BIOLOGY OF ACID-SULFATE-CHLORIDE SPRINGS IN YELLOWSTONE NATIONAL PARK, WYOMING, UNITED STATES OF AMERICA

by

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ABSTRACT

This dissertation investigated the role of biology in several biogeochemical cycles in acid sulfate chloride (ASC) geothermal springs in Yellowstone National Park (YNP). Elemental sulfur (S°) is associated with many geothermal springs, yet little is known about the organisms involved in its cycling. The aqueous and solid phase geochemistry near the source of Dragon Spring, an ASC spring in the Norris Geyser Basin (NGB) of YNP, was used to guide the enrichment and isolation of two novel S°-reducing Crenarchaeota affiliated with the order *Desulfurococcales*. Both isolates are chemoorganotrophs, dependent on complex peptide-containing carbon sources, S°, and anaerobic conditions for respiration-dependent growth. Physiological characterization suggests the isolates are adapted to the physicochemical conditions of Dragon Spring which is supported by quantitative PCR analysis which indicates that the isolates represent a significant fraction of the microbial community associated with S° precipitates in several ASC geothermal springs in the NGB in YNP. Both isolates are capable of utilizing naturally-occurring, complex forms of carbon as a carbon and energy source, and naturally-formed S° as terminal electron acceptor for respiration-dependent growth, suggesting a role for these microbes in the biological cycling of carbon and sulfur in these environments.

Our understanding of the flow of carbon, energy, and other materials between microbial producer species and heterotrophic consumers is limited, in particular in geothermal systems. Novel invertebrate larvae related to *Odontomyia* sp. (Stratiomyidiae: Diptera) were observed grazing microbial mat biomass in several ASC geothermal springs in the NGB. DNA-based methods were used to demonstrate that stratiomyid larvae graze interstitial algal populations within the mat biomass. Results also indicate that the biomass grazed by larvae contained elevated levels of mono-methylated Hg (MeHg) and total mercury (THg). As a consequence of grazing the mat biomass, larvae biomagnified MeHg in their tissues at 2.7- to 5.5-times the concentrations measured in mat biomass. The results of this analysis indicate the Killdeer contained MeHg at a concentration 4.64-fold greater than larval tissue. Collectively, this data suggests that larval grazing behavior represents a key pathway for the transfer of MeHg to species within a geothermal mat-based food web.

CHAPTER 1

THE SCOPE OF THE THESIS

The goal of this thesis was to investigate the role of biology in a variety of understudied geochemical cycles in ASC geothermal springs. Chapter 2 introduces organisms that have been identified in ASC geothermal springs in the Norris Geyser Basin of Yellowstone National Park and introduces the putative role that these organisms may have in the ASC geothermal ecosystems. The primary objective of the work presented in chapter 3 was to identify the organisms involved in reductive S° cycling in the source waters of ASC geothermal springs. S° forms in copious amounts near the source of many ASC springs in YNP. Despite the prevalence of S° solid phase in ASC geothermal springs in YNP, little is known concerning its cycling. Microbes have been shown to be important in the cycling of sulfur compounds in a variety of other aquatic environments. Thus, it was hypothesized that microbes were at least partially responsible for catalyzing the cycling of S° in ASC springs. To identify and characterize ecologically-relevant microbes of potential importance in S° reduction, enrichment media was designed based on the aqueous and solid phase chemistry of several ASC springs in NGB. Through this approach, two novel S°-reducing *Crenarchaea* were isolated.

The second objective of this thesis was to determine the ecological relevance of these *Crenarchaeal* isolates in terms of S° and carbon cycling within the source water S° precipitate-associated community in ASC geothermal springs. To address this question, isolated *Crenarchaea* were subjected to a variety of physiological and genetic assays to

identify their putative role and relevance in the cycling of S° and carbon in ASC environments. Both isolates appeared to be physiologically adapted to the geochemical conditions present in the source waters of various ASC geothermal springs in NGB. Analysis of the abundance of the organisms indicated the isolates represent a significant fraction of the S° precipitate associated-microbial community. In addition, both isolates were capable of growth on naturally-occurring complex carbon sources and naturally-formed S°. Collectively, these data suggest a role for these microbes in the cycling of carbon and sulfur in these environments.

The primary objective of the work presented in Chapter 4 was to identify and characterize novel trophic level interactions that occur in phototrophic mat communities. Previously undocumented invertebrate larvae were observed in the phototrophic portions of ASC geothermal springs throughout the NGB. Since larvae appeared to inhabit autotrophic microbial mats, it was hypothesized that invertebrate larvae were utilizing the mat biomass as a food source and in the process, contributing to the cycling of carbon in these environment. DNA-based methods were used to identify the larvae, the autotrophic populations within the foregut of larvae, and the autotrophic mat populations within the phototrophic mat community. A comparison of the populations in the foregut of larvae with those inhabiting the microbial mat indicated that larvae were grazing algal populations within the microbial mat biomass and suggested a role for larvae in the cycling of carbon in the phototrophic mat environments.

The second objective of the work presented in chapter 3 was to identify the possible implications of microbial mat grazing to higher trophic structures in terms of

MeHg biomagnification in the phototrophic zone food web. THg and MeHg are elevated in concentration in phototrophic mat biomass and previous studies have shown that consumption of MeHg-containing biomass can lead to biomagnification of MeHg. Thus, it was hypothesized that larvae, as a consequence of consuming MeHg-containing microbial mat biomass as a food source, would contain MeHg at a concentration greater than that determined in the microbial mat biomass as a result of biomagnification. Examination of tissue recovered from larvae revealed MeHg concentrations that were greater than that determined in microbial mat biomass, indicative of biomagnification. Avian populations morphologically resembling *Charadrius* sp. were observed preying upon larvae in ASC springs in the NGB, suggesting that these organisms may also biomagnify MeHg. Analysis of a feather discarded by a *Charadrius* sp. observed grazing larvae revealed MeHg concentrations greater than determined in the larvae, suggesting the MeHg biomagnification extends to higher trophic levels in ASC food webs. In addition, since stratiomyid larvae appear to be important herbivores in ASC geothermal springs of NGB, it is likely that their grazing behavior represents a key pathway for the transfer of MeHg to species that range beyond this particular geothermal system to other areas within YNP and beyond.

In summary, this dissertation highlights the importance of biology in the geochemical cycling of elements such as sulfur, carbon, and mercury in ASC geothermal springs. In addition, this body of work demonstrates the value of field-scale observations in generating and testing hypotheses that are of ecological significance.

CHAPTER 2

BIOLOGY OF ACID-SULFATE-CHLORIDE GEOTHERMAL SPRINGS

LITERATURE REVIEW

Introduction to ASC Springs

Acid sulfate chloride (ASC) geothermal springs are common features in the Yellowstone National Park (YNP), Wyoming, USA geothermal complex (69). ASC springs are named for their distinctive chemical signature consisting of low pH and high

Figure 2.1. Beowulf Spring, NGB, YNPillustrating the transition of the sulfur zoneand the iron/arsenic zone. Photo by E. Boyd, October 20, 2005.

concentrations of chloride and sulfate (66, 68, 69, 88, 113, 152). The low pH of ASC springs likely results from the oxidation of reduced sulfur species (hydrogen sulfide, elemental sulfur) near the spring surface or the hydrolysis of SO_2(g), both of which yield sulfuric acid (152). The acidic geothermal water then mixes with chloride-rich water

yielding the signature ASC spring chemistry (69, 113). ASC spring source waters in the Norris Geyser Basin (NGB) geothermal area typically contain nanomolar concentrations of H_2(aq), millimolar concentrations of CO_2(aq), and micromolar concentrations of H_2S(aq). In addition to the aforementioned dissolved gases, ASC spring waters typically contain micromolar concentrations of dissolved organic carbon, $Fe^{(II)}$, and $As^{(III)}$; all of which can support microbial respiration (66, 68, 69, 88, 113).

As geothermal source water effluent flows down the channel draining the stream, it slowly cools creating a thermal gradient. In addition, geochemical gradients are created from biological and abiological chemical transformations. For example, as ASC geothermal spring source water flows down the first few meters of an effluent channel, H_2S is oxidized which results in a gradient in both H_2S concentration and S° (resulting from H_2S oxidation) (69, 88, 113). Following the sulfur depositional zone, $As^{(III)}$ and $Fe^{(II)}$ are oxidized, corresponding to the deposition of an amorphous brown ferric oxyhydroxide solid phase containing sorbed arsenate (Figure 2.1) (88). At approximately 46°C, the brown mat transitions to a vibrant green photosynthetic mat which at 32°C transitions to a purple mat, which most likely consists of phototrophic organisms (21, 88).

The changes in aqueous phase chemistry and solid phase chemistry as ASC source water flows down the thermal transect extending from the source are well-documented in many ASC springs in the NGB (69, 88, 98). The focus of the current review is to assess the changes in microbial composition and physiology that accompany or directly influence the changes in geochemistry of ASC springs in the NGB. The vast majority of research conducted in NGB ASC springs to date has included 16S rRNA gene

diversity surveys. While it is acknowledged that inferring physiology from 16S rRNA gene phylogeny is inherently biased (74), the putative physiology and identity of all organisms reported in this literature review will be inferred from the physiology of the closest cultivated 16S rRNA gene relative, unless otherwise stated.

The biota subject to the current review were primarily detected in three ASC springs located in the One Hundred Springs Plain of the NGB: Beowulf Spring (NHSP35; lat 44°43'53.4"N, long 110°42'40.9"W), Succession Spring (lat 44°43'75.7"N, long 110°42'74.7"W), and Dragon Spring (NHSP106; lat 44°43'54.8"N, long 110°42'39.9"W). Detailed descriptions of the geochemistry of Beowulf Spring (68, 69), Succession Spring (69, 98), and Dragon Spring (69, 88) have been described previously and thus will not be reported in this review unless in the context of a particular physiology.

Finally, the biota presented in this review will be divided into four sections: the sulfur zone, the iron/arsenic zone, the phototrophic zone, and ASC food webs (Figure. 2.1). A brief description of important physiologies that may be involved in geochemical cycles that may be important in each zone (food webs are discussed in context of nutrient cycling) will be briefly introduced followed by a description of the prominent organisms that have been identified in each zone. For organizational purposes, organisms present in each zone will be divided into classes based on spring-relevant physiologies such as S°-reduction and H_2S oxidation in the sulfur zone, etc. Following the description of organisms within each physiological class, a synthesis of the community comprising each zone will be presented and discussed in relation to biogeochemical cycling.

Sulfur Zone

Introduction

The natural reduction and oxidation of sulfur compounds is known as the sulfur cycle, which consists of three common oxidation states: sulfide (S^{2-}), elemental sulfur (S°), and sulfate (S^{6+}) (4). The importance of microorganisms in sulfur cycling is evident by the multitude of organisms that utilize either oxidized or reduced sulfur compounds in

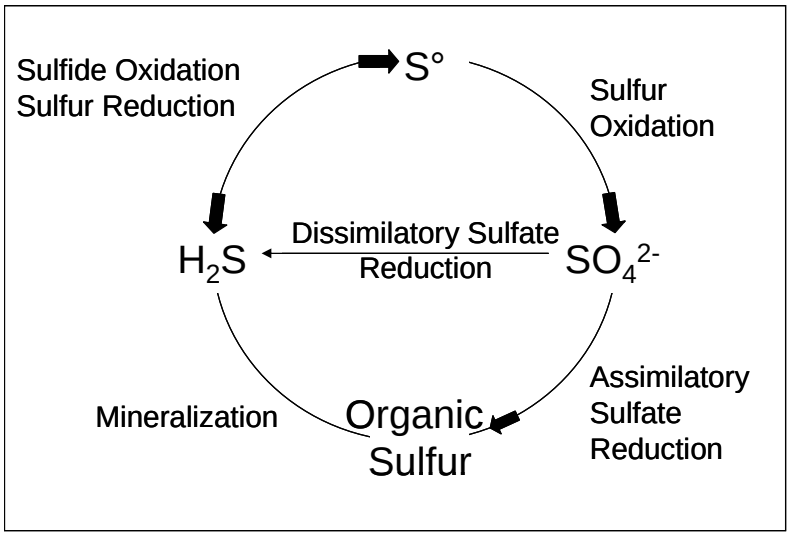

Figure 2.2. Key reactions in the sulfur cycle. Modified from Kleinjan et al (84).

their metabolism (Figure 2.2). Sulfide serves as an energy source for a diversity of heterotrophic and chemo(litho)- and photo-trophic microorganisms distributed throughout the *Archaea* and *Bacteria*. In marine hydrothermal environments, the majority of primary production is driven by the oxidation of H_2S, yielding S° (4). Similarly, recent studies suggest H_2S to be important in primary productivity in ASC

geothermal environments (37), which presumably also generate the metabolic by-product S°.

S° can be utilized both as an energy source by organisms such as *Thiobacillus* (108) and *Sulfolobus* (24) or as a terminal electron acceptor for organisms such as *Desulfuromonas* (117) and *Pyrodictium* spp. (139). S° enhances the growth rate of a variety of thermophilic or hyperthermophilic *Bacteria* and *Archaea* that ferment monomeric or polymeric organic carbon sources (4, 5, 138). In such cultures, it has been suggested that S° serves as an additional electron sink and the observed growth enhancement is attributable to decreased hydrogen production (50) which is often inhibitory to fermentative organisms (90). The versatility of elemental sulfur in biology is further revealed by organisms such as *Acidianus infernus* (131) and *Desulfurolobus ambivalens* (155), both of which respire S° under anaerobic conditions and oxidize S° when grown aerobically. In addition, S° can simultaneously serve as an electron donor and acceptor in a process termed sulfur disproportionation. Sulfur disproportionation can be described as an inorganic fermentation in which sulfur compounds with an intermediate oxidation state serve as both electron donor and electron acceptor in a metabolic, energy-generating process (135). Intermediate oxidation state sulfur compounds that have been identified as electron donor/acceptor substrates for sulfur disproportionating chemolithoautotrophic organisms include thiosulfate and S° (51, 73). The process of sulfur disproportionation was first identified in *Desulfovibrio sulfodismutans* (8, 9) and since has been documented in a number of phylogenetically diverse organisms (94, 123). The importance of this metabolism in nature is unknown;

however, 16S rRNA gene sequences related to organisms capable of this metabolism and $\Delta^{34}S$ stable isotope signatures in sulfur compounds have been recovered from a variety of environments (45, 59), suggesting that this may be an important process in the global sulfur cycle.

Sulfate reduction is catalyzed by a multitude of phylogenetically diverse organisms distributed throughout the *Bacteria* and *Archaea* (29) such as *Desulfovibrio* (85) and *Archaeoglobus* (137). Sulfate is the most abundant form of sulfur in the environment and therefore represents a sink of sulfur, suggesting that the activity of sulfate-reducing microbes may be the basis of the biological sulfur cycle. Sulfate reducers have been identified from a variety of environments including hypersaline microbial mats (28), marine sediments (79, 80), and ASC geothermal sediments (48, 52), thereby illustrating the ubiquity and potential importance of these organisms in the cycling of sulfur compounds in the environment.

Microorganisms Involved In S° Reduction

Thermocladium-like sp. *Thermocladium*-like organisms have been identified in association with source water S° precipitates collected from Succession Spring where the temperature ranged from 78-84°C and the pH was nearly constant (pH 3.1) (98). The type strain, *Thermocladium modestius*, is an acidophilic hyperthermophile isolated from solfataric waters and sediments in Japan (70). *T. modestius* grows over a pH range of 2.6-5.9 (pH_{opt} = 4.0) and over a temperature range of 45-82°C (T_{opt} = 75°C), consistent with the temperature and pH of the Succession Spring thermal transect where *T.*

modestius-like 16S rRNA gene sequences were recovered. Carbon sources utilized by the microaerophilic *T. modestius* include glycogen, starch, gelatin, and complex proteinaceous substrates coupled with the reduction of S°, thiosulfate, or cystine, forming H_2S as a metabolic by-product (70). The presence of DOC and S° in the sulfur zone of Succession Spring suggests this to be a suitable environment for the growth of *T. modestius*-like organisms.

<u>*Caldisphaera*-like sp.</u> *Caldisphaera*-like 16S rRNA gene phylotypes have been recovered from S° precipitates collected from just downstream from the source of Succession Spring where the temperature ranged from 65-75°C and the pH was constant at 3.1 (69, 98). *Caldisphaera lagunensis* grows over a temperature range of 45-80°C (T_{opt} 70-75°C) and over a pH range of 2.3-5.4 (pH_{opt} 3.5-4.0) (71). The cardinal growth temperatures of *C. lagunensis* are consistent with those of Succession Spring, suggesting this environment to be favorable for this organism in regards to temperature and pH. *C. lagunensis* is a facultative anaerobe that grows heterotrophically on complex sugars and peptide-containing substrates coupled with the reduction of S°, fumarate, and sulfate. In addition, *C. lagunensis* can also couple organic carbon oxidation with the reduction of oxygen. The aerobic or microaerophilic phenotype of *C. lagunensis* demarcates it from other S°-respiring organisms found in ASC springs (see below).

The genus *Caldisphaera* is in the order *Desulfurococcales*, phylum *Crenarchaeota*. Within the *Desulfurococcales*, *C. lagunensis* clusters in the 'Acidilobus group' as determined by comparative 16S rRNA gene analysis (122). The defining

characteristic of the 'Acidilobus lineage' is the acidophilic nature of all of the constituent members as compared to other members of the order *Desulfurococcales* which are neutraphilic to moderately acidophilic. The heterotrophic, S°-reducing phenotype of *Caldisphaera* sp. is consistent with the recovery of these phylotypes from the sulfur zone in Succession Spring which contains 40 μM DOC and S°. Alternatively, *Caldisphaera* sp. could be coupling hetertrophic growth with sulfate reduction given the abundance of sulfate (1.3 mM) in Succession Spring (69, 98).

Caldococcus-like sp. Sequences related to '*Caldicoccus noboribetus'* strain NC12 have been recovered from the source of Succession Spring, where the temperature ranged from 78-84°C (98). *C. noboribetus* is a hyperthermophilic acidophile that was first isolated from the Noboribetsu geothermal spring in Japan (6); however, this strain was never completely characterized and therefore has not been officially recognized as a novel genus or species by the international community. *C. noboribetus* grows over a temperature range of 70-96°C (T_{opt} 92°C) and over a pH range of 1.5-4.0 (pH_{opt} of 3.0), both of which are consistent with the recovery of *C. noboribetus*-like 16S rRNA gene phylotypes from the sulfur zone in Succession Spring (78-84°C, pH 3.1) (69, 98). In addition, *C. noboribetus* grows by obligatory S° reduction coupled to the oxidation of complex organic carbon substrates, suggesting the sulfur depositional zone in Succession Spring, which contains DOC (40 μM) and S° as a predominant solid phase, to be an environment favorable for this organism (68, 69, 98).

C. noboribetus clusters within the 'Acidilobus group' of the *Desulfurococcales* as determined by comparative 16S rRNA gene analysis. As previously mentioned, all members of the 'Acidilobus group' are extreme acidophiles (pH_{opt} <4.5); a phenotype shared by *C. noboribetus*. Within the 'Acidilobus group', all hyperthermophilic members including *Acidilobus aceticus* and *C. noboribetus* cluster within the same lineage while the sole thermophile, *C. lagunensis* forms a separate lineage (122).

Thermofilum-like sp. 16S rRNA gene sequences related to *Thermofilum* were recovered from the source water S° precipitates at Succession Spring over a temperature range of 78-84°C (98). The type strain, *Thermofilum pendens*, was isolated from solfataric fields in Iceland (154). *T. pendens* is a moderately acidophilic, hyperthermophile that couples peptide oxidation to S° reduction (154), suggesting the sulfur zone within Succession Spring to be a favorable habitat for *T. pendens*-like organisms. 16S rRNA analysis places *T. pendens* in the *Thermoproteales* (27) along with *Thermoproteus tenax*, a related acidophilic hyperthermophile. Interestingly, the growth of *T. pendens* was dependent on the presence of lipids derived from *T. tenax*, an organism that has never been identified in the ASC springs using molecular 16S rRNA gene diversity approaches (68, 69, 72, 98). If the *T. pendens*-like organism inhabiting Succession Spring requires lipids, this requirement may be met by lipids produced by *T. modesties*, a *Thermoproteale* whose sequence has been recovered from S° flocs sampled from Succession Spring (69, 98).

Stygiolobus-like sp. *Stygiolobus*-like 16S rRNA gene sequences were recovered near the source of Succession Spring, where the temperature ranged from 78-84°C and S° was abundant. In addition to Succession Spring, 16S rRNA gene sequences and cultivated isolates related to *Stygiolobus* have been recovered from a variety of acidic environments including terrestrial and marine solfataric fields and acidic coal refuse piles (23, 138). The type strain, *Stygiolobus azoricus*, was isolated from acid geothermal springs in the Azores (132). Growth of *S. azoricus* was observed over a temperature range of 57-89°C (T_{opt} 80°C) and over a pH range of 1.0-5.5 (pH_{opt} 2.5-3.0). *S. azoricus* is an anerobic chemolithoautotroph that exhibits an obligate requirement for both S° and H_2. Succession Spring source water contains 1.8 mM dissolved CO_2, 55 nM H_2, and S° as a solid phase, suggesting this environment to be favorable for the growth of *Stygiolobus*-like organisms. Despite being a strict anaerobe, *S. azoricus* is a member of the *Sulfolobales*, an order comprised of facultative anaerobes and obligate aerobes (132). The optimum temperature (78-84°C) and pH (3.1) for growth of the type strain, *S. azoricus*, are similar to the temperature and pH where 16S rRNA gene sequences related to this organism were recovered, further suggesting that the physicochemical conditions present in the sulfur zone of Succession Spring to be favorable for growth.

Desulfurella-like sp. *Desulfurella*-like 16S rRNA gene phylotypes have been recovered in Dragon Spring at distinct thermal transects corresponding in temperature to 51-60°C where S° is abundant. The type strains of *Desulfurella* sp. (*D. multipotens* (105), *D. kamchatkensis* (106), *D. acetivorans* (18), and *D. propionica* (106)) were

isolated from cyanobacterial mats and sediments collected from thermal areas in New Zealand and Kamchatka, Russia. *Desulfurella* spp. exhibit similarities in their cardinal temperatures and pH: all are thermoneutraphiles. For example, the T_{opt} for all cultivated and characterized *Desulfurella* spp. is between 55-60°C and the pH_{opt} is between 6.0-7.0. Similarly, all *Desulfurella* spp. are phylogenetically similar at the 16S rRNA gene level (>99% sequence homology) and utilize S° as a terminal electron acceptor. The substrates utilized by *Desulfurella* spp. differ slightly: all are capable of chemoorganotrophic growth; however, *D. multipotens* can also grow chemolithotrophically with H_2 (105). The presence of CO_2 (4.4 mM), H_2 (60 nM) and S° in Dragon Spring suggest this to be an environment favorable for *Desulfurella*-like organisms growing chemolithotrophically (69, 72). Alternatively, *Desulfurella* spp. are capable of utilizing organic compounds such as fatty acids (105) which may be a constituent of the 80 μM dissolved organic carbon (DOC) present in the source of Dragon Spring (69, 88). Based on the presence of S°, DOC, dissolved CO_2, and H_2, it is not surprising that 16S rRNA gene sequences related to *Desulfurella* accounted for 3% of the total bacterial clone library constructed from S° DNA extracts obtained from the sulfur zone by Jackson et al. (72).

None of the *Desulfurella* type strains characterized to date are capable of growth in the acidic (pH 3.1) conditions present in Dragon Spring. Thus, the organism(s) yielding the 16S rRNA genes recovered from the Dragon Spring sulfur zone may represent the first acidophilic taxon within the *Desulfurella* genus.

Microorganisms Involved In H_2S Oxidation

Hydrogenobaculum-like sp. *Aquificales* have been identified as important constituents of a variety of geochemically-diverse geothermal communities (37, 43, 69, 72, 102, 124-127, 136). *Aquificales*, represented by the genus *Hydrogenobaculum*, are also important in the Beowulf, Succession, and Dragon Spring ecosystems (37, 43, 69, 72, 98, 136). *Hydrogenobaculum acidiphilum* was origionally isolated from a solfataric field in Japan (133). *H. acidiphilum* grows over a temperature range of 50-70°C (T_{opt} 65°C) and a pH range of 2.0-6.0 (pH_{opt} 3.0-4.0). *H. acidiphilum* couples H_2 oxidation with CO_2 fixation using oxygen (O_2) as an oxidant. Similar to *H. acidiphilum*, *Hydrogenobacculum* sp. NOR3L3B is a hyperthermoacidophilic *Aquifex* isolated from Mud Hole, NGB (44). While the physiology of this isolate has not been reported in full, *Hydrogenobacculum* sp. NOR3L2B was cultivated using Allen-medium (2) with the pH adjusted to 3.0 under a gas phase of H_2-CO_2-O_2 and the cultures were incubated 65°C (44). Thus, *Hydrogenobacculum* sp. NOR3L3B may be similar to *H. acidiphilum* in that it likely couples the oxidation of H_2 with the fixation of CO_2 using O_2 as an oxidant.

Aquificales are chemolithoautotrophic thermophilic bacteria that couple CO_2 fixation with the oxidation of reduced compounds such as H_2, H_2S, or S° (37, 124, 136). Both Dragon and Succession Springs contain abundant CO_2 (4.4 and 1.8 mM, respectively), H_2 (60 and 55 nM, respectively), and H_2S (60 and 7-80 uM, respectively), suggesting these environments to be favorable for *Aquificales* such as *H. acidiphilum* or *Hydrogenobacculum* sp. NOR3L3B (69, 88, 98). Recent cultivation efforts have

successfully enriched and isolated a number of *Hydrogenobacculum*-like isolates that couple CO_2 fixation with H_2 and H_2S oxidation (37). These data suggest that CO_2 fixation in ASC springs in NGB may be driven at least in part by H_2S. This is in contrast to Spear et al (136), which concluded that H_2 was the energy source driving chemolithotrophic CO_2 fixation in geothermal springs exceeding 70°C in Yellowstone National Park. However, the conclusions of Spear et al (136) were based solely on inferred physiology based on 16S rRNA gene homology to cultivated *Hydrogenobaculum* spp., which were reported to only couple CO_2 fixation to H_2 oxidation (43, 44, 133). The discovery of *Hydrogenobaculum* spp. that coupled CO_2 fixation with H_2S oxidation were reported (37) following the publication of Spear et al (136).

Sulfur Zone Synthesis

With few exceptions, the *Aquificales* are the only bacterial lineage that contains hyperthermophilic autotrophs (62). With the exception of *Hydrogenobacter subtgerraneus*, all cultivated members of the *Aquificales* are microaerophilic chemolithotrophs (124). Cultivation efforts targeting *Hydrogenobaculum* spp. inhabiting NGB ASC geothermal springs have resulted in the isolation of fifteen phylogenetically different, obiligate H_2S-oxidizing organisms and two phylogenetically different, obligate H_2-oxidizing organisms (37). The preponderance of *Hydrogenobacculum*-like organisms associated with the S° precipitates in Dragon and Succession Springs (69, 88, 98) may be indicative of the importance of these organisms as primary producers in ASC environments and furthermore, suggests their putative importance in the oxidation of H_2S

and concomitant deposition of S°. Alternatively, *Hydrogenobacculum* sp. may be driving CO_2 fixation by the oxidation of S°, suggesting their importance in S° cycling.

With the exception of *Thermocladium*- and *Stygiolobus*-like sp., the inferred physiology (based on 16S rRNA gene sequence analysis) of *Archaea* inhabiting the S° depositional zone in ASC springs in NGB is that of a heterotroph, dependent on reduced forms of carbon for growth. Previous studies conducted in alkaline geothermal environments indicate the importance of photosynthetically-produced organic carbon for heterotrophic consumers in these environments (11, 22). Within the Beowulf, Succession, and Dragon Springs geothermal environments, DOC is present at concentrations ranging from 40-80 μM (69). *Hydrogenobacculum*-like populations, like the phototrophs inhabiting alkaline environments, may be important contributors to the DOC pool that supports heterotrophic *Archaea*.

Organisms that couple CO_2 fixation with the oxidation of H_2S produce S° as a metabolic by-product (84, 118). If *Hydrogenobacculum*-like populations associated with S° precipitates at the source of ASC geothermal springs are indeed coupling CO_2 fixation with the oxidation of H_2S, then a fraction of the S° formed at the source may be biogenic in origin. Alternatively, H_2S oxidizes abiotically to S° upon exposure to O_2, a reaction which also may contribute to the copious amounts of S° in the source waters of ASC geothermal springs. The S° produced by biotic and abiotic oxidation would be available for reduction by any of the following organisms found associated with S° precipitates in the sulfur zone of ASC geothermal springs: *Thermocladium* sp., *Caldisphaera* sp.,

Caldococcus sp., *Thermofilum* sp., *Stygiolobus* sp., and/or *Desulfurella* sp. H_2S produced via S° reduction by these organisms would then be available for oxidation by H_2S-oxidizing *Hydrogenobacculum* sp., completing a truncated sulfur cycle. A similar truncated sulfur cycle has been reported previously in a laboratory-based consortium of H_2S-oxidizing green-sulfur phototrophs grown in mixed culture with S°-reducing prokaryotes (118).

The relative abundance of closely-related microbial populations in other thermal spring microbial communities also reflects how closely their T_{opt} for growth, based on laboratory studies (fundamental niche), coincides with their distribution along thermal gradients in hot springs (realized niche) (3, 46, 103, 149). For example, in an alkaline hot spring microbial mat, *Synechococcus* strain A (T_{opt} 55°C) was detected in an area of the mat exposed to a temperature of 56°C, while *Synechococcus* strain B (T_{opt} 50°C) was not. In contrast, *Synechococcus* strain B was detected in an area of the mat exposed to a temperature of 53°C, whereas *Synechococcus* strain A was absent (3, 46). In addition to differences in carbon source and electron donor properties, the T_{opt} for growth of *S. azoricus* (80°C), *C. noboribetus* (92°C), and *T. modestius* (75°C) are also different, suggesting that these organisms may occupy different temperature niches within the sulfur zone of Succession Spring. Thus, dominance among populations with similar physiologies may be determined by how closely their T_{opt} coincide with the temperature of the environment.

Sulfate-reducing (SR) microorganisms often couple sulfate reduction with the oxidation of organic carbon (DOC) or H_2. Despite the presence of DOC (40-80 μM), H_2

(55-60 nM), and sulfate (1.3 mM) in Succession and Dragon Spring, the sole 16S rRNA gene sequence related to an organism capable of SR activity was *C. lagunensis* (69, 98); however, SR activity of these organisms remains to be determined in the field. Previous SR activity measurements from low pH sites with comparable temperatures to those present in Succession and Dragon Springs sulfur zones yielded SR activity values that were at the limits of detection (52), suggesting that a combination of high temperature and low pH may exclude or severely hamper SR activity.

Based on inferred physiology, seven of the eight 16S rRNA gene phylotypes recovered from the sulfur zone in Succession and Dragon Springs appear to be involved in reductive sulfur cycling. Despite the presence of copious amounts of S° in the sulfur zone of ASC springs throughout YNP, organisms capable of S° reduction have never been cultivated from ASC springs in YNP. Thus, despite the apparent importance of this physiological class of organisms in ASC environments (69, 98) their ecology and physiology remain uncharacterized.

