

MICROBIAL INTERACTIONS WITH ARSENITE, HYDROGEN AND SULFIDE IN
AN ACID-SULFATE-CHLORIDE GEOTHERMAL SPRING

by

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ABSTRACT

The work presented in this thesis investigated the importance of hydrogen, sulfide and arsenite in microbial community structure and function within a model Acid-Sulfate-Chloride (ASC) spring in Yellowstone National Park. Previous studies in this spring found that microbial arsenite [As(III)] oxidation is absent in regions of the spring outflow channel where H₂S exceeds ~5 μM. Ex situ assays with microbial mat samples demonstrated immediate As(III) oxidation activity when H₂S was absent or in low concentrations, suggesting the presence of functional As(III) oxidase enzymes in regions of the spring where arsenite oxidation had not been previously observed. Cultivation efforts resulted in the isolation of an As(III)-oxidizing chemolithotroph phylogenetically related to the α-proteobacterium *Acidicaldus*. H₂S concentration appeared to be the most important constraint on spatial distribution of this organism. This was verified with pure culture modeling and kinetic experiments.

Additionally, a study is presented that addresses the relative importance of dissolved hydrogen and sulfide for primary production in the same spring. Throughout the outflow channel where these gases could be detected, biological H₂S consumption rates exceeded those of H₂ by at least three orders of magnitude. Molecular analysis showed that *Hydrogenobaculum*-like organisms dominate the microbial community in this region of the spring. Culturing efforts resulted in 30 *Hydrogenobaculum* isolates belonging to three distinct 16S rRNA gene phlotypes. The isolates varied with respect to electron donor (H₂S, H₂) and oxygen tolerance and requirement. These metabolic physiologies are consistent with in situ geochemical conditions. An isolate representative of the dominant 16S phylotype was used as a model organism for controlled studies to determine whether an organism capable of utilizing either of these substrates demonstrated preference for H₂S or H₂, or whether either electron donor exerted regulatory effects on the other. The organism studied utilized both H₂S and H₂ simultaneously and at rates roughly comparable to those measured in the ex situ field assays. Major conclusions drawn from this study are that phylogeny cannot be relied upon to predict physiology, and that, in ASC springs, H₂S clearly dominates H₂ as an energy source, both in terms of availability and apparent consumption rates.

CHAPTER 1

THE SCOPE OF THE THESIS

The goal of this thesis was to explore microbial interactions with three important electron donors; hydrogen, sulfide, and arsenite, in a model Acid-Sulfate-Chloride (ASC) geothermal spring in Yellowstone National Park. Previous studies in springs of this type have demonstrated several consistent physical and chemical gradients. Substantial microbial communities inhabit all regions of these outflow channels and the composition of these communities is strongly influenced by physical and chemical parameters. Concurrently, the chemical changes observed in these systems are a result of both abiotic reactions and the activities of the microorganisms inhabiting these unique thermal features.

Chapter 2 introduces the typical geothermal spring types found in Norris Geyser Basin of Yellowstone National Park. Particular attention is paid to the common characteristics of ASC-type springs and the continuing biogeochemical research in these environments. The primary focus of this chapter is to explore the occurrence, impacts and microbial activities related to hydrogen, sulfide and arsenic. Natural and anthropogenic sources of each of these constituents in the surface environment are presented, followed by a summary of microbial metabolisms that involve these potential electron donors in a variety of environmental settings including, when possible, geothermally influenced systems. Additionally, characterized microbes that are capable of these metabolisms are introduced and briefly discussed, particularly in the context of chemolithotrophic primary production. The potential inhibitory effects of hydrogen,

sulfide and arsenic on microbial metabolisms are also introduced in this chapter as a means of exploring the role that in situ chemistry can play in microbial population distribution.

The primary objective of the work presented in chapter 3 was to explore in more detail the effect of sulfide on As(III) oxidation. Previous work in Dragon Spring, a model ASC spring in Norris Geyser Basin, indicated that biological arsenite oxidation does not occur in regions of the spring that contain $>5\mu\text{M}$ sulfide. To further our understanding of this relationship, ex situ assays were performed that showed that microbial mats taken from high-sulfide regions of the spring are capable of arsenite oxidation under low-sulfide conditions. Culturing efforts were also undertaken to isolate an arsenite-oxidizing, aerobic chemolithotroph from this environment. This isolate was found to be phylogenetically related to *Acidicaldus* at the 16S level, and was characterized with respect to its ability to oxidize arsenite and the inhibitory effect of sulfide on this activity. In order to assess the impact of spring chemistry on the distribution of this organism in situ, sulfide and oxygen concentration gradients in the sulfur deposition zone of Dragon Spring were examined both in the direction of spring discharge as well as perpendicular to water flow. Quantitative PCR and DGGE analysis revealed a negative correlation between sulfide concentration and the cell density of *Acidicauldus*-like organism in situ despite otherwise adequate environmental conditions for its growth.

Chapter 4 is primarily concerned with the relative importance of hydrogen and sulfide as electron donors for chemolithoautotrophic growth in the sulfur deposition zone of Dragon Spring. Chemical analysis of this region of the spring indicates that the

concentrations of both of these dissolved gasses decrease as a function of distance from the spring source. Ex situ assays show that biotic processes are responsible for at least a portion of this change in spring water chemistry. Additionally, the rate of apparent sulfide consumption in these experiments proceeded at a rate roughly three orders of magnitude faster than that of hydrogen. Ex situ work in Dragon Spring was supplemented by isolating chemotrophic microbial community members from the sulfur deposition zone that are capable of sulfide and/or hydrogen oxidation. All isolates obtained through enrichment belong to the genus *Hydrogenobaculum*, which is consistent with clone library data obtained previously and within the current study. These thirty isolates belong to 3 distinct 16S phylotypes that encompass widely varying electron donor/acceptor pair physiologies. One of these isolates was characterized in further depth regarding its sulfide- and hydrogen-oxidizing activity and was found to exhibit similar properties to those observed whole mat. These results suggest that sulfide may play a more important role in primary production in ASC springs than hydrogen.

The research presented in this thesis is focused on highlighting the interactions between microbes and three important spring water constituents in a model ASC spring. Firstly, $\text{As}^{(\text{III})}$ is a potent electron donor in this spring, supporting the chemoautotrophic growth of an *Acidicaldus*-like organism. The presence of sulfide at relatively high concentrations in some portions of the spring determines the spatial distribution of not only this organism but of the $\text{As}^{(\text{III})}$ oxidation metabolism potentially carried out by members of the overall microbial community. Furthermore, while H_2S inhibits chemotrophic $\text{As}^{(\text{III})}$ oxidation by some members of the mat community, it acts as an

important electron donor for an ecologically relevant group of organisms also found in the spring. The upstream, high sulfide reaches of this geothermal feature are numerically dominated by members of the genus *Hydrogenobaculum*, several isolates of which were obtained through culturing efforts. Ex situ assays with whole-mat samples and Controlled culture studies with these isolates confirm that H₂S utilization and energy yield under spring-relevant conditions are several orders of magnitude higher than those observed for dissolved H₂ and that these metabolisms are simultaneously active. Additionally, 16S-based phylogeny failed to accurately predict important physiological traits of the isolates regarding substrate utilization. This work emphasizes the benefits of combining detailed chemical analysis with molecular methods, microbial culturing, and spring-side assays in order to gain a robust understanding of microbial activities in a complex environmental setting.

CHAPTER 2

MICROBIAL INTERACTIONS WITH HYDROGEN, SULFIDE, AND ARSENITE
LITERATURE REVIEWIntroduction to the Study SiteNorris Geyser Basin

Norris Geyser Basin (NGB) in Yellowstone National Park (YNP) contains the most diverse assortment of thermal features of any defined geyser basin in the park (138). With the sole exception of travertine-depositing springs, every type of hot spring, geyser, fumarole and mud volcano observed in the park as a whole can be found in the relatively small Norris Geyser Basin. A nearly neutral water type, high in Cl and SiO₂, is predominant throughout the basin and is the result of contact between high Cl waters and SiO₂ rich minerals at high temperatures (138). Circumneutral springs have also been found commonly in the basin that contain high levels of SO₄ and Cl. The concentration of Cl in these waters has been used as an indicator of meteoric water dilution of the subsurface waters. A minor water type, Acid-SO₄, is generally observed in features with near-boiling temperatures at relatively high altitudes. The spring type of interest to this review is Acid-Sulfate-Chloride (ASC), which is abundant in the southwestern part of the NGB and contains high concentrations of several reduced compounds as well as CO₂ which can be used for chemolithoautotrophic primary production (61, 75)

Acid-Sulfate-Chloride Springs

ASC springs are classified as such due to their low pH and high (typically millimolar) concentrations of sulfate and chloride (58-61, 75, 96, 141). This chemical signature is likely the result of the mixing of chloride-rich subsurface waters with thermally impacted acidic waters. The low pH evident in these waters is most likely a function of two processes that yield sulfuric acid; the hydrolysis of dissolved SO_2 , and the oxidation of reduced sulfur compounds including H_2S and S^0 (60, 96, 141). Those ASC springs located within the NGB are also typically supersaturated with respect to dissolved CO_2 and contain nanomolar concentrations of $\text{H}_{2(\text{aq})}$, and micromolar concentrations of $\text{H}_{2\text{S}(\text{aq})}$. Additionally, ASC spring waters typically contain micromolar concentrations of dissolved $\text{Fe}^{(\text{II})}$ and $\text{As}^{(\text{III})}$; all of these reduced compounds have been shown to support microbial growth in acidic systems (58-60, 75, 96).

These systems also exhibit characteristically steep physical and chemical gradients as waters flow downstream from the source. The primary physical gradient observed in these springs is decreasing temperature from the often $>70^\circ\text{C}$ source water (25, 59-61, 75). Chemical gradients apparent at ASC springs include substantial changes in dissolved gas composition and oxidation of reduced compounds in the spring water. CO_2 , H_2 and H_2S concentrations decrease as a function of off-gassing and microbial activities. Oxygen concentrations increase gradually as water flows downstream from the terrestrial source and equilibrates with the ambient air (25, 60, 61, 75). Further downstream, reduced constituents such as $\text{Fe}^{(\text{II})}$ and $\text{As}^{(\text{III})}$ are oxidized through abiotic mechanisms and microbial activity (61, 75, 96). These changes in spring chemistry

correspond to major transitions in spring appearance. The upstream portion of these springs is often yellow in color as a result of H_2S oxidation and S^0 precipitation (60, 75, 96). Downstream of this zone, iron oxidation results in the deposition of a brown-colored, amorphous ferric oxyhydroxide solid phase that contains high levels of sorbed $\text{As}^{(\text{V})}$ (81). As temperatures drop below 46°C , a green microbial mat becomes evident that is comprised primarily of photosynthetic eukaryotes (60, 61, 78). The physical and chemical gradients found in ASC springs are well documented (14, 25, 60, 61, 75) and relatively consistent (78). This review focuses on three important chemical constituents; H_2 , H_2S , and $\text{As}^{(\text{III})}$ and the potential roles that these electron donors play in primary production in ASC springs.

Hydrogen

Occurrence of Hydrogen

H_2 is ubiquitous on Earth above the upper mantle (16, 42). In particular, H_2 is found in igneous rocks and volcanic gasses (23) and found above predicted equilibrium in surface waters of the ocean (17). Apps and Van de Kamp (4) suggested six abiotic mechanisms that result in H_2 in subsurface systems: (1) reaction between dissolved gases in the C–H–O–S system in magmas, especially in those with basaltic affinities; (2) decomposition of methane to carbon (graphite) and hydrogen at $T \geq 600^\circ\text{C}$; (3) reaction between CO_2 , H_2O , and CH_4 at elevated temperatures in vapors; (4) radiolysis of water by radioactive isotopes of uranium, thorium, and their daughters, and potassium; (5) cataclasis of silicates under stress in the presence of water; and (6) hydrolysis by ferrous

minerals in mafic and ultramafic rocks. Additionally, Neal and Stanger (92) proposed that H₂ may be formed through water decomposition in the presence of some metal hydroxides under strongly reducing conditions. The hydrolysis of water by ferrous iron under strongly reduced conditions is the most important abiogenic process of the six listed (4). Additionally, solid-state redox reactions have been demonstrated to occur between silicates and water or CO₂ trapped in the mineral lattice during cooling of igneous melts producing H₂, H₂O₂, and organic acids(35, 36).

H₂ loss from soils was first reported in 1839 by de Saussure (27, 87) and this was shown to be a result of biological activity in 1892 by Immerdorf (56, 87, 93, 128). Systems impacted by geothermal features, such as those in the YNP complex are known to have elevated concentrations of H₂ in both the gaseous and aqueous phases (93, 128).

Hydrogen-based Chemolithotrophy

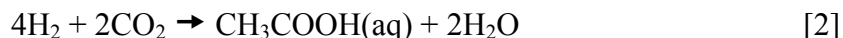
Hydrogen is a potent energy source that is utilized by microorganisms in a variety of ecological settings(120). There are over 110 characterized hydrogen-oxidizing bacteria that belong to 15 bacterial orders including the *Aquificales* (28, 68, 73, 123), four orders of the *Cyanobacteria* (32, 52, 74), all orders of the *Firmicutes*, (47, 120), the α -, β -, δ -, γ -, and ϵ -*Proteobacteria* (31, 100, 140, 142), and archaeal representatives from the *Thermococales* (34, 95, 143). Despite the wide distribution of hydrogen-oxidizing organisms throughout the *Archaea* and *Bacteria*, all of these organisms have remarkably similar enzymes that are responsible for this metabolism. There are three unique groups of hydrogenases presently classified based on metal content: [FeFe] hydrogenases, [NiFe] hydrogenases (including the [NiFe(Se)] hydrogenases), and [Fe] hydrogenases (formerly

referred to as “metal-free hydrogenases). These enzymes are all capable of carrying out the same reactions and constitute a remarkable example of convergent evolution (120).

Dissolved H₂ has been shown to be a particularly important electron donor in oligotrophic, anoxic subsurface systems (131) and potentially the keystone electron donor in subsurface lithotrophic microbial ecosystems (20). Anoxic systems such as these are dominated by methanogenic *Archaea* of the *Methanosarcinales* (20, 49, 50, 103) that catalyze the following reaction:



Methanogenic microbial communities are responsible for a large portion, if not all, of the primary production in these environments and initiate a hydrogen-based food chain (105). Anaerobic H₂ oxidation can also result in the production of acetate by organisms such as *Acetobacterium* (7) and *Clostridium aceticum* (15) through the reaction:



Organisms responsible for these reactions have been found primarily in subsurface aquifers and, like the methanogens, are members of the domain *Archaea*.

In anoxic systems with high S⁰ and H₂ concentrations, some chemolithotrophs are capable of reducing oxidized sulfur species to H₂S. In acidic systems, members of the *Archaea*, including *Stygiolobus*-like organisms, have been detected through 16S-based surveys (75). The type strain of this genus, *Stygiolobus azoricus*, an isolate from an acidic geothermal spring in the Azores (121), as well as other isolates from marine solfataric fields and acidic coal refuse piles (53, 130), have been characterized as obligate anaerobes requiring H₂ and S⁰. More recently, Boyd et al. (13) isolated two sulfur-

respiring, hydrogen-oxidizing members of the *Crenarchaea*; *Caldisphaera draconis* and *Acidilobus sulfurireducens*.

Chemolithotrophic growth based on hydrogen oxidation is also common in aerobic and microaerobic systems, where oxygen is used as a terminal electron donor for the oxidation of dissolved H₂ (also referred to as the Knallgas reaction) (37):



Bowien and Schlegel (13) suggested that the aerobic hydrogen-oxidizing bacteria are the most taxonomically diverse group of chemolithotrophic bacteria. Most bacteria that contain an active hydrogenase are capable of chemotrophic growth on hydrogen provided they possess the ability to form the key enzymes of the Calvin cycle (13). The majority of characterized, aerobic, hydrogen-oxidizing bacteria (Knallgas bacteria), including the most comprehensively characterized hydrogen-oxidizing isolate, *Ralstonia eutropha* (120, 139), are facultative and are capable of chemoheterotrophic growth when sugars or organic acids are present (5). This bacterium contains two energy-generating [NiFe] hydrogenases; a periplasmic membrane-bound type that feeds electrons to into a respiratory chain through a *b*-type cytochrome and a cytoplasmic, soluble tetrameric hydrogenase that reduces NAD⁺ via a connected NADH oxidoreductase module (118, 119). The former enzymatic system is most commonly found in the knallgas bacteria (120) however the second pathway and combinations of both have been found in a number of Gram-positive bacteria (45).

The first isolated aerobic, obligate hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus*, was obtained from geothermally influenced waters in

YNP in the early 1980s (68). Since then, several obligate Knallgas bacteria have been isolated including two strains of the genus *Persephonella* from geographically distinct deep-sea hydrothermal vent sites (43). In ASC terrestrial springs, hydrogen-oxidizing chemolithotrophic aerobic and microaerobic isolates of the *Aquificales* including members of the genera *Hydrogenobacter*, *Hydrogenobaculum* and *Sulfurihydrogenibium* (2, 30, 48) have been isolated. Some of these isolates are facultative H₂ oxidizers that are alternatively capable of aerobic growth on sulfide (48), while others are obligate aerobic hydrogen oxidizers (2, 30). Due to the ubiquity and permeability of hydrogen along with the potential energy to be gained by the oxidation of this reduced gas, it has been suggested that dissolved hydrogen is the “universal energy source” for long-term microbial survival and anabiosis (35, 87).

