The distribution of cultivated and uncultivated cyanobacteria and green non-sulfur bacteria in hot spring microbial mats
by Alyson L Ruff-Roberts

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Oligodeoxynucleotide probes were designed to complement regions of the small subunit ribosomal RNA (SSU rRNA) of uncultivated and cultivated cyanobacteria and green nonsulfur bacteria thought to inhabit hot spring microbial mats. The probes were used to investigate the distribution of SSU rRNA from these species in hot. spring microbial mats of varying temperature and pH, and to measure changes in distribution resulting from in situ changes in temperature, pH, and light intensity. Synechococcus lividus Y-7c-s SSU rRNA was only detected in the mat of a slightly acidic hot spring from which it may have been initially isolated, and when samples from a more alkaline spring were incubated at the acidic spring. S. Iividus SSU rRNA was enriched in a medium used for growing thermophilic cyanobacteria when undiluted mat samples of both the alkaline and acidic springs were used as inocula. However, S. Iividus SSU rRNA was enriched in this medium from highly-diluted mat samples from the acidic spring only. Chloroflexus aurantiacus Y-400 SSU rRNA was only detected in a high temperature mat sample from the alkaline Octopus Spring, or when samples from a lower temperature region of Octopus Spring mat were incubated at the high temperature region. SSU rRNA of the uncultivated species was more widely distributed. Temperature distribution and responses to temperature shifts suggested that three of four uncultivated cyanobacteria might be adapted to high, moderate, and low temperature ranges, whereas an uncultivated green nonsulfur bacterium appears to have broad temperature tolerance. Two of the uncultivated cyanobacteria were enriched from highly-diluted Octopus Spring 50°C mat samples. One was also enriched from highly-diluted samples of the acidic Clearwater Spring mat. Total SSU rRNA, as measured by a universal probe, decreased with depth throughout the upper 10 mm of the 50°C Octopus Spring mat. Specific probe reactions targeting uncultivated species inhabiting this site were highest in the upper I mm and were not detected below 3.5 mm, consistent with their possible phototrophic nature. However, the effects of light intensity reduction on the organisms contributing these SSU rRNAs were variable, indicating the difficulty of establishing phenotype from environmental perturbation experiments.

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Alyson L. Ruff-Roberts

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APPROVAL
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Alyson Louise Ruff-Roberts

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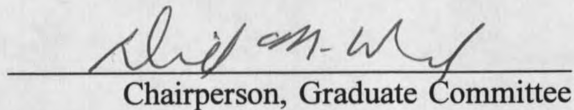
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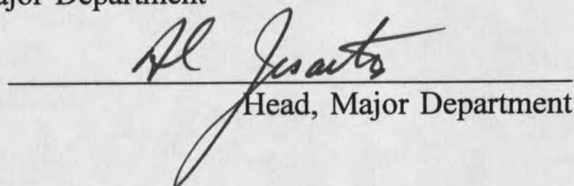
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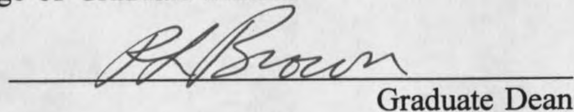
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ABSTRACT

Oligodeoxynucleotide probes were designed to complement regions of the small subunit ribosomal RNA (SSU rRNA) of uncultivated and cultivated cyanobacteria and green nonsulfur bacteria thought to inhabit hot spring microbial mats. The probes were used to investigate the distribution of SSU rRNA from these species in hot spring microbial mats of varying temperature and pH, and to measure changes in distribution resulting from *in situ* changes in temperature, pH, and light intensity. *Synechococcus lividus* Y-7c-s SSU rRNA was only detected in the mat of a slightly acidic hot spring from which it may have been initially isolated, and when samples from a more alkaline spring were incubated at the acidic spring. *S. lividus* SSU rRNA was enriched in a medium used for growing thermophilic cyanobacteria when undiluted mat samples of both the alkaline and acidic springs were used as inocula. However, *S. lividus* SSU rRNA was enriched in this medium from highly-diluted mat samples from the acidic spring only. *Chloroflexus aurantiacus* Y-400 SSU rRNA was only detected in a high temperature mat sample from the alkaline Octopus Spring, or when samples from a lower temperature region of Octopus Spring mat were incubated at the high temperature region. SSU rRNA of the uncultivated species was more widely distributed. Temperature distribution and responses to temperature shifts suggested that three of four uncultivated cyanobacteria might be adapted to high, moderate, and low temperature ranges, whereas an uncultivated green nonsulfur bacterium appears to have broad temperature tolerance. Two of the uncultivated cyanobacteria were enriched from highly-diluted Octopus Spring 50°C mat samples. One was also enriched from highly-diluted samples of the acidic Clearwater Spring mat. Total SSU rRNA, as measured by a universal probe, decreased with depth throughout the upper 10 mm of the 50°C Octopus Spring mat. Specific probe reactions targeting uncultivated species inhabiting this site were highest in the upper 1 mm and were not detected below 3.5 mm, consistent with their possible phototrophic nature. However, the effects of light intensity reduction on the organisms contributing these SSU rRNAs were variable, indicating the difficulty of establishing phenotype from environmental perturbation experiments.

INTRODUCTION

The composition of microbial communities and the interactions between the members of these communities are difficult to determine. Traditionally, microbial ecologists have attempted to characterize a microbial community based on the organisms they could identify, either by morphological characteristics or by isolation of the organisms in pure culture. However, prokaryotes have very few interspecies morphological differences, complicating their recognition, and it has been estimated that less than 20% of the bacteria in natural environments have been cultivated (Wayne, *et al.*, 1987). Molecular methods utilizing slowly-evolving ribosomal RNA (rRNA) molecules have been developed in order to begin to answer fundamental questions about microbial community composition and structure (Olsen, *et al.*, 1986; Pace, *et al.*, 1986; Ward, *et al.*, 1992a). In this thesis, I will discuss the rationale behind the use of small subunit ribosomal RNA (SSU rRNA) as a signature molecule for characterization of microbial communities and present some background data from the application of rRNA-based methods to microbial communities. I will present and discuss data from my application of rRNA-based methodology to study the microbial ecology of cultivated and uncultivated photosynthetic prokaryotes inhabiting microbial mats at Octopus Spring and several other hot springs in Yellowstone National Park (YNP), Wyoming, U.S.A. I thus begin by briefly outlining the previous work

performed on the microbial mat at Octopus Spring which makes this system particularly valuable as a model microbial community for this study.

The Octopus Spring Mat as a Model Community

Hot spring microbial mats are relatively simple communities, mostly restricted to prokaryotic organisms by the extreme environments in which they exist (Brock, 1978). The simplicity of these communities makes them excellent natural models for the study of microbial community ecology. The Octopus Spring microbial mat has been extensively studied for three decades (Brock, 1978; Ward, *et al.*, 1987; Ward, *et al.*, 1989a; Ward, *et al.*, 1989b; Ward, *et al.*, 1992a) as a typical mat community of alkaline hot springs. This luxuriant mat community covers most of a partially barriered region of the spring, adjacent to the source pool, and effluent channels where the temperature is between 42°C and 70°C. The environment in which the mat occurs is relatively stable in temperature and pH. The mat is well-laminated, with a top green layer above an orange layer. The top green layer contains many curved, unicellular cyanobacteria and many long filamentous bacteria, while the orange layer is dominated by the filaments. Previous researchers (Castenholz, 1969; Bauld and Brock, 1973; Pierson and Castenholz, 1974; Bauld and Brock, 1974) suggested, on morphological and culture evidence, that the dominant microorganisms in the mat were the cyanobacterium *Synechococcus lividus* and the green non-sulfur (GNS) bacterium *Chloroflexus aurantiacus*. Later, Tayne, *et al.* (1987), used polyclonal antisera specific for *C. aurantiacus* to show that only one of three morphologically distinct filaments

in the Octopus Spring mat can be antigenically identified as *C. aurantiacus*. Table 1 lists the organisms thought to be present in the mat at Octopus Spring. Some organisms were identified based on morphological evidence, some were cultivated from either low or high dilution samples, and one was identified by characteristic lipid profiles (Ward, *et al.*, 1989a).

There have been numerous studies establishing the *in situ* physiological activity of the cyanobacterium and GNS bacterium thought to dominate microbial mats of alkaline hot springs. Other studies have focussed on the optimal temperature, pH, and light conditions these organisms prefer (for a summary, see Brock, 1978). These studies revealed temperature adaptations, pH effects, and light adaptations, all of which may be important to the survival of these organisms in the microbial mat environment.

The first study of temperature adaptation in strains of *S. lividus* in a hot spring microbial mat was done by Peary and Castenholz (1964) at Hunter's Hot Spring, Oregon, U.S.A. They used samples from the mat collected from sites of different temperatures to inoculate enrichment cultures which were incubated at various temperatures (30°C - 75°C) and cultivated 4 different strains of *S. lividus* that grew best at different optimum temperatures. Later, Brock (1967) provided evidence consistent with the existence of cyanobacterial temperature strains in the Mushroom Spring microbial mat community. Further confirmation was provided by the isolation and characterization of a high temperature strain of *S. lividus* from Hunter's Hot Springs, OR (Meeks and Castenholz, 1971). Temperature strains of *Chloroflexus*

Table 1. Microorganisms thought to be present in the Octopus Spring (OS) microbial mat (adapted from Ward, *et al.*, 1987).

Organism	Type of organism	Evidence for presence at OS
Synechococcus lividus	Cyanobacterium	Microscopic observation
Chloroflexus aurantiacus	Green nonsulfur bacterium	Microscopic observation Enrichment from undiluted inoculum
Thermus aquaticus	Aerobic heterotroph	Enrichment from undiluted inoculum
Isosphaera pallida	Aerobic heterotroph	Microscopic observation
Thermomicrobium roseum	Aerobic heterotroph	Lipid analysis
Thermobacteroides acetoethylicus	Anaerobic fermenter	Enrichment from high dilution inoculum
Thermoanaerobium brockii	Anaerobic fermenter	Enrichment from low dilution inoculum
Thermoanaerobacter ethanolicus	Anaerobic fermenter	Enrichment from undiluted inoculum
Clostridium thermohydrosulfuricum	Anaerobic fermenter	Enrichment from low dilution inoculum
Clostridium thermosulfurogenes	Anaerobic fermenter	Enrichment from low dilution inoculum
Thermodesulfobacterium commune	Sulfate reducer	Enrichment from undiluted inoculum
Methanobacterium thermoautotrophicum	Methanogen	Enrichment from high dilution inoculum

were hypothesized by Bauld and Brock (1973). This hypothesis was supported by their *in situ* experiments which indicated that temperature optima for bacterial photosynthesis at different temperature sites in the mat of Twin Butte Vista spring approximated the environmental temperatures.

Kallas and Castenholz (1982a; 1982b) evaluated the effect of low pH on the growth of *S. lividus* Y-7c-s and found that it showed sustained growth only above pH 6.5. Both *Synechococcus* and *Chloroflexus* are found in mats of neutral and alkaline hot springs (Brock, 1978). Therefore, it is possible that strains of these organisms are adapted for life within a certain pH range, as well as an optimal temperature range. Madigan and Brock (1977) thoroughly evaluated the effects of decreased light on cyanobacterial and photosynthetic bacterial photosynthesis in the mat at Octopus Spring. They found that the photosynthetic prokaryotes carrying out these processes can adapt to relatively low light intensities. When adapted to a light intensity equal to 27% of full sunlight, the light optima for cyanobacterial photosynthesis and bacterial photosynthesis shifted from nearly 100% sunlight (unadapted) to 14% sunlight. When the light was decreased to 2% of full sunlight, cyanobacteria photosynthesized at very low levels, but photosynthetic bacteria continued to be active with an adapted light optimum of <10% of full sunlight. Both types of organism showed some high light inhibition after adaptation to a lower light intensity.

Until recently, most ecological studies of the microbial mat at Octopus Spring have been limited to the evaluation of either (1) a population of organisms (i.e., phototrophs that are microscopically identified as curved unicellular cyanobacteria or

filamentous bacteria which don't necessarily consist of a single species) or (2) a cultivated microorganism which doesn't necessarily represent the most relevant species to study.

Ribosomal RNA as a Signature Molecule for Characterization of Microbial Mat Communities

The development of culture-independent species-specific rRNA methods to evaluate the role of particular organisms in a community was a major advance in microbial ecology, which permits more direct and detailed studies of microbial communities. Ribosomal RNAs are logical choices as signature molecules to identify microorganisms (Woese, 1987). Ribosomes are present in all cells as part of the protein synthesis apparatus. The function of rRNA molecules in protein synthesis is dependent, in part, on their secondary structure, which is similar between organisms. The primary structure of rRNA molecules, however, varies in all but closely related organisms. These differences in primary sequence have evolved relatively slowly and at different rates depending on the position in the molecule. Thus, universally conserved regions of rRNA molecules can be targets for kingdom-specific oligonucleotide probes; semi-conserved regions can be used as signature sequences to identify groups of similar organisms; finally, hypervariable regions of these molecules can be used as targets for species-specific probes. Figure 1 shows the conserved, semi-conserved, and hypervariable regions of SSU rRNA. Individual species can be identified without resorting to culture methods if their rRNA sequences are known (Olsen, *et al.*, 1986; Pace, *et al.*, 1986; Ward, 1989; Ward, *et al.*, 1992a). In addition,

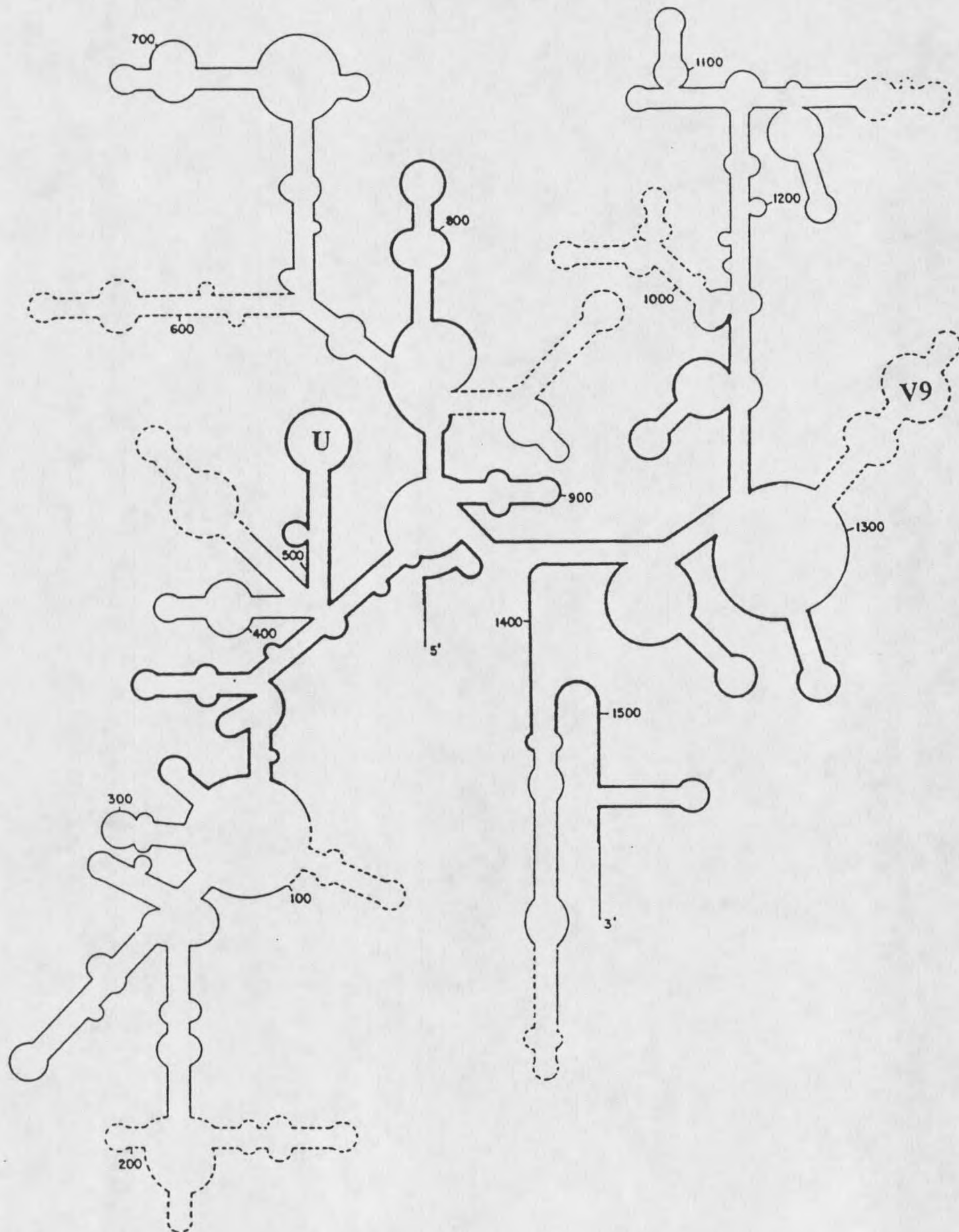


Figure 1. Secondary structure of *Escherichia coli* SSU rRNA indicating regions of low (▬), moderate (—) and high (---) sequence variability (from Ward, *et al.*, 1992a). **V9** indicates the general location of the variable region used as a target for oligodeoxynucleotide probes in this study. **U** indicates a region of universal conservation in all 16S rRNA sequences which was used as a target for the universal probe used in this study.