Iron/Arsenic Zone

Introduction

The iron/arsenic depositional zone is visually distinguished from the sulfur depositional zone by an abrupt change in color from yellow to brown. This transition corresponds to H_2S concentrations decreasing to <5 μM and to the deposition of hydrous ferric oxides (HFO) with sorbed $As^{(V)}$ (68, 69, 88, 98). Notably, iron and arsenic oxidation commence at the start of the HFO phase, possibly as a result of oxygen

concentrations increasing as a result of the decrease in concentration of the oxygen-scrubbing H_2S, a decrease in the oxygen consuming activity of H_2S-oxidizing *Hydrogenobaculum*-like spp. resulting in an increase in available O_2, inhibition of iron and arsenic oxidizing enzymes by H_2S, and/or temperature constraints on $Fe^{(II)}$ and/or $As^{(III)}$-oxidizing organisms.

Both iron and arsenic have received considerable attention recently as substrates for biological activity (92, 114). Iron is one of the most abundant metals on Earth and has been shown to serve as both an electron donor and acceptor for a variety of organisms (15). $Fe^{(II)}$ can be oxidized biotically under acidic conditions by a diversity of organisms such as *Thiomonas* sp. (109), *Acidithiobacillus ferroxidans* (16), *Metallosphaera sedula* (60), and *Ferroplasma acidiphilum* (97) using a variety of oxidants such as O_2 or nitrate (15, 140). Anoxygenic purple non-sulfur phototrophic bacteria can drive CO_2 fixation through the oxidation of $Fe^{(II)}$ using O_2 as an oxidant; however, only under neutral conditions (151). In addition to biotic oxidation, chemical oxidation of $Fe^{(II)}$ can also occur, yielding iron oxides (141). However, in environments with pH <4.0 such as Beowulf, Succession, and Dragon Springs, $Fe^{(II)}$ oxidation is likely mediated primarily by biota (76, 141). 16S rRNA gene sequences with inferred $Fe^{(II)}$-oxidizing physiologies have been recovered from Beowulf, Succession, and Dragon Spring suggesting a microbial role in the oxidation of $Fe^{(II)}$ and the resulting formation of HFO mats in ASC springs (68).

Iron oxides resulting from biological or abiological oxidation precipitate due to their low solubility in aqueous media (141), and thus represent an important solid phase

electron acceptor for a variety of organisms inhabiting anoxic environments (92, 93, 95). The reduction of soluble ferric iron or insoluble iron oxides has been studied in detail in two model organisms: *Shewenella oneidensis* strain MR1 (56, 111) and *Geobacter* spp. (92). In addition to these two model organisms, $Fe^{(III)}$ reduction has been reported in acidophilic *Bacteria* such as *Thiobacillus ferroxidans* (25, 40) and *Sulfolobus* spp. (25) and *Archaea* such as *Pyrobaculum* spp. (81). *Sulfolobus* spp. couple the oxidation of S° with the reduction of $Fe^{(III)}$ (25) while *P. islandicum* couples organic carbon oxidation with the reduction of $Fe^{(III)}$ (92, 95). A variety of insoluble iron oxides can serve as electron acceptors for $Fe^{(III)}$-reducing microbes including crystalline forms such as hematite, goethite, and magnetite in addition to amorphous iron oxides (92). The iron oxides present in ASC springs in NGB are amorphous hydrous ferric oxide (HFO) (ferrihydrite-like) encrustations surrounding microbial filaments that contain iron to arsenic ratios of approximately 0.62 (68).

The predominant form of inorganic arsenic in oxic environments is arsenate, which is strongly sorbed to the surface of a variety of minerals including ferrihydrite and the HFO mats present in Beowulf Spring (68, 114). However, in anoxic waters such as those in the sulfur zone of Dragon Spring, arsenite is the predominant arsenic species (88). Notably, at the interface of the sulfur and iron/arsenic zone, oxidation of arsenite commences (88) as does the oxidation of $Fe^{(II)}$ with the result being the deposition of an arsenic-rich HFO phase.

The biological diversity of arsenite-oxidizing microbes spans both the *Bacteria* and the *Archaea* (114). *Pseudomonas arsenitoxidans* couples the oxidation of arsenite to

energy generation (65) and YNP *Thermus* spp. isolates have also been shown to oxidize arsenite in the laboratory (55). One such YNP *Thermus* isolate related to *Thermus aquaticus* (98% similar) was determined to have the capacity to both oxidize arsenite and to reduce arsenate; reportedly switching metabolisms depending on the ratio of arsenite/arsenate in the media menstruum (54). In addition, a recent study determined the presence and expression of arsenite oxidase genes in microbial mats in YNP suggesting that biological arsenite oxidation is occuring in geothermal springs (67). An arsenite-oxidizing *Hydrogenobaculum* strain (H55) has been isolated from Dragon Spring (43) and 16S rRNA genes related to this organism have been recovered from HFO mats in this spring (72). In addition, 16S rRNA gene sequences related to *Hydrogenobacculum* strain H55 have been recovered from Beowulf and Succession Springs HFO mats and *Thiomonas*-like 16S rRNA gene sequences have been recovered from Succession Spring HFO mats (69, 98). Collectively, these results suggest a role for these organisms in the oxidation of $As^{(III)}$ in these springs. As such, the activity of these organisms may directly be responsible for the $As^{(V)}$ sorbed to HFO mats present in Beowulf, Succession, and Dragon Spring (68, 69, 88, 98).

Microorganisms Involved In $Iron^{(II)}$ Oxidation

Acidimicrobium-like sp. 16S rRNA gene sequences related to *Acidimicrobium* have been recovered from Dragon, Succession, and Beowulf Springs thermal transects ranging in temperature from 48-58°C. The type strain, *Acidimicrobium ferroxidans*, is an acidophilic, moderately thermophilic $Fe^{(II)}$-oxidizing bacterium which was isolated from

an Icelandic geothermal site in a co-culture with *Sulfobacillus thermosulfidooxidans* (32). From co-culture phenotypic characterization studies, it was determined that *A. ferroxidans* grows autotrophically by $Fe^{(II)}$ oxidation and in the process, releases autotrophically-fixed organic carbon into the culture that presumably supports the growth of *S. thermosulfidooxidans*. The maximum generation time (based on OD_{600}) of the co-culture grown autotrophically was observed at 48°C, although growth was observed over a temperature range of 35-57°C. The pH range that permitted growth was not reported (32); however, the pH_{opt} was approximately 2.0 for *A. ferroxidans* and *S. thermosulfidooxidans* co-cultures. *A. ferroxidans* can also grow heterotrophically on yeast extract (32).

Phenotypic characterization of *A. ferroxidans* grown in co-culture suggests Beowulf, Succession, and Dragon Spring HFO mats to be environments favorable for growth of *A. ferroxidans*-like organisms. $Fe^{(II)}$ and dissolved CO_2 are present at the temperature transects where *A. ferroxidans*-like 16S rRNA genes were recovered and the pH of the springs are close (3.0-3.1) to the pH_{opt} (2.0) for growth of *A. ferroxidans.* In addition, the T_{opt} of *A. ferroxidans* is at the lower range of the thermal transect where 16S rRNA gene phylotypes related to this organism were recovered. *Sulfobacillus*-like clones related to *S. thermosulfidooxidans* have also been recovered from ASC springs (66) (See below).

<u>*Sulfobacillus*-like sp.</u> 16S rRNA genes related to *Sulfobacillus* have been recovered from iron/arsenic zones in ASC springs at thermal transects ranging from 50-

65°C (66) and many strains of *Sulfobacillus* have been isolated from acid geothermal springs in YNP by Johnson et al. (78). *Sulfobacilli* (type strain *Sulfobacillus thermotolerans*) have also been cultivated from gold-containing sulfide mineral ores in Siberia (17). Phenotypic characterization of these strains and other *Sulfobacilli* revealed the physiological capacity to oxidize ferrous iron in oxic media and the ability to reduce ferric iron in anoxic media (20, 78). In addition, many YNP *Sulfobacillus* isolates can oxidize pyrite and S°; phenotypes that are similar to those of *Metallosphaera* (See below). *Sulfobacilli* cultivated to date grow more efficiently when supplied with an organic carbon source such as glucose as opposed to glucose-free (ferrous iron/yeast extract) or in autotrophic media (78).

Sulfobacillus isolates from YNP grew at a maximum temperature of 50-65°C; the optimal temperature for growth was not reported. While the optimum pH for growth for the six YNP *Sulfobacillus* isolates was not reported, the minimum pH permitting growth was 1.3 for all YNP isolates (20, 78). Similar to the YNP isolates, *Sulfobacilli* cultivated from Siberia when grown on ferrous iron exhibited a maximum growth temperature of 60°C (T_{min} 20°C; T_{opt} 40°C) and a minimum pH permitting growth of 1.2 (pH_{max} 2.4, pH_{opt} 2.0) (17). Interestingly, the minimum, maximum, and optimum pH for *S. thermotolerans* were higher when grown on S° as compared to ferrous iron: pH_{min} 2.0, pH_{max} 5.0; pH_{opt} 2.5; however, no explanation for this phenomenum was given (17). In contrast to YNP *Sulfobacilli*, *S. thermotolerans* could not grow autotrophically without trace organic carbon (17, 78). *Sulfobacilli* cultivated from both Siberia and from YNP

are mixotrophic, capable of oxidizing Fe^{2+}, S°, tetrathionate, or sulfide minerals in the presence of trace yeast extract (17, 78).

Given the optimal pH and temperature for growth, and considering the metabolic versatility of the YNP *Sulfobacilli* cultivated to date, it would not be unexpected to find 16S rRNA gene sequences related to these organisms in Beowulf, Succession, and Dragon Springs.

Metallosphaera-like sp. *Metallosphaera*-like organisms have been identified in Succession and Beowulf Springs by both cultivation and cultivation-independent approaches (68, 69, 72, 86, 98). The type strain, *Metallosphaera sedula*, was isolated from a solfataric field in Italy (60). *M. sedula* is a facultative autotroph that couples the oxidation of either organic matter or mineral sulfides with the reduction of oxygen. Ferrous iron and S° are also electron donors utilized by *M. sedula*, both of which are present in Beowulf, Succession, and Dragon Springs. In addition, *M. sedula* is capable of growth on pyrite, chalcopyrite, and sphalerite, forming sulfuric acid and releasing metal ions in the process (138).

Metallosphaera hakonensis, was isolated from a geothermal spring in Japan (87, 143). *M. hakonensis* grows over a pH range of 1.0-4.0 (pH_{opt} 3.0) and a temperature range of 50-80°C (T_{opt} 70°C). In contrast to *M. sedula*, *M. hakonensis* is a mixotroph that cannot oxidize ferrous iron (143). Both *M. hakonensis* and *M. sedula* are acidophilic (75) and grow over a temperature range of 50-80°C (60). Similar to both *M. hakonensis* and *M. sedula*, a representative isolate obtained from YNP exhibited a T_{opt} of 65°C and pH_{opt}

of 2.8 (86). Preliminary analysis of the distribution of *Metallosphaera*-like organisms in ASC springs indicates that gradients in dissolved oxygen may influence the distribution of this organism, constraining it to HFO mats where O_2 concentrations are high enough to support $Fe^{(II)}$ oxidation (86). Supporting this hypothesis are previous diversity studies which also localize this organism to iron/arsenic HFO zones in Beowulf and Succession Springs (69, 86, 98).

Based on physiological properties of isolates obtained from Japan and YNP, it is not surprising to find *Metallosphaera*-like organisms in Beowulf and Succession Spring; however, it is surprising that *Metallosphaera*-like clones have not been recovered from HFO mats in Dragon Spring (72). Both CO_2 and $Fe^{(II)}$ are available for microbial activity in the HFO mats of Dragon Spring. Whether O_2 is at a concentration high enough to support *Metallosphaera*-like respiration remains to be determined.

Microorganisms Involved In $As^{(III)}$ Oxidation

Hydrogenobaculum-like sp. Molecular characterization of mats collected from the sulfur zone and from the iron/arsenic zone indicated the presence of *Hydrogenobaculum*-like 16S rRNA gene phylotypes (72). The differences in temperature, aqueous-phase chemistry, and solid-phase chemistry between the sulfur zone and iron/arsenic zone, suggest that these phylotypes may represent different *Hydrogenobaculum*-like sp. Previous studies have isolated *Hydrogenobaculum*-like organisms from the sulfur zone that drive CO_2 fixation through H_2S or H_2 oxidation (37). Similarly, Donahoe-Christiansen et al. (43) successfully isolated *Hydrogenobaculum*

strain H55 from the iron/arsenic zone in an ASC spring in the NGB that drives CO_2 fixation by H_2 oxidation. However, in contrast to many of the *Hydrogenobaculum* spp. isolates cultivated from the sulfur zone by D'Imperio et al (37), strain H55 was unable to grow chemolithotrophically via H_2S oxidation (43). These data suggest that the isolates obtained from the sulfur zone and iron/arsenic zone are different strains within the genus *Hydrogenobaculum*.

Iron/arsenic HFO mats commence when H_2S concentrations drop below 5 μM, and typically contain an appreciable amount of sorbed $As^{(V)}$ (68, 69, 88, 98). As previously mentioned, *Hydrogenobaculum* strain H55 was isolated from NGB ASC spring HFO mats. Phenotypic characterization of strain H55 indicated the physiological capability to oxidize $As^{(III)}$ to $As^{(V)}$; a process that was inhibited by H_2S (69, 88). Incubations of killed control HFO mats indicated that $As^{(III)}$ was not oxidized abiotically, suggesting $As^{(III)}$ oxidation to be a microbially-mediated process in ASC geothermal springs (43). The arsenite-oxidizing activity of *Hydrogenobaculum* strain H55 may be a source of sorbed arsenate in the HFO solid phase.

Strain H55 is an obligate autotroph, exhibiting optimal growth at pH of 3.0 and 55-60°C (43). The phenotypes of strain H55 are consistent with the presence of CO_2 and the pH (3.1) and temperature (50-65°C) where 16S rRNA gene sequences corresponding to the isolate were recovered (72, 98).

Microorganisms Involved In $As^{(III)}$ and $Fe^{(II)}$ Oxidation

Thiomonas-like sp. *Thiomonas*-like 16S rRNA gene sequences have been recovered from HFO solid phases in the iron/arsenic zone of Beowulf and Succession Springs along thermal transects ranging in temperature from 48-58°C (68, 98). Thiomonads have been isolated from a variety of environments including soils and mining wastes (12, 91). The optimal growth temperature for thermophilic thiomonads is approximately 50°C (109) and the optimum pH permitting growth of thermophilic thiomonads is 3.0-6.0 (109); the pH of both Beowulf and Succession Springs (3.1) are at the lower end of this range. Thiomonads are facultative chemolithoautotrophs with optimal growth occurring in media supplemented with reduced sulfur compounds and peptides (61, 109). Autotrophic growth is exhibited in the presence of S°, tetrathionite, or thiosulfate (134). Many studies have implicated thiomonads in the mobilization of metals through the oxidation of sulphide ores such as chalcopyrite, sphalerite, arsenopyrite, and galena (61). In addition, thiomonads can grow chemolithotrophically by $Fe^{(II)}$ (76) and $As^{(III)}$ oxidation (12).

HFO mats in Beowulf and Succession Spring are suitable environments for growth of *Thiomonas*-like organisms given the elevated concentrations of $Fe^{(II)}$ (50-60 μM) and $As^{(III)}$ (35 μM) and the presence of DOC and DIC (68, 98). In addition, the temperature transect where thiomonad 16S rRNA genes were recovered is near the T_{opt} for thiomonads grown in the laboratory, further suggesting these environments to be favorable for these types of organisms. Along with *Hydrogenobaculum*-like organisms,

$As^{(III)}$-oxidizing activity of thiomonads may contribute to the $As^{(V)}$ sorbed to the HFO solid phase.

Microorganisms Involved In Heterotrophy

Alicyclobacillus-like sp. *Alicyclobacillus*-like sp. have been identified in the iron/arsenic zone of ASC springs in the Norris Geyser Basin (19) and have been cultivated from a variety of habitats, including geothermal springs (7, 38), non-geothermal acidic soils (42), and from acidic beverages (116, 153). A survey of a 60 thermoacidophilic *Alicyclobacilli* strains obtained from various culture collections exhibited growth over a temperature range of 35-70°C (T_{opt} 55-65°C) and over a pH range of 2.5-6.0 (pH_{opt} 4.0-5.0) (57, 112). All of the strains tested were obligate aerobic heterotrophs with the exception of a single strain which could couple organic carbon oxidation with the reduction of nitrate to nitrite.

Acidophily is a unifying theme of all *Alicyclobacilli* cultivated to date; however, the optimal growth temperature varies (42, 57, 116, 153). The thermophilic nature of some isolates and the acidophilic nature of all isolates suggest acidic geothermal springs to be environments suitable for growth for *Alicyclobacilli*. In addition, dissolved organic carbon (60-80 μM) is available in Beowulf, Succession, and Dragon Springs which could presumably support the growth of *Alicyclobacillus* sp.

Meiothermus-like sp. 16S rRNA genes related to *Meiothermus* have been recovered from the 50-55°C thermal transect in Dragon Spring. *Meiothermus* spp. have also been detected in a variety of geographically-distinct hot springs in Iceland, Portugal,

and the Azores (31, 120, 145). *Meiothermus* spp. are moderate thermophiles exhibiting T_{opt} for growth ranging from 50-65°C (30, 31) with temperatures permitting growth ranging from 35-68°C (120). Members of the genus *Meiothermus* are aerobic heterotrophs, utilizing monomeric or polymeric carbohydrates as carbon and energy substrates (31). In addition, some *Meiothermus* strains can utilize complex proteinaceous substrates such as casein and elastin (120). The growth of *Meiothermus cerbereus* requires media supplemented with reduced sulfur species such as cysteine, thiosulfate, or thioglycolate. In addition, *M. cerbereus* grows aerobically at neutral to alkaline pH with an optimum of 7.5 (range 5.5-10.0) (31). With the exception of pH, the physicochemical conditions present in Dragon Spring would presumably favor the growth of *Meiothermus*-like organisms.

Marinithermus-like sp. *Marinithermus* like organisms have been identified in Succession and Beowulf Springs by 16S rRNA gene diversity surveys in thermal transects corresponding in temperature to 52-55°C (68, 69, 98). The type strain, *Marinithermus hydrothermalis*, was isolated from a hydrothermal vent chimney near Japan. *M. hydothermalis* grows over a pH range of 6.2-7.7 with an optimum of 7.0 and over a temperature range of 50-70°C with an optimum of 67°C (104, 128). Growth of *M. hydrothermalis* is by aerobic respiration on carbohydrates, peptides, and organic acids. The range of %NaCl permitted for growth ranged from 0.5-4.5%, which is greater than the 0.08-0.09% NaCl present in Succession and Beowulf Springs. Thus, with the exception to pH and %NaCl, the physiocochemical conditions present within Beowulf

and Succession Springs could presumably support the growth of *Marinithermus*-like organisms

Iron/Arsenic Zone Synthesis

The unifying theme of organisms inhabiting the iron/arsenic zone is the involvement of O_2 in their respiration. The solubility of oxygen increases with decreasing temperature (148). Thus, as geothermal source water flows down the thermal gradient and cools, the influx of O_2 presumably increases, enabling O_2-dependent respiration. However, the availability of O_2 for respiration is dependent on the absence of oxygen reactive compounds such as H_2S. The boundary of the sulfur and iron/arsenic zones corresponds to an H_2S "chemocline" with concentrations decreasing to <2.5-5.0 μM prior to significant oxidation of $As^{(III)}$ or $Fe^{(II)}$ (68, 69). As H_2S concentrations decrease, so too would the O_2-consuming activity of *Hydrogenobaculum* sp. present in the sulfur zone, many of which grow autotrophically by the O_2-dependent oxidation of H_2S (37). Thus, the boundary of the S° and HFO mats may represent O_2 reaching a threshold concentration permitting measurable $As^{(III)}$ and/or $Fe^{(II)}$-oxidizing activity.

Results of Langner et al (88) suggest that arsenite oxidation is a biologically-driven process. Two organisms were identified in ASC HFO mats that are putatively capable of $As^{(III)}$ oxidation using O_2 as the oxidant: *Hydrogenobaculum* strain H55 and *Thiomonas*-like sp. *Hydrogenobaculum* strain H55 oxidizes $As^{(III)}$ in the absence of H_2S in a non-energetically conserving process. In contrast, *Thiomonas* sp. can grow chemolithotrophically via $As^{(III)}$ oxidation. The differences in phenotypes may explain the co-existence of the two organisms in the HFO mats. Alternatively, these two

organisms may occupy different thermal transects in the HFO zone. Regardless, the activity of $As^{(III)}$-oxidizing organisms similar to *Hydrogenobaculum* sp. and/or *Thiomonas* sp. are likely responsible for the $As^{(V)}$ species present in HFO solid phases (68, 88).

The oxidation of $Fe^{(II)}$ is not necessarily dependent on O_2 in ASC systems since other terminal electron acceptors such as nitrate have been shown to support $Fe^{(II)}$ oxidation (15, 140). However, $Fe^{(II)}$-oxidizing *Metallosphaera*-like isolates cultivated from YNP ASC springs are aerobes and analysis of the distribution of these isolates suggests that oxygen concentration influences their distribution (86). *Sulfobacillus* also oxidize $Fe^{(II)}$ using O_2. In contrast to the autotrophic *Metallosphaera* isolates, *Sulfobacilli* inhabiting these environments are likely heterotrophic which may explain why both organisms co-exist in the iron/arsenic HFO mats.

It is not known whether abiotic oxidation of ferrous iron occurs in acidic ASC geothermal springs (88). However, in acid mine drainage (AMD) environments with pH values less than 4.0, the oxidation of ferrous iron by chemical means is negligible (141). Thus, iron-oxidizing microbes likely play a pivotal role in the oxidation of ferrous iron and the deposition of HFO mats in ASC environments (76, 77).

Despite the prevalence of HFO solid phases in the iron/arsenic zone in ASC geothermal springs, the sole 16S rRNA gene phylotype related to an organism putatively capable of $Fe^{(III)}$ reduction was *Sulfobacillus* (68, 72, 98) which have been shown to reduce $Fe^{(III)}$ in anoxic media (20, 78). It remains to be determined whether *Sulfobacilli* present in HFO mats in ASC springs in YNP are capable of reducing HFO solid phases.

The diversity of organisms present in the iron/arsenic zone HFO mats that grow by aerobic heterotrophy may be an indicator of the importance of heterotrophic reactions in the iron/arsenic zones of ASC geothermal springs. Heterotrophic activity may be important in the recycling of organic matter, contributing CO_2 to the spring which may be important for the growth of *Hydrogenobaculum*-like sp. and/or *Metallosphaera*-like sp. that also inhabit iron/arsenic mats. In addition, O_2-consuming heterotrophs may influence the activity and the distribution of $Fe^{(II)}$ and $As^{(III)}$-oxidizing chemolithotrophic organisms if these organisms are obligate aerobes. Further research is needed to determine the parameters influencing the distribution of heterotrophic and chemolithotrophic organisms in the iron/arsenic HFO mats, especially in regard to organisms which utilize oxygen.

Phototrophic Zone

Introduction

Phototrophs are important components of the global carbon cycle due to their transformation of inorganic carbon into biomass which supports heterotrophs occupying higher trophic structures. Vibrant photosynthetic mats develop in ASC geothermal springs in the NGB at temperatures of approximately 45°C. Within the photosynthetic zone, two mat types are visually distinguishable: a vibrant green mat type which forms thin mats and a green/purple mat type which forms thick, gelatinous mats. The distribution of the organisms which comprise each of the two visually distinuguishable mat types appear to be controlled largely by water temperature (21, 49, 89, 96). Thus, for

organizational purposes, the organisms inhabiting each mat type will be discussed in two sections: those inhabiting warmer (>39°C) thermal region (thermophilic phototrophs) and those inhabiting cooler (<39°C) thermal regions (non-thermophilic phototrophs).

Both molecular and cultivation approaches have been utilized to characterize the phototrophic communities comprising the warmer (>42°C) geothermal transects of NGB ASC geothermal springs. Using molecular approaches, *Cyanidioschyzon merolae*-like 18S rRNA gene sequences were recovered (49, 89). Yet, examination of the organisms yielding the *C. merolae*-like 18S rRNA gene sequence revealed that they more closely resembled *Galdieria sulphuraria* (49, 89). For this reason, the phenotypes of both organisms will be discussed, compared, and contrasted.

Molecular and cytomorphological approaches have been used to characterize the phototrophic mats inhabiting the cooler (<32°C) transects in NGB ASC springs. Morphological characterization suggests the presence of *Zygogonium* sp. and *Chlamydomonas* sp. in some ASC springs (35, 96) while molecular characterization of 18S rRNA genes indicate the presence of *Chlamydomonas* sp. and *Chlorella* sp. in some ASC springs (49). The non-thermophilic mats appear to grow predominantly in association with sediments in thermal transects where water flow is minimal (35).

Thermophilic Phototrophs

Cyanidioschyzon-like sp. *Cyannidioschyzon merolae*-like 18S rRNA gene sequences have been recovered from Dragon Spring in temperature transects greater than 42°C (89). *C. merolae* is a strict autotroph that inhabits sulfate-rich geothermal springs

(41, 101). The pH_{opt} for *C. merola*e is 1.5 and the T_{opt} is 45°C (1), both of which are similar to the pH and temperature where samples were collected for molecular analyses in Dragon Spring (89) and Nymph Creek (49). *C. merolae* can utilize either ammonium or nitrate as a nitrogen source, both of which are available in Dragon Spring. *C. merolae* is a small (1.5-2.0 μm in length) organism that has a single nucleus, a single mitochondrian, and a single plastid and that lacks a cell wall (107). Due to simple cellular structure relative to other algae, it has been suggested that *C. merolae* may represent a transitional organism bridging the cyanobacteria and the red algae (101, 107, 130).

C. merolae-like sequences dominate ASC phototrophic mats when the spring water temperature is greater than 42°C (49, 89) and are not found at thermal transects less than 38°C (49). Temporal mat decline has been observed in *C. merolae*-like phototrophic mats inhabiting Dragon Spring (89) although Ferris et al (49) did not observe such a change in cyanidia mats in Nymph Creek. Whereas Ferris et al (49) characterized temporal changes in the Nymph Creek mats using the evolutionarily-conserved 18S rRNA gene, Lehr et al (89) employed a much more sensitive technique which targeted differences in single-sequence repeat (SSR) loci. Characterization of SSR loci facilitated the study of changes in *C. merolae* sequences (ecotypes) over a temporal period in Dragon Spring (89). The change in *C. merolae* SSR loci and mat decline were correlated primarily with changes in UV-visible irradiance and day length, both of which are highest in mid summer months. In addition, Lehr et al (89) correlated decreased photosynthetic activity with increased UV exposure. Previous photosynthetic activity studies by Cockell

and Rothschild (34) did not detect a UV effect on cyanidia populations inhabiting Nymph Creek. The UV effects on temporal community structure and photosynthetic activity in cyanidia mats inhabiting Dragon Spring compared to little or no UV effects on Nymph Creek mats may be attributable to shading of the Nymph Creek mats by surrounding trees. Whereas Dragon Spring receives no shade from nearby trees, Nymph Creek is located in a heavily-shaded drainage. Shading would decrease the total irradiance and would therefore decrease the effects of UV on the cyanidia that inhabit these shaded areas.

Galdieria-like sp. Organisms that morphologically resemble *Galdieria sulphuraria* but whose 18S rRNA gene sequence suggests relation to *C. merolae* have been observed in Dragon Spring thermal transects greater than 42°C (89). *G. sulphuraria* T_{opt} is 37°C and the pH_{opt} is 2.0 (1), both of which are lower than thermal transects in Dragon Spring known to contain *C. merolae*-like sequences (89). Whereas *C. merolae* are ovalular with a size of 1.5 x 3.5 μm (1), the organisms observed in Dragon Spring were spherical with a diameter of approximately 5 μm (89). In addition, *G. sulphuraria* can grow as a heterotroph on a variety of sugars and alcohols as the sole carbon source (58), whereas *C. merolae* is an obligate autotroph (1). In contrast to *C. merolae*, *G. sulphuraria* cannot utilize nitrate as a nitrogen source. It remains to be determined if the isolates cultivated by Lehr et al (89), which morphologically resemble *G. sulphuraria* but which are genetically related to *C. merolae*, can utilize nitrate as a nitrogen source.

C. merolae contains one of the smallest eukaryotic genomes sequenced to date (101). Comparative analysis of the genomes of both *G. sulphuraria* and *C. merolae* suggests *G. sulphuraria* to be more broadly distributed given an abundance of membrane transporters involved in the uptake of reduced organic carbon which are absent in *C. merolae* (101, 142). *G. sulphuraria* has been detected in geographically-distinct acidic sulfur springs in YNP (89) as well as in Italy where cryptoendolithic growth was also observed (58, 142). *C. merolae* was not reported as growing cryptoendolithically in the YNP or Italian environments, suggesting that this organism may be constrained to more humid habitats such as within the geothermal spring effluent channel (142). *C. merolae*-like isolates and 18S rRNA genes related to *C. merolae* were recovered from cyanidia mats which inhabit the effluent channel of Dragon Spring (89) and Nymph Creek (49). More research is needed to better define the taxonomy of the organisms which are phylogenetically related to *C. merolae*, but which are morphologically related to *G. sulphuraria*.

Non-Thermophilic Phototrophs

Zygogonium-like sp. *Zygogonium* sp. have been detected in ASC geothermal springs in NGB by morphological methods (35, 96). In addition, *Zygogonium* sp. mats have been identified in acidic springs in YNP including Amphitheater Spring, Mud Volcano, and Sylvan Spring (82, 96). Outside of YNP, *Zygogonium* sp. have been identified in acidic aquatic systems (83). *Zygogonium* mats typically develop on moist sediments near the source of small springs where the flow of water is minimal, resulting

in mat temperatures that often approach ambient temperatures (96). *Zygogonium* sp. are autotrophic green algae that fix CO_2 over a pH range of 1.0-9.0, exhibiting an optimum of 1.0-5.5 (96). CO_2 fixation is maximal at a temperature of 25°C, although fixation can be detected over a temperature range of 18-45°C; however, their growth *in situ* is typically constrained to temperatures less than 32°C (96). The decrease in CO_2 fixation by *Zygogonium* sp. in reduced sunlight suggest a significant influence of total irradiance on fixation: a 50% decrease in full sunlight corresponded to a 40% decrease in CO_2 fixation activity (96). This may help explain why *Zygogonium* spp. are typically found in unshaded areas (53).

Zygogonium sp. grow as filaments, forming thick dark purple layered mats approximately 1 cm to 10 cm thick (35). It has been hypothesized that *Zygogonium* filaments in layers below 1 cm are inactive due to an abrupt change in color from dark green to ligher green to yellow (35, 96). Conversely, it has been suggested that light intensity affects pigment development in organisms comprising lower layers or that filaments in lower layers are in the process of dying or are dead (96). Layers of *Zygogonium* sp. filaments entrap water and serve as a habitat for the development of a community comprised of a variety of algae at temperatures below 30-32°C (21, 96).

