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

© Copyright by Alyson L Ruff-Roberts (1993)

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.

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
Bozeman, Montana

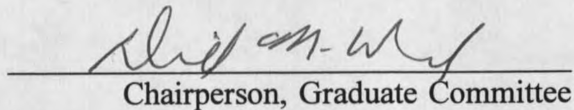
July, 1993

T1378
R8379

APPROVAL
of a thesis submitted by
Alyson Louise Ruff-Roberts

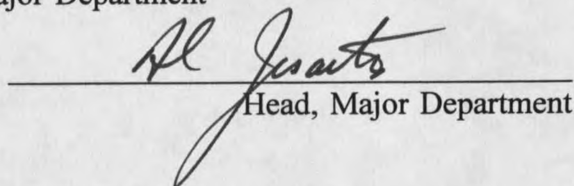
This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

8/4/93
Date


Chairperson, Graduate Committee

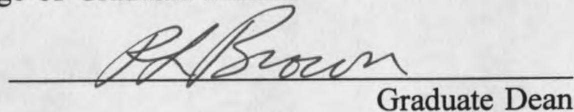
Approved for Major Department

8/6/93
Date


Head, Major Department

Approved for the College of Graduate Studies

8/14/93
Date


Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Dean of Libraries when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature SL Roff-Roberts

Date 7/17/93

TABLE OF CONTENTS

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT ix

INTRODUCTION 1

- The Octopus Spring Mat as a Model Community 2
- Ribosomal RNA as a Signature Molecule for Characterization of Microbial Mat Communities 6
- rRNA Methods Applied to Microbial Ecology 8
- Summary of Experimental Goals 12

METHODS 14

- Organisms and Growth Conditions 14
- Collection and Processing of Microbial Mat Samples 15
- Pigment Concentration 15
- *In Situ* Manipulations of Microbial Mat Samples 17
  - Temperature Shift 17
  - pH/Temperature Shift 17
  - Light Intensity Reduction 18
- Enrichment Cultures from Microbial Mat Samples 18
- Preparation of Target Nucleic Acids 19
  - Lysis Methods 19
    - French Pressure (FP) Cell 19
    - Other Lysis Methods 20
    - Combined Lysis Methods 21
  - Purification of RNA 22
  - Growth of Transformed Cells and Purification of Plasmid DNA 22
  - Quantification of Nucleic Acids 23
- Preparation of Probes 23
  - Probe Design 23
  - Synthesis and Radiolabelling 25

Purification 25
Quantification 26
Hybridizations 27
Denaturation of Target 27
Preparation of Filters 27
Hybridization 28
Autoradiographs 28
Analysis of Results 28

RESULTS 30

Probe Reactivity 30
Probe specificity 30
Quantification of Probe Reaction 32
Effects of Lysis Methods on RNA Yield, Quality, and Probe Response 34
RNA Yields 34
Quality of Extracted RNA 37
Probe Response 38
Distribution of SSU rRNAs Among Microbial Mats from Hot Springs of Different Temperatures and pH 39
Vertical Distribution of SSU rRNAs and Pigments in the Octopus Spring 50°C Mat 42
Effect of Environmental Manipulation on Distribution of SSU rRNAs 42
Temperature Shift at Octopus Spring 42
pH/Temperature Shift at Clearwater Springs 45
Light Reduction at Octopus Spring 47
Enrichment Cultures 48
Octopus Spring, Site 50 49
Clearwater Springs, Site A 49

DISCUSSION 52

Methods 52
Lysis 52
Probe design 53
Quantification 53
Autecology of Cultivated and Uncultivated Community Members 54
Correlation with Cloning Results 54

Distribution of Cultivated
Organisms in Nature 55
Distribution of Uncultivated
Organisms in Nature 57
Phenotypic Characterization from
Phylogenetic Probes 60

CONCLUSIONS 62

LITERATURE CITED 63

APPENDIX 69

LIST OF TABLES

1. Microorganisms thought to be present in the Octopus Spring microbial mat 4

2. Description of samples used in this study 16

3. Sequences, T_m, and T_H values of oligodeoxynucleotide probes used in this study 24

4. Relative probe responses with RNA extracted from mat samples taken from hot spring locations of different temperature and pH 40

