Arsenite oxidation by a Hydrogenobaculum sp. isolated from Yellowstone National Park
by Jessica Donahoe-Christiansen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Land Resources and Environmental Sciences
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Abstract:
A novel Hydrogenobaculum sp. was isolated from an acid-sulfate-chloride geothermal spring in Yellowstone National Park (YNP), WY,USA that had previously been shown to contain microbial populations engaged in arsenite oxidation. Acid-sulfate-chloride thermal springs are a prominent spring type in the Yellowstone geothermal complex and provide unique habitats to study chemolithotrophy where iron, sulfur, arsenic, and perhaps hydrogen gas represent the predominant potential electron donors for generating energy. The organism (designated H55) is an obligate microaerophilic chemolithoautotroph that grows exclusively on hydrogen gas as an electron donor. The optimum temperature and pH for H55 are 55-60°C and 3.0, respectively, and the 16S rDNA sequence of H55 is 98% identical to Hydrogenobaculum acidophilum. Whole cells of H55 displayed Michaelis-Menten type kinetics when oxidizing arsenite, with an estimated Km of 80μM arsenite and a Vmax of 1.47 pM arsenite oxidized / min. (for 1.0 x 10 6 cells per ml). The native habitat of H55 contains large amounts of ferric iron in the stream sediments and high concentrations of aqueous hydrogen sulfide, with concentrations of the latter negatively correlated with arsenite oxidation in the spring. Both chemical species were examined for their contribution to, or influence of, overall (i.e. biotic plus abiotic) stream arsenic redox activity by comparing their effects with arsenite oxidation activities of pure cultures of H55. The abiotic oxidation of arsenite by ferric iron occurred at significantly slower rates than that observed with H55, and aqueous sulfide appeared to inhibit the putative arsenite oxidase of H55. The abiotic reduction of arsenate via aqueous sulfide was negligible under the experimental conditions utilized. The isolation and characterization of this novel Hydrogenobaculum sp. initiates the process of characterizing the microbial populations inhabiting acid-sulfate-chloride thermal springs in YNP, and contributes fundamental information to enhance the general understanding of the biogeochemistry that shapes the YNP geothermal landscape.

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ABSTRACT

A novel *Hydrogenobaculum sp.* was isolated from an acid-sulfate-chloride geothermal spring in Yellowstone National Park (YNP), WY, USA that had previously been shown to contain microbial populations engaged in arsenite oxidation. Acid-sulfate-chloride thermal springs are a prominent spring type in the Yellowstone geothermal complex and provide unique habitats to study chemolithotrophy where iron, sulfur, arsenic, and perhaps hydrogen gas represent the predominant potential electron donors for generating energy. The organism (designated H55) is an obligate microaerophilic chemolithoautotroph that grows exclusively on hydrogen gas as an electron donor. The optimum temperature and pH for H55 are 55-60°C and 3.0, respectively, and the 16S rDNA sequence of H55 is 98% identical to *Hydrogenobaculum acidophilum.* Whole cells of H55 displayed Michaelis-Menten type kinetics when oxidizing arsenite, with an estimated K_m of 80μM arsenite and a V_{max} of 1.47 μM arsenite oxidized / min. (for 1.0 x 10^6 cells per ml). The native habitat of H55 contains large amounts of ferric iron in the stream sediments and high concentrations of aqueous hydrogen sulfide, with concentrations of the latter negatively correlated with arsenite oxidation in the spring. Both chemical species were examined for their contribution to, or influence of, overall (i.e. biotic plus abiotic) stream arsenic redox activity by comparing their effects with arsenite oxidation activities of pure cultures of H55. The abiotic oxidation of arsenite by ferric iron occurred at significantly slower rates than that observed with H55, and aqueous sulfide appeared to inhibit the putative arsenite oxidase of H55. The abiotic reduction of arsenate via aqueous sulfide was negligible under the experimental conditions utilized. The isolation and characterization of this novel *Hydrogenobaculum sp.* initiates the process of characterizing the microbial populations inhabiting acid-sulfate-chloride thermal springs in YNP, and contributes fundamental information to enhance the general understanding of the biogeochemistry that shapes the YNP geothermal landscape.

CHAPTER 1

LITERATURE REVIEW

Arsenic and the Environment

Arsenic is ubiquitous in the environment, and due to its acute and chronic toxicity, it has become one of the most globally relevant pollutants (1). Major natural sources of arsenic in the biosphere derive principally from igneous activity, weathering of parent rock, and leaching of subsurface minerals via geothermal waters (2). It is commonly found in trace amounts in the atmosphere, fresh and marine waters, and in soils in the following concentration ranges: atmospheric dust, 50-400ppm; marine water, 2.6 ppb; fresh water, 0.4 ppb; soils, 0.1-1000ppm (3). Numerous anthropogenic activities have also resulted in the release of arsenic into the environment. These include mining activities, ore smelting, sulfuric acid manufacturing, agricultural pesticide use, wood preservation plants, combustion of fossil fuels, pharmaceuticals industry, semiconductor use, and wastewater from underground geothermal electric power stations (4,5,6,7). As an elemental pollutant, arsenic cannot be mineralized to a harmless gas as is the case with many organic contaminants (i.e. CO_2), and thus there is keen interest for achieving a better understanding of the processes involved in the natural cycling and behavior of arsenic in the landscape.

Properties of Arsenic

The common valence states of arsenic are -3, 0, +3 and +5, corresponding to arsine gas, arsenic metal, arsenite, and arsenate, respectively (4). It is widely distributed in nature as salts of copper, nickel and iron as well as arsenic sulfides or oxides (3). Among these, the most abundant and widespread mineral form of arsenic is arsenopyrite (FeAsS) (7). Examining various geologic materials often reveals some form of arsenic, as it will readily sorb onto certain metals commonly found in aquatic and terrestrial sediments (8). There are also naturally occurring methylated arsenicals that result from biochemical activities of certain bacteria, algae and fungi (3).