Inhibition of Metabolisms by Hydrogen

Dissolved H₂ has been shown to inhibit some important subsurface microbial activities, most notably anaerobic methane oxidation. In anoxic systems where methane is consumed by microbes, H₂ is released as a waste product. When dissolved H₂ concentrations rise, the thermodynamics of methane oxidation become unfavorable for energy generation by this pathway and this activity ceases. In systems such as those found at Hydrate Ridge and anoxic sediments in the Black Sea (91), the highest rates of anaerobic oxidation of methane were observed where sulfate concentrations support the simultaneous growth of sulfate reducing bacteria of the *Desulfosarcina/Desulfococcus* group (12, 70, 83) that are using excess H₂ as an oxidant and decreasing the partial pressure of H₂ in the system.

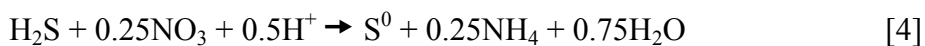
Sulfide

Occurrence of Sulfide

H₂S is found in a variety of terrestrial, subsurface, and marine environments. Like H₂, it is often associated with igneous formations and is a major component of volcanic gasses (138). It has been proposed that much of the H₂S found in surface and marine environments is the result of biogenic sulfur and sulfate reduction (8, 66, 67). Anthropogenic sources of H₂S include wastewater treatment facilities, fossil-fuel production and combustion, and paper production though these inputs are still largely the result of microbial activities (9, 54, 62).

Sulfide-based Chemolithotrophy

Anaerobic sulfide oxidation coupled to nitrate reduction has been observed with colorless sulfur oxidizing bacteria in marine systems (10, 11) and is considered an important process in the control of sulfide production by sulfate reducing bacteria in anoxic marine sediments. In industrial settings, this activity has been exploited to remove dissolved sulfide and nitrate from municipal waters (9, 62) and from water co-produced with petroleum compounds (39, 82) through the addition of nitrate. The anaerobic oxidation of sulfide with nitrate as a terminal electron donor is carried out by a variety of acidophilic autotrophs including members of the *Rhodobacteracea* (24). This reaction yields approximately -139.7 kJ mol⁻¹ (57):



Surrounding many deep-sea hydrothermal vents, robust endemic communities

thrive in environments where H₂S has been measured above 300μM (22). In these systems, where there is no transmitted sunlight for the growth of traditional phototrophs, sulfide is considered the principal electron donor for primary production (22, 113). The tube worms *Alvinella pompejana* (46), *Riftia pachyptila*, (63) and the crustacean *Rimicaris exoculata* (107) are all supported through the growth and activity of sulfide-oxidizing bacterial epibionts. In aquatic systems with a pH ≤5, sulfide exists primarily in the nonionic form (H₂S) and does not rapidly oxidize through chemical means (21, 85). In these systems, microbes are responsible for a considerable portion of total sulfide oxidation (65, 110, 127). Two enzymatic pathways have been identified for the conversion of H₂S to S⁰. The first pathway involves the direct transfer of electrons to cytochromes *c* by *c*-type cytochromes or flavocytochromes *c* (38, 69). In the second pathway, electrons are transferred to the cytoplasmic quinone pool via a membrane-bound sulfide:quinone oxidoreductase (6, 38, 98).

These metabolic pathways result in net energy yield for the proliferation of chemoautotrophic microbes based on the oxidation of reduced sulfur compounds. The aerobic oxidation of H₂S to elemental sulfur;



which yields approximately 213 kJ mol⁻¹, does not yield as much free energy as aerobic H₂ oxidation (-260.7 kJ mol⁻¹) however, it can yield even more energy per mole (-732.3 kJ mol⁻¹) (57) when that metabolism is carried out concurrently with the aerobic oxidation of sulfur to sulfate:



With the net reaction written as:



Inhibition of Metabolisms by H₂S

The nitrogen cycle is acutely influenced by the presence of H₂S in natural and man-made environments. The rate of nitrate reduction by a *Desulfovibrio desulfuricans* isolate from rice paddy soils is reduced by 70% in the presence of 127 μM sulfide even with an excess of nitrate in the system (5mM) (26). Nitrous and nitric oxide reduction by a denitrifying *Pseudomonas fluorescens* isolate is also severely inhibited by the presence of H₂S *in vitro* (126) as is large-scale nitrification in estuarine sediments (64). In the *Cyanobacteria* and chloroplasts of higher plants including tobacco, oxygenic phototrophism (particularly photosystem II) is severely impacted by the presence of sulfide (102). *Cyanobacteria* in sulfidic habitats, including those in YNP, have adapted to tolerate varying sulfide concentrations and the degree to which they can tolerate this inhibitor has been used as an indicator for strain differentiation (84).

The arsenite oxidation activity of a *Hydrogenobaculum* isolate (30) and an arsenite-oxidizing chemolithotrophic *Acidicaldus*-like isolate (25) from an ASC spring in YNP are strongly inhibited by the presence of H₂S at concentrations as low as 2.0 μM. This inhibition appears immediately reversible and has been characterized as uncompetitive by whole-cell assay, suggesting that sulfide does not influence the transcription or translation of arsenite oxidase genes but rather binds to the substrate:enzyme complex (25).

Arsenite

Occurrence of Arsenite

Arsenic is a commonly found transition metal that has natural and anthropogenic sources in surface and subsurface environments. Igneous activity is the primary source of arsenic on the Earth's surface (88). It is found commonly in crustal, marine sedimentary, and weathered volcanic rocks (132). The anthropogenic release of arsenic is mainly due to industrial and agricultural activities including the melting of metalliferous ores, burning of fossil fuels, and application of agricultural pesticides (86). Additionally, arsenic, typically as $\text{As}^{(\text{III})}$, is released from mineral phases through the processes of acid rock drainage both in naturally occurring systems and as a result of hard rock mining and ore extraction (18, 97).

Arsenic occurs in the environment in several oxidation states: arsine [$\text{As}^{(-\text{III})}$], arsenic [$\text{As}^{(0)}$], arsenite [$\text{As}^{(\text{III})}$] and arsenate [$\text{As}^{(\text{V})}$], and it is this valence state that determines its solubility, mobility, sorption behavior, bioavailability and toxicity (29, 33, 133). In oxygenated waters, $\text{As}^{(\text{V})}$ is the most common form (80, 132) and is thermodynamically stable as H_2AsO_4^- , HAsO_4^{2-} , and AsO_4^{3-} in the pH range from 5-12 (132). $\text{As}^{(\text{III})}$ is the prevalent oxidation state in anoxic waters with the nonionic form (H_2AsO_3) most common below pH 9.22 and the anionic form (HAsO_3^-) found more commonly above that pH. Abiotic oxidation of $\text{As}^{(\text{III})}$ progresses very slowly and it is believed that in anoxic systems, arsenic oxidation is primarily a biological process (133). In biological systems, $\text{As}^{(\text{III})}$ is reported to be on average 100 times more toxic than the pentavalent form (89, 94) and more difficult to remove from aqueous systems through

conventional means (90, 132).

Arsenite-based Chemolithotrophy

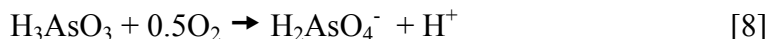
Microbial arsenite oxidation was first reported in 1918 (44) and has since been shown to occur in a wide variety of environments (25, 30, 40, 41, 55, 58, 76, 77, 101, 104, 106, 116, 117, 134-137) by a phylogenetically diverse group of organisms including members of the *Bacteria* and *Archaea* (Table 1.1). Arsenite oxidation is often considered to be a component of detoxification pathways in heterotrophs such as *Alcaligenes* strains and *Cenibacterium*, allowing these organisms to tolerate higher levels of $\text{As}^{(\text{III})}$ (19, 124, 125). As more bacterial genomes are fully sequenced, it has become apparent that genes coding for arsenite oxidases are ubiquitous (88). The heterodimeric molybdopterin arsenite oxidase from *Alcaligenes faecalis* (77) was purified and characterized (3) and is capable of transferring electrons to either azurin or cytochrome *c*. The ability to transfer electrons from the oxidation of $\text{As}^{(\text{III})}$ to a cytochrome *c*, a common component of electron transport chains, has led to the search for and isolation of microbes capable of chemotrophic growth based on $\text{As}^{(\text{III})}$ oxidation metabolism.

Table 2.1. Summary of documented arsenic-oxidizing microorganisms.

<u>Microorganism</u>	<u>Isolation Origin</u>	<u>Oxidation Type</u>	<u>Growth Condition</u>	<u>Ref</u>
<i>Bacillus arsenoxydans</i>	Cattle-dipping fluids	Detoxification	NR	(44)
<i>Pseudomonas arsenoxydans</i>	Cattle-dipping fluids	Detoxification	40°C, pH 6.4	(134)
<i>Alcaligenes faecalis</i> str. YE56	Raw Sewage	Detoxification	32-37°C, pH 7.4	(106)
<i>Alcaligenes faecalis</i> str. ANA	Soils	Detoxification	25-37°C, pH 7	(104)
<i>Pseudomonas arsenitoxidans</i>	Mine waters	Autotrophic	28-35°C, pH 7.5	(55)
<i>Pseudomonas putida</i> str. 18	Gold-Arsenic deposits	Autotrophic	4-28°C, pH 6-9	(1)
<i>Sulfolobus acidocaldarius</i> str. BC	Mineral leaching	Detoxification	65°C, pH 2-4	(122)
<i>Alcaligenes faecalis</i> str. 8687	Previously isolated	Detoxification	32-37°C, pH 7.4	(3)
<i>Cenibacterium</i> str. ULPAs-1	Contaminated water	Detoxification	25°C, pH 7.2	(137)
<i>Agrobacterium</i> str. NT-26	Gold mine	Autotrophic	28°C, pH 8	(116)
<i>Thermus</i> str. HR13	Soil	Autotrophic	70°C, pH 7.5	(41)
<i>Agrobacterium albertimagni</i>	Macrophyte surfaces	Detoxification	30°C, pH 7-8	(114)
<i>Hydrogenobaculum</i> str. H55	ASC Spring	Detoxification	55°C pH3.1	(30)
<i>Alcaligenes faecalis</i> str. O1201	Soils	Detoxification	30°C, pH 7	(132)
<i>Hydrogenophaga</i> str. YED 1-8	Geothermal Water	Autotrophic	30°C, pH 8	(115)
<i>Azoarcus</i> str. DAO1	Contaminated soil	Autotrophic	30°C pH 7.2	(109)
<i>Sinorhizobium</i> str. DAO10	Contaminated soil	Autotrophic	30°C pH 7.2	(109)
<i>Thermus thermophilus</i> str. HB8	NR	Autotrophic	NR	(108)
<i>Bosea</i> str. WAO	Arsenic-laden Shale	Autotrophic	30°C, pH 7.2	(108)
<i>Ancyclobacter</i> str. OL1	Freshwater sediment	Autotrophic	30°C, pH 7.2	(108)
<i>Thiobacillus</i> str. S1	Industrial soil	Autotrophic	30°C, pH 7.2	(108)
<i>Hydrogenophaga</i> str. CL3	Petroleum refinery	Autotrophic	30°C, pH 7.2	(108)
<i>Alkalilimnicola ehrlichii</i> MLHE-1	Mono Lake water	Autotrophic	20°C, pH 9.8	(51)
<i>Acidicaldus</i> str. AO5	ASC Spring, YNP	Autotrophic	55°C, pH 3	(25)

NR: Not reported

Nearly three decades have passed since Ilyaledtinov and Abdrashitova first isolated an autotrophic arsenite-oxidating, aerobic, mesophilic *Pseudomonas arsenitoxidans* from mine waters of a gold-arsenic deposit containing high levels of dissolved arsenic (55). Since then, several additional arsenite-oxidizing chemolithotrophic organisms have been isolated from high-arsenic environments with widely varying pH, temperature and oxygen requirements and sensitivities. A number of characterized arsenite-oxidizing autotrophs are obligate aerobes (25, 55, 108, 116) that couple the oxidation of As^(III) to As^(V) with the reduction of dissolved O₂:



While the $\text{As}^{(\text{III})}:\text{O}_2$ couple has the potential to yield considerable energy to microbial metabolism (109), several denitrifying organisms have been recently isolated (101, 108, 109) from anoxic systems that are capable of coupling the oxidation of $\text{As}^{(\text{III})}$ to the reduction of NO_3^- for chemolithotrophic growth:



An *Alkalilimnicola ehrlichii* isolated from the bottom waters of Mono Lake was also shown to be capable of anaerobic nitrate reduction coupled to $\text{As}^{(\text{III})}$ oxidation, however it is unable to reduce nitrite to ammonia (51, 101):



This reaction yields less energy per mole $\text{As}^{(\text{III})}$ oxidized than reaction (8) but still supports growth of this strain in anoxic waters.

In a survey of YNP, Stauffer et al. (129) observed rapid oxidation of arsenite to arsenate in several geothermal springs. In a model ASC spring in YNP, arsenite oxidation occurs in regions of the spring with little to no dissolved H_2S present (25, 75). Ex situ assays, however, revealed that organisms present in high-sulfide regions of the outflow channel are capable of arsenite oxidation and that this activity is strongly but reversibly inhibited by H_2S in situ (25). An isolate, *Acdidicaldus* strain AO5, from this environment is capable of chemolithoautotrophic growth on $\text{As}^{(\text{III})}$ under aerobic conditions at elevated temperatures ($T_{\text{opt}} = 55^\circ\text{C}$) (25).

Inhibition of Metabolisms by Arsenite

While Kobayashi et al. (72) noted that arsenite inhibits the activity of a sulfite reductase from *Desulfovibrio vulgaris*, very little work has examined the effects of arsenite on prokaryotic metabolisms. The toxicity of arsenic to both eukaryotic and prokaryotic organisms, on the other hand, is well documented. Arsenite toxicity derives from its high affinity to essential sulfhydryl groups of proteins and dithiols such as glutaredoxin (3, 71, 99). Arsenite, in its ionic form, is also a known mutagen and, intensifying this effect on microbial cells, inhibits DNA repair mechanisms (112). It is believed that $\text{As}^{(\text{III})}$ enters bacterial cells not in the commonly found nonionic form but as a polymer that is a structural analog to hexoses through aquaglyceroporins and hexose permeases(79). Many bacteria, including *E. coli*, are capable of extruding $\text{As}^{(\text{III})}$ from the cell through the ArsB transmembrane channel or, more effectively by the ArsAB complex which couples the $\text{As}^{(\text{III})}$ transport component to an ATPase (111).

Synthesis

ASC springs represent a common thermal feature type in the Norris Geyser Basin of Yellowstone National Park. The distinctive chemistry of these springs is stable and predictable (61, 75), and results in the formation of pronounced physical and chemical gradients in a variety of scales and dimensions. The high concentrations of reduced chemical constituents in ASC spring waters, along with high dissolved CO_2 concentrations and elevated temperatures make these features ideal environments for the study of chemolithoautotrophic primary production by thermophilic organisms. (25)

Chief among the reduced species available to microbes as electron donors are hydrogen, sulfide, sulfur, ferrous iron, and arsenite. Organisms have been isolated from ASC springs that are capable of growth based on each of these energy sources (14, 25, 30) and continued work reveals remarkable phylogenetic and metabolic diversity in the microbial communities inhabiting ASC springs.

Hydrogen, sulfide, and arsenic metabolism in these features are closely linked and, in some cases, interdependent. For example, sulfide, which is an important electron donor for some members of the microbial community in the ASC spring studied in this thesis, strongly inhibits $\text{As}^{(\text{III})}$ oxidation and is the major determinant of the distribution of another community member. Biological activity in these systems is both dictated by and responsible for observed environmental conditions.

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CHAPTER 3

AUTECOLOGY OF AN ARSENITE CHEMOLITHOTROPH: SULFIDE
CONSTRAINTS ON FUNCTION AND DISTRIBUTION
IN A GEOTHERMAL SPRINGAbstract

Previous studies in a acid sulfate chloride spring in Yellowstone National Park found that microbial arsenite [As(III)] oxidation is absent in regions of the spring outflow channel where H₂S exceeds ~5 μM and served as a backdrop for continued efforts in the current study. Ex situ assays with microbial mat samples demonstrated immediate As(III) oxidation activity when H₂S was absent or in low concentrations, suggesting the presence of As(III) oxidase enzymes that could be reactivated if H₂S is removed. Cultivation experiments initiated with mat samples taken from along the H₂S gradient in the outflow channel resulted in the isolation of an As(III)-oxidizing chemolithotroph from the low H₂S region of the gradient. The isolate was phylogenetically related to *Acidicaldus* and was characterized in culture experiments for spring relevant properties, which were then compared to its distribution pattern in the spring as determined by denaturing gradient gel electrophoresis and quantitative PCR. While neither temperature nor oxygen requirements appeared related to the occurrence of this organism within the outflow channel, H₂S concentration appeared to be an important constraint. This was verified with pure culture modeling and kinetic experiments. In summary, the studies reported

herein illustrate that H₂S is a potent inhibitor of As(III) oxidation and will influence niche opportunities and population distribution of As(III) chemolithotrophs.

Introduction

Microbial arsenite [As(III)] chemolithotrophy was first reported by Ilyaletdinov and Abdrashitova (18), and then more recently by Santini et al. (38), Oremland et al. (34), and Rhine et al. (37). Progress towards understanding the genetics and physiology of As(III) oxidation is at an early stage, being limited to a few definitive papers that describe the biochemical and structural features of one of the two identified As(III) oxidases (4, 11, 36), and recent studies that have identified the structural genes encoding these As(III) oxidases (31, 40).