rRNAs are relatively easy to recover from cells (Pace et al., 1986), can be sequenced directly (Lane, et al., 1985), and, since rRNA molecules are produced as a function of growth rate, the abundance of rRNA from a particular organism should represent the protein synthesis capacity of an actively growing species in the community (DeLong, et al., 1989b).

rRNA Methods Applied to Microbial Ecology

This laboratory has applied rRNA methods to the study of the microbial mat community at Octopus Spring (Figure 2, left side). First, a database of the SSU rRNA sequences of relevant organisms was accumulated (Bateson, *et al.*, 1989; Bateson, *et al.*, 1990; Ward, *et al.*, 1990b). This included organisms cultivated from Octopus Spring (Table 1). Second, SSU rRNAs from the mat were cloned, screened, sequenced, and phylogenetically analyzed in order to identify the SSU rRNA sequences and phylogeny of naturally occurring organisms (Weller and Ward, 1989; Ward, *et al.*, 1990a; Ward, *et al.*, 1990b; Weller, *et al.*, 1991; Ward, *et al.*, 1992a; Weller, *et al.*, 1992). The results of these studies are quite interesting. So far, fifteen distinct SSU rRNA sequence types, which must be attributed to fifteen distinct community members, have been identified (Ward, *et al.*, 1992a). The sequences do not correspond to those of any previously cultivated microorganisms. Five of these sequence types were identified and phylogenetically characterized before the experimental work for this thesis project began: types designated as A, B, I, and J were phylogenetically characterized as cyanobacteria; the type designated C was

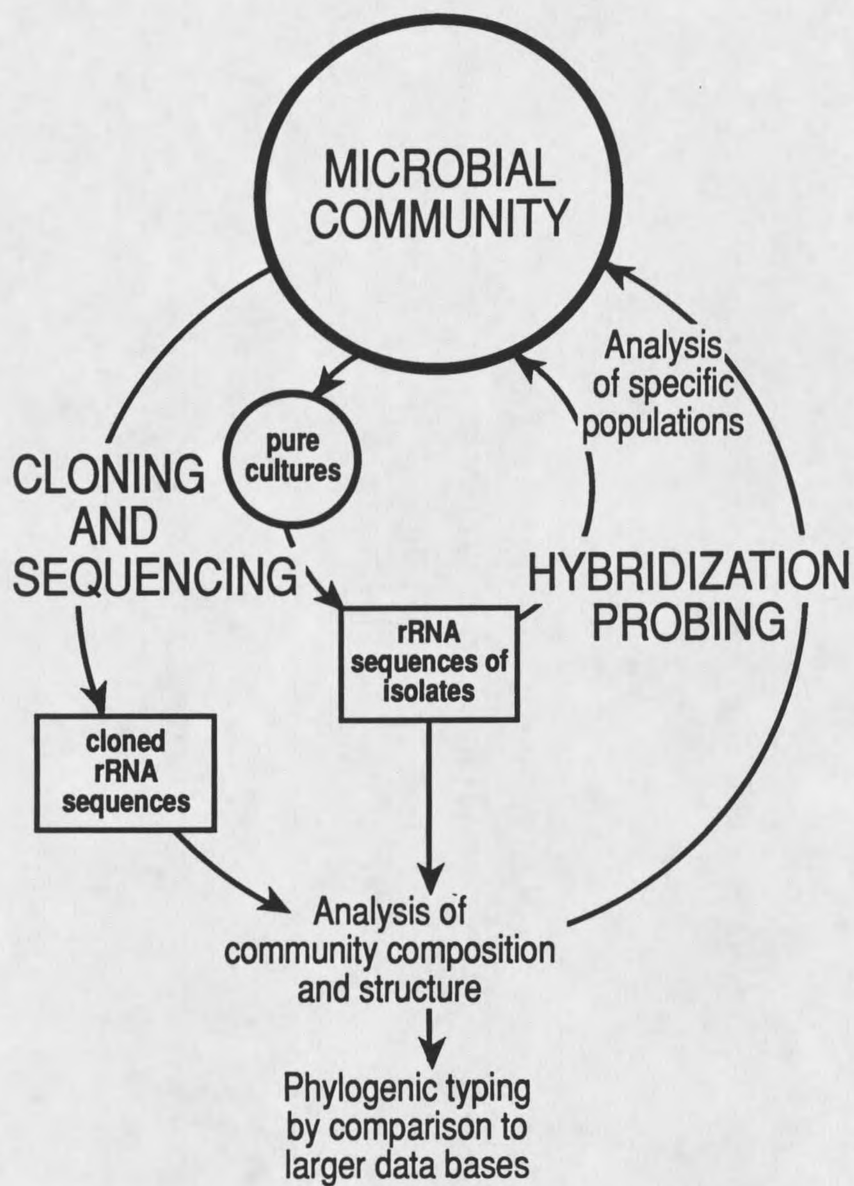


Figure 2. General approaches to the use of rRNA methods in microbial ecology (from Ward, *et al.*, 1992a).

phylogenetically characterized as a green nonsulfur (GNS) bacterium (Weller, et al., 1991; Weller, *et al.*, 1992). Other sequence types, characterized after this study began, belong to a second GNS bacterium, two spirochete-like bacteria, another cyanobacterium, and a proteobacterium (Weller, *et al.*, 1992; E.D. Kopczynski and M.M. Bateson, personal communication). The identification of so many previously uncharacterized cyanobacterial and GNS bacterial sequences challenges the assertion that *S. lividus* and *C. aurantiacus* are the dominant phototrophs in the Octopus Spring mat.

Others have also found that culture-independent, rRNA-based studies result in identification of SSU rRNA sequence types representing numerous uncultivated microorganisms. Giovannoni, *et al.* (1990), used a cloning technique based on the polymerase chain reaction to identify cyanobacterial SSU rRNA sequences in the surface waters of the Sargasso Sea. They found six unique sequence types, all of which could be established phylogenetically as cyanobacteria. As with the Octopus Spring study, none of the recovered sequences matched with sequences of any cultivated cyanobacteria thought to be dominant in this particular environment. DeLong, *et al.*, (1989a) and Schmidt, *et al.*, (1991), used yet another approach to evaluate a microbial community in the surface waters of the central Pacific Ocean (Aloha site). They extracted DNA from the sample, prepared a "shotgun" cloned library, screened the library with SSU rRNA to identify cloned SSU rRNA genes and identified 16 unique sequence types from 38 clones bearing SSU rRNA genes. Four of the types were identified phylogenetically as cyanobacteria, which were remarkably

similar to those recovered in the Sargasso Sea study (DeLong, *et al.*, 1989a; Giovannoni, *et al.*, 1990; Britschgi and Giovannoni, 1991). Eleven of the unique sequence types were identified as belonging to the proteobacterial phylum. Again, none of the sequences were identical to sequences of cultivated microorganisms.

Oligonucleotide probes specific for cultivated or uncultivated microorganisms have been used to investigate the distribution of particular microbial populations. For instance, group-specific and species-specific oligonucleotide probes were used by Stahl, *et al.*, (1988), to study the microbial ecology of certain rumen bacteria. The dynamics of the populations of cultivated microorganisms were followed using oligonucleotide probes -- the first ecological application of rRNA probe methods. This study showed that the cultivated organism thought to be dominant in the rumen (*Bacteroides succinogenes*) actually represented only 1-2% of the rumen microbial community (as measured by the abundance of its SSU rRNA relative to total SSU rRNA). Universal and group-specific probes have also been used to determine the vertical position of physiological groups of microorganisms within marine sediments (Devereux, *et al.*, 1990). Sulfate-reducing bacteria form a phylogenetically coherent group, therefore group-specific, fluorescently labelled oligonucleotide probes have also been used as intracellular stains to identify sulfate-reducers (Devereaux, *et al.*, 1991). Ribosomal RNA-based oligonucleotide probes have also been used to identify and study the distributions of bacterial symbionts in marine invertebrates and in plants (Distel, *et al.*, 1991; Pelletier, *et al.*, 1991; Hahn, *et al.*, 1989, 1990a, 1990b).

It is obvious from both cloning and sequencing and probe studies of rRNAs that the organisms which have been cultivated from the natural environment are not necessarily the organisms most relevant within the environment. The more specific rRNA methods allow an evaluation of the microbial community, which may include both uncultivated and cultivated organisms. In the case of the Octopus Spring microbial mat community, microscopic observation and culture work led to the conclusion that *S. lividus* and *C. aurantiacus* were the dominant phototrophs. However, molecular cloning of SSU rRNA from the mat revealed four cyanobacterial-type sequences and one GNS-type sequence, all from previously uncultivated microorganisms. This project was designed to apply species-specific oligonucleotide probing of SSU rRNA in natural mat samples (Figure 2, right side) to identify the presence and relevance of cultivated and uncultivated microorganisms in Octopus Spring and other hot spring microbial mats. To that end, experimental goals were established that would not only exhibit the effectiveness of culture-independent, species-specific oligonucleotide probe methods for community analysis, but would also result in ecologically interesting data regarding distributions of phototrophic community members.

Summary of Experimental Goals

This project involved analysis of a SSU rRNA sequence database to identify appropriate target regions for designing oligonucleotide probes, development of lysis procedures that enable the unbiased recovery of SSU rRNAs from members of

microbial mat communities, and the development of hybridization protocols that resulted in unambiguous detection of the SSU rRNAs of targeted species. The methods devised were applied to answer the following questions about uncultivated and cultivated phototrophs in hot spring microbial mat communities:

> 1. Are the uncultivated phototrophs in the 50-55°C Octopus Spring microbial mat, whose SSU rRNA sequences were recovered by cloning (types A, B, I, J, and C), more abundant than the cultivated organisms thought by morphological examination to be dominant (*S. lividus*, *C. aurantiacus*)?
>
> 2. What are the distributions of SSU rRNAs of these seven cultivated and uncultivated microorganisms among hot springs mats of different pH and/or temperature?
>
> 3. What is the vertical distribution of SSU rRNAs of these organisms in the Octopus Spring microbial mat?
>
> 4. What is the response of these phototrophs to environmental perturbation of light, pH, or temperature?
>
> 5. Does enrichment culture favor the cultivation of a less-dominant cyanobacterium which thrives under laboratory culture conditions? Does enrichment culture bias against the recovery of dominant cyanobacteria?

METHODS

Organisms and Growth Conditions

An axenic culture of *Synechococcus lividus* Y-7c-S, a cyanobacterium isolated from a pH 5.5 pool in Clearwater Springs, YNP (Kallas and Castenholz, 1982a), was obtained from Dr. Richard Castenholz, (University of Oregon, Eugene, OR). Stock cultures were maintained at 54°C in unbuffered liquid Medium D, pH 8.2, (Castenholz and Pierson, 1981) in an incubator illuminated with cool-white fluorescent bulbs (21 $\mu Em^{-2}s^{-1}$; all light intensities were measured with a Li-Cor, Inc. quantum photometer model LI185B equipped with a LI190SB quantum sensor). For RNA isolation, 800 ml cultures were grown in aerated flasks to late log phase as previously described (Bateson and Ward, 1988). A frozen pellet of *Chloroflexus aurantiacus* Y-400-fl was obtained from Dr. Michael Madigan (Southern Illinois University, Carbondale, IL). *Escherichia coli* Q358 was obtained from Dr. Norman Pace (Indiana University, Bloomington, IN), stored in 30% glycerol/50 μM $MgSO_4$/12.5 μM Tris-HCl (pH 8) at -70°C, and grown at 37°C in Luria-Bertani broth (Sambrook, *et al.*, 1989).

Collection and Processing of Microbial Mat Samples

Samples were obtained from hot spring microbial mats in YNP. Table 2 lists the samples according to experiments described below, relevant environmental information and the method of collection. Most samples were collected using a #6 (11 mm i.d.) or #15 (22 mm i.d.) brass cork borer. For the vertical profile study, a 44 mm (i.d.) steel core sampling tube was used and the sample was dissected with a spatula. Some shallow mats were sampled by scraping with a spoon or by dissection with a spatula. Immediately after collection, samples were removed from the sampling device, placed in labelled 50 ml polystyrene tubes (Falcon) and frozen in liquid nitrogen. Frozen samples were transported to the laboratory on dry ice then stored at -70°C until removed for lysis.

Pigment Concentration

In vivo absorption spectra were measured from Octopus Spring vertical profile samples to determine chlorophyll a and bacteriochlorophyll a and c composition essentially as described by Trüper and Pfennig (1981). Each vertical profile sample (Table 2) was suspended in 10 ml STE buffer (100 mM NaCl/10 mM Tris-HCl, pH 7.6/1 mM EDTA; Sambrook, et al., 1989) and homogenized. Then, 200 μl of each homogenate was added to 9.8 ml of 55% sucrose (empirically determined to maximize clarity of cell suspension), mixed, and read against 55% sucrose between 350 and 900 nm on a Gilford 2600 absorption spectrophotometer (Gilford Instrument

Table 2. Description of samples used in this study.