In water, arsenic is primarily found as arsenate ($As^{V}[H_2AsO_4^-]$) or arsenite ($As^{III}[H_3AsO_3]$) (8). The predominant arsenic specie in marine waters is arsenite (As[III]), while the dominant arsenic specie in freshwater systems is quite variable (3). Under oxidizing conditions (0.2V-0.5V at pH 7), the anionic arsenate (As[V]) form is thermodynamically favored, whereas the uncharged As(III) often predominates in reducing environments (0-0.1V at pH 7) (9). However, it is important to note that predicting arsenic chemical speciation based solely on environmental redox potential (i.e. oxic or anoxic conditions) is not always accurate. For example, there are documented cases of higher than predicted levels of As(III) in oxic surface waters (10,11). The inconsistency between what is predicted and what is actually observed is an important issue that has slowed progress in understanding arsenic behavior in the environment.

Numerous processes can influence the bioavailability of arsenic in the environment, including sorption/desorption, oxidation/reduction, and

precipitation/dissolution reactions. When arsenic is introduced into an aquatic system, it either binds to the sediments or cycles in the surface water (4). It has been well established that As(V) sorbs strongly to surfaces of Fe, Mn or Al oxides (12). The removal of As(V) from the water column via ferric iron (Fe[III]) has been shown to be greatest under mildly acidic conditions (2,13). Under well-oxidized conditions, As(V) species are relatively immobile in aquatic systems containing sufficient amounts of Fe, Mn or Al oxides (14). Adsorption and co-precipitation reactions bind the aqueous As(V) to particles that settle through the water column, accumulating in sediments, and thus explaining why arsenic concentrations in the sediments are often higher than those in the overlying water (10). Arsenite has also been shown to adsorb to metal oxide surfaces, although not at the magnitude observed with As(V) species (4,15). Consequently, As(III) is generally considered to be much more mobile *in situ* (4,8,16,17).

There are specific conditions that result in the release of As(V) from the sediment back into the water column. For example, a substantial addition of organic carbon will often result in oxygen depletion, thus lowering the redox potential (4). Sedimentary Fe(III) could then be reduced by anaerobic Fe(III)-reducing microorganisms, resulting in the release of sorbed arsenic into pore waters (reductive dissolution) (17,18,19). A fraction of the desorbed As(V) may diffuse downward through the sediment profile into anaerobic sulfate-reducing zones, providing opportunity for it to be cycled back to As(III) via As(V) respiring bacteria (see below) and precipitating as arsenic sulfides (i.e. orpiment [As_2S_3] or realgar [AsS]) under acidic conditions (20). All of these potential

states contribute to the complexity of arsenic behavior in soils, groundwater and sediments.

Understanding the cycling and movement of arsenic in the environment is critical to efforts aimed at predicting endpoint accumulations or potential toxicity. Arsenic is able to induce cell transformations resulting in intensive gene damage (1,3,21). Long-term arsenic exposure induces neoplasia (tumorigenic potential) often leading to cancers of the lung, skin, bladder, kidney, and liver (1,6). Arsenite is viewed as ranging from twenty-five to one hundred times more toxic than As(V), and several hundred times more toxic than methylated arsenicals, particularly to microbial cells (1,8). Arsenite toxicity derives from its affinity for protein sulfhydryl groups, initiating such problems as destabilization of proteins and interference of protein function (e.g. enzymatic reactions) (1,21). Arsenate is an analog of phosphate, thus it is capable of disrupting phosphorylating reactions involved in nucleic acid synthesis or energy metabolism (i.e. ATP cycling) (1,3). Because of the types of cellular metabolic and synthesis activities that are disrupted and the introduction into basic cellular building blocks, As(III) and As(V) can be found in numerous cellular proteins and lipids, resulting in arsenic accumulation in living tissues (1).

The damaging affects of arsenic are being witnessed globally. Members of a small community in Georgia, USA are experiencing neurological damage due to inhalation of high levels of arsenic in air-borne soil particles (22). In several villages in Taiwan, long-term arsenic exposure has now been linked to an extraordinarily high incidence of non-insulin-dependent diabetes mellitus (23). The largest single

documented arsenic mass poisoning disaster involves the contamination of groundwater in Bangladesh. Thousands of exposed people have died and millions more are currently enduring numerous cancers (24). Ironically, while the toxicity of arsenic is well understood, there are examples where it has been included as an experimental drug in specific medical treatment protocols. In short term trials, arsenic trioxide (As_2O_3) has been used with positive results to treat patients stricken with acute promyelocytic leukemia (25).

Arsenic Microbiology

Microorganisms in natural environments are continuously exposed to metals and metalloids, some being necessary as micronutrients (i.e. magnesium, copper and zinc), while others often have no apparent function except to exert toxic effects (i.e. mercury, arsenic and lead) (3). For arsenic, the most frequently studied microbial interactions are those that are viewed to be involved with either arsenic resistance or for energy generation, with As(III) oxidation and As(V) reduction involved in both categories of reactions (2,3,10,17,26,27). Biologically based arsenic redox activity has been known for some time, with the first microorganisms reported to oxidize or reduce arsenic isolated from cattle dipping tanks in the early 1900's (28). As subsequent research has shown, these microbial transformations are an integral facet of arsenic behavior and mobility in aqueous and soil environments (4).