Chlamydomonas-like sp. Little is known concerning the physiology of *Chlamydomonas* sp. inhabing acidic geothermal springs. Acidophilic chlamydomonads have been isolated from a variety of environments including acidic soils, peat bogs, and acidic lakes that range in temperature from 23.5-50°C and pH from 0.8-3.3 (26, 121,

129). Other studies suggest acidiphilic chlamydomonads from marine systems to be more temperature sensitive; with growth severly impeded at temperatures exceeding 32°C (146). *Chlamydomonas*-like sp. have previously been detected in NGB ASC geothermal springs in association with *Zygogonium* sp. filamentous mats (35, 96). In these environments *Chlamydomonas* spp. are found infused in *Zygogonium* sp. filaments in cooler thermal transects (<30°C) (35). The acidophilic phenotypes exhibited by *Chlamydomonas* spp. and their occurrence in association with *Zygogonium* sp. mats suggest lower temperature thermal transects in the acidic Beowulf, Succession, and Dragon Spring to be favorable environments for the growth of these organisms.

Chlorella-like sp. *Chlorella* are common constituents of acidic phototrophic mat communities where the temperature is less than 30°C (21, 26, 63, 146); however results of Ferris et al (49) suggest *Chlorella* sp. can survive at temperature of up to 39°C. Within the NGB ASC geothermal springs, 18S rRNA genes related to *Chlorella* sp. were detected using molecular techniques over a range of temperatures. *Chlorella* spp. (both types II and III) predominate in Nymph Creek photosynthetic mats when the temperature is less than 39°C, with the maximum sequence diversity observed at 35°C (49), suggesting that these more thermotolerant organisms are different species of *Chlorella* than those studied previously. Two different lineages of *Chlorella* sequences were identified in the work of Ferris et al.(49), both of which seemed to be adapted to different temporal seasons. Type II *Chlorella* sequences were more abundant in late summer and type III *Chlorella* sequences were more abundant in the Spring (49). In addition, type II

Chlorella sequences were more thermotolerant exhibiting maximum diversity and abundance at 35°C as opposed to type III *Chlorella* sequences which exhibited maximum diversity and abundance at 30°C (49).

Chlorella spp. grow over a range of temperatures with a maximum temperature for growth of 39°C and a maximum pH for growth of 5.5 (49, 64). The generalized phenotypes of all cultivated *Chlorella* spp. suggest the lower temperature transects of NGB ASC springs to be favorable habitats for growth of *Chlorella*-like organisms.

Phototrophic Zone Synthesis

Photosynthetic organisms inhabiting ASC geothermal springs appear to be structured primarily by two physical parameters: temperature and irradiance (49, 89). With the exception of cyanidia, temperature constrains eukaryotes to lower temperature transects (<35°C) (49) and irradiance seems to influence the interspecies composition of *C. merolae*-like organisms in the thermal transects corresponding to ~35-46°C (89). The temperature constraints observed in eukaryotes may be due to the inability of some eukaryotes to form thermally stable, yet functional intercellular organelles (144).

While photosynthetic mats forming in ASC springs with temperatures >35°C can be thought of as phototrophic monocultures, (89), it remains to be determined whether slight differences in *C. merolae* SSR loci that arise over a temporal period correspond to organisms with different physiologies (ecotypes). *Synechococcus* ecotypes that are similar at the 16S rRNA gene level but that exhibit markedly different physiology have been identified in alkaline systems (3, 46, 147). These *Synechococcus* ecotypes, like the

putative *C. merolae* ecotypes, seem to be adapted to different light intensities (3). In addition, *Synechoccus* sp. ecotypes also seem to be adapted to different thermal niches (3). Further work is needed to determine if *C. merolae*-like sp. which exhibit differences in SSR loci have adapted at the physiological level to inhabit different thermal niches.

Zygogonium sp. filaments support a community comprised of single-celled algae related to *Chlorella* sp. and *Chlamydomonas* sp. (35, 96). *Chlorella* sp. and *Chlamydomonas* sp. are found in a layer just below (~ 1 mm) the surface of the mat where they occupy the interstitial space between *Zygogonium* sp. filaments (35, 96). One possible reason for these organisms inhabiting lower layers of the *Zygogonium* sp. mats may be light sensitivity. Alternatively, these organisms may be adapted to harvest different wavelengths of light, resulting in a vertical distribution of phototrophs similar to what is observed in alkaline cyanobacterial mats (47). Further research is needed to determine the environmental or physiological parameters that influence the structure of *Zygogonium* sp. mat communities in ASC environments.

The product of photosynthesis is fixed carbon. In alkaline environments, fixed carbon is released into the aqueous phase when photosynthetically-produced O_2 becomes too high (11). This photosynthetically-produced organic carbon supports a variety of heterotrophic consumers in alkaline environments (11, 13, 22). It has been hypothesized that prokaryotic consumers themselves are insufficient in recycling the organic carbon back to inorganic CO_2 in these environments, resulting in a buildup of organic carbon in the form of thick mats, the majority of which are likely inactive or dead phototrophs (22).

Similar to the previously mentioned alkaline algal mats, cyanidia studied in the laboratory were shown to excrete up to 6% of the fixed CO_2 in various organic forms including polysaccharides and peptides when grown at optimal growth temperatures (14, 110). The percent of fixed carbon excreted increased dramatically as the incubation temperature was increased above the optimum growth temperature. In natural mat samples, the percent of fixed carbon that was excreted was much lower (14). Shifts in spring flow and temperature thus could have dramatic effects on CO_2 fixation efficiency by cyanidia and the consumers that co-inhabit these environments.

The fate of phototrophically-fixed carbon in *Zygogonium* sp. mats inhabiting ASC geothermal springs remains largely unknown also. The standing crop of *Zygogonium* sp. mats in some transects of streams is up to 10 cm thick (96), yet in others, the mats are much thinner. The differences in mat thickness has been hypothesized to be a function of the presence or absence of invertebrate grazers: thicker mats occur in the absence of grazers and thinner mats in the presence of grazers (21, 96). Other than these conjectures, nothing is known on the processes that influence mat (carbon) recycling in acidic geothermal springs.

Food Webs

Introduction

In 1859, Darwin stated that plants and animals 'were bound together by a web of complex relations' (39). Today, a food web can be described as the pattern of energy and material flow between organisms (trophic levels), or simply, the feeding relationships

(trophic level interactions) between members within a defined community (115). Traditional food webs involve interactions between living organisms that consume other living organisms; however, these food webs often also include interactions between living organisms and the abiotic environment such as in the case of organic detritus consumption by decomposers.

Food webs often do not include microbial populations due to difficulties in assigning trophic status to a given member of the microbial community. However, food webs should include all potential inputs of energy and should include interactions involving organisms that derive energy from the transformation of inorganic compounds such as sulfur, iron, or arsenic. Such lithotrophic interactions appear to be important in ASC geothermal mat ecosystems based on the inferred physiology of the biota presented in preceding sections. These lithotrophic interactions represent important inputs of energy and carbon to ASC geothermal spring food webs which should be considered in ASC geothermal food webs.

Little is known on the trophic level interactions occuring in ASC geothermal springs. The primary food web that has been researched in ASC springs involves *Zygogonium* sp. mats which develop at temperatures less than 32°C and invertebrate grazers (21, 35). Studies of this food web have included detailed descriptions of the population biology of *Ephydra thermophila*, which inhabit these mats. From these studies, it was shown that the density of interstitial algae which inhabit *Zygogonium* sp. filaments and the sites chosen for *E. thermophilia* oviposition are positively correlated.

This suggests that interstitial algae influence some aspect of the biology of *E. thermophila*.

Invertebrates Involved in ASC Food Webs

Ephydra thermophila. *E. thermophila* (Diptera: Ephydridae) has been studied in a single ASC spring located in the northern portion of NGB. The pH of the water inhabited by *E. thermophila* was 2.15 to 2.70 while the major anions were chloride (430 ppm), silica (215 ppm), and sulfate (120 ppm) (35). In ASC geothermal springs, *E. thermophila* is found inhabiting *Zygogonium* sp. mats infused with interstitial algae (96). Discrepencies between abundant sources of food, (*Zygogonium* sp. and interstitial algae) and low population densities of the herbivore, *E. thermophila*, prompted investigation into the population biology of this organism.

E. thermophila adults preferentially lay eggs in areas of mats where interstitial algae are present at high density, suggesting that algae influenced fecundity (35). Subsequent laboratory-based experimentation revealed that *E. thermophila* adults fed a diet rich in interstitial algae laid, on average, 2.6 times the number of eggs as compared to adults fed a diet rich in *Zygogonium* sp. filaments. Based on this observation, the authors hypothesized that interstitial algae offer greater nutritional benefit than *Zygogonium* sp. filaments and may be the preferred food source *in situ* (35).

Bezzia setulosa. Larvae of the predatory midge, *B. setulosa* Loew (Diptera: Heleidae), feed on the eggs and immature larvae of *E. thermophil*a. This predatory behavior decreases the survirorship of immature *E. thermophila* larvae (35). For

example, the probability of *E. thermophila* egg survivorship in the presence of *B. setulosa* was dependent on egg density. The probability of egg survivorship decreased with increasing egg density for mats up to 1000 eggs/m^2. However, at increased egg densities ranging from 1000-4500 eggs/m^2, the probability of egg survivorship increased, yet only slightly. This data suggests a stabilizing influence of *B. setulosa* on *E. thermophila* population density in ASC geothermal spring mat ecosystems.

Food Web Synthesis

Very little is known about the food webs in ASC springs. The sole food web studied in the field occurs in the *Zygogonium* sp. mat communities. Results of Collins (35) suggest that *E. thermophila* oviposition is influenced by the composition of the microbial mat and that *E. thermophila* egg survivorship is influenced by the predatory midge, *B. setulosa*. Importantly, the results of Collins for the first time suggest that *E. thermophila* may utilize interstitial algae as a food source in ASC springs. However, despite the inference, the importance of mat grazing by invertebrate larvae on the recycling of photosynthetically-produced mat material and excreted carbon remains largely unknown in acid geothermal environments.

E. thermophila also inhabit alkaline geothermal springs. Previous studies indicate that the standing crop biomass and the concentration of bacteria in mats are influenced by mat grazing by ephydrid larvae (22, 150). Total mat biomass and the concentration of bacteria were shown to increase in treatments where grazing was permitted; however on resumption of natural flow, the standing crop decreased to approximately 20% of its starting value; likely a result of the physical disruption of the structured biomass during

grazing. In thermal transects which permitted larval grazing (<50°C), photosynthetic efficiency was significantly greater than in thermal transects which were too hot for invertebrate grazers, a result attributed to increased nutrient cycling (22). Future research should focus on identifying the food source supporting invertebrate larvae in ASC springs and also should aim at generating a better understanding of the ecological effects of grazing on mat microbial populations and communities in both alkaline and acidic geothermal springs.

Food webs involve the transfer of materials between organisms (trophic structures) as a result of consuming organisms to meet nutritional demands. For example, the biomagnification of methylmercury (MeHg) in food webs results from the consumption of biomass containing MeHg (33, 99, 100). MeHg is formed primarily by the activity of sulfate-reducing microorganisms (36) which have been identified in *Zygogonium* sp. mats in other acidic springs in YNP (48, 52), although other potential mercury methylation sources exist such as humic and fulvic acids (10). Elevated total mercury (THg) has been determined in the waters and soils surrounding ASC springs in the Norris Geyser Basin including Dragon Spring (82, 119). In addition, recent results suggest that *Zygogonium* sp. biomass collected from acidic springs in YNP contain elevated concentrations of Hg and MeHg (82). This raises the question of whether MeHg biomagnification is occuring in invertebrate larvae which putatively graze MeHg-containing *Zygogonium* sp. mat biomass.

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CHAPTER 3

ISOLATION, CHARACTERIZATION, AND ECOLOGY OF SULFUR-RESPIRING *CRENARCHAEA* INHABITING ACID-SULFATE-CHLORIDE GEOTHERMAL SPRINGS IN YELLOWSTONE NATIONAL PARK

Abstract

Elemental sulfur (S°) is associated with many geochemically diverse hot springs, yet little is known about the phylogeny and physiology of the organisms involved in its cycling. Here we report the isolation, characterization, and ecology of two novel, S°-reducing *Crenarchaea* from an acid geothermal spring referred to as Dragon Spring. Isolate 18U65 grows optimally at 70-72°C and at pH 2.5-3.0, while isolate 18D70 grows optimally at 81°C and pH 3.0. Both isolates are chemoorganotrophs, dependent on complex peptide-containing carbon sources, S°, and anaerobic conditions for respiration-dependent growth. Glycerol dialkyl glycerol tetraethers (GDGTs) containing 4-6 cyclopentyl rings were present in the lipid fraction of isolates 18U65 and 18D70. Physiological characterization suggests the isolates are adapted to the physicochemical conditions of Dragon Spring and can utilize the natural organic matter in the spring as a carbon and energy source. Quantitative PCR analysis of 16S rRNA genes associated with the S° flocs recovered from several acid geothermal springs using isolate-specific primers indicate these 2 populations together represent 17-37% of the floc-associated DNA; suggesting a role for these microbes in the biological cycling of sulfur at these sites. The physiological characteristics of isolates 18U65 and 18D70 are consistent with

their potential widespread distribution and putative role in the cycling of sulfur in acid geothermal springs throughout the Yellowstone National Park geothermal complex. Based on phenotypic and genetic characterization, the designations *Caldisphaera draconis* sp. nov. and *Acidilobus sulfurireducens* sp. nov. are proposed for isolates 18U65 and 18D70, respectively.

Introduction

Sulfur compounds are common constituents of the gaseous, aqueous, and solid phases of acid geothermal fluid of volcanic origin (32). At the point of surface discharge, gas phase components typically include sulfur dioxide (SO_2), hydrogen sulfide (H_2S), and elemental sulfur vapor (35, 44), while the solid phase is typically comprised of flocculent S° (35, 38). The origin of solid phase S° near the point of discharge in acidic geothermal springs can be attributed to biotic oxidation of H_2S as well as abiotic H_2S oxidation to thiosulfate ($S_2O_3^{2-}$), which disproportionates under acidic conditions to form sulfite (SO_3^-) and S° (48). Abiotic H_2S oxidation by metal oxidants (vanadate, ferric iron, cupric copper, etc.) may also contribute to the precipitation and accumulation of S° in geothermal environments (57); however, these reactions are less important due to the reducing conditions and the absence of these oxidants in source water in many high temperature acidic geothermal systems such as those found in Norris Geyser Basin, Yellowstone National Park (YNP), Wyoming (35, 38, 48). The S° formed from H_2S oxidation by these mechanisms accumulates in many acid geothermal springs due to its

slow reactivity with water below 100°C (47), and thus represents a significant source of electron donor and acceptor for microorganisms in these systems (2, 25).

S° has been shown to support respiratory metabolisms for a variety of organisms distributed throughout the *Bacteria* and *Archaea* (8, 21, 33, 56). Within the *Archaea*, S°-reduction has been identified in both the *Euryarchaeota* (13, 18) and *Crenarchaeota* (27, 28, 53, 66) and is typically coupled with the oxidation of complex organics and/or hydrogen (21). Within the order *Desulfurococcales*, phylum *Crenarchaeota*, the dominant metabolism of acidophiles is the fermentation of simple and complex organic compounds (28, 53), with significantly enhanced growth rates in the presence of S°. Many acidic geothermal springs within YNP possess geochemical conditions that should support organisms with metabolisms similar to those of the order *Desulfurococcales*.

The study summarized herein primarily focused on an acidic geothermal spring referred to as Dragon Spring (29, 35), which is located in Norris Geyser Basin, YNP. Dragon Spring is classified as an acid-sulfate-chloride spring (ASC), so-named because of its distinctive chemical signature (6, 35, 63). The source water of Dragon Spring (pH 3.1, 66-73°C) contains significant dissolved organic carbon (80 μM) and various inorganic energy sources such as H_2S, $Fe^{(II)}$, and $As^{(III)}$ (35). In addition, the entire outflow channel is visually distinguished by copious amounts of a solid phase comprised almost exclusively of S° (35) (Figure 3.1), suggesting this as an appropriate habitat for biotic S° oxidation and reduction. While the geochemical conditions at the source of Dragon Spring suggest an environment favorable for biological S°-reduction, phylogenetic analysis of bacterial and archaeal community 16S rRNA genes obtained

from S° precipitates failed to identify phylotypes related to known S°-reducers (unpublished data), thereby precluding efforts to use phylogeny to identify cultivation conditions necessary to culture such organisms; an approach previously utilized in other studies (54, 60). Alternatively, we analyzed the geochemistry of the spring water to identify the laboratory culture conditions representative of *in situ* conditions for the enrichment and isolation of microorganisms capable of S°-reduction. Here we report the isolation, characterization, and ecology of two novel, S°-respiring heterotrophs that cluster both phylogenetically and phenotypically within the "Acidilobus group" of the order *Desulfurococcales*, phylum *Crenarchaeota*. The physiological properties which could convey pertinent information on the ecological role of each isolate in ASC geothermal springs were determined. Results suggest that both isolates are adapted to the geochemical conditions present in several ASC springs in the Norris Geyser Basin, YNP

Materials and Methods

Sample Collection and Enrichment and Isolation of Spring Microbial Populations

Samples of precipitated (flocculent) S° were collected using sterile syringes and serum bottles from the source of Dragon Spring (44°43'55" N, 110°42'39" W), Beowulf Spring (44°43'53.4" N, 110°42'40.9" W), and Succession Spring (44°43'75.7" N, 110°42'72.7" W); all of which are ASC thermal springs located in the Hundred Springs Plain area of Norris Geyser Basin, YNP, Wyoming. Samples were collected from the point of discharge at the source of Dragon (66°C, pH 3.1), Succession (69°C, pH 3.1), and Beowulf (68°C, pH 3.0) Springs. Upon collection, samples were stored at 75°C

under anaerobic conditions during the 90-minute transit to Montana State University (MSU), Bozeman. Separate samples were placed on dry ice during transit to MSU, where they were stored at -20°C until processed for DNA extraction. Peptone-S° (PS) medium was used to enrich for S°-reducing thermophiles.

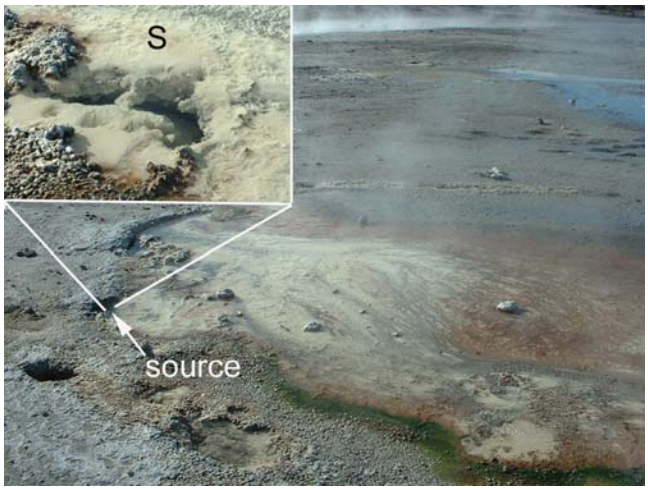

Figure 3.1. Dragon Spring source with abundant S° flocs (labeled S in picture inset)

PS medium consisted of NH_4Cl (0.33 g L^{-1}), KCl (0.33 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.33 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.33 g L^{-1}), and KH_2PO_4 (0.33 g L^{-1}), peptone (0.05% w/v), S° (5 g L^{-1}), Wolfe's vitamins (1 mL L^{-1}) (5), and SL-10 trace metals (1 mL L^{-1}) (62). Following autoclave-sterilization of the peptone and salts components in serum bottles, filter-sterilized Wolfe's vitamins, filter-sterilized SL-10 trace metals, and S° (sterilized by baking at 100°C for 24 h) were added and the bottles and contents were deoxygenated by purging with sterile nitrogen gas passed over heated (210°C) copper shavings. The serum bottles were sealed with butyl rubber stoppers and heated to 80°C prior to replacement of the headspace with H_2 and addition of sterile and anaerobic sodium

ascorbate to a final concentration of 50 μM. The serum bottles were then inoculated with S° precipitates recovered from the source of Dragon Spring and were incubated at 65°C or 70°C. A pure culture of isolate 18U65 was obtained from the 65°C enrichment by dilution to extinction. A pure culture of isolate 18D70 was obtained from the 70°C enrichment by dilution to extinction and increased incubation temperature (80°C). Multiple rounds of dilution to extinction were performed for each isolate to ensure pure culture status. Enrichment isolation progress was monitored using epifluorescence microscopy and denaturing gradient gel electrophoresis (DGGE) (see below).

DNA Extraction and Denaturing
Gradient Gel Electrophoresis

Enrichment progress was monitored using DGGE analysis of PCR-ampified 16S rRNA gene fragments recovered from enrichment cultures. CS_2 was added prior to DNA extraction to remove S° and to improve the recovery of DNA. Biomass for DNA extraction was concentrated by centrifugation (14,000 x g, 10 min., 4°C) and was resuspended in a minimal volume of sterile PS medium (lacking S°). CS_2 was added to a final concentration of 10% v/v and following phase separation, the top aqueous phase containing cells was collected and concentrated by centrifugation (14,000 x g, 10 min., 4°C). Preliminary experiments indicate that CS_2 treatment did not significantly affect the integrity or recovery (79 ± 9.9% recovery) of cells as determined by epifluorescence microscopy but did enhance the recovery of DNA by ~20% (data not shown). The cell pellet was subjected to DNA extraction using the FastDNA Spin for Soil Kit (MP Biomedicals, Solon, Ohio) according to the manufacturer's directions with the following

exception: 500 μL of tris-buffered phenol (pH 8.0) (Sigma, St. Louis, MO) was substituted for 500 μL of the 978 μL of sodium phosphate buffer. Following extraction, the DNA concentration was determined using the High DNA Mass Ladder (Invitrogen, Carlsbad, CA) for use in PCR. Each PCR contained the following reagents: 10 ng of DNA template, 1X PCR buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 200 μM of each deoxynucleotide triphosphate (Eppendorf, Hamburg, Germany), 0.5 μM of each forward and reverse primer, 0.4 μg μL^{-1} molecular-grade bovine serum albumin (Roche, Indianapolis, IN), 0.25 U Taq DNA Polymerase (Invitrogen), and nuclease-free water (Sigma) to a final volume of 50 μL. Primers used in PCR for DGGE analysis were archaeal 931F (5'- AAGGAATTGGCGGGGGAGCA -3') or bacterial 1070F (5'-ATGGCTGTCGTCAGCT-3'), in conjunction with universal 1392R (5'-ACGGGCGGTGTGTRC-3') or 1492R (5'-GGTTACCTTGTTACGACTT-3'). Each of the reverse primers were conjugated with a 40 bp GC-clamp attached to the 5' terminus (Integrated DNA Technologies, Coralville, IA). PCR was performed according to the following conditions for all primer sets: initial denaturation (94°C, 4 min.) followed by 30 cycles of denaturation (94°C, 1 min.), annealing (55°C, 1 min.), and elongation (72°C, 1 min.) with a final extension step at 72°C for 20 min. DGGE (60V, 60°C, 18 h) was performed according to previously published protocols (29) in a vertical gradient of 50-70% (archaeal amplicons) or 35-55% denaturant (bacterial amplicons).

Characterization of Physicochemical Properties of Isolates

The cardinal temperatures, pH, and Cl^- concentrations were determined for each isolate to assess how well adapted the isolates were to the physicochemical conditions of Dragon Spring. The minimum, optimum, and maximum temperatures for growth of each isolate were determined in PS medium adjusted to pH 3.0. Minimum, optimum and maximum pH for growth of isolates 18U65 and 18D70 were determined in PS medium incubated at 65 and 80°C, respectively. The requirement for and tolerance to Cl^- for each isolate was determined in PS medium with the pH and incubation temperature adjusted to the optimum for each isolate. Cl^- concentrations were adjusted by additions of NaCl.

Carbon Source and Electron Donors

Identification of carbon sources that support growth of the isolates when using S° as the terminal electron acceptor (TEA) was evaluated using PS medium adjusted to pH 3.0, where the candidate carbon source was substituted for peptone. Carbohydrate and polymeric carbon sources were added to a final concentration of 0.1% w/v; alcohols, alkanes, alkenes, and ketones to a final concentration of 10 mM; amino acids to a final concentration of 0.02% w/v; a mixture of 20 essential amino acids mixed at equal molar concentrations to a final concentration of 0.02% w/v; and fatty acids to a final concentration of 4 mM in media containing a 1:1 H_2 gas:aqueous phase ratio with the pH and incubation temperature adjusted to the optimum for each isolate.

An extract of lodgepole pine needles was also tested as a carbon source for the isolates since needle litter is a potential source of organic carbon *in situ* (unpublished

observations). Extract was prepared by incubating 10 g of fresh lodgepole pine needles in a 150-mL serum bottle containing 100 mL of pH 3.0 peptone-free PS medium at 81°C for 24 hr, after which the aqueous phase was recovered, filter-sterilized, and frozen. One mL of thawed extract was added to 100 mL of peptone-free PS medium as the sole carbon and energy source for the isolates.

Growth of isolates 18U65 and 18D70 on the different carbon sources was determined at 65°C or 81°C, respectively, in cultures that initially contained 100% H_2 in the headspace. After a carbon source was determined to support growth of the isolates when using S° as the TEA, fermentative growth with that carbon source was assessed by deleting S° from the medium (beef extract, glycogen, and gelatin were not evaluated).

The effect of the spring-relevant lithoautotrophic substrates H_2 and CO_2 was evaluated in the presence and absence of peptone. H_2 was tested as a potential electron donor by comparing growth rates and final cell densities in PS medium containing 100% H_2 in the headspace at the time of inoculation to that observed when the headspace contained 100% N_2. In addition, H_2 was also used as an electron donor in tests for chemolithoautotrophic growth with CO_2 as a sole C source in serum bottles containing peptone-free PS medium and a headspace gas mixture of 20%:80% CO_2:H_2 (headspace:aqueous phase ratio of 1:1).

Alternative Terminal Electron Acceptors

The ability of the isolates to utilize TEAs other than S° for growth was determined in S°-free PS medium. Sodium sulfate, sodium sulfite, sodium thiosulfate, potassium nitrate, and potassium nitrite were added at a concentration of 10 mM; $Fe^{(III)}$

citrate and $FeCl_3$ were added at a concentration of 500 μM; sodium arsenate was added at a concentration of 100 μM; and cystine was added at a concentration of 0.02% w/v.

Evaluation of Growth

Growth of each isolate on different carbon sources and electron donors and electron acceptors was quantified both in terms of generation time (T_n) and H_2S production. Generation time was determined by collecting a sample of suspended cells at different times following inoculation, adding SYBR Gold (Invitrogen) diluted 1/4000 v/v, collecting the cells on black 0.22 μm polycarbonate filters (Millipore, Billerica, MA), and enumerating the cells by direct epifluorescence microscopy using an Olympus B-max microscope with a WIBA filter cube combination (Olympus, Center Valley, PA).

The total sulfide produced by cultures respiring S° was determined using the methylene blue reduction method (19). Triplicate measurements of dissolved sulfide for each culture were averaged for three replicate cultures to calculate the average sulfide concentration and the standard deviation among replicates. Temperature-corrected Henry's law coefficients for H_2S were calculated using Eq. 1:

$$K_H = K_H^\circ \times e^{\left(\frac{-d\ln KH}{dT} \times \left(\frac{1}{T} - \frac{1}{T^o}\right)\right)} \qquad \text{Eq. 1}$$

where K_H° is the Henry's constant with the unit M/atm at 298° K, $-d\ln K_H/dT$ is the temperature dependence on the solubility constant, and T is the temperature in Kelvin (K). A Henry's constant (K_H°) of 0.087 and temperature dependence on solubility constant of 2100 were used to calculate temperature-corrected Henry's coefficients (11).

The ratio of the concentration of H_2S in the aqueous-phase and gas phase (head space) was calculated using Eq. 2:

$$\frac{c_a}{c_g} = K_H \times RT \qquad \text{Eq. 2}$$

where K_H is the temperature corrected Henry's coefficient, c_a is the aqueous phase concentration at equilibrium, c_g is the gas phase concentration at equilibrium, R is the gas constant 8.21 x 10^{-2} L atm K^{-1} mol^{-1} and T is the temperature in K. Since the pKa of H_2S deprotonation is 6.8-7.0 at 65-81°C, H_2S was considered to be a molecular species at the pH values of <5.5 used in this study (16). The activity of H_2S as a molecular species was calculated according to Eq. 3:

$$\log \gamma_i = K_i I \qquad \text{Eq. 3}$$

where K_i is the salting out coefficient and I is the ionic strength of PS medium. Ionic strength (I) was calculated according to Eq. 4:

$$I = \frac{1}{2}\sum(m_i z_i^2) \qquad \text{Eq. 4}$$

where m_i are the concentrations expressed in molality and z_i are the charges of the ions. The salting out coefficient for H_2S of 0.020 (42) and the calculated ionic strength of PS medium (24 mM) were used to calculate an activity (γ) for H_2S of 1.0011. Based on these calculations, the activity of H_2S was considered equal to the concentration. The headspace volume/aqueous phase volume was 1.0 in culture media used for the various growth studies.

Influence of Dissolved O_2
on Growth of Isolates

The influence of oxygen concentration on the growth of isolates was determined by replacing different volumes of headspace H_2 with air in serum bottles containing PS medium with the pH and incubation temperature adjusted to the optimum for each isolate. A Henry's constant of 0.0013 and temperature dependence on solubility constant of 1500 (36) were used to calculate temperature-corrected Henry's coefficients. The ratio of oxygen in the gas and aqueous phase was calculated according to Eq. 2.

Metabolic By-Products

The production of the metabolic by-products acetate, lactate, NH_4^+, and H_2, was determined for both isolates during growth at optimal pH and temperatures in PS medium. Following inoculation, cultures were sampled for acetate, lactate, and NH_4^+ daily for 10 days. Culture samples were filtered through a 0.22 μm membrane and the filtrate was analyzed using a Bioprofile 300A Biochemistry Analyzer (Nova Biomedical Corporation, Waltham, Massachusetts) calibrated with Nova Level 6 and 7 certified calibration standards (Nova Biomedical Corporation). Culture headspace H_2 concentration was determined during logarithmic growth using a GC8A gas chromatograph (Shimadzu Columbia, MD) equipped with an 80/100 ProPak N column (Supelco, St. Louis, MO). Total sulfide was determined as described above.

Amino Acid Analysis

Amino acid analysis was performed on culture supernatant collected from isolate 18D70 grown in PS medium with the pH and incubation temperature adjusted to the

optimum. Amino acids were determined in filtered (0.22 μm) culture supernatant collected at the time of inoculation and following 120 h of growth using an Applied Biosystems 420A derivatizer coupled to an Applied Biosystems 130A separator system. Samples were hydrolyzed in 6M HCl plus trace phenol in HCl vapors for 1 h and then in a vacuum at 150°C. After hydrolysis, norleucine was added as an internal standard.

Calculation of Cell Yields

Cell yields were calculated using 100 fg/μm^3 as the carbon content to biovolume conversion factor (45). Cell yields were computed during log-phase growth using the average cell diameter as determined by transmission electron microscopy (see below). The cell yield for *A. aceticus* was calculated from data extrapolated from figure 5 of Prokofeva et al (53).

