Attempts to Cultivate Red Layer Bacteria Using Site-Specific Water Chemistry Data

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Left: Terry at Hillside Spring (September 2004); Right: Terry at Mound Spring (July 2005); Terry at Fairy Spring (July 2004)

Abstract
My research focuses on novel filamentous red Chloroflexi bacteria that form mats in Yellowstone hot springs (pH 7.5-9, 37-65°C), Red Layer Microbial Observatory (RLMO) sites. Based on 16S rRNA sequence analysis, red Chloroflexi bacteria are 92-95% similar to *Roseiflexus castenholzii*, a red filamentous phototroph isolated from comparable hot springs in Japan. Despite these similarities, culturing of Yellowstone red Chloroflexi using medium PE, which was used to culture *Roseiflexus*, has been unsuccessful. Given that RLMO sites showed distinct water chemistry profiles (using 15 salts and metals), I designed media based on these data, focusing on one RLMO site, Hillside Springs (pH 8, 54°C). I hypothesized that (A) the growth of red Chloroflexi bacteria would be dependent on chemical differences at that are site-specific, (B) a different array of microbial isolates would be retrieved using each medium, and (C) media made using site-specific chemical data would support more diversity than broad-based media such as medium PE. Hillside red Chloroflexi samples were inoculated into: (1) site-specific media plus high yeast extract (0.1 g/L), (2) site-specific media plus low yeast (0.01 g/L), (3) *Roseiflexus* media (medium PE), (4) and sterilized source water from Hillside Springs. Growth was assessed once a week for four weeks. Microscopic, pigment, DGGE, and 16S rRNA sequence analyses were used to compare diversity enriched by each medium. Although site-specific media did not support the culture of target red Chloroflexi bacteria, these media enriched for different populations of new Proteobacteria, Gram Positives, and green Chloroflexi. Medium PE enriched for new Proteobacteria, Gram Positives, and Nitrospira - but not any red Chloroflexi. Sterilized source water supported the most visible growth of red filaments. Additionally, a co-culture derived from source water enrichments retrieved *Chloroflexus* species 396-1, a Yellowstone green Chloroflexi. Given that the use of sterilized source water media appeared to be the most promising, the potential environmental impact of source water-based cultivation could be substantial; thus, future cultivation studies should include improved research into the composition of the source water and how to more closely replicate it.

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INTRODUCTION

History of Culturing in Microbiology:

Ferdinand Cohn first pioneered sterile culture media in his work with *Bacillus*. In 1876, Cohn described the entire life cycle of *Bacillus* and in doing so he devised simple yet effective ways to prevent contamination of sterile media (8). These techniques were then used by Robert Koch, a physician who studied *Bacillus anthracis,* to develop pure culture methods and the Germ Theory of Disease (13, 14, 15). Koch postulated that some diseases came from the presence of specific microorganisms that caused them. In order to prove this, he had to obtain a sample of only one specific organism and inject it into study animals to show that the specific organism caused the disease. To do this he realized that the culture must be pure, so in 1881 he developed methods for obtaining pure cultures (13). A pure culture is a culture containing only one species of bacteria. One important insight that Koch had was that each colony on a solid surface represented a pure culture. At the time he was growing bacteria using potato slices. He found that upon leaving the potato slices in the open air that bacterial colonies of different morphologies and phenotypes would grow. He inferred that each colony must stem from one bacterial cell that fell on the surface, found suitable nutrients, and multiplied. Koch was also instrumental in the development of solid media. He realized that with a solid medium cells were immobilized and would form distinct colonies which were pure cultures. He also realized that not all bacteria grew on potato slices. After using gelatin to solidify his media, he realized this substance turned to liquid at body temperature. Thus it was not applicable to culture pathogens which thrive at body temperature. With the help of Walter Hesse, who first used agar for solidifying media for bacteriology, Koch had found a new way to make a medium that would allow him to culture pathogens (16). These basic methods and tools are still the basis for microbial culturing today.

Martinus Beijerinck was a Dutch microbiologist who pioneered the enrichment culture technique in 1901. The enrichment culture technique is a method used to isolate pure cultures from complex microbial communities. Most microorganisms live in communities rather than in isolation. The enrichment culture technique is a method that has been used to successfully isolate and characterize individual species that make up the microbial communities. This method is based on designing a medium that selects for the target microorganisms and counterselects for undesired organisms (16). Beijerinck first isolated *Azotobacter* using this technique. Beijerinck knew that *Azotobacter* was uniquely capable of fixing atmospheric nitrogen in aerobic conditions, and by designing a medium without fixed nitrogen (e.g. ammonia and nitrate), he would select for this organism (3, 4). Consequently, non-nitrogen fixing and anaerobic nitrogen fixing bacteria were counterselected. As evident from Beijerinck's work, it is important to know the type of metabolism and the conditions in which desired organisms grow in order to employ the enrichment culture technique.
Another key microbiologist who has impacted culture techniques is Sergei Winogradsky. The Winogradsky column was developed to create a miniature anoxic environment to enrich for anoxygenic phototrophic bacteria and sulfur lithotrophs (21). From the columns, enrichment culture techniques can be used to isolate species. This process has led to the isolation of several anoxygenic phototrophs, including purple and green sulfur bacteria.