Arsenate Reduction. Microorganisms appear to reduce arsenic primarily for resistance/detoxification strategies (nondissimilatory reduction) or for energy generation

(dissimilatory reduction) (4). Nondissimilatory As(V) reduction is observed in resistance or detoxification strategies, where no energy is gained via the reduction process. In this case, arsenic resistance is genetically encoded by the *ars* genes, which are widely distributed throughout the prokaryotes and may be plasmid- or chromosomal-encoded (3,29). Plasmids containing *ars* genes have been documented for *Escherichia*, *Staphylococcus*, *Pseudomonas* and *Rhodococcus* species (30,31). Chromosome-located *ars* genes have been reported for numerous bacterial species including *Erwinia carotovora, Citrobacter freundii, E. coli, P. aeruginosa, Enterobacter cloacae, Shigella sonnei, Klebsiella pneumoniae,* and *Salmonella arizonae* (32).

The basic features of the *ars* encoded As(V) resistance model typically includes active transport of As(V) into the cell via a phosphate transporter, reduction to As(III) by a soluble As(V) reductase, followed by active extrusion by a cytoplasmic membrane efflux pump (3,33). This pump efficiently provides the cell protection from As(V), As(III) and antimonate (34,35). The *ars* operons of *E. coli* and *Staphylococcus* sp. are representative of *ars* operon arrangements typically found in bacteria (35,36,37). *E. coli* plasmid R773 contains an *ars* operon consisting of five genes named *arsA, arsB, arsC, arsD* and *arsR*, while the *ars* operons of *S. aureus* plasmid pI258 and *S. xylosus* plasmid pSX267 contain only the *arsB, arsC* and *arsR* genes (3,36,38).

The *arsR* gene encodes a negative-acting regulatory protein that controls the expression of the *ars* operon, which is only induced in the presence of As(III), antimonite and bismuth (39). The *arsD* gene is also a regulatory protein that is independent of the inducer and responsible for controlling the maximum expression of the *ars* operon (40).

The *arsC* gene encodes an As(V) reductase that reduces intracellular As(V) to As(III) (2,3,41). The K_m's of purified As(V) reductases for As(V) are quite variable, ranging from 1μM for *S. aureus*, 300μM for *Chrysiogenes arsenatis*, to 8mM for the *E. coli* enzyme (2,41,42,43). The As(V) reductases of *E. coli* and *S. aureus* require other proteins (glutaredoxin and thioredoxin, respectively) to successfully complete the reduction reaction, showing no evidence of independent activity (3,43).

The ArsA ATPase contains two ATP binding sites that are both necessary for ATPase activity, and is bound to ArsB, an integral membrane protein (44). Together, they form a complex that is the first system described to be an anion-transporting ATPase conferring resistance to As(III) and antimonite (45). In certain cases, ArsB can utilize membrane potential energy to pump As(III) out of the cell in the absence of the ArsA ATPase (36). However, it has been shown that expressing the *E. coli* ArsA with the *S. aureus* ArsB substantially increases the degree of As(III) resistance when compared to that of ArsB alone (3).

An *ars*H gene was discovered in *Yersinia* and *Thiobacillus ferrooxidans*. The function of ArsH is unknown, but it is required by these particular bacteria for maximal resistance of arsenic (46,47).

Under anaerobic or microaerobic conditions, As(V) can serve as an electron acceptor to couple with electron transport and generate energy via anaerobic respiration (2,4,27). Dissimilatory As(V) reduction in the *Bacteria* appears obligatorily coupled to the oxidation of organic substrates and is catalyzed by a terminal reductase which is typically associated with a cytochrome (2). Arsenate respiration was first demonstrated

with environmental isolates that exhibited anaerobic growth in the presence of As(V) as the sole potential electron acceptor and that oxidized stoichiometric amounts of lactate, formate, or acetate (27,48,49). Dissimilatory As(V) reduction has since been characterized in various bacteria, including *Sulfurospirillum barnesii*, *Bacillus arsenicoselenatis*, *Chrysiogenes arsenatis*, *Desulfomicrobium* strain Ben-RB, *B. selenitireducens* and *Desulfotomaculum auripigmentum* (4,27,48,50). Arsenate-respiring *Archaea* have also been isolated. The hyperthermophile *Pyrobaculum* was shown to be capable of anaerobic chemolithoautotrophic As(V) reduction using carbon dioxide as a carbon source and hydrogen as the electron donor (51).

Finally, regulation of reducing equivalents is another, albeit less understood, mechanism of As(V) reduction that is utilized by organisms such as *Rhodobacter sphaeroides*, which apparently maintain intracellular redox potential via continuous electron dumping (30). A pool of reducing equivalents accumulates during anaerobic growth in the light, eventually to a threshold that negatively affects photosynthesis (2). One mechanism to reduce this pool of reductant, and thus alleviate the negative feedback on photosynthesis, is thought to occur through the reduction of heavy-metal oxyanions, including As(V) (2). The enzyme that carries out this reaction is potentially a membrane-bound, $FADH_2$-independent metal reductase (52).

