ecotypes, groups of organisms using the “same or similar ecological resources, such that an adaptive mutant from within the ecotype out-competes to extinction all other strains of the same ecotype; the adaptive mutant does not, however, drive to extinction strains from other ecotypes”. In a similar manner to that of the Galapagos finches, adaptive mutation and periodic selection events might lead to the development of microbial ecotypes specifically adapted to the unique niches of the ecosystems they inhabit.

Another evolutionary force molding species diversity (both at macroscopic and microscopic levels) is geographic isolation. In this case, a geographic barrier of some

kind (e.g. mountains, rivers, or oceans) separates individuals of a population. The two populations evolve separately and eventually become so different that they can no longer be considered the same species (Rosenzweig, 1995). In many cases, geographic isolation is coupled with adaptive radiation. For instance, the adaptive divergence of Darwin's Galapagos finches occurred only after the original mating pair was geographically isolated from the mainland populations on islands where niches were not colonized by competing bird species.

Examples of these evolutionary and ecological processes and the diversity that stems from them can be found in several different microbial systems. However, to understand them, it is necessary to first consider how microbial diversity analyses are made. Traditionally, microbiologists have used different cultivation techniques to determine the individual populations comprising a natural microbial community. With the development of molecular techniques such as the polymerase chain reaction (PCR) and DNA sequencing, it was discovered that traditional cultivation approaches were biased and did not give appropriate estimates of diversity. Therefore, microbial ecologists are now utilizing molecular techniques to improve estimates of diversity. One widely used approach is to compare nucleotide sequences of the 16S rRNA molecule to identify different organisms. As first demonstrated by Woese and Fox (1977), the molecule is universal in all prokaryotes and highly conserved, making it a useful tool for constructing evolutionary histories (phylogenies) for all microorganisms and analyzing microbial diversity in a natural environment (Olsen et al., 1986; Pace et al., 1986).

Through the use of the molecular techniques mentioned above, microbiologists can gather microbial 16S rRNA sequences from diverse environments and compare the

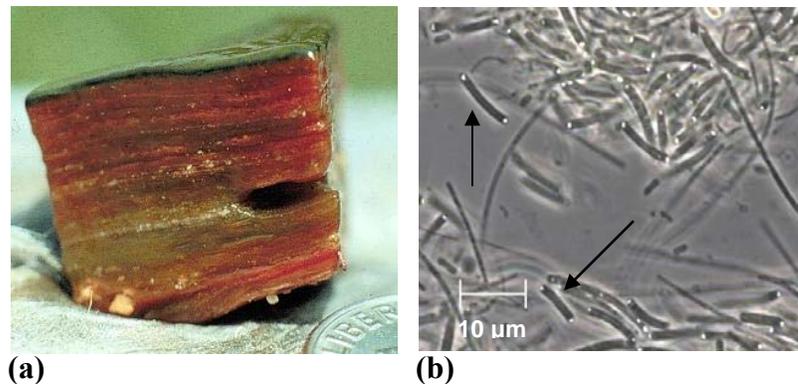
sequences to others contained in databases (such as the Ribosomal Database Project, RDP (Maidak et al., 1997)) in order to identify the phylogenetic relationships between the natural and database collections. Based on this technique, 40 major bacterial divisions are currently recognized (Pace, 1997), compared to the 10 or 12 originally identified through cultivation work (Woese, 1987).

One early study involving the application of such molecular techniques was carried out on a 55°C cyanobacterial mat from Octopus Spring in Yellowstone National Park (Figure 1). This mat is constructed by unicellular cyanobacteria of the morphologically defined genus *Synechococcus*, which dominate the upper 1 mm thick photic zone (Figure 2). Eight different 16S rRNA sequence types (genotypes) unrelated to the 16S rRNA sequences of any organism previously cultivated from the hot spring mat were revealed (Ward et al., 1990); some were from cyanobacteria quite unrelated to the one typically cultivated (see below). A similar study examining the diversity of Sargasso Sea bacterioplankton also identified a novel cluster of eight sequences distantly related to those of any previously cultivated heterotrophic marine organism (Giovannoni et al., 1990). A few novel sequences related to marine oxygenic phototrophs (cyanobacteria, prochlorophytes) were also discovered.

Microbial ecologists continue to find incredible molecular diversity, which leads to the question of how much prokaryotic species diversity truly exists in nature. According to Ward (2002), estimating community diversity requires knowledge about the number of species in the system and the number of individuals in each species. Data of this kind are often represented in a species abundance curve; the number of species is



**Figure 1.** Octopus Spring in Lower Geyser Basin, Yellowstone National Park. The arrow identifies the effluent channel where cyanobacterial mat studies reported herein have been conducted.



**Figure 2.** Octopus Spring microbial mat ecosystem. (a) A typical mat sample from Octopus Spring. *Synechococcus* cells are found within the top 1 mm (green layer). For scale, a dime is depicted in the right corner; lettering on the dime is ~1.5 mm tall. (b) Microscopic image of *Synechococcus* cells (identified by arrows) and other filamentous bacteria that inhabit the mat.

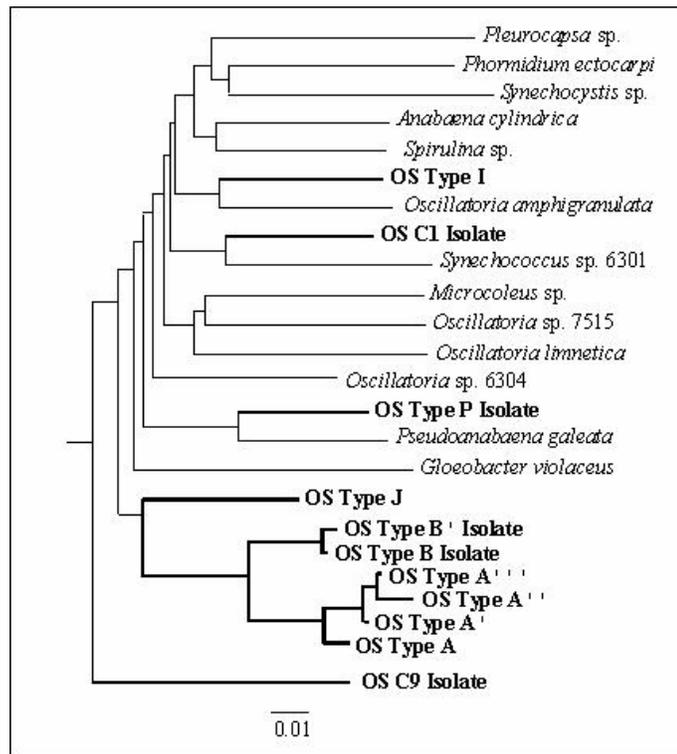
plotted against the number of individuals per species. To calculate this, however, scientists need to determine what constitutes a bacterial species. Currently, species are demarcated using phenotypic properties and one of two genetic criteria: (i) the degree of DNA-DNA association or (ii) the percent identity in the 16S rRNA molecule. DNA-DNA association is a measure of the amount of total genomic DNA from two different

organisms that can hybridize, or re-associate, after being melted apart. Two organisms are considered to be different species if less than 70% of their DNA will hybridize (Johnson, 1984; Wayne et al., 1987). The second molecular criterion often used to define bacterial species involves comparing the 16S rRNA sequences from two different organisms. It is generally accepted that two organisms are considered different species if there is greater than a 3% difference in their 16S rRNA sequences (Ward et al., 2002). Using such genetic criteria, microbial ecologists have attempted to calculate the amount of species diversity in a given ecosystem. Using the 3% 16S rRNA criterion, Curtis et al. (2002) calculated approximately 163 different species of bacteria in 1 ml of seawater and approximately 6,300 different species in 1 gram of soil! This figure is similar in magnitude to Torsvik et al.'s (1990) estimation of 4,000 bacterial genomes per gram of soil (based on the re-association of DNA sequences obtained from soil). Dykhuizen (1998) used Torsvik et al.'s data, the 70% DNA-DNA hybridization species cutoff, and additional considerations to argue that there may be as many as 40,000 to 500,000 different prokaryotic species in 30 grams of soil. These are only estimates however, and it is generally agreed upon that the actual degree of species diversity is unknown and cannot be determined at this point in time due to a lack of agreement about what bacterial species are (Curtis et al., 2002; Ward, 2002). Given that the criteria for distinguishing prokaryotic species are based on phenotypic properties combined with genetic differences, rather than ecological differences, it is important to understand how genetic diversity correlates with ecotype diversity.

Answering the question about what species are is extremely important. Species “are the basic units of ecology...no ecosystem can be fully understood until it has been

dissected into its component species and until the mutual interactions of these species are understood” (Mayr, 1982). This question is being investigated in the Octopus Spring microbial mat ecosystem; over 30 unique 16S rRNA sequences unrelated to those of any previously cultivated isolates have been detected (Ward et al., 1998). Of these sequences, nine are related to cyanobacterial sequences, but none of them to the sequence of the readily cultivated Yellowstone cyanobacterial isolate *Synechococcus lividus* (Ward et al., 1998), which was previously believed to be the sole cyanobacterial species present in such mat systems around the world (Castenholz, 1973). In fact, the Octopus Spring cyanobacterial sequences are very distantly related to that of *S. lividus* as shown in Figure 3 (*S. lividus* is represented by OS C1 Isolate). Six of the genotypes (A, A', A'', A''', B, and B') are closely related and some exhibit less than 3% difference in their 16S rRNA sequences. Although closely related, the sequences occur as two distinct phylogenetic clusters: A-like (types A, A', A'', and A''') and B-like (B and B')(both shown in Figure 3)(Ward and Castenholz, 2000).

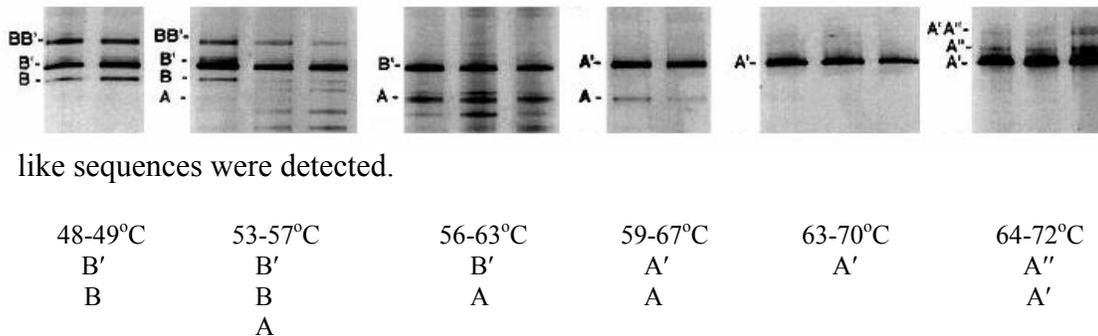
In addition to identifying new sequences in the mat system, a pattern in the distribution of these sequences was also discovered. This pattern was first found using oligodeoxynucleotide hybridization probes specific to the 16S rRNA sequences of the A-like and B-like organisms to study the distribution of these genotypes along a thermal gradient in the Octopus Spring effluent channel (Ruff-Roberts et al., 1994). A-like sequences were distributed at higher temperatures and the B-like sequences at lower temperatures. In addition, shifting samples from low to high temperatures for one week resulted in a disappearance of the B-like sequences, but a rise in the A-like sequences, providing evidence that distribution patterns may reflect adaptation. A more detailed



**Figure 3.** Distance matrix phylogenetic tree depicting cyanobacterial 16S rRNA sequences including those from Octopus Spring. The genetic distance between any two sequences is represented by the length of the horizontal line segments connecting them; longer lines signify a greater genetic difference between sequences (scale bar corresponds to 0.01 substitutions per sequence site). *Synechococcus lividus* is represented by the Octopus Spring C1 Isolate. The cyanobacterial sequences detected in the Octopus Spring mat are shown in bold (Ward et al., 1998).

pattern was revealed when PCR-amplified 16S rRNA gene segments from different temperature sites were examined with denaturing gradient gel electrophoresis (DGGE)(Ferris and Ward, 1997). This is a technique often used to examine the community members of a microbial ecosystem. DGGE gels contain a gradient of denaturants that cause DNA to melt apart. Depending on the sequence, the double-stranded DNA will melt apart at a certain position in the gel and create a band. Therefore,

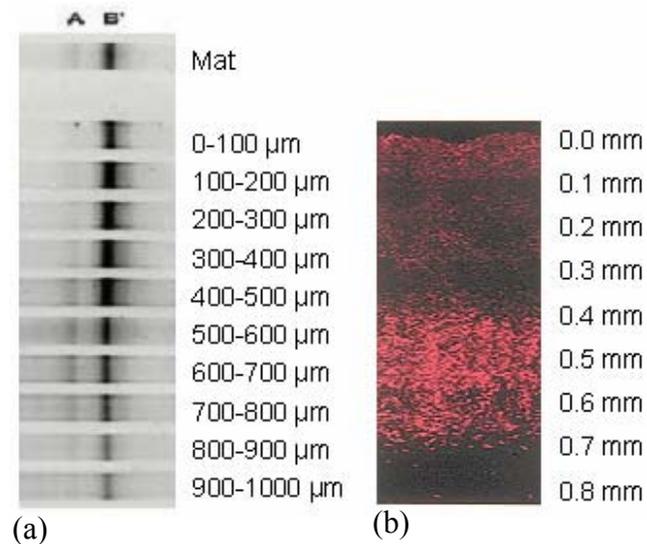
each band represents a different sequence, which may represent a different organism in the community. The bands can be PCR-amplified and sequenced to determine which organism the DNA belongs to. The results of the DGGE analysis showed a change in the distribution of A- and B-like sequences with increasing temperature (Figure 4). At lower temperatures, only sequences for B and B' were detected, whereas at higher temperatures, only the A, A', and A'' sequences were found; at mid-range temperatures both A- and B-



**Figure 4.** Distribution of 16S rRNA gene segments of cyanobacterial populations inhabiting the Octopus Spring mat detected by DGGE (denaturing gradient gel electrophoresis). Cyanobacterial mat samples were collected from temperature-defined sites along the thermal gradient in the Octopus Spring effluent channel. Individual bands were sequenced to identify the contributing organism; bands with double letters are heteroduplex artifacts (modified from Ferris and Ward, 1997).