Even less is known about the ecology of As(III) chemolithotrophs. Drainage waters originating from commercial mining operations often contain appreciable amounts of As(III), and thus are potential habitats for organisms capable of using As(III) as an energy source. Such was the case for the As(III) chemolithotrophs isolated from gold mines (38, 39). Also, waters originating from geothermal sources often carry significant As(III) (5, 28, 44). The facultative anaerobic As(III) chemolithotroph isolated by Oremland et al. (34) was obtained from anaerobic bottom waters of Mono Lake, a meromictic lake containing high concentrations of As(III) derived from geothermal inputs (33). Thermophiles capable of oxidizing As(III) have been isolated (10, 14), and a recent PCR-based survey documented the presence and expression of As(III) oxidase structural genes in geothermal springs in Yellowstone National Park (YNP), Wyoming (21).

However, to date, the lone documentation of a thermophile capable of As(III) chemolithotrophy is a brief notation that *Sulfurihydrogenibium azorense* can use As(III) as an electron donor (1).

Our previous studies in the Norris Geyser Basin in YNP have focused on an acidic geothermal feature referred to as Dragon Spring (22, 28). Chemical analysis of Dragon Spring found the As(III) concentration in the source waters to be 33 μM (28), which has been documented on subsequent occasions to be nearly invariable. At a constant flux, this level of As(III) should be adequate for supporting growth of As(III) chemolithotrophs. And, while biological As(III) oxidation has been documented in this spring, it was only found in specific regions of the outflow channel where H₂S was absent or at low concentrations (28). In subsequent in vitro experiments with a pure culture of an As(III)-oxidizing *Hydrogenobaculum*-like organism isolated from this spring, H₂S inhibited As(III) oxidation (10). However, other environmental factors may also contribute to the specific localization of As(III) oxidation in this spring. In the current study, As(III) oxidation in Dragon Spring was re-examined to assess the potential for As(III) chemolithotrophs to inhabit this geothermal feature, and to gain a more thorough understanding of the H₂S inhibition phenomenon with respect to how it may relate to microbial function and population distribution in the environment.

Materials and Methods

Study Site and Chemical Analysis

All field experiments were conducted at Dragon Spring (44°43'54.8" N, 110°42'39.9" W, spring number NHSP106 in the YNP thermal inventory) located in Norris Geyser Basin, YNP. Water temperature and pH were routinely monitored throughout the two-year study. At the point of discharge, pH was a near constant 3.1 and temperature ranged from 68-72°C during the course of the study, with changes in the latter being unpredictable. Dissolved H₂S was measured using the amine-sulfuric acid methodology previously described (28) except that the spring water was assayed within seconds of sampling and without prior filter sterilization. Dissolved oxygen was assayed using a Hach OX-2P test kit (Hach Company, Loveland, CO).

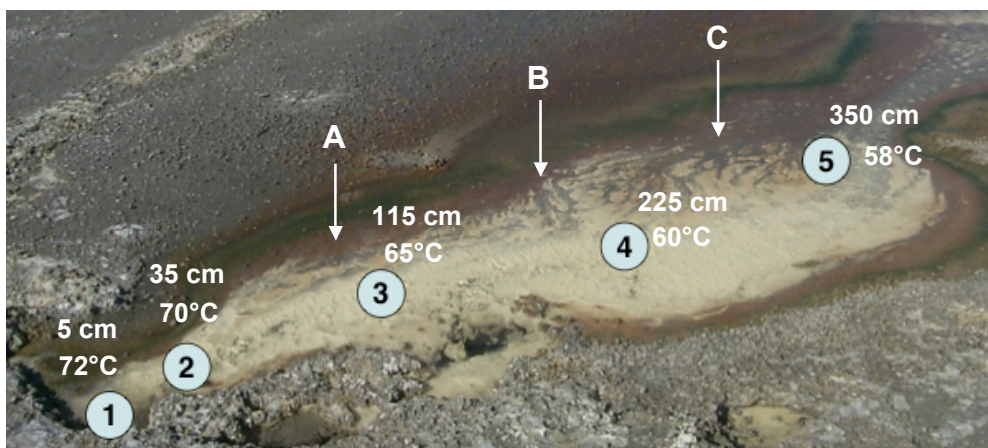


Figure 3.1 Color image of Dragon Spring. Almost all sampling occurred in the yellow zone where the solid phase material is comprised of S⁰. Main transect points are indicated with numbers 1-5, although sampling for some experiments occurred at many locations not always corresponding exactly with the primary transect points. Typical temperatures observed at each transect point are also shown and illustrate the temperature gradient in this portion of the outflow channel. Water temperatures shown were measured on June 15th 2003. Vertical arrows indicate the sampling sites for DNA used for PCR-DGGE analysis and correspond to lanes A, B, and C in Figure 3.8.

As^(III) Oxidation Assays

Mat samples were collected using autoclaved tools or sterile pipet tips and placed into sterile 15 ml conical tubes, homogenized by mixing, then split into two sub-samples. One sub-sample was used to measure As^(III) oxidation, while the other was boiled for 20 minutes to serve as a killed control. Mat samples were aseptically transferred into 150 ml serum bottles containing 40 ml of filter-sterilized spring water that was amended to 60 μM As^(III) (added as NaH_2AsO_3). Assay bottles were closed with butyl rubber septa, pressure sealed with an aluminum ring, and then incubated at in situ temperatures by placing the bottles in the spring at the same location from which the mat material was sampled. Water samples were withdrawn at 10 min intervals to assay for As^(III) oxidation using sodium borohydride speciation as described previously (10). Samples were stored at 4°C until analysis by hydride generation atomic absorption spectrometry (24). As^(V) formation and H₂S disappearance data were normalized based on mat sample dry weight, which was determined by collection of all mat material from each serum bottle onto pre-weighed 0.2 μm filters, drying overnight at 65°C, and then weighing to determine the mat dry weight contained in each serum bottle. Maximum mat dry weight variation between samples was 7.6%.

Cultivation Work

Approximately 2 g of mat material was aseptically collected using wide-bore pipette tips along five transect sites shown in Figure 3.1. Mat material was stored for transportation to the laboratory in 15 ml conical tubes and maintained at 55°C in a Thermos™ bottle filled with site water. Mat samples were resuspended in 10 ml spring

water collected directly above the mat sampling site and 100 μl of this suspension was used to inoculate 16 ml serum bottles containing 4.9 ml of filter sterilized spring water amended with 1mM As(III) (supplied as NaH_2AsO_3) and degassed to remove the H_2S . The bottles were sealed as described above, with the headspace made 50% CO_2 and 50% air using filter-sterilized gases. Enrichments were incubated at the same temperature as recorded for each sample in the field, and the headspace gasses were replaced every third day via syringe. Every 30 days, 100 μl of culture was transferred to similarly prepared serum bottles. In latter stages of the enrichment process, the addition of a dilute synthetic, defined medium, pH 3.1 (see 15), was found to enhance growth yields and thus a 1:1 mixture of filter sterilized spring water and synthetic media was the composition of late-stage enrichment media. Throughout, enrichment cultures were monitored for growth by phase-contrast microscopy. After 120 days, cultures were serially diluted and used as inocula for spread plates containing the same medium solidified with 1.8% Gelrite (Research Products International, Mt. Prospect, IL). A pure culture was established by repeated sub-culturing isolated colonies and is referred to herein as strain AO5.

Further experiments with the pure culture found the organism would grow on the defined medium without spring water and thus the undiluted defined medium was used in all subsequent experiments. Cardinal temperatures were determined in water bath shakers (containing glycerol) set at varying temperatures. The sealed serum bottles contained 20 ml media, and 80 ml of headspace gasses (50% CO_2 and 50% air, both filter-sterilized) that were replaced daily to maintain optimum levels of CO_2 and O_2 . The

same culture conditions were used for As^(III) oxidation and sulfide inhibition kinetics studies. For anaerobic culture work, the liquid medium was amended with sulfate (as FeSO₄), nitrate (NaNO₃⁻), or ferric iron (FeCl₃) as representative dominant spring-relevant electron acceptors previously documented in this spring (28), and all at 1 mM. The media and headspace in each autoclaved sealed serum bottle was then purged for 2 h with filter-sterilized certified O₂-free N₂ gas, being vented via a sterile syringe needle. All gassing treatments were conducted aseptically in a Labconco biosafety cabinet (Labconco Corp., Kansas City, MO).

As^(III) Oxidation Kinetics

Late log phase AO5 cells (1.0×10^6 total) were added to sealed serum bottles containing 20 ml sterile media amended with 500 μ M citric acid (pH 3.0) as a pH buffer and 30 μ M As^(III). Na₂S was added to a final concentration of 0, 3, 15, 30, or 60 μ M, and then 100 μ l samples removed at 10 min intervals starting immediately upon addition of the Na₂S (time = 0) and continuing for 60 min. Samples were immediately frozen in liquid nitrogen and stored at -80°C until speciation, with the analysis carried out as described above for the ex situ assays. To model the mode of enzyme inhibition, As^(III) oxidation rates were calculated from AO5 cultures incubated in the same fashion except that As^(III) was added to a final concentration ranging from 3.75 to 30 μ M in media amended with 0, 2.91 or 5.82 μ M Na₂S.

PCR and Phylogenetic Analysis

DNA was extracted from the pure culture using methods previously described (6). Near full-length 16S rDNA was amplified using *Bacteria*-specific primers 8F and the universal primer 1492R (3). The PCR contained 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 μM of each primer, approximately 10 ng template DNA, 0.5 mg ml⁻¹ BSA, 2.5 units Taq polymerase and 1x buffer (Promega, Madison, WI) in a total volume of 50 μl. The amplification program consisted of 2 min at 95°C, then 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 45° C, 1 min extension at 72°C, followed by a final extension of 7 min, at 72°C. Sequencing was carried out at the Ohio State University Plant Microbe Genomics Facility using primers 8F, 533F, 533R, and 1492R. The 16S rDNA sequence for isolate AO5 is available as GenBank accession EF151282. Sequences were aligned using ClustalX (46) and then edited and trimmed using Se-AL v2.0a11 (<http://evolve.zoo.ox.ac.uk>). Phylogenetic analysis of the aligned sequences was performed using the Maximum Likelihood algorithm and bootstrap values for 100 pseudoreplicates were generated using the PAUP* v4.0b10 software package (45). *Hydrogenobaculum acidophilum* (GenBank accession AJ320225), a member of the deeply branching family *Aquificaceae*, was used as an outgroup for these analyses.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR primers and conditions, and techniques employed for DGGE analysis were the same as previously described (32). Briefly, primers were *Bacteria*-specific 1070F (13) and the universal reverse primer 1392R (3) containing a GC-clamp (32). DGGE was performed using a Bio-Rad DCode system (Bio-Rad, Hercules, CA), and an 8%

polyacrylamide gel containing a 40-70% denaturant concentration (where 100% denaturant contains 7 M urea and 40% (vol/vol) formamide). DNA was extracted from DGGE gels by excising bands from the gel and crushing them in a 1.5 ml microcentrifuge tube. Extraction solution (150 μ l of 500 mM ammonium acetate, 0.1% w/v SDS, 0.1 mM EDTA) was added to the crushed gel slice and the mixture was incubated at 75°C for 2 hours. The tube was centrifuged at 14,000 x g for 10 minutes and the supernatant was removed to a separate tube. DNA was precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 volumes of 95% ethanol.

Quantitative PCR

Quantitative PCR was used to study the in situ distribution pattern of the As(III) chemolithotroph isolated and characterized in this study. Nucleic acids were extracted from the mat material using the method of Botero *et al.* (7). Each quantitative PCR (Q-PCR) contained a total of 100 ng DNA. Standards contained 0, 0.001, 0.01, 0.1, 1, or 10 ng DNA extracted from isolate AO5 in a background of sheared herring sperm DNA. Each 25 μ l reaction contained 2.5 μ l Mg-free buffer, 2.5 μ l 25mM MgCl₂, 2 μ l dNTP mix (2.5 mM each dNTP), 0.1 μ l *Taq* DNA polymerase (Promega, Madison, WI), 1 μ l 20X Sybr-Green (Invitrogen, Carlsbad, CA) and 1 μ l each of primers AO5-965F (5'-ATCGGTCGTCCCGCAAC-3') and AO5-1181R (5'-CTGTCACCGCCATTGTAGCA-3'). Q-PCR cycling conditions were as follows: 95°C for 10 min; 35 cycles of 60 sec at 95°C, 60 sec at 53.5°C, 45 sec at 72°C followed by a final melting step gradually ramping the temperature up to 105°C. Cycling and fluorescence monitoring were carried out in a

Rotor-Gene RG-3000 (Corbett Research, Mortlake, NSW). Results were analyzed using the Rotor Gene software package Version 6.0.

Primer specificity was evaluated first by *in silico* methods using both the probe match function at the Ribosomal Database Project II (<http://rdp.cme.msu.edu>) and the BLAST (2) search for short, nearly identical matches at the National Center for Biotechnology Information (<http://ncbi.nlm.nih.gov>) (42). In addition, the Q-PCR primer design took into account all 16S sequences cloned using two different primer sets from a previous molecular analysis of Dragon Spring (22) and sequences derived from the DGGE analysis (above) conducted in the current study. The closest identity of any PCR clones derived from this spring deviated by at least 6 nts in the 18mer primers. As expected, testing the primers against various *Hydrogenobaculum* PCR clones that dominate the clone libraries derived from the yellow S⁰ zone this spring (22) did not result in an amplicon (data not shown). However, these primers could not differentiate AO5 from clone YNPFFP86 (accession AF391980; a single nt mismatch in AO5-965F) that was originally obtained by reverse transcriptase PCR from RNA extracted from a geothermally heated soil at a geographically distant (~50 km) YNP location (7), but thus far not detected in Dragon Spring.

Results

Mapping Spring H₂S Gradients

H₂S gradients were defined in two dimensions, with all work focused in the yellow S⁰ zone (Figure 3.1). A spatially intensive sampling transect of the S⁰ zone demonstrated a H₂S gradient consistent with that observed by Langner et al. (28), showing that aqueous H₂S concentration decreases as a function of distance from the spring source (Figure 3.2A), and that such gradients in this spring are stable over time (years). H₂S levels decreased to roughly 5 μM at sampling transect site 5 in the yellow S⁰ zone (Figures 3.1 and 3.2A), and then to undetectable levels at a distance of approximately 5 m and beyond (Figure 3.2A). Additional measurements quantified H₂S levels across the width of the yellow S⁰ zone to assess H₂S concentrations in a second dimension. H₂S was highest at mid-channel coinciding with highest flow rates, but sharply declined to below detection (~1 μM) at the interface between the yellow S⁰ mat area and the brown band zone (Figures 3.1 and 3.2B).

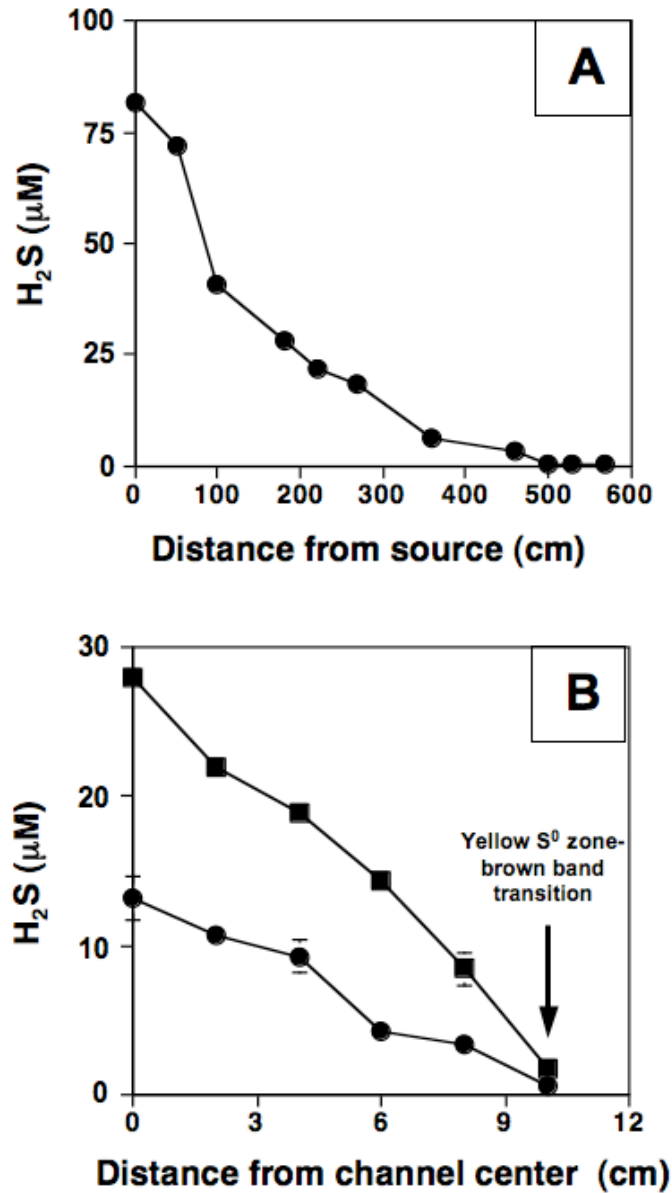


Figure 3.2. Dragon Spring H₂S gradients. (A) H₂S concentration decreasing with increasing distance from the spring source along the main flow channel. (B) H₂S concentration as a function of distance perpendicular to stream flow beginning at the center of the outflow channel and corresponding to transect site 4 (■) and transect site 5 (●). For both panels, data points represent the mean of three samples, with error bars (where visible) representing one standard deviation of the mean. Samples for panel B were taken on a separate sampling trip and thus maximum values at the channel center do not exactly correspond to those plotted in panel A.