Arsenite Oxidation. There have been several examples in the literature of organisms capable of oxidizing As(III) to As(V), including the genera *Alcaligenes*, *Pseudomonas*, *Archromobacter* and *Xanthomonas* (10,53,54). The oxidation of As(III) has often been viewed to be a detoxification strategy. Such theory stems from As(III)

being much more toxic than As(V) and that despite the fact that the reaction is exergonic [$2H_3AsO_3 + O_2 \rightarrow HAsO_4^- + 3H^+$ ($G^{o'}$=-256 kJ/Rx)], most As(III) oxidizers could not be shown to gain energy from the process (but see below). The vast majority of the As(III) oxidizing microorganisms isolated thus far are heterotrophic (requiring the presence of organic materials for growth) (26,54,55,56). There have been only two microorganisms reported in the literature that grow chemolithoautotrophically with As(III) as the electron donor, oxygen as the electron acceptor, and carbon dioxide as the carbon source. The first was *Pseudomonas arsenitoxidans*, which exhibited extremely long doubling times (approximately two days) and poor growth yields (57). More recently, other organisms (two closely related strains named NT-25 and NT-26) capable of chemolithotrophic growth on As(III) were isolated from a gold mine in the Northern Territory of Australia. Phylogenetic analysis placed these bacteria within the *Agrobacterium-Rhizobium* branch of the α-Proteobacteria (26). Growth by these organisms with As(III) as the sole electron source was relatively rapid, with a doubling time of 7.6 hours (26).

Unlike the better characterized *ars* genes and the biochemistry of As(V) reduction, the genes encoding proteins involved in As(III) oxidation have not been identified. Two mechanisms of As(III) oxidation have been characterized, one enzyme driven and the other associated with peroxide formation initiated by membrane exposure to arsenic (53,58). An As(III) oxidase has been purified from the outer surface of the cytoplasmic membrane of *Alcaligenes faecalis* strain NCIB 8687 and then characterized (53). *Alcaligenes faecalis* was found to have As(III) oxidizing activity only when the

cells were grown in the presence of As(III), consistent with other reports of inducible As(III) oxidation (35). The enzyme is an 85 kDa monomer consisting of five or six iron atoms, one molybdenum atom and one sulfide atom (53). Both azurin and a c-type cytochrome were established as electron acceptors for the As(III) oxidase, potentially forming a periplasmic electron transfer pathway resulting in As(III) detoxification (53). Using azurin as the oxidizing substrate, a K_m of 8μM As(III) was estimated (53). This As(III) oxidase is the first known oxyomolybdoenzyme to contain a HiPIP (4Fe-4S), 3Fe, and/or Rieske-type (Fe-S) reaction center (53). The HiPIP and Rieske-type centers have high reduction potentials of 300-400 and 200-300 mV respectively, making them feasible components of an As(III) oxidase given that the oxidation of As(III) to As(V) has the reduction potential of 60mV (53,59). One of the *Agrobacterium-Rhizobium*-like As(III) oxidizing organisms was also found to have an As(III) oxidase, purified from the periplasm of the cell, that is currently being characterized with the gene(s) encoding the enzyme being cloned and sequenced (26).

In an organism referred to as *Pseudomonas pudita*, As(III) oxidation was shown to initiate the process of free radical-mediated lipid peroxidation, resulting in elevated levels of peroxides of unsaturated fatty acids (UFA) and As(V) in the culture medium (60). Certain multivalent metals, and metalloids such as arsenic, can initiate the formation of active forms of oxygen, changing valence states in the process (60,61). Transition metals are capable of initiation reactions due to their ability to donate or accept electrons, resulting in the formation of free radicals of the UFA hydroperoxides and contributing to the following reaction:

$$ROOH + Me^{2+} \Rightarrow Me^{3+} + RO^{\bullet} + OH^{-} \quad (61).$$

In additional experiments with this organism, lipid synthesis increased upon exposure to As(III) (60). The increased lipids resulted in decreased membrane permeability, which is thought to offer added protection against cell lysis (60). The cells that were oxidizing As(III) were also reported to have a marked increase in the activity of the antioxidant enzymes, catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase, all of which aid in the consumption of free radicals (60).

Arsenic Oxidation and Reduction. A microorganism isolated from the Growler Hot Spring in northern California, USA has the ability to both oxidize As(III) and reduce As(V) (62). The isolate, designated HR13, is closely related to the genus *Thermus* and exhibited an interesting oscillating arsenic redox cycle (62). A 16h period of As(III) oxidation (at a rate of 0.15mg/L/min) was followed by a 12 hour reduction phase of As(V) back to As(III), after which the entire cycle re-commenced (62). Growth of this organism was not observed when As(III) was supplied as the only potential electron source, indicating that the oxidation of As(III) was utilized as a detoxification strategy (62). The strain did have the ability to grow via As(V) respiration, with As(V) acting as the electron acceptor for lactate oxidation (62). The culture conditions during the experiments were discovered to be oscillating between aerobic and anaerobic, with As(III) oxidation occurring under oxic conditions followed by As(V) respiration during anoxic periods (62). Under continuous aeration, As(V) reduction was not observed (62). The effect that *Thermus* HR13 has on arsenic cycling *in situ* would likely depend on the oxygen availability in the particular environment.

Other Arsenic Transformations

Numerous marine organisms such as macroscopic algae, crustaceans, mollusks, phytoplankton, and certain fish will assimilate arsenic (63,64,65). Several of these marine organisms are able to incorporate arsenic into carbon skeletons to form organoarsenical compounds (66). For example, an arsenic atom can replace a nitrogen atom in the compound glycine betaine, forming arsenobetaine which aids in cellular osmotic pressure regulation (2). One study reported a Gram-negative bacterium isolated from activated sludge as capable of utilizing an organoarsenical as the sole carbon source required for growth (67). This particular bacterium had the ability to break the carbon-arsenic bond in arsonoacetate and arsonochloroacetate, utilizing the carbon from the acetate accompanied by a quantitative extracellular release of As(V) (67).

Arsenic volatilization pathways are particularly common in anaerobic environments such as freshwater sediments, active compost piles and sewage sludge (68,69). Gaseous arsenicals derive from methylation reactions, and have been documented in a variety of bacteria and fungi (63). A strain of *Methanobacterium* was shown to form volatile dimethylarsine from As(V), As(III), and methanearsonic acid (70), while anaerobically respiring *Pseudomonas* and *Alcaligenes* will synthesize arsine gas from either As(III) or As(V) (71).