LIST OF FIGURES

1. Secondary structure of *Escherichia coli* SSU rRNA 7

2. General approaches to the use of rRNA methods in microbial ecology 9

3. Oligodeoxynucleotide probe specificity of first generation probes used in this study 31

4. Specificity of oligodeoxynucleotide probes used in this study 33

5. Dependence of universal probe reactivity on target *Chloroflexus aurantiacus* RNA or type-C SSU rcDNA concentration 34

6. Lysis experiments I 35

7. Lysis experiments II 37

8. Vertical distribution of SSU rRNAs and pigments in the Octopus Spring Pool mat 43

9. Temperature shift at Octopus Spring 44

10. pH/Temperature shift at Clearwater Springs 46

11. Light reduction at Octopus Spring 48

12. Enrichment at Octopus Spring Site 50 50

13. Enrichment at Clearwater Springs, Site A 51

14. Diagram of Octopus Spring sampling sites 69

15. Diagram of Clearwater Spring sampling sites 70

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,

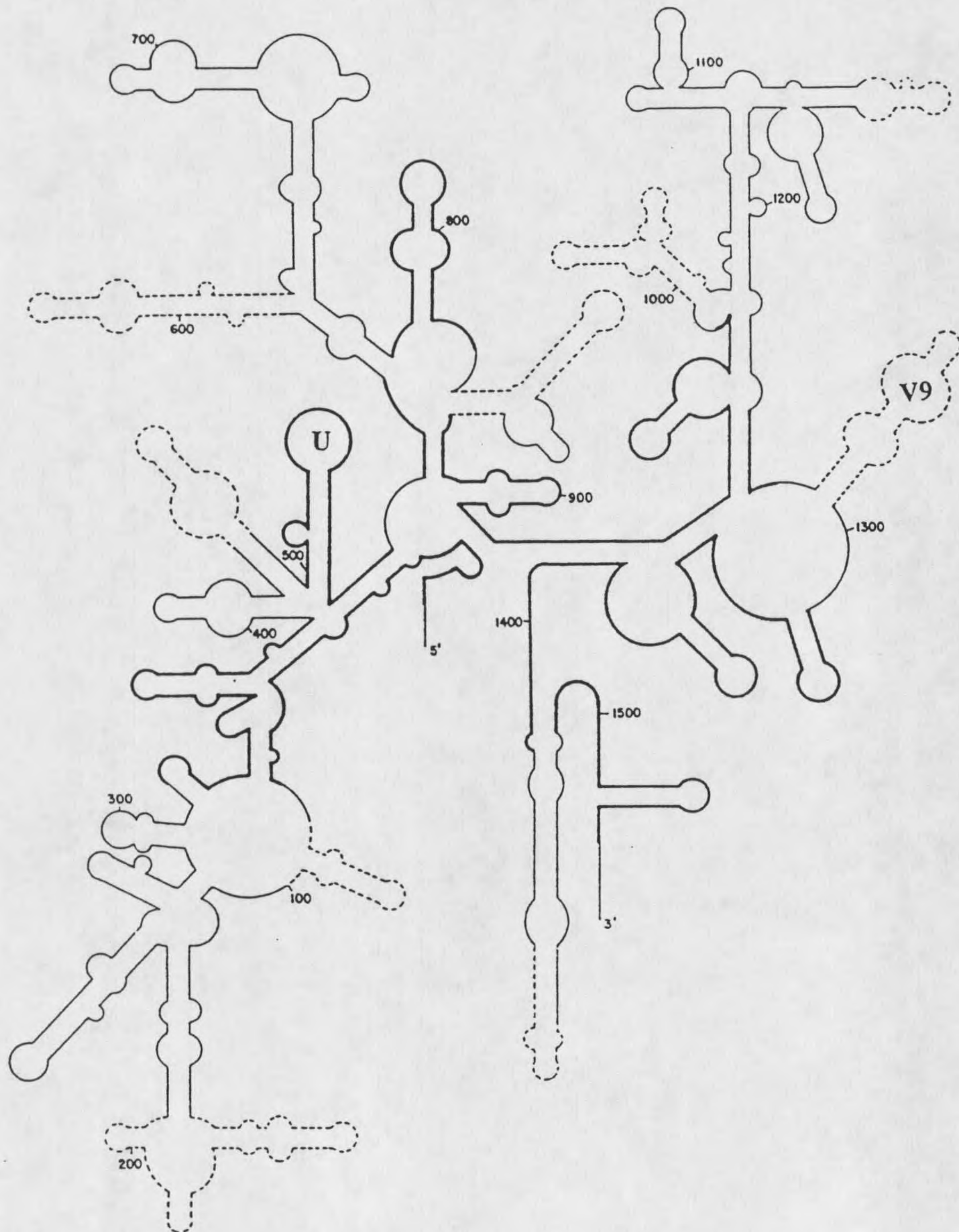


Figure 1. Secondary structure of *Escherichia coli* SSU rRNA indicating regions of low (▬), moderate (—) and high (---) sequence variability (from Ward, *et al.*, 1992a). **V9** indicates the general location of the variable region used as a target for oligodeoxynucleotide probes in this study. **U**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

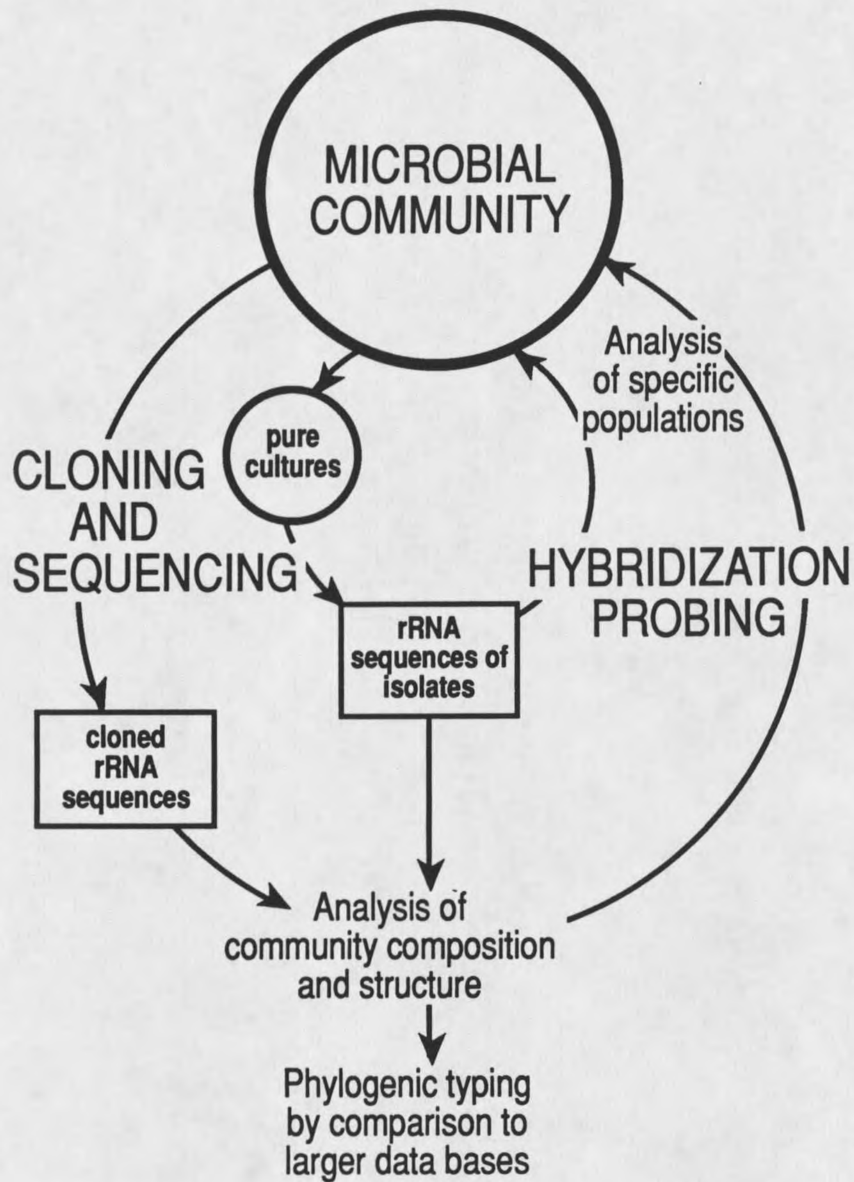


Figure 2. General approaches to the use of rRNA methods in microbial ecology (from Ward, *et al.*, 1992a).

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