16S rRNA Gene Analysis

PCR for determination of the 16S rRNA gene sequence was performed as described above for DGGE analysis using archaeal 21F (5'-TTCCGGTTGATCCYGCCGGA-3') and universal 1492R (Integrated DNA Technologies) primers. Amplicons were purified using the Wizard PCR Preps DNA Purification System (Promega) and were subsequently cloned, assembled, and analyzed according to previously published protocols (10) using the pGEM-T Easy Vector (Promega). Pairwise alignment of isolate 18U65, isolate 18D70, and archaeal reference 16S rRNA gene sequences was performed using Clustal w (v1.83) (59). Pairwise evolutionary distances were computed employing the correction of Jukes & Cantor (31).

The neighbor-joining algorithm and bootstrap resampling were used to construct and evaluate the phylogenetic tree, respectively, using programs within the TREECON package (61) with *Methanococcus vannielii* as the outgroup.

qPCR Analysis

qPCR was used to determine the relative abundance of isolate 18U65 and 18D70 DNA associated with S° floc materials sampled from the sources of Dragon, Succession, and Beowulf Springs. At the laboratory, samples of S° floc were treated to remove residual S° and subjected to total DNA extraction as described above for DGGE analysis. DNA was quantified using the nucleic acid binding fluorochrome SYBR Green I (Invitrogen) added to achieve a final concentration of 1/1000 v/v, a ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, DE), and Lambda phage DNA (Promega) as the standard. Strain-specific forward primers for isolate 18U65 and 18D70 were designed from the near-full length 16S rRNA gene. To ensure that qPCR amplicons generated in each reaction arose from the template of either isolate 18U65 or 18D70, forward primers were designed to contain 3-4 bp sequence mismatches when compared to the 16S rRNA gene sequence of *A. aceticus* and *C. lagunensis*, respectively. Primer 18U65-1062F (5'- GCTCTTAGTTGCTATCCC -3') and 1392R and primer 18D70-1073F (5'-CTGCGGGCGACCGTG-3') and 1392R were used to amplify segments of the 16S rRNA gene from isolates 18U65 and 18D70, respectively. For each set of sequence-specific primers, a series of qPCRs were performed over a range of annealing temperatures, primer concentrations, and SYBR Green I concentrations to optimize the qPCR. qPCRs contained the same concentrations of reagents as used above

for PCR with the following exceptions: primers were added to a final concentration of 400 nM and SYBR Green I was added to a final dilution of 1.6X (diluted 1:6250). A range of DNA template concentrations were used in the qPCRs and the final reaction volume was then adjusted to 25 μL with nuclease-free H_2O (Sigma). qPCRs were performed in 0.5 mL optically pure PCR tubes (Corbett, Sydney, Australia) in a RotorGene3000 quantitative real-time PCR machine (Corbett) according to the following cycling conditions: initial denaturation (95°C, 10 min.) followed by 35 cycles of denaturation (95°C, 10 sec.), annealing (56°C, 15 sec.), and extension (72°C, 20 sec.). Standards were prepared from genomic DNA for both isolates and serial dilution of these standards were used for qPCR resulting in a standard curve relating DNA template concentration to the qPCR threshold amplification signal generated for that amount of template. Abundance values for each isolate in each spring were generated by comparing qPCR threshold amplification signals from reactions containing known quantities of spring DNA extract to isolate-specific standard curves. Negative control qPCRs for isolate 18U65-specific primers contained isolate 18D70 genomic DNA as template and negative control qPCRs for 18D70-specific primers contained isolate 18U65 genomic DNA as template. Criteria for selecting a given template dilution for reporting in the present study included: 1) the threshold amplification signal must have been generated between PCR cycle 10-25; 2) the degree of similarity of DNA abundance values for a given template dilution must have resembled the DNA abundance values from the other template dilutions; and 3) the template dilution that yielded the lowest variability among replicates within a single template dilution. The standard deviation reported for qPCR

results reflects the standard deviation of 3 replicate qPCRs for the selected template concentration.

G + C Analysis

The genomic DNA G + C mol% content for each isolate was determined for taxonomic purposes. G + C mol% content was determined according to the methods of Gonzalez and Saiz-Jimenez (20) on a RotorGene3000 quantitative real-time PCR machine (Corbett) using *Pseudomonas aeruginosa* strain PAO1 and *E. coli* strain K12 as reference strains. The standard deviation reported for G + C analysis reflects the deviation of 3 replicate determinations.

Glycerol Dialkyl Glycerol Tetraether Analysis

Isolates 18U65 and 18D70 were cultivated in PS medium with the pH and incubation temperature adjusted to the optimum for each isolate to determine the GDGT lipid composition of the isolates. Cells (2.5 L) were harvested in mid-exponential growth phase by centrifugation (10,000 x g, 20 min) and were resuspended in a minimal volume of S°-free PS medium. Cells were treated with CS_2 to remove residual S° as described above for DNA extraction. The S°-free cell pellet was then subjected to lipid analysis as described previously (50, 64). The GDGT ring index was calculated as the weighted average number of rings according to the formula RI = GDGT-1 + 2(GDGT-2) + 3(GDGT-3) + 4(GDGT-4+GDGT-4') + 5(GDGT-5') + 6(GDGT-6+GDGT-6') (50).

Electron Microscopy

Isolate morphology was evaluated for taxonomic purposes. Logarithmic phase cultures of isolates 18U65 and 18D70 were fixed in 0.5% glutaraldehyde for 8 h at their respective cultivation temperatures. Fifty mL of each fixed cell suspension was centrifuged (5,000 x g, 15 min.) and the cell pellet was resuspended in 1 mL of glycine-HCl buffer (0.05 M, pH 3.0), transferred to a 1.0 mL microcentrifuge tube, and centrifuged (13,000 x g) to form an intact cell pellet. The cell pellet was dehydrated by overlaying a series of solutions of ethanol (30%, 50%, 70%, 100%) and 100% propylene oxide (PO) for 30 min in each solution. The cell pellet was embedded using different mixtures of PO and Spurr's embedding resin (1:1, 1:2, and finally, pure resin). The pellets were then transferred to vial block molds, overlaid with additional resin, and the resin polymerized (70°C, 8 h). The polymerized resin blocks were removed from the molds, sectioned with an ultramicrotome, and the sections stained with uranyl acetate and lead citrate before examination with a Zeiss LEO 912 AB electron microscope.

Nucleotide Sequence Accession Numbers

Nucleotide sequence accession numbers for 16S rRNA gene sequences used as reference for 16S rRNA gene qPCR primer design and phylogenetic analysis are as follows: *Desulfurococcus amylolyticus* (AF250331), *Desulfurococcus fermentans* (AY264344), *Thermosphaera aggregans* (X99556), *Sulfophobococcus marinus* (X99560), *Pyrolobus fumarii* (X99555), *Pyrodictium occultum* (M21087), *Hyperthermus butylicus* (X99553), *Stetteria hydrogenophila* (Y07784), *Aeropyrum camini* (AB109559), '*Caldococcus noboribetus*' strain NC12 (D85038), *Acidilobus aceticus* (AF191225),

Caldisphaera lagunensis (AB087499), *Pyrobaculum islandicum* (L07511), *Caldivirga maquilingensis* (AB013926), *Thermocladium modesties* (AB005296), *Stygiolobus azoricus* (X90480), *Sulfolobus metallicus* (D85519), *Acidianus brierleyi* (X90477), *Metallosphaera sedula* (X90481), *Ignisphaera aggregans* strain AQ1 (DQ060321), *Methanococcus vannielii* (M36507), *Geogemma pacifica* (DQ492259), uncultured *Aquificaceae* bacterium clone 1 (DQ398776), and uncultured *Aquificaceae* bacterium clone 27 (DQ398794). The sequences of the 16S rRNA genes for isolates 18U65 and 18D70 have been deposited in the GenBank, DDBJ, and EMBL databases under accession numbers EF057392 and EF057391, respectively.

Results

Site Description

The aqueous and solid phase chemistry of Dragon Spring has been reported previously (35). Spring source water temperature ranged from 66-73°C, while the pH remained relatively stable at 2.9-3.1 over the course of the study. The spring contains abundant precipitated (flocculant) S°, extending from the source to approximately 4 meters down the central transect of the main flow channel (Figure 3.1). The aqueous and solid phase geochemistry of Succession Spring (69°C, pH 3.1) and Beowulf Spring (68°C, pH 3.0) have been reported previously (26).

Enrichment and Isolation

Incubation (65°C or 70°C) of S° flocs collected from the source of Dragon Spring in PS medium resulted in the production of H_2S, indicating the presence of active, S°-

respiring populations. DGGE analysis of the 65°C and 70°C enrichment cultures indicated the presence of 3 and 8 unique 16S rRNA gene phylotypes, respectively (data not shown). Following dilution to extinction (isolate 18U65) or increased incubation temperature (80°C), DGGE analysis of PCR-amplified 16S rRNA genes generated using multiple primer sets indicated a single 16S rRNA gene phylotype from cultures subjected to these different conditions (data not shown). Epifluorescence microscopic analysis of enrichments yielded a single morphotype in each culture, further indicating the presence

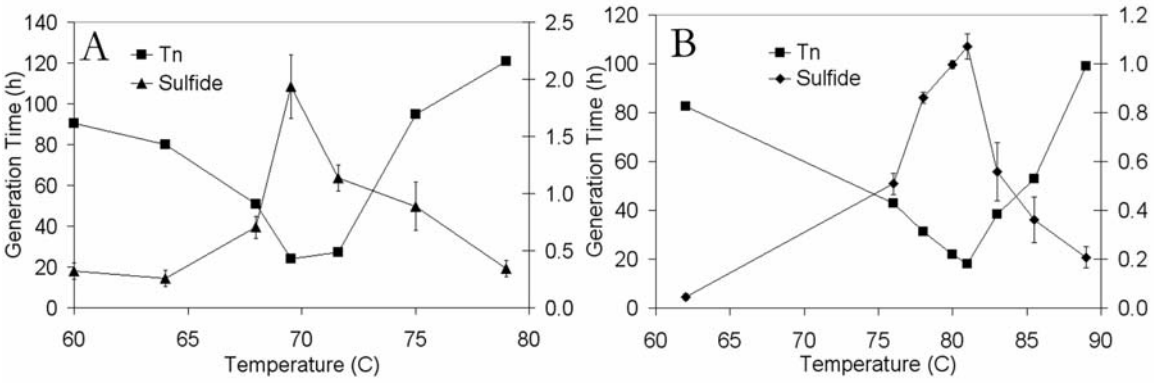

Figure 3.2. Influence of temperature on generation time (Tn) and total sulfide production for isolate 18U65 (A) and 18D70 (B).

of a clonal population of cells. Prior to characterization, cultures of each isolate were maintained at 65°C (isolate 18U65) or 80°C (isolate 18D70) through serial transfer (5% v/v) of log-phase cultures and routine (twice weekly) replacement of the headspace gas phase with H_2.

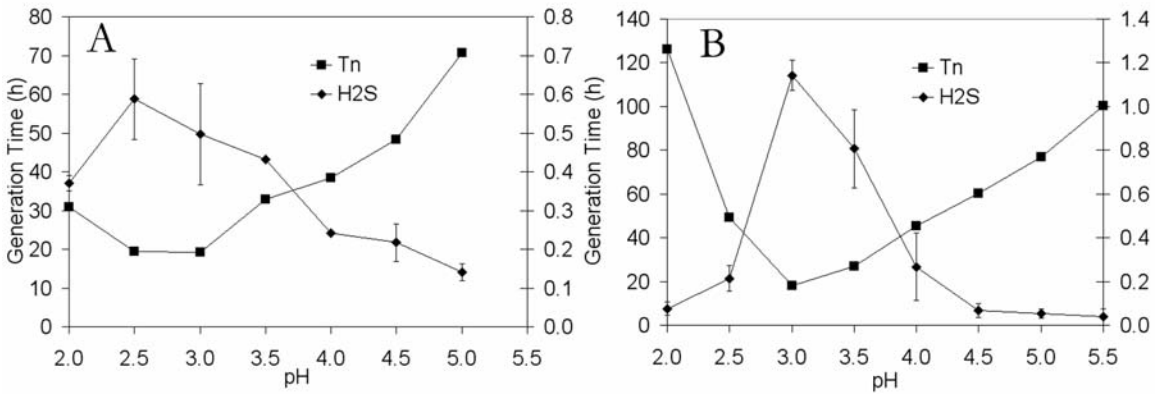

Figure 3.3 Influence of pH on generation time (Tn) and total sulfide production for isolate 18U65 (A) and 18D70 (B).

Physiological Properties

Isolate 18U65 grew over a temperature range of 60-79°C with an optimum of 70-72°C (Figure 3.2) and over a pH range of 2.0-5.0 with an optimum of 2.5-3.0 (Figure 3.3), whereas isolate 18D70 grew over a temperature range of 62-89°C with an optimum of 81°C (Figure 3.2) and over a pH range of 2.0-5.5 with an optimum of 3.0 (Figure 3.3).

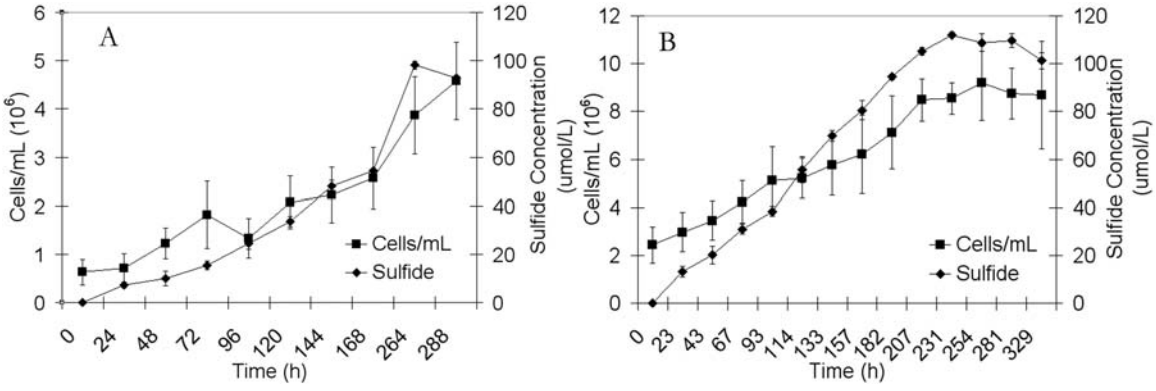

Figure 3.4. Growth curve of isolates 18U65 (A) and 18D70 (B) grown at optimal temperature and pH.

Isolate 18U65 grew over a Cl^- concentration range of 0-86 mM, with an optimum of 17-34 mM, while isolate 18D70 grew over a Cl^- concentration range of 0-128 mM, with an

optimum of 12 mM. The shortest generation times for isolates 18U65 and 18D70 coincided with maximal H_2S production (S°-reducing activity) (Figures 3.2 and 3.3). The generation times of isolates 18U65 and 18D70, grown at optimal pH and temperature, were 19 and 17 hours, respectively. Sulfide production paralleled culture growth (Figure 3.4), with maximum culture cell densities for isolates 18U65 and 18D70 being 4.6×10^6 and 2.1×10^7 cells mL^{-1}, respectively.

Both isolates required complex, organic carbon sources for growth (Table 3.1). Isolate 18U65 could utilize yeast extract, peptone, tryptone, casamino acids, beef extract, glycogen, and gelatin while isolate 18D70 could utilize yeast extract, peptone, glycogen, and gelatin. Both isolates grew on pine needle extract in the absence of peptone, but neither isolate could utilize monomeric or polymeric carbohydrates, fatty acids, alkanes, alkenes, alcohols, ketones, organic acids, individual amino acids, or an equal molar mixture of amino acids (Table 3.1). When isolate 18D70 was grown in PS medium, the combined free amino acid concentration decreased from 116.4 to 93.9 pmol/µL following 120 h of growth (data not shown). Following peptide hydrolysis, the combined free amino acid concentration decreased from 1017.7 pmol μL^{-1} at the time of inoculation to 520.3 pmol μL^{-1}, suggesting that peptides are preferred over free amino acids to satisfy a nutritional requirement. The sole TEA utilized by both isolates was S° (Table 3.2), although 18U65 could ferment yeast extract and peptone (Table 3.2).

Due to the abundance of dissolved inorganic carbon in the source of Dragon Spring (35), the isolates were evaluated for their ability to grow as autotrophs and/or as

Table 3.1. Carbon source utilization[a] of isolates and selected members of the *Crenarcheota*

Carbon Sources	Organisms			
Carbohydrates	18U65	18D70	*A. aceticus* (48)	*C. lagunensis* (26)
glucose	-	-	-	-
galactose	-	-	-	-
fructose	-	-	-	-
ribose	-	-	ND	-
lactose	-	-	ND	-
maltose	-	-	-	-
xylose	-	-	-	-
sucrose	-	-	ND	-
arabinose	-	-	-	-
mannose	-	-	ND	-
Polymeric Sources				
yeast extract	+	+	+	+
peptone	+	+	ND	+
casamino acids	+	-	ND	+
tryptone	+	-	ND	ND
pine needle extract	+	+	ND	ND
glycerin	-	-	ND	ND
cellobiose	-	-	ND	ND
cellulose	-	-	-	ND
starch	-	-	+	+
soya extract	-	-	+	ND
beef extract	+	-	+	+
gelatin	+	+	ND	+
glycogen	+	+	ND	+
Organic Acids			ND	ND
butyrate	-	-	ND	-
propionate	-	-	-	-
succinate	-	-	ND	-
lactate	-	-	ND	-
pyruvate	-	-	-	-
acetate	-	-	-	-
citrate	-	-	ND	-
malate	-	-	-	-
formate	-	-	-	-
fumarate	-	-	ND	-
benzoate	-	-	ND	ND
Amino Acids			ND	ND
20 amino acid equal molar mixture	-	-	-	ND
each individual amino acid	-	-	-	ND

Table 3.1 Continued

Alkanes/Alkenes/	Organisms			
Alcohols/Ketones	18U65	18D70	*A. aceticus* (48)	*C. lagunensis* (26)
methane	-	-	ND	ND
propane	-	-	ND	ND
propylene	-	-	ND	ND
ethanol	-	-	ND	ND
butanol	-	-	ND	ND
propanol	-	-	ND	ND
isopropanol	-	-	ND	ND
methanol	-	-	-	-
acetone	-	-	ND	ND

[a](-), no growth; (+) growth; (ND), not determined

Table 3.2. Growth[a] of isolates 18U65 and 18D70 on various carbon (C) sources and terminal electron acceptors (TEA)

	Carbon Source					
	18U65				18D70	
TEA	Peptone	Yeast Extract	Tryptone	Casein	Peptone	Yeast extract
thiosulfate	-	-	-	-	-	-
sulfate	-	-	-	-	-	-
sulfur	+	+	+	+	+	+
nitrate	-	-	-	-	-	-
nitrite	-	-	-	-	-	-
arsenate	-	-	-	-	-	-
ferric chloride	-	-	-	-	-	-
ferric citrate	-	-	-	-	-	-
cystine	-	-	-	-	-	-
fermentative	+	+	-	-	-	-

[a](-), no growth; (+), growth

mixotrophs. Neither isolate grew under the autotrophic conditions tested in this study. Supplementation of PS medium with H_2 or CO_2 did not enhance or inhibit S°-respiring activity of either isolate. S°-respiration and growth were inhibited when the concentration of oxygen in the aqueous phase was greater than 62 and 54 nM for isolate

18U65 and 18D70, respectively. Metabolic by-products detected in the cultures of the isolates grown in PS medium include H_2S (both isolates) and NH_4^+ (isolate 18D70 only). Lactate, acetate, and H_2 were not detected as metabolic products in the culture supernatant of either isolate grown in PS medium.

GDGT Composition of *Crenarchaeal* Isolates

GDGTs containing 4-6 cyclopentyl rings were present in isolates 18U65 and 18D70 (Table 3.3). GDGTs containing 4 rings (GDGT-4) represented 34% and 4% of the total GDGTs for isolates 18U65 and 18D70, respectively. GDGT-5', which contains 5 cyclopentyl rings, represented 41 and 36% of the total GDGTs in isolate 18U65 and 18D70, respectively. The 6-ring GDGT (GDGT-6) common to both isolates represented 25 and 55% of the total GDGT in the lipid fraction of isolate 18U65 and 18D70, respectively while the second 6-ringed GDGT (GDGT-6') was found only in isolate 18D70 (5% of total GDGTs).

Table 3.3. Abundance of glycerol diacyl glycerol tetraether (GDGT) lipids with different numbers of rings[a] in isolates 18U65 and 18D70

Sample	Temp (°C)	GDGT-4 m/z 1294[c]	GDGT-5' m/z 1292[c]	GDGT-6 m/z 1290[c]	GDGT-6' m/z 1290[c]	Ring Index[b]
Isolate 18U65	70	0.34	0.41	0.25	0	4.91
Isolate 18D70	81	0.04	0.36	0.55	0.05	5.56

[a]GDGT-4, 4 ring cyclopentane; GDGT-4', 4-ring cyclopentane isomer; GDGT-5', 5-ring cyclopentane (not crenarchaeol); GDGT-6, 6 ring cyclopentane; GDGT-6', 6-ring cyclopentane isomer.
[b]Ring Index = Weighted average number of rings, calculated as [GDGT-1 + 2(GDGT-2) + 3(GDGT-3) + 4(GDGT-4+GDGT-4') + 5(GDGT-5') + 6(GDGT-6+GDGT-6')]
[c]mass/charge

Genetic Properties of Isolates

Near full-length 16S rRNA gene sequence analysis (corresponding to positions 27-1479 of the *E. coli* 16S rRNA gene) was determined for use in phylogenetic analysis of isolates 18U65 and 18D70. Both isolates clustered within the "Acidilobus group" (28) (phylum *Crenarchaeota*) with isolate 18U65 clustering within the *C. lagunensis* lineage (100% bootstrap support) and isolate 18D70 clustering within the *A. aceticus* lineage

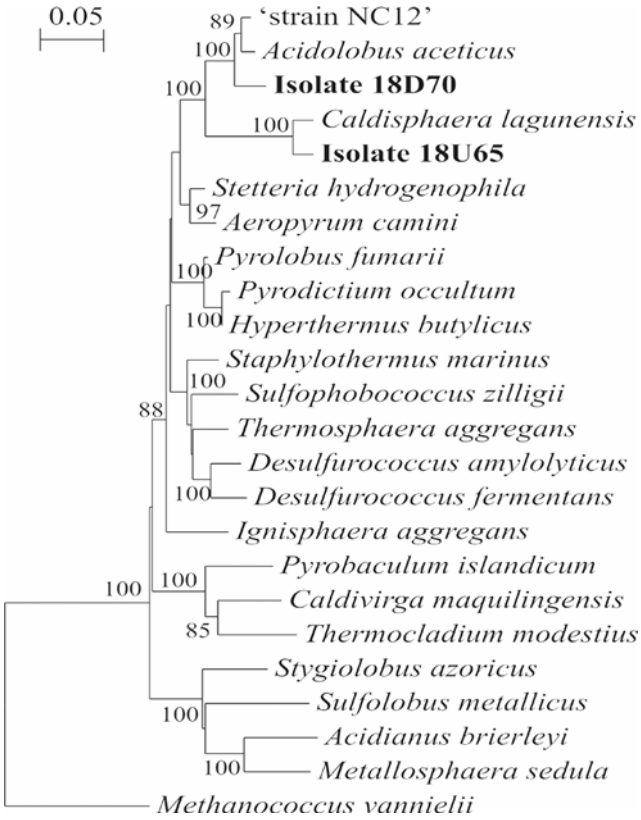

Figure 3.5 Phylogenetic relationship of isolate 18U65 and 18D70 and members of the *Crenarchaeota* based on comparison of 16S rRNA gene calculated using the neighbor-joining method. Bootstrap values (100 re-samplings) are shown at branch points; values greater than 80 are reported. Bar represents 5 nucleotide substitutions per 100 nucleotides. *Methanococcus vannielii* used as the outgroup.

(100% bootstrap support) (Figure 3.5). The 16S rRNA gene of isolate 18U65 was determined to be 96% similar to the 16S rRNA gene from *Caldisphaera lagunensis*, whereas the 16S rRNA gene of isolate 18D70 was determined to be 94% similar to the 16S rRNA gene from *Acidilobus aceticus*. The 16S rRNA gene from the uncharacterized crenarchaeotal strain NC12 ('*Caldococcus noboribetus*', D85038) was 96 and 89% similar to the 16S rRNA gene of isolates 18D70 and 18U65, respectively. The 16S rRNA genes from isolate 18U65 and 18D70 were 88% similar to each other. The G + C content of DNA from isolates 18U65 and 18D70 were found to be 53.9 ± 0.0 and 59.9 ± 0.4 mol %, respectively

Abundance of Isolates Associated With S° Precipitates

Primers specific to the 16S rRNA gene of each isolate were used in qPCR to determine the abundance of their DNA relative to that of other members of the S° precipitate-associated microbial communities of Dragon Spring where the organisms were isolated, and of two other ASC springs in the Norris Geyser Basin: Beowulf and Succession Springs. The DNA of isolates 18U65 and 18D70 represented 20.2 ± 0.6% and 7.9 ± 0.1% of the DNA associated with Dragon Spring S° precipitate, respectively; 32.3 ± 1.9% and 5.3 ± 0.6% of the DNA associated with S° precipitate from Beowulf Spring, respectively; and 12.2 ± 0.8% and 5.6 ± 0.3% of the DNA associated with S° precipitate from Succession Spring, respectively. There was no indication of a matrix inhibitory effect in the qPCR assay using S° precipitate DNA extracts from Dragon, Beowulf, and Succession Springs diluted 2000-fold or more (data not shown). Treatment

of S° precipitate with CS_2 prior to DNA extraction did not alter the results of the qPCR assay (data not shown) suggesting that CS_2 treatment did not significantly alter the apparent community composition.

Morphology and Ultrastructure

Cells of isolate 18U65 and 18D70 were examined by epifluorescence and electron microscopy. Both isolates exhibited similar coccoid morphology and ultrastructure (Figure 3.6). Cells of isolate 18U65 were 0.8-1.0 µm in diameter while cells of isolate 18D70 were 0.4-0.6 µm in diameter (Figure 3.6). Both isolates were routinely observed singly or in pairs. Electron microscopy of thin sections revealed a cell envelope for both isolates that contained both a cytoplasmic membrane and an outer S-layer (Figure 3.6).

Preservation

Cultures of isolates 18U65 and 18D70 remained viable at room temperature when the headspace was purged with N_2 and they were kept in the dark. Isolate 18D70 could be frozen (-80°C) in the presence of 10% v/v glycerol. Attempts to cryo-preserve cultures of 18U65 have been unsuccessful to date.

Discussion

S°-reducing *Crenarchaea* have been detected by 16S rRNA gene-based diversity surveys in a variety of geographically distinct, sulfur-rich geothermal springs including those in Yellowstone National Park (38, 40), the Philippines (28), and Japan (3). Despite

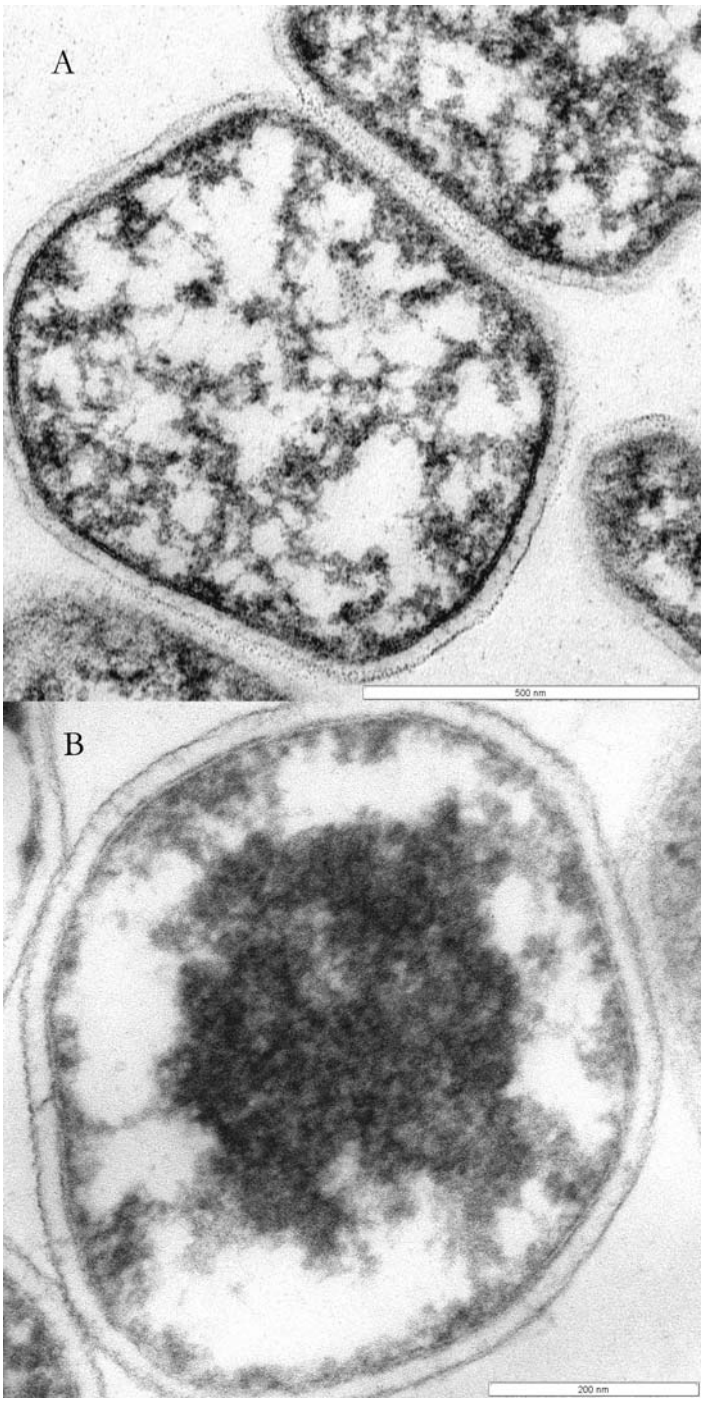

Figure 3.6. Electron micrograph of isolate 18U65 (A) and 18D70 (B). Scale bars represent 500 nm (A) and 200 nm (B)

geochemical analyses which suggested the source of Dragon Spring to be an environment favorable for S°-reducing populations (35), 16S rRNA gene-based diversity analyses of the S° floc-associated community conducted with universal (non-specific) primer sets failed to recover sequences related to known S°-reducing organisms. Therefore, a traditional laboratory cultivation and enrichment strategy based on aqueous and solid phase geochemistry was utilized; an approach that has proven successful in isolating ecologically-relevant organisms from acid mine drainage (60) and acid hydrothermal environments (54). This approach, when applied to floc material from Dragon Spring yielded enrichment cultures exhibiting biological S° reduction which led to the subsequent isolation of two novel S°-reducing *Crenarchaea.*

Comparative sequence analysis of the 16S rRNA gene from the isolates suggested that they each represent novel species within the 'Acidilobus group' in the order *Desulfurococcales* (phylum *Crenarcheota*) (55): isolate 18U65 clustering within the *Caldisphaera lagunensis* lineage (28) and isolate 18D70 clustering within the *Acidilobus aceticus* lineage (53). To date, the *A. aceticus* lineage is comprised of the type strain *A. aceticus* (53) and the uncharacterized strain NC12 ("*Caldococcus noboribetus*") (3), while the *C. lagunensis* lineage is comprised solely of the type strain (28).