Additional studies were conducted to examine the vertical structure of the top 1 mm of the 60°C Mushroom Spring mat system. [Mushroom Spring is located 0.5 km from Octopus Spring in the Lower Geyser Basin of Yellowstone National Park. The two springs have a similar chemical composition and many of the same cyanobacterial sequences are found in the Mushroom Spring mat that are found in Octopus Spring mat (Ferris et al., 2003).] PCR and DGGE performed on 16S rRNA sequences obtained from 100 µm-thick horizontal sections revealed a distinct pattern of A- and B-like genotypes with depth; the B' sequence was detected through the entire 1 mm depth interval, but the

A sequence was only found at depths between 400 and 800  $\mu\text{m}$  (Figure 5a)(Ramsing et al., 2000). Further examination of an entire vertical section using epifluorescence microscopy revealed a layer of dimly autofluorescent *Synechococcus*-shaped cyanobacterial cells extending from the surface to a depth of 400  $\mu\text{m}$  and a second layer of brightly autofluorescent cells extending from 400 to 700  $\mu\text{m}$  (Figure 5b)(Ramsing et al., 2000). [Cyanobacterial cells contain chlorophyll *a*, which fluoresces red when excited by UV light, and the amount of the fluorescence is a function of the amount of pigment.]



**Figure 5.** Evidence of vertical distribution of distinct *Synechococcus* genotypes in the Mushroom Spring 60°C mat. (a) DGGE gel depicting the distribution of A and B' sequences with depth (direction of electrophoresis gel is right to left). (b) Epifluorescence microscopy image demonstrating dim and bright autofluorescent layers of *Synechococcus*-like cyanobacterial cells. (modified from Ramsing et al., 2000).

Although many factors may influence the vertical distribution of these organisms, light may be an especially important factor since light intensity rapidly decreases with depth into the mat. At a depth corresponding to the deep pigment-rich *Synechococcus* layer, only 5% of the incident light is available (Ferris et al., 2003). Higher per-cell pigment

concentration and a shift from random to vertical orientation of *Synechococcus* cells at the top of the brightly autofluorescent layer near noon suggested possible adaptation to low light (Ramsing et al., 2000). A second example of vertical distribution comes from studies of a 68° C mat in Mushroom Spring (Ferris et al., 2003), where distinctly pigmented populations (found at different depths) were so closely related that it was necessary to use the more rapidly evolving internal transcribed spacer (ITS) region separating the 16S and 23S rRNA genes to identify genetic variability. The ITS region is less conserved than the 16S rRNA gene, and therefore, it accrues genetic changes more rapidly. This makes it an appropriate marker for identifying organisms that may have diverged from one another more recently.

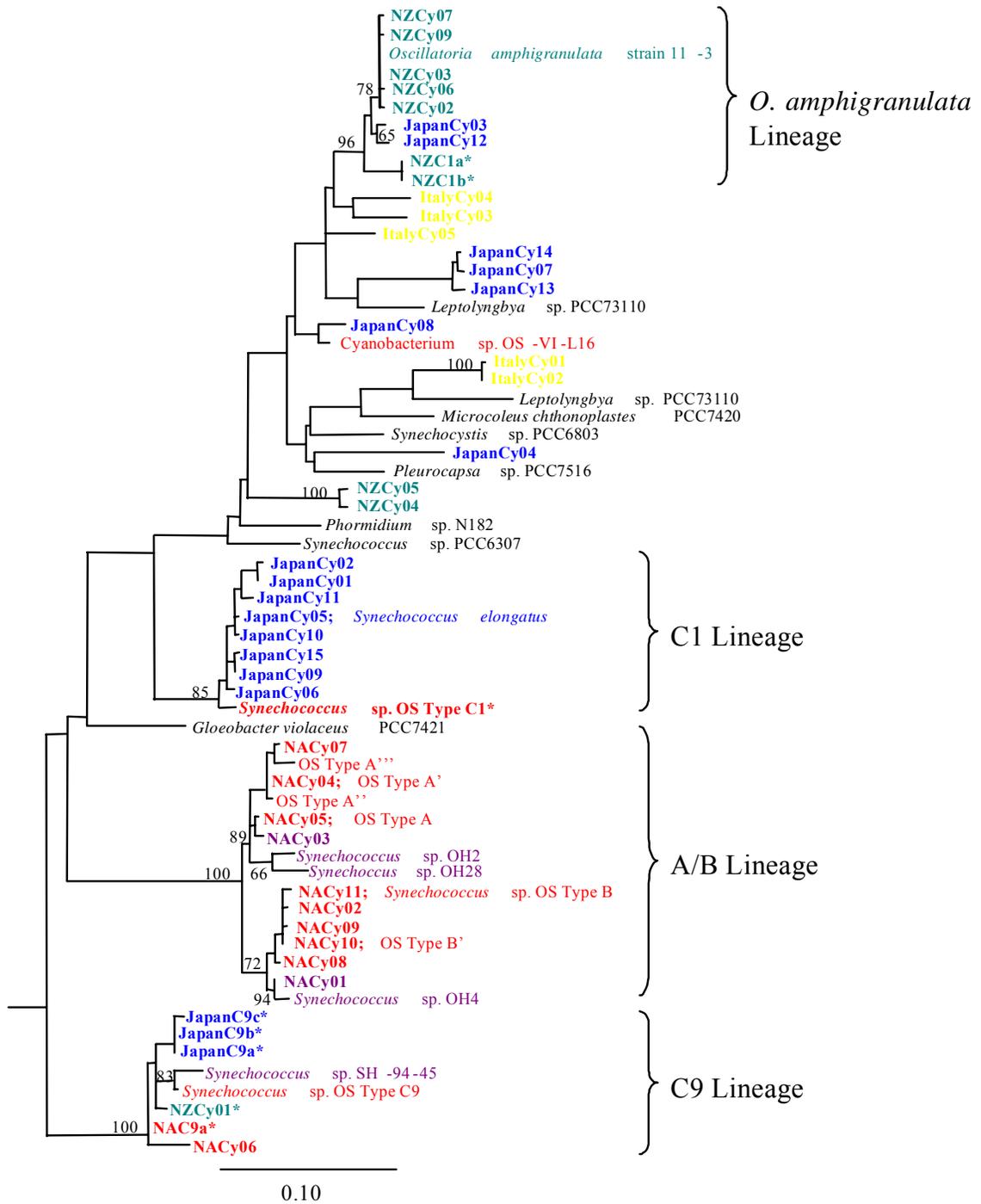
Similar types of distribution patterns have been found for populations of two different types of marine oxygenic photosynthetic prokaryotes, *Prochlorococcus* and *Synechococcus*. Although these organisms can be found throughout the ocean environment, gene sequences from surface and deep-water populations form two distinct phylogenetic clades (Ferris and Palenik, 1998; West and Scanlan, 1999). Another example is the proteorhodopsin genes recently discovered in marine bacterioplankton (Béjà et al., 2001). Genes from these organisms recovered from either shallow depths or deep in the water column seem to belong to distinct evolutionary clades; the gene products appear to be adapted to wavelengths of light found at the different depths.

The results of these distribution studies suggest that very closely related 16S rRNA or ITS-defined genotypes might correspond to unique ecotypes that are adapted to different temperature and/or light intensity conditions. If an ecological species concept was applied (i.e., species are ecologically specialized populations that occupy unique

niches (Simpson, 1961; Van Valen, 1976)), it would challenge the notion that a 3% difference in 16S rRNA sequences should be used to demarcate bacterial species.

Patterns in distribution have also been found on a global scale and have lead microbial ecologists to believe that geographic isolation is another important factor driving speciation in microorganisms (Papke and Ward, 2004). In a recent study conducted by Papke et al. (2003), cyanobacterial gene sequences from hot springs in North America, Japan, Italy, and New Zealand were compared. The survey revealed a specific distribution of 16S sequences according to geographic location, both among and within countries (Figure 6). For instance, the A/B lineage appears to be endemic to North America; other lineages dominate in Japan and New Zealand. Within North America the A/B genotypes for the Greater Yellowstone Ecosystem and Oregon form separate clades. Further analysis of the ITS region provided evidence for localized geographic patterning. For example, only one B'-like 16S rRNA genotype was detected in Yellowstone National Park, but nine different B'-like ITS variants were discovered (Papke et al., 2003), some with distinct distributions within Yellowstone National Park. Geographic isolation has also had an effect on the diversity of *Sulfolobus solfataricus*, an archaeon that is found in water and mud from acidic hot springs at temperatures between 50° C and 87° C. Phylogenetic analysis of gene segments from *Sulfolobus* chromosomal loci identified five distinct groups, each group corresponding to the specific geographic region sampled (Whitaker et al., 2003).

Although these patterns in distribution exist in many microbial ecosystems, it is impossible to determine from distributions alone if organisms are indeed adapted to specific environmental conditions. However, adaptation can be studied with isolates in a



**Figure 6.** 16S rRNA gene tree depicting the relationships of cyanobacterial clones retrieved from hot spring mats in North America, Japan, New Zealand, and Italy relative to other cyanobacterial 16S rRNA gene sequences, including *Synechococcus* spp. Scale bar indicates 0.10 substitutions per site. Color highlighting: green, New Zealand; blue, Japan; yellow, Italy; red, Greater Yellowstone Ecosystem; purple, Oregon (Papke et al., 2003).

laboratory setting. Adaptation experiments have been performed with cultures of *Synechococcus* spp. from Hunter's Hot Springs in Oregon. In one study, different temperature-adapted strains of *Synechococcus* resembling *S. lividus* were recovered from different temperatures in the effluent channel (Peary and Castenholz, 1964). When new *Synechococcus* isolates from Hunter's Hot Springs were examined later using molecular techniques, four different 16S rRNA-defined sequence groups were discovered. Three of the four groups were related, but not identical, to the Octopus Spring A- and B-like *Synechococcus* sequences (Miller and Castenholz, 2000). Experiments testing the growth response of these isolates to different temperature conditions demonstrated that each was a distinct ecotype adapted to a specific temperature condition. Similar results have been found with marine *Prochlorococcus* populations. Moore et al. (1998) showed that isolates from the same water column in the Sargasso Sea and the Gulf Stream had very different light adaptations, in that some isolates grew at intensities that completely inhibited the growth of other isolates, despite showing a 97% similarity in their 16S rRNA sequences. These isolates were also found to correspond to populations that dominate at different depths. Moore et al. (1998) concluded that there were at least two distinct ecotypes co-existing in the ecosystem, and that this permitted the survival of the population as a whole since the ecotypes were able to withstand a broad range of environmental conditions.

Based on the distribution patterns of Octopus Spring A-like and B-like *Synechococcus* genotypes relative to temperature and light gradients, and the results observed in similar ecosystems, I hypothesized that:

16S rRNA-defined *Synechococcus* genotypes in the Octopus Spring microbial mat correspond to distinct ecotypes that are adapted to specific temperature and/or light conditions.

In order to test this hypothesis, I developed four objectives. The first objective was to obtain *Synechococcus* cultures from the Octopus Spring mat system. Following cultivation, the cultures were characterized genotypically using DNA extraction, PCR-amplification of 16S rRNA and ITS genes, and sequencing techniques to determine whether relevant genotypes had been retrieved (A, A', A'', B, and B'). The third objective was to establish pure cultures. The fourth objective was to determine if possible adaptations to different temperature and light conditions existed by measuring the growth rate and photosynthesis rate responses of isolates representing the relevant genotypes.

## MATERIALS AND METHODS

Study Site

Octopus Spring is an alkaline hot spring located in Lower Geyser Basin, Yellowstone National Park, WY (Figure 1). The spring consists of a boiling source pool and adjacent effluent channels containing cooler water. The cooler water temperatures in these channels have allowed for the development of extensive microbial mat systems (Figure 2). These systems are free of macroinvertebrate grazing above 42°C, creating a community composed entirely of microorganisms.