Hot Springs Location	Sample	Temp (°C)	pH	Sample type[a]	Date collected
Octopus Spring[b] Temperature Distribution	Pool	48 - 51	8.7	1 #15, top	8/8/91
	Site 50	50	8.5 - 8.6	1 #15, top	9/19/91
	Site 55	55	8.3 - 8.4	1 #15, top	9/19/91
	Site 60	60 - 66	8.3	1 #15, top	9/19/91
	Site 65	63 - 67	8.3	1 #15, top	9/19/91
	Site 70	61 - 70	8.3 - 8.4	1 #15, top	9/19/91
Octopus Spring Vertical Distribution	0-1.0 mm	48 - 51	8.7	1 44, dissect	8/8/91
	1-2.5 mm	48 - 51	8.7	1 44, dissect	8/8/91
	2.5-3.5 mm	48 - 51	8.7	1 44, dissect	8/8/91
	3.5-5.0 mm	48 - 51	8.7	1 44, dissect	8/8/91
	5.0-7.0 mm	48 - 51	8.7	1 44, dissect	8/8/91
	7.0-10.0 mm	48 - 51	8.7	1 44, dissect	8/8/91
Octopus Spring Light Intensity Reduction	100% illum.	48 - 51	8.7	2 #15, top	8/16/91
	7% illum.	48 - 51	8.7	2 #15, top	8/16/91
	0% illum	48 - 51	8.7	2 #15, top	8/16/91
Octopus Spring Temperature Shift	50→50	50	8.5 - 8.6	1 #6, full	9/26/91
	50→65	50 → 60-67	8.6→8.3	1 #6, full	9/26/91
	65→65	60 - 67	8.3	1 #6, full	9/26/91
	65→50	60-67 → 50	8.3→8.6	1 #6, full	9/26/91
Octopus Spring Site 50 Enrichments	"undiluted" 10^{-6}-diluted	50	8.5 - 8.6	2 #15, top	8/8/91
Clearwater Springs[c] Distribution	Site A	54	6.2	1 #15, top	7/31/91
	Site B	45 - 51	5.4	scrape, top	7/31/91
	Site C	65	6.7	scrape, top	7/31/91
	Site D	64	7.8	2 #15, top	7/31/91
	Site E	50	5.0	scrape, floating	7/31/91
Clearwater Springs pH/Temperature Shift	A→A	54	6.2	1 #6, full	8/8/91
	A→D	54 → 64	6.2→7.8	1 #6, full	8/8/91
	D→D	64	7.8	1 #6, full	8/8/91
	D→A	64 → 54	7.8→6.2	1 #6, full	8/8/91
Clearwater Springs Site A Enrichments	"undiluted" 10^{-6}-diluted	54	6.2	2 #15, top	7/31/91
Mushroom Spring[d]	MS	54	8.3	2 #15, top	8/8/91
Twin Butte Vista Spring	TBV	62	9.1	2 #15, top	8/8/91
Nymph Creek	NC	47	2.8	scrape, top	7/31/91
Roland's Well	RW	54	6.2	dissect, top	7/31/91
New Pit	NP	56	6.3	dissect, top	7/31/91

[a] Sample types:

"1" or "2" indicate the number of core samples placed in each replicate tube

"#6", "#15", or "44" indicate the device used to take core samples (see text)

"top" indicates that only the top 5mm of the mat was sampled

"dissect" indicates a sample that was sectioned out of the mat or from a core sample using a spatula

"full" indicates that the sample consisted of the full depth of the mat down to the sediment

"scrape" indicates a sample that was taken by scraping with a spoon because of insufficient biomass for use of cork borers

"floating" indicates that the sample was taken from biomass floating on the surface of the pool

[b] Octopus Spring sampling sites are diagrammed in Appendix, Figure 14.

[c] A map of Clearwater Springs and sampling sites at Clearwater Springs is in Appendix, Figure 15.

[d] Locations of other hot springs are given in Brock, 1978, and Ward, *et al.*, 1989b.

Laboratories, Inc., Oberlin, Ohio). Relative concentrations of pigments were determined by dividing the height (mm) of the absorption maxima peak relevant to each pigment by the height of the greatest absorption maxima peak obtained from all vertical profile samples (Barer, 1955; Doemel and Brock, 1977; Trüper and Pfennig, 1981).

In Situ Manipulations of Microbial Mat Samples

Temperature Shift

Two sets of triplicate samples were removed from each of two temperature regions in the shoulder area of Octopus Spring, Site 50 (50°C) and Site 65 (63 - 67°C), and placed in uncapped 2 dram vials (Kimble) so the sample would be exposed to the spring water at its respective incubation site. Half of the vials from each temperature site were immediately returned to the site from which they were removed (50→50, 65→65) as *in situ* controls. The remaining Site 50 vials were placed at Site 65 (50→65) and the remaining Site 65 vials were placed at Site 50 (65→50). Samples were incubated *in situ* for one week, collected, frozen, and stored as above.

pH/Temperature Shift

Two sets of triplicate samples were removed from both Clearwater Springs Site A (pH 6.2, 54°C) and Clearwater Springs Site D (pH 7.8, 64°C) and each core was placed in an uncapped 2 dram glass vial so it would be exposed to the spring water at

its respective incubation site. Half of the Site A samples were placed in Site D (A→D); the remaining Site A samples were immediately returned to Site A (A→A) as *in situ* controls. Half of the Site D samples were placed in Site A (D→A); the remaining Site D samples were immediately returned to Site D (D→D) as *in situ* controls. Samples were incubated *in situ* for one week, then collected and treated as above.

Light Intensity Reduction

At time zero, triplicate mat samples were removed from the center of a small 48-51°C pool located in the southernmost effluent channel of Octopus Spring and referred to as "Pool" (Table 2). Then a wooden frame containing neutral density filters (after Madigan and Brock, 1977) was placed over a portion of the pool. The incident light intensity at the time the filters were installed (1630 h.) was 1700 $\mu Em^{-2}s^{-1}$ with no light reduction, 130 $\mu Em^{-2}s^{-1}$ (93% reduced intensity) under a muslin filter, and 0 $\mu Em^{-2}s^{-1}$ (100% reduced intensity) under a black plastic filter. At 1200 h. the incident light was 2150 $\mu Em^{-2}s^{-1}$ and light was reduced by the same percentages under the neutral density frame. The frame was left in place one week, removed, and samples were collected and treated immediately as above.

Enrichment Cultures from Microbial Mat Samples

The top 5 mm of two #15 cores from the mat at Clearwater Springs Site A were placed in a 50 ml polystyrene tube, volume was brought to 15 ml with 54°C

water from Site A, and the sample was transported to the laboratory in a thermos filled with 54°C spring water. At the laboratory the sample was homogenized in a 40 ml Dounce tissue grinder (Corning). A 5 ml portion of the homogenate was used to inoculate a tube containing 5 ml of either Medium D, pH 6.5, or Medium D, pH 8.2. A ten-fold dilution series was prepared from these primary tubes (termed "undiluted") in tubes containing the same type of medium. All tubes were incubated at 54°C in an incubator illuminated with cool-white fluorescent bulbs (21 $\mu Em^{-2}s^{-1}$). The "undiluted" and 10^{-6}-diluted tubes (the highest dilution to show growth after two months incubation) were maintained by subculturing 10% of the total volume into tubes with 5 ml Medium D of the same pH. Eight to ten replicate subcultures of each enrichment were pooled to obtain sufficient biomass for RNA extraction. Enrichments from Octopus Spring Site 50 were processed as for Clearwater Springs Site A enrichments, except that only medium of pH 8.2 was used.

Preparation of Target Nucleic Acids

Lysis Methods

French Pressure (FP) Cell. Frozen mat samples were removed from the 50 ml Falcon tubes by striking each tube against a hard surface to detach the frozen sample from the walls of the tube. The frozen pellet was transferred immediately to a 15 ml Dounce tissue grinder (Corning), 3.25 ml lysis buffer (80 mM NaCl/8 mM Tris-HCl, pH 7.6/0.8 mM EDTA/50 mg lysozyme; Sigma Chemical Company) was added and the sample was thawed quickly at 50°C. After the sample was thoroughly

homogenized, it was transferred to the chamber of a FP Mini-Cell (SLM Aminco) that was previously rinsed with 10% H_2O_2 to remove RNase. The sample was passed through the FP cell three times at 20,000 psi; each time the sample was collected in a chilled 30 ml Corex tube (Corning). After the final passage, the tissue grinder was rinsed with 0.5 ml STE which was also French pressed. Proteinase digest buffer (1 ml; 2.5 M NaCl/5% sodium dodecyl sulfate) was added and the samples were digested with 2 mg proteinase K (Sigma Chemical Company) for 1 hour at 50°C before RNA purification as described below.

Other Lysis Methods. For enzymatic lysis of cells (ENZ), frozen samples were removed from Falcon tubes, thawed at 50°C in 2.25 ml lysis buffer, then frozen in a dry ice/ethanol slurry. Each sample was put through three freeze-thaw cycles, then 0.5 ml proteinase digest buffer and 1 mg proteinase K were added, the sample was digested at 50°C for 1 hour, and RNA was purified as described below.

Several samples were lysed mechanically in the 30 ml chamber of a Bead-Beater (referred to as BB; Biospec Products, Bartlesville, OK). These samples were thawed and resuspended in 6 ml lysis buffer, then combined with 6 ml STE-saturated phenol and 15 ml sterile 0.1 mm glass beads in the BB chamber, which had been previously treated to remove RNases by soaking 15 minutes in 10% H_2O_2. Each sample was homogenized for 2 minutes in the BB. The temperature of the sample was kept low by an ice water-filled outer chamber surrounding the sample chamber. After lysis, the sample was transferred to a chilled 30 ml Corex tube and the RNA was

purified as described below, except that the first phenol extraction also removed the glass beads from the sample.

Single step lysis and purification by acid guanidinium thiocyanate-phenol-chloroform extraction (GITC) was performed on several samples essentially as described by Chomczynski and Sacchi (1987).

Combined Lysis Methods. Two lysis methods involved the combination of FP and different GITC solutions. For the first set of samples (lysis method GITC-FP-1), 1 ml guanidinium solution D (4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarcosyl/0.1 M 2-mercaptoethanol; Chomczynski and Sacchi, 1987), 1 ml H_2O-saturated phenol, 100 μl 2M sodium acetate (pH 4.0), and 200 μl chloroform:isoamyl alcohol (24:1) were added to the frozen cell pellet in a 7 ml Dounce tissue grinder. Cells were thawed in this solution at 50°C, homogenized, passed three times through the FP Mini-Cell at 20,000 psi, and the nucleic acids were precipitated as previously described (Chomczynski and Sacchi, 1987). The second set of samples (lysis method GITC-FP-2) were thawed in a 7 ml Dounce tissue grinder with 1 ml guanidinium solution D then passed three times through the FP Mini-Cell at 20,000 psi. Next, sodium acetate, phenol, and chloroform were added sequentially, in the amounts designated above, with mixing after each addition, the tube was incubated on ice for 15 minutes, and the nucleic acids were precipitated as above.

Purification of RNA

Nucleic acids were purified by one extraction of the lysed sample with one volume of STE-saturated phenol, one extraction with one-half volume STE-saturated phenol and one-half volume chloroform:isoamyl alcohol (24:1), one extraction with one volume chloroform:isoamyl alcohol, and an overnight ethanol precipitation, according to standard protocols (Sambrook, et al., 1989). DNA was removed from samples using RQ1 DNase according to the protocol supplied by the manufacturer (Promega).

Growth of Transformed Cells and Purification of Plasmid DNA

Transformed *E. coli* Q358 cells containing cloned SSU rcDNA suspected to be from uncultivated phototrophs of the Octopus Spring microbial mat (collected from 50-55° sites in the shoulder region; Ward, *et al.*, 1990a; Weller, *et al.*, 1992) and *E. coli* Q358 cells for RNA extraction were obtained from Roland Weller and David Ward (Montana State University, Bozeman, MT) as frozen glycerol stocks. Clones used for rcDNA extraction were: OS III-3 (type A), OS II-18 (type B), OS VL-16 (type I), OS VL-13 (type J), and OS II-23, III-15, and VI-L11 (type C). Ten ml Luria-Bertani broth containing either 125 μg tetracycline (pBR322 cloning vector) or 500 μg ampicillin (pGEM cloning vector) was inoculated with 100 μl from a glycerol stock of the selected transformant and incubated 6-8 hours at 37°C in a shaking incubator at 180 rpm. This culture was used to inoculate 200 ml of the same medium which was incubated overnight under the same conditions. Cultures were centrifuged

for 20 minutes (10,000 rpm, 4°C, Sorvall RC5B centrifuge, GSA rotor), the supernatant was discarded, and the pellet was resuspended in 5 ml LB broth. Cells were lysed and plasmid DNA was extracted and purified with a Qiagen <Plasmid> Kit following instructions provided by the manufacturer (The Qiagenologist, 3rd Ed, Qiagen, Inc., Studio City, CA).

Quantification of Nucleic Acids

DNA and RNA were quantified by absorption spectroscopy as described by Sambrook, et al. (1989). The quality of RNA was determined by formaldehyde gel electrophoresis using a Hoefer HE 33 Horizontal Submarine unit with an EC 600 power supply (E-C Apparatus Corp., St. Petersburg, FL) as previously described (Sambrook, et al., 1989).

Preparation of Probes

Probe Design

A SSU rRNA sequence database was assembled, using software obtained from Dr. Gary Olsen (University of Illinois), which included sequences from cultivated Octopus Spring mat community members (Table 1), uncultivated mat community members investigated in this study, and representatives from all eubacterial phyla (Woese, 1987). The V9 region (see Figure 1), as defined by Gray, *et al.* (1984), was evaluated as a possible target because in this region complete sequence data for all organisms analyzed were available and there were few ambiguous bases. Target regions for oligodeoxynucleotide probes were selected to maximize the number of

mismatches between the most phylogenetically similar sequences. Probe sequences, melting temperatures (T_m), and hybridization temperatures (T_H) are given in Table 3. The number of mismatches between cyanobacterial probes and targets ranged from one (first generation A-type with B-type) to nine (A-type with I-type). The A-type and B-type probes designed to improve specificity between these two closely related sequence types had four mismatches with B- and A-type rcDNA respectively. There were six mismatches between the C-type and *C. aurantiacus* probes and targets. A universal probe targeting a conserved region (labelled U in Figure 1) was designed to complement eubacterial and archaebacterial SSU rRNA (Table 3).

Table 3. Sequences, T_m, and T_H values of oligodeoxynucleotide probes used in this study.

Target Organism	Region[1]	Probe sequence[2]	T_m[3]	T_H[4]
I. Cyanobacteria				
A-type	1254-1275	5'TCCCGCTCGCGCGTTCGCTGCC	78°C	68°C
B-type	1254-1275	5'TCACGCTCGCGCGCTCGCGACC	78°C	68°C
First generation A	1273-1294	5'CGCRGTTTTTGGGATTGGCTCC	69°C	58°C
I-type	1254-1275	5'TTCGTCTCGCGAGCTTGCAACC	70°C	58°C
J-type	1240-1281	5'ATTATCCCTCTGTCCACGCCA	65°C	54°C
S. lividus Y-7c-S	1273-1294	5'CGTGGTTTAAGAGATTAGCTCG	64°C	54°C
II. "Green nonsulfur bacteria"				
C-type	1273-1294	5'SCCCSTTT-GGCGATTRGCATC	67°C	58°C
C. aurantiacus Y400-fl	1273-1294	5'CCACGTTTAGGCGATTAGTTCC	66°C	58°C
III. Universal probe				
Universal	519-536	5'GTATTACCGCGGCKGCTG	59°C	47°C

[1]Target regions correspond to *E. coli* position numbers (Gutell, et al., 1985).
[2]Sequences are written with IUPAC abbreviations.
[3]T_m (melting temperature) = 2°C (A+T) + 4°C (G+C); (Meinkoth and Wahl, 1984).
[4]T_H (hybridization temperature) was determined empirically. See text for hybridization conditions.

Synthesis and Radiolabelling

Oligonucleotide probes complementing the target regions were synthesized by the Veterinary Molecular Biology Laboratory (Montana State University, Bozeman, MT) or by American Synthesis, Inc. (Pleasanton, CA). Probes (275-340 pmol) were labelled by 5′ kination using 80 units T4 polynucleotide kinase (Promega Corp., Madison, WI), 200 pmol 5′-[γ-^{32}P]-adenosine triphosphate (New England Nuclear, Boston, MA), and 1X T4 polynucleotide kinase buffer (Promega) in a final volume of 140 μl. The reaction was incubated for 2 hours at 37°C, then 40 more units of T4 polynucleotide kinase were added, and the reaction continued at 37°C for 1 hour. The reaction was stopped by heating for 10 minutes at 75°C. Urea was added to a final concentration of ≈7M, and the reaction mixture was stored at -20°C until purification.