The above methylation reactions are perhaps easiest to rationalize as general detoxification mechanisms or as toxicity avoidance strategies. Another potentially important toxicity avoidance scheme might also be viewed to occur at the cellular uptake level. For As(V) to exert its full toxic effect, it must first enter the cell via an active

anion transporter in order to achieve lethal or inhibitory concentrations. Arsenate is an analog of phosphate, and thus enters the cell via phosphate transporters. Phosphate transporters have been well studied in *E. coli* and *A. johnsonii*, where two systems, each with varying degrees of phosphate affinity, have been described (72,73). The Pit transport system catalyzes the transport of soluble, neutral metal phosphates ($MeHPO_4$), but kinetic analysis has found that it will also transport As(V), exhibiting a K_m of about 25μM for both phosphate and As(V) (73). The latter property results in the failure of the Pit transporter to significantly discriminate between phosphate and As(V), and thus similar uptake rates occur for each (3,72). In these particular bacteria, the Pit system is constitutively expressed, showing no alteration in expression levels during phosphate deprivation (73). The second phosphate transporter is referred to as the Pst (phosphate specific) transporter, and is highly specific for phosphate, having a 100-fold higher affinity for phosphate (K_m of 0.25 μM P) than for As(V) (72,73). The Pst system has been shown to mediate the transport of $H_2PO_4^-$ and HPO_4^{2-} in *A. johnsonii*, and is up-regulated during phosphate limitation or starvation (73). The form and amount of phosphate present in the environment dictates which transport system will dominate. In a natural system that is phosphate stressed, a bacterial cell will have a decreased amount of As(V) uptake due to the Pst transport system's high affinity for PO_4 relative to As(V). Complete As(V) resistance is typically observed in those mutants defective in the Pit pathway (3). However, if there is an excess of phosphate available, the Pit transport system will dominate resulting in an increased amount of cellular As(V) uptake, provided the P:As ratios are not excessive.

Abiotic Arsenic Oxidation and Reduction

Metals in water sediments and soils have long been studied for their arsenic sorbing abilities (16,74,75). However, metals can also participate in abiotic As(III) oxidation. Particular attention has been devoted to iron (Fe) and manganese (Mn) oxides due to their abundance in sediments/soils. The oxidation of As(III) by Mn(IV) and Fe(III) oxides is thermodynamically favorable as viewed by the following reactions:

$$MnO_2 + H_3AsO_3 + 2H^+ \Rightarrow Mn^{2+} + H_3AsO_4 \quad (E^o=0.67v)$$

$$2Fe^{3+} + H_3AsO_3 + 2H_2O \Rightarrow 2Fe^{2+} + H_3AsO_4 + 2H^+ \quad (E^o=0.21v)$$

(76). It was found that at near neutral pH, the Mn dioxides birnessite, cryptomelane, and pyrolusite act as strong As(III) oxidants, while Fe(III) species showed extremely slow oxidation rates (77). Arsenite was also observed to bind to Fe and Mn oxide surfaces without oxidation occurring (75). However, As(V) appears to preferentially sorb to Fe-oxides more readily than to Mn-oxides (74). The sorption of arsenic onto Fe and Al oxides, as well as onto clay mineral edges, occurs via a ligand exchange mechanism (12).

The pH of a system is a driving force in the sorption equilibrium of arsenic to metals and in redox reactions involving Fe(III) and sulfide (16,78,79). Under lower pH conditions (e.g. pH 1.0-4.0), sorption of As(V) onto metal oxide surfaces increases due to the net positive charge gained on sediment surfaces and the net negative charge of As(V) (16). Acidic environments have also been shown to increase the oxidation of As(III) to As(V) by Fe(III) (79). Over a one week period, no discernable As(III) oxidation occurred via Fe(III) at pH 7, while As(III) was nearly quantitatively oxidized to As(V) at pH 2

(79). This differential Fe effect is viewed to be caused by the low solubility of Fe(III) ions at higher pH values (79).

Another pH dependent abiotic arsenic redox reaction is the reduction of As(V) by dissolved sulfide as depicted in the following equation:

$$H_2AsO_4^- + H_2S + H^+ \Rightarrow H_3AsO_3 + 1/8S_8 + H_2O \quad (78).$$

This is a potentially common reaction occurring particularly in the deeper, anoxic sediments (80). Aqueous sulfide (H_2S or HS^-) was found to be 300 times more effective as a reductant at pH 4 than at pH 7 (78). Arsenite may not be the direct reaction product, with arsenic-sulfide complexes forming that may persist for several days until dissociating to As(III) (78).

Abiotic As(III) oxidation by hydrogen peroxide (H_2O_2) in combination with other metals has also been examined (81,82). There are numerous *in situ* studies indicating the presence of H_2O_2 at varying amounts in freshwater, rainwater, and in marine environments (81). It was reported that, depending on pH, ionic strength and temperature, H_2O_2 has the capability of increasing abiotic As(III) oxidation rates (82). The rate of H_2O_2 catalyzed oxidation was noted to be substantially higher in freshwaters vs. seawaters (82). This was determined to be due to the increased amount of particular metals (i.e. Cu^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}) present in the freshwaters examined (81). Specifically, copper and iron, combined with H_2O_2, resulted in substantial increases in abiotic As(III) oxidation, while lead appeared to have the most effect in heavily polluted areas (81).