Until the 1980's, microbiologists mainly used phenotypic, morphological, and metabolic data from a pure culture to characterize and identify new species. With the use of a microscope, different species could be identified based on their size, shape, and bacterial cytoplasmic structures, such as sulfur granules. Furthermore, they could be characterized using media and variable growth conditions based on metabolism and whether they needed oxygen to grow or not. However, recent advances in science have shown that the differences in morphology among microbial species may be too small to show distinct species (2). Many species may have been misinterpreted as another species because of the similar morphologies and metabolisms. Thus morphology and metabolism, on the scale of microorganisms, is not a sound way to classify and identify microbial species.

DNA methods currently provide more reliable means of classifying microbial species (2, 12, 16). Two important DNA methods are 16S rRNA cloning and sequencing and Denaturing Gradient Gel Electrophoresis (DGGE). 16S rRNA analysis is a labor intensive procedure that uses PCR to replicate full length (1500 bp) DNA of the 16S rRNA gene including both conserved and variable regions. This highly informative gene can then be compared to other 16S rRNA genes to infer identity (16). In order to derive DNA sequences, PCR products must be cloned into a host and screened for insert. To characterize a mixed population, a significant number of clones containing vector insert must be analyzed. A newer and less labor intensive method called DGGE uses PCR to amplify an internal 300 bp of the 16S rRNA gene. A SDS acrylamide gradient gel separates the mixed population of PCR fragments based on the percentage of guanine and cytosine (%G/C) nucleotides. In theory, every single band on a DGGE gel represents a different population member and DGGE can resolve bands that differ by only one nucleotide (16, 20).

DNA analysis has shown the limitations of what pure culture methods can retrieve. Studies have shown that only 0.25-5% of extant microorganisms are amenable to pure culture techniques (2, 12). For example, it is estimated that only 0.25% of freshwater microorganisms can be cultured (2). This suggests that existing media is inadequate, which is not surprising since microorganisms live in communities and in symbiotic relationships where independence simply may not be possible. In response, microbiologists are trying to develop new ways to retrieve and classify the remaining 99.75% of freshwater microbial species. One approach is to develop new media using habitat-specific data.

History of RLMO:
The Red Layer Microbial Observatory (RLMO) aims to characterize red filamentous bacteria that grow in alkaline mat communities throughout Yellowstone National Park (YNP). These filamentous bacteria are most closely related to *Roseiflexus castenholzii,* which is an atypical red member of the phylum Chloroflexi (formerly, Green Nonsulfur) (7). Red Chloroflexi from Yellowstone thrive in microbial mats that have three layers. The most superficial layer is inhabited by oxygenic phototrophic Cyanobacteria, while the middle layer is composed of anoxygenic phototrophic *Chloroflexus*. Below the green layer of *Chloroflexus* lies the dark red layer of the red Chloroflexi.

Due to the unique ecology and metabolism of the Chloroflexi lineage, these organisms have been scientifically interesting in evolutionary and environmental studies (5, 6, 7, 16). The anoxygenic phototrophic metabolism exhibited by Chloroflexi is thought to be similar to the first organisms on primordial earth. In addition, analysis of stromatolites, dating 3.6 billion years ago, have shown striking similarities to filamentous phototrophic bacteria that grow in mats, such as *Chloroflexus* (5).

The Chloroflexi lineage includes cultured species such as *Chloroflexus, Chloronema*, and *Oscillochloris* which are all green filamentous phototrophs that use bacteriochlorophyll (Bchl) *c* and Bchl *a.* Of these, *Chloroflexus aurantiacus* was the first to be cultured by Pierson

et. al. in 1974. Pierson used simple non-selective chemically defined medium, named medium D, with yeast extract as the carbon source. Inoculations were incubated in semi-aerobic screw cap tubes at 60˚C under incandescent light (17). Hanada first cultured *C. aggregans* in 1995 also using a simple non-selective chemically defined medium which was named medium PE using yeast extract as the carbon source. Inoculations were incubated in screw cap tubes at 55˚C under incandescent light (10).

Among the Chloroflexi lineage are unique organisms that differ from the typical green Chloroflexi. These organisms include orange *Heliothrix* and red *Roseiflexus* which are both anoxygenic phototrophs that use only Bchl *a*. Pierson *et. al*., isolated *Heliothrix oregonensis* in co-culture with *Isoshpaera pallida*. *I. pallida,* a member of the Planctomycete lineage, is a heterotrophic thermophile that was cultured before *H. oregonensis* but cohabitates with *H. oregonensis* in some Oregon hot spring mats (18). The medium used to obtain this co-culture was a simple non-selective chemically defined medium called medium IMC using glucose as the carbon source (18). Hanada cultured *R. castenholzii* in a similar manner to that of *C. aggregans* using medium PE with yeast extract as the carbon source (11). Here the inoculated media was incubated in screw cab tubes anaerobically at 50˚C. Despite the phylogenetic relatedness between *R. castenholzii* and Yellowstone red Chloroflexi bacteria, attempts at culturing the red Chloroflexi bacteria using medium PE and *Roseiflexus* methods have been unsuccessful (7). Furthermore, attempts at cultivating red Chloroflexi using Pierson media methods and a wide variety of carbon sources have failed at retrieving a pure culture (5). This suggested that new methods needed to be developed in order to improve retrieval of red Chloroflexi bacteria.