Sample Collection

Sampling sites were chosen on the basis of the temperature range. The range was determined by holding a thermometer in the water flowing directly above the mat for at least 10 minutes to record the temperature variation caused by surging of the spring. Samples were obtained at four different temperature sites: 52-65°C (Site 1), 51-61°C (Site 2), 58-65°C (Site 3) and 59-70°C (Site 4)(Figure 7). Samples were collected from Sites 1 and 2 on July 10, 2002; from Site 3 on July 25, 2002; and from Site 4 on October 30, 2002 and January 13, 2003. A no. 4 cork borer (7 mm diameter) was used to remove cylindrical cores samples. Two cores were collected from each site. The cores were put into 6 ml Falcon tubes with spring water and then placed into a Whirl Pak bag. The bags were stored in a Thermos containing spring water at approximately 65°C until they reached the laboratory. The temperature in the Thermos was ~48°C by the time the cores were removed. In situ light intensity measurements were taken using a LI-250 light meter



**Figure 7.** Sampling locations used in this study.

with a LI-190SA quantum sensor (LI-COR, Lincoln, Nebr.); summer mid-afternoon intensities were approximately  $1450 \mu\text{mol m}^{-2} \text{s}^{-1}$  (no measurements were recorded in winter).

### *Synechococcus* Cultivation

#### Filter Cultivation

A filter cultivation approach was chosen because it could be used to isolate organisms difficult to obtain through typical cultivation methods (DE Bruyn et al., 1990) and because it had proven successful for isolating *Synechococcus* by Tony Scotti, a previous student. Immediately upon arrival at the laboratory, each core was placed into a Petri dish and the top green layer (approximately 1-2 mm) was removed using a sterile razor blade. This layer was placed in a Dounce tissue grinder containing 10 ml of Octopus Spring water and homogenized until no visible cell clumps remained. Cells were then serially diluted ten-fold in pre-autoclaved Octopus Spring water to  $10^{-7}$  of the cell

density in the original suspension. All dilution work was carried out in a water bath set to a temperature matching that of the sampling site. Cell material from Sites 1 and 2 was diluted at 55°C and material from Sites 3 and 4 was diluted at 60°C. The cells were then filtered through pre-sterilized 47 mm diameter Nucleopore polycarbonate filters using a Millipore vacuum system. Since it was impractical to sterilize the filtration unit between samples, filtration was performed in the order of highest dilution ( $10^{-7}$ ) to lowest ( $10^0$ ) to minimize cross contamination of samples with rare individuals that might be particularly well suited to laboratory culture. Inoculated filters were transferred to 47 mm diameter Petri dishes (Millipore) containing Whatman glass fiber filters pre-saturated with 1.5 ml of medium DH (Castenholz's medium D (Castenholz, 1969) supplemented with HEPES buffer at 1.2 g/liter). The Petri dishes were put into plastic bags with wetted paper towels and incubated at 55°C with  $\sim 47 \mu\text{mol m}^{-2} \text{s}^{-1}$  of light (Sites 1, 2, and 3) or 60°C with  $\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$  of light (Site 4) provided by cool white fluorescent bulbs. Microcolonies of *Synechococcus* approximately 0.2 mm in diameter that could be seen without magnification developed about 2 weeks after inoculation. Colony growth was maintained by adding 0.5 ml of medium DH every 2-3 days.

#### Revival of Frozen Isolates

Isolates of *Synechococcus* were also obtained from frozen stocks created by Tony Scotti in 2000. Cell material obtained from filtration work during 1999 had been frozen in 0%, 5%, 8%, 12% or 15% dimethyl sulfoxide (DMSO) concentrations in distilled water and stored in a -80°C freezer. Revival of frozen isolates was performed in a manner similar to that described by Brand on the Purdue University cyanobacteria website

(<http://www-cyanosite.bio.purdue.edu/protocols/cryo.html> on May 2002). Samples were removed from the freezer and quickly warmed to room temperature. The suspension was centrifuged in a microcentrifuge at the lowest setting for approximately 5-10 seconds. The supernatant was quickly removed and 1 ml of sterile medium DH was added to the cell pellet. The tube was lightly mixed using a vortex mixer to resuspend the cell pellet and then centrifuged again as above. The wash liquid was removed and another 1 ml of medium DH was added. The tubes were then incubated at either 50°C or 55°C under  $\sim 30 \mu\text{mol m}^{-2} \text{s}^{-1}$  of light (cool white fluorescent bulbs) for several weeks until growth was observed. Liquid medium was added every 2-3 days to prevent drying of cultures.

### Purification Attempts

*Synechococcus* cells could be recognized microscopically by their large size and autofluorescence due to chlorophyll *a*. However, all microcolonies also contained smaller, non-fluorescing cells. These contaminants were isolated in pure culture and subcultured on 0.1% wt/vol tryptone yeast extract dextrose (TYD) medium (broth or solidified with 1.6% wt/vol Difco agar)(Nold and Ward, 1996). In an attempt to obtain pure *Synechococcus* cultures, four different techniques were used: streaking for isolation, dilution and filtration, antibiotic treatment, and medium supplements.

### Streaking for Isolation

*Synechococcus* cultures were streaked onto 0.8% wt/vol GELRITE medium DH plates. Cell material was collected from liquid cultures using a flame-sterilized inoculating loop or from microcolonies on filters using a sterile pipette tip. Plates were

divided into four quadrants; cell material was placed in the first quadrant and then subsequently streaked out into the remaining quadrants using an inoculating loop that was flame-sterilized between each transfer. This method was repeated approximately 5 times with each *Synechococcus* culture.

### Dilution and Filtration

Repeated serial dilution and filtration was also tried for separating *Synechococcus* cells from contaminant bacterial cells. Dilution and filtration was performed in the same manner as described above. Cell material obtained from a well-isolated colony originally cultivated on polycarbonate filters was used to prepare a dilution series (in medium DH) and dilutions were filtered onto polycarbonate filters. As soon as microcolonies were detected using a dissecting microscope, they were picked and used to carry out the dilution and filtration process again. This was repeated at least 5 times for each isolate and in some cases more.

### Antibiotic Treatment

The antibiotic primaxin (imipenem and cilastatin; obtained by physician's prescription; Merck Pharmaceuticals) was chosen for use on the basis that it had previously shown the strongest results in reducing heterotrophic numbers in cyanobacterial cultures (Ferris and Hirsch, 1991). The procedures for applying primaxin to cultures were adapted from Ferris and Hirsch (1991) and were used in conjunction with plating and dilution/filtration techniques. Contaminated *Synechococcus* cultures were grown in 250 ml capped Bellco flasks for two weeks prior to treatment. 27 ml was transferred to a sterile 250 ml flask containing 3 ml of 1.0% wt/vol TYD (to yield a 0.1%

concentration) and 600  $\mu\text{l}$  of primaxin (final concentration 100  $\mu\text{g}/\text{ml}$ ). The flasks were incubated in the dark (to encourage heterotrophic growth while preventing cyanobacterial growth) for 24 hours at 55°C and shaken at 150 rpm. Following incubation, the cultures were centrifuged at 12,000 rpm (17,300  $\times g$ ) for 15 minutes at room temperature. The cells were washed twice with 30 ml of fresh medium DH to remove the antibiotic. Approximately 10  $\mu\text{l}$  of cell material was streaked out onto 1.6% wt/vol washed agar plates containing medium DH. The plates were placed in clear Tupperware containers and incubated at 55°C with 70  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of light. In addition, 1 ml of primaxin treated culture was used in a dilution series out to  $10^{-9}$  of the original cell density and then each tube in the series was filtered onto polycarbonate filters following the procedures described above. These plates were placed in clear plastic bags with wetted paper towels under the same conditions as the agar plates.

### Medium Supplements

Several different media and solidifying agents were evaluated to test their suitability for growing and isolating *Synechococcus* cells. Castenholz's medium D, medium DG (Castenholz's medium D buffered with glycyl-glycine at 1.6 g/liter), and medium DH were used in conjunction with either 0.8% wt/vol or 1.0% wt/vol GELRITE (Kelco gellan gum polysaccharide; Schweizerhall, Inc, N.J.) or 1.6% wt/vol washed agar (Difco). Once it was determined that medium DH and 0.8% GELRITE plates yielded the best growth, seven different combinations of supplements were tested in an attempt to obtain isolated *Synechococcus* colonies:

- i. Wolin's B vitamin solution (5 ml/liter)(Wolin et al., 1964) plus vitamin B<sub>12</sub> (1 ml/liter)

- ii. Wolin's vitamin B solution plus vitamin B<sub>12</sub> at the concentrations stated above plus sodium bicarbonate (Na<sub>2</sub>HCO<sub>3</sub>) at 1 g/liter. Medium DH does not contain any sources of CO<sub>2</sub>; therefore, *Synechococcus* cultures must obtain CO<sub>2</sub> from the air or from contaminants that produce it. Sodium bicarbonate was added as an additional source of CO<sub>2</sub> possibly substituting for any CO<sub>2</sub> that might be obtained from the contaminant organisms.
- iii. Autoclaved contaminant supernatant at 33% vol/vol. This supplement type was tested in case the contaminants were providing unknown nutrients to the *Synechococcus* cells. The three contaminants were grown separately in 0.1% TYD broth. Liquid and cell material from each contaminant culture was combined and the mixture was autoclaved for 30 minutes. It was then used as a supplement at the concentration stated above.
- iv. Filter sterilized supernatant from contaminant cultures at 33% vol/vol. This supplement was also tested in case of dependence on a heat-labile nutrient. Similar procedures were used as those for iii, except the mixture of all three contaminants was first centrifuged at 12,000 rpm (17,300 x g) for 15 minutes at room temperature and then the supernatant was removed and passed through a sterile 0.2 µm filter.
- v. Filter sterilized contaminant supernatant at 33% vol/vol plus Wolin's B vitamin solution, vitamin B<sub>12</sub>, and sodium bicarbonate (at the concentrations stated in i and ii).
- vi. Filtered *Synechococcus* culture supernatant at 4 ml/100 ml. This was tested to determine if supernatant from *Synechococcus* cultures could provide autoinducers for growth and thereby aid in isolating cells on the GELRITE plates. Actively growing cultures of *Synechococcus* were centrifuged at 12,000 rpm (17,300 x g) for 15 minutes and the supernatant was passed through a sterile 0.2 µm filter before being used as a supplement.
- vii. Contaminant supernatant at 33% vol/vol and *Synechococcus* supernatant at 4 ml/100 ml.

Pour plates and spread plates of each supplement type were inoculated with serially diluted contaminated *Synechococcus* cultures (to 10<sup>-3</sup> of the original cell density); pour plates received 100 µl and spread plates received 20 µl. All plates were incubated in clear Tupperware containers at 55°C with approximately 40 µmol m<sup>-2</sup> s<sup>-1</sup> of light. A few pour plates produced contaminant colonies with surrounding halos of *Synechococcus* cells,

indicating a potential relationship between *Synechococcus* cells and a specific contaminant. A micromanipulator was used to remove this contaminant cell material with a sterilized drawn Pasteur pipet. The cells were added to 0.1% TYD broth and cultivated separately. The culture supernatant from this contaminant was then used as a supplement in the manners described in iii and iv above.

### Genotypic Analysis

DNA extraction, PCR amplification and sequencing were performed on (i) frozen isolates collected by Tony Scotti, (ii) *Synechococcus* colonies retrieved from sampling sites described above, and (iii) contaminant cultures to determine if any relevant 16S rRNA and/or ITS genotypes existed in the collection.

### Nucleic Acid Extraction

DNA was extracted using a method similar to that described by Moré et al. (1994). *Synechococcus* cell material was transferred into 2.0 ml centrifuge tubes with 800 µl of phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>; 120mM; pH 8) and 260 µl of sodium dodecyl sulfate solution (10% sodium dodecyl sulfate, 0.1 M NaCl, 0.5 M Tris-HCl, pH 7.5). Zirconium beads of 0.1 mm diameter (Biospec Products, Bartlesville, Okla.) were added to a volume of 0.5 ml and the tubes were shaken for 45 seconds in a FastPrep bead beater (Savant Instruments Inc., Farmingdale, N.Y.) at the maximum setting of 6.5. Samples were cooled on ice and then centrifuged for 5 minutes at 14,000 rpm (16,000 x g) in a tabletop microcentrifuge. Following centrifugation, 600 µl of the supernatant was transferred to a new tube and 240 µl of ammonium acetate (10 M) was added. Proteins

were precipitated on ice for 5 minutes and tubes were centrifuged again for 6 minutes. Approximately 700 µl of supernatant was transferred to another tube and 490 µl of isopropanol was added to precipitate nucleic acids at 4°C overnight. The samples were then centrifuged for 30 minutes and the pellets were rinsed with 70% ethanol and air dried in a vacuum for 9 minutes. The pellets were resuspended in 100 µl Tris (10mM Tris, pH 8.0).