The rate constants determined may have the potential application as a predictive model for the amount of abiotic As(III) oxidation associated with H_2O_2 and certain metals in particular systems, thus aiding in the tedious task of monitoring arsenic transformations and movement in nature (80). However, confounding variables must also be taken into account when designing models meant to predict the movement and behavior of arsenic in the environment. Chief among these are the competing inorganic and organic ligands, which may dissolve the metal-As(III) complexes releasing arsenic back into the water, and the influence of microbial oxidation/reduction activities that affect the net arsenic speciation (4,82).

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CHAPTER 2

INTRODUCTION

Arsenic contamination in the environment has become the focus of great concern due to its well known toxicity and the frequency at which it has been found at unacceptably high concentrations. It is ubiquitous in the biosphere, found naturally at varying concentrations in almost every environment. However, in some locations anthropogenic sources such as hard rock mining or the use of arsenic-containing herbicides have contributed significantly to arsenic contamination of surface and ground waters used for irrigation and/or drinking (1,2).

The arsenic content of hydrothermal springs is also well documented, and potentially can contribute significant arsenic loading into streams and rivers that drain geothermally active areas (3,4). Arsenic concentrations in these geothermally-derived waters normally range from 1-3 mg l^{-1}, but have been documented to be as high as 150 mg l^{-1} in some of the thermal environments located in Yellowstone National Park, WY, USA (3). The arsenic found in these thermal systems exists predominantly as the inorganic species arsenite (As[III]) and arsenate (As[V]) (4). The dominant species emerging in geothermal waters is thought to be As(III), while As(V) is thermodynamically favored in most aerobic systems (5,6).

Several prokaryotes have the ability to reduce and/or oxidize arsenic (1,2,7,8,9). Arsenate reduction can occur via a dissimilatory route, in which As(V) is utilized as an electron acceptor under anaerobic or microaerobic conditions, or by a detoxification

strategy whereby As(V) is taken into a cell via a phosphate transporter, reduced by an As(V) reductase and extruded from the cell by an As(III) efflux pump (10,11). Microbial oxidation of As(III) has also been viewed as an arsenic detoxification mechanism, until recently where it has been found to be used for generating energy to support chemolithoautotrophic growth (7,12).

The thesis work described below follows previous studies that characterized the microbial populations inhabiting an acidic-sulfate-chloride thermal spring in Yellowstone National Park, WY, USA (13). An important parallel and companion study (Langner et al. 2001) completed the initial characterization of this spring by examining the basic biogeochemical features and activities occurring along the outflow channel of the spring (14). One important observation in these initial experiments was that As(III) oxidation occurred at very high rates, but only at specific locations. Therefore the primary goal of this study was to initiate isolation and characterization efforts aimed at describing the organisms contributing to the As(III) oxidation measured previously at the whole community level. Dilution-extinction enrichment procedures were targeted for any organism(s) that oxidized As(III) under environmentally relevant conditions. Pure culture characterization work with the resulting isolate sought to describe basic features relevant to identification and classification, and to semi-quantitatively examine the As(III) oxidation capability to help establish the organism's ecological relevance with respect to overall microbial community As(III) oxidation activity.

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CHAPTER 3

MATERIALS AND METHODS

Site Description

The research site was a geothermal spring located in Norris Geyser Basin, Yellowstone National Park, WY, USA (44°43'54.8" N, 110°42'39.9" W, Spring No. NHSP106, thermal inventory of YNP). The spring is approximately 0.5m wide and 1-2 cm deep and is fed by a constant 63°C geothermal source having a pH of 3.1. There are at least two substantial temperature gradients present. The first occurs with distance from the source, resulting in a decrease in temperature as the water flows through the channel. The second occurs at right angles to the first along the entirety of the channel, with the highest temperature in the middle of the channel and then cooling towards the bank. Distinctive microbial mats can be found along the spring floor, correlating to regions along the temperature gradient. A yellow mat is present at the warmest sites (63-60° C), and transitions to a brown mat that is observed at the intermediate temperatures (55-51° C), which then changes to a green color when temperatures decline further (50-45° C). The pH remains constant at 3.1 throughout the entirety of the spring. There are high amounts of dissolved chloride (16.0 mM), sulfate (1.3mM), sodium (16.3mM) and dissolved inorganic carbon (DIC) (4.4mM) carried in the spring source water, while lower concentrations of sulfide (0.063mM) and dissolved organic carbon (DOC) (0.08mM) are also present (1). Total aqueous arsenic concentration appears to remain

relatively constant at 0.033mM throughout the area of the spring studied (1). Previous work demonstrated that the proportion of arsenic as arsenate (As[V]) abruptly increases at the transition from yellow to brown zones (1). This area of rapid arsenite (As[III]) oxidation (the brown microbial mat) is the site from which the investigation originated.

Sampling and Enrichment Cultures

Brown microbial mat utilized for enrichment cultures was aseptically sampled with autoclaved tools, placed into 70mL serum bottles and submerged with 35mL of the spring water that was flowing directly above (vertical orientation) where the sample was taken. Killed controls were prepared the same way except that 4% (w/v) formaldehyde was added as a biocidal agent. The bottles were also outfitted with scuffed glass rods to provide surfaces for microbial colonization and which could be easily manipulated for subculture. The bottles were then sealed on site with butyl rubber stoppers and transported to the laboratory for further preparation and incubation.

Three different head space gas phases were selected based on inferred physiology of the microbial community populations identified in previous work on the basis of 16S rDNA gene sequence analysis (2). One gas phase was filter sterilized air, a second was filter sterilized air enriched with 10% of CO_2, and the last contained a filter sterilized mixture composed of 85% H_2 + 10% CO_2 + 5% air. Enrichments with each gas phase, and their appropriate killed controls, were incubated at 55° C in the light and dark.