My Project:
The goal of this project was to develop and examine alternative media that would promote the culture of RLMO bacteria. Instead of using broad-based media which were used to culture other phototrophic Chloroflexi, I hypothesized that designer medium based on site specific chemical parameters measured in the mat would better lend to the cultivation of the red Chloroflexi bacteria. These mat chemistry data were determined using ampoule based tests and a portable colorimeter according to the manufacturer's protocol (Hach DR/890). Hillside Springs, see Figure 1, is located on a prominent hillside between the Biscuit and Black Sand Basins (GPS coordinates: N 44.28.52, W 110.51.98). Mat pH was measured using colorpHast pH 5-10 pH indicator strips. Temperatures were measured using a Traceable universal digital thermometer (Friendswood, TX). Figure 1 and Table 1 shows Hillside Spring and summarizes the mat chemical data.

Figure 1: Hillside Springs; red layer was 52.2˚C, pH 9.

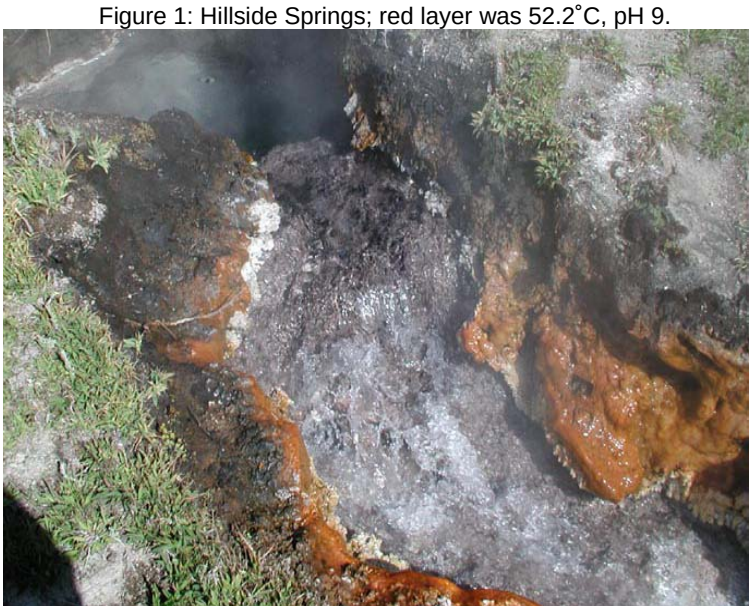


Table 1: Hillside Mat Chemistry

Chemical	Amount (mg/L)
Zinc	18.75
Molybdenum	22.5
Total Chlorine	2.25
Copper	3
Manganese	1.2
Hydrogen Sulfide	3
Sulfate	75
Aluminum	18.53
Silica	88.5
Total Iron	6
Ferrous Iron	0
Nitrate	105
Reactive Phosphorus	21
Calcium Hardness	1.5
Magnesium Hardness	1.5

Given observed chemical data, I set out to make site-specific media and compare it with medium PE and sterilized source water addressing the following hypotheses:

Hypothesis A	***Hypothesis B***	***Hypothesis C***
The growth of red Chloroflexi bacteria is dependent on site-specific chemical differences at each particular hot spring.	A different array of microbial isolates will be retrieved using each medium.	Media made based on site-specific chemical data will support more diversity than broad-based media (e.g. PE).

MATERIALS AND METHODS

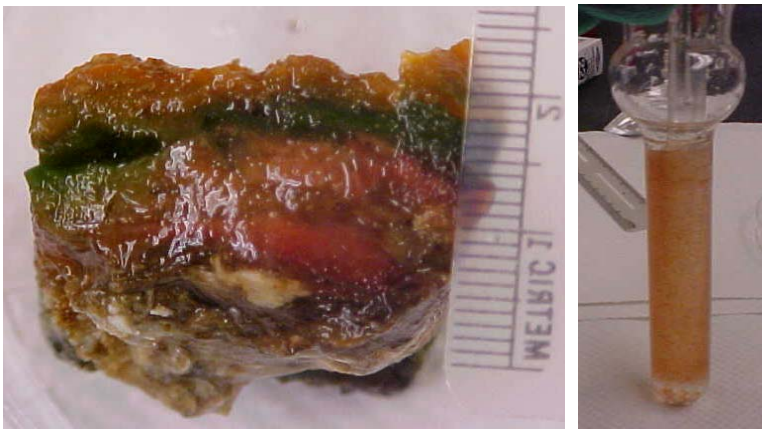
Media Design:
Based on previous attempts to cultivate the RLMO bacteria, four media were tested: medium PE, site specific high yeast concentration (SSH), site specific low yeast concentration (SSL), and sterile source water (SSW). Medium PE was prepared according to the Hanada recipe (10). Site specific medium was prepared using Hillside mat chemical data from 2002 (Table 1). Table 2 shows the amount and source of each chemical used to mimic the chemical data. Since mat chemistry analyses did not include an accurate test for carbon, the carbon source and concentration present in the mat for the red Chloroflexi is not known. To compensate, yeast extract was used at two concentrations 1.0 g/L (SSH) and 0.01 g/L (SSL). In addition to SSH and SSL, 2 L of geothermal source water was collected on site and sterilized, providing the fourth medium (SSW). Collection of SSW was executed by dipping a sterile funnel that had 8 ft of sterile