Purification

Radiolabelled probes were purified by electrophoresis on a 19% acrylamide/4.75% bisacrylamide/0.5M urea denaturing polyacrylamide gel run for 12-14 hours at 100V in 1X TBE buffer (90 mM Tris, pH 8.0/90 mM boric acid/2.5 mM EDTA) according to standard protocols (Sambrook, et al., 1989). A dye solution (0.05% bromphenol blue) and a solution containing 15-25 μg of unlabelled probe were electrophoresed as markers. Reaction mixtures and marker solutions were heated 40 seconds at 90°C before loading. Following electrophoresis, the gel was removed from the gel unit, wrapped in plastic wrap, and the marker probe was visualized by short-wave ultraviolet shadowing (UVS-11, Ultra-Violet Products, Inc., San Gabriel, CA)

against an intensifying screen (Cronex Lightning Plus, E.I. DuPont DeNemours & Co., Wilmington, DE). The locations of radiolabelled probes in the gel were determined by an autoradiograph exposed for 2 seconds (see next section), by fluorescence of the intensifying screen in response to the presence of ^{32}P, and by the correspondence between the position of the marker probe and the radiolabelled probe. The band corresponding to radiolabelled probe was excised from the gel with a flame-sterilized razor blade, placed in a 15 ml Corex tube, and macerated with a flame-sterilized spatula. Probe was eluted from gel particles in five successive changes of 1 ml dH_2O, over a period of 16 hours. All five eluates were shaken constantly at 300 rpm in a shaking water bath at room temperature to promote efficient elution. Gel particles were removed from the pooled eluates by filtration through two 9 mm filters (#591, Schleicher and Scheull) and the purified probe was stored at -20°C.

Quantification

The radioactivity (cpm) of eluted probes was determined by liquid scintillation counting (Tri-Carb 460 CD, Packard Instrument Co., Meriden, CT) in Aquasol-2 (New England Nuclear Research Products). Based on unpublished results from this lab (J.G. Kuenen, A.L. Ruff-Roberts, and D.M. Ward) involving purification, elution, and recovery of labelled and unlabelled probe at various concentrations, virtually all of the probe was labelled and 60-75% was recovered after gel purification.

Hybridizations

Denaturation of Target

RNA targets (0.5 pmol) were denatured by incubation for 15 minutes at 65°C in 100 μl 5X SSPE (1X SSPE is 0.18 M NaCl/10 mM NaH_2PO_4/1 mM EDTA; Sambrook, et al., 1989) containing 7.5% formaldehyde. DNA targets (0.8 pmol) were denatured by incubation for 1 hour at 65°C in 1 M NaOH. After denaturation, all targets were diluted to 0.1 pmol/200 μl in 5X SSPE.

Preparation of Filters

Slot blots were prepared using a Minifold II (Schleicher and Schuell, Keene, NH) essentially as described by the manufacturer. One piece of 0.1 μm Nytran (Schleicher and Schuell) and two pieces of GB002 gel blot paper (Schleicher and Schuell) were wetted with distilled water, soaked for 30 minutes in 5X SSPE, and applied to the manifold. Vacuum was supplied by a faucet aspirator. Wells were rinsed with 600 μl 5X SSPE, 200 μl sample containing 0.1 pmol target (unless otherwise specified) was applied, and finally wells were rinsed with 400 μl 5X SSPE. The filter was baked for 30 minutes at 80°C then nucleic acids were UV crosslinked to the filter (Nierezwicki-Bauer, et al., 1990) for 10 minutes under a germicidal hood (SterilGARD Hood, The Baker Company, Inc., Sanford, Maine).

Hybridization

Unless otherwise specified, filters were prehybridized for 2 hours at T_H (Table 3) within sealed plastic bags (Seal-a-Meal) containing 250 μl hybridization buffer [5X SSPE, 0.5 mg/ml polyadenylic acid (P9403, Sigma), 0.1 mg/ml tRNA (R8759, Sigma), 10X Denhardt's solution (Sambrook, et al, 1989), 0.1% sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, CA)] per cm^2 of filter. After prehybridization, the filters were hybridized at T_H for 10-12 hours within sealed plastic bags containing 100 $\mu l/cm^2$ fresh hybridization buffer and approximately 30-40 pmol of labelled probe. After hybridization, filters were washed three times for 10 minutes at T_H in 2.5X SSPE/0.1% SDS and dried at room temperature.

Autoradiographs

Kodak X-OMAT AR film was directly exposed on filters for up to 120 hours. Each filter was exposed for an empirically determined time period that resulted in film images between 0.1 and 1.0 A_{540} units (Sambrook, et al., 1989) to ensure film linearity.

Analysis of Results

Mat samples were obtained, hybridized, and analyzed in triplicate except for the enrichment studies where pooling of low biomass samples precluded replication. The degree of reaction of probes with various targets was quantified by scanning autoradiographs with a GS-300 scanning densitometer (Hoefer Scientific Instruments, San Francisco) and the peaks were integrated using the Hoefer GS-350 Data System

software supplied with the instrument. Specific probe reactions (in integration units, IU) were normalized by dividing by the universal probe reaction value (also in IU) determined from replicate samples. This provided a concentration-independent measure of relative probe reaction. Yield data, from universal probe reactivity (IU) were calculated using the formula:

$$\frac{\textit{universal probe response (IU)}}{\textit{percent of sample on filter}} = \textit{yield (IU)}$$

Yield data determined by absorption spectroscopy were calculated using the formula:

$$\frac{\textit{concentration } (\mu g/\mu l)}{\mu l \textit{ of sample}} = \textit{yield } (\mu g)$$

Differences in means were tested for significance by a paired student's t-test (Sigmaplot, Jandel Scientific).

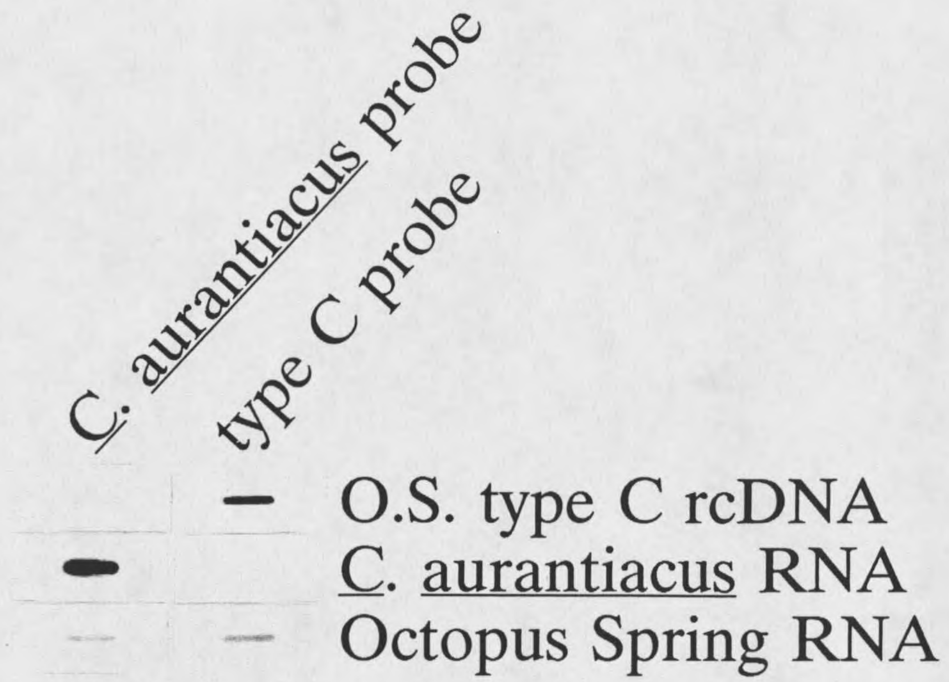
RESULTS

Probe Reactivity

Probe Specificity

The first generation A-type probe, the C-type probe, and probes for *S. lividus* and *C. aurantiacus* were tested for cross-reaction with non-homologous targets (Figure 3). Initially 6X SSPE was used in the hybridization solution and 5X SSPE was used in the wash solution. The first generation A-type, *C. aurantiacus*, and C-type probes exhibited cross-reactions with non-homologous targets under these preliminary hybridization conditions. When the salt concentrations were decreased to 5X SSPE and 2.5X SSPE respectively, cross-reaction of the *C. aurantiacus* and C-type probes was eliminated (Figure 3A). However, it was never possible to completely eliminate all cross-reaction of the first generation A-type probe with B-type rcDNA (Figure 3B). When the hybridization stringency was increased by further lowering salt concentrations to 3X SSPE the non-homologous reaction of the first generation A-type probe with the B-type target was decreased, but the strength of the homologous reaction also decreased. Design of more specific A-type and B-type probes, and of probes targeting the SSU rRNA of two other cyanobacterial SSU rRNA sequence types, I-type and J-type, was based on improved sequence data provided by

A.



B.

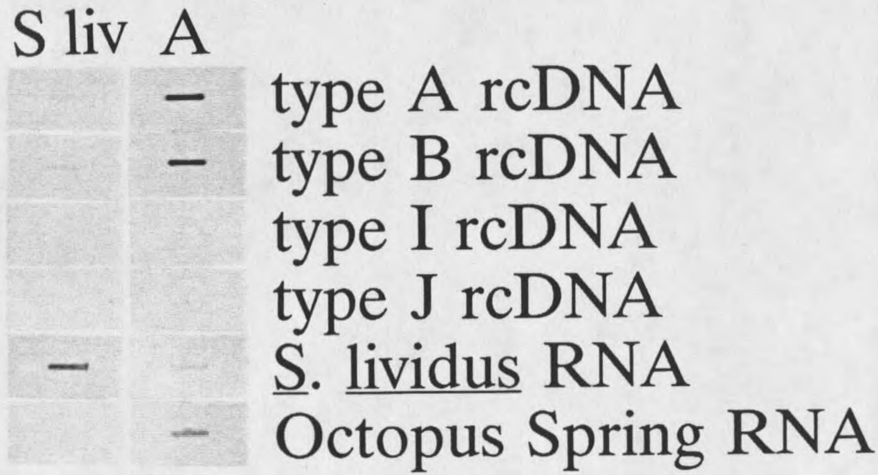


Figure 3. Oligodeoxynucleotide probe specificity of first generation probes used in this study. A. *Chloroflexus aurantiacus* and C-type probes. B. *Synechococcus lividus* and A-type probes. rcDNA = cloned SSU rcDNA.

Dr. J.G. Kuenen (Delft University of Technology, The Netherlands) and Mary Bateson (Montana State University) and was performed in collaboration with Dr. Kuenen. Preliminary experiments led to determination of conditions under which each of these probes hybridized only to its homologous target (Figure 4). The universal probe reacted with all target molecules except the type A and type B SSU rcDNA clones which did not contain that target region.

Quantification of Probe Reaction

The linearity of probe response was evaluated using the universal probe with *C. aurantiacus* RNA and C-type SSU rcDNA as targets. The probe response was linear between 0.0 and 1.0 pmol target (Figure 5). Universal probe response with these targets was not linear between 1.0 and 15.0 pmol, possibly due to film saturation or saturation of the target with probe. The universal probe responses with the same picomolar amounts (as determined by absorption spectroscopy) of different targets were slightly different, despite several repeat experiments with other targets (Octopus Spring RNA, *E. coli* SSU rRNA, *E. coli* SSU rcDNA). This result is attributed to the inaccuracy of absorption spectroscopy in the determination of absolute concentration, since the probe reaction with different concentrations of the same target was always linear. Because I could not obtain SSU rRNA of uncultivated species and could not be assured that SSU rcDNA and the corresponding SSU rRNA gave identical probe responses, I was unable to determine an absolute probe response. Therefore, specific probe reactions were normalized to universal probe responses and reported as "relative probe responses", a concentration-independent measure of reaction.

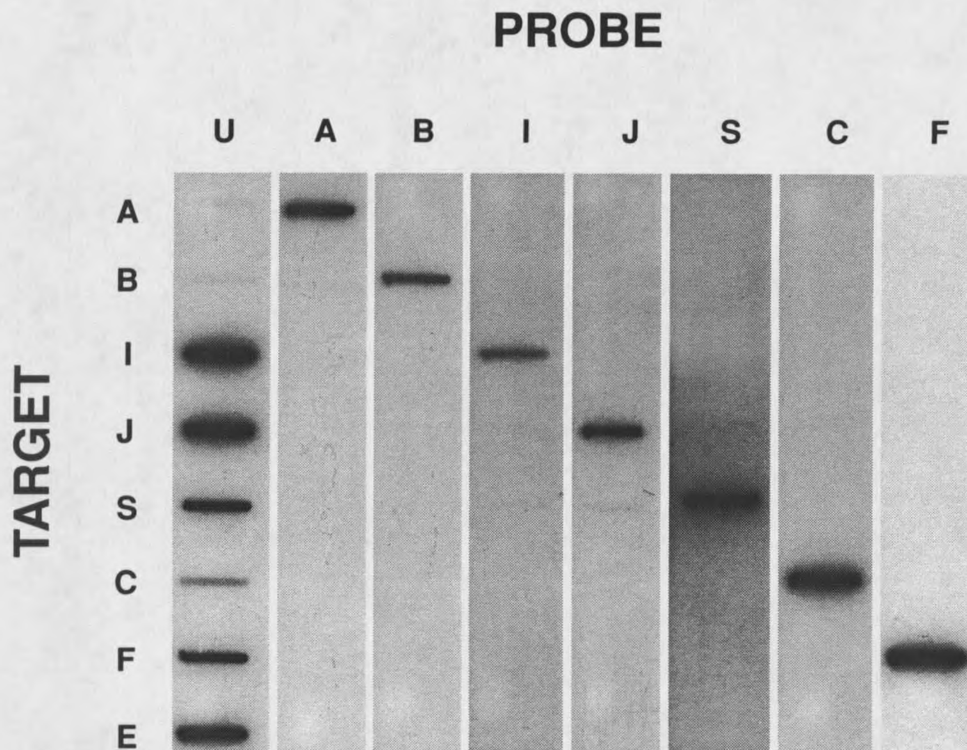


Figure 4. Specificity of oligodeoxynucleotide probes used in this study. A, B, I, J, C = cloned SSU rcDNA of Octopus Spring sequence types or probes targeting those sequence types. S = RNA or probe targeting SSU rRNA of *Synechococcus lividus* Y-7c-s. F = RNA or probe targeting SSU rRNA of *Chloroflexus aurantiacus* Y-400-fl. E = *Escherichia coli* RNA. U = universal probe. Universal probe does not react with A-type or B-type targets because these cloned SSU rcDNAs do not contain the universal probe target region (see Ward, *et al.*, 1990a).