Media

Additional spring water was also brought back to the laboratory for use as growth medium in the enrichment and dilution-extinction cycles that followed. Prior to use, however, it was filter sterilized sequentially through Whatman 1 Qualitative Filter Paper, 0.45 Protran nitrocellulose filter paper, and finally through a Nalgene 0.20 PES bottle top filter. In addition, a minimal medium was also developed which consisted of (per liter) 5.0g of elemental sulfur, 1.0g of $(NH_4)_2SO_4$, 1.0g of K_2HPO_4, 1.0g of NaCl, 0.3g of $MgSO_4 \cdot 7H_2O$, 1.0mg of $CaCl_2$, 0.06mg of $NiSO_4 \cdot 6H_2O$ and 2.0mL of a trace elements solution (3). The pH was adjusted to 3.0 with HCl. The trace elements solution contained (per liter) 1.0mg of MoO_2, 7.0mg of $ZnSO_4 \cdot 7H_2O$, 0.5mg of $CuSO_4 \cdot 5H_2O$, 1.0mg of H_3BO_3, 1.0mg of $MnSO_4 \cdot 5H_2O$ and 1.0mg of $CoCl_2 \cdot 6H_2O$ (3). Another version of this medium that lacked elemental sulfur was also used. Solid medium was prepared by adding 1.8% Phytagel (Sigma Chemical Co., St. Louis, MO, USA) to filter sterilized spring water or to synthetic medium.

Isolation Procedures

Dilution-extinction routines were incorporated as part of the enrichment process. For subculture from the serum bottles initiated at spring side (see above), glass rods as well as aqueous samples were transferred to fresh medium at 7 day intervals. At each subculture, the highest dilution that still demonstrated arsenite oxidation was diluted to extinction based on total cell concentration counts of the positive bottle. For transfer of

biofilm material proliferating on glass rods, biofilm cells were scraped from the rods, homogenized by vortexing, and then diluted to extinction based on total cell counts derived from staining and direct microscopic counts. Cell counts for both aqueous and biofilm samples was accomplished by collecting 1.0 mL of culture or homogenate onto a 25mm diameter 0.22μm polycarbonate filter (Osmonics, Inc.), and staining with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (final concentration of 100mg/L, 30 minute incubation). After five washes with sterile water, the filter was placed onto a microscope slide then viewed with a Zeiss Axioskop 2 epifluorescence microscope outfitted with a 10x10mm, 1mm square eyepiece grid (Fisher Scientific) to quantify cells in a known area for calculating cell concentration in the original suspension.

Microscopy

Routine examination of cell cultures was accomplished with a Zeiss Axioskop 2 microscope equipped for brightfield, phase contrast, and epiflourescence work, and for some experiments cultures were also examined with a JSM-6160 scanning electron microscope (Jeol USA). For the latter, cells were washed with sterile water, transferred onto a small silica wafer, then coated with Au for analysis using the cryostage scanning electron microscope.

Nucleic Acid Extraction

Mat material from the geothermal spring study site was aseptically placed into 2mL micro-centrifuge tubes and flash frozen on site in a dry ice-ethanol bath. The

samples were transported back to the laboratory on dry ice and kept at -80° C until used. Two different nucleic acid extraction methods were utilized, both with consistent reproducibility. The first method is based on three freeze-thaw cycles followed by a high salt and sodium dodecyl sulfate (SDS)-based extraction, as outlined by Zhou et al. (1996) (4). The DNA was obtained via chloroform extraction followed by isopropanol precipitation and then resuspended in either Tris-EDTA (TE) buffer, or sterile water. The second method is based on that utilized by Schmidt-Goff and Federspiel (1993) (5). A solution of microbial mat material, or culture pellet, was made by mixing with a 1:1 (w/v) ratio of sterile water. Approximately 0.5ml of this solution was transferred into a 2.0 mL screw cap tube, snap frozen in liquid nitrogen, and kept at -80° C for at least one hour. The following was then added to the frozen sample: 0.5g 106 micron glass beads (Sigma Chemical Co.), 33.3μL 20% SDS, 167μL 3% diatomaceous earth, and 583μL tris-buffered phenol (pH 8.0). The samples were then homogenized on the high speed setting for 160 sec in a mini-beadbeater (Biospec Products) then centrifuged for 15 minutes at 13,000g. The top aqueous layer was transferred to a sterile 1.5ml Eppendorf tube where a 0.0612X volume of 3M sodium acetate and a 0.7967X volume of 95% ethanol were added. Nucleic acids were precipitated for 2 hours to overnight at -20° C. The DNA was again centrifuged for 15 minutes at 13,000g. The pellet was washed with 70% ethanol, dried under a vacuum for 2 minutes and resuspended in 25-40 μL of nuclease-free water.

DGGE Analysis of Partial PCR Amplified 16S rDNA Gene Products

A 323 bp section of the 16S rRNA gene was amplified using primers previously described by Ferris et al. (1996) (6). The forward primer was the *Bacteria*-specific Bac1070f (5'-ATGGCTGTCGTCAGCT-3') and the reverse was the universal Univ1392r (5'-ACGGGCGGTGTGTAC-3'). The reverse primer was modified to contain a 40 bp GC clamp at the 3' end, and allowed for separation of the resulting PCR products in a denaturant gradient gel. The final PCR reaction mixture consisted of 0.4 μM of each primer, 0.2 mM of each dNTP (deoxyribonucleotide triphosphate), 2.5 mM $MgCl_2$, buffer containing 10mM Tris-HCl, 50mM KCl and 0.1% Triton X-100, and 1.0 U of Taq polymerase (Promega). The 50μL reactions were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems) using the following program: 2 min at 95°C, followed by 26 cycles of 95°C for 45s, 43°C for 45s, 72°C for 45s, and ending with 7 min at 72°C. PCR fidelity (i.e. single product of correct size) was assessed by electrophoresing the reaction products in a 1.2% agarose gel, staining with ethidium bromide (Bio Rad), and then viewing under UV light. The PCR products were then analyzed by denaturant gradient gel electrophoresis (DGGE) using the DCode Universal Mutation Detection System (BioRad). The products were electrophoresed through an 8% acrylamide gel containing an increasing denaturant gradient of 40-70% urea-formamide. The gels were run at 60°C at 60V for 15-18 h, then stained with SYBR green (Bio Rad), allowing for examination via UV light.