#### Sequence Acquisition and Analysis

PCR was performed according to the method described by Papke et al. (2003), using primers 1070F (Ferris et al., 1996) (5'-ATGGCTGTCGTCAGCT) and L23cyR (5'-TGCCTAGGTATCCACC). These primers are biased towards cyanobacteria and amplify the 16S rRNA gene, the internal transcribed spacer region (ITS), and the beginning of the 23S rRNA gene. Samples were considered to be unicyanobacterial if only 1 band of DNA appeared on the electrophoresis gel; samples with >1 band were not sequenced. The PCR products were purified using a QIAquick PCR purification kit (Qiagen). Amplified DNA was sequenced on an Applied Biosystems 310 genetic analyzer using primers 1070F to obtain 16S rRNA sequences and 1505F (5'-GTGAAGTCGTAACAAGG) to obtain ITS sequences, and Big Dye v3.1 terminators (Applied Biosystems, Foster City, Calif.). 16S rRNA and ITS sequences were analyzed using Sequencher 3.0 (Gene Codes) software. Culture sequences were compared to a Sequencher database created by M. Ferris and T. Papke containing sequence information for all *Synechococcus* genotypes directly retrieved from Yellowstone microbial mats. 16S rRNA sequences from the contaminant organisms were identified on the National Center for Biotechnology Information (NCBI)

website using the BLAST nucleotide-nucleotide pairwise alignment program version 2.2.8 ([www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/), Spring 2004). Sequence data have been deposited with the NCBI GenBank sequence database.

### Phenotypic Analysis

#### Growth Studies

The growth of *Synechococcus* isolates with respect to different light intensities and temperatures was assessed by inoculating triplicate tubes containing 9 ml of medium DH with cell material from stock cultures of each isolate to yield an initial cell density of  $1.0\text{-}1.6 \cdot 10^6$  cells/ml. Stock cultures were pre-grown in 50 ml of medium DH in a dry  $55^\circ\text{C}$  incubator with  $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$  (provided by cool white fluorescent bulbs). For light intensity adaptation experiments, tubes were placed at a slight angle in clear plastic boxes under different layers of plastic screen mesh in a  $55^\circ\text{C}$  incubator. Six separate light conditions were created: 8-10, 40-45, 66-70, 118-120, 272-280, and 378-385  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (cool white fluorescent lights). Temperature adaptation experiments were conducted in a similar manner. Tubes were placed in boxes under  $48\text{-}55 \mu\text{mol m}^{-2} \text{s}^{-1}$  of cool-white light and the growth rate was measured at seven temperatures: 39, 45, 50, 55, 60, 65, and  $70^\circ\text{C}$ . In both types of growth studies, the tubes were removed from the incubator (at 24-hour intervals), thoroughly mixed, and 100  $\mu\text{l}$  was withdrawn from each tube. The liquid sample was transferred into a 0.5 ml tube and 10  $\mu\text{l}$  of 37% formaldehyde was added to preserve the sample for cell counting. Cell counts were performed using a Petroff-Hausser counting chamber (Sperm/Bacteria counter, Hausser Scientific, P.A.). The same pattern of 80 small grid squares was counted. Each sample was counted three times,

generating a total of 9 counts per isolate for each temperature and light condition.

### Photosynthetic $^{14}\text{CO}_2$ Fixation Studies

With the exception of preliminary studies (see below), cultures were pre-grown with constant bubbling of 5%  $\text{CO}_2$  to down-regulate carbonic anhydrase synthesis, thereby reducing  $^{14}\text{CO}_2$  uptake due to this  $\text{CO}_2$  concentrating mechanism rather than by true photosynthetic incorporation (Badger and Price, 2003). In all experiments, 2 ml of culture was transferred in triplicate into Kimble autosampler vials (distributed by Cole-Palmer Instrument Co., Vernon Hills, IL) and 1  $\mu\text{Ci}$  of  $^{14}\text{C}$ -labeled sodium bicarbonate (57 mCi/mmol; Moravek Biochemicals, Inc., C.A.) was added to each vial. Vials were incubated for 30-35 minutes (except for preliminary work). Following incubation, cells were killed with 0.2 ml of 37% formaldehyde. Cells were centrifuged for 8 minutes at 16,000 x g and washed once with sterile water (Sigma, pH 8.5) to remove unincorporated  $^{14}\text{C}$ -bicarbonate. The radioactivity of each sample was then determined by liquid scintillation counting as described by Nold and Ward (1996) and adjusted to a per cell basis using cell density estimates measured on each culture prior to experimentation.

- i. *Preliminary experiments:* These were conducted to evaluate the extent to which  $^{14}\text{C}$ -bicarbonate fixation in contaminated *Synechococcus* cultures was due to photosynthesis. Uptake of  $^{14}\text{CO}_2$  under both light and dark conditions was tested on three contaminants and two contaminated *Synechococcus* cultures. All cultures were pre-grown in a dry 55°C incubator under 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of light. Samples incubated in the dark were wrapped with black electrician's tape and covered with aluminum foil. Formalin killed controls of both *Synechococcus* and contaminant cultures were run in both the light and dark treatments. Sample vials were placed on their side in a dry 55°C incubator and allowed to incubate for 2, 4, or 6 hours.  $^{14}\text{CO}_2$  incorporation was measured at different time intervals to determine if uptake was linear over time.

- ii. *Temperature adaptation experiments:* Photosynthetic activity was measured at 6 different temperatures: 45, 50, 55, 60, 65, and 70°C. A bank of cool white fluorescent lights was placed above the water baths and provided approximately 90-110  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of light. Due to equipment limitations, temperature experiments were performed in two sets of three; one set of vials was incubated first at 45°C, 55°C, and 65°C and then a second set of vials was incubated at 50°C, 60°C, and 70°C. The experiment was repeated and the two sets were carried out in reverse order. No more than 2 hours passed between sets in both experiments. Two separate cell counts were made to account for any growth between the first temperature set and the second temperature set.
- iii. *Light intensity adaptation and acclimation experiments:* Light intensity studies were conducted both outside under natural sunlight and inside under cool white fluorescent light. Cultures used in the outdoor experiments were grown in 100 ml of medium DH at 55°C with 90-120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of fluorescent light. Immediately after radiolabel addition, vials were inserted into envelopes made of different numbers of layers of plastic screen mesh creating up to 7 different light intensity conditions: 9-12, 30-60, 80-115, 250-340, 440-650, 730-800, and 1100-1230  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Cultures used in the indoor experiments were pre-grown under either relatively high (150-210  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) or relatively low (25-43  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) light. Light was reduced to a similar degree as stated above for outdoor experiments by using screen mesh. The highest light intensity achieved in the incubator was approximately 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Both outdoor and indoor light intensity studies were carried out at 55°C.

### Statistical Analyses

#### Growth Studies

Simple linear regression was used to fit the data for each genotype incubated at a particular temperature or light condition over time. Slope estimates were derived from a linear model for each experiment; from the estimated slopes a doubling time/day with standard error was calculated by the following formula:  $\text{doublings/day} = \text{slope} \cdot 24/\log(2)$ . The slopes within individual genotypes were compared at different temperature and light intensity conditions using Tukey's multiple comparison test to give

95% confidence interval values. In addition, at each temperature (or light intensity) the doubling times/day were compared between specific genotypes with Tukey's multiple comparison test. Analyses were performed with R statistical software, version 1.8.1 (R Development Core Team, 2003).

### Photosynthetic $^{14}\text{CO}_2$ Fixation Studies

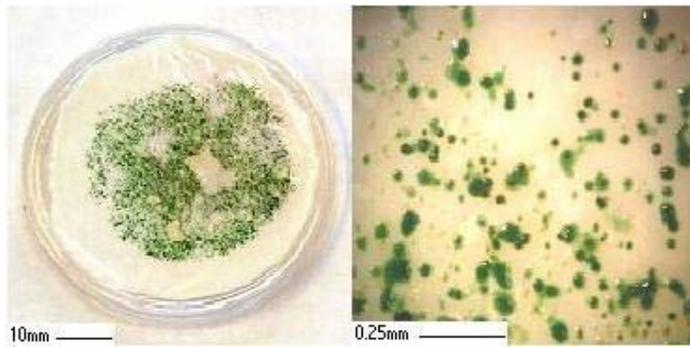
Two-factor analysis of variance (ANOVA) was used to analyze data from the temperature and outdoor light intensity fixation experiments. In the temperature experiment, the effects of temperature and genotype on photosynthetic uptake were examined and in the outdoor light intensity fixation experiments the effects of light intensity and genotype on uptake were determined. For indoor light intensity experiments, broken line regression was used in combination with two-factor ANOVA to examine the effects of pre-growing the genotypes under high or low light. Broken line regression analysis is used when two regression lines fit the data better than a single line (e.g. linear, cubic, or quadratic). With this type of regression, a break point in the data is first determined, and then one linear model is fit to the left of the break point and another to the right, with both lines in agreement at the actual point. For the indoor light intensity experiments, it was empirically determined that the high and low pre-treatment samples had a break point of  $180 \mu\text{mol m}^{-2} \text{s}^{-1}$ . This allowed for comparisons among cultures pre-grown under high and low light across all light intensities. All analyses were performed using R software, version 1.8.1 (R Development Core Team, 2003).

All of the statistical analyses were conducted in collaboration with Jim Robison-Cox and Melinda Yager at Montana State University, Bozeman, Montana.

## RESULTS

*Synechococcus* Cultivation and Genotypic Analysis

Several *Synechococcus* isolates were obtained using the filter cultivation method. Site 4 (59-70°C) was the only site from which isolates were not retrieved. Colony growth was highest on filters from the first two tubes in the dilution series ( $10^0$  and  $10^{-1}$ ), typically resulting in a dense collection of microcolonies (Figure 8). Comparisons



**Figure 8.** Photographic images of a filter containing *Synechococcus* colonies;  $10^{-1}$  dilution.

between direct counts and enumeration of microcolonies revealed that up to 10% of cells inoculated onto the filters were growing. Well-isolated colonies appeared on filter plates from higher dilutions ( $10^{-2}$  to  $10^{-5}$ ), but colony growth was never achieved from samples diluted greater than  $10^{-5}$  and colony frequency was less than expected based on dilution. Microscopy revealed that microcolonies contained unicellular cyanobacteria resembling *Synechococcus* spp. Sequencing of microcolonies revealed that isolates were identical or nearly identical at the 16S rRNA locus to *Synechococcus* genotypes previously detected in the mat (Table 1). In addition, most of the isolates were genetically relevant compared to mat genotypes at the ITS locus, but others had ITS sequences not yet found in the mat.

Site	No. of Isolates Retrieved	Genotype Identification	16S rRNA locus			ITS locus		
			Closest 16S rRNA Relative	No. of Base Differences	Percent Similarity	Closest ITS Relative	No. of Base Differences	Percent Similarity
Cultivated Isolates:								
51-61°C	1	B	B	0	100%	Unknown	-----	----
	4	B'	B'	0	100%	NACy10b*	0	100%
	1	<b>B'</b>	<b>B'</b>	<b>0</b>	<b>100%</b>	<b>NACy10b-1diff</b>	<b>1</b>	<b>99%</b>
	1	B'-1diff	B'	1	99%	NACy10b	3	99%
	5	<b>B'-1diff</b>	<b>B'</b>	<b>1</b>	<b>99%</b>	<b>NACy10j</b>	<b>4</b>	<b>99%</b>
	2	B'-2diff	B'	2	99%	NACy10k,n, 11b1017	Several	70%
52-65°C	5	A	A	0	100%	NACy05a	0	100%
	9	B'	B'	0	100%	NACy10b	0	100%
58-65°C	3	<b>A</b>	<b>A</b>	<b>0</b>	<b>100%</b>	<b>NACy05a</b>	<b>0</b>	<b>100%</b>
Frozen Isolates: *								
49-56°C	5	A	A	0	100%	NACy05a	0	100%
	10	<b>B</b>	<b>B</b>	<b>0</b>	<b>100%</b>	<b>P2</b>	<b>0</b>	<b>100%</b>
	2	B'	B'	0	100%	Unknown	-----	----
	4	B'-2diff	B'	2	99%	NACy10k,n, 11b1017	Several	70%
58-65°C	14	A	A	0	100%	NACy05a	0	100%
	7	A	A	0	100%	NACy05a-1diff	1	99%
	8	B'	B'	0	100%	NACy10b*	0	100%
	1	B'-1diff	B'	1	99%	NACy10b	1	99%
	9	<b>B'-2diff</b>	<b>B'</b>	<b>2</b>	<b>99%</b>	<b>NACy10k,n, 11b1017</b>	<b>Several</b>	<b>70%</b>

\* Collection created by Tony Scotti

**Table 1.** Sequencing results of *Synechococcus* isolates. Genotypic analysis on each isolate revealed the closest relative at both the 16S rRNA and internal transcribed spacer (ITS) loci. Genotypes labeled with an ITS prefix of NACy have previously been detected by Papke et al.(2003). Genotypes used for adaptation experiments are shown in bold. (B'-1diff & 2diff names may change in publication.)

Sequencing of Tony Scotti's frozen isolates demonstrated that his collection also contained relevant genotypes (Table 1). All isolates were given a laboratory designation to distinguish them from one another (Table 2). In addition to genetic variation, phenotypic variation was also detected. Microscopic examination revealed slight differences among the genotypes with regards to cell morphology. Cells of genotypes A, B', and B'-2diff were typically ~8-9  $\mu\text{m}$  long and rod-shaped, sometimes with slight curvature. Genotype B cells were longer (~10-20  $\mu\text{m}$ ) and had a more curved shape; the greater length might be due to a lack of separation after cell division. B'-1diff cells were usually 8-10  $\mu\text{m}$  long and had a propensity to form small clusters (Figure 9).