clear tubing into the Hillside source, collecting 2 L in the sterile bottles. For each kind of media, both solid and liquid media was prepared. The solid media was prepared by adding 15 mg of granulated agar for each liter of medium.

Table 2: Site Specific Medium

Chemical	Amount Needed (mg), Source
Mg	7.43 $MgSO_4$
Ca	49.3 $CaCO_3$, $CaCl_2$
Sulfide	22.56 Na_2S—9 H_2O
Chlorine	4.73 $CaCl_2$
Nitrate	123.90 KNO_3, NH_4NO_3, $FeNO_3$
Sulfate	75 $MnSO_4$, $MgSO_4$, $CuSO_4$, $Al_2(SO_4)_3$
Silica	92 mL – 85% Silica Solution
Ammonia	13.04 NH_4 NO_3
Phosphorus	3.4 mL KH_4PO_4 Solution
Molybdenum	56.7 Na_2MoO_4
Aluminum	175.24 $Al_2(SO_4)_3$, $AlPO_4$
Iron	25.97 $FeNO_3$
Manganese	3.6 $MnSO_4$
Zinc	36.06 $ZnCO_3$
Copper	11.81 $CuSO_4$—5 H_2O

Figures 2 and 3: Mat and Mat Homogenate



Mat Collection and Preparation:
A sterile scalpel was used to cut a 1 inch square sample of mat (Figure 2) that, upon excision, had a visible red layer. The samples were placed in clear screw top Nalgene cups and placed on dry ice for transport back to Monmouth, Oregon. The environmental sample was carefully dissected after the 16 hour traverse to the WOU lab using sterilized forceps and scalpels. 0.1g of dissected red layer was diluted in 10 ml of sterile water. The sample was then homogenized using a sterile mortar and pestle (Figure 3). Of this original dilution, 5 ul were placed on a glass slide for microscopic analysis.

Inoculation:
10 ml homogenized red layer was then added to 40 ml of double distilled water. Of this 50 ml solution, 1 ml was inoculated into a tube of each test media. These tubes were then diluted 10 fold 8 times, with 2 replicates for each dilution. Each final sample dilution was grown in 10 ml tubes with screw tops with 5 ml of the respective medium. In order to mimic the environmental conditions of the red Chloroflexi, tubes were grown anaerobically using Bio-Bag Environmental Chamber Type A™ bags sealed using Seal-a-Meal™ vacuum machine (provided my grandmother). All bags were placed in a lighted incubator at the environmental mat temperature of 50˚C. Each week for four weeks a sample from each medium was photo-documented and collected for further analysis. 0.2 ml of the first weeks growths were plated on the respective solid media.

Sample Processing:
A replicate of the 10^{-1} sample dilution from each medium was chosen at the week 1 and week 4 time points to create 16S rRNA libraries and to undergo DGGE analysis. This was done to compare initial growth versus final growth. Samples were chosen for further processing based on the extent of visible growth with an emphasis on red filamentous growth.

Pigment Analysis and Light Microscopy:
Pigment analysis was performed on liquid SSW growth after 14 days of growth and on solid SSW growth after 54 days of growth. *In vivo* absorption spectra of pigments were performed on homogenized suspensions of each growth as previously described (7). Methanol absorption spectra were performed on homogenized suspensions of each growth as previously described (7). All microscopy was performed with an Olympus BX41 with a DP11 digital camera system. Microscopy was used to identify filamentous growth and to decide which enrichments to pass from liquid media to solid media (7).

DNA Analysis:
Genomic DNA extraction was performed on the samples illustrated in Table 3 and on the final solid media subculture for 16S rRNA library and DGGE analysis. All extractions were done using a phenol-chloroform based extraction as previously described (7). 16S rRNA libraries were made from the 10^{-1} dilution from each medium at the week 1 and week 4 time points. PCR amplifications were done using Promega Mastermix PCR Optimization Kit (Epicentre Technologies, Madison, Wis.). Each reaction was composed of the following mix: 25 ul of MasterMix; which contains a 2X buffer, Taq polymerase, and DNA monomers, 22 ul of water, 1 ul of 8FPL forward primer, 1 ul of 1492 RPL reverse primer, and 1 ul of extracted DNA (7, 19). The reactions were set up using UV sterilized plasmid free materials. Thermal cycling was carried out as previously described using an Eppendorf Mastercycler with 35 cycles as follows: 1 min at 94˚C for denaturation, 1 min at 60˚C for annealing, and 3 min at 72˚C for extension (7). The amplified products were cloned as previously described (7).