Phenotypic characteristics determined for both isolates 18U65 and 18D70 support the 16S rRNA gene-based phylogenetic assessment placing them in the *Crenarchaeota* within the *Archaea* (Table 3.4). The recovery of tetraether-linked GDGTs from the lipid fraction of both isolate 18U65 and 18D70 further supports their placement in the *Archaea* (15, 34). Within the *Archaea*, the majority of thermoacidophiles belong to the

Table 3.4. Growth properties of selected members of the *Crenarcheota*

Genus species[a]	Temp[b]	pH[b]	Met[c]	e-Acceptor
Isolate 18U65	60-79 (70-72)	2.0-5.0 (2.5-3.0)	H/F	S°
Isolate 18D70	62-89 (81)	2.0-5.5 (3.0)	H	S°
'strain NC12' (3)	70-96 (92)	1.5-4.0 (3.0)	H/F	S°
A. aceticus (53)	60-92 (85)	2.0-6.0 (3.8)	H/F	S°
C. lagunensis (28)	45-80 (70-75)	2.3-5.4 (3.5-4.0)	H/F	S°, $C_4H_4O_4$, O_2
A. camini (46)	70-97 (85)	6.5-8.8 (8.0)	H	O_2, $S_2O_3^{2-}$
S. hydrogenophila (30)	70-102 (95)	4.5-7.0 (6.0)	M	S°, $S_2O_3^{2-}$
H. butylicus (65)	75-110 (95-106)	ND (7.0)	H/F	S°,
P. fumarii (7)	90-113 (106)	4.0-6.5 (5.5)	A	NO_3^-, $S_2O_3^{2-}$, O_2
D. fermentans (51)	63-89 (80-82)	4.8-6.8 (6.0)	H/F	S°
D. amylolyticus (9)	68-97 (90-92)	5.7-7.5 (6.4)	H/F	S°

[a]Genus species and literature reference
[b]Cardinal temperature and pH values: minimum - maximum (optimum)
[c]Metabolism: H, heterotroph; F, fermentative; M, mixotroph; A, autotroph
[d]Org, organic carbon; H_2, hydrogen
[e]S°, elemental sulfur; $C_4H_4O_4$, fumarate; $S_2O_3^{2-}$, thiosulfate

Crenarchaeota (53), consistent with the clustering of both thermoacidophilic isolates in the *Crenarchaeal* lineage. Within the *Crenarchaeal* order *Desulfurococcales*, the predominant metabolism is the oxidation of complex organic compounds coupled with the reduction of S° (12), a phenotype shared by both isolates. In addition, all characterized members of the order *Desulfurococcales* that are extreme acidophiles (pH_{opt} <4.5) cluster solely within the 'Acidilobus group' lineage, supporting phylogenetic characterization which placed both acidophilic isolates within this lineage.

A number of phenotypic traits of isolate 18U65 more closely resemble those of the *C. lagunensis* lineage than those of the *A. aceticus* lineage. Cardinal temperatures and pH of isolate 18U65 more closely resemble those of *C. lagunensis* than of those *A. aceticus*. The range of carbon sources utilized by and cellular morphology of isolate 18U65 also more closely resemble those of *C. lagunensis* than those of *A. aceticus*.

While similar to *C. lagunensis* in many ways, a few phenotypic traits distinguish isolate 18U65 from *C. lagunensis*. Whereas, *C. lagunensis* can utilize oxygen as a terminal electron acceptor, isolate 18U65 is a strict anaerobe incapable of growth in media containing nanamolar concentrations of oxygen. Furthermore, *C. lagunensis* can respire fumarate and sulfate in addition to oxygen (28), while isolate 18U65 can only respire S°.

A number of phenotypic traits of isolate 18D70 more closely resemble those of the *A. aceticus* lineage than those of the *C. lagunensis* lineage. Like *A. aceticus*, but in contrast to *C. lagunensis*, isolate 18D70 is a strict anaerobe incapable of growth in media containing nanamolar concentrations of oxygen. Cardinal temperatures and pH of isolate 18D70 more closely resemble those of *A. aceticus* than those of *C. lagunensis*. However, isolate 18D70 exhibits phenotypes which distinguish it from *A. aceticus*. For example, isolate 18D70 is unable to couple the oxidation of starch, soya extract, or beef extract to S° respiration, all of which support S° respiration in *A. aceticus* (53). Furthermore, unlike *A. aceticus*, isolate 18D70 is unable to support growth through fermentation pathways. Isolate 18D70 also differs from *A. aceticus* in cell morphology: cells of *A. aceticus* occur as irregular cocci with a diameter of 1-2 μm (53), whereas, cells of isolate 18D70 occur as regular cocci with a much smaller diameter of 0.4-0.6 μm.

Lipid data is not reported in the characterization of *A. aceticus* (53), and while both acyclic and cyclic tetraethers are reported in the lipid fraction of *C. lagunensis*, a detailed description of the structures of these tetraethers is not provided (28). Previous studies have shown that genetically-related organisms have similar GDGT profiles (37). While the GDGT composition of isolates 18U65 and 18D70 are similar, it remains to be

determined whether the GDGT composition of isolates 18U65 and 18D70 corroborates genetic and phenotypic properties which support the clustering of these organisms in the *C. lagunensis* and *A. aceticus* lineages, respectively.

On the basis of phylogenetic and physiological properties, it is proposed that isolates 18U65 and 18D70 represent distinct taxa within the *C. lagunensis* and *A. aceticus* lineages, respectively. We propose that isolate 18U65 be assigned to a new type species – *Caldisphaera draconis*, and that isolate 18D70 be assigned to a new type species – *Acidilobus sulfurireducens*.

Lodgepole pines are a predominate form of macrovegetation in the Norris Geyser Basin ecosystem (49) and needle litter is often found in the waters of ASC geothermal springs in this area (personal observation). Thus, needle litter represents a natural source of carbon and energy for heterotrophic consumers inhabiting ASC geothermal environments. Both isolates were capable of coupling S° reduction with the oxidation of pine needle extract, suggesting a role for these microbes in the mineralization of complex natural organic matter (NOM) in geothermal environments. Recent stable isotope and radioisotope studies suggest NOM utilization by some members of the planktonic marine *Archaea* community (22, 23). Furthermore, the generation time of a haloarchaean (*Halobacterium salinarum*) grown on fish protein hydrolysates was reduced by 50% during the first 20 hours of growth relative to cultures grown on synthetic medium (DSM 97), suggesting NOM to be a suitable carbon and energy source for this organism (39). While NOM utilization has been demonstrated in other archaeal phyla, the results of the

current study represent the first time that NOM has been shown to be used as the sole carbon and energy source in the *Crenarcheaota*.

qPCR has been used with phylotype-specific primers to assess the abundance of *Archaea* in a variety of environments (43, 58). The greater abundance of isolate 18U65-like phylotypes than isolate 18D70-like phylotypes in the S° precipitate-associated microbial communities sampled in the present study may be due in part to their different laboratory-derived optimum temperatures (T_{opt}) for growth. Whereas, the T_{opt} (70-72°C) of isolate 18U65 was within a few degrees Celsius of the temperature of the spring water where the isolates were recovered (66-69°C), the T_{opt} (81°C) of isolate 18D70 was over 10°C higher than the highest spring water temperature recorded in the spring during the duration of the study (66-73°C). In fact, the spring temperature was closer to the minimum temperature (62°C) for growth of this isolate. Efforts to collect subsurface spring source water with temperatures closer to the T_{opt} of isolate 18D70 have not yet been successful.

The relative abundance of closely-related microbial populations in other thermal spring microbial communities also reflects how closely their T_{opt} for growth, based on laboratory studies, coincides with spring temperature (1, 17, 41). In an alkaline hot springs, *Synechococcus* strain A (T_{opt} 55°C) was detected in an area of the mat exposed to a temperature of 56°C, while *Synechococcus* strain B (T_{opt} 50°C) was not. In contrast *Synechococcus* strain B was detected in an area of the mat exposed to a temperature of 53°C, whereas *Synechococcus* strain A was absent (1, 17). Thus, dominance among

populations with similar physiologies may be determined by how closely their T_{opt} coincide with the temperature of the environment.

The differences in relative abundance of the two isolates in the S° precipitate-associated microbial communities in the springs sampled in the present study may also reflect differences in their cell yields when using S° as TEA. Cell yields of isolates 18U65 (344±186 pmol C • nmol $S^{\circ -1}$) and 18U70 (39±18 pmol C • nmol $S^{\circ -1}$) respiring on S° were significantly different. By comparison, the cell yield of isolate 18U65 was not significantly different from that (195±27 pmol C • nmol $S^{\circ -1}$) reported for *Acidilobus aceticus*, a closely-related species (53). However, when the cell yield of the latter was recalculated using total H_2S produced instead of aqueous phase H_2S, according to the method described in the current study, a value of 71.3±9.9 pmol C • nmol $S^{\circ -1}$ was obtained, which is significantly different from the values obtained for either isolate in the present study. The greater cell yield of isolate 18U65 compared to that of isolate 18U70 could contribute to the greater abundance of the former in the springs sampled in this study if these laboratory-based values apply to the spring environment.

The results of the laboratory studies reported here indicate that both isolates 18U65 and 18D70 require S° for respiration. Nine of the twelve recognized genera within the *Desulfurococcales* are capable of coupling the oxidation of organic carbon or hydrogen with S° reduction. Two genera, *Ignicoccus* (24) and *Staphylothermus* (4), can only use S° as TEA for respiration. Both *Ignicoccus* and *Staphylothermus* are thermophiles that inhabit marine hydrothermal vent ecosystems where S° precipitates

from H_2S-containing vent fluids similar to that observed at the source of ASC springs in the Norris Geyser Basin (29, 35). Habitats such as these with a consistent supply of S° likely promote the establishment of microbial populations that depend on S° for respiration such as *Ignicoccus* and *Staphylothermus* and their freshwater counterparts such as the isolates described above.

Our unpublished studies indicate that chemolithotrophic *Hydrogenobacculum* spp. affiliated with the *Aquificales* are also abundant members of the microbial community associated with the S° flocs in Dragon Spring. Isolates of *Hydrogenobacculum* spp. from Dragon Spring are capable of H_2S oxidation, forming S° external to the cell as a metabolic by-product (14). Thus, S° floc-associated populations of *Hydrogenobacculum* spp. and the *Crenarchaeal* isolates described in the present study together appear to be capable of cycling the H_2S and S° forms of sulfur. A similar truncated sulfur cycle has been reported in a laboratory-based consortium of H_2S-oxidizing green-sulfur phototrophs grown in mixed culture with S°-reducing prokaryotes (52). The extent to which the H_2S-oxidizing activities of *Hydrogenobacculum* spp. and S°-reducing activities of the *Crenarchaeal* isolates described here contribute to the overall sulfur cycling in source waters of the ASC thermal spring environment remains to be determined.

In summary, two novel S°-reducing *Crenarchaea* were isolated that together represent a significant fraction of the microbial community associated with S° precipitates in several ASC geothermal springs of Norris Geyser Basin in Yellowstone National Park. Both isolates are capable of utilizing a naturally-occurring complex form of carbon as a carbon and energy source and naturally-formed S° as terminal electron

acceptor for respiration-dependent growth. Incorporation of other naturally-occurring substrates from this environment in enrichment media may facilitate the isolation of additional novel, biogeochemically-relevant microorganisms from this and similar habitats.

Description of *Caldisphaera draconis* sp. nov.

Caldisphaera draconis (dra.co' nis. L. gen. masc. n. draconis of /from dragon, as the organism was isolated from Dragon Springs, Yellowstone). *Caldisphaera dracosis*: hot spherical cell from Dragon Spring. Growth is anaerobic. Cells are coccoid, 0.8-1.0 μm in diameter, and are found singly or in pairs. Cell envelope contains a cytoplasmic membrane and an outer S-layer. Chemoorganotrophic metabolism capable of growth on tryptone, casamino acids, peptone, yeast extract, beef extract, glycogen, gelatin, and pine needle extract with S° as sole TEA. Fermentative growth on peptone and yeast extract. Growth over a pH range of 1.5-5.0, a temperature range of 60-79°C, and a Cl^- range of 0-86 mM. Optimal growth occurs at pH 2.5-3.0, 70-72°C, and 17-34 mM Cl^-. Generation time under optimal conditions is 19 h, yielding a maximum cell density of 4.6×10^6 cells mL^{-1}. Core lipid fraction contains glycerol dialkyl glycerol tetraethers containing 4-6 cyclopentyl rings. Genomic DNA G+C content is 53.9 mol%. The type strain 18U65 was isolated from Dragon Spring, Norris Geyser Basin, YNP, Wyoming, USA.

Description of *Acidilobus sulfurireducens* sp. nov.

Acidilobus sulfurireducens (sul.fu.ri.re' du.cens L. n. sulfur sulfur, L. part. adj. reducens leading back, reducing, N.L. part. adj. sulfurireducens reducing sulfur.).

Acidicoccus sulfurireducens: acidiphilic coccus that reduces sulfur. Growth is anaerobic. Cells are coccoid, 0.4-0.6 μm in diameter, and occur singly or in pairs. The cell envelope contains a cytoplasmic membrane and outer S-layer. Growth occurs over a temperature range of 62-89°C, a pH range of 2.0-5.5, and a Cl^- range of 0-128 mM. Optimal growth conditions are 81°C, pH 3.0, and 12 mM Cl^-. Generation time under optimal conditions is 17 h, yielding a maximum cell density of 2.1×10^7 cells mL^{-1}. Growth on peptone, yeast extract, glycogen, gelatin, and pine needle extract as carbon and energy source coupled to obligate S° respiration. Ammonia is produced and excreted into culture medium. Strict anaerobe. Genomic DNA G+C content is 59.9 mol%. Core lipid fraction contains GDGTs containing 4-6 cyclopentyl rings. The source of isolation was Dragon Spring, Norris Geyser Basin, YNP, Wyoming, USA. The type strain is *Acidilobus sulfurireducens* strain 18D70.

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CHAPTER 4

SELECTIVE GRAZING OF MICROBIAL MATS BY DIPTERAN LARVAE LEADS TO BIOMAGNIFICATION OF METHYLMERCURY IN A GEOTHERMAL FOOD WEB

Abstract

Complex networks of species interacting on different trophic levels underlie the flow of energy in all natural ecosystems, yet our understanding of such processes involving microbial producer species and higher trophic structure is limited, in particular in geothermal systems. Here we employ DNA-based methods to demonstrate that stratiomyid larvae selectively graze algal populations of a microbial mat community in geothermal springs with naturally elevated levels of Hg. Analysis of microbial community structures determined from DNA obtained from microbial mat and larval foregut contents revealed the presence of shared phylotypes (populations) aligning most closely with *Chyamydomonas reinhardtii* and *Trebouxia decolorans*, suggesting that larvae are grazing algal populations within the microbial mat community. Phylotypes that were abundant in the microbial mat were absent in the foregut of larvae, suggesting population-specific grazing. Sampled larvae contained MeHg in their tissues at 2.7- to 5.5-times the concentrations measured in mat biomass, suggesting that invertebrate grazing of microbial mat populations serves as a mechanism for MeHg biomagnification in food webs within these geothermal ecosystems. In addition, the results of the present

study may allude to evolutionary adaptations in organisms subjected to prolonged exposure to THg, which may decrease the bioaccumulation efficiency of MeHg and THg.

Introduction

Chemolitho- and phototrophic microorganisms (autotrophs) constitute the base of complex food networks present in microbial mat ecosystems common to many geothermal environments. Autotrophs use energy from reduced inorganic compounds or light to fix and assimilate inorganic carbon into soluble organic compounds and particulate biomass that support production of heterotrophic prokaryotes (2-4) and invertebrate grazers (9, 10, 16, 17, 19). While the transfer of carbon between autotrophic producers and heterotrophic consumers inhabiting geothermal systems have been characterized (2-4), the transfer of carbon and toxic chemicals between autotrophic producers and invertebrates occupying higher trophic structures is poorly understood.

Four families of insects within the order *Diptera* (phylum *Arthropoda*) can be found in thermal springs including the *Ephydridae* (brine flies), the *Chironomidae* (midges), the *Tabanidae* (horse flies), and the *Stratiomyidae* (soldier flies) (11). Larva of the family *Ephydridae* have been observed in hot springs located in Utah, Nevada, and California over a temperature range of 35-43°C and a pH range of 8.2-9.6 (13) as well as in hot springs in Iceland (>40°C), where it was hypothesized that the larvae were feeding on the algal mats within the spring (6). Brock et al. (9) reported larvae of two ephydrid (Ephydridae: Diptera) species in the alkaline Mushroom and White Creek hot springs in the Lower Geyser Basin of Yellowstone National Park (YNP), WY that range in

temperature from 30-45°C. Stratiomyid larvae (Stratiomyidae: Diptera) have been reported in physicochemically diverse hot springs located in both Nevada and California over a temperature range of 28-50°C and a pH range of 5.7-8.6 (13) and in the Lake Taupo thermal area in New Zealand (34°C, pH 6.5) (46). While these and other studies reveal the range of habitats in which stratiomyid larvae occur, little is known of the food resources available to larvae of these insects inhabiting thermal environments.

Mat composition was shown to influence the growth characteristics of *Ephydra thermophila* collected from an acid hot spring located in the Norris Geyser Basin of YNP (16). *E. thermophila* larvae maintained under laboratory conditions exhibited higher growth rates when reared on algae that infuse filaments of the rhodophyte *Zygogonium* than when reared on living or dead filaments of the *Zygogonium* sp. lacking the interstitial algae. In addition, mats containing greater densities of interstitial algae were preferred oviposition sites for adult ephydrids. For these reasons, Collins (16) proposed that the interstitial algae were a preferred source of nutrition for the immature larvae.

The first direct evidence that dipteran larvae assimilate microbial mat biomass was obtained through laboratory-based radioisotope tracer experiments. Brock et al. (9) incubated microbial mats collected from an alkaline thermal spring in YNP in the presence of $NaH^{14}CO_3$ and then exposed the mat biomass containing autotrophically-fixed, radiolabeled-carbon to ephydrid larvae collected from the same spring. Recovery of radiolabeled-carbon from larval tissue confirmed that the larvae assimilated the photosynthetically-fixed carbon. While the results of the laboratory studies described

above provide compelling evidence that ephydrid larvae utilize microbial mat biomass as a food source in geothermal environments, it remains to be demonstrated in the field.

We have observed high densities of soldier fly (Diptera: Stratiomyidae) larvae in numerous acid-sulfate-chloride (ASC) springs in the Norris Geyser Basin of YNP (Figure 4.1). These springs contain autotrophic microbial mats in reaches where the pH is

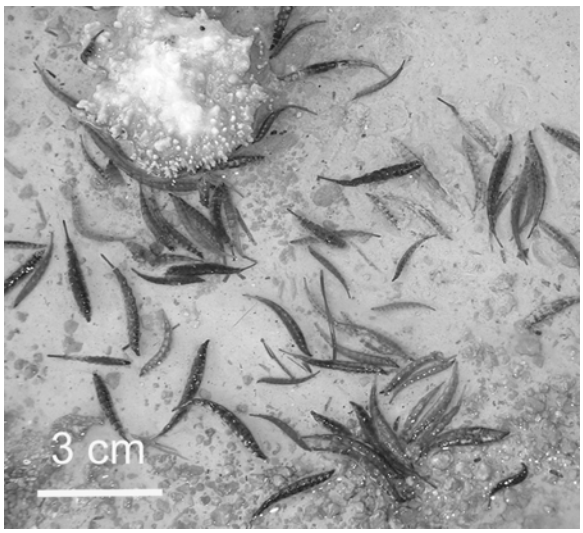

Figure 4.1. Abundance of stratiomyid larvae in Larval Spring

approximately 3.0 and the temperature ranges from 25ºC to 45ºC (29). In the study presented below, polymerase chain reaction (PCR)-based terminal-restriction fragment length polymorphism (T-RFLP) of the ribulose 1-6-biphosphate carboxylase (RuBisCO) gene (*rbcL*) was used to investigate whether stratiomyid larvae, like those of the family *Ephydridae*, utilize algal populations associated with the mats as a food source.

Thermal soils in many parts of YNP contain naturally elevated levels of Hg (Figure 4.2) (36). Recently, elevated levels of total Hg (Hg) were detected in the water of thermal springs in areas of YNP with Hg-enriched soil (34), and mono-methylated Hg

(MeHg) has been detected in the mat microbial biomass of these springs (28). In view of the aforementioned evidence of larval grazing of microbial mat populations in thermal springs, the present study included an investigation of the transfer of MeHg to higher tropic levels of the food web of ASC thermal springs where microbial mats and soldier

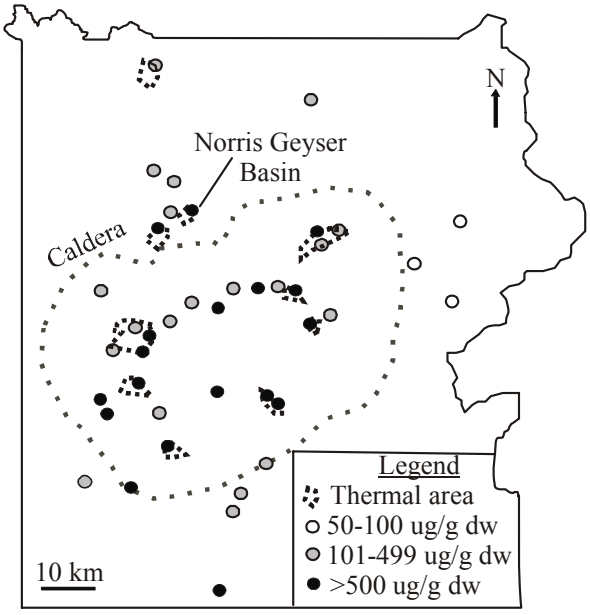

Figure 4.2. Soil mercury concentrations in Yellowstone National Park, Wyoming. Only the major thermal areas are indentified. Figure adapted from Phelps and Busick (1980)

fly larvae coexist. While numerous studies have documented biomagnification of MeHg in food webs exposed to anthropogenic sources of Hg (32, 33, 41), this process has not been extensively studied in geothermal habitats containing high levels of naturally-occurring Hg.

Materials and Methods

Site Description

Three springs, unofficially named Larval Spring (44°43.756' N, 110°42.774' W), Dragon Spring (44°43.919' N, 110°42.656' W), and Hazle Lake Spring (44°45.050 N, 110°42.700 W) served as sampling sites for this study. Dragon and Larval Springs are located in Norris Geyser Basin, Yellowstone National Park (YNP), Wyoming, USA. Both springs drain to the North, lack observable obstacles which might influence total radiance, have similar elevations (2289 and 2288 m for Larval and Dragon Springs, respectively), and have separate source and drainage areas. The chemistry and physical features of Dragon Spring have been described previously (26). Hazle Lake Spring is an acidic thermal spring within the boundaries of YNP in a watershed that extends along a north-south fault between Norris Geyser Basin and Mammoth Hot Springs. Hazle Lake Spring is drained by the Gibbon River.

Spring Water Chemical Analysis

Samples of spring water were collected at the site at which mat and larval specimens were sampled. Water temperature and pH were determined with a model 59002-00 field thermometer and pH meter (Cole Parmer, Vernon Hills, IL). Water samples for chemical analysis were collected with pipettes, transferred to plastic screw-cap vials, and stored on ice for transfer to the laboratory.

Sample Collection and
Processing for DNA Analysis

Larval specimens and samples of mat material adjacent to the grazing larvae were collected on June 2, 2004 from Larval Spring. Samples were collected with sterile forceps (larval specimens) or sterile syringes (mat specimens), transferred to sterile 1.5 mL tubes, and were placed on dry ice for transport back to the laboratory.

Taxomomic Identification of Larvae

Stratiomyid larvae are morphologically indistinguishable. Since adult forms of the larvae typically used for identification were rarely observed at these springs, the 28S rRNA and the cytochrome c oxidase subunit I-tRNA-leucine (COI-UUR) genes were PCR-amplified for use in taxonomic characterization of the larvae. A small specimen (approximately 20 mg) of tracheal tissue was aseptically removed and subjected to bead beating nucleic acid extraction according to the manufacturer's directions (FastDNA Spin Kit for Soil, Q-Biogene, Irvine, CA). DNA concentration was determined using the High DNA Mass Ladder (Invitrogen, Carlsbad, CA) and 20 ng of larval DNA was used as template for PCR amplification of a 500 bp fragment of the 28S rDNA using primer pair rc28C and 28P (45). In addition, an 831 bp segment of COI-UUR genes was amplified with primers CI-J-2183 (5'-CAACATTTATTTTGATTTTTTGG-3') and TL-2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA-3') (39). PCR, cloning, and sequencing were performed according to previously published protocols (7). The COI-UUR and 28S rRNA gene sequences have been deposited in the GenBank, DDBJ, and EMBL databases under the accession numbers DQ782814 and DQ782815, respectively.

Reference sequences used in analysis of the stratiomyid 28S rDNA sequence were *Odontomyia occidentalis* (DQ168747), *Stratiomys laticeps* (DQ168767), *Hedriodiscus binotatus* (DQ168748), *Dieuryneura stigma* (DQ168768), *Pseudocyphomyia mimetica* (DQ168740), *Leucoptilum* sp. CB0326 (DQ168771), *Euryneura propinqua* (DQ168791), *Diaphorostylus nasica* (DQ168765), *Auloceromyia* sp. CB0435 (DQ168798), *Dysbiota parvula* (DQ168801), and *Xylomya parens* (AY456143). Reconstruction of phylogenetic relationships were performed according to previously published protocols (7) using *Xyloma parens* as the outgroup.

Extraction and PCR Amplification of
Microbial DNA for T-RFLP Analysis

Terminal-restriction fragment length polymorphism (T-RFLP) was used to compare ribulose-1,5-carboxylase/oxygenase (*rbcL*) genes recovered from the contents of the foregut of larvae with those recovered from the microbial mat on which the larvae grazed. Intact larval foreguts were aseptically removed from specimens and deposited in sterile bead-beating tubes. Triplicate foregut and mat biomass from each spring were subjected to bead beating nucleic acid extraction according to the manufacturer's directions (FastDNA Spin Kit for Soil, Q-Biogene). Equal volume aliquots of each of the triplicate larval foregut and microbial mat extractions were pooled for further molecular analysis. DNA concentration was determined using the High DNA Mass Ladder (Invitrogen) and 20 ng of each larval foregut and pooled mat DNA extract was used as template for PCR amplification of *rbcL* genes. Primers used for *rbcL* gene amplifications were 595F (5'-GACTTCACCAAAGACGACGA-3') and 1387R (5'-

TCGAACTTGATTTCTTTCCA-3') (21) conjugated with fluoroscein (FAM) at the 5' terminus (Invitrogen). PCRs were performed in triplicate as described above using an annealing temperature of 52°C. Forty μL of each reaction were pooled yielding a total volume of 120 μL. Pooled PCR products were purified using Wizard PCR Preps DNA Purification System (Promega) and DNA concentration was determined using the Low DNA Mass Ladder (Invitrogen).

DNA Digestion and Fragment Purification

Approximately 200 ng of PCR-amplified DNA from the contents of larval foregut and microbial mat were subjected to digestion at 37°C for 12 h using the restriction enzyme MspI (C^CGG) (New England BioLabs, Ipswich, MA) according to previously published protocols (7). A fifty-fold excess of enzyme and twelve-fold excess digestion time (based on an activity of 1 μg DNA digested/h/U enzyme at 37°C) were used to ensure complete digestion of amplicons. Restriction digests were purified and analyzed according to previously published protocols (7)

Analysis of Restriction Digests

Terminal-restriction fragment (T-RF) profiles were analyzed using Genescan version 2.1 (Applied Biosystems). T-RFs that migrated to within 0.5 bp of each other were considered to have originated from the same template. Only T-RFs present in 2 of the triplicate digests were retained for community characterization. Total fluorescence (TF) contributed by the sum of all T-RFs in each digest was standardized to a value of 10,000 units (5). Composite T-RF community profiles were generated from the triplicate

digests by averaging peak migration distances and the percent TF for each T-RF. Analysis of composite community T-RF profiles indicated that the Jaccard similarity coefficient for the mat communities from Larval and Dragon Springs depended on the minimum fluorescence value selected to establish whether a T-RF peak, and hence a phylotype, was included as a community member. A minimum fluorescence value that increases the Jaccard similarity of two communities while simultaneously decreasing phylotype richness simplifies the community structure thereby promoting the analysis of only the dominant members within the community. Using the aforementioned criteria, it was empirically found that a minimum fluorescence value of 2.0% of TF decreased the species richness while simultaneously increasing the Jaccard similarity of the microbial mat community profiles. Thus, all phylotypes representing <2.0% TF were discarded without further consideration and the total community TF was again adjusted to 10,000 units.

Construction of *rbcL* Gene Clone Library

In order to assign taxonomic affiliation to individual phylotypes (T-RFs) that were grazed by larvae, a clone library was constructed of *rbcL* gene amplicons generated from DNA extracted from the Dragon Spring microbial mat. PCR, amplicon purification, and determination of DNA concentration were performed as described above for T-RFLP reactions; however, the reverse primer used in PCR was not conjugated with FAM. Amplicon cloning and plasmid purification were performed as described above for taxonomic identification of larvae. Primers used in sequencing were M13F and M13R. Sequencing reactions and assemblies were also performed as described above. Processed

sequences were aligned using ClustalW (http://clustalw.genome.jp/) and were grouped (100% sequence homology threshold) using the Sequence Grouper program (Andrew Shewmaker, Idaho National Laboratory, Idaho Falls, Idaho). A representative of each group was subjected to translated query-translated database (tblastx) analysis (1) against the non-redundant database provided by the National Center for Biotechnology Information (NCBI). All clone nucleotide sequences have been deposited in the GenBank, DDBJ, and EMBL databases under the accession numbers DQ398806-DQ398847.

Clone T-RFLP

A representative clone from each grouping was subjected to T-RF analysis in order to establish the taxonomic affiliation of each T-RF generated from the contents of the larval foregut and microbial mat on which the larvae grazed. Purified plasmids were subjected to PCR amplification as described above for community amplifications for T-RFLP with primers 595F and 1387R-FAM using 20 ng of purified plasmid as template. PCR amplicons were purified, quantified, and subjected to digestion as described above using 25 ng of DNA for each digest. Clone T-RF profiles were generated as described above for construction of community T-RF profiles. Clone T-RFs and T-RFs from foregut and mat samples were considered to be the same if they migrated to within 0.5 bp of each other.

Statistical Analysis of
T-RFLP Electropherograms

For the purposes of this study, unique T-RFs represent distinct operational taxonomic units (OTU), the unit by which individual phylotypes (populations) are demarcated. Jaccard and Bray-Curtis similarity coefficients were computed using PAST freeware software (24).