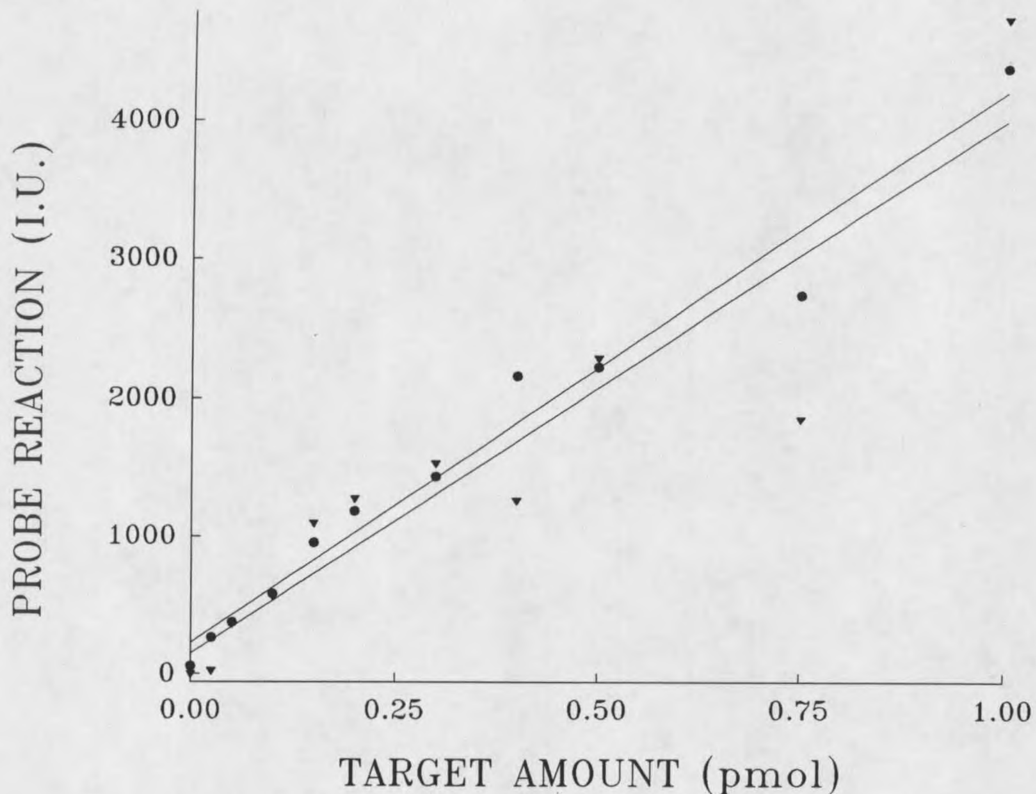


Figure 5. Dependence of universal probe reactivity on target *Chloroflexus aurantiacus* RNA (●) or type C SSU rcDNA (▾) concentration. Linearity of probe reactivity is indicated by regression lines through data. R=0.98 for RNA. R=0.96 for SSU rcDNA.

Effects of Lysis Methods on RNA Yield, Quality and Probe Response

RNA Yields

In order to evaluate whether the lysis method affected RNA yield or biased against recovery of SSU rRNA from particular organisms, Octopus Spring Site 50 mat samples, with or without *C. aurantiacus* and *S. lividus* cells added, and pure samples of *C. aurantiacus* or *S. lividus* cells were lysed (Figure 6). SSU rRNA yield was

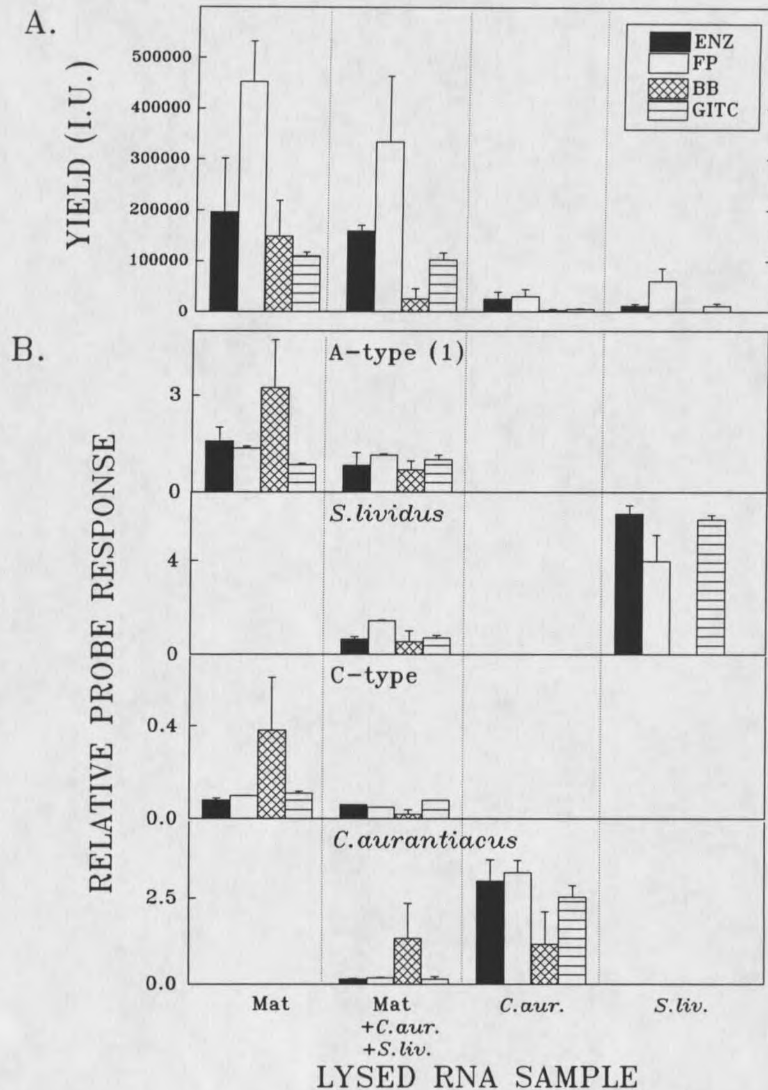


Figure 6. Lysis experiments I. Mat = RNA extracted from Octopus Spring Site 50 (50°C, pH 8.5-8.6). Mat/*C.aur.*/*S.liv.* = RNA extracted from a homogenized mixture of mat cells from Octopus Spring Site 50, a pure culture of *Chloroflexus aurantiacus* Y-400-fl, and a pure culture of *Synechococcus lividus* Y-7c-s. *C.aur.* = RNA extracted from a pure culture of *C. aurantiacus* Y-400-fl. *S.liv.* = RNA extracted from a pure culture of *S. lividus* Y-7c-s. A. Yields of SSU rRNA obtained by lysing different samples by either enzymatic (ENZ), French Press (FP), Bead-Beater (BB), or guanidine thiocyanate (GITC). Yields were determined from universal probe responses. B. Relative response of oligodeoxynucleotide probes with RNA extracted from different mixtures of mat and/or organisms. A-type (1) = first generation A-type probe. C-type = C-type probe. Bars indicate standard error.

quantified using the universal probe. The effectiveness of ENZ, FP, BB, and GITC lysis methods were compared (Figure 6A). The total RNA yield was highest when samples were lysed by FP. Samples of *C. aurantiacus*, but not *S. lividus* cells and mat samples were as effectively lysed (ie., had statistically similar yields) using ENZ. Yields from BB-lysed samples were often particularly low; no RNA was recovered from the *S. lividus* samples. Lysis with GITC resulted in relatively low yields, but resulted in the recovery of high quality rRNA (see next section). Lysis with the combinations of FP and GITC (GITC-FP-1 and GITC-FP-2) was attempted to determine whether high yields of good quality rRNA could be achieved. Although GITC-FP-1 resulted in slightly decreased yields compared to FP (data not shown) GITC-FP-2 resulted in much lower yields (Figure 7A).

Yields extrapolated from universal probe responses with 0.1 pmol of each target and from absorption spectroscopy of portions of the RNA obtained in the lysis experiments were linearly correlated (R=0.96) in the range between $\approx$30 and 400 μg RNA. Linear correlation was also found for all samples used to make quantitative comparisons of relative probe responses (Octopus Spring sites, Clearwater Springs Site A and D). In other cases (Nymph Creek, Clearwater Springs Site E, Mushroom Spring, and Twin Butte Vista Spring) linear correlation was not found and no quantitative comparisons were attempted.

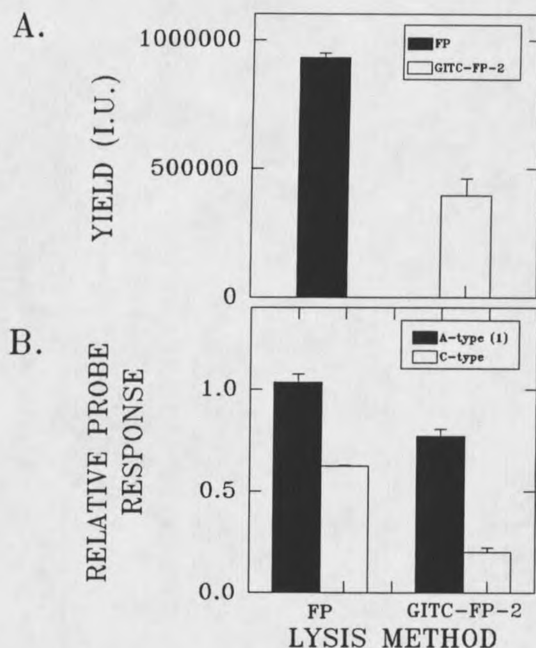


Figure 7. Lysis experiments II. A. Yields of SSU rRNA obtained by lysing samples from Octopus Spring Site 50 (50°C, pH 8.5-8.6) by either French Press (FP) or a combination of FP and guanidine thiocyanate extraction (GITC-FP-2). Yields were determined from universal probe responses. B. Relative response of oligodeoxynucleotide probes with RNA extracted by FP or GITC-FP-2. A-type (1) = first generation A-type probe. C-type = C-type probe. Bars indicate standard error.

Quality of Extracted RNA

The quality of the RNA extracted using different lysis methods was determined by agarose gel electrophoresis. Enzymatically lysed samples (ENZ) resulted in recovery of RNA exhibiting distinct 23S, 16S, and 5S bands. Samples lysed by BB showed no rRNA bands, even though low concentrations were detected by spectroscopy. This could indicate that absorbance was affected by the presence of

contaminating DNA or some other UV light-absorbing material. FP lysis in the presence of guanidinium (GITC-FP-1 and -2) resulted in recovery of RNA with a distinct 16S band. However, the samples were contaminated with material which was visible as fluorescence in the wells of the gel. FP lysis resulted in the recovery of a smear of RNA between 5S and 16S in size (≈ 120 - 1540 bases long).

Probe Response

The first generation A-type probe, the C-type, *S. lividus*, and *C. aurantiacus* probes were used to test the possibility of bias against recovery of specific SSU rRNAs in different lysis protocols. *S. lividus* and *C. aurantiacus* SSU rRNAs were not detected in the mat samples but were detected by nearly all lysis methods in each pure culture and after mixing cells of each type with the mat samples. There was no statistically significant difference ($p > 0.05$) in recovery of SSU rRNA of any of the targeted organisms for most methods. However, BB lysis failed to recover SSU rRNA from *S. lividus* (Figure 6B). The first generation A-type probe and the C-type probe hybridized with RNA extracted from all mat samples after lysis by all methods. There was a significant bias ($p < 0.05$) against recovery of both cyanobacterial and GNS-like RNA with the combined method GITC-FP-2 (Figure 7B), but the RNA recovery with the other combined method (GITC-FP-1) was statistically similar to FP ($p > 0.05$; data not shown). All environmental samples were subsequently lysed by FP, since it resulted in a large yield of relatively long RNA (compared to the probe target sites) and exhibited no apparent bias against cyanobacteria (A/B-type, *S. lividus*) or green nonsulfur bacteria (C-type, *C. aurantiacus*).

Distribution of SSU rRNAs Among Microbial Mats from Hot Springs of Different Temperatures and pH

RNA samples from microbial mats at several hot springs differing in temperature and pH were hybridized with the A-type, B-type, I-type, J-type, *S. lividus*, C-type, and *C. aurantiacus* probes in order to evaluate the range over which these organisms contribute their specific SSU rRNAs in nature (Table 4). Since the I-type probe did not hybridize with RNA from any sample, it was omitted from the table.

Only the universal probe reacted with RNA from biomass of the more acidic hot springs (pH $\leq$ 5.4; data not shown). This included Clearwater Springs Site B, which contains *Chlorogloeopsis*-like filamentous cyanobacteria (microscopic observations), Site E, which also contains *Chlorogloeopsis*-like cyanobacteria as well as short, straight, unicellular cyanobacteria, and the Nymph Creek mat, which is dominated by the alga *Cyanidium caldarium* and apparently lacks cyanobacteria.

Mats in more alkaline springs contained RNA which reacted with one or more of the cyanobacterial probes used in this study (Table 4). The distributions of these SSU rRNAs may indicate patterns of natural competitiveness of each cyanobacterium with respect to temperature and pH, with the effect of temperature more clearly exhibited in Octopus Spring where pH was more constant (Table 4, III). To summarize:

1.) SSU rRNA from the A-type organism was detected only in mats of hot springs above pH 6.7 and between 48°C and 70°C, with the strongest probe reactions occurring above 60°C. The A-type organism was the only cyanobacterium whose SSU rRNA was detected above 67°C.

Table 4. Relative probe responses with RNA extracted from mat samples taken from hot spring locations of different temperature and pH.

	RELATIVE PROBE RESPONSES[b,c]					
TARGET[a]	A	B	J	*S.liv.*	C	*C.aur.*
I. Acidic hot springs						
NC, 47°C, pH 2.8	---	---	---	---	---	---
CW E, 50°C, pH 5.0	---	---	---	---	---	---
CW B, 45-51°C, pH 5.4	---	---	---	---	---	---
CW A, 54°C, pH 6.2	---	0.55 ± .17	---	0.64 ± .10	---	---
CW C, 65°C, pH 6.7	4.73 ± .29	---	---	---	---	---
II. Alkaline hot springs						
MS, 54°C, pH 8.3	---	2.52 ± .20	---	---	2.43 ± .20	---
TBV, 62°C, pH 9.1	---	1.54 ± .17	0.55 ± .09	---	1.22 ± .21	---
CW D, 64°C, pH 7.8	1.70 ± .15	1.86 ± .25	---	---	---	---
III. Temperature distribution at Octopus Spring						
OS 50, 50°C, pH 8.5-8.6	---	0.50 ± .02	0.64 ± .05	---	0.10 ± .01	---
OS Pool, 48-51°C, pH 8.7	0.16 ± .02	0.68 ± .03	1.73 ± .08	---	0.37 ± .21	---
OS 55, 55°C, pH 8.3-8.4	---	0.71 ± .04	---	---	0.53 ± .13	---
OS 60, 60-66°C, pH 8.3	1.27 ± .22	0.12 ± .06	---	---	1.83 ± .08	---
OS 65, 63-67°C, pH 8.3	0.18 ± .06	0.40 ± .02	---	---	1.43 ± .16	---
OS 70, 61-70°C, pH 8.3-8.4	2.64 ± .17	---	---	---	1.49 ± .05	1.30 ± .15

[a] NC = Nymph Creek; CW A, B, C, D, E = Clearwater Springs sites; MS = Mushroom Spring; TBV = Twin Butte Vista Spring; OS Pool, 50, 55, 60, 65, 70 = Octopus Spring temperature sites

[b] ± standard error based on triplicate samples

[c] A = A-type; B = B-type; J = J-type; *S.liv.* = *Synechococcus lividus*; C = C-type; *C.aur.* = *Chloroflexus aurantiacus*

2.) SSU rRNA from the B-type organism was detected only in mats of hot springs above pH 6.2 and between 48°C and 67°C, with the strongest probe reactions occurring with RNA from hot springs of pH 7.8 - 9.1 and temperatures between 54°C - 64°C.

3.) SSU rRNA from the J-type organism was detected only in mats of hot springs above pH 8.5 and between 48°C and 62°C, with the strongest probe reaction occurring with RNA from a hot spring of pH 8.7, 48°C - 51°C.

4.) SSU rRNA from *S. lividus* was found only in the slightly acidic mat from which it may have been originally isolated, Clearwater Springs Site A.

These data suggest the possibility that types A, B, and J are cyanobacteria adapted to different temperatures within neutral to alkaline springs, while *S. lividus* may be adapted to lower pH.