PCR amplifications for DGGE were composed using the same genomic and plasmid free materials as above. For DGGE, instead of the full length 16S primers, the following DGGE internal 16S primers were used: 1070F and U1392GC (9). Thermal cycling was performed in the following steps: 1 min denaturing at 94˚C, 1 min of annealing, and 3 min of primer extension at 72˚C (9). PCR products were separated using DGGE with a linear gradient of urea and formamide (35 to 80%) in a 6% acrylamide gel at 180 V and 60˚C for 22 hours (9). Gels were analyzed after ethidium bromide staining using Foto/Analyst Investigator PCImage software (Fotodyne Inc.). *Roseiflexus* and *Chloroflexus* controls were run as references. For resulting DGGE bands that did not correspond with *Chloroflexus* or *Roseiflexus* controls, “touch PCR” followed by sequence analysis was used. This method was executed by viewing the ethidium-stained gel under

UV light, which illuminated the DGGE bands. A sterile pipette tip was touched to the corresponding bands and then placed in the PCR cocktail described above (9). Thermal cycling and cloning were also done as above for full-length 16S rRNA (7).

Nucleotide sequence analysis was performed on all final 16S rRNA library products as previously described using a Li-Cor 4200 Gene ReadIR Single Dye system (Li-Cor Inc., Lincoln, Ne)(7). Sequence analysis was executed using e-Seq V2.0 (Li-Cor Inc., Lincoln, Ne). Edited and trimmed sequences were then compared to the National Center for Biotechnology Information (NCBI) database by using the Basic Local Alignment Search Tool (BLAST) to infer possible identity of sequences (1).

RESULTS

Liquid Enrichment Results:
Figure 4 shows the growth of each respective medium dilution series at week 1 and week 4 time points. Figure 5 provides a close up of the growth of each respective medium at the week1 and week 4 time points. In weeks 1 and 4, SSH, PE, and SSW all showed dark red growth. The SSW growth was dense with filaments migrating up the side of the tube. The SSL medium had green growth. Medium PE showed a layer of film growing at the meniscus of the medium in the tube (see Figure 5; PE week 4 10^{-1} dilution). *In vivo* pigment analysis of the liquid SSW media showed absorption maxima for Bchl *c* at 741 nm. The methanol extraction yielded maxima for Bchl *a* at 767 nm, and for Bchl *c* at 668.

Figure 4: Liquid Dilutions at Weeks 1 and 4 With Close-Up of 10^{-1} Dilution

	Week 1 Dilution Series; Close-Up10^{-1} Dilution	Week 4 Dilution Series
SSH		
SSL		
PE		
SSW		

DNA Analysis:
All 16S rRNA sequence analysis data is summarized in Table 4 and further described in Appendix 1. 16S rRNA analysis of the liquid enrichment media retrieved no red Chloroflexi sequences.

Table 4: Summary of Full-Length Bacteria 16S rRNA Results

Medium	Week 1		Week 4	
	% Clones, Phylum	Significance	% Clones, Phylum	Significance
SSH	100% Gram Positive	Thermophilic Chemotroph	84% Gram Positive 8% Proteobacteria 8% Unknown Artesian Basin	Thermophilic Chemotroph Thermophilic Environmental Thermophilic Australian Springs
SSL	37.5% Acidobacter 37.5% Nitrospira 25% Unknown Octopus	Anaerobic Environmental Aquatic Environmental Thermophilic, Yellowstone	Not Done	
PE	100% Gram Positive	Thermophilic Isolates	43% Proteobacteria 24% Nitrospira 24% Gram Positive 9% Candidate OP10	Thermophilic Environmental Aquatic Environmental Thermophilic Thermophilic, Yellowstone
SSW	42% Spirochete 32% Acidobacter 16% Unknown Octopus 5% NKB19 5% Proteobacteria	Anaerobic Environmental Hydrothermal Enviromental Thermophilic, Yellowstone Environmental Thermophilic Environmental	35% Nitrospira 20% Candidate OP10 15% Proteobacteria 10% Chloroflexi 10% CFB 5% Acidobacter 5% Unknown	Thermophilic Microbial Mats Thermophilic, Yellowstone Thermophilic Phototrophic Green Chloroflexi, Yellowstone Anaerobic Thermophilic Hydrothermal Enviromental Environmental

DGGE results are summarized in Figure 5. Bands from the enrichment samples in the DGGE gel did not match the *Chloroflexus* and *Roseiflexus* controls; therefore, touch PCR was used to identify the populations represented by the bands of the gel. Figure 5 indicates which DGGE bands were sequenced and what the results indicated. In the Figure 5 gel, A = *Chloroflexus* control; B = SSH-Week 1; C = PE-Week 1; D = SSW-Week 1; E = SSH-Week 4; F = PE-Week 4; G = SSW-Week 4; H = *Roseiflexus* control.