Sample Collection and
Processing for Hg Analysis

Samples of microbial mat, interstitial spring water, and larvae were collected at Larval, Dragon, and Hazle Lake Spring in Yellowstone National Park, WY on April 15, 2006. Mat samples and live larval specimens were collected with acid-washed Teflon forceps and transferred to certified Hg-free vials. Water samples were collected in acid-washed Teflon bottles and preserved with low-Hg hydrochloric acid at room temperature. Mat and larval samples were immediately frozen on dry ice for transport to the lab where they were stored at -50°C until processed. Larvae were thawed and dissected with acid-washed dissecting instruments in a sterile laminar-flow hood. The foregut, midgut, and hindgut and their contents from 3 larvae from Hazle Lake Spring, 4 larvae from Larval Spring, and 8 larvae from Dragon Spring were pooled into a single sample for MeHg analysis. Air tube tissue (trachea) from the same larvae was similarly pooled into single, site-specific samples for MeHg analysis. Separate larval specimens collected from each spring were retained for whole-organism MeHg analysis. A third set of larvae from each spring were used to determine the dry weight/wet weight ratio for larval biomass. The average dry weight of a single larva was 20 ± 10 mg. A single feather molted from an

unidentified avian species observed grazing larvae in Dragon Spring was collected with sterile Teflon forceps and was frozen immediately. Aqueous phase MeHg was analyzed using chromatographic separation with cold vapor atomic fluorescence spectrometry according to previously published protocols (18). Tissue MeHg was extracted by additions of KBr, $CuSO_4$, and methylene chloride according to previously published protocols (18). THg in aqueous samples was analyzed according to previously published protocols (35). THg in tissues was analyzed according to Olsen et al (35) with the following modifications: samples were digested with HNO_3/H_2SO_4 prior to addition of BrCl. All MeHg and THg analyses were performed by Frontier GeoSciences Inc. (Seattle, WA).

Results

Larval Abundance

During a sampling trip to One Hundred Springs Plain in the Norris Geyser Basin of YNP in the spring of 2003, insect larvae whose morphological characteristics resembled those of the soldier fly (Diptera: Stratiomyidae) were observed in abundance in numerous acidic thermal springs in the area (Figure 4.1). In Larval Spring, the larvae appeared to originate from autotrophic mats growing in a section of the spring where the pH was 2.9 and temperatures ranged from 28-32°C (Table 4.1). Stratiomyid larval abundance was determined on two occasions in Larval Spring. On one occasion (May 23, 2003), 169 larvae (live + dead) were observed along a 10-m section (~10 m^2) of the spring. A survey of this same transect on May 25, 2003 yielded a total of 213 live + dead

larvae. Based on an individual mean dry weight of 0.02 ± 0.01 g, the larvae contributed 0.38 ± 0.17 g dry weight m^{-1} and 0.43 ± 0.21 g dry weight m^{-1} in this section of the spring at these two sampling times.

Analysis of Larval 28S rRNA and COI-UUR Genes

The taxonomic affiliation of the larvae was determined through sequence analysis of the 28S rRNA and COI-UUR genes. The 28S rRNA gene sequence clustered within the subfamily *Stratiomyinae*, with the closest affiliated sequence belonging to *Odontomyia occidentalis* (98.6% nucleotide sequence homology) followed by *Stratiomys*

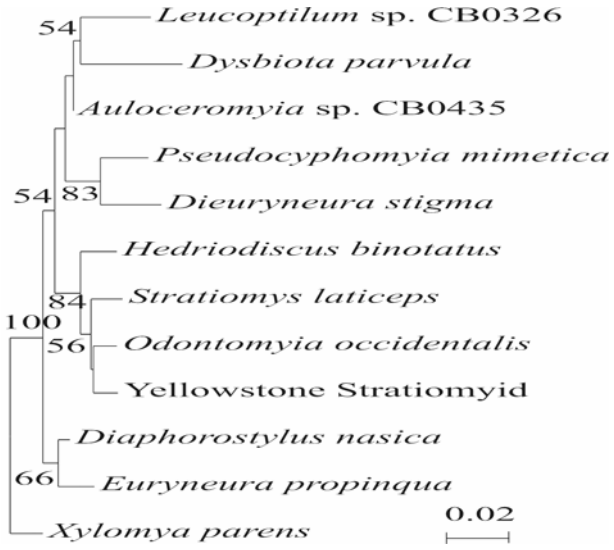

Figure 4.3. Taxonomic affiliation 28S rRNA genes of unclassified Yellowstone stratiomyid larvae and selected members of the *Stratiomyidae* calculated by the neighbor-joining method using Jukes and Cantor correction. The bar represents 0.02% nucleotide substitution. Bootstrap values of >50% are shown.

laticeps (98.0%) and *Hedriodiscus binotalus* (97.6%) (Figure 4.3). The closest affiliate COI-UUR gene sequence was from the dipteran *Bactrocera depressa* (96% inferred amino acid sequence identities (IAASI)). The apparent discrepancy in taxonomic affiliations based on the different genes may be a result of many more 28S rRNA gene sequences than COI-UUR gene sequences in the NCBI database for members within the subfamily *Stratiomyinae*.

Aqueous Chemistry

The physicochemical properties of the spring water at sites from which mat and larval samples were collected are presented in Table 4.1. Major cations were present at similar concentrations in Larval and Dragon Springs. Cations were not measured at Hazle Lake Spring. Water temperature, pH, and THg concentration were the measured parameters that varied most between the three springs.

TABLE 4.1. Aqueous water chemistry at mat and larval sampling sites.

				Dissolved Cations (mg L^{-1})[b]							
Site	pH	T (°C)	EC[a]	As	Fe	Na	K	Al	B	Ca	Hg[d]
Larval	2.9	28	2.02	1.8	6.5	296	58.0	2.6	7.1	4.5	38.1
Dragon	3.1	32	2.08	1.6	2.6	284	54.3	3.7	6.8	5.0	94.1
Hazle Lake	2.6	14	ND[c]	ND	ND	ND	ND	ND	ND	ND	2.1

[a]Electrical conductivity (mmhos/cm)
[b]Dissolved components determined using inductively coupled plasma emission spectrometry
[c]ND, not determined
[d]Total Hg (ng L^{-1})

Microbial Community T-RFLP Analysis of *rbcL* Genes.

rbcL gene-based T-RFLP analysis yielded T-RFs of 11 and 9 different lengths from DNA obtained from the foregut of larval specimens collected from Larval and

Dragon Spring, respectively (Table 4.2). *rbcL* gene-based T-RFLP analysis also yielded T-RFs of 9 and 10 different lengths from mat material on which the larvae grazed in Larval and Dragon Springs, respectively (Table 4.2). The 16S rRNA gene was also initially targeted for T-RFLP. However, T-RFLP analysis of 16S rRNA gene amplicons yielded microbial communities with low phylotype richness (data not shown). T-RF profiles based on variations in the *rbcL* gene sequence were therefore used to compare microbial phylotypes from the larval foregut and mat material grazed by the larvae in both springs.

Table 4.2. Comparison of community structures revealed by T-RF analysis of PCR-amplified *rbcL* genes. T-RF abundance (%) relative to total community fluorescence (TF).

	Larval Spring		**Dragon Spring**	
T-RF (bp)	Larval Foregut	Microbial Mat	Larval Foregut	Microbial Mat
160		9.5		6.1
233	5.2			
239		5.8		
244	6.3		5.7	
263		13.0		5.8
390	8.4		8.0	
417	6.6	17.8		11.8
419	11.2		11.5	
420	7.7		11.9	
437		4.4		4.1
465		4.8		5.5
529	4.9		5.1	
532	6.0		6.6	
534	6.4		7.1	
593	9.1	15.1	7.8	24.5
644		5.1		11.6
668	28.4	24.6	36.4	23.5
692				3.7
737				3.5
Total	100.0	100.0	100.0	100.0

Comparison of Larval and Dragon Springs Mat Microbial Communities

T-RF profiles of the *rbcL* genes amplified from Larval and Dragon Spring mat microbial communities exhibited a high degree of similarity (Table 4.2). Of the 9 and 10 *rbcL* gene T-RFs recovered from the mat communities from Larval and Dragon Springs, respectively, 8 were shared (Table 4.2), yielding a Jaccard similarity coefficient of 0.73 for the two mat communities. These 8 shared *rbcL* gene phylotypes contributed 94 and 93% of the total fluorescence of the T-RF profiles of the Larval and Dragon Spring microbial mat communities, respectively (Table 4.2). Taking into account the relative abundance of each *rbcL* gene phylotype based on the fluorescence contribution of each T-RF to total profile fluorescence, the mat communities from the two different springs exhibited a weighted Bray-Curtis similarity coefficient of 0.76.

Comparison of Microbial Populations in Larval Foregut to Those Associated With the Microbial Mats Grazed by Larvae.

rbcL gene phylotypes associated with the microbial mats of Larval and Dragon Springs were compared with those recovered from the foregut of the larvae collected from the same location. Three of the 11 *rbcL* gene phylotypes detected by T-RFLP analysis of DNA from the foregut of the larvae from Larval Spring were among the 9 phylotypes recovered from the microbial mat on which the larvae grazed (Table 4.2). These 3 shared *rbcL* gene phylotypes contributed 44% and 58% of the total fluorescence of T-RF profiles of the microbial populations from larval foregut and mat material, respectively (Table 4.2). Similarly, 2 of the 9 *rbcL* gene phylotypes detected by T-RFLP

analysis of DNA from the foregut of the larvae from Dragon Spring were among the 10 phylotypes recovered from the microbial mat on which the larvae grazed (Table 4.2). These 2 shared *rbcL* gene phylotypes contributed 44% and 48% of the total fluorescence of T-RF profiles of the microbial populations from larval foregut and microbial mat, respectively (Table 4.2).

Eight of the 11 *rbcL* gene phylotypes recovered from the foregut of larvae grazing the microbial mat of Larval Spring and 7 of the 9 phylotypes recovered from the foregut of larvae grazing the mat of Dragon Spring were not recovered from microbial mat samples grazed by the larvae (Table 4.2). However, nine of the foregut-specific *rbcL* gene phylotypes were shared by the larval specimens collected from the different springs (Table 4.2). The absence of these *rbcL* gene amplicons from the microbial mat DNA samples suggests the possibility that these phylotypes represent the indigenous foregut microflora in these larval specimens.

There were also *rbcL* gene phylotypes recovered from the microbial mat communities of both springs that were not found in the foregut of the larval specimens grazing the microbial mats. Six *rbcL* gene phylotypes recovered from the Larval Spring microbial mat were not found in the foregut of the larval specimen grazing the mat (Table 4.2). These 6 *rbcL* gene phylotypes contributed 43% of the total fluorescence of the T-RF profile for the Larval Spring mat community. Similarly, 8 *rbcL* gene phylotypes recovered from Dragon Spring microbial mat were not found in the foregut of the larval specimen grazing the mat. These 8 *rbcL* gene phylotypes contributed 52% of the total fluorescence of T-RF profiles for the Dragon Spring microbial mat community.

Comparison of Microbial Populations in the Foregut of Larvae From Larval and Dragon Springs

Many of the same *rbcL* gene phylotypes were detected in the foregut of larvae recovered from mats of different springs (Table 4.2). Of the 11 total *rbcL* gene phylotypes recovered from the foregut of larval specimens from Larval and Dragon Springs, 9 were shared, yielding an unweighted Jaccard similarity coefficient of 0.82 and a weighted Bray-Curtis similarity coefficient of 0.86. Two of the *rbcL* gene phylotypes present in the foregut of larvae recovered from both springs were among those present in the mats of both springs. These 2 *rbcL* gene phylotypes contributed 38 and 44% of the total fluorescence of T-RFLP profiles of foregut microbial populations in the larval specimens from Larval and Dragon Springs, respectively. These results indicate that *rbcL*-based communities in the foregut of larvae in these two springs have highly similar structures.

Phylogeny of Dragon Spring Microbial Mat Community

Microbial mat DNA extract from Dragon Spring was used to construct a *rbcL* gene clone library in order to assign taxonomic affiliation to grazed phylotypes. *rbcL* gene amplicons generated from DNA extracted from Dragon Spring microbial mat reflected a community comprised of green algae (Eukaryota: Chlorophyta) and *Proteobacteria*. Of the 40 *rbcL* gene sequences analyzed, 16 were affiliated with the green alga *Chlamydomonas reinhardtii rbcL* gene (98.1% IAASI), while 9 were affiliated with the green alga *Trebouxia decolorans rbcL* gene (97.2% IAASI). β-*Proteobacteria*

affiliated with *Thiobacillus intermedius rbcL* gene (99.6% IAASI) accounted for 5 of the 40 *rbcL* gene sequences while γ-*Proteobacteria* affiliated with *Nitrobacter winogradskyi* (95.7% IAASI) and *Acidothiobacillus ferroxidans* (95.7% IAASI) accounted for 8 and 2 of the 40 *rbcL* gene sequences, respectively. Based on this analysis, eukaryotic green algae represented a larger fraction (62%) of the *rbcL* gene clone library than *Proteobacteria* (38%).

Phylogeny of Phylotypes Grazed by Larvae

T-RF analysis of individual *rbcL* gene sequences facilitated the determination of the taxonomic affiliation of grazed mat phylotypes. One phylotype present in both the larval foregut and mat microbial communities in both Larval and Dragon Springs was most closely affiliated with *C. reinhardtii* (668 bp, Table 4.2). *C. reinhardtii* represented 28% and 36% of total fluorescence of the *rbcL* gene T-RF profile of Larval and Dragon Springs larval foregut microbial communities, respectively, and 25% and 24% of the total fluorescence of the T-RF profiles of the respective microbial mat communities. A second phylotype shared by larval foregut and microbial mat communities from both springs was most closely affiliated with *T. decolorans* (593 bp, Table 4.2). *T. decolorans* contributed 9% and 8% of the total fluorescence of *rbcL* gene T-RF profiles of Larval and Dragon Springs larval foregut communities, respectively, and 15% and 25% of the total fluorescence of T-RF profiles of the respective microbial mat communities. No transformant was recovered from the library that produced a T-RF corresponding in size to the third *rbcL* gene sequence phylotype (417 bp, Table 4.2) recovered from the larval foregut collected from Larval Spring and the microbial mat specimens from both springs.

Total and Mono-Methyl Hg
Levels in Thermal Food Web Organisms

Since a recent study revealed high levels of THg and MeHg in microbial mat biomass in other acid springs in YNP, and the results presented above suggest larval grazing of the mat biomass in Larval and Dragon Springs, whose water contained high concentrations of THg, a study was conducted to determine whether the mats of Larval and Dragon Springs contained THg and if so, whether it accumulates as MeHg in the larvae. Two microbial mat samples from Larval Spring averaged 18,043 ± 5,312 ng THg g^{-1} dry weight mat, while two other samples from the same mat averaged 4.29±3.08 ng MeHg g^{-1} dry weight (Table 4.3). Pooled trachea (tissue) from 4 larvae grazing the mat contained 23.5 ng MeHg g^{-1} dry weight of organ, respectively (Table 4.3).

Table 4.3. Mercury concentrations in material recovered from thermal springs of Yellowstone National Park

		Hg concentration		
Sample type	Hg form	Larval Sp.	Dragon Sp.	Hazle Lake Sp.
water[a]	total	94.1±25.3	38.1	2.13
	methyl	<0.025	<0.025	0.032
microbial mat[b]	total	18043±5312	14934±4714	157±46
	methyl	4.29±3.08	7.25	2.93±0.86
larva tissue[b,c]	methyl	23.50 (5.48)[d]	33.65 (4.64)[d]	7.90 (2.69)[d]
bird feather[b]	methyl		200.00 (27.86)[d]	

[a]ng L^{-1}
[b]ng g^{-1} dry weight
[c]Reported values calculated from gut/tissue sampled from multiple individuals and pooled for analysis
[d]MeHg fold increase relative to microbial mat in parentheses

Similarly, two mat samples from Dragon Spring averaged 14,934± 4,714 ng THg g^{-1} dry weight mat, while 1 other sample from the same mat contained 7.25 ng MeHg g^{-1} dry weight mat (Table 4.3). Pooled trachea from 8 larvae grazing the mat contained

33.65 ng MeHg g^{-1} dry weight of organ, respectively (Table 4.3). A feather of an unidentified avian species observed grazing larvae was found at the edge of Dragon Spring. The feather contained 200 ng MeHg g^{-1} dry weight.

In contrast, two microbial mat samples from Hazle Lake Spring, whose water contained greater than an order of magnitude less THg (Table 4.1 and 4.3), averaged approximately 2 orders of magnitude less THg than mats from Larval and Dragon Springs (Table 4.3). Two other samples from the same mat in Hazle Lake Spring, surprisingly contained levels of MeHg similar to those detected in mats from Larval and Dragon Springs (Table 4.3). Pooled trachea from 3 larvae grazing the mat contained 7.90 ng MeHg g^{-1} dry weight, respectively (Table 4.3).

Discussion

Stratiomyid larvae appear to be abundant in some acid geothermal springs in the Norris Geyser Basin, YNP representing a significant portion of the spring biomass. Previous studies have detected straiomyid larvae in aquatic environments that ranged in pH from 5.7-8.6 (13). The present study extends the pH range of larvae within the family stratiomyidae to 2.9, nearly three orders of mangitude more acidic than previously observed. The acid tolerant larvae subject to the present study were identified as a novel taxon within the family stratiomyidae exhibiting close relation to *Odontomyia* sp., an invertebrate common to aquatic environments (27).

In Larval Spring, stratiomyid larvae were present in densities of 17-21 larvae/m^2; within the range of densities of 10-100 larvae/m^2 reported for ephydrid larvae inhabiting

acid geothermal algal mats in YNP (16). Reports of stratiomyid larvae in geothermal springs first appeared in 1895 (23); yet, studies on their foraging behavior and prey base in these habitats have not been investigated. In the present study, PCR-based T-RFLP and sequence analysis of *rbcL* functional genes provided direct evidence that stratiomyid larvae preferentially graze algal populations aligning most closely with *Chlamydomonas reinhardtii* and *Trebouxia decolorans*.

Chlamydomanads are common to acid geothermal environments in YNP where they are often found associated with *Zygogonium* mats (16, 30). In contrast, *Trebouxia* have not been reported in acid geothermal environments; however, the related alga *Chlorella* is common to the acid geothermal environments (22). Sequence comparisons of the *rbcL* gene clone recovered in the present study to *rbcL* gene sequences from *T. decolorans* and *Chlorella pyrenoidosa* reveals a difference of only 1.0% IAASI, suggesting that the organism yielding the *rbcL* gene in question is a moderately thermophilic relative of both organisms.

Selective grazing of algal populations present in the Larval and Dragon Spring mat microbial community by dipteran larvae may reflect the evolutionary history of the fly. Ephydrid larvae inhabit both acidic and alkaline environments; however, they are more prevalent in alkaline springs (47) suggesting that alkaline springs ephydrids may be the progenitors of acidic springs ephydrids (16). In alkaline geothermal springs, ephydrid larvae graze small (~10 μm in length) single-celled cyanobacteria (9, 10, 43, 44). Similarly, ephydrids inhabiting acidic geothermal springs also appear to graze the smaller, less abundant interstitial algae rather than the larger *Zygogonium* filaments (~40

μm in length) (16). The common grazing of small algae by both acidophilic and alkaliphilic ephydrids may reflect preadaptation of mouth part structures which restrict grazing of the larger *Zygogonium* sp. filaments. Like ephydrid larvae, stratiomyid larvae inhabit both acidic (present study) and circumneutral to moderately acidic (pH 5.7) thermal springs (13, 46) the latter of which would be expected to contain cyanobacteria rather than *Zygogonium* sp. (30) Thus, acidiphilic stratiomyid larvae, like the related ephydrid larvae, may have evolved similar mouth structures, restricting their diet to the smaller interstitial algae.

While the mats sampled from Larval and Dragon Spring morphologically resembled *Zygogonium* sp. mats, the *rbcL* gene clone sampling in the present study did not contain sequences related to this genus. Bias in diversity studies is common due to primer mismatches in multi-template PCR, often resulting in obscured template to product ratios (38, 40). The forward and reverse primers utilized in the present study were only 35 and 85% homologous with *Zygogonium* spp. *rbcL* gene sequences deposited in the GenBank database, respectively; resulting in a bias against *Zygogonium* sp. sequences in the Dragon Spring microbial mat *rbcL* gene clone library.

Not all of the T-RFs present on the community T-RF profiles were identified. It has been suggested that PCR can generate single-stranded amplicons; each of which may contain restriction sites that differ from the parent template, resulting in pseudo-T-RFs (pT-RFs) (20). The number of pT-RFs formed increases with increased numbers of PCR cycles. Thirty PCR cycles were utilized to generate the *cbbL* gene amplicons for T-RFLP

analysis of the Larval and Dragon Springs communities in the present study, raising the concern that the unidentified T-RFs in each community profiles may be pT-RFs.

Previous studies indicate that chlamydomonads bioaccumulate MeHg (37) and elevated levels of MeHg have been detected in *Zygogonium* microbial mat biomass collected from acid geothermal springs in YNP (28). Since populations closely related to chlamydomonads were grazed by stratiomyid larvae, a study was undertaken to explore trophic level MeHg biomagnification in ASC geothermal food webs.

The ratio of MeHg to THg in microbial mat biomass sampled from springs with high (>10 ng L^{-1}) THg in the aqueous phase were lower than in mat biomass sampled from springs with low aqueous phase THg (Table 4.3). With few exceptions, the MeHg/ THg ratio in biomass from geothermal sites with high aqueous phase THg concentrations are several orders of magnitude lower than those in biomass from non-geothermal sources with low THg (14, 49). For example, a survey of 22 physicochemically-diverse YNP geothermal springs with high aqueous phase THg concentrations revealed a comparably low MeHg/THg ratio in mat biomass sampled from 20 of those springs as was observed in the high THg springs subject to the present study (28). However, an examination of MeHg/THg ratios in recently contaminated aquatic environments with aqueous phase THg ranging from 0.2 to 63 ng L^{-1} revealed MeHg/THg ratios in algal biomass that were still over an order of magnitude greater than in algal biomass collected from springs with elevated THg of the present study. The differences in MeHg/THg ratios between biomass collected from environments recently contaminated with THg when compared with environments subjected to prolonged exposure to naturally elevated

THg suggests that adaptations that influence the bioaccumulation efficiency of MeHg and THg likely evolve over geologic time scales.

To determine if MeHg biomagnification extends to larvae grazing mat biomass, larvae were dissected and the tracheal tissue was removed and pooled prior to MeHg analysis. Pooled larval tissue contained 2.69- to 5.48-fold greater concentrations of MeHg than the respective microbial mat biomass grazed by the larvae (Table 4.3). Numerous studies have documented MeHg biomagnification in herbivorous organisms positioned at trophic levels similar to that of stratiomyid larvae as a result of the consumption of biomass containing MeHg (15, 25, 42). Such studies report MeHg biomagnification values (1.6- to 35-fold) that are generally greater than what was observed in the present study. While the degree to which MeHg transfers between trophic levels may be influenced by a variety of environmental (pH, temperature, DOC) and ecological (food web complexity) parameters (31, 42), it may also reflect evolutionary adaptation. The prolonged exposure to THg over geological time scales may select for adaptations which decrease the efficiency of trophic-level MeHg transfer or mechanisms that decrease the toxicity of food sources such as harboring gut microflora capable of MeHg demethylation.

Accumulation of MeHg in larval biomass may have important consequences for species at higher trophic levels of this geothermal food web. Previous studies have determined that ephydrid larvae inhabiting thermal springs in YNP are a food source for birds such as the Killdeer (*Charadrius vociferous*) as well as for a variety of invertebrates and spiders (17, 44). Based on previous evidence that MeHg bioaccumulates in avian

species as a result of predation on MeHg-containing invertebrates (8, 48), it is possible that avian species which prey on the stratiomyid larvae in Norris Geyser Basin also bioaccumulate MeHg. A molted feather discarded by an individual Killdeer observed grazing stratiomyid larvae in Dragon Spring contained MeHg at a concentration of 200 ng g^{-1} dry weight. Although this represents the result from a single sample, and should be interpreted with caution, it does suggest that bioaccumulation of MeHg extends to higher tropic levels in this ecosystem. Furthermore, since stratiomyid larvae appear to be an important herbivore in acid geothermal springs of Norris Geyser Basin (16), it is likely that their grazing behavior represents a key pathway for the transfer of MeHg to species that range beyond this particular geothermal system to other areas within the Park and beyond. Since dipteran larvae have been observed in geothermal springs in various areas of the Park (9, 12, 16, 17), as have elevated levels of total Hg in sediments which commonly exceed 500 μg g^{-1} dryweight (Figure 4.2), it is likely that the biomagnification of MeHg described in the thermal springs of the Norris Geyser Basin occurs in other areas of the Park and in other geothermal areas around the world that have naturally elevated levels of Hg.

In summary, the present study extends the pH range that stratiomyid larvae can tolerate, provides evidence of selective grazing of algal mat populations by larvae of these invertebrates in a geothermal spring environment, and documents the consequences of this grazing activity in Hg-containing geothermal ecosystems in terms of MeHg biomagnification through a phototrophic mat-based food web. Further study of food webs subjected to prolonged THg exposure will aid in predicting the response of other

food webs to the sustained release of Hg currently retained (90% of total anthropogenic input) in Earth's terrestrial biosphere (31).

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CHAPTER 5

THESIS SYNTHESIS

The current thesis focused on developing an understanding of the biological component influencing the biogeochemical cycling of sulfur, carbon, and mercury in ASC springs. The research presented in chapter 2 sought a better understanding of the biota involved in the reductive cycling of S°. Aside from inferred physiology from 16S rRNA gene phylogeny, nothing was known on the organisms involved in the reduction of the copious amounts of S° in ASC springs throughout YNP prior to the present body of work. The geochemistry of Dragon Spring was used to guide the cultivation, enrichment, and isolation of two novel *Crenarchaeal* organisms; both of which exhibited an obligate requirement for S° as the sole terminal electron acceptor in respiration-dependent growth. A comprehensive phenotypic characterization of the isolates revealed they were both anaerobic heterotrophs, dependent on pre-reduced forms of carbon for growth. Attempts to cultivate both *Crenarchaeal* isolates on a natural extract of organic carbon derived from pine needle litter were successful, suggesting that these organisms may be important in the mineralization of natural organic carbon sources derived from pine needle litter in situ.

Careful characterization of cardinal temperature and pH of both isolates suggested that *Caldisphaera draconis* may be better adapted to the temperatures near the source of Dragon Spring as compared to *Acidilobus sulfurireducens*. Using qPCR, the abundance of each isolate in association with source water S° flocs was determined. qPCR

abundance results reflected the results of the phenotypic characterization: *C. draconis* was in greater abundance in the S° floc associated communities sampled from Beowulf, Succession, and Dragon Spring than *A. sulfurireducens*. These results have raised intriguing new questions into the preferred habitat for *A. sulfurireducens*. One such habitat may be subsurface, where spring water temperatures are likely warmer than near the surface. Presumably, the growth of organisms in subsurface environments may influence the geochemistry of surficial geothermal spring outflows. Future research should be directed at characterizing the activity of organisms inhabiting subsurface geothermal springs with the goal of uncovering the origin of compounds such as hydrogen or hydrogen sulfide that emanate from the source of many geothermal springs.

Characterization of the core lipids of both isolates grown at their T_{opts} (70°C for *C. draconis*, 81°C for *A. sulfurireducens*) indicated that they both produced glycerol dialkyl glycerol tetraether (GDGT) lipids. The composition of GDGT lipids often reflects the temperature from where the sample was recovered (11); GDGT-producing organisms sampled from warmer environments typically contain GDGTs containing greater numbers of cyclopentyl rings than GDGT-producing organisms from cooler environments. As expected, the number of cyclopentyl rings was greater in *A. sulfurireducens* than *C. draconis*, thereby supporting previous research.

Most importantly, the results presented in chapter 2 highlight the value of studying the environment for natural substrates prior to designing enrichment and isolation strategies. Both isolates grew on substrates naturally-present in Dragon Spring: dissolved organic carbon in the form of pine needle extract and S°. Given that the

organisms grew on these substrates which are naturally present in the ASC springs, it is perhaps less surprising that they represented such a large percentage of the microbial community present in these environments. To increase the probability of cultivating ecologically-relevant organisms, future enrichment and isolation techniques should include substrates naturally present in the environment.

To our best knowledge, the results presented in chapter 3 represent the first report of stratiomyid larvae in Yellowstone National Park. Stratiomyid larvae were identified through 28S rRNA gene sequencing and were found to be related (98.6% similar) to *Odontomyid* sp., a stratiomyid which also inhabits aquatic environments. The primary goal of chapter 3 was to determine the food source utilized by stratiomyid larvae that inhabit acid geothermal springs. Larvae were observed in the photosynthetic portions of the mats in a variety of ASC springs and thus, it was speculated that larvae were utilizing mat biomass as a source of nutrients and energy. The *rbcL* gene, which encodes for the ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme utilized in all of photosynthesis, was employed as a biomarker for tracking the fate of mat DNA. Results of the DNA-based analysis indicate that stratiomyid larvae selectively graze the algal portion of the autotrophic microbial mat in two geographically-distinct ASC springs in the NGB. These results suggest that invertebrate grazing may have a fundamental role in mineralizing phototrophically-fixed microbial mat biomass. In addition, these results suggest a role of stratiomyid larvae in influencing the standing crop biomass in ASC geothermal photosynthetic mat communities.

Previous research has indicated elevated THg in the soils surrounding geothermal springs in NGB (8) and elevated levels of THg and MeHg have been determined in microbial mat biomass in YNP (6). Therefore, a study was undertaken to investigate THg and MeHg bioaccumulation in photosynthetic mats. Results of this analysis revealed bioaccumulation of THg and MeHg in photosynthetic mats in NGB ASC geothermal springs. However, the ratio of MeHg to THg concentrations in ASC photosynthetic mats exposed to elevated concentrations of THg were orders of magnitude lower than mat biomass and aquatic biomass that was exposed to lower THg concentrations. In addition, the concentration of MeHg in *Zygogonium* mat biomass (4.29-7.3 ng g^{-1} dry weight) collected from springs with elevated THg are generally lower than biomass of comparable trophic level collected from aquatic ecosystems containing low concentrations of THg. Such observations raise important questions into the mechanisms which have evolved in mat populations in response to prolonged exposure to naturally elevated levels of THg in these environments.

Since it was previously determined that mat biomass served as an important food source for stratiomyid larvae and that mat biomass contained elevated MeHg, a study was conducted to determine if MeHg biomagnification extended to higher trophic structures in the NGB geothermal food webs. As expected, stratiomyid larvae which grazed MeHg-containing microbial mat biomass accumulated MeHg in tissue. The fold increases in MeHg between the autotrophic and stratiomyid trophic levels were also low compared to similar trophic level MeHg biomagnification events in freshwater environments. The lower biomagnification fold increases determined in this trophic level interaction may be

a result of many factors one of which may be adapatation to the naturally elevated THg levels in the ASC geothermal food webs present over geologic time periods.

To determine if grazing of microbial mat biomass by larvae served as a mechanism for MeHg biomagnification in food webs not confined to NGB geothermal complex in terms of MeHg, a feather of a Killdeer observed preying upon larvae was analyzed for MeHg. Analysis of a feather molted by a Killdeer observed grazing larvae in Dragon Spring indicated MeHg was biomagnified in this trophic level relative to stratiomyid prey. These results suggest that grazing of algal mats by larvae may serve as a mechanism for MeHg entry into broader-based food webs in the NGB. Because THg is elevated in geothermal springs throughout the Park which also have invertebrate grazers, this mechanism may prove to be an important entry point for MeHg in food chains throughout the Park.