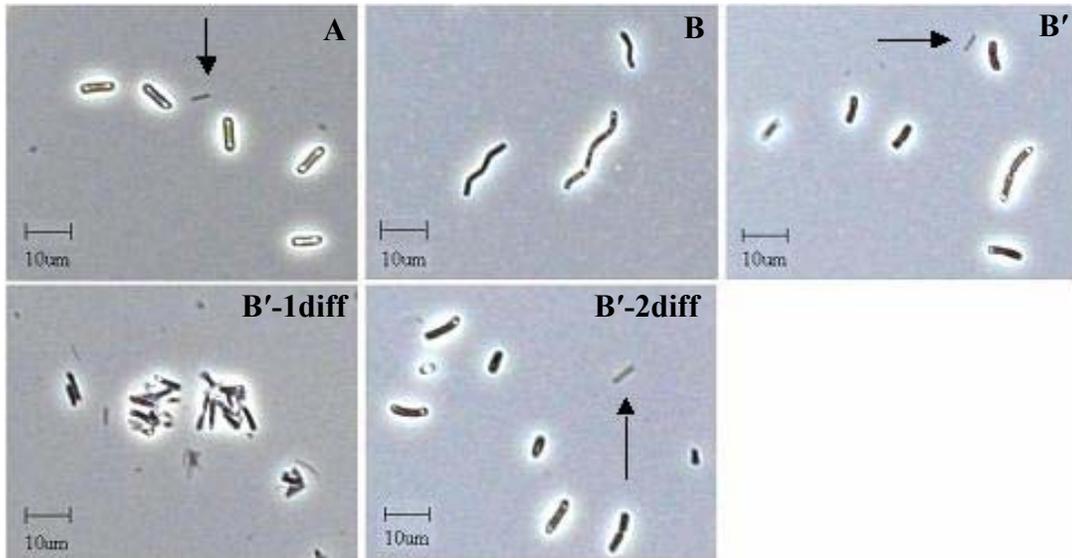
#### Contaminant Identification

*Synechococcus* cultures contained heterotrophic contaminants (see Figure 9); these organisms were pure-cultured and sequenced to find an appropriate method for eliminating them. Three distinct and differently colored contaminant colonies were retrieved through cultivation: red colonies (JA-Red), pink colonies (JA-Pink), and white colonies (JA-White). The colonies showed slight differences in morphology, with pink colonies being smaller with smooth edges than the larger red and white colonies, which had fuzzy edges. BLAST analysis of contaminant 16S rRNA sequence segments revealed a 100% identity match to the following organisms (~300bp sequence size): red colonies corresponded to *Rubrobacter taiwanensis* (GenBank accession number AF465803), pink colonies to *Meiothermus taiwanensis* (AF18001), and white colonies to *Geobacillus uralicus* (AY079151). The sequence matching *Geobacillus uralicus* was also 100% identical to the 16S rRNA sequences of nine other organisms.

Site	16S rRNA Identification	Closest ITS Identification	Laboratory Designation *
<b>Cultivated Isolates:</b>			
51-61°C	B	Unknown	2-3Ba
	B'	NACy10b	2-3B'a, 2-3B'b, 2-5B'd
	B'	NACy10b	2-4B'c
	<b>B'</b>	<b>NACy10b-1diff</b>	<b>2-13</b>
	B'-1diff	NACy10b	2-3B'(1 diff)a
	<b>B'-1diff</b>	<b>NACy10j</b>	2-3B'(1 diff)b, 2-F4, 2-F5, <b>2-F6</b> , 2-18
	B'-2diff	70% match	2-3B'(2 diff)a, 97JR
52-65°C	A	NACy05a	1-3Aa, 1-3Ac, 1-3Ad, 1-5Ae, 1-5Af
	B'	NACy10b	1-3B'a, 1-3B'b, 1-3B'c, 1-3B'g, 1-5B'e, 1-5B'h, 1-5B'i, 1-5B'j, 1-5B'p
58-65°C	<b>A</b>	<b>NACy05a</b>	3-3Aa, <b>3-3Ab</b> , 3-3Ac
<b>Frozen Isolates:</b>			
49-56°C	A	NACy05a	37, 39, 41, 47, 49
	<b>B</b>	<b>P2</b>	11, 12, <b>15</b> , 17, 19, 28, 29, 30, 34, 35
	B'	Unknown	4, 6
	B'-2diff	70% match	42, 44, 45, 46
58-65°C	A	NACy05a	64, 65, 66, 67, 71, 72, 75, 77, 83, 85, 88, 89, 94, 96
	A	NACy05a-1diff	62, 70, 76, 78, 81, 87, 93
	B'	NACy10b	107
	B'	NACy10b	97, 98, 99, 100, 101, 102, 103
	B'-1diff	NACy10b	104
	<b>B'-2diff</b>	<b>70% match</b>	63, 73, 80, 82, 84, 86, 90, <b>91</b> , 92

\* All of the designated names for cultivated isolates are preceded by JA for Jessica Allewalt; similarly, designated names for frozen isolates are all preceded by TS for Tony Scotti (in order to identify the person who cultivated the isolates).

**Table 2.** Culture designations. With a few exceptions, cultivated isolates were named in the following manner: the first number signifies the sampling site, the second number identifies the dilution from which the microcolony was attained (i.e.  $10^{-3} = 3$ ,  $10^{-4} = 4$ , etc.), the first letter describes the genotype, and the second letter denotes the number of replicates of that genotype. For example, 1-3Ad is a *Synechococcus* colony retrieved from Site #1. It developed on a filter plate from a  $10^{-3}$  diluted sample. It is an A genotype and the 4<sup>th</sup> (d) genotype of that kind to be retrieved from the temperature site. Frozen isolates were sequenced in order starting at 1; once genotype information was known, the isolates were simply referred to by number. Genotypes used for adaptation experiments are shown in bold. (The names for B'-1diff and B'-2diff may change in a publication.)



**Figure 9.** Phase contrast photomicrographs of each *Synechococcus* genotype. Arrows identify contaminant organisms.

### Purification Attempts

#### Streaking for Isolation, Dilution and Filtration, and Antibiotic Treatment

Repeated streaking of cultures for isolation on 0.8% wt/vol GELRITE-solidified medium DH plates did not result in pure cultures. In almost every case, the contaminants grew on the entire plate and the *Synechococcus* cells grew only at the beginning of the streak (first quadrant)

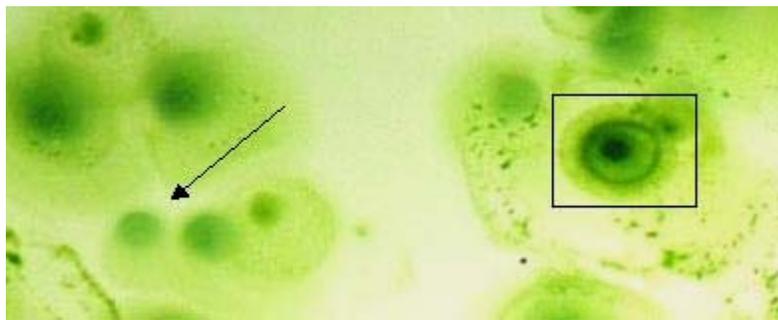
All cultures were routinely diluted and re-filtered, but this did not result in any axenic *Synechococcus* cultures. Contaminant colonies repeatedly diluted out to extinction as expected whereas *Synechococcus* colonies did not dilute out past the first few dilutions ( $10^{-5}$ ).

Cultures treated with primaxin did not grow on the washed agar plates and only one polycarbonate filter developed microcolonies. The microcolonies were picked and

examined microscopically, but contaminant cells could still be detected in each one.

### Medium Supplements

Medium supplements were first tested with a B' culture (culture 2-3B'a, (2-13)). Growth was supported on pour plates of medium DH and pour plates supplemented with B vitamins or filter-sterilized supernatant from the contaminant cultures. In these plates, *Synechococcus* microcolonies grew first only as halo colonies surrounding contaminant colonies (Figure 10). Once the halo colonies were established,



**Figure 10.** Photographic image of B' *Synechococcus* growth on a medium DH pour plate supplemented with Wolin's B vitamin solution and vitamin B<sub>12</sub>. The box outlines a halo colony; the contaminant colony is in the center with the cyanobacterial cells surrounding. The arrow depicts *Synechococcus* microcolonies that appeared after the halo colonies were established.

other individual *Synechococcus* microcolonies appeared between the halo colonies.

*Synechococcus* microcolonies between halo colonies from these plates were picked with a sterilized micromanipulator and streaked out onto GELRITE plates containing the same supplements, but no growth occurred. In addition, plates supplemented with the contaminant supernatant developed from growth of a halo colony into 0.1% TYD did not support growth of *Synechococcus* colonies. Pour plates with other types of supplements

did not yield any colonies and spread plates of all supplement types only supported growth of the contaminants.

### Growth and Photosynthesis Responses of Different Genotypes

Given the difficulty of purifying *Synechococcus* cultures it was reasoned that adaptations could nevertheless be studied by two approaches. First, growth studies based on microscopic cell counts were possible because of the distinctive morphologies of *Synechococcus* and contaminant cells (Figure 9). Growth rate would be expected to be the best measure of adaptation, since this determines overall competitive fitness. Second, since the photosynthetic apparatus is unique to *Synechococcus*, it should be possible to study light-driven  $^{14}\text{CO}_2$  incorporation as a measure of photosynthetic activity. This would only be possible if contaminants did not exhibit light-driven  $^{14}\text{CO}_2$  fixation; preliminary fixation experiments were first conducted to determine if this was true (results are shown below). For the growth and photosynthesis experiments, one isolate of each genotype was chosen for analysis of adaptation to temperature and light (Tables 1 and 2). Three of these isolates were retrieved through filter cultivation: A (from Site 3, 58-65°C), B' (from Site 2, 51-61°C), and B'-1diff (also from Site 2, 51-61°C). The other two were revived from Tony Scotti's collection of frozen isolates: B (49-56°C site) and B'-2diff (58-65°C). In the graphs below, data are displayed separately for each genotype, with those most relevant to the Octopus Spring microbial mat system listed above genotypes of unknown importance to the ecosystem.

### Preliminary $^{14}\text{CO}_2$ Experiments

In *Synechococcus* cultures of genotypes A and B'  $^{14}\text{CO}_2$  incorporation was light-dependent, with only 0.2-1.6% of light incorporation measured in the dark (Table 3). Contaminant cultures did not exhibit light-dependent  $^{14}\text{CO}_2$  assimilation. Furthermore, incorporation by contaminants was only 0.2-1.5% of that detected in *Synechococcus* cultures incubated in the light, even though contaminant cell densities were approximately equivalent to *Synechococcus* cell densities (*Synechococcus* =  $3.8 \cdot 10^7$  cells/ml; contaminants =  $2.45 \cdot 10^7$  cells/ml) at the time of the experiment. Cells treated with formalin demonstrated very little incorporation (both *Synechococcus* and the contaminants). Uptake of  $^{14}\text{CO}_2$  in light by *Synechococcus* cells pre-grown with 5%  $\text{CO}_2$  was linear along a 2, 4, and 6 hour incubation time course.

Treatment	Culture	Incorporation x $10^3$ (dpm) (mean $\pm$ SD; n = 3)
Light	A	585.6 $\pm$ 68.1
	B'	594.9 $\pm$ 15.1
	JA-Red	4.7 $\pm$ 0.1
	JA-White	1.8 $\pm$ 0.1
	JA-Pink	7.8 $\pm$ 0.3
Dark	A	7.7 $\pm$ 0.8
	B'	2.0 $\pm$ 0.6
	JA-Red	5.0 $\pm$ 0.3
	JA-White	1.7 $\pm$ 0.2
	JA-Pink	10.6 $\pm$ 0.7
Formalin	A	0.24 $\pm$ 0.09
	B'	0.21 $\pm$ 0.1
	JA-Red	0.14 $\pm$ 0.15
	JA-White	0.06 $\pm$ 0.02
	JA-Pink	0.06 $\pm$ 0.01

\* Background = 24.24 dpm

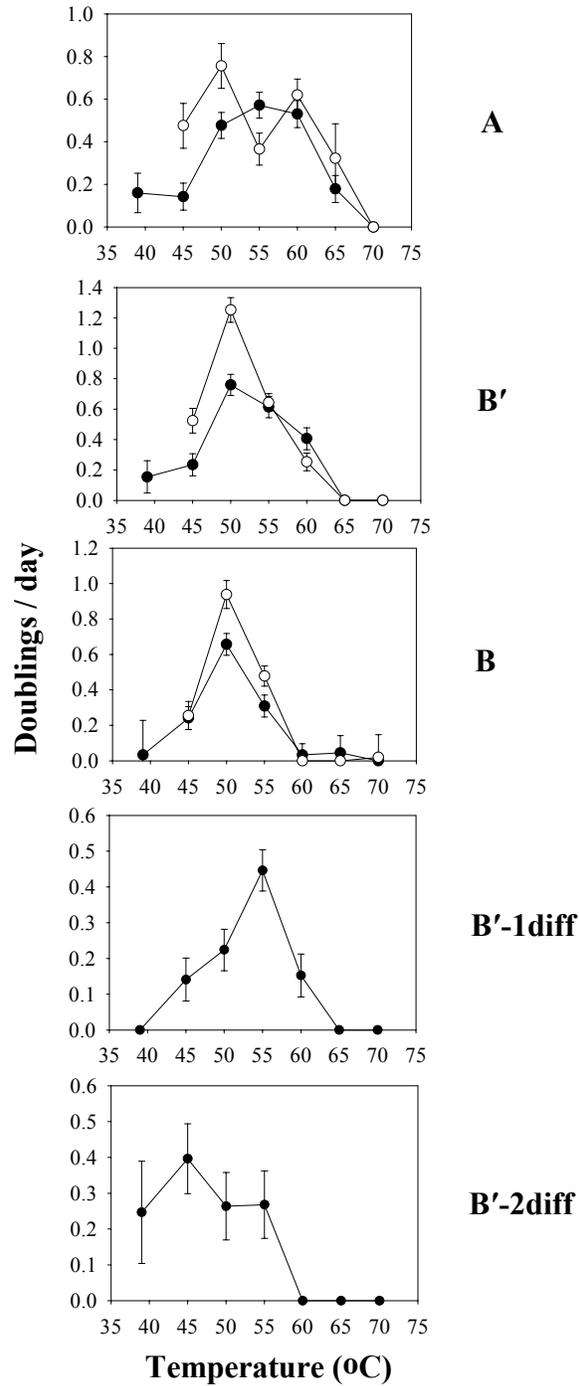
**Table 3.** Results of preliminary  $^{14}\text{CO}_2$  experiment; 6-hour incubation time.