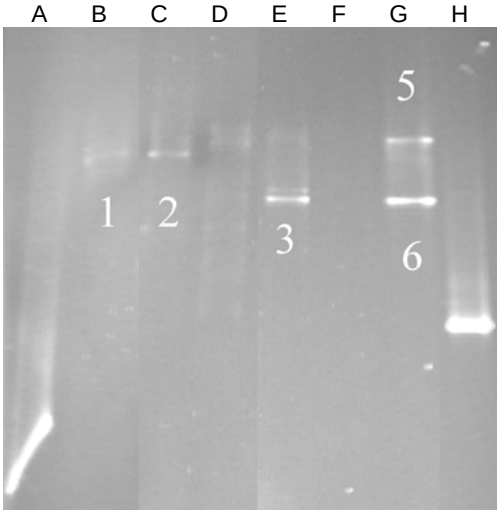
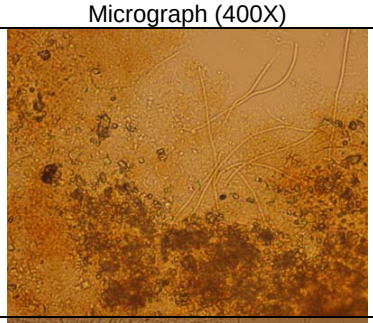
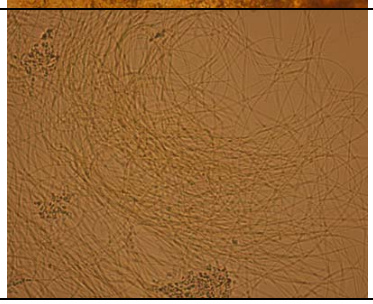


Figure 5: DGGE and Band Sequencing Results

Band	Sequence Analysis
1: SSH week 1	Proteobacteria: 10 Gram Positive: 1
2: PE week 1	Green Chloroflexi: 1 Gram Positive: 3 Proteobacteria: 8
3: SSH week 4	Green Chloroflexi: 2 Proteobacteria: 1 Gram Positive: 3 Red Chloroflexi: 2
4: SSW week 4	Proteobacteria: 7
5: SSW week 4	Green Chloroflexi: 1 Proteobacteria: 7
6: SSW week 4	Green Chloroflexi: 1 Proteobacteria: 4 Red Chloroflexi: 1

Solid Medium Results:
The enrichment with the most visible red Chloroflexi like growth was liquid SSW week 3. Therefore, the liquid 10^{-1} dilution of SSW week 3 was inoculated onto the solid media. Figure 6 compares solid medium growth at 14 days and at the final 54 days and shows representative microscopy photos of the solid growth. The growth was lighter orange then the typical red layer, but the growths were filamentous and did not fluoresce, a sign of red Chloroflexi Bchl *a*. DNA analysis at 54 days showed these enrichments were 56% green *Chloroflexi* and 44% *Anoxybacillus*. The micrograph of the 54 day sample in Figure 8 illustrates the filamentous *Chloroflexus* and the rod *Anoxybacillus* morphologies.

Figure 6: Solid Media Attempts and Results

Time Point	Plate	Micrograph (400X)
14 Days Growth		
54 Days Growth		

DISCUSSION

Project hypotheses that this discussion will address are as follows:

Hypothesis A	***Hypothesis B***	***Hypothesis C***
The growth of red Chloroflexi bacteria is dependent on site-specific chemical differences at each particular hot spring.	A different array of microbial isolates will be retrieved using each medium.	Media made based on site-specific chemical data will support more diversity than broad-based media (e.g. PE).

The array of 16S rRNA genes obtained from each liquid medium supports hypothesis C. In week 1, the chemically derived media SSL showed more diversity than medium PE. The SSW media showed the most diversity at week 1, with 5 different phyla represented. Of these, most were thermophilic species of each phylum. SSW media is more similar to the chemically derived media than to medium

PE. Thus it too should be considered as supporting the hypothesis that site-specific chemically derived media would show more diversity than medium PE. For all media, the week 4 results show an increase in diversity from week 1. Of all the liquid enrichments, only SSW had full-length 16S rRNA Green Chloroflexi sequences.

Phenotypically, the four media differed greatly in retrieved growth. SSH showed orange colored growth, SSL showed green growth, medium PE showed red growth, while SSW showed dark red growth. Since SSW growth appeared most similar to the red layer mat growth, a sample from week 2 was plated. Pigment analysis showed Bchl *a* and *c*, and 16S rRNA sequence data showed that the plate subculture was *Chloroflexus* and *Anoxybacillus*.

16S rRNA sequences retrieved from liquid enrichments supported hypothesis B that a different array of isolates would be found on the different media. SSH and medium PE showed similar isolates (mostly dominated by thermophilic Gram Positives) probably because of their similar yeast concentration. Yet not all of their isolates were the same. SSH showed novel representatives from unknown bacteria from the Artesian Basin while medium PE showed Nitrospira and Candidate OP10 representatives. Interestingly, SSW and SSL media showed no Gram Positive isolates. SSW and SSL media showed thermophilic, anaerobic, and YNP derived representatives. As Table 4 shows, the representatives from SSW and SSL tend to be more closely related to hot spring microbial mat species than media SSH and PE. The lower yeast concentration of SSW and SSL probably accounts for this.