Future Directions

Influence of Physicochemistry on *Crenarchaeal* GDGT Composition

Future work in the area of this dissertation includes a broader analysis of the physicochemical parameters that influence glycerol dialkyl glycerol tetraether (GDGT) development in *A. sulfurireducens*. In addition to the stability afforded by ether linkages in GDGT core lipids, the presence of cyclopentyl rings has been shown to increase the thermal stability of lipids in pure culture isolates; the greater the number of rings equates to greater thermal stability (3). The presence of cyclopentyl rings in planktonic marine biomass is correlated positively with oceanic temperature (11) and has been used to

estimate the temperature of marine environments (9). Because GDGT biomarkers are resistant to (bio)degradation and are readily preserved in the geologic record, GDGT profiles can potentially be used to assess the temperature of the ocean that marine archaea inhabited over geologic time. Coupled with radiocarbon dating, this data could potentially be used in paleoclimatology.

However, previous research suggests that parameters other than temperature, such as pH, ionic stength, etc., may also influence GDGT development in *Archaea*. Since it is difficult, if not impossible, to decouple these physicochemical parameters in most environments (13), the influence of any single parameter on GDGT development cannot be assessed with confidence. In addition, it is conceivable that the changes in GDGT profiles in environments sampled over a gradient of one of the above parameters may represent a change in the populations and their associated GDGTs rather than an acclimation of the organism and their associated GDGTs. The study of GDGT development in a pure culture isolate can circumvent these problems and can provide GDGT profile datasets as a function of any single physicochemical parameter for use in interpreting GDGT profiles sampled from complex environments. Future work will focus on characterizing changes in GDGT profiles as a function of temperature, pH, and ionic strength in pure cultures of *A. sulfurireducens*.

Sulfur Intermediates Involved in Sulfur Reduction Under Acidic Conditions

Because of the low solubility of elemental sulfur (5 μg/L at 25°C) (2), it is unlikely that S° serves as the direct substrate for S°-reducing microbes (10). Indeed,

microscopic analysis of the S°-dependent *Crenarchaeal* isolate *A. sulfurireducens* indicates that this organism is not in direct contact with S° when grown under laboratory conditions, yet S° is being reduced. These observations are consistent with other S°-reducing hyperthermophiles which also do not associate with the surface of the S° solid phase when grown under laboratory conditions (4, 10). In addition, physiological studies suggest *A. sulfurireducens* does not require direct contact with the S° solid phase. For example, *A. sulfurireducens* cultivated with S° sequestered in dialysis membranes (3.5-14 kDa pore size) still produce H_2S (unpublished data). Collectively, these results suggest that *A sulfurireducens* is reducing a soluble form of S° when grown under laboratory conditions.

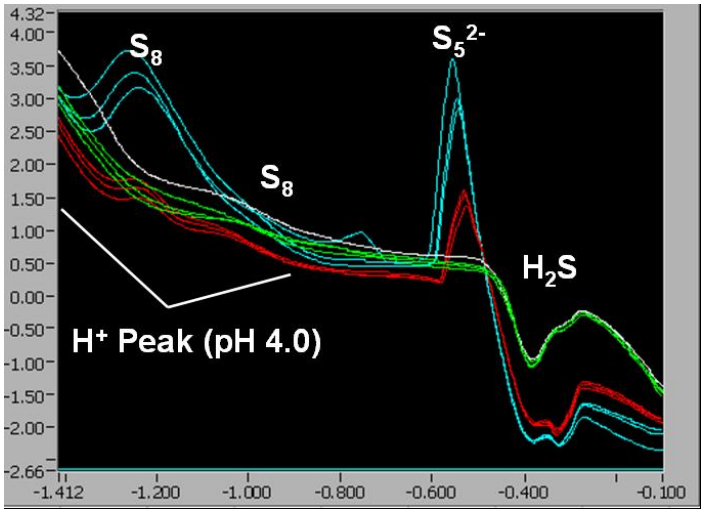

Figure 5.1. Differential pulse voltamogram of *A. sulfurireducens* (isolate 18D70) grown in the presence of S°. Green line, 12 hrs; red line, 24 hrs; white line, 36 hrs; blue line, 72 hrs. X-axis is volts (mV), y-axis is amperage (μA)

Polysulfide is one possible soluble form of sulfur which has previously been shown to serve as an electron acceptor for neutralphilic to alkaliphilic organisms such as *Wolinella succinogenes* (5) and *Pyrococcus furiosis* (1). Polysulfide is formed according to the following reaction:

$$S_8 + HS^- \rightarrow S_n^{2-} + H^+$$

where S_8 is elemental sulfur, and S_n^{2-} is polysulfide (n = 5 at pH 3.0). However, polysulfide is labile under acidic solutions with the maximum concentration of S° dissolved as polysulfide in solution at 90°C and pH 3.0 estimated to be less than 10^{-9} molar; too low to support microbial respiration (10). Under acidic conditions, polysulfide disproportionates rapidly to S° and sulfide (12). The S° resulting from polysulfide hydrolysis may form temporarily soluble colloids (7), which may serve as the electron acceptor for acidophilic sulfur-reducing microbes. Alternatively, S° may be soluble at a significantly higher concentration at 80°C than was determined at 25°C, and thus may serve as the electron acceptor.

Preliminary voltametric analysis of isolate *A. sulfurireducens* grown at pH 4.0 and 80°C indicate that polysulfides are being formed at near stoichmiometric concentrations (amperage is positively correlated with concentration) with the concentration of H_2S produced (Figure 5.1). The voltamagram presented in Figure 5.1 indicates a proportional increase in S_5^{2-} with increasing H_2S concentration. In addition, by day 3 of the incubation, the concentration of polysulfide disproportionation products (S°) are at high enough concentration to now be detected voltametrically. Future work will focus on

studying the kinetics of the formation and disproportionation of polysulfide in cultures of *A. sulfurireducens* as well as studying the kinetics of S° formation from polysulfide hydrolysis. Work will also focus on characterizing the particle size and particle size growth of S° formed from the disproportionation of polysulfide. In addition, the concentration of dissolved S° will be determined at elevated temperature. Together, the results of the aforementioned research thrusts should yield new insight into the soluble or colloidal electron acceptor(s) utilized by *A. sulfurireducens*.

Genome-Enabled Studies of Sulfur Reduction

As has been mentioned previously, very little is known concerning the organisms, genes, or enzymes, involved in acidophilic S° reduction. Two acidophilic, S°-reducing organisms have been cultivated from ASC geothermal springs in YNP as part of this dissertation. Because of low biomass yields (approximately 10 mg wet weight L^{-1} culture) we were not able to biochemically characterize the enzymes involved in sulfur reduction. As an alternative, the genome of *Crenarchaeal* isolate *A. sulfurireducens* is currently being sequenced. This genome will be one of the first genomes available in the order *Desulfurococcales* (phylum Crenarchaea) and will aid in the identification of genes and gene products involved in sulfur reduction, opening the door to new studies concerning acidophilic sulfur reduction at high temperature.

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APPENDICES

APPENDIX A

MINERALOGY INFLUENCES STRUCTURE AND DIVERSITY OF BACTERIAL COMMUNITIES ASSOCIATED WITH GEOLOGICAL SUBSTRATA IN A PRISTINE AQUIFER

MINERALOGY INFLUENCES STRUCTURE AND DIVERSITY OF BACTERIAL COMMUNITIES ASSOCIATED WITH GEOLOGICAL SUBSTRATA IN A PRISTINE AQUIFER

Abstract

Our understanding of mineralogical influences on subsurface microbial community structure and diversity has been difficult to assess due to difficulties in isolating this variable from others in the subsurface environment. In this study, biofilm coupons were used to isolate specific geological substrata from the surrounding geological matrix during colonization by microorganisms suspended in the surrounding groundwater for an 8-week period. Upon retrieval, the structure and diversity of the microbial community associated with each type of substratum was evaluated using 16S rDNA-based terminal-restriction fragment length polymorphism (T-RFLP). Phylogenetic affiliations of the populations associated with each type of substratum were established based on sequence analysis of near full-length 16S rDNA obtained through construction of a clone library. Hematite, quartz, and saprolite each harbored a community dominated

by members of the division *Proteobacteria* (>67% of community). However, the different substrata selected for different subdivisions of bacteria within the *Proteobacteria*. After accounting for the influence exerted by substratum type on recovery of DNA from the attached populations, both phylogenetic data and Jaccard and Bray–Curtis similarity indices derived from terminal-restriction fragment (T-RF) profiles suggested a strong mineralogical influence on the structure and composition of the solid phase-associated community. The results suggest that mineralogical heterogeneity influences microbial community structure and diversity in pristine aquifers.

Introduction

It has recently been estimated that microbial biomass in subsurface environments greatly exceeds the combined biomass of all other microbial reservoirs (31, 44). According to the literature, most of the microbial biomass in the subsurface is associated with surfaces of minerals and other solid-phase constituents that make up the geological substrata (1, 4, 20). Our current knowledge of the structure and diversity of the microbial community associated with the solid phase is based largely on analysis of bulk core material, which is often geologically heterogeneous (35). It is unclear whether geological heterogeneity at this scale exerts an influence on the structure and diversity of the solid phase-associated microbial community since it has been difficult to isolate substratum effects from other variables present in the system.

Using phospholipid fatty acid signatures as an indicator of microbial diversity, Peacock et al. (32) demonstrated that the microbial community associated with Bio-Sep

beads (a composite of aramid polymer and powdered activated carbon) was more diverse than that associated with glass wool incubated in a nuclear fuel-contaminated zone of a subsurface aquifer. While the artificial substrata used in the aforementioned study permitted characterization of the composition and activities of microbial communities in the subsurface, the communities selected by these substrata may not resemble or behave like those that develop on the geological substrata comprising the aquifer.

Recently, Reardon et al. (37) exposed different geological substrata to the groundwater microbial community in the saturated zone of a pristine aquifer in a manner that suggested a mineralogical influence on the structure and diversity of the solid-phase-associated microbial community. Analysis of terminal-restriction fragments (T-RFs) of PCR-amplified community 16S rRNA genes using Jaccard silimarity coefficients and Euclidean distance revealed differences in the communities that developed on the different geological substrata after an 8-week exposure period. However, Reardon et al. (37) used a different DNA extraction method to recover DNA from the communities associated with hematite than that used to recover DNA from communities associated with the other geological substrata under investigation, making comparisons difficult.

The purpose of this study was to determine the influence of different types of geological substrata on the structure and diversity of substrata-associated subsurface microbial communities using a common DNA extraction technique. A secondary goal of the study was to determine the extent to which the structure and diversity of a substratum-associated community varied with different DNA extraction techniques. Analysis of T-RF profiles of communities associated with hematite, quartz and saprolite

substrata, the same types of geological substrata used in the aforementioned study, yielded robust, new evidence of a geological substratum influence on the structure and diversity of microbial communities at the particle scale. Furthermore, the use of different methods to extract DNA from a substrata-associated community resulted in a different community T-RF profile.

Methods

Site Description

Well FW303 at the Field Research Center (FRC), Oak Ridge Reservation, Oak Ridge, TN was used as the subsurface environment for evaluating the influence of geological substrata on microbial community structure and diversity. Well FW303 (latitude 35.94113923; longitude 84.33627935) penetrates a pristine aquifer in a weathered shale composed of unconsolidated clay-rich saprolite supported by a bedrock of interbedded shales, siltstones, and limestones residing 5-10 m below the land surface. Overlying the saprolite is a thin veneer of organic- and clay-rich soil ranging from 0.5 to 3 m in thickness. The water table varies from <1 m to 3 m below the land surface. The predominant minerals in the shales include illite, quartz, kaolinite, chlorite, calcite, and plagioclase feldspar. Calcite, goethite, and kaolinite occur as coatings on fractures (38) while low-Mg calcite, dolomite, and ferroan dolomite are contained within the carbonates (19). Groundwater flux in the saturated zone occurs primarily within an interval defined by the interface between the competent bedrock and overlying highly-weathered saprolite (porosity of 30-50%) (23, 24, 45). It is believed that bacteria reside only in the high-

permeability fractures due to size exclusion from the matrix (http://public.ornl.gov/nabirfrc/sitenarrative.cfm). Aquifer geochemistry has been described elsewhere (Reardon et al. (37); URL: http://public.ornl.gov/nabirfrc/sitenarrative.cfm#Anchor1).

Biofilm Coupon Preparation and Incubation

Biofilm coupons, composed of capped stainless steel mesh cylinders (25.4 cm x 1.27 cm; 1-mm mesh size) (Figure 1), were prepared with approximately 10 g of the following minerals: quartz, specular hematite, and saprolite. Quartz sand (98-99% SiO_2; 1.0-1.4 mm particle size) was obtained from Ricci Bros. Sand Co. Inc., Port Norris, NJ. Specular hematite particles, obtained from Minas Gerais, Brazil (kindly provided by K. Rosso, Pacific Northwest National Laboratory, Richland, WA) and saprolite from the FRC subsurface (a gift from P. Jardine, Oak Ridge National Laboratory) were crushed and sieved. Only particles in the 1-3 mm diameter size range were used in an attempt to minimize differences in particle surface area, porosity, and permeability. All substrata

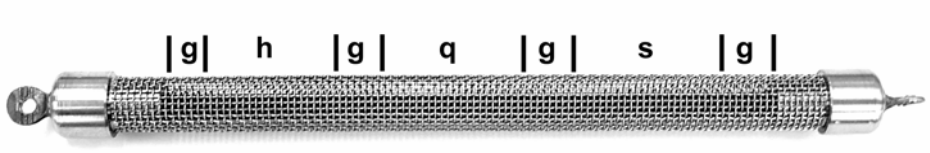

Figure 1. Biofilm coupon (25.4 x 1.27 cm) used to retain and isolate geologic substrata during incubation in monitoring wells. Glass wool spacer (g), hematite (h), quartz, (q), saprolite (s).

were separated within the coupon by a plug of glass wool and mineral-loaded coupons were sterilized by combustion (550°C, 6 h). Coupons were lowered by fishing line into

the saturated zone of the well and incubated for an 8-week period to promote colonization of substrata by those microorganisms with a propensity for attachment. The large coupon mesh size allowed access of the particle surfaces to bacteria suspended in the surrounding groundwater. Following incubation, coupons were retrieved, frozen and shipped on dry ice to the Idaho National Laboratory (Idaho Falls, ID), and stored at -80°C for molecular analyses.

Community DNA Extraction and
Amplification of 16S rRNA Genes
From Colonized Mineral Substrata

Total community DNA extraction was performed using the Bio101 FastDNA SPIN Kit for Soil (Q-Biogene, Irvine, CA). In order to account for heterogeneity in community diversity and structure as well as variability in DNA extraction efficiency, each type of geological substratum from the biofilm coupon was sampled in triplicate (0.5 g) and extracted along with positive and negative controls according to the manufacturer's instructions with the following exceptions: silica matrix-bound DNA was allowed to settle for 30 min and the SPIN Filter was allowed to air-dry in a sterile PCR hood for 30 min following the salt-ethanol wash. Equal parts of each triplicate extraction from each substratum type were combined and quantified using the High DNA Mass Ladder (Invitrogen, Carlsbad, CA). Approximately 1.5-2.0 ng of DNA was used as template under the following cycling conditions: initial denaturation at 94^{o}C (4 min) followed by 30 cycles of denaturation at 94^{o}C (1 min), annealing at 55^{o}C (1 min), primer extension at 72^{o}C (1.5 min), and a final extension step at 72^{o}C (20 min). The final reaction mixture (50 µL) contained 2 mM $MgCl_2$ (Invitrogen), 200 µM of each

deoxynucleotide triphosphate (Eppendorf, Hamburg, Germany), 0.5 μM of each forward and reverse primer, 0.4 μg μL^{-1} molecular-grade bovine serum albumin (Roche, Indianapolis, IN), and 0.25 U Taq DNA Polymerase (Invitrogen) in 1X PCR buffer (Invitrogen). Conserved regions of the 16S rRNA gene were targeted with bacterial forward primer 8F-FAM (5-AGAGTTTGATCCTGGCTCAG-3') modified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) at the 5' end (Invitrogen) and the universal reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Integrated DNA Technologies, Coralville, IA). Following electrophoresis of amplicons in a matrix of 1.2% agarose, it was decided that a semi-nested PCR reaction was necessary to generate a higher yield of DNA product for T-RFLP analysis. PCR products were quantified using the Low DNA Mass Ladder (Invitrogen) and 10-20 ng of DNA was used as template for the semi-nested amplification using the bacterial forward primer 8F-FAM and bacterial reverse primer 907R (5'-CCGTCAATTCMTTTRAGTTT-3' where M = A or C and R = A or G). The following conditions were used for semi-nested PCR: initial denaturation 94^{o}C (4 min) followed by 25 cycles of denaturation at 94^{o}C (1 min), annealing at 55^{o}C (1 min), and primer extension at 72^{o}C (1.5 min.) with a final extension step at 72^{o}C (20 min). Each primary reaction was subjected to semi-nested PCR amplification once, including three individual negative controls, yielding triplicate semi-nested PCR reactions of each. Successful PCR was verified following the semi-nested reaction on a 1.2% agarose gel prior to preparation for T-RFLP.

Intact Biofilm Polymerase Chain Reaction
From Hematite and Quartz Surfaces.

Intact biofilm-polymerase chain reaction (IB-PCR) was used to identify microbial populations through direct amplification (*i.e.* no extraction step) of DNA from the communities associated with hematite and quartz substrata. IB-PCR was performed in triplicate following previously published protocols (37). Briefly, colonized particles of hematite or quartz were added directly to PCR tubes containing nuclease-free water and 1X PCR buffer (Invitrogen) yielding a 25 μL total "pre-mix" aqueous volume. The tubes and their contents were heated at 99^{o}C for 15 min to lyse cells. The temperature was lowered to 80^{o}C, before subjecting the DNA to the same PCR conditions as used to amplify DNA extracted by bead-beating in order to maintain the same PCR bias for DNA extracted by the different methods. Following electrophoresis of amplicons in a matrix of 1.2% agarose, it was determined that a semi-nested PCR was necessary to generate sufficient DNA product for T-RFLP analysis. Semi-nested IB-PCR was performed using the same conditions as those used for semi-nested PCR of DNA extracted by the bead-beating method.

T-RFLP Analysis of PCR-
Amplified 16S rRNA Genes

T-RFLP was used to compare the subsurface microbial communities colonizing the different geological substrata in the biofilm coupon. Forty μL of each triplicate semi-nested PCR reaction were pooled, yielding a total volume of 120 μL. Pooled PCR amplicons were purified using the Wizard PCR Preps DNA Purification System

(Promega, Madison, WI) and DNA concentration was determined using the Low DNA Mass Ladder (Invitrogen) in a matrix of 1.2% agarose.

Approximately 200 ng of PCR-amplified DNA from each type of substratum was subjected to digestion at 37°C for 12 h in an Eppendorf Mastercycler Gradient thermocycler using the 4-base-recognition restriction enzyme MspI (C^CGG) (New England BioLabs, Ipswich, MA). The digestion mixture (50 µL total volume) contained 10 U of MspI (20U/µL), 5 µL 10X NEB2 Buffer (New England BioLabs), 200 ng DNA, and nuclease-free water (Sigma, St. Louis, MO). Digestion was performed in triplicate for each substratum type. Restriction digests were purified with 3 µL of sodium acetate (3 M, pH 5.2) and 66 µL ethanol (70%), air-dried, and re-suspended in 10 µL nuclease-free water (Sigma). Nucleic acids were denatured by heating to 95°C for 3 min followed by submersion in an ice bath. The denatured DNA (2 µL), along with the internal Rox 1000 standard (Applied Biosystems, Foster City, CA), was loaded onto a model 377 DNA sequencer (Applied Biosystems) employing a Cambrex Long Ranger XL 5% polyacrylamide denaturing gel. Terminal restriction fragments (T-RF) were separated by electrophoresis (51°C, 3 kV) for 4.5 h.

T-RFs generated by gel electrophoresis were analyzed using Genescan version 2.1 (Applied Biosystems). T-RFs that migrated to within 0.5 bp of each other were, for the purposes of this study, considered to have originated from the same phylotype. Only T-RFs present in all 3 of the triplicate digests were retained for community characterization. T-RFs greater than 900 bp were discarded in order to reduce the number of errors associated with fragment drift due to increasing T-RF length (6, 26). Total fluorescence

(TF) contributed by the sum of all T-RFs in each digest was standardized to a value of 10,000 units (6). Composite community profiles were generated from the triplicate digests by averaging peak migration distances and the percent TF. The same screening criteria used for substrata-derived community structure analysis was applied to electropherograms produced by nested negative T-RFLP reactions. T-RFs present in the composite profiles of both the semi-nested negative T-RFLP reaction and semi-nested substrata associated reactions were discarded without further consideration and all remaining T-RFs were again standardized to 10,000 units.

Construction of Bacterial
16S rDNA Clone Library

In order to identify individual phylotypes that contributed T-RFs, a clone library was constructed from IB-PCR-amplified 16S rDNA from hematite mineral surfaces. Primary PCR, nested PCR, amplicon purification, and DNA concentration determination were carried out as described above for the IB-PCR amplification of hematite-associated microbial communities. However for the clone library, the forward primers used in primary and nested PCR were not conjugated with FAM. Amplicons and pGEM-T Easy Vector (Promega) were ligated at room temperature for 1 h followed by ligation overnight at 4°C according to the manufacturer's directions. Competent *Escherichia coli* JM109 cells were transformed with the vector construct as recommended by the manufacturer. Transformed cells were plated on S-GAL agar (Sigma) using 100 μg mL^{-1} ampicillin sodium salt (Fluka, Seelze, Germany) as the selection agent. Plates were

incubated at 37°C for 18 h and a total of 100 clones were selected for sequencing and T-RF analysis.

Transformants were grown in 3 mL LB (100 μg mL^{-1} ampicillin sodium salt) at 37°C for 18 h. Cells were harvested by centrifugation and the plasmids were purified (QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA). Primers used in sequencing were 8F, 515F (5'-GTGCCAGCMGCCGCGGTAA-3', where M = A + C), 519R (5'-ATTACCGCGGCTGCTGG-3'), and 907R. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 3700 automated DNA sequencer (Applied Biosystems). Sequences were assembled using the BioEdit sequence alignment editor freeware (version 7.0.1, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (17). Sequences were examined for chimeric artifacts using the CHIMERA_CHECK function of the Ribosomal Database project II (RDP) (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) and were aligned using ClustalW (http://clustalw.genome.jp/). Aligned sequences were grouped using a 97% similarity threshold (40) using the Sequence Grouper program (Andrew Shewmaker, Idaho National Laboratory, Idaho Falls, Idaho). A representative of each group was subjected to nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) analysis (2) against the "nr" database provided by the National Center for Biotechnology Information (NCBI).

Nucleotide Sequence Accession Numbers

All clone sequences have been deposited in the GenBank, DDBJ, and EMBL databases under the accession numbers DQ003152-DQ003205 as well as DQ004244 and DQ004245.

Clone T-RFLP

A clone from each group was subjected to T-RF analysis in order to assign a phylotypic identity to T-RFs generated from the microbial community associated with each type of geological substrata. Purified plasmids from a representative clone from each group were subjected to PCR amplification as described above using the primers 8F-FAM and 907R using 20 ng of purified plasmid as template. Reaction conditions were: initial denaturation at 94^{o}C (4 min) followed by 30 cycles of denaturation at 94^{o}C (1 min), annealing at 55^{o}C (1 min), primer extension at 72^{o}C (1.5 min), and a final extension step at 72^{o}C (20 min). Successful PCR was determined on a 1.2% agarose gel. Reaction products were purified, quantified, and subjected to digestion as described above except that 25 ng of DNA was digested. Clone T-RF lengths were determined as described above and corresponding peaks from the clone T-RF and community T-RF electropherograms were considered to be the same if they migrated to within 0.5 bp of each other.

Statistical Analysis of
T-RFLP Electropherograms

For the purposes of this study, distinct T-RFs were considered to be unique operational taxonomic units (OTU) and were the unit by which individual phylotypes

were demarcated. Richness (R) was calculated as the total number of phylotypes. Shannon-Weaver diversity indices (H) were calculated using the formula $H = -\sum P_i(\ln P_i)$ where P_i represents the proportional abundance of individuals or phylotypes (i) and an increase in H indicates an increase in diversity. Shannon equitability (J) was calculated according to the formula $J = -H(\ln S^{-1})$ resulting in values of 0-1.0, with 1.0 indicating maximal diversity (5). Simpson Index (dominance) values were calculated using the formula $D = \sum P_i^2$ which results in values from zero (infinite diversity) to 1.0 (no diversity), and an increase in the inverse ($1/\sum P_i^2$) indicates an increase in diversity (5). Cluster analysis and calculations of Jaccard and Bray-Curtis (Odum) similarity coefficients were completed using PAST freeware software (18).

Results

The influence of mineralogy on the structure and diversity of microbial communities that associate with different mineral phases in subsurface environments was evaluated using T-RFLP of PCR-amplified community 16S rDNA extracted from different geological substrata following 8-week incubation in a subsurface aquifer. Before conducting this evaluation, it was first necessary to 1) establish an appropriate minimum fluorescence for T-RFs representing populations comprising a community, and 2) determine whether the nature of the substratum influenced the efficiency of extraction of DNA from the different substratum-associated populations.

Criteria for Including a Population
in a Substratum-Associated Community

A range of baseline values by which a T-RF is considered real or background noise are reported in the literature including 0.5% TF (37), and 1.0% (10, 27). In the present study, the similarity of the microbial communities associated with different substrata was found to be influenced by the threshold fluorescence value used to select T-RF peaks representing the populations that comprise each community. The highest unweighted Jaccard similarity coefficient obtained for communities associated with hematite and quartz using DNA extracted by either IB-PCR or bead-beating methods was achieved when a minimum fluorescence value of 3% of total profile fluorescence (TF) was used to filter out background fluorescence and populations in low abundance. Of the 18 phylotypes recovered from hematite and quartz substrata using the IB-PCR nucleic acid extraction, 7 were shared, resulting in a Jaccard similarity coefficient of 0.389 (Table 1).

Of the 15 phylotypes recovered from hematite and quartz substrata using bead-beating nucleic acid extraction, 5 were shared (Table 1), resulting in a Jaccard similarity coefficient of 0.333 (Table 2). When threshold fluorescence values were set above or below 3.0%, a smaller fraction of shared phylotypes were recovered from the hematite and quartz substrata, yielding lower Jaccard similarity coefficients (data not shown). The minimum fluorescence value that maximizes the similarity of the communities associated with different substrata should facilitate detection of only the most significant substratum

influences on community structure. Consequently, all subsequent community comparisons were conducted using communities defined by a 3% TF threshold value.

Bias of Different Methods of DNA Recovery from Substratum-Associated Microbial Populations.

The influence of the substratum on recovery of DNA from the substratum-associated populations was examined by first identifying two DNA extraction methods

Table 1. Relative abundance[a] of T-RFs revealed by bead beating DNA extraction from various substrata

T-RF[b]	Hematite	Quartz	Saprolite	Glass Wool
1			7.7	
3		9.6	5.0	5.0
4			38.4	
5	10.8	13.5	8.4	9.1
6	5.9	5.5		6.4
7			25.5	
10	3.9			
12	3.9	5.9		
13		5.2		
14	5.1			
16		6.8		7.6
17				3.7
20	15.6	46.4		24.4
21	27.6			30.4
22	4.7			
23	4.1	7.0		3.2
24			4.2	
25			7.0	
26				4.9
27	3.8			
28			3.8	
30	11.0			
32				5.2
33	3.7			
Total	100.0	100.0	100.0	100.0

[a]Percent of total fluorescence (TF) of phylotypes representing greater than 3.0% TF

[b]Terminal-restriction fragment (T-RF)

that exhibit unique biases for a specific substratum-associated microbial community and then evaluating whether the uniqueness of the bias varies with the type of substratum. Bias uniqueness was determined by comparing T-RF profiles of the same substratum-associated community whose DNA was extracted by IB-PCR and bead-beating methods. Profile similarity was evaluated using both unweighted Jaccard and weighted Bray-Curtis similarity coefficients. The Jaccard similarity algorithm accounts only for the presence/absence of a phylotype, whereas the Bray-Curtis similarity algorithm accounts not only for presence/absence but also relative abundance of a phylotype. If the two extraction methods exhibited no bias or the same bias in extracting DNA from members of the same substratum-associated community, both the Jaccard and Bray-Curtis similarity coefficients should be 1.00 for a given substratum. A value less than 1.00 describes the uniqueness of the bias.

Bead-beating and IB-PCR nucleic acid extraction methods exhibited unique biases for recovery of DNA from the hematite- and quartz-associated microbial communities. IB-PCR and bead-beating together yielded 17 unique phylotypes from the hematite-associated community, only 5 of which were recovered by both extraction methods (Table 3), yielding an unweighted Jaccard similarity coefficient of 0.294 and a weighted Bray-Curtis similarity coefficient of 0.391 (Table 2). For the quartz-associated community, the two extraction methods together yielded 19 unique phylotypes, only 4 of which were shared (Table 3), yielding a Jaccard similarity coefficient of 0.211 and a Bray-Curtis similarity coefficient of 0.250 (Table 2). Thus, the IB-PCR and bead-beating

Table 2. T-RFLP-based Jaccard and Bray-Curtis similarity coefficients for bacterial communities associated with different geological substrata subjected to IB-PCR or bead-beating nucleic acid extraction.

Jaccard Similarity Coefficients							
		IB-PCR		Bead-Beating Extraction			
		Hematite	Quartz	Hematite	Quartz	Saprolite	Glass Wool
IB-PCR	Hematite	1.000	0.389	0.294	0.385	0.059	0.429
	Quartz		1.000	0.174	0.211	0.095	0.190
Bead-Beating Extraction	Hematite			1.000	0.333	0.053	0.294
	Quartz				1.000	0.143	0.500
	Saprolite					1.000	0.125
	Glass Wool						1.000

Bray-Curtis Similarity Coefficients							
		IB-PCR		Bead-Beating Extraction			
		Hematite	Quartz	Hematite	Quartz	Saprolite	Glass Wool
IB-PCR	Hematite	1.000	0.460	0.391	0.576	0.084	0.512
	Quartz		1.000	0.212	0.250	0.124	0.214
Bead-Beating Extraction	Hematite			1.000	0.399	0.084	0.614
	Quartz				1.000	0.134	0.541
	Saprolite					1.000	0.134
	Glass Wool						1.000

methods of DNA extraction methods met the criteria for their subsequent use in assessing the influence of substratum properties on the efficiency of recovery of DNA from the different phylotypes present.