The distributions of SSU rRNA from the green non-sulfur bacterium *C. aurantiacus* and from the suspected green non-sulfur bacterium contributing C-type SSU rRNA exhibit a more simple pattern. Ribosomal RNA from these organisms was not detected in microbial mat samples from any of the acidic hot springs. The C-type probe hybridized at some level with RNA from all alkaline hot spring microbial mats with the exception of Clearwater Springs Site D, which has the lowest pH of the alkaline hot springs sampled for this study. The strongest hybridization with the C-type probe was with RNA from Mushroom Spring, although the reactions with Twin Butte Vista Spring and with Octopus Spring Sites 60, 65, and 70 were also strong. The *C. aurantiacus* probe hybridized only with RNA from the high temperature Octopus Spring Site 70.

Vertical Distribution of SSU rRNAs and Pigments in the Octopus Spring 50°C Mat

Chlorophyll a and bacteriochlorophyll a decreased with depth, but bacteriochlorophyll c maximized at 1-2.5 mm (Figure 8A). Most of the changes with depth were not statistically significant ($p > 0.05$). The relative responses of the A, B, J, and C-type probes also decreased with depth and no probe response was detected below the 2.5 - 3.5 mm level (Figure 8B). There was no reaction with the I-type, *S. lividus*, or *C. aurantiacus* probes. SSU rRNA decreased significantly ($p < 0.05$) with depth over the upper 10 mm of the mat (Figure 8C).

Effect of Environmental Manipulation on Distribution of SSU rRNAs

Temperature Shift at Octopus Spring

Samples were shifted between sites of different temperatures *in situ* at Octopus Spring to evaluate whether population shifts would occur because organisms were adapted to a particular temperature range. The results of this manipulation are summarized in Figure 9. The mean probe responses of time zero and *in situ* controls were qualitatively similar despite shifts that might relate to temporal changes in SSU rRNA level.

For samples from Octopus Spring Site 50, there was a significant increase in A-type and *C. aurantiacus* SSU rRNA in the sample shifted to the Octopus Spring Site 65 ($p < 0.01$). Conversely, there were significant decreases in B-type, J-type, and C-type SSU rRNA in the Site 50 samples shifted to Site 65 ($p < 0.01$). For samples

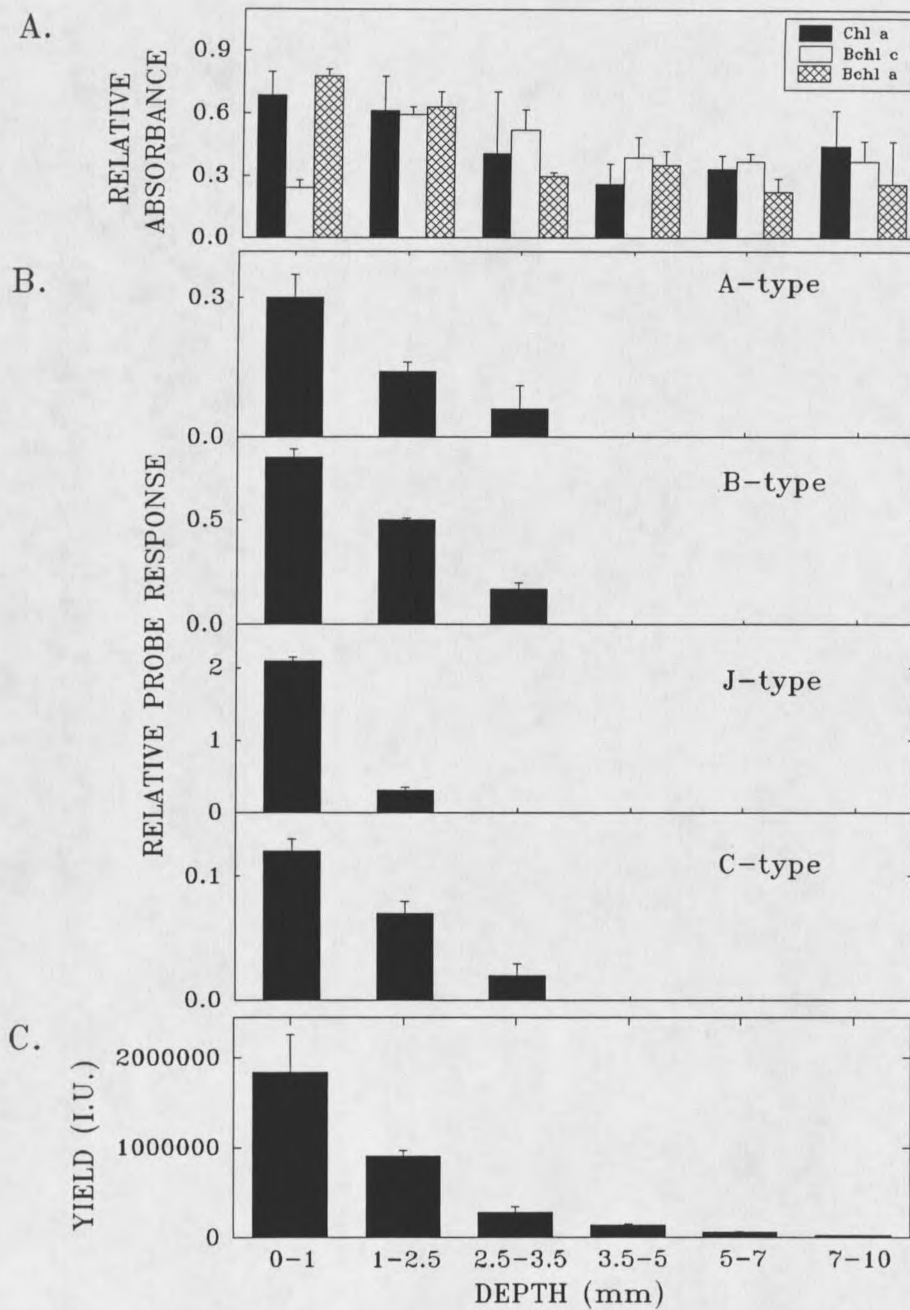


Figure 8. Vertical distribution of SSU rRNA and pigments in Octopus Spring Pool mat samples. Bars indicate standard error. A. Relative absorbances of chlorophyll a, bacteriochlorophyll a, and bacteriochlorophyll c B. Relative responses of specific probes. C. Universal probe response.

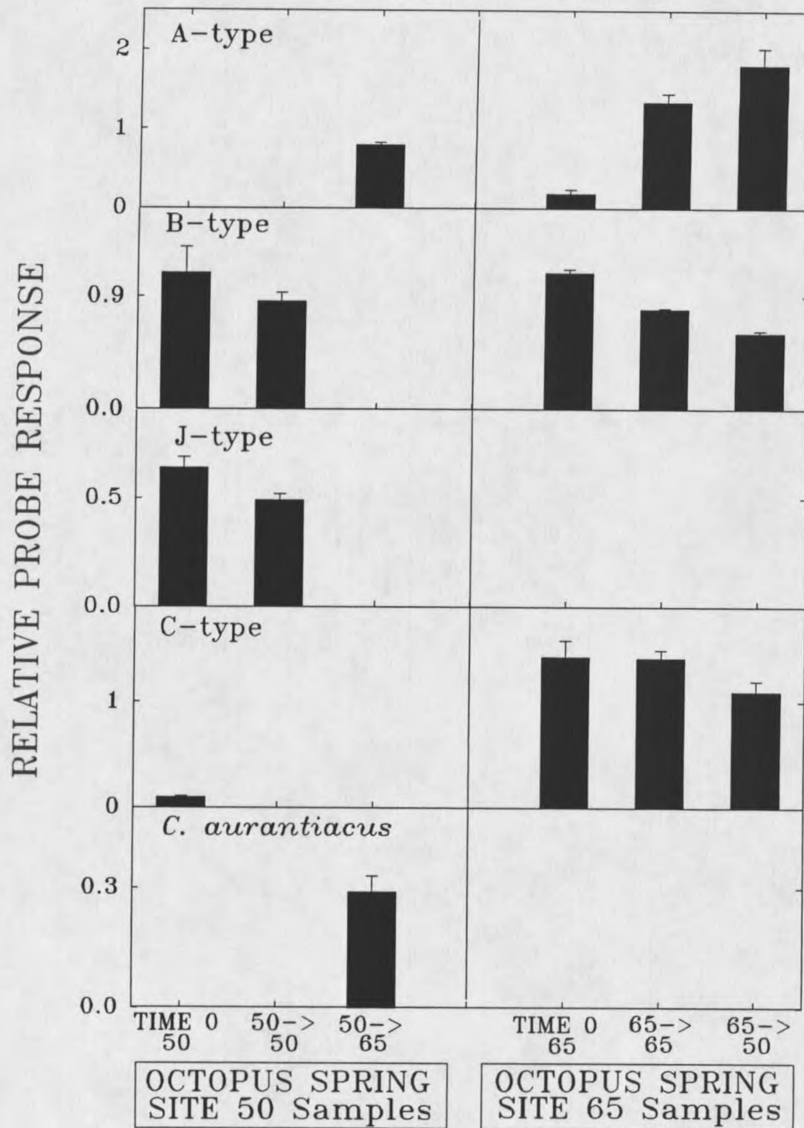


Figure 9. Temperature shift at Octopus Spring. Relative responses of oligodeoxynucleotide probes with RNA extracted from samples taken from Octopus Spring Site 50 [50°C, pH 8.5-8.6] and incubated at Site 65 [63-67°C, pH 8.3] (50→65), or collected at Site 65 and incubated at Site 50 (65→50). Controls were incubated at the collection site (50→50, 65→65). Results after one week *in situ* incubation are compared with those for mat samples collected before incubation (TIME 0). Bars indicate standard error.

from Octopus Spring Site 65 there was a significant increase in A-type SSU rRNA between the time zero samples and the shifted samples ($p < 0.05$), but an increase in the *in situ* control suggests that the increase may not have been due to temperature reduction. There was a significant decrease in the presence of B-type SSU rRNA in the Site 65 samples shifted to Site 50 ($p < 0.01$), which is interesting since B-type SSU rRNA also decreased in the Site 50 samples shifted to Site 65. The difference between mean responses for C-type SSU rRNA in Octopus Spring Site 65 samples shifted to Site 50 was insignificant ($p > 0.05$).

pH/Temperature Shift at
Clearwater Springs

Samples were shifted between different springs at Clearwater Springs in order to evaluate whether the cyanobacterial inhabitants are adapted to a particular pH (Figure 10). However, I was unable to locate springs of different pH with similar temperatures, so in addition to the 1.6 pH unit difference between the springs (pH 6.2 → 7.8), there was also a 10°C temperature difference (54°C → 64°C) which may have affected population dynamics. Furthermore, during *in situ* incubation a layer of colonizing bacteria developed (≈0.5-1.5 mm thick) around the tops and sides of the glass incubation vials; in the samples incubated at Site D this colonization resulted in a very thin green "veil" of microbes covering the tops of several vials. It was impossible to exclude these films so that the measured effects may have been due to colonization as well as to changes within the original sample.

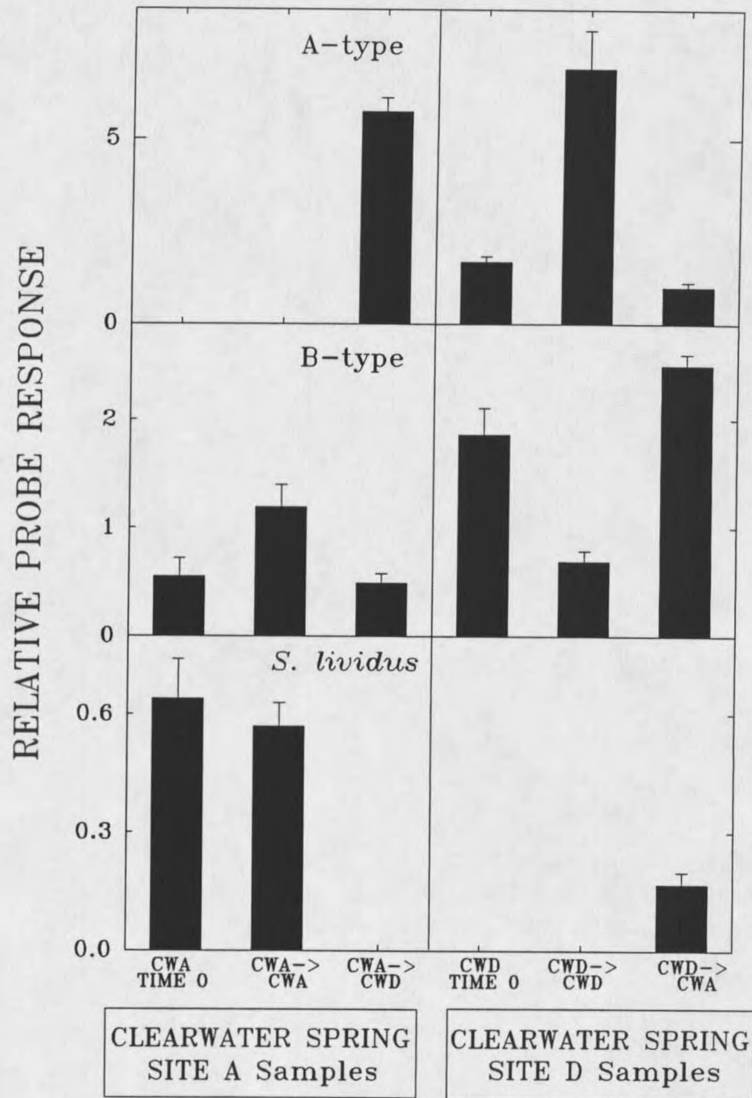


Figure 10. pH/Temperature shift at Clearwater Springs. Relative responses of oligodeoxynucleotide probes with RNA extracted from samples taken from Clearwater Springs Site A [54°C, pH 6.2] and incubated at Site D [64°C, pH 7.8] (CWA→CWD), or collected at Site D and incubated at Site A (CWD→CWA). Controls were incubated at the collection site (CWA→CWA, CWD→CWD). Results after one week *in situ* incubation are compared with those for mat samples collected before incubation (TIME 0). Bars indicate standard error.

When samples were shifted from Site A to the hotter, more alkaline Site D there was a significant increase ($p < 0.01$) in A-type SSU rRNA, which had not been detected in the Site A sample before the shift. For the B-type sequence, there was no significant difference in the mean responses between time zero or *in situ* controls and samples shifted from Site A to Site D ($p > 0.05$). *S. lividus* SSU rRNA decreased to below detection level in the Site A sample incubated at Site D ($p < 0.01$).

For samples from Clearwater Springs Site D, there was a significant difference between time zero and *in situ* control samples ($p < 0.05$). A-type SSU rRNA did not significantly decrease compared to the time zero control when shifted to Site A ($p > 0.05$), but there was a significant decrease compared to the *in situ* control ($p < 0.05$). Shifting Site D samples to Site A resulted in a significant increase of B-type SSU rRNA from the *in situ* control ($p < 0.01$), but the difference from time zero was not statistically significant ($p > 0.05$). *S. lividus* SSU rRNA was present in the samples shifted from Site D to Site A, though it was not detected in the control Site D samples.