As(III) Oxidation Potential in the H₂S Zone

In previous work with a *Hydrogenobaculum* sp. isolate obtained from this spring (10), As(III) oxidase activity was shown to be constitutive; i.e. As(III) oxidation activity profiles were the same for cultures pre-exposed to As(III) or for As(III)-naïve cells. As previous molecular-based analyses also demonstrated that the yellow S⁰ mat area is heavily dominated by *Hydrogenobaculum*-like populations (22; unpublished data), additional experiments were undertaken to determine whether the presence of As(III) oxidase activity could be demonstrated in this region of the spring if H₂S concentrations were manipulated. In experiments where microbial mat material sampled from transect site 2 (Figure 3.1) were suspended in filter-sterilized spring water from the same location, As(III) oxidation was not observed until aqueous H₂S concentrations decreased to below 5 μM (Figure 3.3). In contrast, As(III) oxidation was observed to occur within 10 min. (the first sampling time point) in incubations where mat material from site 2 was incubated with filter-sterilized spring water taken at 5 m from the source (i.e., H₂S was below detection, Figure 3.2A) (Figure 3.3). Similarly, As(III) oxidation was detected at the first sampling point with site 5 mat samples incubated in spring water taken from that same location. There was no As^(V) production observed in the heat-killed samples (Figure 3.3).

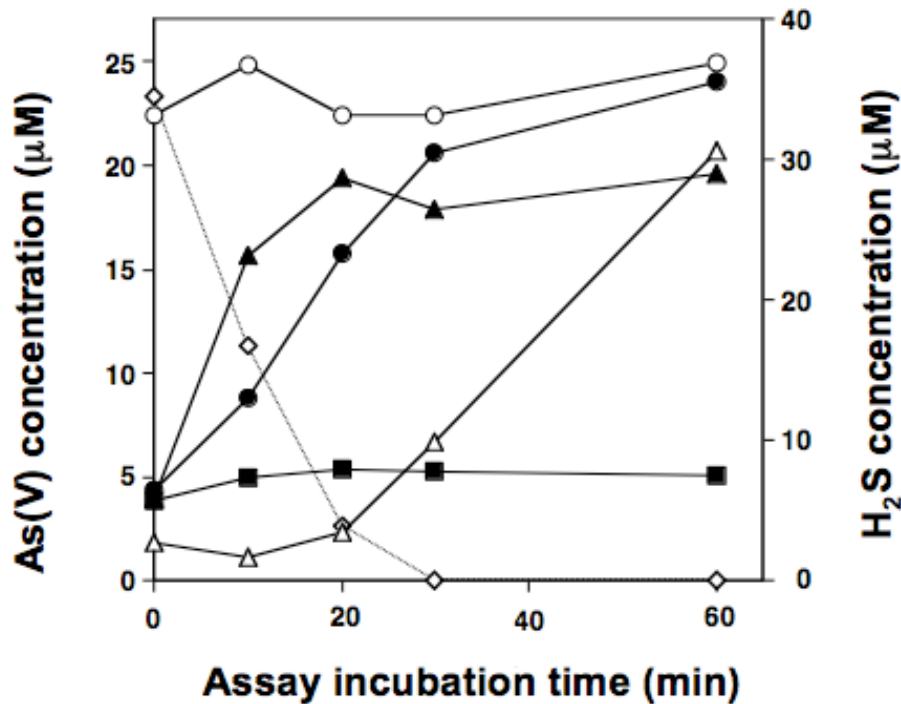


Figure 3.3. *Ex situ* assays of As(III) oxidation by microbial mat from the yellow S⁰ zone in Dragon Spring. ○, total dissolved As in transect site 2 mat assays showing no appreciable change in total dissolved As concentrations during these assays; ◇ with dashed line, dissolved H₂S measured in assays containing transect site 2 filter-sterilized water with mat collected from transect site 2; △, As(V) formation in assays containing mat material from transect site 2 suspended in spring water from transect site 2; ●, As(V) formation in assays containing mat material from transect site 2 suspended in filter-sterilized spring water collected from transect site 5; ▲ As(V) formation in assays containing mat material from transect site 5 in filter-sterilized spring water collected from transect site 5; ■ As(V) concentration in heat-killed mat material from transect site 2 suspended in filter-sterilized spring water from transect site 5. Data are from one representative field trip experiment, with symbols representing the average of duplicate assays at each time point for each treatment. Error bars (where visible) represent one standard deviation of the mean. In all assays, H₂S concentrations were lower than shown in Figure 3.2A because volatile losses occurred during filter sterilization of spring water.

Isolation of an As(III) Chemolithotroph

Given the apparent potential for As(III) oxidation activity in the yellow S⁰ zone, subsequent experiments sought to determine whether As(III) chemolithotrophs could be cultivated from this spring. Only enrichments inoculated with mat material from transect site 5 (Figure 3.1) and corresponding to ~ 5 μ M H₂S (Figure 3.2A) generated a positive enrichment, which yielded pure culture strain AO5. In liquid defined media, AO5 oxidized As(III) at a rate of approximately 1 nmole \cdot min⁻¹ \cdot 10⁶ cells⁻¹. No growth was observed in the absence of As(III) (Figure 3.4A) and, although somewhat variable, final culture density was positively correlated with incremental increases of As(III) in the medium (Figure 3.4A). Growth rates declined as As(V) accumulated in the culture fluids (Figure 3.4B), perhaps reflecting As(V) toxicity as As(V):phosphate ratios increased or the exhaustion of another of the defined nutrients in this medium. With extended incubation (14 days), As(III) was nearly quantitatively oxidized (results not shown).

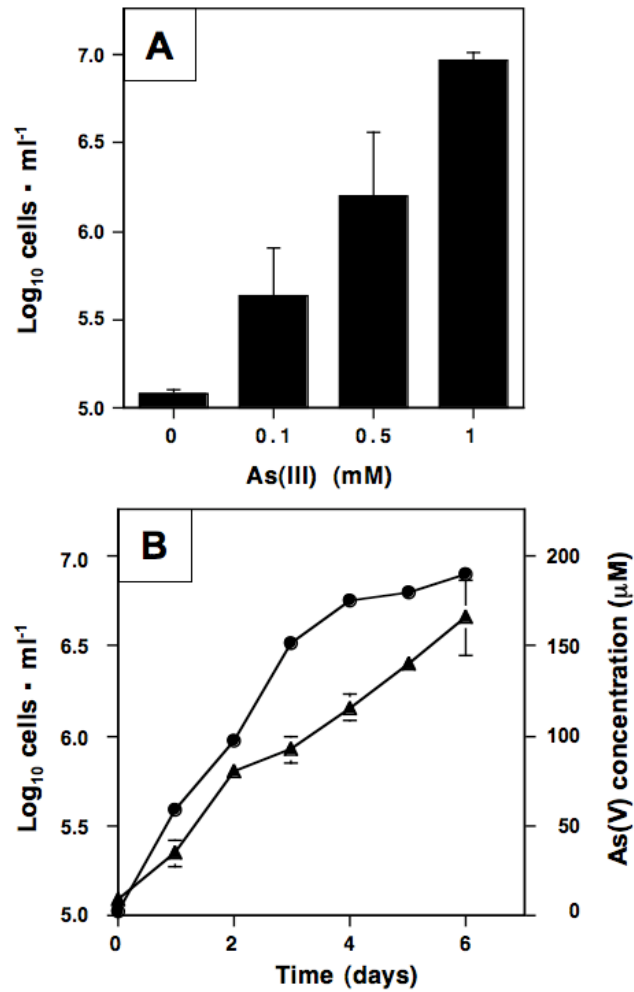


Figure 3.4. As(III) chemolithoautotrophic growth by isolate AO5. (A) Culture cell density after ten days of growth at 55°C with varying amounts of As(III) (shown as initial concentrations). Cell number recorded for 0 mM As(III) reflects starting inoculum cell density. (B) Growth of isolate AO5 (●) with 1mM As(III); (▲) As(V) accumulating in culture media during growth of isolate AO5. For both panels, data represent the mean of three replicate cultures, with error bars (where visible) representing one standard deviation.

Near full-length (1320 bp) sequence analysis of the 16S rRNA gene showed strain AO5 was 97.8% identical to *Acidicaldus organivorus* strain Y008^T, an acidothermophilic heterotrophic α -proteobacterium also isolated from YNP (23). AO5 was 99.2% identical to the above mentioned YNPFFP86 (accession AF391980), and 98.9-99.2% identical to nine distinct 16S rDNA environmental clones (GenBank Accessions: AY882679, AY882680, AY882682, AY882695, AY882794, AY882795, AY882809, DQ834208, DQ834209), retrieved from two other geographically distant YNP springs, Joseph's Coat (~32 km) and Rainbow Springs (~48 km), where As(III) oxidation has also been documented (20). Phylogenetic comparison with other α -proteobacteria showed that AO5 and *A. organivorus* share a common node distinct from other characterized α -proteobacteria (Figure 3.5).

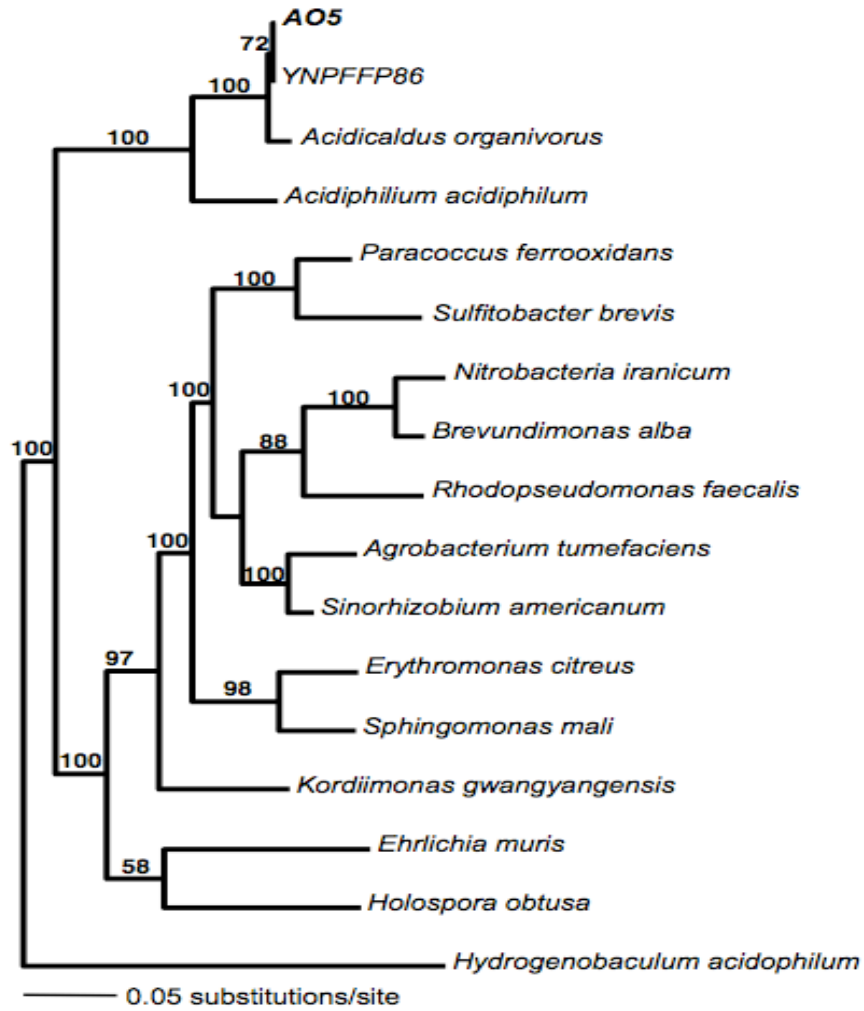


Figure 3.5. Maximum Likelihood tree showing phylogenetic placement of isolate AO5. *Hydrogenobaculum acidophilum*, a deep branching bacterium of the family *Aquificaceae*, was used as an outgroup. Numbers at nodes indicate bootstrap values for 100 pseudoreplicates.

Given the relatedness of AO5 to *Acidicaldus organivorius* strain Y008^T, AO5 was examined for growth on various carbon sources and with spring-relevant alternative electron acceptors. Best growth was observed with xylose [doubling time of ~18 h as compared to 23.4 h with As(III)], with slower growth on fructose and ethanol, and very weak growth with glucose (doubling time > 7 days). AO5 would not grow anaerobically with spring relevant electron acceptors such as SO₄²⁻, NO₃⁻, or Fe(III), regardless of whether As(III) or xylose were used as the electron donor. AO5 also failed to demonstrate growth when incubated in filter-sterilized spring water without the addition of As(III), showing that any organic carbon present in the spring water was incapable of supporting measurable growth. Previous work found dissolved organic carbon in the spring source water to be 80 μM (28).

Reaction of Strain AO5 to H₂S

In liquid cultures with a starting As(III) concentration of 30 μM and with 0-60 μM H₂S, half maximal inhibition of As(III) oxidation was modeled to occur at approximately 5.8 μM H₂S (Figure 3.6A). Additional kinetic analysis demonstrated that the H₂S inhibition was uncompetitive in nature (Figure 3.6B). However, inhibition was determined to be unique to growth on As(III), as H₂S (added at 0 to 60 μM) had no inhibitory effect on AO5 when cultured heterotrophically on xylose (Figure 3.7).

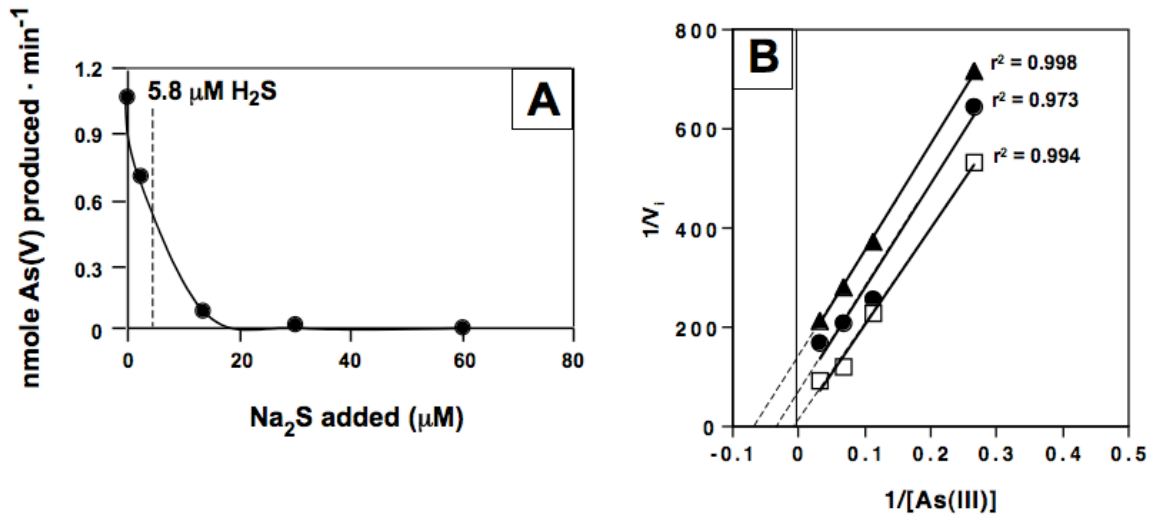


Figure 3.6. Kinetic analysis of H₂S inhibition of As(III) oxidation in isolate AO5. (A) Determination of half maximal arsenite oxidation rate as a function of H₂S concentration. (B) Lineweaver-Burk plot of H₂S inhibition of As(III) oxidation by whole cells of strain AO5. □, no Na₂S added to assays; ●, 2.01 μM Na₂S added; ▲, 5.82 μM Na₂S added. Solid lines indicate best fit linear functions of measured data, with dashed portions indicate extrapolated data. r² values for each line are given. Initial velocity (V_i) = 0.0287 μmoles As(V) produced per minute with 30 μM initial As(III) and no added Na₂S.