### Temperature Effects on Growth

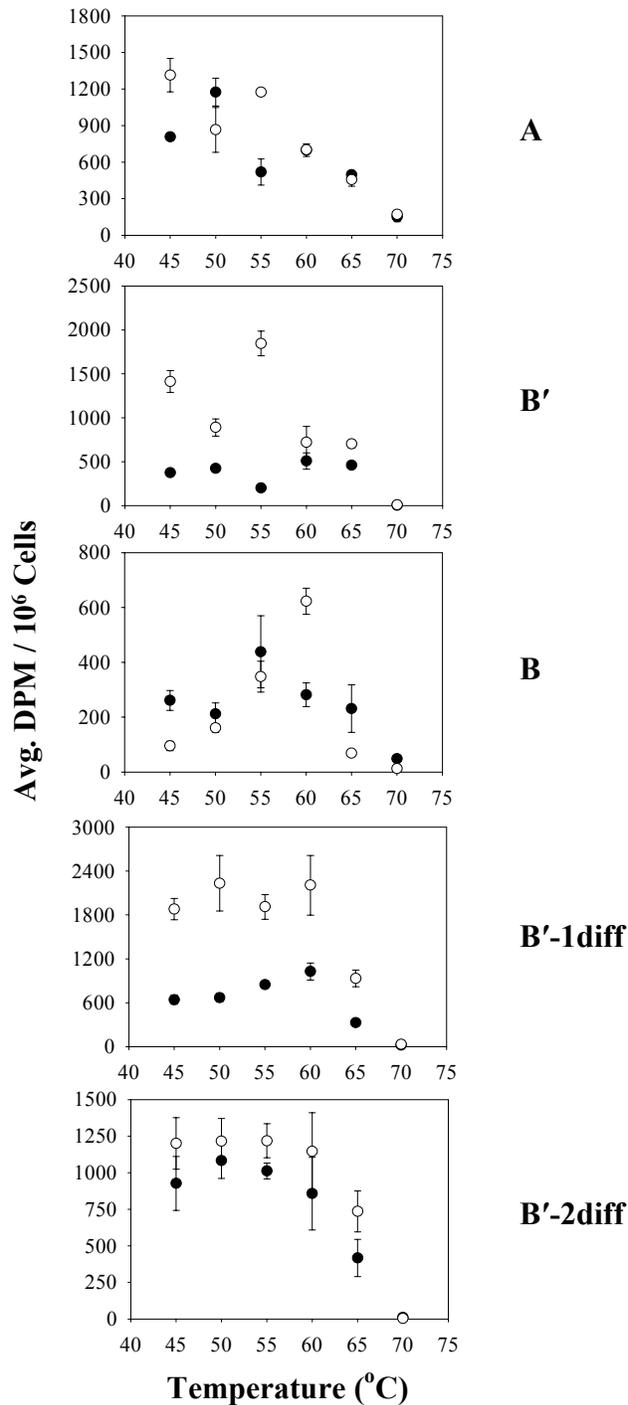
The growth rate of each *Synechococcus* genotype was measured between 40°C and 70°C at 5°C intervals (Figure 11, filled circles). The genotypes exhibited differences in the temperature ranges supporting growth. Genotype A grew at temperatures from 40°C to 65°C, B' at 40-60°C, B'-1diff at 45-60°C and B and B'-2diff at 40°C to 55°C. Tukey's multiple comparison test demonstrated that genotype A achieved optimal growth between 50-60°C; B' at 50-55°C; B at 50°C; and B'-1diff at 55°C. An optimum temperature for growth could not be determined for B'-2diff. Genotypes typical of those observed in situ (A, B', B) grew at faster rates than did the genotypes discovered in this study (B'-1diff, B'-2diff). In addition, the growth rates of A, B, and B' were compared between 50-60°C. At 50°C, genotypes B and B' grew significantly faster than genotype A, whereas at 55°C genotypes A and B' grew faster than genotype B. At 60°C genotype A grew significantly faster than both B' and B. Genotype A was the only one capable of growth at 65°C. Similar results were found with data collected from an additional growth experiment involving A, B, and B' (Figure 11, open circles).

### Temperature Effects on Photosynthesis

Given that  $^{14}\text{CO}_2$  incorporation in contaminated cultures was light-dependent and that pure cultures of contaminant bacteria could not incorporate this substrate in a light-dependent fashion, the photosynthetic activity of representative genotypes was measured.  $^{14}\text{CO}_2$  incorporation was measured in all five *Synechococcus* genotypes at temperatures between 45° and 65°C (Figure 12). All showed a reproducible trend towards lower incorporation at higher temperatures. Only genotype A demonstrated activity at 70°C.



**Figure 11.** Growth rate of each *Synechococcus* genotype incubated at different temperatures and constant light intensity of  $48-55 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; different symbols represent data from separate experiments. Error bars correspond to  $\pm 1$  standard error.



**Figure 12.**  $^{14}\text{CO}_2$  fixation for each *Synechococcus* genotype at different temperatures and a constant light intensity of  $90\text{-}110 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; different symbols represent data from separate experiments (Filled circles lie behind open circles at  $70^{\circ}\text{C}$ ). Error bars correspond to  $\pm 1$  standard deviation from the mean.

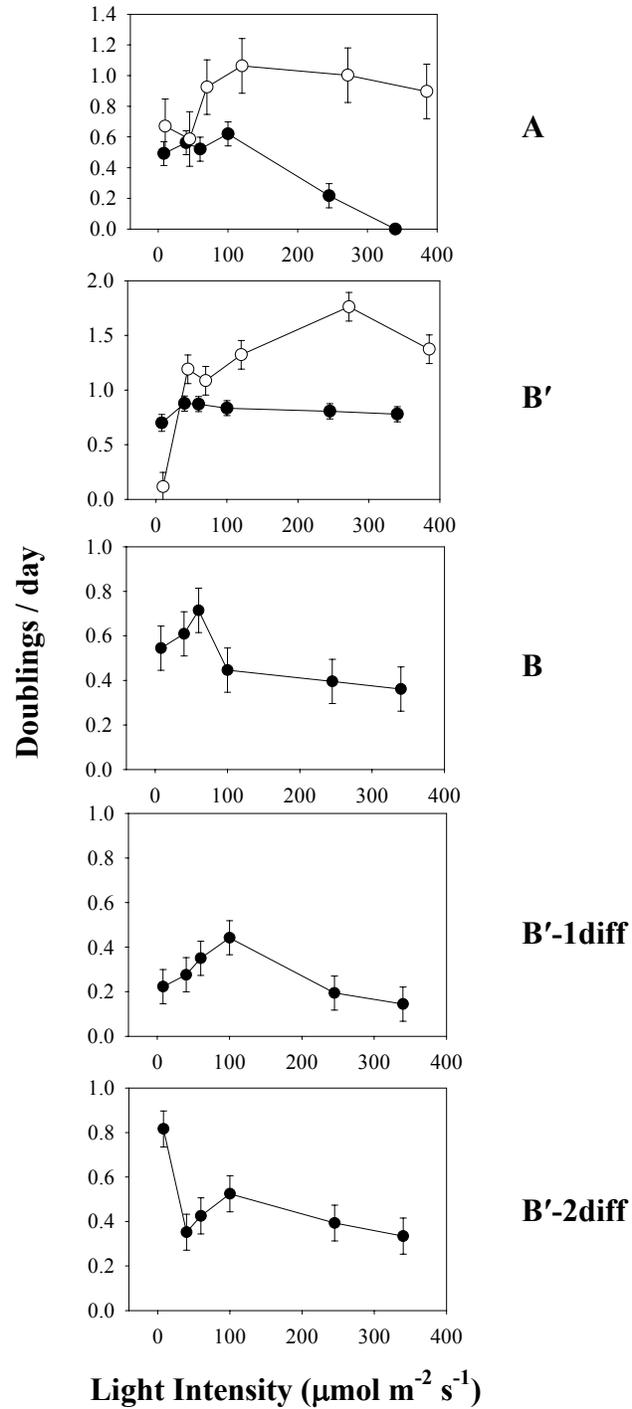
Results of two-factor ANOVA demonstrated that there was a high degree of variability between experiments and therefore no specific temperature optimum for photosynthesis could be detected for any of the isolates ( $P > 0.0001$ )(Table 4).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value	Probability (>F)
Exp	1	5881360	5881360	400.254	2.2E-16
Factor (Temp)	5	18693722	3738744	254.439	2.2E-16
Genotype	4	15207113	3801778	258.728	2.2E-16
Exp:Factor(Temp)	5	1703435	340687	23.185	2.2E-16
Exp:Genotype	4	6078682	1519671	103.421	2.2E-16
Factor(Temp):Genotype	20	6355028	317751	21.624	2.2E-16
Exp:Factor(Temp):Genotype	20	3750880	187544	12.763	2.2E-16
Residuals	132	1939619	14694		

**Table 4.** An example of a two-factor analysis of variance (ANOVA) table. This table provides analysis results for the experiments examining the effects of temperature on photosynthesis.

#### Light Effects on Growth

The effects of light intensity on growth rate were first examined with *Synechococcus* genotypes A and B' at six different light intensities: 10, 45, 70, 120, 272, and 385  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Figure 13, open circles)(385  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was the highest light intensity that could be achieved in the laboratory incubators). Tukey's multiple comparison test revealed that, in this experiment, genotype A grew faster than B' at the lowest light intensity (10  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), but B' grew faster than A at the higher light intensities (45, 272, and 385  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). In a repeat experiment involving all five *Synechococcus* genotypes, A and B' did not show the same results (Figure 13, filled circles); genotype A had lower doublings/day than B' across all light intensities. With rare exceptions, growth occurred for all genotypes at all light intensities investigated and, in most cases, growth at low light intensities was surprisingly high. In this experiment, A



**Figure 13.** Growth rate of each *Synechococcus* genotype incubated at different light intensities and a constant temperature of 55°C; different symbols represent data from separate experiments. Error bars correspond to  $\pm 1$  standard error.

grew significantly better at 8, 40, 60 and 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  than at the two highest intensities, and genotype B'-2diff achieved the best growth at the lowest light intensity (8  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). An optimum light intensity for growth could not be determined for genotypes B, B', and B'-1diff due to a lack of significant differences in the doublings/day values. It appeared that B'-1diff and B'-2diff grew more slowly at all light intensities as compared to A, B', and to some extent, B. It was also noted that all genotypes showed visual evidence of acclimation to light, in that cells were greener when incubated at low light intensities, indicating higher chlorophyll content per cell (Figure 14).