Light Reduction at Octopus Spring

The effect of decreased light on the presence of SSU rRNA from the targeted organisms was investigated to indicate light-dependent behavior consistent with phototrophic phenotype (Figure 11). The level of B-type SSU rRNA present was not significantly affected by the decrease in light intensity or complete darkening ($p > 0.05$). The amounts of J-type SSU rRNA in the samples incubated at 7% and 0% of normal light intensity were significantly decreased from the time zero samples

($p < 0.01$). Although there was a minimal response between the A-type probe and the initial time zero sample, there was no reaction with the *in situ* control or light intensity reduced samples (data not shown). Type C SSU rRNA reacted weakly with both control and decreased light intensity samples (data not shown); the reactions were not significantly different ($p > 0.05$)

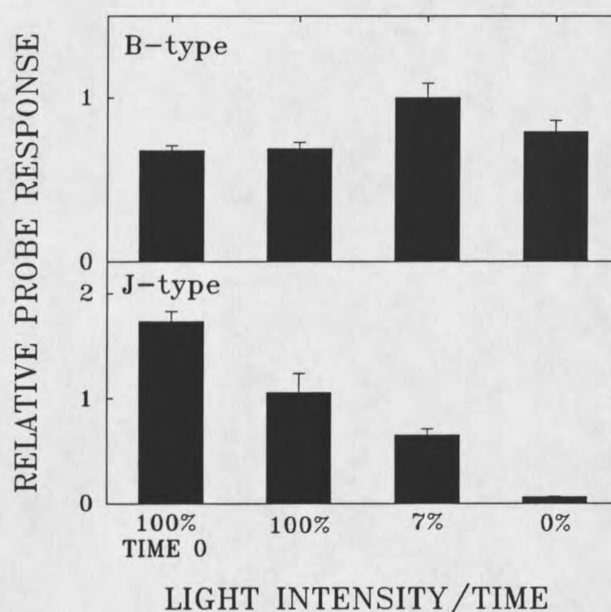


Figure 11. Light reduction at Octopus Spring. Relative responses of oligodeoxynucleotide probes with RNA extracted from samples taken from Octopus Spring Pool (48-51°C, pH 8.7) before (TIME 0) and after one week *in situ* incubation at 100%, 7%, and 0% of natural light intensity. Bars indicate standard error.

Enrichment Cultures

Enrichment cultures were examined in order to determine the effect of enrichment on species selection.

Octopus Spring, Site 50. A-type SSU rRNA was detected in an enrichment inoculated with a 10^{-6} dilution of an Octopus Spring Site 50 sample, despite the fact that A-type SSU rRNA was not detected in the time zero sample or the enrichment inoculated with an undiluted sample (Figure 12). B-type SSU rRNA was detected in the time zero sample and the enrichment inoculated with an undiluted sample, and was greatly enriched in the culture inoculated with a 10^{-6}-diluted sample. *S. lividus* SSUrRNA was not detected in the time zero sample or the diluted culture but was strongly enriched in the undiluted culture. The SSU rRNA of both the J-type and C-type organisms was not detected in either enrichment sample despite the fact that type-J and type-C SSU rRNA was detectable in the time zero sample. *C. aurantiacus* SSU rRNA was not detected in any Octopus Spring enrichments.

Clearwater Springs, Site A. Enrichments of phototrophs from Clearwater Springs, Site A, were attempted using the pH 8.2 Medium D originally used to isolate *S. lividus* Y-7c-s from this mat (Kallas and Castenholz, 1982a), and using Medium D of pH 6.5, closer to the pH of the spring (pH 6.2). A-type SSU rRNA was enriched in cultures inoculated with both undiluted and 10^{-6}-diluted samples in pH 6.5 medium; its SSU rRNA was not detected in the pH 8.2 enrichments or in the time zero samples (Figure 13). B-type SSU rRNA was detected in the time zero sample as well as in enrichments of diluted and undiluted samples incubated in either pH 6.5 or pH 8.2 media, with the largest enrichment occurring in the diluted sample incubated in pH 6.5 medium. *S. lividus* SSU rRNA was detected at time zero and was enriched from both undiluted and diluted samples by incubation in pH 6.5 and 8.2 media.

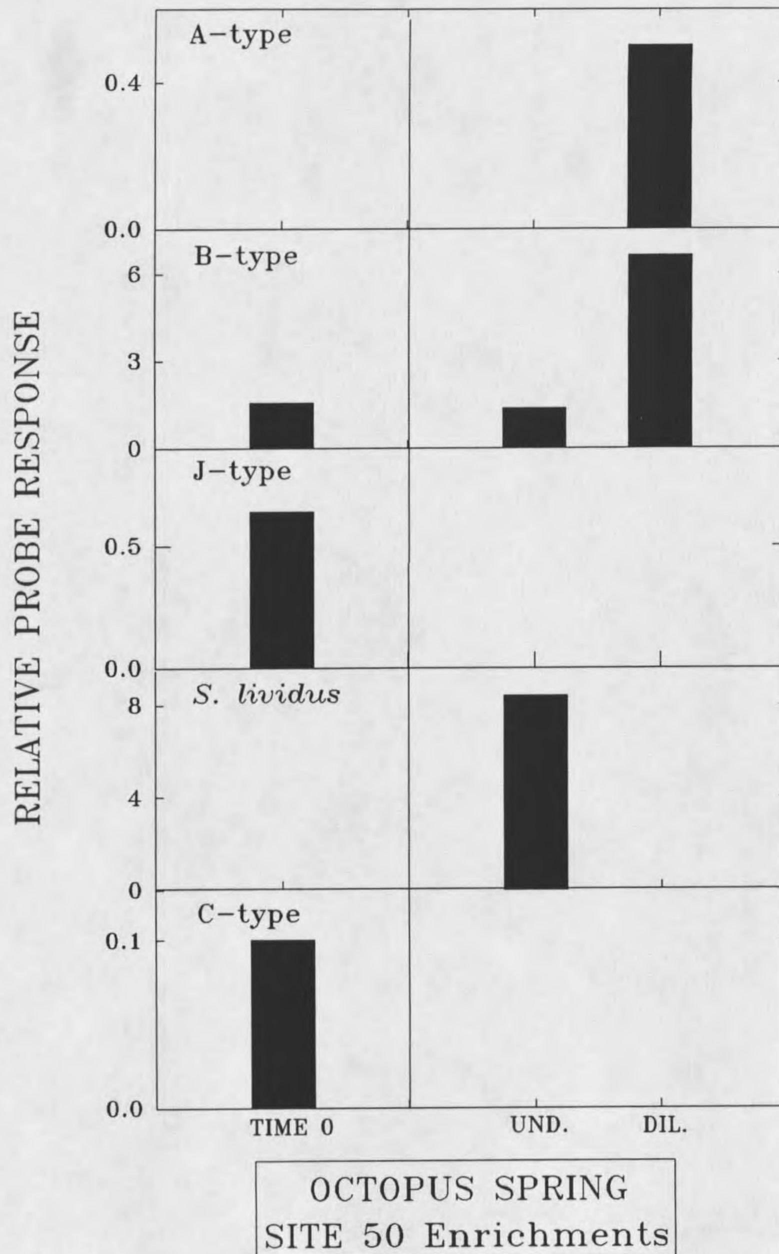


Figure 12. Enrichment at Octopus Spring Site 50. Relative responses of oligodeoxynucleotide probes with RNA extracted from a homogenized mat sample from Octopus Spring Site 50 (50°C, pH 8.5-8.6; TIME 0) or from phototrophic enrichment cultures in Medium D inoculated with relatively undiluted (UND.) or 10^{-6}-diluted (DIL.) mat samples.

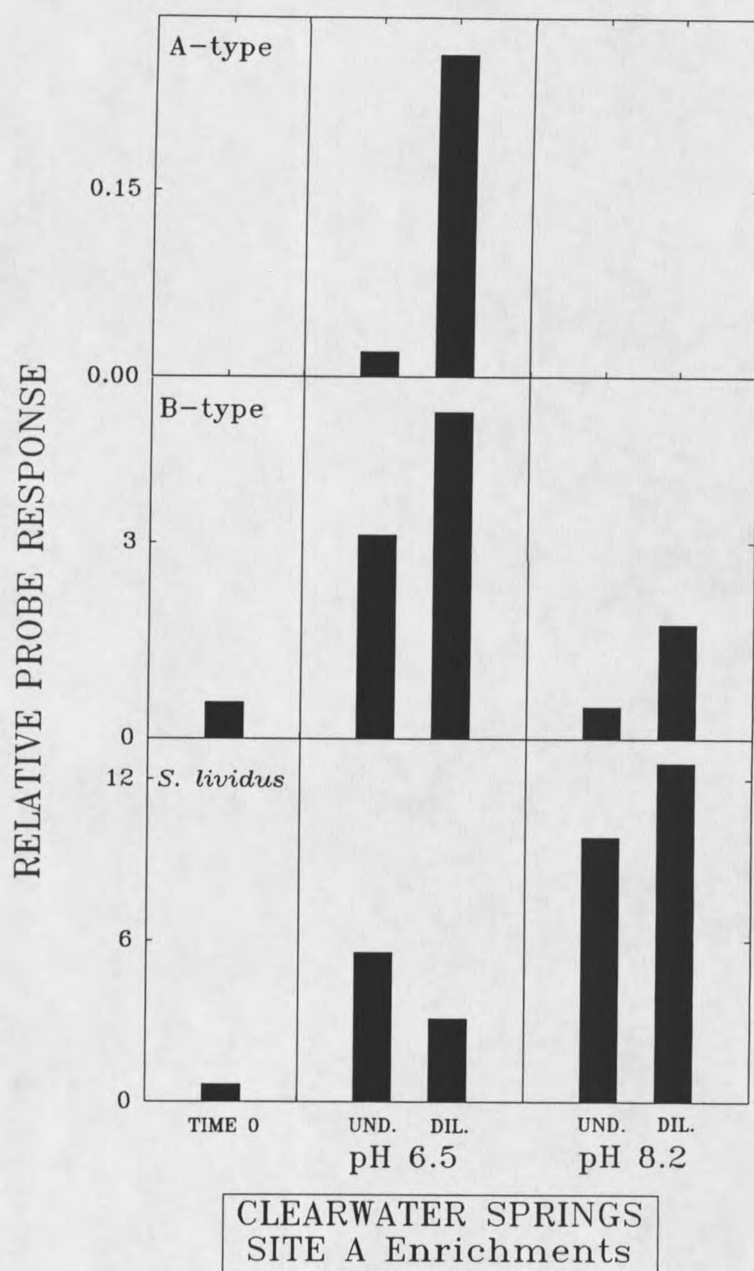


Figure 13. Enrichment at Clearwater Springs Site A. Relative responses of oligodeoxynucleotide probes with RNA extracted from a homogenized mat sample from Clearwater Springs Site A (54°C, pH 6.2; TIME 0) or from phototrophic enrichment cultures inoculated with relatively undiluted (UND.) or 10^{-6}-diluted (DIL.) mat samples in either pH 6.5 or pH 8.2 Medium D.

DISCUSSION

Methods

Lysis

The choice of lysis method for these experiments was most important. The method had to result in the unbiased recovery of relatively high yields of good quality RNA from all organisms of interest in the mat. The FP method satisfied these requirements: (1) it yielded the greatest amount of RNA; (2) the recovered RNA was of sufficiently large size to ensure that the target region for probes was intact; and (3) there was no bias against the recovery of either *S. lividus* or *C. aurantiacus* RNA. The detection of *S. lividus* and *C. aurantiacus* SSU rRNA in the mat sample seeded with these cells eliminates the possibility that the failure to detect these sequences in earlier cloning and sequencing work may have been due to an inability of the FP lysis procedures to recover RNA from mat samples. The importance of choosing the appropriate lysis method was underscored by the discovery that mechanical lysis using the Bead-Beater resulted in inadequate recovery of *S. lividus* RNA from both pure cultured *S. lividus* cells, where no *S. lividus* SSU rRNA was detected, and mat samples seeded with *S. lividus* cells, where *S. lividus* SSU rRNA was detected in only one of the triplicate samples. Another important aspect of the lysis study is that it revealed

that SSU rRNA of reproducible yield and probe reactivity (ie., SSU rRNA target amount) could be reliably obtained from relatively small samples of mat.

Probe Design

The design and optimization of oligodeoxynucleotide probes specific for their targets was a central part of this study. Some SSU rRNA sequence types are highly similar; for example, the percent similarity of the A-type and B-type sequences in unrestricted analysis is 96.9% (Ward, *et al.*, 1992a). The first generation A-type probe had only a single mismatch with the B-type sequences and it was impossible to eliminate cross-reaction by increasing stringency (Figure 3A). Improved sequence data obtained by subsequent cloning and sequencing enabled the design of A-type and B-type probes that reacted specifically with their respective targets (Figure 4). Probes complementing the other highly similar cyanobacterial sequences were also specific to their targets (Figure 4). The difficulty in designing specific probes to complement the A-type and B-type sequences underscores the importance of obtaining accurate and complete sequence data from a community that one wishes to study using oligodeoxynucleotide probes.

Quantification

The quantification of RNA for these experiments was done both by absorption spectroscopy and by SSU rRNA-specific universal probe response because there were doubts regarding the accuracy of absorbance readings on extracted nucleic acids. Absorption spectroscopy overestimated the amount of RNA in samples from several

hot springs (data not shown) probably due to UV absorbance by contaminating material.

The inability to compare differences in probe responses between organisms studied was unfortunate. However, absolute quantitation of probe responses with uncultivated species is not possible with current methods. In order to construct concentration curves to determine specific probe responses with SSU rRNA from specific organisms, one would need the RNA, which is impossible to obtain from the uncultivated species. Work is progressing in this lab to cultivate the organisms which contribute the SSU rRNA sequence types found in Octopus Spring. If the organisms can be cultivated, quantitative comparisons of importance in the community and response to environmental perturbation can be made through hybridization probe analysis.

Autecology of Cultivated and Uncultivated Community Members

Correlation with Cloning Results

Previous research in this laboratory involving cloning and sequencing of naturally occurring SSU rRNAs (Ward, *et al.*, 1990a; Ward, *et al.*, 1990b; Ward, *et al.*, 1992a) indicated that uncultivated cyanobacteria and GNS-like bacteria dominate the 50-55°C microbial mat at Octopus Spring. SSU rRNA sequences of the cultivated organisms previously thought to be dominant, the cyanobacterium *S. lividus* and the GNS bacterium *C. aurantiacus*, were not found. These cloning results were confirmed by the sequence-specific oligonucleotide probe results reported in this paper. *S. lividus*

and *C. aurantiacus* SSU RNA were not directly detected in 50-55°C Octopus Spring mat samples. On the other hand, SSU rRNAs from uncultivated organisms, A-type, B-type, J-type, and C-type, were detected. It is obvious that morphological and pure culture analyses have given a biased view of the cyanobacterial and GNS-like bacterial populations of this mat.

Distribution of Cultivated Organisms in Nature

S. lividus SSU rRNA was directly detected only in the mat sample from Clearwater Springs Site A, the pH 6.2 pool from which it was apparently originally isolated (Kallas and Castenholz, 1982a). However, *S. lividus* was heavily enriched in an enrichment culture begun with an undiluted sample from Octopus Spring Site 50 (pH 8.2), which indicates that it is present in that mat, even though its SSU rRNA was not directly detected using oligonucleotide probes. The failure to detect *S. lividus* SSU rRNA in enrichments inoculated with highly-diluted samples suggest that the *S. lividus* population in the 50°C Octopus Spring mat is relatively low. *S. lividus* was also greatly enriched in the cultures begun with undiluted or highly-diluted samples from Clearwater Springs Site A, most notably in the pH 8.2 medium enrichment of the diluted sample. The presence of *S. lividus* SSU rRNA in an enrichment culture inoculated from a highly-diluted sample suggests that it is a numerically significant member of the mat community at Clearwater Springs Site A. It is interesting that the enrichment of *S. lividus* from Clearwater Springs Site A in a pH 6.5 medium was not as great as that which occurred in pH 8.2 medium. Contradicting the idea that

S. lividus is more competitive at higher pH is the fact that its SSU rRNA was not directly detected in the Clearwater Springs Site A (pH 6.2) sample that was incubated at Site D (pH 7.8) or in any alkaline mat sample. The fact that *S. lividus* SSU rRNA was directly detected only at Clearwater Springs Site A, pH 6.2, yet can be enriched in a pH 8.2 medium from both Clearwater Springs Site A and Octopus Spring Site 50, suggests that pH may not be the only driving mechanism for dominance of *S. lividus* in an environment.