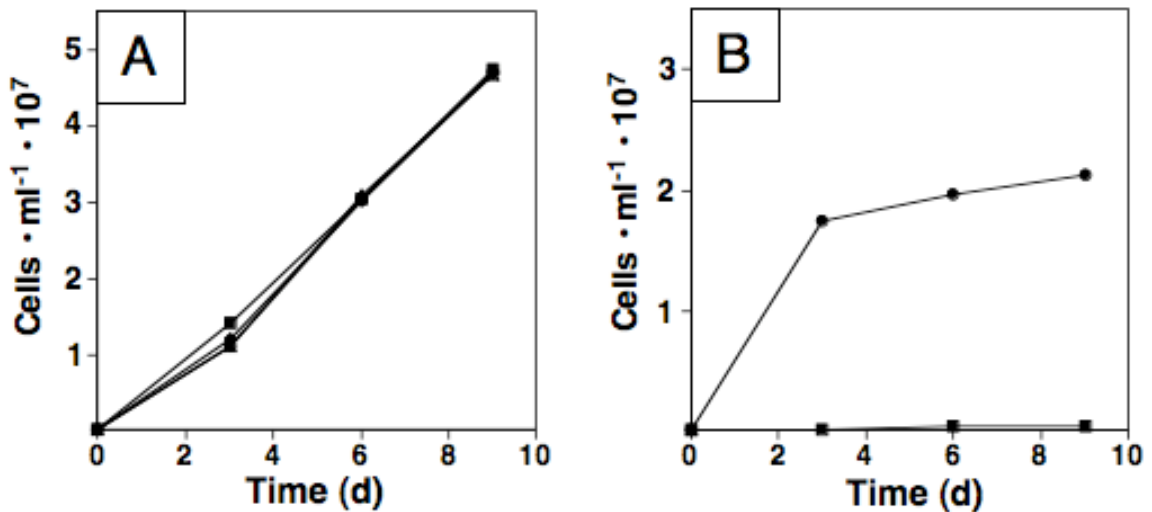


Figure 3.7. Growth of isolate AO5 in the presence of H₂S. (A) Grown on 5mM xylose. (B) Grown on 1mM As(III). ●, no Na₂S added to culture medium; ■, 1 μM H₂S in culture medium; ▲, 10 μM H₂S in culture medium; ◆, 60 μM H₂S in culture medium.

Localization of Strain AO5
Within Dragon Spring

Localization of AO5 in the spring was established by two different culture-independent approaches. First, DGGE was used to assess the microbial diversity present at outflow channel locations where chemical analysis indicated H₂S was absent (Figure 3.2B). DGGE profiles of three separate sites (A, B, and C) along the interface between the yellow S⁰ and brown band zones (see Figure 3.1) were essentially identical (Figure 3.8). Band purification and sequencing identified strain AO5 (100% sequence match) at each location (Figure 3.8).

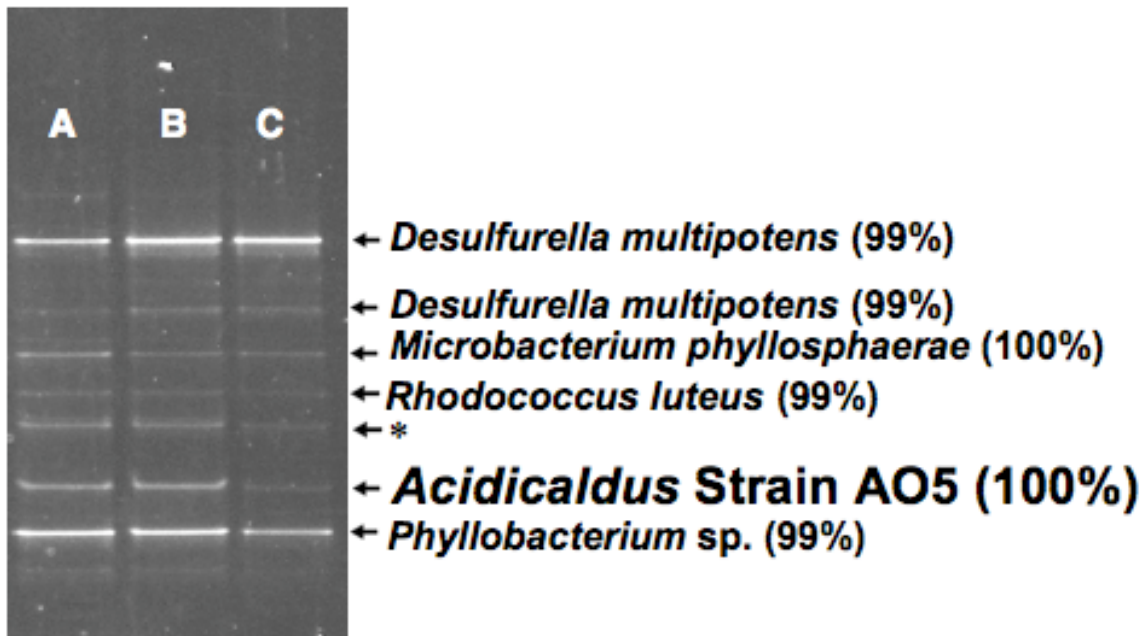


Figure 3.8. Denaturing gradient gel electrophoresis analysis of microbial diversity found at the interface between the yellow S⁰ zone and the brown band zone. Lanes A, B, and C correspond to sites A, B, and C, respectively, in Figure 3.1. BLAST identification of band sequences are shown with % identity scores. The band marked with an * was recalcitrant to isolation and sequencing.

DNA sequences representing other organisms identified in the DGGE profiles provided information that was also important for designing primers for Q-PCR, which was the second approach taken to locate AO5 in the spring. Of particular interest was the distribution of AO5 in relation to H₂S concentrations. Mat samples were taken for DNA extraction from sampling points along the outflow channel as well as perpendicular to the water flow in the channel center (5-10 cm apart). Water samples were taken at the same mat sample locations and immediately measured for H₂S content. The Q-PCR detection limit for the AO5 primers was determined to be 0.001 ng AO5 DNA in 100 ng total DNA from either sheared herring sperm DNA or from total DNA extracted from the microbial mat in the center of the yellow S⁰ zone. This corresponds to approximately 3 cells, assuming 0.39 pg DNA per cell as was estimated by the method of Hermansson and Lindgren (17), and assuming one copy of a 3.5Mb genome per cell (average for all genome sequenced α -proteobacteria), and a single rRNA operon per genome. In addition to determining the sensitivity limits of the primers, these experiments also demonstrated that lack of an AO5 Q-PCR signal in the yellow S⁰ zone was not due to technical problems with the Q-PCRs or to template bias. As approximated with Q-PCR, AO5 cell numbers were $\sim 2.5 \times 10^6$ cells \cdot g⁻¹ dry mat (Figure 3.9) where the yellow S⁰ zone transitioned to the brown band zone (Figure 3.1), and which corresponded to low or undetectable levels of H₂S (Figure 3.2B). On the basis of the threshold cycle number and as derived from standard curves, the AO5 DNA was calculated to represent roughly 18% (w/w basis; 100 ng total DNA in each Q-PCR) of the total template population at this location. The AO5 template concentration was significantly reduced ($\sim 2.5\%$ of total)

where H_2S concentrations increased above approximately $2 \mu\text{M}$ (Figure 3.9), and fell below the detection limit at roughly $15 \mu\text{M}$ H_2S .

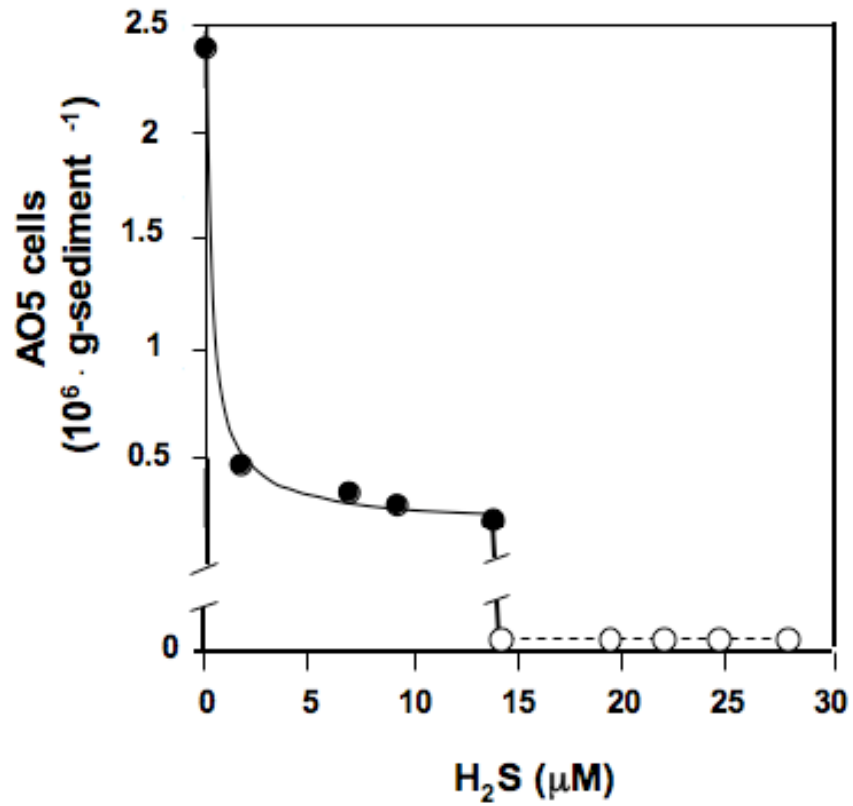


Figure 3.9. Quantitative PCR estimate of AO5 (or AO5-like organisms) in mat samples in the yellow S^0 zone in relation to dissolved H_2S . For locations where AO5 DNA was not detectable, the counts are shown as zero.

To determine whether other environmental factors might also be important in establishing AO5 distribution patterns, AO5 temperature limits were examined. AO5 was capable of chemolithotrophic growth at 50-70°C, with T_{opt} determined to be 55-60°C. The T_{opt} of AO5 corresponded to temperatures typically observed at transect sites 4 and 5 (Figure 3.1) and at the S^0 – brown zone interface, where water temperature was consistently 58°C at sites A, B, and C (Figure 3.1) measured during several sampling trips. Thus, it was concluded that while the temperature at the yellow S^0 – brown zone interface area was near optimum, water temperatures well into the yellow S^0 zone should have also allowed for near optimum growth and thus would not limit AO5 distribution.

Since AO5 characterization experiments suggested this organism would not grow without oxygen (above), dissolved O_2 measurements in the spring outflow channel were made to determine whether O_2 would limit AO5 distribution. O_2 was undetectable in the initial 100 cm of the channel center flow region (Figure 3.10), corresponding to transect sites 1, 2, and 3 (Figure 3.1). However, O_2 content increased rapidly in the more distal reaches of the yellow S^0 zone center channel and O_2 gradients perpendicular to water flow in the channel center were quite steep, suggesting that adequate electron acceptor would be available in much of the yellow S^0 zone where AO5 was not detectable by Q-PCR and from where cultivation efforts failed to isolate AO5 (or any As(III) chemolithotroph).

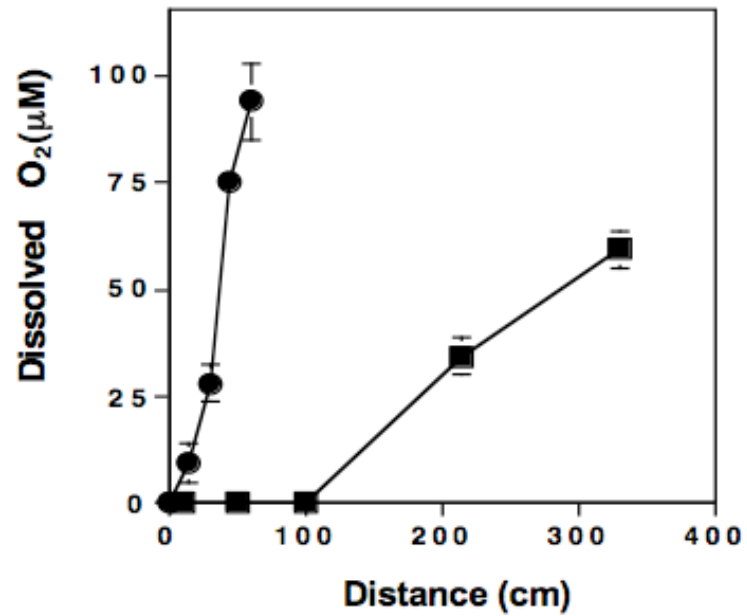


Figure 3.10. Dissolved oxygen in the Dragon Spring yellow S⁰ zone. ■, as a function of distance from the spring source; and ●, as a function of distance from the center of the flow channel with sampling increments perpendicular to the direction of spring flow at transect site 3. Symbols represent the average of three replicate samples, with error bars (where visible) representing one standard deviation.

Discussion

The study summarized herein is one of a continuing series of investigations of a geothermal spring in YNP and the microbial populations that occur along its geochemical and thermal gradients. During the course of this study, an organism was isolated that is contributing to a prominent feature of the spring biogeochemical profile and that could also be detected using culture-independent techniques. These features, along with its sensitivity to H₂S and the apparent uniqueness of its 16S rRNA gene (relative to the balance of the microbial community) suggested this organism would be an interesting model for exploring fundamental questions in microbial ecology; namely the relationship between a microbe's functional attributes and its environment. Dragon Spring is ideally suited for such autecological studies as it is characterized by superimposable chemical and physical gradients that are measurable and definable (Figure 3.1, Figure 3.2, Figure 3.9, Figure 3.10), and that correspond to distinct color changes (Figure 3.1). These gradients define a continuum of potential niche opportunities, with the H₂S gradient and its effect on As^(III) oxidation being the focal point in the present study. Presumably due primarily to off-gassing, aqueous H₂S concentrations decline rapidly as a function of distance from the spring source (Figure 3.2A), and even more rapidly in relation to distance from the center of the spring channel (Figure 3.2B). In the latter case, the spring waters are shallow and quiescent, and thus allow for rapid gas equilibration with the air.

While experimental evidence clearly demonstrated that H₂S inhibits As^(III) oxidation (Figure 3.3, Figure 3.6), the high flux of As^(III) and extremely high

concentrations of CO₂ in the source water of this spring (28; unpublished data) nevertheless provide otherwise ideal conditions for the proliferation of As(III) chemolithoautotrophs. The *Acidicaldus*-like organism characterized in the current study constitutes the first detailed description of thermophilic As(III) chemolithoautotroph. Phylogenetically, AO5 is closely related to the heterotrophic α -proteobacterium *A. organivorus* and could also grow on some of the same carbon compounds. However, *A. organivorus* Y008^T was reported to be capable of anaerobic growth with Fe(III) as an electron acceptor, but incapable of As(III) oxidation (23). Along with differences in 16S rRNA gene sequence, these physiologic features distinguish isolate AO5 from *A. organivorus* Y008^T. Additional characterization would be required to assess whether AO5 represents a new species of *Acidicaldus*.

The environmental features of the spring correlate well with the properties of AO5 and its distribution within the spring outflow channel. DGGE analysis (Figure 3.8), Q-PCR determinations (Figure 3.9), and enrichment and isolation success document the presence and numerical dominance of AO5 at the interface of the yellow and brown band zones (Figure 3.1). H₂S is depleted at this location (Figure 3.2B), which agrees with the H₂S sensitivity of As(III) oxidation in this organism and suggests that H₂S is a major niche determinant for AO5. Conversely, the T_{opt} and O₂ requirement of this organism were less predictive as the temperature gradients (Figure 3.1) and aqueous O₂ measurements (Figure 3.10) inferred that AO5 could thrive in a significant portion of the yellow S⁰ zone. The microgeographical boundaries of the AO5 niche along the gradients may be very focused. The 16S rDNA signature of this organism was not encountered in

a previous molecular survey of this spring (22) that sampled regions of the yellow S⁰ zone and the middle of the brown band zone but not the interface between these two major gradient zones.

Sulfide inhibition of other microbial processes has also been documented. This includes nitrate reduction by *Desulfovibrio desulfuricans* (8), nitric and nitrous oxide reduction by *Pseudomonas fluorescens* (43), oxygenic photosynthesis in cyanobacteria (35), and nitrification in estuarine sediments (25). Sulfide has also been shown to apparently influence microbial species composition in hot springs in Iceland (41), in marine coastal systems (16), and in salt marsh sediments (9). Miller and Bebout (30) also showed that sulfide tolerance among different cyanobacteria was positively correlated with sulfide levels in the environments from which they were isolated. The current autecology study extends beyond these general observations to more specifically quantify sulfide influence on a microbial process in nature, combining in situ and culture-based experiments to demonstrate the importance of such environmental constraints on function and how sulfide apparently correlates very closely with the distribution of the microorganism studied.

The H₂S sensitivity of As^(III) oxidation in isolate AO5 was similar to our observations with a *Hydrogenobaculum*-like isolate (10), and prompted us to examine an *Agrobacterium tumefaciens* soil isolate that has been used as a model organism for studying the genetics and physiology underlying As^(III) oxidation (26, 27). Sulfide inhibited As^(III) oxidation in this bacterium as well (results not shown), suggesting that sulfide inhibition of As^(III) oxidation may be common among As^(III)-oxidizing

microorganisms. However, there may be exceptions. For example, the facultative As(III) chemolithotroph isolated by Oremland *et al.* (34) was enriched from the anaerobic bottom waters of Mono Lake where sulfide levels exceed 1 mM (33). Thus, either sulfide is not an inhibitor of As(III) oxidation in that organism, or it may use As(III) as an energy source in regions of the Mono Lake water column where sulfide is absent or at least at lower concentrations. Another example comes from YNP itself, where Inskeep *et al.* (20) reported increasing As(V):As(III) ratios occurring in circumneutral geothermal springs containing 30-50 μ M sulfide.