The only evidence of red Chloroflexi in any enrichment was found using DGGE analysis. SSH media showed Gram Positive, red Chloroflexi, and green Chloroflexi sequences. Medium PE showed Proteobacteria, Gram Positive, and green Chloroflexi sequences, while SSW media showed Proteobacteria, green Chloroflexi, and red Chloroflexi sequences. Interestingly, both site-specific media (SSH and SSW), showed red Chloroflexi sequences, while non-site-specific medium PE did not supporting hypothesis A.
Another noteworthy finding is that full-length 16S rRNA analysis of the liquid media enrichments showed different representatives than those from the DGGE bands. The most significant difference is that the DGGE analysis retrieved red Chloroflexi representatives, while the full length 16S rRNA analysis did not. This is probably because each primer set amplifies a different region of the 16S rRNA gene and likely has some unique biases.

The lack of comparable DGGE results for all media in week 1 and 4 is a problem for this project. It is hard to make conclusive arguments without consistent and comparable data. Recent unpublished data from the Boomer lab has shown that this problem may be due to inefficient cell lysis. Phenol chloroform lysis/extraction alone has been shown to be less effective than combining this method with bead beating. At the time of this project, a bead beating apparatus was not available.
A second problem presented by this project is that, in theory, one band in the DGGE gel is supposed to be composed of one species. Upon sequence analysis of the DGGE touch PCR, this was not found to be so. Instead, each band was composed of from 1 to 4 species. Since DGGE works by separating DNA based on the G/C content, perhaps the species retrieved have very similar G/C contents. Furthermore, DGGE methods in general were problematic in this project, so incorrect voltage, gradient selection, or other method parameters may account for this finding.

If red Chloroflexi bacteria are not part of the hypothesized 99.75% of freshwater bacterium that cannot be cultured, future attempts to culture this microbe should clearly be based on site specific chemistry. The 16S rRNA comparison data supporting hypotheses A and B suggest red Chloroflexi growth is dependent on the chemistry of each site. The key to improving site-specific media would be to better understand the carbon source and concentration used by the red Chloroflexi. Changes in inoculation procedures and physical conditions could also improve enrichment of red Chloroflexi. For example, enrichment of red Chloroflexi may be aided by pre-filtering the inoculum as to select for only larger filaments. This physical manipulation would decrease the competition for red Chloroflexi to flourish and provide a more red Chloroflexi concentrated inoculums. Lastly, one possible method would be to devise an environment in the lab that would provide a constant cycling and splashing of the source water or site-specific media on the mat.

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Appendix 1: Full Length 16S Clones Submitted to GenBank

Week 1			
Accession	**Clone**	**Medium**	**Inferred Identity**
AY508232	HH11_1	SSH	Caloramator
AY508233	HH11_8	SSH	Caloramator
AY508234	HH11_9	SSH	Caloramator
AY508239	HH11_2	SSH	Caloramator
AY508242	HH11_3	SSH	Caloramator
AY508243	HH11_4	SSH	Brevibacillus
AY508245	HH11_6	SSH	Caloramator
AY508246	HH11_7	SSH	Caloramator
AY508248	HC21_1	PE	Clostridium
AY508249	HC21_2	PE	Clostridium
AY508250	HC21_3	PE	Clostridium
AY508251	HC21_4	PE	Clostridium
AY508252	HC21_5	PE	Clostridium
AY508253	HC21_6	PE	Clostridium
AY508254	HC21_7	PE	Clostridium
AY508255	HC21_8	PE	Clostridium
AY508256	HC21_9	PE	Clostridium
AY508257	HC21_10	PE	Caloramator
AY508269	HW21_1	SSW	Spirochete
AY508270	HW21_2	SSW	Alphaproteobacteria
AY508271	HW21_3	SSW	Spirochete
AY508272	HW21_4	SSW	Acidobacterium
AY508273	HW21_5	SSW	Alphaproteobacteria
AY508274	HW21_6	SSW	Eubacterium
AY508275	HW21_7	SSW	Alphaproteobacteria
AY508276	HW21_8	SSW	Spirochete
AY508277	HW21_9	SSW	Alphaproteobacteria
AY508278	HW21_10	SSW	Alphaproteobacteria
AY508279	HW21_11	SSW	Eubacterium
AY508280	HL21_1	SSL	Nitrospira
AY508281	HL21_2	SSL	Acidobacteria
AY508282	HL21_3	SSL	Nitrospira
AY508283	HL21_4	SSL	Nitrospira
AY508284	HL21_5	SSL	Acidobacteria
AY508285	HL21_6	SSL	Eubacterium
AY508286	HL21_7	SSL	Eubacterium
AY508348	HC21_11	PE	Clostridium
AY508349	HC21_12	PE	Clostridium
AY508350	HC21_13	PE	Clostridium
AY508351	HC21_14	PE	Clostridium
AY508352	HC21_15	PE	Clostridium
AY508353	HC21_16	PE	Clostridium
AY508354	HC21_17	PE	Clostridium
AY508355	HC21_18	PE	Clostridium
AY508356	HW21_12	SSW	Eubacterium
AY508357	HW21_13	SSW	Deltaproteobacteria
AY508358	HW21_14	SSW	Spirochete
AY508359	HW21_15	SSW	Eubacterium
AY508360	HW21_16	SSW	Proteobacteria
AY508361	HW21_17	SSW	Alphaproteobacteria
AY508362	HW21_18	SSW	Eubacterium
AY508363	HW21_19	SSW	Spirochete
AY508364	HW21_20	SSW	Spirochete
AY508365	HH11_10	SSH	Caloramator