Similarity of Communities Associated With Different Geologic Substrata Using a Common DNA Extraction Method

In this study, the influence of substratum type on the efficiency of extracting DNA from microbial communities associated with different substrata was investigated by comparing the similarity of the T-RF profiles of communities associated with hematite

Table 3. Relative abundance[a] of T-RFs revealed by IB-PCR or bead beating DNA extraction from hematite- and quartz-associated communities

	Relative Abundance of Fragment on Hematite		Relative Abundance of Fragment on Quartz	
	Extraction Method Yielding Fragment		Extraction Method Yielding Fragment	
T-RF[b]	IB-PCR[c]	BB[c]	IB-PCR[c]	BB[c]
2			10.7	
3			6.5	9.6
5	11.2	10.8	7.4	13.6
6		5.9		5.5
8			4.1	
9	8.3		5.0	
10		3.9		
11			5.0	
12	11.1	3.9	7.6	5.9
13				5.2
14		5.1		
15			7.1	
16	5.6			6.8
17	7.6		6.2	
18	4.9		8.7	
19			5.6	
20	28.3	15.6	5.2	46.4
21		27.6		
22	9.8	4.7	12.4	
23	6.7	4.1		7.0
27		3.8		
29			4.1	
30		11.0		
31			4.6	
32	6.7			
33		3.7		
Total	100.0	100.0	100.0	100.0

[a]Percent of total fluorescence (TF) of phylotypes representing greater than 3.0% TF

[b]Terminal-restriction fragment (T-RF) reference number corresponding to clone library (see supplemental information)

[c]Intact biofilm-PCR (IB-PCR) or bead beating (BB)

and quartz whose DNA was extracted by IB-PCR to the similarity of the profiles of communities associated with the same two substrata whose DNA was extracted by bead-beating. T-RF profiles of hematite- and quartz-associated communities whose DNA was extracted using IB-PCR yielded a Jaccard similarity coefficient of 0.389 (Table 2). A similar Jaccard coefficient (0.333) was obtained from T-RF profiles of the same two communities when their DNA was extracted by bead-beating (Table 2). The similarity of the profiles from the two communities based on the Bray-Curtis algorithm was 0.460 and 0.399 when their DNA was extracted by IB-PCR and bead-beating, respectively (Table 2). The difference in the Bray-Curtis similarity values (0.460-0.399 = 0.061) was similar to the difference in the Jaccard similarity values (0.389-0.333 = 0.056).

Since not all populations gave rise to a T-RF of unique size (Table 4), the similarity of T-RF profiles generated from DNA extracted by the two methods could be a result of different populations within the community contributing the same T-RF. However, it is unlikely that the different extraction methods recovered different populations from the same substratum that not only produced a T-RF of the same size, but that were also present in approximately the same relative abundance as indicated by the Bray-Curtis similarity coefficient. Since it was demonstrated above that each extraction method has unique biases for the same populations, the most reasonable interpretation of these results is that hematite and quartz exert a similar, yet minimal influence on the efficiency in which the two extraction methods recover DNA from the populations associated with these substrata.

Substratum Influence on Community Diversity and Structure

Assuming that other geological substrata exert no more of an influence on community DNA extraction efficiency than hematite and quartz, then any substrata exerting a greater influence on their community's structure and diversity than that imposed by these two minerals should produce Jaccard or Bray-Curtis similarity coefficients of less than 0.333 or 0.399, respectively, when the bead-beating method is used to extract DNA from the communities. The communities associated with all substrata evaluated, with the exception of quartz and glass wool, when subjected to the bead-beating method of DNA extraction, yielded Jaccard similarity coefficients lower than those of the communities associated with hematite and quartz (Table 2). The high similarity of the communities associated with quartz and glass wool is not unexpected since both are silica-based substrata, whereas, hematite is an iron oxide mineral. The communities associated with hematite and saprolite shared a single phylotype (Table 1) and therefore exhibited the lowest Jaccard similarity coefficient (0.056) (Table 2).

When phylotype abundance was factored into the determination of community similarity using the Bray-Curtis algorithm, trends similar to those produced by the unweighted Jaccard algorithm were preserved (Table 2). The exception was a higher similarity coefficient for the communities associated with hematite and glass wool (0.614) than for the communities associated with quartz and glass wool (0.541). This reflected the fact that the communities associated with hematite and glass wool shared

two dominant phylotypes (>15% TF), whereas, the quartz- and glass wool-associated communities shared only one dominant phylotype (Table 1). This resulted in a different branch order in dendograms constructed with Jaccard and Bray-Curtis similarity coefficients (Figure 5.2). Except for the communities associated with saprolite and quartz, Bray-Curtis similarity coefficients for communities associated with different substrata were higher than Jaccard similarity coefficients for the corresponding communities (Table 2). In spite of the differences in community similarity when phylotype abundance was taken into account, the results strongly suggest that the structure and diversity of solid phase-associated microbial communities are influenced by the nature of the solid-phase substratum.

The influence of geologic substrata on the structure and diversity of the microbial

Table 4. Results of IB-PCR extracted hematite-associated community clone library

GenBank No.	T-RF[a]	Freq[b]	Closest Affiliation[c]	Putative Division
DQ003179	5	9	*Novosphingobium hassiacum* (97)	*α-Proteobacteria*
DQ003156	21	7	*Pseudomonas putida* (100)	*γ-Proteobacteria*
DQ003174	20	5	*Acidovorax* sp. (99)	*β-Proteobacteria*
DQ003169		4	*Haliangium tepidum* (92)	*δ-Proteobacteria*
DQ003153		3	*Flexibacter* sp. MG5 (94)	*Sphingobacteria*
DQ003180	5	2	*Novosphingobium hassiacum* (98)	*α-Proteobacteria*
DQ003181		2	*Catellibacterium nectariphilum* (90)	*α-Proteobacteria*
DQ003175	3	2	*Comamonadaceae* bacterium MWH55 (98)	*β-Proteobacteria*
DQ003170	14	2	*Bdellovibrio bacteriovorus* BEP2 (91)	*δ-Proteobacteria*
DQ003154		2	*Flexibacter aggregans* (88)	*Sphingobacteria*
DQ004244		2	*Flavobacterium ferrugineum* (92)	*Flavobacteria*
DQ003200	6	2	*Arthrobacter* sp. Ellin110 (99)	*Actinobacteria*
DQ003182	5	1	*Sphingomonas* sp. WSCII (93)	*α-Proteobacteria*
DQ003183		1	*Mesorhizobium* sp. rops-2 (91)	*α-Proteobacteria*
DQ003184	15	1	*Aminobacter aminovorans* (97)	*α-Proteobacteria*
DQ003185	10	1	*Bradyrhizobium* sp. Cmey 1 (94)	*α-Proteobacteria*
DQ003186		1	*Bradyrhizobium* spec. (LMG 10689) (93)	*α-Proteobacteria*
DQ003187		1	*Catellibacterium nectariphilum* (93)	*α-Proteobacteria*

Table 4 Continued

GenBank No.	T-RF[a]	Freq[b]	Closest Affiliation[c]	Putative Division
DQ003188		1	*Rhizobium* sp. CJ2 (94)	*α-Proteobacteria*
DQ003189	8	1	*Caulobacter* sp. A1 (99)	*α-Proteobacteria*
DQ003190	28	1	*Mesorhizobium* sp. rops-7 (86)	*α-Proteobacteria*
DQ003191	5	1	*Oligotropha carboxidovorans* (96)	*α-Proteobacteria*
DQ003192		1	*Afipia* genosp. 12 (92)	*α-Proteobacteria*
DQ003193		1	*Caulobacter* sp. (97)	*α-Proteobacteria*
DQ003194	4	1	*Bosea thiooxidans* (89)	*α-Proteobacteria*
DQ003195		1	*Rhodopseudomonas palustris* (92)	*α-Proteobacteria*
DQ003196		1	*Sphingomonas* sp. HTCC399 (98)	*α-Proteobacteria*
DQ003197	5	1	*Sphingomonas adhaesiva* (96)	*α-Proteobacteria*
DQ003198	5	1	*Sphingomonas* sp. (94)	*α-Proteobacteria*
DQ003199		1	*Bradyrhizobium* sp. ORS135 (97)	*α-Proteobacteria*
DQ003176	5	1	*Comamonadaceae* bacterium MWH55 (95)	*β-Proteobacteria*
DQ003177		1	*Comamonadaceae* bacterium MWH55 (98)	*β-Proteobacteria*
DQ003178	20	1	*Acidovorax* sp. UFZ-B517 (99)	*β-Proteobacteria*
DQ003171		1	*Haliangium tepidum*(92)	*δ-Proteobacteria*
DQ003172	27	1	Uncultured sludge bacterium A9 (95)	*δ-Proteobacteria*
DQ003173	30	1	*Bdellovibrio bacteriovorus* (95)	*δ-Proteobacteria*
DQ003157	24	1	*Pseudomonas* sp. Fa2 (97)	*γ-Proteobacteria*
DQ003158	23	1	*Pseudomonas* sp. CG20106T (95)	*γ-Proteobacteria*
DQ003159	16	1	*Pseudoalteromonas* sp. SM9913 (98)	*γ-Proteobacteria*
DQ003160	21	1	*Pseudomonas putida* (95)	*γ-Proteobacteria*
DQ003161	17	1	*Pseudomonas* sp. Clb01 (94)	*γ-Proteobacteria*
DQ003162	25	1	*Pseudomonas alcaligenes* (89)	*γ-Proteobacteria*
DQ003163	10	1	*Pseudomonas putida* (97)	*γ-Proteobacteria*
DQ003164	22	1	*Pseudomonas alcaligenes* (89)	*γ-Proteobacteria*
DQ003165		1	*Pseudomonas* sp. HF3/S21027 (100)	*γ-Proteobacteria*
DQ003166		1	*Pseudomonas putida* strain AJ (98)	*γ-Proteobacteria*
DQ003167	23	1	*Pseudomonas* sp. 6C_12 (88)	*γ-Proteobacteria*
DQ003168		1	*Pseudomonas fluorescens* (98)	*γ-Proteobacteria*
DQ003155	5	1	*Flexibacter* cf. sancti (94)	*Sphingobacteria*
DQ004245		1	*Flavobacterium ferrugineum*(93)	*Flavobacteria*
DQ003201		1	*Actinobacterium* 4a-2 (97)	*Actinobacteria*
DQ003202		1	*Arthrobacter aurescens* (98)	*Actinobacteria*
DQ003203		1	*Arthrobacter* sp. Ellin110 (96)	*Actinobacteria*
DQ003204	2	1	*Leifsonia poae* (98)	*Actinobacteria*
DQ003205		1	*Actinobacterium* 4a-2 (97)	*Actinobacteria*
DQ003152		1	*Holaphaga foetida* strain TMBS4-T (96)	*Acidobacteria*

[a]Terminal-restriction fragment.
[b]Frequency of sequence type out of 86 clones (97% similarity grouping)
[c]Closest identified sequence match in the GenBank database followed by percent similarity

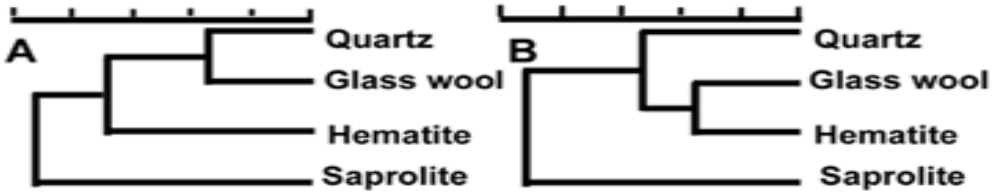

Figure 2. Cluster analysis based on Jaccard (A) or Bray-Curtis (B) similarity analysis of bacterial communities associated with different substrata revealed by bead-beating DNA extraction. Tick marks represent similarity units of 0.2 over a range of 0-1.0.

communities whose DNA was extracted by bead-beating was further investigated using various mathematical algorithms that describe richness, dominance, equitability, and diversity. Community richness (S), a measure of the total number of individual phylotypes, was found to depend on the substratum on which the community developed (Table 1 and 5).

Table 5. T-RFLP-based diversity statistics[a] of bacterial communities associated with different geological substrata subjected to bead-beating DNA extraction.

Geologic	Diversity Statistics			
Substratum	S[b]	J[b]	D[b]	H[b]
Hematite	12	0.883	0.135	2.194
Glass Wool	10	0.825	0.236	1.991
Quartz	8	0.815	0.257	1.695
Saprolite	8	0.865	0.184	1.715

[a]Calculated using peaks >3.0% total community fluorescence
[b]S, Richness; J, Shannon Equitability; D, Dominance;
H, Shannon-Weaver Index.

The hematite-associated community contained 50% more phylotypes than the community associated with saprolite, indicating a substratum influence on community structure. The ability to deduce substratum effects can be obscured by the richness index since this index does not take into account phylotype abundance or community evenness. Shannon

equitability (J), a measure of the relative proportion of individuals among a given set of phylotypes (*i.e.*, phylotype abundance) is a standardized numerical representation of community evenness. An equitability value of close to 0 indicates low species evenness or a high degree of dominance by a single phylotype. Equitability values approaching 1 indicate equal abundance of all phylotypes or maximal evenness. The equitability value was higher for the phylotypes associated with hematite and saprolite than for the phylotypes associated with quartz and glass wool, indicating that the phylotypes comprising the hematite- and saprolite-associated communities were more evenly distributed than the phylotypes associated with quartz and glass wool communities (Table 5). Dominance (Simpson index) describes the probability that any two members of a community chosen at random will belong to the same phylotype. Higher dominance values indicate an uneven distribution among individual phylotypes. Dominance indices ranged from 0.257 for the community associated with quartz to 0.135 for the community associated with hematite (Table 5). No one index offers a comprehensive measure of community structure and diversity. The Shannon-Weaver index of diversity (H), however, sums phylotypes weighted by their abundance and therefore takes into account both community richness and equitability. Shannon-Weaver indices varied from 2.194 for the hematite-associated community to 1.695 for the quartz-associated community indicating that the former was more diverse than the latter (Table 5). The high Shannon-Weaver index associated with the hematite community is consistent with the high phylotype richness and the high equitability (evenness) of the community. Correspondingly, the quartz-associated community had the lowest Shannon-Weaver

index, phylotype richness, and equitability. Similar trends between Shannon-Weaver indices, richness, and equitability were also observed when the glass wool- and saprolite-associated communities were analyzed (Table 5). In summary, the differences in richness, evenness, and Shannon-Weaver index values obtained for the communities associated with the different substrata support the results of the Jaccard and Bray-Curtis similarity analyses indicating that both community structure and diversity are strongly influenced by the nature of the geological substratum with which the community is associated.

Phylogenetic Affiliations of Substratum-Associated Community Members

A 16S rDNA clone library was constructed from the hematite-associated microbial community whose DNA was extracted using IB-PCR in order to establish the identity of phylotypes contributing T-RFs to the community profiles. DNA extracted from the hematite-associated community by IB-PCR was chosen for library construction since T-RFLP analysis indicated that it provided the greatest Shannon-Weaver index of diversity, thereby offering the greatest opportunity to relate the maximum number of phylotypes associated with this and other geological substrata to phylotypes in the ribosomal RNA database. Phylogenetic analysis of 16S rDNA clones indicates that very few of the clones exhibited close affiliation with any cultured *Bacteria* (Table 4). Furthermore, phylogenetic and T-RF analysis of rDNA clones indicate that distinct populations that belonged to the same class did not always yield unique T-RFs. For these reasons, only class-level taxonomic data are reported here. Sixty seven to 96% of the TF

of T-RF profiles generated by bead-beating extraction of the different substrata were phylogenetically identified by T-RF analysis of 16S rDNA clones (Figure 5.3).

A smaller fraction of the TF of community T-RF profiles generated by IB-PCR extraction of hematite and quartz were identified by individual clone T-RF analysis than for communities generated by bead-beating extraction (Figure 5.3). This difference was attributed to 4 unidentified phylotypes associated with hematite and 6 unidentified phylotypes associated with quartz that each contributed greater than 4%TF (data not shown).

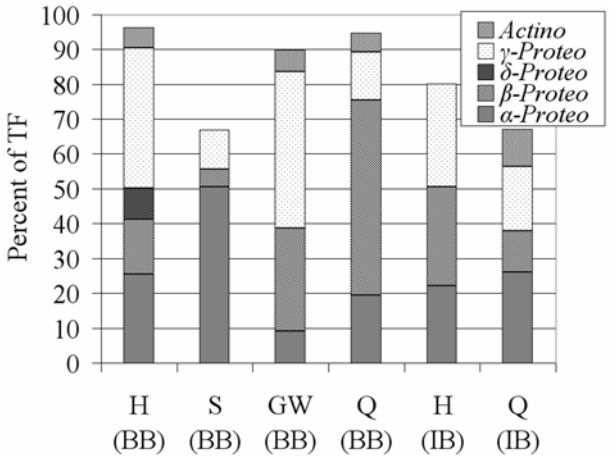

Figure 3. 16S rRNA-based phylogenetic composition of bacterial communities associated with hematite (H), Saprolite (S), quartz (Q), glass wool (GW) subjected to bead-beating (BB) or IB-PCR (IB) nucleic acid extraction. Abbreviations: TF, total fluorescence; *Actino*, *Actinobacteria*; *Proteo*, *Proteobacteria*

Members of the *Proteobacteria* dominated the communities associated with all geologic substrata evaluated in this study regardless of whether T-RF profiles were based on DNA extracted by bead-beating or IB-PCR. The different geologic substrata each

selected for a microbial community dominated by a different subdivision of *Proteobacteria* when bead-beating was used to extract DNA from the substrata-associated communities. Hematite-associated communities were dominated by phylotypes aligning with the γ-*Proteobacteria* (40% TF) and the α-*Proteobacteria* (26% TF) (Figure 3). β-*Proteobacteria* (56% TF) and α-*Proteobacteria* (19% TF) dominated the community associated with the quartz substratum (Figure 3). The glass wool-associated community was dominated by γ- and β-*Proteobacteria* (45 and 30% TF, respectively) (Figure 3). The phylogenetic structure of the saprolite-associated community was markedly different from that of the communities associated with the other geologic substrata with 51% of the phylotypes falling within α-*Proteobacteria* (Figure 3). These results indicate that not only do different geological substrata select for different phylotypes, but that the majority of phylotypes associated with each substratum align within different classes of the *Proteobacteria*.

Interestingly, the bias introduced by DNA extraction method on the composition of the communities associated with the different substrata was evident at the subdivision-level. The hematite-associated bacterial community whose DNA was extracted using IB-PCR contained a larger fraction of β-*Proteobacteria* than the same community whose DNA was extracted by bead-beating (Figure 5.3). Like the hematite-associated community, the quartz-associated bacterial community whose DNA was extracted using IB-PCR contained a greater fraction of non-*Proteobacteria* and γ-*Proteobacteria* than the same community whose DNA was extracted by bead-beating (Figure 5.3). Unlike the hematite-associated community, the quartz-associated bacterial community whose DNA

was extracted using IB-PCR contained a smaller fraction of β-*Proteobacteria* than the same community whose DNA was extracted by bead-beating (Figure 5.3). The results support similarity coefficient-based data indicating that different DNA extraction methods have different biases for recovering DNA from *Proteobacteria* and non-*Proteobacteria* as well as from the different subdivisions of *Proteobacteria* associated with a substratum-associated bacterial community. These results also support the similarity coefficient-based data suggesting that geological substrata influence bacterial community structure.

Discussion

Subsurface sediments often contain a heterogeneous mixture of minerals suitable for colonization by groundwater microbial populations (35). The present study employed T-RF analysis of PCR-amplified 16S rRNA genes extracted from communities that developed on different minerals during subsurface incubation to demonstrate a substratum influence on community structure and diversity. The unweighted Jaccard coefficient provided a quantitative assessment of the similarity of the microbial communities associated with different minerals based on the presence or absence of distinguishable populations. As expected, communities associated with substrata with similar properties such as quartz and glass wool yielded a higher similarity coefficient than communities associated with substrata with different properties such as hematite and quartz. Using an experimental approach and methodology similar to those used in the present study, Reardon et al. (37) obtained Jaccard coefficients for the communities

associated with quartz and glass wool (0.464-0.600) and with quartz and hematite (0.345) similar to those reported in the present study (0.550 and 0.333, respectively). A similar coefficient was anticipated from the two studies for the communities associated with quartz and glass wool since both studies used the same bead-beating DNA extraction protocol following incubation in the same well at the same depth for equivalent periods of time, but in different years.

The similarity of the coefficients reported in this study and by Reardon et al. (37) for the communities associated with hematite and quartz was not expected since Reardon et al. (37) used IB-PCR and bead-beating to obtain DNA from the hematite- and quartz-associated communities, respectively, while the present study used bead-beating to extract DNA from both communities. Different extraction techniques are known to have their own unique biases for recovering DNA from different populations (13, 28, 29). The results of the present study suggest that any bias experienced by Reardon et al. (37) from the use of different DNA extraction methods to recover DNA from hematite- and quartz-associated communities was masked by the substratum influence on the Jaccard-based measure of community similarity. The similar Jaccard coefficients (0.389 and 0.33) obtained in the present study for communities associated with these two substrata when DNA was extracted using either IB-PCR or bead-beating protocols, respectively support this conclusion. While seasonal variations in community composition may have occurred during the 1-year period that elapsed between the two studies, the results suggest that conditions in the well were at least sufficiently similar during the respective incubations

to preserve structure and diversity of the microbial communities associated with each mineral phase.

Jaccard similarity coefficients for communities associated with saprolite and the other substrata evaluated in this study were consistently lower than those reported by Reardon et al. (37). For example, the similarity coefficient of 0.143 obtained in the present study for the communities associated with saprolite and quartz was lower than the 0.419 value obtained by Reardon et al. (37). The difference in this case is difficult to explain since the same bead-beating protocol was used in both studies to extract DNA from the communities associated with these two substrata.

The low similarity of the saprolite-associated community and other substratum-associated communities in the present study compared to those reported by Reardon et al. (37) may be due, in part, to the different stringencies employed by the two studies for including a T-RF in a community profile. Whereas Reardon et al. (37) included a T-RF if it contributed greater than 0.5% TF and appeared in two of three replicate T-RFLP digestions, the present study included a T-RF only if it contributed greater than 3.0% TF and occurred in all three replicate digestions. The higher stringency employed in the present study tends to decrease the probability that a particular T-RF is shared by two communities, which likely yields a lower Jaccard similarity coefficient than when lower stringencies are used. Whereas 17 phylotypes qualified for inclusion in the hematite-associated community using the criteria applied in the present study, 28 phylotypes qualified when the criteria of Reardon et al. (37) were used (data not shown). Of the 39 total phylotypes associated with the saprolite and hematite communities compiled using

the criteria of Reardon et al. (37), 7 were shared, resulting in a Jaccard similarity of 0.179. This value is only modestly larger than the value obtained using the 3.0 %TF threshold used to establish a community profile in the present study (0.143), and is much lower than the value of 0.370 reported by Reardon et al. (37). In spite of the Jaccard-based differences in similarity between the saprolite-associated community and the communities associated with other substrata used in the two studies, Jaccard coefficient-based cluster analysis yielded similar dendogram topologies for the microbial communities associated with hematite, quartz, saprolite, and glass wool spacer material [Fig 2 this study; Reardon et al. (37)].

Factors such as relative abundance of phylotypes are not accounted for by the Jaccard similarity algorithm. A similarity index such as Bray-Curtis, which factors in relative abundance along with presence/absence of a phylotype should produce a lower coefficient value than that produced by the Jaccard index if there is a significant difference in the relative abundance of the phylotypes shared between two communities. Bray-Curtis similarity coefficients were higher than the corresponding Jaccard coefficient for communities associated with different substrata, the exception being those that described the similarity between the saprolite-associated community and the communities associated with other substrata. The higher similarity coefficient between substrata-associated communities when abundance is taken into account can be attributed to the sharing of dominant phylotypes between communities. For example, the quartz and glass wool communities, which exhibited a significantly higher coefficient when abundance was accounted for in the similarity algorithm, shared 5 T-RFs that each contributed >5%

TF (Table 1). Together, these 5 phylotypes accounted for 81.8% of the quartz-associated community and 52.5% of the glass wool-associated community, which is reflected in the similarly high dominance indices for the two substrates (Table 5). Furthermore, the hematite and glass wool-associated communities which also showed a marked increase in community similarity when abundance was accounted for, shared a total of 4 T-RFs that each contributed >5% TF (Table 1). Together, these 4 phylotypes comprised 59.9% of the hematite-associated community and 70.3% of the glass wool-associated community. Consequently, the similarity indices for the quartz- and the glass wool- and the hematite- and the glass wool-associated communities increased by 0.041 and 0.320, respectively, when phylotype abundance (%TF) was included in community similarity calculations.

The reason for the higher similarity achieved between the hematite- and glass wool-associated communities than between the quartz- and glass wool-associated communities using the Bray-Curtis algorithm is not readily apparent. Since this study focused on the effect of geologically relevant substrata on microbial community structure and the purpose of the glass wool was primarily to isolate these substrata during colonization by the groundwater populations, this anomaly was not investigated further.

A potentially important difference between the hematite and quartz substrata that could have contributed to the development of different microbial communities on these minerals is that hematite can serve as a terminal electron acceptor for respiration by some subsurface microorganisms during anaerobic conditions, whereas quartz cannot (21). Therefore, if anaerobic conditions had developed within the coupons during the incubation, hematite but not quartz should have selected for subsurface microbial

populations capable of Fe transformations. However, no 16S rDNA sequences were recovered from either the hematite-associated community or the communities associated with the other substrata that aligned with those of known Fe-reducers or Fe-oxidizers deposited in the Ribosomal RNA Databases. Instead, phylotypes were recovered from the various substrata whose 16S rDNA aligned most closely with *Sphingomonas* spp., *Pseudomonas* spp., *Caulobacter* spp., and *Novasphingobium* spp. These genera are known to be physiologically diverse and have a propensity to colonize surfaces (3, 25, 36, 42). These traits, however, provided little indication of the basis of the observed substratum influence on community structure and diversity.

The dependence of bacterial community composition on the nature of the geological substratum that was observed in the present study is consistent with results of previous studies, which used artificial instead of geological substrata to capture subsurface microbial communities (19). *Proteobacteria* comprised >67% of the total microbial community associated with the three geologic substrata (hematite, quartz, saprolite) evaluated in the present study. *Proteobacteria* were also the primary phylotypes recovered from Bio-Sep beads incubated in a different part of the same aquifer by Peacock et al. (32). α-*Proteobacteria* with 16S rDNA sequences that most closely aligned with *Sphingomonas* (93-96% similarity) accounted for >10% TF of the communities associated with all three geological substrata evaluated in the present study. α-*Proteobacteria* (*Sphingomonas* spp. and *Rhodopseudomonas* sp.), β-*Proteobacteria*, and δ-*Proteobacteria* aligning with the genus *Frateuria* were also detected on Bio-Sep beads by Peacock et al. (32). The results reported here are also consistent with previous

results indicating a highly diverse groundwater community comprised exclusively of α-, β-, γ-, and δ-*Proteobacteria* as well as high and low G+C gram-positive bacteria in the pristine portion of the FRC aquifer (12).

Proteobacteria also dominated the 16S rDNA clone library (96% of total clones) constructed from the hematite-associated community by Reardon et al. (37) using the IB-PCR nucleic acid extraction method. At the subdivision level, however, composition of the IB-PCR-extracted, hematite-associated community obtained in the present study differed from that reported by Reardon et al. (37). Whereas, the β-*Proteobacteria*, γ-*Proteobacteria*, and α-*Proteobacteria* contributed 69%, 21% and 3.5% of the clones, respectively, in the Reardon et al. (37) study, they contributed 28%, 30%, and 22% of the total community T-RF fluorescence, respectively, in the present study. DNA used for construction of clone libraries in both studies was extracted using IB-PCR from the same types of substrata incubated in the same well for identical (8-week) periods of time, but in different years. However, similar Jaccard coefficients generated from T-RF community profiles in the two studies for quartz- and glass wool-associated communities and for quartz- and hematite-associated communities suggested that the structure and diversity of the communities associated with these substrata had not changed during the intervening 1-year period in which the two studies were conducted.

The community composition differences noted above likely resulted from the different approaches used by the two studies to assemble community structure information. The T-RFLP method used in the present study is independent of sample size and thus offers the opportunity to achieve greater coverage of the true community

structure than the clone library sub-sampling (n = 95 clones) approach used by Reardon et al. (37). However, T-RFLP is limited by the fact that only those members divergent enough in the 16S rRNA gene yield different length T-RFs. The use of different or multiple restriction enzymes could enhance diversity of a community T-RF profile. However, in order to achieve this result, the recognition and cut site for each enzyme cannot be conserved among all members of a community. Furthermore, T-RFLP does not provide phylogenetic information for those T-RFs that are resolved unless complemented with a 16S rDNA clone library that yields a unique clone for each T-RF.

Denaturing gradient gel electrophoresis (DGGE) is an alternative approach that has been widely used to describe microbial community structure and diversity (11, 30, 32). This approach has the advantage of analyzing all members of a community 16S rDNA pool rather than only those members whose 16S rDNA gene yields T-RFs of unique size. However, those phylotypes whose 16 rRNA gene sequence share similar melting characteristics may not be resolved, leading to an underestimation of community richness (22). Thus, it may be necessary to employ multiple approaches in order to achieve a higher degree of certainty of the community diversity associated with the geological media utilized in the present study. Nevertheless, the approaches employed here permitted detection, quantification, and characterization of the influence of mineralogy on the structure and diversity of the microbial communities that develop on subsurface geological media.

The results of the present study also serve as a reminder that community structure and diversity data obtained using different DNA extraction methods may not be directly

comparable due to the unique biases of each method (6, 13, 28, 29). The different DNA extraction techniques used in this study clearly influenced subdivision-level community composition (Figure 3). For example, IB-PCR recovered proportionally fewer γ-*Proteobacteria* of the hematite-associated community and proportionally fewer β-*Proteobacteria* in the quartz-associated community than did the bead-beating extraction method. The results of the current study suggest that the type of substratum which the community is associated influences the detection of certain microbial populations by the PCR-based T-RFLP method since other biases such as those associated with the PCR (33, 41) should be the same for both bead beating and IB-PCR extracts. The results also suggest that the substratum-derived bias is quantifiable and distinguishable from an actual substratum influence on community structure and diversity.

A potentially important consideration not investigated in the present study is how efficiency of extraction of DNA from the different substratum-associated populations is influenced by the amount of biomass present (13, 34, 43). Unfortunately, the efficiency of chemically-based methods used to quantify microbial biomass in subsurface environments such as phospholipid fatty acid analysis may also be influenced by substratum properties, precluding acquisition of absolute biomass values (39). Direct microscopic methods of biomass determination are hampered by particle masking of cells and uncertainties associated with assigning carbon content to populations of cells that vary in size and physiological state (7, 8, 15).

In summary, 16S rDNA-based T-RFLP and clone library analysis provided new evidence supporting previous studies that different geological media are colonized by

communities of microorganisms with unique structure and diversity. The substratum influence on community structure and diversity was isolated from the substratum influence on efficiency of extraction of DNA from the substratum-associated community. To our knowledge, this is the first study that has determined the influence of both the type of substratum and extraction method on the diversity and phylogenetic composition of communities associated with subsurface geological media. The present study described the structure and diversity of microbial communities associated with different geological media at the particle scale. In the subsurface, however, geological heterogeneity occurs over a wide range of scales (9, 14, 16, 35). We presently have little understanding of how microbial community structure and diversity at the particle scale relates to that which exists at the aquifer scale where these community parameters likely control important processes such as contaminant fate and transport. The results presented here represent a first step toward achieving this goal.

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