While previous chemical analysis of solid phases associated with the yellow S⁰ zone mat material failed to detect As-S solid phases (28), the studies of Wilkin *et al.* (47) suggest the potential for thioarsenite complexes to occur in Dragon Spring. The consequences for enzymatic As(III) oxidation are unknown, and therefore As:S complexation cannot be ruled out as a potential factor influencing or controlling microbial As(III) oxidation *in situ*. However, the results of the kinetic analyses suggests such As:S complexes may be of little significance. The periplasmic location of the As(III) oxidase enzyme (4, 40) provides the opportunity to examine the impact of inhibitors using whole cells as opposed to purified enzyme preparations. The precision of enzyme catalysis rates would be influenced by the requirement for the substrate [As(III)] and product [As(V)] to diffuse in/out of the periplasm; however, assays with whole cells would nevertheless still provide an opportunity to gather evidence as to the mechanism of inhibition. As(III) oxidation was reduced by roughly 50% with an As(III):H₂S ratio of approximately 5:1, and nearly completely inhibited at a As(III):H₂S ratio of 2.0 (Figure

3.6A). The K_m for As(III) under these conditions was estimated to be roughly 0.109 μM (extracted from the Lineweaver-Burke plots), and thus in at least the culture-based assays, $\text{H}_2\text{S}:\text{As(III)}$ complexing should be of little consequence as there still should have been As(III) available for oxidation at near maximum rates. Therefore, the outcomes of the kinetic analysis (i.e. uncompetitive inhibition, Figure 3.6B) are more indicative of the true basis of H_2S inhibition. The complete lack of H_2S inhibition of AO5 heterotrophic growth is also probably informative in that it implies that if AO5 were growing heterotrophically on organic carbon in situ, then its distribution would not be expected to correspond so closely with H_2S concentration. Furthermore, it would also imply that the inhibitory effect does not derive from H_2S binding to iron centers of cytochromes in electron transport (12).

Finally, the ex situ in-field assays yielded important information on two separate issues. First, by extending the As(III) oxidation assay incubations beyond the time frame employed by Langner et al. (28), As(V) formation coinciding with the disappearance of H_2S (Figure 3.3) became apparent, and thus illustrated agreement between in situ- and in vitro-based observations. Second, these assays also generated data that suggested constitutive expression of the As(III) oxidase enzyme(s) in situ. Microbial mat As(III) oxidation was observed in the earliest measurement (10 min.) with mat samples incubated in spring water that contained undetectable H_2S (Figure 3.3). The occurrence of measurable As(V) within such short time frames suggest that As(III) oxidation observed with mat organisms inhabiting the high sulfide zone was not due to gene induction and de

novo As(III) oxidase enzyme synthesis. Rather, the As(III) oxidase(s) appear to be present in the yellow S⁰ zone mat, with the removal of sulfide resulting in rapid reactivation.

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CHAPTER 4

A COMPARISON OF H₂ AND H₂S AS ENERGY SOURCES FOR PRIMARY
PRODUCTION IN GEOTHERMAL SPRINGSAbstract

Geothermal waters contain numerous potential electron donors capable of supporting chemolithotrophic growth. Thermodynamic predictions of energy yields for specific electron donor and acceptor pairs in such systems are available, although direct assessment of these predictions are rare. This study assessed the relative importance of dissolved H₂ and H₂S as energy sources for support of chemolithotrophic growth in an acidic geothermal spring in Yellowstone National Park. H₂S and H₂ concentration gradients were observed in the outflow channel, and vertical H₂S and O₂ gradients were evident within the microbial mat. H₂S levels and microbial consumption rates were approximately three orders of magnitude greater than H₂. *Hydrogenobaculum*-like organisms dominated the bacterial component of the microbial community and isolates representing three distinct 16S rRNA gene phylotypes (phylotype = 100% identity) were isolated and characterized. Within-phylotype, O₂ requirement varied as did energy source utilization; some isolates could grow with H₂S, some only H₂, while others could utilize either as an energy source. These metabolic phenotypes were consistent with *in situ* geochemical conditions measured using aqueous chemical analysis, and in-field measurements using gas chromatography and microelectrodes. Culture experiments with an isolate that could utilize H₂S and H₂ and that represented the dominant phylotype

(70% of the *Hydrogenobaculum* PCR clones) found that H₂S and H₂ were used simultaneously, without evidence of induction or catabolite repression, and at rates relatively comparable to those measured in the *ex situ* field assays. Under in situ relevant concentrations, growth of this isolate with H₂S was better than H₂. Major conclusions drawn from this study are that phylogeny cannot be relied upon to predict physiology, and that H₂S can dominate H₂ as an energy source in terms of availability, apparent in situ consumption rates, and growth-supporting energy.

Introduction

Thermophiles dominate the deepest and shortest branches of the *Bacteria* and *Archaea* domains in global phylogenetic trees, suggesting they are likely ancestors to Earth's contemporary microbial populations (8, 44). Consequently, these organisms have attracted considerable attention due to interest in the origin of enzymes and metabolic pathways that are thought to have evolved from such organisms. Chemolithotrophic metabolism, in particular, is foundational to primary productivity in environments where temperatures exceed the limit of photosynthesis. Bioenergetics of geothermal systems has been extensively examined from the perspective of theoretical energy yield as a way of discussing the relative importance of the various electron donors and acceptors that could support chemolithoautotrophy (3-5, 27). Other studies have sought to link the inferred physiology of microbial populations in specific environments with the predicted energy yields obtainable from the inorganic constituents present (4, 21, 32, 34, 42).

Geothermal features will vary considerably with respect to temperature, pH, and the presence and concentrations of energy sources such as H_2 , H_2S , S^0 , $Fe(II)$ and $As(III)$ (23, 37). Acid-sulfate-chloride (ASC) springs (e.g. Figure 4.1) are common throughout the Yellowstone geothermal complex, although are considerably more concentrated in and around the Norris Geyser Basin area, which is located on the northeast edge of the Yellowstone caldera boundary (35, 51). ASC springs are characterized by mM concentrations of Na^+ , Cl^+ , SO_4^{2-} , and acidic pH at the point of discharge (23, 26, 52), and are intriguing from a bioenergetics standpoint because they offer a virtual buffet of energy sources for chemolithoautotrophs, including a constant flux of μM concentrations of dissolved H_2S , $Fe(II)$ and $As(III)$, nM concentrations of H_2 , and conspicuous amounts of S^0 . (11, 22, 26, 32, 46). Discussions regarding the relative importance of these electron donors for supporting primary production in such systems sometimes center on comparisons of potential energy released from their oxidation, with the H_2 / O_2 couple perhaps being favored (46), whereas others urge caution in making such predictions, pointing out that in low pH systems the oxidation of H_2S could yield nearly as much energy (21).

ASC springs clearly are in the latter category and are the focus of the current study, where we examined microbial utilization of H_2S and H_2 using a combination of in-field ex situ assays, microelectrodes, and gas chromatography to document the presence, concentrations, gradients, and consumption of these energy sources. These analyses were combined with molecular community analysis, and with cultivation techniques that isolated and characterized ecologically relevant organisms that represent the dominant

phylotypes. These experiments can be summarized by two important conclusions: 1) microbial phylogeny cannot necessarily be relied upon to predict physiology, and 2) H_2S can dominate H_2 in terms of availability, apparent consumption rates, and growth-supporting energy.

Materials and Methods

Study Site

Field experiments were conducted at Dragon Spring ($44^{\circ}43'54.8''$ N, $110^{\circ}42'39.9''$ W, spring number NHSP106 in the YNP thermal inventory) located in Norris Geyser Basin, Yellowstone National Park (Figure 4.1).

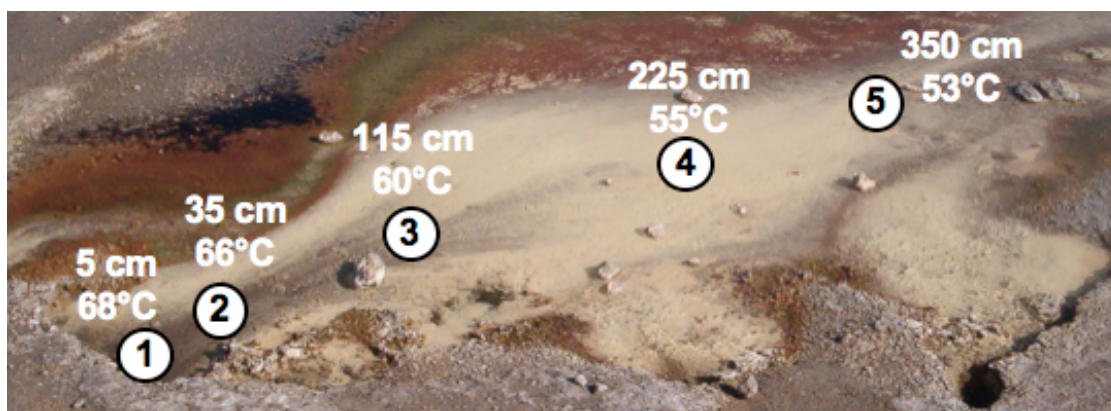


Figure. 4.1. Color image of Dragon Spring in Norris Geyser Basin. The picture depicts changes in the spring structure relative to previous studies (Langner et al. 2001; Jackson et al. 2001; D'Imperio et al. 2007), including additional sources emerging downstream from the main source. Sampling transect sites are numbered and shown at their approximate distance from the spring source. Representative temperatures occurring during most of the current study reflect the thermal gradients.

In situ Chemical Analysis

Water temperature and pH were routinely monitored throughout the two-year study. The pH of the source water ranged from 2.8-3.1 and temperature ranged from 68-72°C.

Dissolved H₂S was measured using the methylene blue method (9). Spectrophotometric analysis was performed at spring-side using a USB2000 portable spectrophotometer (Ocean Optics, Dunedin, FL). H₂ was determined using a portable Varian gas chromatograph (model CP2900) using Ar and N₂ as carrier gases. H₂(aq) concentrations in each sample were determined using the headspace gas chromatography method and temperature-corrected Henry's Law constants described previously (21).

Ex situ Measurements of H₂S and H₂ Consumption Activity

Mat samples were collected using sterile wide-bore pipette tips and placed into sterile 15ml conical tubes, homogenized by mixing, and split into two sub-samples. One sub-sample was maintained at *in situ* temperature and was used to assay for microbial H₂ or H₂S consumption, while the other was boiled for 20 minutes to serve as a killed control. Sub-samples of live and heat-killed mat material were aseptically transferred into 150 ml serum bottles containing 40 ml of filter-sterilized spring water. Assay bottles were immediately closed with rubber septa, pressure sealed with an aluminum ring, and incubated at *in situ* temperatures by placing the bottles in the spring at the same location from which the mat material was sampled. Water samples were withdrawn at 2 min intervals to assay for H₂S(aq) concentration, whereas headspace gas samples were taken to measure H₂. Dissolved H₂S and H₂ data were normalized based on mat sample dry weight, which was determined by collecting all mat material from each serum bottle onto

pre-weighed 0.2 μm filters, drying overnight at 65°C and then weighing to determine the mat dry weight. Maximum mat sample dry weight variation between samples was 4.1%

Cultivation and Isolation

Microbial mat samples were collected from sites 35, 115, and 350 cm from the point of spring discharge using sterile wide bore pipette tips. The mat material was stored for transportation in 15 ml conical tubes and maintained at 65°C in a Thermos™ bottle filled with site water. Mat samples were resuspended in 10 ml spring water collected directly above the mat sampling site and 100 μl of this suspension was used to inoculate 16 ml serum bottles sealed with Teflon-coated butyl rubber septa (National Scientific, Rockwood, TN). For aerobic H_2S chemolithotroph enrichments, the serum bottles contained 4.9 ml of filter sterilized spring water buffered to pH 3.1 with 5mM citric acid, 200 μM Na_2S , and with a headspace containing 50% ambient air 50% CO_2 headspace for aerobic culture conditions, or 90% CO_2 and 10% ambient air for microaerobic conditions. For aerobic H_2 chemolithotroph enrichments, serum bottle set-up was the same except Na_2S was omitted and headspace gas composition consisted of 50% CO_2 , 25% H_2 , and 25% ambient air. Microaerobic H_2 enrichments were the same except the headspace composition was 50% CO_2 , 40% H_2 , and 10% ambient air. Enrichment cultures were incubated at temperatures ranging from 55-70°C corresponding to *in situ* temperature where the mat sample inoculum was collected. Every 30 days, 100 μl of culture was transferred to similarly prepared serum bottles and enrichment cultures were monitored for growth by phase-contrast microscopy and progress towards a clonal culture was monitored using denaturing gradient gel electrophoresis (DGGE, described below). After

120 days, cultures were diluted to $\sim 2 \text{ cells} \cdot \text{ml}^{-1}$ and 500 μl of this dilution was used as inocula for 20 similarly prepared serum bottles for each enrichment showing growth (theoretical final cell density of 1 cell per bottle). These bottles were monitored for growth after 100 days as above and positive cultures were again diluted and used as inocula for a second round of dilution-to-extinction enrichment. The resulting cultures were considered clonal based on single DGGE bands for each.

DNA Extraction, PCR, DGGE, and Clone Libraries

Total DNA was extracted from enrichment cultures and isolates using the method of Pitcher *et al.* (39). Near full-length 16S rRNA genes were amplified using PCR primers and protocols we have previously described (12, 24). Sequencing for these clones as well as all other clones (below) was carried out at the Ohio State University Plant Microbe Genomics Facility. DGGE analysis of enrichment cultures was also carried out as previously described (12). Internally Transcribed Spacer (ITS) regions were amplified using the primers and cycling conditions described in Ferrera *et al* (15).

Mat 16S rDNA clone libraries were constructed from DNA extracted (6) from microbial mat taken from the Dragon Spring S^0 deposition zone (yellow area in Figure 4.1) at sites 35 and 115 cm from the point of discharge. Near full-length 16S rRNA genes were amplified as described above, cloned into pCR2.1 (Invitrogen, Carlsbad, CA), with 45 clones from each sampling site full-length sequenced. Sequences were aligned using ClustalX (49) and edited and trimmed using Se-A1 v2.0a11 (<http://evolve.zoo.ox.ac.uk>). Phylogenetic analysis of the aligned sequences was performed using the Maximum

Likelihood algorithm and bootstrap values for 100 pseudoreplicates were generated using the PAUP* v4.0b10 software package (48). *Hydrogenothermus marinus* (GenBank accession AJ292525.1), a member of family *Aquificaceae*, was used as the outgroup.

Culture-Based Studies

An isolate representative of the major 16S rRNA gene phylotype recovered from the spring was used for studies that compared growth on H₂S and H₂. Serum bottles were set-up as described above, with cell growth rates on these substrates determined by phase-contrast microscopy cell counts. H₂S and H₂ consumption rates were determined by pre-growing separate cultures on either H₂S or H₂ to acclimate the cells to a single energy source, and then 2x10⁶ cells from late log phase cultures were transferred to 10 ml sealed serum bottles containing 5ml of filter-sterilized, de-gassed, spring water buffered at pH 3.0 with 5mM citric acid, and containing H₂S [added as 30 μM NaS₂ (aq)] and H₂ [provided at 30 nM (aq) by amending the headspace to 0.1% H₂ and allowing bottles to equilibrate at 55 °C in the laboratory with periodic shaking for 24 hours], which are spring-relevant concentrations. H₂ consumption was immediately measured for a t=0 sample, and then subsequently at 20 min intervals, with the samples analyzed on a Shimadzu GC-8A ECD with a Stainless Steel column (Shimadzu Scientific Instruments Inc., Columbia, MD). 500 μl samples were removed at 15 min intervals for dissolved H₂S analysis as described above. Killed controls were obtained by autoclaving cultures at 121°C for 45 min.

Production of sulfate by isolate 3684 was monitored by growing 10 ml of cells under standard conditions to 5 • 10⁵ cells ml⁻¹ then concentrating the cells by

centrifugation at 5000xg for 5 min. Cells were resuspended in 100 μ l of a synthetic medium (10mM $(\text{NH}_4)_2\text{PO}_4$; 0.2mM KH_2PO_4 ; 2mM NaCl; 1mM MgCl; 0.5mM CaCl_2 ; 50 μ M $(\text{NH}_4)_2\text{SO}_4$; 1.2 μ M FeCl_3 ; 0.5 ml Nitsch's trace element solution (2); 5mM Citric Acid, pH 3.0) and used to inoculate 59 ml of the same medium. Na_2S was added to a final concentration of 200 μ M and 5.5ml samples were removed at 12h intervals, measured for H_2S utilization as described above, with 5.0 ml filtered through a 0.2 μ m PTFE filter and measured for SO_4 using a Dionex ion chromatograph.

In-field Microelectrode Assays

Electrochemical field analyses of H_2S was accomplished using a field portable DLK-60 potentiostat (Analytical Instrument Systems, Inc.) controlled by a laptop computer using Analytical Instrument Systems, Inc. software. A glass Au-amalgam solid state working electrode (5mm glass tube drawn to approximately a 500 μ m diameter tip contained a 100 μ m diameter Au-amalgam electrode surface), a platinum wire counter electrode, and a Ag/AgCl reference electrode were used for analysis, constructed after Brendel and Luther (7). Peak assignments for the H_2S signal was made after Lorenson (29) and calibrated using the same electrodes in the labs at the University of Vermont with freshly prepared standards (19). The glass Au-amalgam working electrode was inserted vertically through the water column and into the fluffy yellow mat material using a micromanipulator for spatial positioning. At each position, at least 10 replicate cyclic voltammetry scans were made at 1000 mV/sec with an initial holding potential of -0.1 V between -0.1 and -1.8 V (vs. Ag/AgCl).