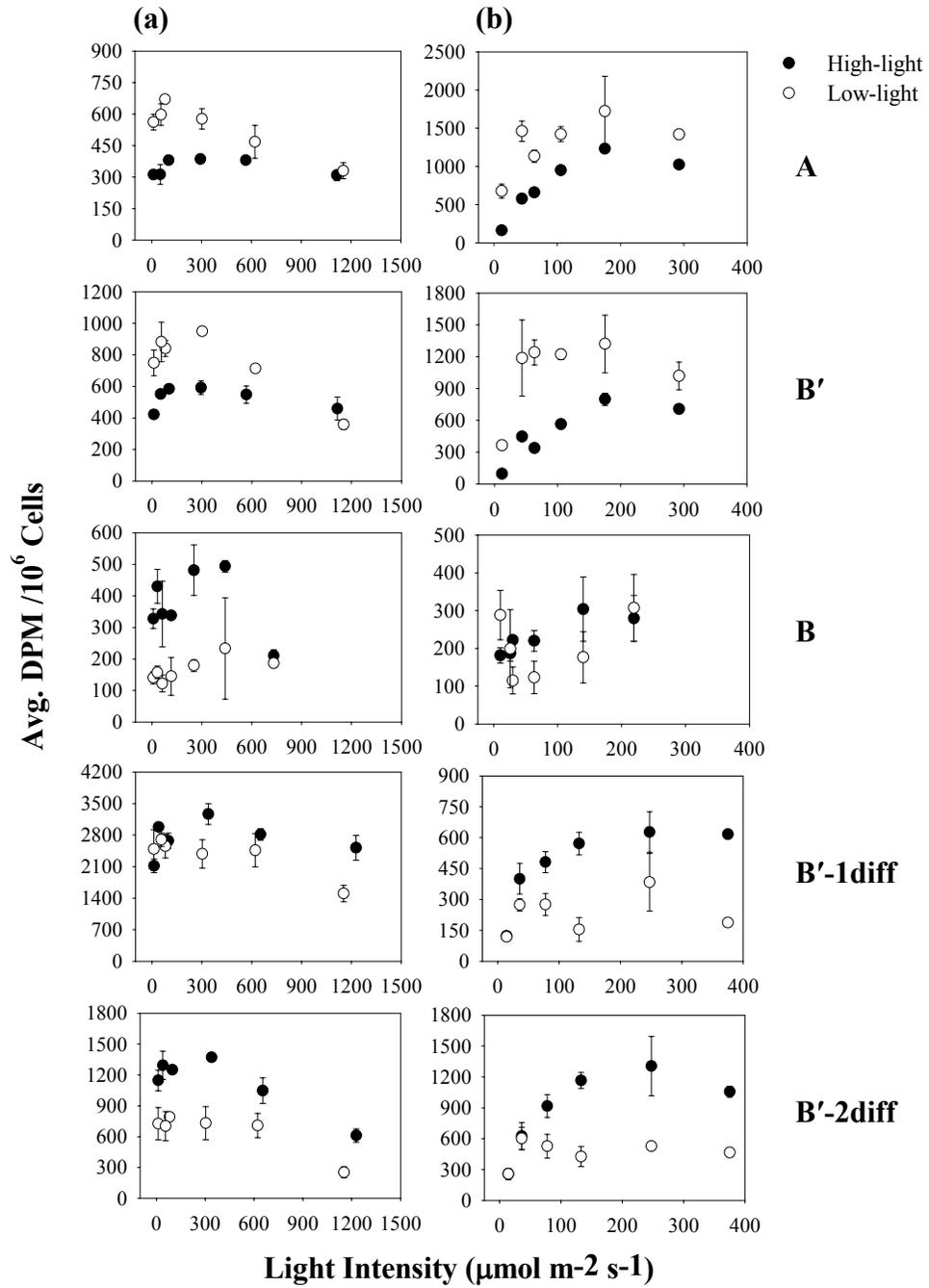


**Figure 14.** Visual evidence of *Synechococcus* acclimation to light intensity. Photographic image of genotype A following the light intensity vs. growth experiment (taken at day 7). Light intensity during growth (from left to right) was: 8, 40, 60, 100, 245, and 340  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

#### Light Effects on Photosynthesis

The photosynthetic activity of *Synechococcus* genotypes was also measured in response to light intensity at 6 outdoor light intensities ranging from 10 to 1300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (genotype B was examined under 7 different light intensities)(Figure 15a).

Photosynthetic activity data was collected for all genotypes at all light intensities. The highest mean  $^{14}\text{CO}_2$  uptake occurred at relatively low light intensities (ca. 100-450  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), with lower uptake above and below these values. Light intensity had an effect on



**Figure 15.**  $^{14}\text{CO}_2$  fixation for each *Synechococcus* genotype at different light intensities and a constant temperature of  $55^\circ\text{C}$ . (a) Outdoor experiments; different symbols represents data from separate experiments. (b) Indoor experiments;  $\bullet$  represents pre-growth at  $150\text{-}210 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\circ$  represents pre-growth at  $25\text{-}43 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Error bars correspond to  $\pm 1$  standard deviation from the mean.

$^{14}\text{CO}_2$  fixation ( $P < 0.0001$ ; no significant interactions between factors), but the level of  $^{14}\text{CO}_2$  uptake depended upon genotype ( $P < 0.0001$ ). Although light intensity had an effect on  $^{14}\text{CO}_2$  fixation, a statistically significant optimum light intensity value for photosynthesis could not be determined for any of the genotypes.

For the indoor experiments, cultures were pre-grown under relatively high (150-210  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) or low (25-43  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) light to determine how pre-growth conditions influence acclimation to light in each genotype. Similar to the outdoor experiments,  $^{14}\text{CO}_2$  fixation was measured for each genotype at 6 different light intensities between 5 and 385  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Figure 15b; filled circles – high light pre-growth, open circles – low light pre-growth). The light intensity range for the indoor experiments was a subset of the range examined in the outdoor experiments, i.e. indoor intensities were within the lower range of outdoor light intensities. All genotypes showed similar trends to those observed in outdoor experiments, with highest measured mean rates ca. 100-300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Also, an increase in photosynthesis with increasing light intensity was more evident. However, a statistically significant optimum light intensity for photosynthesis could not be found for any of the genotypes. Broken line regression analysis demonstrated that pretreatment conditions had a significant effect on all genotypes ( $P < 0.0001$ ), although the effects varied by genotype ( $P < 0.0001$ ). The genotypes did exhibit different patterns of acclimation in terms of photosynthetic uptake. Genotypes A and B' showed increases in uptake at all light intensities, with a possible broadening of uptake toward lower light intensities as compared to genotypes pre-grown under high light. Genotype B showed increased uptake only at the lowest light intensity.

Genotypes B'-1diff and B'-2diff showed reduced uptake at higher intensities and possible optimization at lower light intensities.

## DISCUSSION

Cultivation of Relevant Mat *Synechococcus*

Cultivation is a necessary and essential tool to microbial ecologists, but it can be a biased method for isolating organisms (Ward et al., 1998). Enrichment cultures may not always provide the exact nutrients and conditions needed by the targeted organisms, and therefore, it is possible to select for organisms that do not play a key role in the community from which they were obtained. The dilution and filtration cultivation technique proved to be a reliable method for isolating relevant *Synechococcus* genotypes in this study, perhaps because physical isolation of cells on the filters prevented mutual exclusion (i.e., overgrowth) by less relevant organisms. Genotypes A, B, and B' were previously detected by Ferris and Ward (1997)(with respect to the 16S rRNA locus) and isolates A and B' also had ITS sequences that corresponded to genotypes recovered by Papke et al. (2003). This cultivation method also allowed a previous student, Tony Scotti, to retrieve genetically relevant isolates, further demonstrating that it was an appropriate technique. Entire genomic sequences are now being obtained for genotypes A and a B', and these may provide insight into the genetic character of these dominant mat phototrophs. This information will anchor direct genomic studies of this mat community and enable future microarray studies of gene expression directly in the mat (<http://landresources.montana.edu/FIBR/>).

It must be acknowledged that selection bias could have occurred in this study. *Synechococcus* isolates were enriched at temperatures very close to those in situ, but at light intensities that might have favored either high-light or low-light adapted strains.

Low light intensities could have selected against organisms requiring high light. For instance, Meeks and Castenholz (1971) found that a high temperature *Synechococcus* strain from Hunter's Hot Springs (Oregon) required higher light intensities to reach growth saturation in the laboratory. Under adequate nutrient conditions, growth at 72°C only occurred at intensities greater than 1,000 foot candles ( $146 \mu\text{mol m}^{-2} \text{s}^{-1}$  of light when provided by a cool white fluorescent bulb). The inability to cultivate high-temperature genotypes A' and A'' from Site 4 (59-70°C) could be related to the need for higher light intensities in the laboratory. Site 4 samples were placed under only  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , much lower than the intensities Meeks and Castenholz used to grow their high temperature isolates. On the other hand, if lab light intensities are too high they may select against low-light adapted forms. Ramsing et al. (2000) showed evidence that subsurface *Synechococcus* may orient vertically at the brightest part of the day, possibly because ca.  $72.5 \mu\text{mol m}^{-2} \text{s}^{-1}$  of light is too much. The light intensity used in enrichments was near this level.

#### Difficulty in Recovery of Pure *Synechococcus* Cultures

Less than 10% of the *Synechococcus* cells visible by microscopy were recovered in culture. Furthermore, *Synechococcus* growth occurred only on densely inoculated filters and did not follow the dilution of the sample. Coupled with these observations, the inability to obtain pure cultures could suggest that there is a specific nutrient that the *Synechococcus* genotypes are dependent upon for growth in the laboratory. Despite using several medium supplements, including B vitamins, sodium bicarbonate, and supernatant

from contaminant cultures, axenic cultures could not be achieved. It is possible that the correct nutrient was not discovered.

The lack of colony growth on higher dilution plates and streak plates might also be attributed to quorum-sensing. This is a process by which bacteria “communicate” with one another through chemical signals. A signaling molecule (autoinducer) is produced, the concentration of which is dependent upon the population density of the organism producing it. Other cells sense the signaling molecule and the entire population can then initiate a concerted action once a critical autoinducer concentration has been reached. This has been shown to be a common occurrence in several Gram-positive and Gram-negative bacteria (Miller and Bassler, 2001; Whitehead et al., 2001). Quorum-sensing may have also been detected in filamentous cyanobacteria, which use a diffusible peptide molecule as a signaling molecule for heterocyst formation (Yoon and Golden, 1998) [Heterocysts are nitrogen-fixing cells that develop among the photosynthetic cells of the filament. For example, in *Anabaena*, every tenth cell differentiates into a heterocyst; these cells allow this cyanobacterium to perform both photosynthesis and nitrogen-fixation]. Quorum-sensing could explain the lack of colony growth on higher dilution plates, in that a certain density of cells must be present in order to signal additional colony growth. This phenomenon may have had a consequential effect on the ability to obtain pure cultures. If colonies had been able to grow on filter plates of higher dilution, then perhaps *Synechococcus* could have been separated from the contaminant organisms. The only isolated microcolonies to develop were on plates from  $10^{-3}$  to  $10^{-5}$  dilutions (i.e., at low dilutions where the contaminant cell concentrations were still fairly high). Addition of *Synechococcus* supernatant did not permit recovery of pure *Synechococcus*

cultures, suggesting that, if quorum sensing is a part of the purification problem, the amendment did not supply an autoinducer in sufficient amount. Currently, there is no literature on quorum-sensing in thermophilic *Synechococcus* spp.; therefore, it will be interesting to explore the new *Synechococcus* genomes for evidence of quorum-sensing genes.

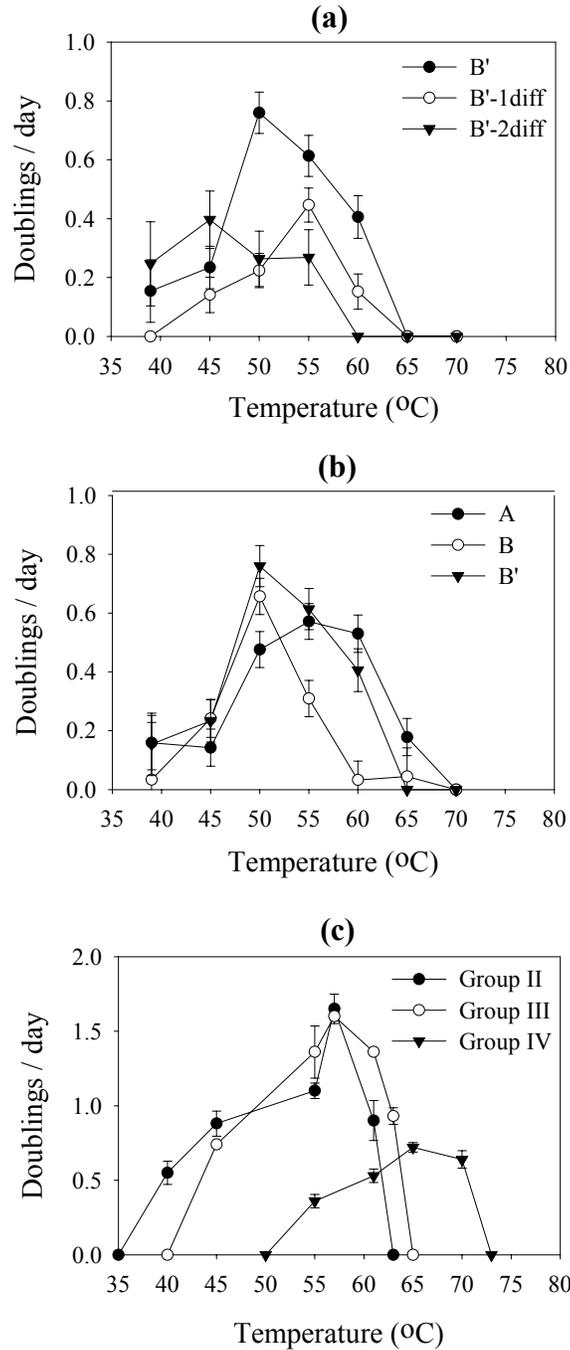
### Temperature Adaptation

#### Growth

It was hypothesized the *Synechococcus* genotypes would correspond to distinct ecotypes that were adapted to specific temperature and/or light conditions. Experiments examining the growth rate of each genotype with temperature provided support for this hypothesis, in that a specific temperature range and optimum for growth was detected for each genotype except one. Genotype A grew at temperatures from 40-65°C with optimal growth between 50-60°C; B' exhibited growth at 40-60°C with optimal growth between 50-55°C; B showed a temperature range of 40-55°C with optimal growth at 50°C; B'-1diff grew at the temperature range of 45-60°C and achieved optimal growth at 55°C; and B'-2diff grew between 40-55°C. Genotypes typically observed in situ, A, B, and B', grew faster at temperatures typical of the collection sites than did genotypes B'-1diff and B'-2diff, which have never been detected in direct molecular analysis of the mat and are presumed to be less numerically relevant. If this is true in situ, as well as in Medium DH, it could explain why genotypes A, B, and B' are more prevalent in the Octopus Spring microbial mat. It is interesting that less predominant strains with only 1-2 differences in their 16S rRNA sequences nevertheless have quite different thermal relations (compare,

for instance, B' with B'-1diff and B'-2diff – Figure 16a) and are not representative of the temperature relations of the predominant genotype. The higher upper temperature limits and broadened temperature optima of genotypes A and B' relative to genotype B (Figure 16b) support the hypothesis that these predominant *Synechococcus* genotypes are different temperature-adapted ecotypes and this is consistent with, and may explain, their distribution in the Octopus Spring thermal gradient (Figure 4).