C. aurantiacus Y-400-fl was originally cultivated from Octopus Spring at a site where the temperature was 59°C (Pierson and Castenholz, 1974). The original enrichment involved the selection of rapidly gliding filaments from a mat inoculum plated on 1.5% agarose/Medium D, pH 8.2 and incubated at 45°C in the light. Therefore, I was surprised that oligonucleotide probe hybridization with mat RNA detected *C. aurantiacus* Y-400-fl only at Site 70, where the temperature ranged between 61 and 70°C, and not at any of the other sites at Octopus Spring (48-67°C). However, this result may explain why the *C. aurantiacus* SSU rRNA sequence has not yet been identified by cloning of Octopus Spring mat SSU rRNAs; all samples for cloning were taken from 50°C - 55°C sites. Although enrichment of Octopus Spring Site 50 cells did not result in detection of *C. aurantiacus* SSU rRNA (*C. aurantiacus* is thought to be a member of cyanobacterial enrichments; Castenholz and Pierson, 1981), there is evidence that *C. aurantiacus* is present in the mat at Site 50. Samples taken from Site 50 and incubated at Site 65 contained RNA which hybridized with the *C. aurantiacus* probe. Since new colonization was not evident in this experiment, it

seems probable that there was a rise of a *C. aurantiacus* population indigenous to the 50°C mat, but below detection limits by direct probing. These results, in concert with the observations of Bauld and Brock (1973) regarding the existence of temperature strains of *C. aurantiacus*, suggest that *C. aurantiacus* Y-400-fl may be a temperature strain of *C. aurantiacus* which has its temperature optimum above 61°C and outcompetes other GNS bacteria in the highest temperature region of the mat, Site 70. It may be present at lower temperatures, but cannot grow to the level detectable by hybridization with oligonucleotide probes. It is unknown why *C. aurantiacus* Y-400-fl could not be detected in samples from regions of the mat with temperatures around 59°C. One of these sites must have been similar to the area from which *C. aurantiacus* Y-400-fl was isolated. The selection for rapidly gliding filaments may have biased toward the recovery of a more minor GNS community member. Future investigators could attempt enrichment of *C. aurantiacus* Y-400-fl from Octopus Spring Site 70 and determine its temperature optimum.

Distribution of Uncultivated Organisms in Nature

One of the most interesting applications of sequence-specific probes is to provide insight into the nature of uncultivated species. The distributions of SSU rRNA of A-type, B-type, and J-type organisms suggest that they may be temperature strains of unicellular thermophilic cyanobacteria, possibly some of those identified by Peary and Castenholz (1964). For example, type A SSU rRNA was strongly detected only in mats from higher temperature hot springs, while SSU rRNAs from the B-type

organism and, to a greater extent, the J-type organism were most strongly detected in mats from lower temperature hot springs. The temperature and pH/temperature shift experiments also lend evidence to this interpretation. SSU rRNAs from the putative low temperature strains, types B and J, were not detected in the Octopus Spring Site 50 sample incubated at Site 65. There was also no increase in J-type RNA in the Site 65 sample incubated at Site 50, but if the J-type truly is a low temperature strain, it may not be present at 65°C. SSU rRNA from the putative high temperature strain, type A, was not detected in samples from Octopus Spring Site 50, but was detected in the Site 50 samples incubated at Site 65. Also, when the Clearwater Springs Site A (54°C) sample was incubated at Site D (64°C), SSU rRNA from the B-type organism decreased while type A SSU rRNA increased. The opposite shift, Site D samples incubated at Site A, resulted in the opposite effect: B-type SSU rRNA increased and A-type SSU rRNA decreased.

SSU rRNA from the A-type organism was detected in the enrichment samples from highly-diluted inoculum of Octopus Spring Site 50 mat and Clearwater Springs Site A mat even though A-type SSU rRNA was not directly detected in the mats at those sites. The putative lower temperature strain, type B, was detected in enrichment samples inoculated with both highly-diluted and undiluted mat samples from both Octopus Spring Site 50 and Clearwater Springs Site A. The fact that B-type SSU rRNA was detected in the highly-diluted cultures from both sites and was directly detected in mat samples from each site suggests that it is a numerically significant member of both communities. Ribosomal RNA from the J-type organism was not

detected in either the Octopus Spring or Clearwater Spring enrichments, although J-type SSU rcDNA was directly detected in the Octopus Spring Site 50 mat samples, suggesting its numerical importance. This suggests that this organism is not as competitive in medium D as the A-type cyanobacterium, the B-type cyanobacterium, or *S. lividus*.

The results from the vertical profile samples for types A, B, J, and C were consistent with these sequences being from phototrophic and/or oxygen-dependent microorganisms considering what is known about light penetration (Doemel and Brock, 1977) and O_2 (Revsbech and Ward, 1984) in the Octopus Spring 50°C mat. SSU rRNA of all four types decreased with depth. Light intensity reduction gave further evidence that the J-type organism requires light, since J-type SSU rRNA decreased when light was reduced or eliminated. The fact that B-type SSU rRNA did not decrease in the 7% full light or 0% full light samples compared to the full light control sample corresponds with the findings of Madigan and Brock (1977) that mat cyanobacteria can adapt and grow at low light intensities. The fact that type B SSU rRNA persists in the dark samples could have been due to heterotrophic growth of this cyanobacterium as is true of some thermophilic cyanobacteria (Richardson and Castenholz, 1987). Alternatively, perhaps one week in the dark was not long enough for degradation of B-type SSU rRNA (ie., this species has good potential to survive adverse environmental conditions). Type A SSU rRNA was not detected in the *in situ* control or after decreased light intensity incubation, although it was present in the time zero sample, suggesting that the population level of the A-type organism is relatively

low and unstable. Type C SSU rRNA was detected after exposure to 7% full light and darkness at essentially the level of the time zero control. Madigan, *et al.* (1974), evaluated the physiological versatility of *Chloroflexus* species and found that *Chloroflexus* is capable of growing not only anaerobically as a photoautotroph or a photoheterotroph (with the ability to adapt to low light intensity) but also aerobically as a heterotroph. Since Doemel and Brock (1977) showed that *Chloroflexus* migrates upwards at night, presumably for heterotrophic aerobic growth, it would be interesting for some future investigator to evaluate aerobic heterotrophic behavior by the C-type organism by examining vertical profiles after light intensity reduction. Another possibility is that the C-type SSU rRNA is contributed by a heterotrophic bacterium closely related to GNS bacteria. Weller, *et al.*, (1992) showed that there are heterotrophic GNS relatives to which the C-type SSU rRNA sequence is related.

Phenotypic Characterization from Phylogenetic Probes

It is clear that determining phenotype from results obtained using phylogenetically-designed probes may be difficult. There was no information obtained by using the probe designed to complement the cyanobacterial I-type sequence. It is possible that this organism is present at a level undetectable by the oligodeoxynucleotide probe method. SSU rRNA from the B-type organism, which is clearly from a cyanobacterium in terms of phylogenetic analysis (Weller, *et al.*, 1991), is detectable and seems to exhibit the vertical distribution expected of a cyanobacterium but does not respond to light as expected of a cyanobacterium, since

B-type SSU rRNA persists in the dark for one week. Clearly, the characterization of phenotype from perturbation experiments can be complicated if an organism has (1) persistent SSU rRNA, (2) alternative metabolisms expressed under different environmental conditions, (3) metabolisms with similar responses to perturbation (i.e., aerobes and phototrophs), or if (4) perturbations lead to secondary changes in the environment caused by the experimental manipulation (for example, elimination of light affects O_2 levels). However, the ability to use oligonucleotide probes to follow the response of an uncultivated organism to environmental manipulation should prove useful for providing initial insight into some phenotypic characteristics. The possible temperature and pH preferences of the cultivated and uncultivated organisms I observed serves as an example. Such information enables the design of new strategies for enrichment culture that might lead to cultivation of more relevant species. Sequence-specific oligonucleotide probes could also become a useful tool when isolating a particular organism. The probes could be used to follow the response of the SSU rRNA of an organism to variations in enrichment culture medium and incubation conditions, thus increasing the chance of enriching for the organism of choice.

CONCLUSIONS

From the data obtained with oligodeoxynucleotide probing of SSU rRNA from hot springs microbial mats, the following conclusions are made:

1. Oligodeoxynucleotide probes, specific for the SSU rRNA of cultivated and uncultivated species, can be developed and used reproducibly on small samples to determine the distributions and responses of the organisms contributing these rRNAs to the microbial community.

2. Oligodeoxynucleotide probe results confirm the observation from cloning and sequencing data that the Octopus Spring 50-55°C microbial mat is dominated by uncultivated phototrophs, rather than the cultivated phototrophs thought to dominate.

3. The cultivated phototrophic species, *S. lividus* and *C. aurantiacus*, are found in relatively few hot spring environments. *S. lividus* appears to be competitive in nature in slightly acidic hot springs and is highly competitive in enrichment cultures with Medium D. *C. aurantiacus* may be a strain adapted to high temperature regions of alkaline hot springs.

4. Oligodeoxynucleotide probe results provide insight into the possible phenotypes of some uncultivated species. Sequence type A, B, and J may be cyanobacterial temperature strains.

5. It may be difficult to obtain phenotypic information for some uncultivated species using oligodeoxynucleotide probes targeting SSU rRNA. For instance, the distribution and response to perturbation of C-type SSU rRNA is consistent with more than one type of physiology. Also, in some species, the persistence of SSU rRNA under adverse environmental conditions may complicate association with changing environmental parameters.

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APPENDIX

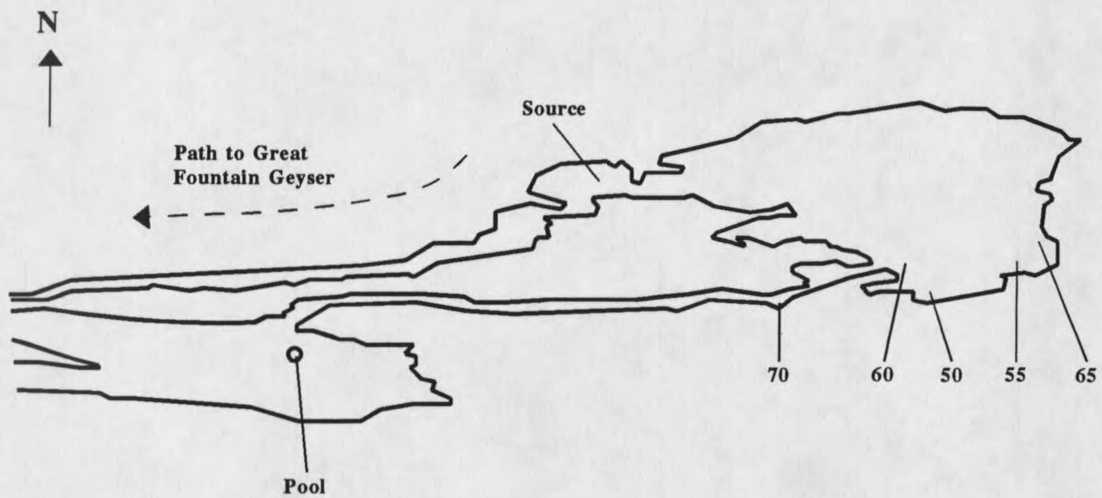

Figure 14. Diagram of Octopus Spring sampling sites (adapted from Ward, *et al.*, 1992b). Octopus Spring is located in the Lower Geyser Basin of Yellowstone National Park, Wyoming, near Great Fountain Geyser (Brock, 1978). 50, 55, 60, 65, 70 = sampling sites.

410602

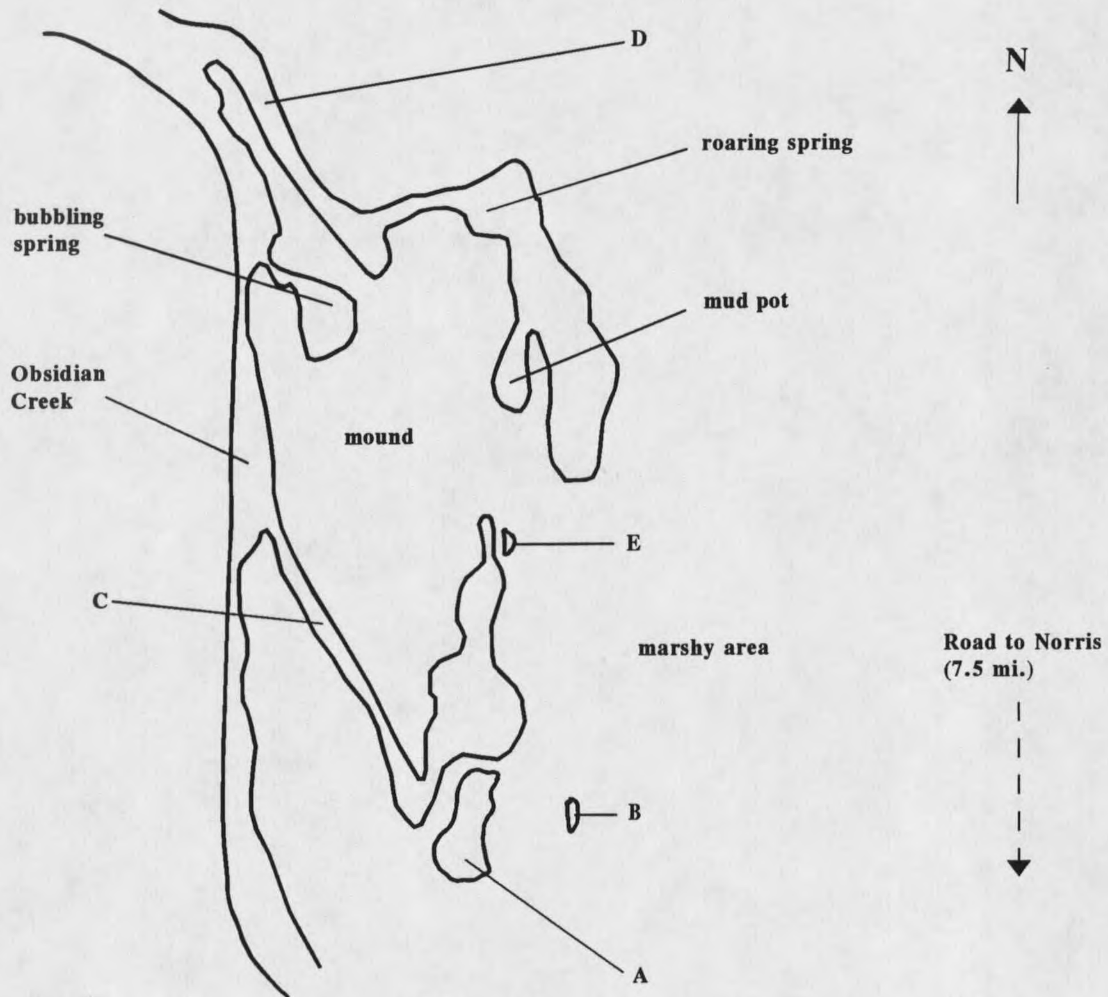

Figure 15. Diagram of Clearwater Springs sampling sites (drawn from a photograph). Clearwater Springs is located next to Obsidian Creek, 7.5 miles north of Norris junction, on the west side of the road between Norris junction and Mammoth Hot Springs in Yellowstone National Park, Wyoming. A, B, C, D, E = sampling sites.

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