Vertical measurements of O₂ were carried out with a Clark-type microsensor (40) connected to a picoammeter (Unisense A/S, Denmark). The O₂ microsensor had a tip diameter of 10 μm, a stirring sensitivity of 1%, and a response time, t_{90} , of 0.4 s. The O₂ microsensor was linearly calibrated in the experimental setup by a two-point calibration using readings of microsensor current in spring water that was air saturated at *in situ* temperature and pressure via a handheld pump (100% air saturation) and in the anoxic part of the mats (0% O₂). Dissolved O₂ concentrations of air saturated spring water at experimental temperatures were calculated according to García and Gordon (17). The electrode was mounted on a micromanipulator for precise movement into and through the microbial mat.

SEM/EDX

Cultures were grown under the conditions described above and the yellow precipitate that is common to these cultures was removed and separated from culture media by centrifugation at 13,000 x g for 5 min then dried under vacuum. The resulting material was applied to carbon tape on a sample stage and analyzed on a JEOL model 6100 scanning electron microscope (JEOL, Tokyo, Japan) equipped with an x-ray detector. The sample was subjected to 20 keV incident energy and measurements were made over a 50 s interval.

Results

Spring Chemistry

In a preceding study in this spring, we examined geochemical constraints on the population distribution of an As(III) chemolithotroph (11), where H₂S gradients were mapped and showed that measurable H₂S was primarily found in a yellow solid phase zone (Figure 4.1) that is a microbial mat comprised of filamentous microorganisms (primarily *Hydrogenobaculum* (24)) interwoven with a mineral phase made of up S⁰ (26). Aqueous H₂S at the point of spring discharge was found to be 75-80 μM, declined to 5-10 μM at a distance of 3.5 m in the outflow channel (transect position 5 in Figure 4.1), and then was below detection at approximately 5 m (11). In that study, O₂ was measured to determine its availability as an electron acceptor. O₂ gradients were essentially opposite to that of H₂S; i.e. aqueous O₂ was undetectable in the first 100 cm in the outflow channel, but then increased rapidly thereon.

The study described herein occurred approximately 18 months subsequent and H₂S levels were checked to verify the same gradient patterns were still present (results not shown). In addition, to better understand potentially important geochemical gradients that may be of value for understanding in situ microbial metabolism and for designing ecologically relevant cultivation conditions, microelectrodes were used to examine H₂S and O₂ concentrations in the vertical dimension (Figure 4.2). At a location corresponding to transect site 2 (Figure 4.1), H₂S concentrations in the water and within the upper 1 mm of mat appeared to remain near constant at ~ 50μM, but then decreased sharply reaching

the detection limit of the microelectrode within ~ 2.5 mm (Figure 4.2A). Vertical O_2 profiles were similarly steep, with O_2 levels being saturated 4 mm above the mat surface and rapidly changing to anaerobic conditions 2-3mm inside the mat (Figure 4.2B). Dissolved H_2 concentrations at the spring source were ~ 13 nM, but decreased steadily for approximately 50cm at which point H_2 increased briefly due to mixing with geothermal water from a second, slightly downstream, source (Figure 4.1), but then again declined with distance (Figure 4.3), presumably due to off-gassing and or microbial consumption.

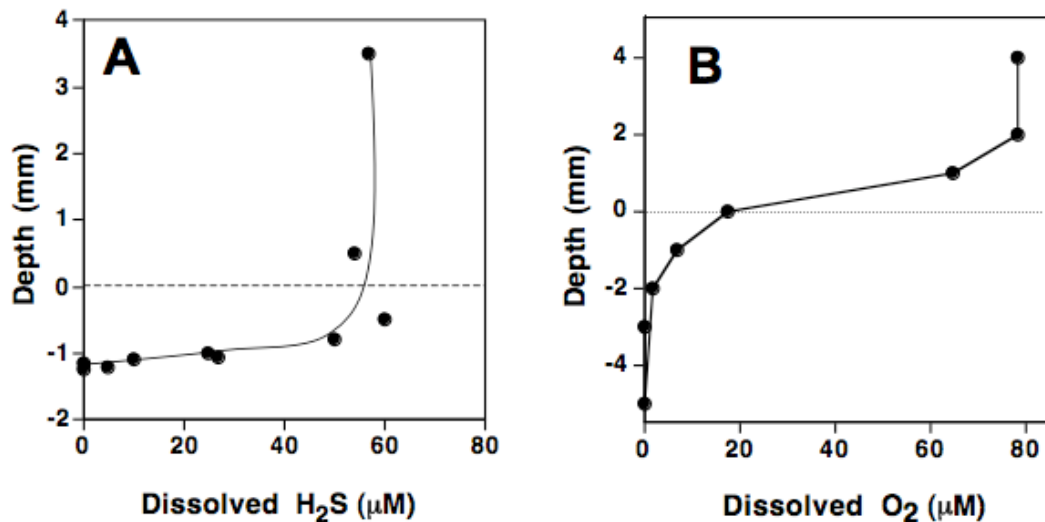


Figure. 4.2. Vertical H_2S and O_2 gradients in Dragon Spring microbial mats. (A) Cyclic voltammetry was used to estimate H_2S concentrations in the center of the outflow channel approximately 100 cm downstream of the spring source. (B) Vertical profile of dissolved O_2 at a point 100 cm downstream of the spring source and 60 cm from the center of the outflow channel. Dashed line represents the approximate top of the microbial mat.

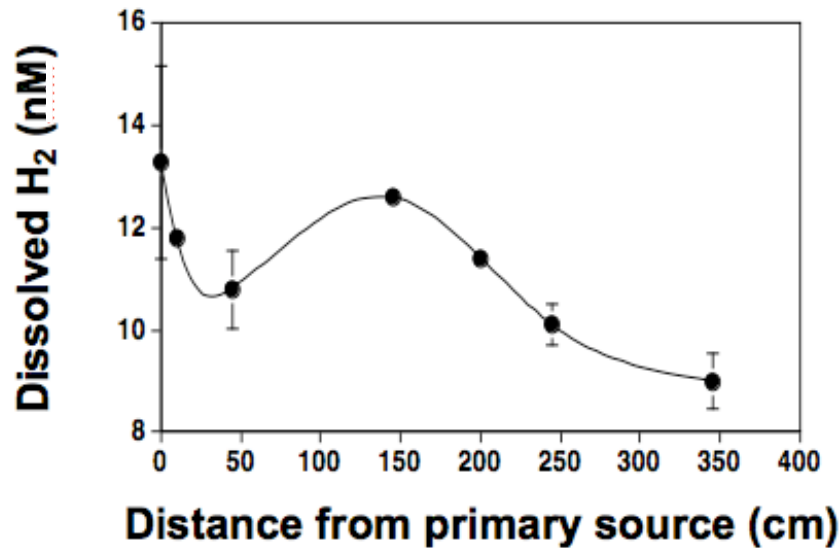


Figure 4.3. Aqueous H₂ concentrations measured along the center of the outflow channel. The increase at 150cm corresponds to input from a second spring source that contributes to the Dragon Spring outflow channel at approximately 140cm. Data points indicate the mean of three samples at each location. Error bars represent one standard deviation of the mean.

Ex situ Assays

H₂S and H₂ consumption rates were studied to determine their relative importance as electron donors to the microorganisms inhabiting the S⁰ deposition zone. H₂S loss (i.e. apparent consumption) from the sealed assay container proceeded at rates from 35-50 $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{g dry mat material}$, whereas H₂ depletion rates were approximately three orders of magnitude lower, 1-4 $\text{nmoles} \cdot \text{min}^{-1} \cdot \text{g dry mat material}$ (Figures 4.4 and 4.5). In ex situ assays using heat-treated mat samples, apparent H₂ and H₂S consumption was negligible or absent. Incomplete heat killing cannot be ruled out, although much slower abiotic H₂S oxidation likely also occurred due to the presence of oxygen unavoidably introduced during sample preparation at spring side.

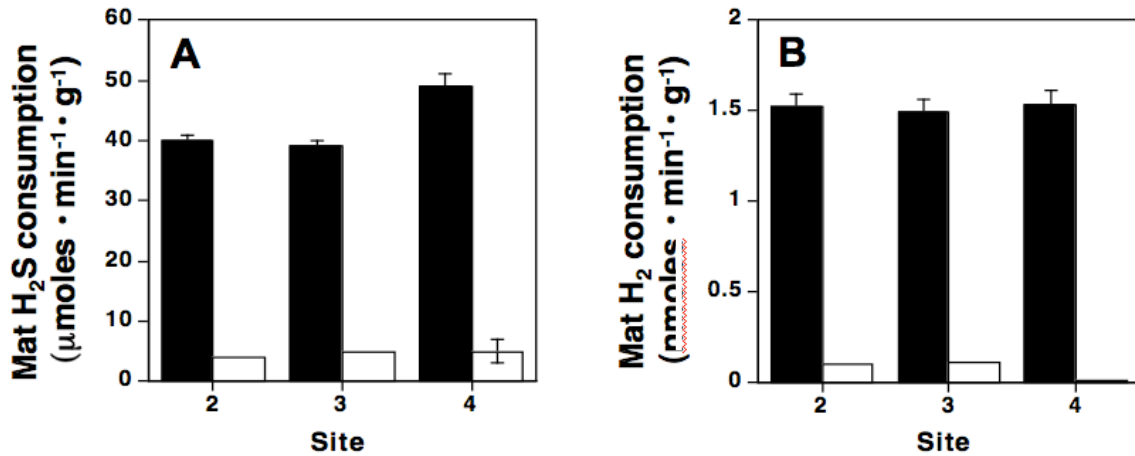


Figure 4.4. Ex situ assays of apparent H₂S and H₂ consumption by whole mat samples. (A) H₂S and (B) H₂. Solid bars represent the activity of untreated mat maintained at in situ temperatures. Open bars represent assays with heat-killed mat material. Site numbers refer to three discrete mat sampling locations in the center of the Dragon Spring outflow channel with respect to distance from the spring source: 2 = 35cm, 3 = 115cm, 4 = 225cm. Error bars represent one standard deviation of the mean from three replicate assays.

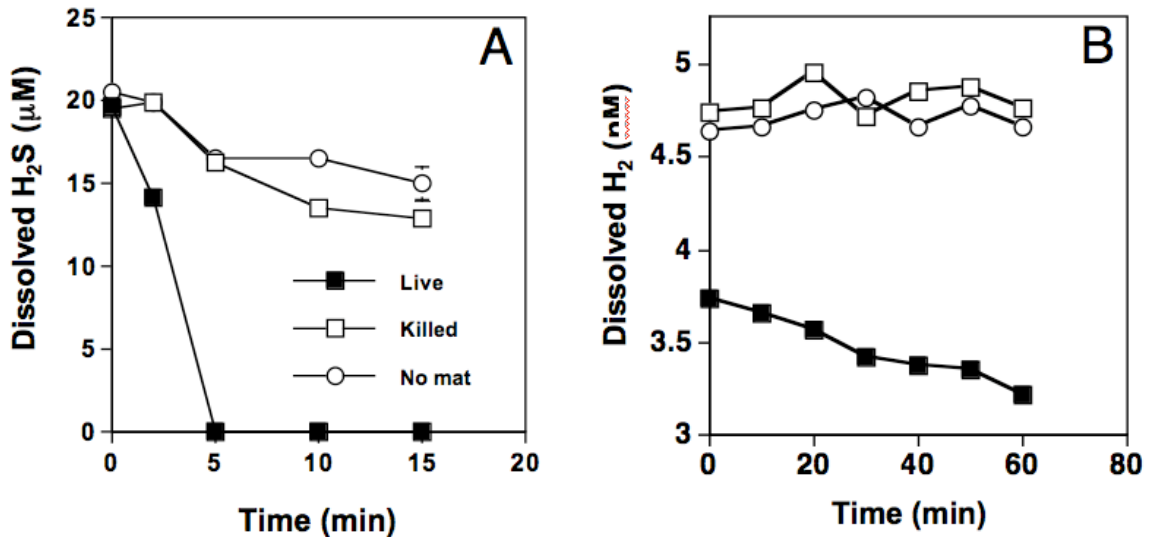


Figure 4.5. Examples of ex situ assays showing apparent H₂S (A) and H₂ (B) consumption by spring mat samples taken from site 3 (115 cm downstream of the spring source). (■) Live mat sample added to assay vial; (●) Heat-killed mat sample added to assay vial; (○) No mat included in assay. Symbols indicate the mean of triplicate samples. Error bars represent one standard deviation of the mean.

Phylogenetic Analysis

A previous phylogenetic survey of this spring that included S⁰ mat material from a position equivalent to site 5 (Figure 4.1) found *Hydrogenobaculum*-like signatures to dominate the 16S rDNA clone libraries (24). To better understand the microbial community structure in the regions of the spring where H₂S and H₂ consumption were being studied in the current study, additional 16S rRNA gene clones were amplified from mat DNA extracted from transect sites 2 and 4 within the central flow channel of the S⁰ deposition zone (Figure 4.1). Of the 90 near full-length clones sequenced, 94% represented *Hydrogenobaculum*-like organisms (99% identity to *Hydrogenobaculum* sp. Y04AAS1, accession AM259504 falling into two distinct phylotypes; phylotype I comprised 71% of the *Hydrogenobaculum*-like clones, with the remainder phylotype II.

Culturing and Isolation

Filter-sterilized spring water was used as a basal medium in cultivation experiments aimed at isolating H₂S and H₂ chemolithotrophs from the mat material exhibiting H₂S and H₂ consumption. The O₂ electrode experiments (Figure 4.2B) indicated a continuum of aerobic, microaerobic and anaerobic environments, although only aerobic and microaerobic enrichments resulted in growth. Following several subcultures, clonal cultures were derived from positive enrichments by three rounds of dilution to a theoretical 1/2 cell · ml⁻¹. Denaturing gradient gel electrophoresis (DGGE) analysis was used to monitor isolation progress throughout; upon continued sub-culture DGGE profiles became progressively less complex, reducing to a single band in each of

late stage enrichment subcultures and remained as such following the dilution to extinction subcultures. A total of 30 isolates were obtained that were phylogenetically very similar to *H. acidophilum* (99% identity) and that formed three distinct 16S phylotypes (Figure 4.6). Isolate phylotypes I and II were identical to the above mentioned phylotypes amplified from total mat DNA, whereas the 16S rRNA signature of phylotype III isolates was novel relative to total community DNA amplicons. Within each phylotype, all isolates were identical with respect to 16S rRNA gene sequences and the 350bp intergenic transcribed sequences (ITS). In addition, cell morphology was similar for all phylotypes; rounded rods, approximately 5µm in length occurring singly or as two cells presumably in the process of division. However, within each phylotype, the isolates displayed widely varying growth phenotypes. Some isolates would only grow on H₂S, some only on H₂, whereas others could grow with either energy source (Table 4.1). Further, some were found to grow best under microaerobic conditions, whereas others preferred fully aerobic conditions. Phylotype II contained isolates having all combinations of H₂S/H₂/O₂ utilization/requirement patterns. An aerobic isolate (3684) capable of growth on either electron donor was maintained on sulfide for a period of 2 years encompassing 32 culture transfers in the absence of H₂. Following this period of extended dilution and culturing, isolate 3684 retained its ability to grow autotrophically on H₂.

Phylotype	# of Isolates	Electron Donor/Acceptor Pair					
		$\text{H}_2\text{S}/\text{O}_2$	$\text{H}_2\text{S}/\mu\text{O}_2$	H_2/O_2	$\text{H}_2/\mu\text{O}_2$	$\text{H}_2\text{S}/\text{H}_2/\text{O}_2$	$\text{H}_2\text{S}/\text{H}_2/\mu\text{O}_2$
I	9	3	0	1	1	4	0
II	18	9	6	0	0	3	0
III	3	2	0	0	0	1	0
Total:	30	14	6	1	1	8	0

Table 4.1. Physiological differences of *Hydrogenobaculum* isolates comprising three distinct 16S phylotypes. Isolates listed under an individual electron donor (H_2 or H_2S) were incapable of growth using the other substrate while those listed under “ $\text{H}_2/\text{H}_2\text{S}$ ” were capable of growth using either electron donor. Where the electron acceptor is listed as “ O_2 ” isolates exhibited fastest growth when the headspace contained 5.5% O_2 . Isolates listed under “ μO_2 ” were incapable of growth when the headspace contained $>2\%$ O_2 .