AY508366	HH11_11	SSH	Caloramator
AY508367	HH11_12	SSH	Caloramator
AY508368	HH11_13	SSH	Brevibacillus
AY508370	HH11_15	SSH	Caloramator
AY508371	HH11_16	SSH	Caloramator
AY508375	HL21_8	SSL	Eubacterium
AY508376	HL21_9	SSL	Eubacterium
AY508377	HL21_10	SSL	Eubacterium
AY508378	HL21_11	SSL	Acidobacteria
AY508379	HL21_12	SSL	Nitrospira
AY508380	HL21_13	SSL	Eubacterium
AY508381	HH11_17	SSH	Caloramator
AY508382	HH11_18	SSH	Caloramator
AY508383	HH11_19	SSH	Caloramator
AY508384	HL21_14	SSL	Eubacterium
AY508385	HL21_15	SSL	Acidobacteria
AY508386	HL21_16	SSL	Eubacterium
AY508387	HC21_19	PE	Clostridium
AY508388	HC21_20	PE	Clostridium
AY598369	HH11_14	SSH	Caloramator

Week 4			
Accession	**Clone**	**Medium**	**Inferred Identity**
AY508235	HH81_1	SSH	Bacillus
AY508236	HH81_2	SSH	Clostridium
AY508237	HH81_3	SSH	Eubacterium
AY508238	HH81_4	SSH	Clostridium
AY508240	HH81_5	SSH	Betaproteobacteria
AY508241	HH81_6	SSH	Clostridium
AY508247	HH81_7	SSH	Clostridium
AY508258	HC71_1	PE	Candidate OP10
AY508259	HC71_2	PE	Candidate OP10
AY508260	HC71_3	PE	Betaproteobacteria
AY508261	HC71_4	PE	Betaproteobacteria
AY508262	HC71_5	PE	Clostridium
AY508263	HC71_6	PE	Clostridium
AY508264	HC71_7	PE	Eubacterium
AY508265	HC71_8	PE	Nitrospira
AY508266	HC71_9	PE	Nitrospira
AY508267	HC71_10	PE	Candidate OP10
AY508268	HC71_11	PE	Clostridium
AY508298	HW71_3	SSW	Nitrospira
AY508299	HW71_4	SSW	Candidate OP10
AY508300	HW71_5	SSW	Candidate OP10
AY508308	HW71_1	SSW	Chloroflexi-green
AY508309	HW71_20	SSW	Candidate OP10
AY508310	HW71_2	SSW	Eubacterium
AY508311	HW71_6	SSW	Nitrospira
AY508312	HW71_7	SSW	Eubacterium
AY508313	HC71_12	PE	Nitrospira
AY508314	HC71_13	PE	Betaproteobacteria
AY508315	HC71_14	PE	Nitrospira
AY508316	HC71_15	PE	Betaproteobacteria
AY508317	HC71_16	PE	Betaproteobacteria
AY508318	HC71_17	PE	Nitrospira
AY508319	HC71_18	PE	Clostridium
AY508320	HC71_19	PE	Betaproteobacteria
AY508321	HC71_20	PE	Betaproteobacteria
AY508322	HW71_8	SSW	Deltaproteobacteria
AY508323	HW71_9	SSW	Deltaproteobacteria
AY508324	HW71_10	SSW	Chloroflexi-green
AY508325	HW71_11	SSW	Eubacterium
AY508326	HW71_12	SSW	Candidate OP10
AY508327	HW71_13	SSW	Eubacterium
AY508328	HW71_14	SSW	Eubacterium
AY508329	HW71_15	SSW	Nitrospira
AY508330	HW71_16	SSW	Nitrospira
AY508372	HH81_8	SSH	Eubacterium
AY508373	HH81_9	SSH	Clostridium
AY508374	HH81_10	SSH	Clostridium
AY508393	HC71_21	PE	Clostridium
AY508394	HC71_22	PE	Betaproteobacteria
AY508395	HW71_17	SSW	Nitrospira
AY508396	HW71_18	SSW	Chloroflexi-green
AY508397	HW71_19	SSW	Candidate OP10
AY508398	HH81_11	SSH	Clostridium
AY508399	HH81_12	SSH	Betaproteobacteria
AY508400	HH81_13	SSH	Clostridium
AY508401	HH81_14	SSH	Clostridium
AY508402	HH81_15	SSH	Clostridium
AY508403	HH81_16	SSH	Clostridium