These results can be compared to data collected on similar organisms from a different hot spring mat (Figure 16c). Both Peary and Castenholz (1964) and Miller and Castenholz (2000) have demonstrated that *Synechococcus* isolates from Hunter's Hot Springs, Oregon have distinct temperature ranges and sometimes optima for growth. In the more recent study, growth was observed for B-like (their group II) organisms between 40°C and 61°C (optimum 57°C), for A-like (their group III) organisms between 45°C and 63°C (optimum 57°C), and for A'-like (their group IV) organisms between 55°C and 70°C (optimum 65°C). [The correspondence with Octopus Spring genotypes was based on the number of nucleotide differences with closest known relatives at the time (Miller and Castenholz, 2000).] Miller and Castenholz noted that as the upper temperature limit for growth was extended, the minimum temperature shifted to higher values as well, leading them to suggest that increases in thermal specialization resulted in a decrease in the overall temperature range for growth. The Octopus Spring genotypes exhibited a different pattern. The genotypes had equivalent lower temperature limits and only the upper temperature limit was extended. For the *Synechococcus* genotypes of Octopus Spring, it does not appear that increased thermal adaptation signifies a reduction



**Figure 16.** Comparison of *Synechococcus* growth rates with respect to temperature: (a) Octopus Spring genotypes B', B'-1diff, and B'-2diff; (b) Octopus Spring genotypes found predominating in situ A, B, and B'; (c) *Synechococcus* groups II, III, and IV from Hunter's Hot Springs, Oregon (modified from Miller and Castenholz, 2000). Error bars correspond to  $\pm 1$  standard error.

in the ability to grow at lower temperatures. In both studies, however, it seems that isolates with an extended temperature limit have a broad optimum. Perhaps this is a characteristic of high-temperature isolates and imparts an ecological advantage over other organisms; a broad optimum could provide more versatility than being specialized to a single temperature.

In general, results for Octopus Spring and Hunter Spring make sense relative to what is known of the distribution of genotypes in Octopus Spring mat. The A'-like Oregon Group IV genotype shows a higher temperature optimum and range than does the type-A genotype observed in both studies, consistent with DGGE results showing a higher temperature distribution for genotype A' than A (Figure 4). Results for the A-like and B-like Oregon sequences (Groups II and III) resemble results for the Octopus Spring A and B' (as opposed to B) isolates in the sense that one is able to grow at slightly higher temperature than the other. The Oregon Group II isolates were judged to be most closely related to the Octopus Spring B genotype, but one must be careful in making direct assignments of comparable genotypes between the two systems. Papke et al. (2003) showed that while Oregon genotypes fell into A-like and B-like clades together with Yellowstone A-like and B-like genotypes, divergence that is likely due to geographic isolation prevents assignment of the specific closest relatives within each clade (Figure 6). Furthermore, Papke et al. (2003) showed that the genotypes cultivated by Miller and Castenholz were not identical in 16S rRNA sequence to those cloned directly from the same mat during the same time period (purple entries in Figure 6, NACy01 and NACy03 were recovered by molecular analysis, whereas *Synechococcus* entries were from the isolates used to generate the data of Figure 16c). Hence, given the observations on

Octopus Spring isolates (Figure 16a), one cannot be certain that the temperature relations of their isolates reflect those of the predominant in situ genotypes from Hunter Spring. The cultivated genotypes did have growth rates comparable to the predominant Octopus Spring isolates, which might, however, argue for their relevance to the system.

### Photosynthesis

$^{14}\text{CO}_2$  fixation experiments involving photosynthetic rate in response to temperature did not identify any distinct differences in temperature adaptations, but they nevertheless provided additional insight regarding the activity of the genotypes at various temperatures. Photosynthesis occurred for all genotypes between 45°C and 65°C, with only genotype A showing activity at 70°C. All of the genotypes demonstrated the same pattern – a decline in photosynthesis with increasing temperature. Independent experiments using oxygen microsensors to measure the photosynthetic rate of genotypes A, B, and B' at temperatures between 40°C and 75°C (5 degree increments) showed similar results (unpublished data of Niels Peter Revsbech and Mary Bateson). Genotype A was able to perform photosynthesis at higher temperatures (70°C, with some activity at 75°C) than B and B'. In addition, all of the genotypes showed peak photosynthetic rates around 65°C. Both  $^{14}\text{CO}_2$  fixation and oxygen production studies revealed high levels of photosynthetic activity at temperatures that are not permissive of growth. This suggests that photosynthetic activity may not be a good determinant of growth rate adaptations; the photosynthetic apparatus may not be restricted by temperature in the same manner as the organism as a whole.

## Light Adaptation

### Growth

Experiments of growth rate with respect to light intensity were also used to test the hypothesis of light adaptations in *Synechococcus* genotypes. In the first experiment, genotypes A and B', A grew faster than B' at lower light intensities and B' grew faster than A at higher light intensities. Although this pattern was not repeated in the second experiment, genotype A grew best at the four lowest light intensities (8, 40, 60, 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), with a sharp decline in growth at light intensities greater than 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Genotype B' maintained a higher growth rate across all light intensities, but did not exhibit much of a decline at higher intensities. These results might provide weak support for the hypothesis that genotype A is adapted to low light intensity and genotype B' to high intensity. This would fit the distribution model observed in the Ramsing et al. (2000) DGGE study (Figure 5). At the Mushroom Spring 56-63°C site, they detected only the B' sequence at the surface of the mat and the A sequence deeper in the mat, where incident light levels are much lower. However, the lack of reproducibility raises doubt about such an inference. In general, genotypes B, B'-1diff and B'-2diff appeared to grow at slightly lower rates than A and B'. Optimum light intensities for growth were not detected in these experiments.

### Photosynthesis

The various genotypes showed a common pattern in their responses to light intensity, in that  $^{14}\text{CO}_2$  uptake increased with light intensity below 100-300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (especially apparent in indoor experiments) and often decreased at higher intensities

(especially evident in outdoor experiments). However, statistically significant optimum light intensities for photosynthesis could not be detected. The decrease in uptake at higher intensities could be attributed to photoinhibition, a phenomenon that causes functional damage to the reaction centers as a result of exposure to high light (Grossman et al., 2003). The accrued damage can be repaired, but if the rate of damage exceeds the ability to repair, then the organism cannot continue to perform photosynthesis at a constant rate (Kim et al., 1993). It is likely the genotypes underwent photoinhibition when exposed to the natural sunlight levels, causing a decline in the rate of photosynthesis. Similar patterns of photosynthesis relative to light intensity were observed in an oxygen microsensor experiment (Revsbech and Bateson, unpublished), except that no inhibition of oxygen production was observed at high light intensities. Also, genotype B' required higher light intensities to saturate photosynthesis than did genotypes A and B, possibly consistent with subtle adaptation of the A isolate to lower light intensity.

The light experiments were performed to test the hypothesis that distinct *Synechococcus* genotypes might be either low-light or high-light adapted. Such adapted populations have been found in the marine environment; Moore et al. (1998) showed that *Prochlorococcus* populations isolated from the top of the water column could grow and photosynthesize at higher light intensities than could isolates from deeper in the water column. The evidence obtained in this study suggesting differently light-adapted strains is weak at best and certainly nothing like the clear-cut adaptation to light observed in the *Prochlorococcus* study. If genotype A is adapted to grow and photosynthesize better than genotype B' at low light intensities, consistent with the distribution data (Figure 5), the difference in adaptation is subtle and not easily reproduced.

Another possibility is that multiple differently adapted *Synechococcus* ecotypes exist that share the A genotype and cultivation favored the isolation of a particular ecotype that is not low-light adapted. The existence of high- and low-light adapted *Synechococcus* ecotypes of the same 16S rRNA genotype has been suggested. Ferris et al. (2003) discovered separate phylogenetic clades of genotype A'-like *Synechococcus* from a Mushroom Spring 68°C site (based on ITS sequences) that correlated with their location in the mat – either surface or subsurface. At 65°C in Mushroom Spring, several *Synechococcus* A genotypes with identical ITS sequences were observed in both surface and subsurface samples (Ferris and Ward, unpublished). Nevertheless, distinctly pigmented populations occur at different depths, as in Figure 5b. If such closely related ecotypes exist, the cultivation process could have affected their isolation. Microbial ecologists working with marine populations have the luxury of their differently adapted populations existing decameters apart, whereas in Octopus Spring microbial mat, hypothesized light-adapted ecotypes exist less than a millimeter apart. Sampling procedures were such that the entire 1 mm layer of the mat was homogenized; samples were not separated based upon upper and lower sections. As discussed above, it is possible the light intensities during enrichment may have excluded isolates preferring higher or lower light intensities.

#### Light Acclimation

In each of the light intensity experiments, the genotypes displayed an acclimation response to the light treatments. Photoacclimation, defined as “the reversible light-induced alterations in the physiological or morphological characteristics of a population”

(Moore et al., 1995), was detected visually in the growth studies. Under low light, the genotypes increased their cellular concentrations of chlorophyll causing the cultures to appear darker green, whereas at high light, they decreased their chlorophyll content and became more pale. This is an acclimation response, as opposed to adaptation, which is not reversible. This is consistent with the results of the  $^{14}\text{CO}_2$  fixation experiments, in which photosynthetic uptake was altered as a result of pretreatment under high or low light.

### Conclusions

Understanding the basic ecology of an organism is fundamental to gaining insight into the community in which it exists. By cultivating the *Synechococcus* strains with genotypes identical to those observed in situ and examining their responses to different temperature and light conditions, it is now possible to make inferences about the Octopus Spring microbial mat in terms of how these organisms interact with one another and how they affect the overall structure of the ecosystem. This study yielded clear evidence of temperature-adapted ecotypes that are consistent with predominant genotypes found in situ at corresponding temperatures. Some of these ecotypes exhibit <3% difference in 16S rRNA sequence (e.g., B, B'), providing another example of the problem with quantitative molecular cut-offs as species concepts. A surprising level of diversity in phenotypic adaptations among the isolates was also shown; isolates with genotypes not predominant in situ grew more slowly than the isolates with predominant genotypes and are presumably less fit. Furthermore, their temperature relations were not representative of closely related predominant populations. The difference in growth rates among these

genotypes probably has an important outcome in the Octopus Spring mat system. Competition and niche partitioning most likely play a major role in determining how the *Synechococcus* genotypes function in response to the conditions of temperature and possibly light. At temperatures where all genotypes can exist (i.e., 50-60°C), the ability to grow faster ensures the success of one genotype over another. At these temperatures, slower growing genotypes may be forced to occupy a different niche, perhaps at a lower depth where light levels are lower, or to persist, awaiting more opportune conditions. Although growth rate experiments demonstrated temperature adaptations among the genotypes, activity measurements did not correspond to temperature relations for growth. They did, however, depict slight adaptive differences (i.e., genotype A active at higher temperatures).

No conclusive evidence for light adaptation was obtained in this study. It is possible light adaptations do not exist, are subtle, or that isolates with different light adaptations were not cultivated. Evidence for light acclimation was detected in each of the genotypes, which does demonstrate that these organisms respond to changes in the light environment.

It should be acknowledged that by isolating only a single variable (either temperature or light) when the experiments were performed, a complete picture of how the genotypes react to these conditions might not have been obtained (for instance, the light experiments were not always performed at the optimal temperature for each genotype). Furthermore, other environmental parameters in addition to temperature and light may influence the overall fitness (i.e. growth rate) of these organisms. Nutrient availability, pH, and interactions with other microorganisms could affect their

distribution in the mat. Temperature and light are only two of the many environmental factors that could be studied. Genomic analyses of genotypes A and B' may be important in revealing further evidence of adaptation to temperature (and light?), and could give insight into the other environmental factors affecting the niche adaptations of these organisms in situ. Despite targeting single variables, the results obtained in this study provided support for the hypothesis that closely related 16S rRNA-defined *Synechococcus* genotypes in the Octopus Spring microbial mat correspond to distinct ecotypes that are adapted to specific temperature and/or light conditions.

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