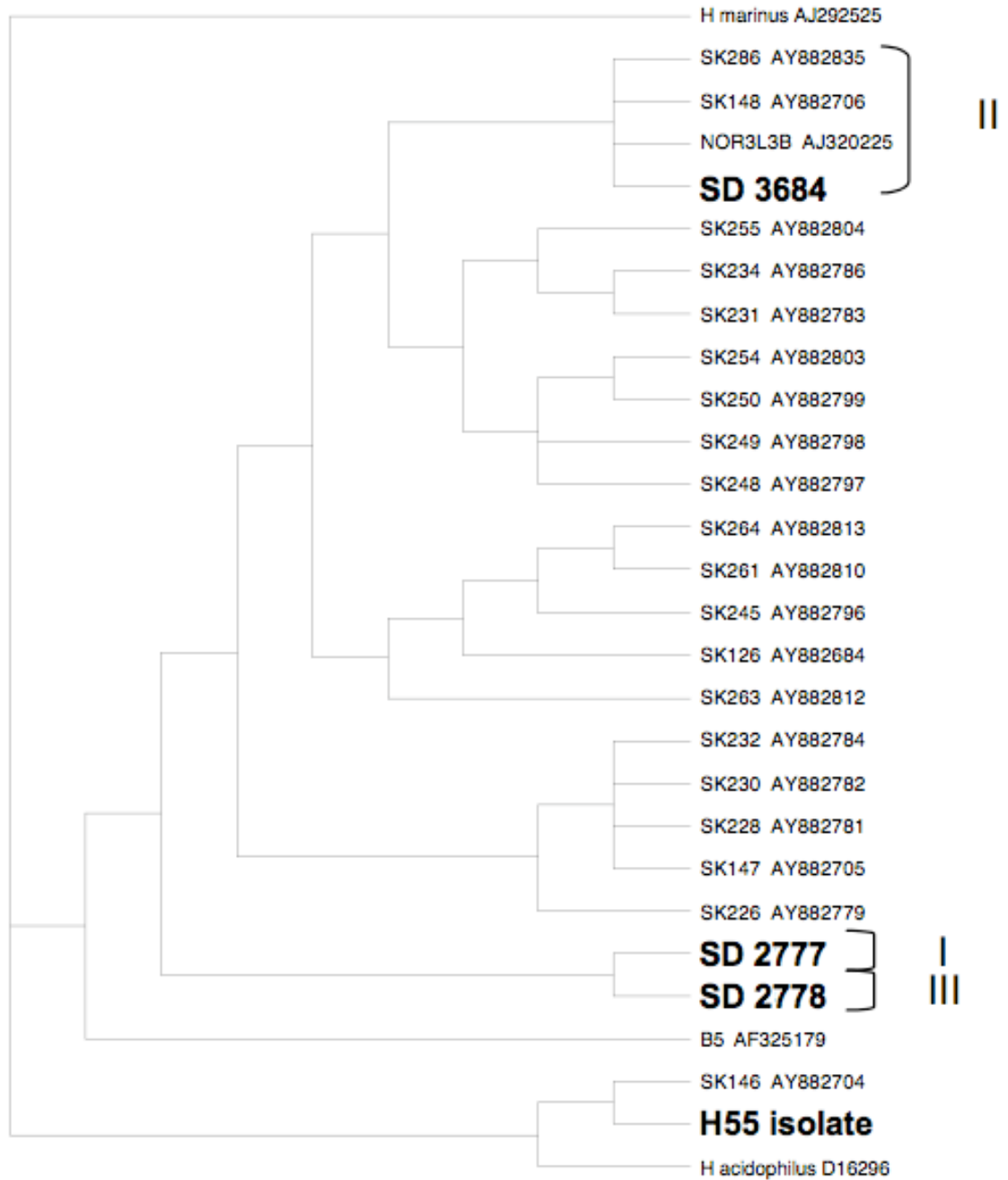


Figure 4.6. Cladogram illustrating the phylogenetic relationships of the *Hydrogenobaculum*-like isolates obtained in the current study relative to isolates NOR3L3B, *A. acidophilum*, SK-annotated PCR clones obtained from various locations in Yellowstone. Isolates representing phylotypes I, II, and III are shown in bold and strain H55 was previously isolated from brown iron mat zone in Dragon Spring (12).

In Vitro Studies

Isolate 3684 was selected for additional characterization studies. Phylogenetically, this organism was identical to the dominant community sequence type and was capable of growth using either H₂S or H₂ as an electron donor and CO₂ as a carbon source. Based on these criteria, this organism was viewed to be ecologically relevant and to represent an opportunity for modeling studies that would more closely examine H₂S or H₂ utilization under spring relevant conditions. One set of experiments examined whether H₂S or H₂ exerted catabolite regulatory controls over the other and thus potentially influence in situ utilization patterns. Isolate 3684 was first cultured separately with H₂S or H₂ (in filter-sterilized spring water) to acclimate the cells to a single energy source and then late log phase cells transferred to the same medium except that now contained H₂S (30 μM) and H₂ (30 nM). In such experiments, consumption of both H₂S and H₂ was immediate and without a discernable lag (Figure 4.7A). Initial consumption rates (first three time points) were 0.25 μmoles · min⁻¹ · 10⁶ cells and 0.32 nmoles · min⁻¹ · 10⁶ cells for H₂S and H₂, respectively. H₂S consumption appeared to remain nearly linear for 50 min where H₂S levels were ~ 5 μM, whereas the H₂ consumption rate showed evidence of decline after 30 min (0.03 nmoles · min⁻¹ · 10⁶ cells) when aqueous H₂ decreased to <15 nM, a concentration roughly equal to that encountered in the spring source waters (Figure 4.3).

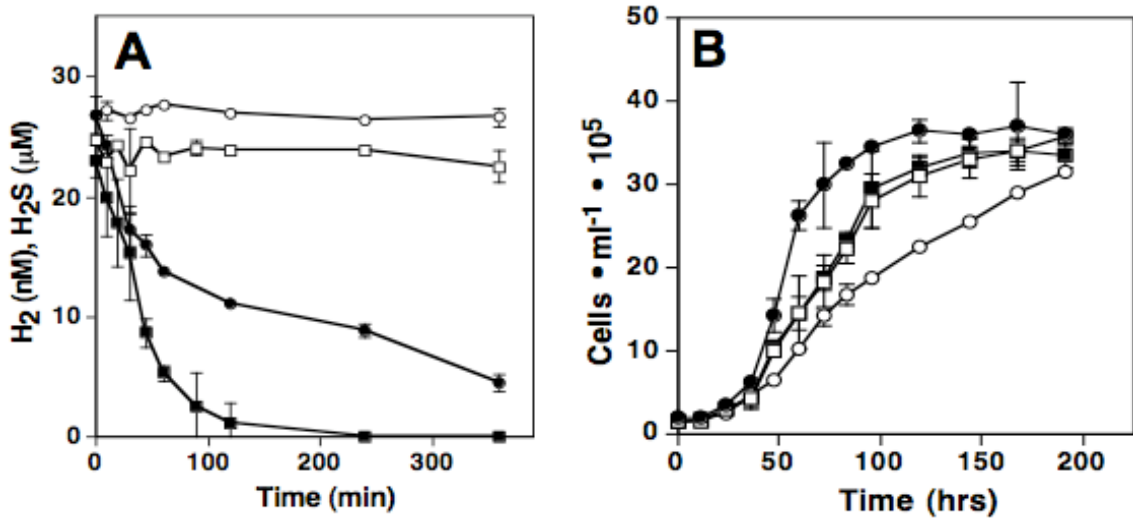


Figure 4.7. H₂ and H₂S consumption and growth by *Hydrogenobaculum* strain 3684. (A) Consumption (○) Dissolved H₂ after the addition of autoclave-killed 3684 culture. (□) Dissolved H₂S after the addition of autoclave-killed 3684 culture. (●) Dissolved H₂ with 2x10⁶ cells ml⁻¹ (■) Dissolved H₂S with 2x10⁶ cells ml⁻¹. (B) Growth (●) 726 nM H₂ (■) 25 nM H₂ + 60 μM H₂S (○) 60 μM H₂S (□) 25 nM H₂. Symbols indicate the mean of triplicate samples. Error bars represent one standard deviation of the mean.

Subsequent experiments then examined 3684 growth with H₂S and H₂ separately. Best growth (doubling time ~ 9.1 h) was observed with saturating levels of H₂ (723nM as per Crozier and Yamamoto (10)) (Figure 4.7B), although overall poorest growth (doubling time ~ 18 h) occurred with 30 nM H₂, a concentration that still exceeded in situ levels in Dragon Spring. Growth rates with spring relevant levels of H₂S were intermediate between these extremes (doubling time ~ 11.5 h), and the addition of H₂ did not significantly improve growth beyond that which occurred with H₂S alone (Figure 4.7B).

Because background levels of S⁰ (solid phase, Figure 4.1) and SO₄ (1.2 mM; Langner et al. 2001) in the spring prohibited experiments designed to track the fate of H₂S in the ex situ assays, experiments were undertaken to determine the fate of H₂S in

batch cultures. Ion chromatography of culture fluids indicated that ~80% of added H_2S was converted to sulfate after 24 hours incubation with a cell culture at $1 \cdot 10^5$ cells ml^{-1} (Figure 4.8A). Culture fluids were visibly yellow when grown on H_2S , whereas uninoculated media remained clear, suggesting S^0 may be an intermediate. SEM/EDX analysis of solid phase culture constituents confirmed the presence of S^0 (Figure 4.8B).

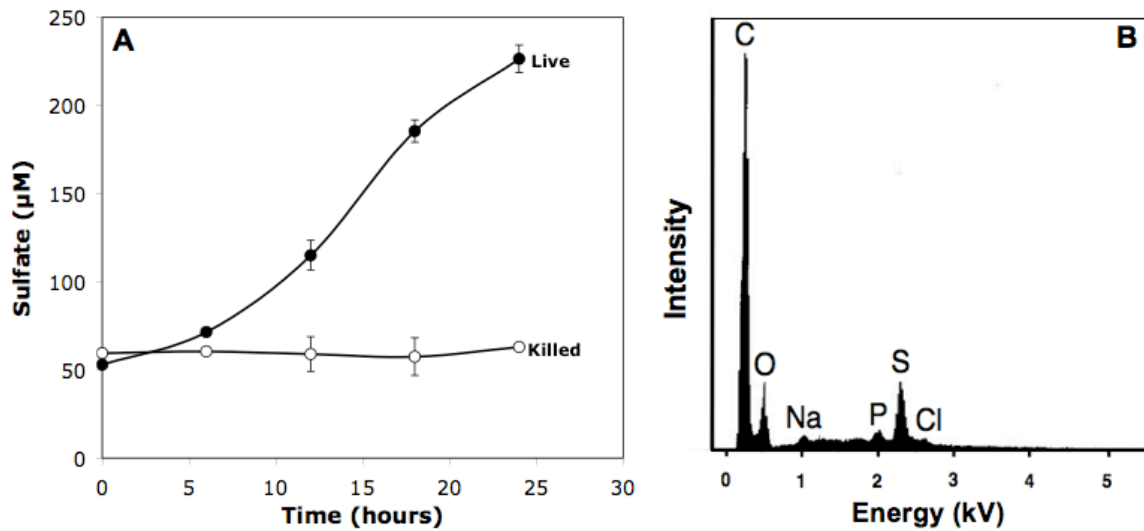


Figure 4.8. (A) Production of sulfate by *Hydrogenobaculum* isolate after two additions Na_2S to a final concentration of $100\mu\text{M}$. (B) Energy Dispersive X-ray analysis of solid phase recovered from 10-day-old cultures of isolate 3684. Samples showed a predominance of carbon and sulfur.

Discussion

Significant effort concerning the geobiology of high temperature environments has focused on determining the underlying sources of energy that fuel primary production (4, 21, 33, 36, 41, 42, 46). Most studies have been predictive, typically based on inferential type evidence, and have been very useful as first approximations. rRNA gene sequence based approaches estimate in situ metabolism based on physiologies inferred from phylogenetically closely related organisms that have been cultured and characterized (34, 42, 46). Thermodynamic predictions, on the other hand, derive from potential free energy yields calculated from the ion activities of electron donors and acceptors found in the various geothermal systems under study (4, 5, 21, 33, 42, 46). The present study had similar goals while focusing on H_2 and H_2S , however the experiments were designed to take a more directed approach; i.e. quantify presence, measure utilization, and examine the characteristics of microorganisms capable of growth on these highly exergonic chemolithotrophic substrates.

Aqueous chemical analysis and microelectrode work demonstrated significant H_2S gradients in the horizontal (11) and vertical (Figure 4.2A) dimensions. Time course ex situ assays provided evidence of rapid microbially-based H_2S consumption (Figure 4.4). H_2S and O_2 sensitive microelectrode experiments illustrated that H_2S penetration into the mat was limited, rapidly decreasing to below detection within the oxygenated region of the mat (Figure 4.2) and suggesting that the H_2S consumption is linked to the presence of O_2 . Similar experiments also demonstrated H_2 gradients (Figure 4.3) and biologic consumption (Figure 4.4B). The vertical and horizontal gradients of H_2S , H_2 ,

and O₂ translates into a continuum of chemical energy gradients, providing numerous niche opportunities for populations having specialized or flexible metabolic needs. The latter was confirmed by the organisms obtained in the isolation exercises (Table 4.1), and indeed demonstrated optimum O₂ requirements exceeding that previously documented for *Hydrogenobaculum* (1, 13, 45).

The *Hydrogenobaculum* 16S rDNA signatures that dominated the community clone libraries were 99% identical to *H. acidophilum*, which was characterized as requiring H₂ and S⁰ for growth (45). Thus, physiologic inference would predict that organisms inhabiting the yellow, S⁰ deposition zone would be engaged in H₂ oxidation and utilize the abundant S⁰ present in this region of the spring outflow channel (Figure 4.1, (26)). Results of the cultivation work, however, illustrate the potential problems associated with physiologic inference in general and, more specifically, incorrect conclusions drawn if applied to the *Hydrogenobaculum* populations inhabiting this spring. Isolates sharing identical 16S rRNA gene sequences (and indeed ITS sequences) differed in important ways with respect to the ecologically relevant electron donors they were capable of utilizing (Table 4.1). Such observations affirm those previously reported for organisms that are phylogenetically closely related (47) or indeed identical (25), but which exhibit widely varying physiologies.

The isolates obtained in this study were also of importance for other reasons. First, we draw attention to the observation that the overwhelming majority (90%) of the *Hydrogenobaculum* isolates were phylogenetically identical to the two major phlotypes (95% of all clones) obtained from total community DNA. The use of filter-sterilized

spring water as the basal growth medium and O₂ concentrations predicted from the microelectrode work likely provided a more natural growth environment as compared to synthetic media. Cultivation of what appears to be the numerically dominant populations (assuming no PCR biases) challenges current views [e.g. Felske et al. (14)] that ecologically relevant microorganisms cannot be isolated from the environment.

Such isolates are useful for culture-based modeling studies, where results of ex situ experiments can be studied in more detail and under more controlled conditions. When isolate 3684 was cultured in spring water with average spring H₂S concentrations (30 μM) but H₂ at levels approximately twice (30 nM) the maximum value observed at any location in the spring, H₂S consumption rates exceeded that of H₂ by three orders of magnitude; an observation completely consistent with the ex situ measurements (Figure 4.4). Regardless of which substrate to which the cells had been preconditioned, utilization of both substrates was immediate and without a lag phase (Figure 4.7A), suggesting constitutive expression of the enzymes involved, complete lack of catabolite repression between these two specific energy sources, and that microbes in natural settings may not exhibit classical diauxic growth (18). Rather, organisms utilize all available metabolites simultaneously and constitutively.

Another important observation derived from the pure culture experiments speaks to the significance of enzyme kinetics when considering the relative importance of various energy sources in natural settings. Theoretical energy yields derived from calculating $\Delta G^{\circ}_{\text{rxn}}$ fail to account for enzyme properties, the importance of which cannot be overstated. H₂ concentrations in the range of 5-10 nM are adequate to support

microbial metabolism (30, 31, 46), however these values are considerably lower than the estimated H₂-uptake hydrogenase K_ms for H₂, which range from 0.92 μM for *Pyrodictium brockii* (38) to 19 μM for an *Anabaena* sp. (20) and that far exceed H₂ concentrations documented for several geothermal features in Yellowstone (46). The significant change in H₂ consumption rates by isolate 3684 when H₂ levels fell below ~15 nM (Figure 4.7A) perhaps reflects the K_m properties of the hydrogenase enzyme(s) in this organism. If so, this would suggest this particular organism is well adapted to the low H₂ levels in most of the Yellowstone hot springs studied (46). However, the H₂ and H₂S consumption profiles by this organism (Figure 4.7A) also suggests that over the range of concentrations of both substrates measured in the spring, in situ utilization of H₂S trumps that of H₂. Further, consumption rate appeared closely related to actual growth; when spring relevant concentrations of H₂ and H₂S were provided, this particular organism grew better with H₂S (Figure 4.7B). Superior growth with H₂ was observed only with saturating concentrations of H₂, levels that should be considered unrealistic given concentrations measured in Dragon Spring and elsewhere in Yellowstone (46). Based on the above-mentioned apparent K_m considerations (Figure 4.7A), we speculate that growth at H₂ concentrations measured in the outflow channel (9-13 nM, Figure 4.3) would result in even slower growth. Technically, these low levels were difficult to maintain experimentally and thus were not examined.

While it is clear that the aerobic respiration of hydrogen can provide substantial energy to chemolithotrophs, the potential energy gained from the oxidation of sulfide depends on the extent to which the sulfur is oxidized and may, in fact, be quite similar to

that gained through H₂ oxidation under the spring conditions examined in this study (Inskeep et al., 2005). Several organisms including *Thiobacillus thioparus* (28, 50) and *Desulfobulbus propionicus* (16) are capable of oxidizing H₂S to SO₄ under aerobic conditions. S⁰ production by this *Hydrogenobaculum* isolate (Figure 4.8) appears to be an intermediate as SO₄ production was also observed. While Rowe *et al.* (43) suggested that the presence of elemental sulfur was a major niche determinant for *Hydrogenobaculum*-like organisms they encountered in thermal tributaries feeding Lemonade Creek, our results indicate that the occurrence of S⁰ is at least partially due to the activity of the organisms present. (Figure 4.4).

This study directly examined the relative importance H₂S and H₂ as energy sources in support of primary productivity in a geothermal spring. Field experiments quantified both and measured consumption rates, and were combined with cultivation and culture-based assays to determine the dynamics of electron donor usage by an environmentally relevant isolate. Major conclusions drawn from this study are that phylogeny cannot be relied upon to predict physiology, and that H₂S can dominate H₂ as an energy source in terms of availability, apparent in situ consumption rates, and growth-supporting energy.

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