Full 16S rDNA Amplification and Sequencing

DNA extracted from the pure culture was used as the template for amplification of the near full length 16S rRNA gene using the primer set Bac8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and Univ1492r (5'-GGT TAC CTT GTT ACG ACT T-3'), which were designed based upon the probes previously described by Amann et al. (1995) (7). The PCR reagent composition and concentrations were the same as described above for the DGGE primer set. The thermocylcer program for these amplifications was: 2 min at 95°C, followed by 30 cycles of 95°C for 1 min, 45°C for 1 min, 72°C for 3 min, and a final 7 min at 72°C. PCR products were examined as described above, purified using the Microcon PCR centrifugal filter devices (Millipore), and then quantified by electrophoresing through an agarose gel alongside a mass ladder (Low DNA Mass Ladder, GibcoBRL). For sequencing these PCR products, the primer set consisted of Bac8f, Univ533f (5'-GTG CCA GCM GCC GCG GTA A-3', where M is A or C), Bac785f (5'-GGA TTA GAT ACC CTG GTA G-3'), Bac1068f (5'-GCA TGG CYG YCG TCA G-3', where Y is C or T), Univ519r (5'-GWA TTA CCG CGG CKG CTG-3', where W is A or T and K is G or T), 907r (5'-CCG TCA ATT CCT TTR AGT TT-3', where R is A or G), Bac1100r (5'-AGG GTT GCG CTG GTT G-3'), and Univ1492r. Sequences were assembled with Sequencher 3.1.1 (Gene Codes Corporation) and compared to sequences in GenBank using BLAST to reveal the closest match as a first step in phylogenetic analysis.

Arsenic Speciation Assays

The arsenic redox activity of the enrichments and of the isolate was monitored using chemical speciation and analytical techniques described previously by Langner et al. (2001). Briefly, aliquots of cell cultures were centrifuged to pellet the cells, the supernatant was filtered through a .22μm in-line disposable syringe filter (Osmonics, Inc.), then separated into two 15 mL HDPE plastic bottles (5mLs into each bottle). The first aliquot was acidified with 0.1mL of 12.1 M HCl and stored at 4°C until analyzed for total arsenic levels. The second aliquot was prepared to determine the concentration of aqueous As(V). For the latter, 1.0 mL of 2M Tris buffer (pH 6) was added to 5mL of filtered sample. The sample was then sparged with N_2 while 1mL of 3% $NaBH_4$ (w/v in 0.1% NaOH) was added in 0.2 mL increments over a 4 min. time period. Any As(III) present in the sample is reduced to arsine gas by the $NaBH_4$, and the arsine gas is then removed when the sample is sparged with N_2. $NaBH_4$-treated samples were further purged with N_2 for an additional 3 minutes, acidified with 0.1mL of 12.1 M HCl and stored at 4°C. All samples were analyzed by hydride generation atomic absorption spectrometry (HG-AAS). Concentrations of As(III) were determined by the difference between total arsenic [As(ts)] and As(V) detected by this analytical method.

Kinetics Assays

Kinetic parameters (K_m and V_{max}) of As(III) oxidation by the pure culture were estimated using substrate saturation assays. Each assay contained a standard number of cells (1.0×10^6 cells per mL) as determined in preliminary experiments that established a

standard curve that compared culture cell number to culture total cellular protein. Cell number of washed cultures was determined using a Petroff-Hauser counting chamber and culture protein concentration was assayed using the Bradford assay (Biorad Protein Assay, BioRad Laboratories). Washed cells were suspended in the synthetic growth medium (described above) that was amended with nine different As(III) (as $AsNaO_2$) concentrations (5, 10, 25, 50, 75, 100,150, 200 and 250μM), and samples were taken after 1.0, 1.5, 2.0 and 2.5 hours of incubation at 55°C. Culture filtrate samples were then analyzed for As(ts) and As(V) using the HG-AAS. Linear plots of the As(III) oxidation activity measured in the different assays were used to establish rates, which were then plotted against substrate concentration and analyzed via non-linear regression (statistical computer software, Origin V6.0; Microcal, MA) to estimate the K_m and V_{max}.

Effects of Abiotic Chemistry on Bacterial Arsenite Oxidation

The effect of aqueous sulfide (S^{2-}) on As(III) oxidation was determined in cultures incubated under conditions that mimicked *in situ* conditions; i.e. 55°C, pH 3.0, microaerobic gas phase, and *in situ* relevant concentrations of As(III) and sulfide. As with the kinetic experiments described above, assays were conducted in the synthetic growth medium, but with the following modifications: cultures either contained no S^{2-}, were amended with 60μM S^{2-} (as Na_2S) at the beginning of the experiments, or were spiked with S^{2-} at the midway point (with each condition containing 30μM As(III)). Identical amounts of cells were added to each culture and every condition was replicated in triplicate. Abiotic (non-inoculated) controls consisted of 30μM As(III) alone or of

30μM As(V) (as $AsHNa_2O_4 \cdot 7\ H_2O$) + 60μM S^{2-}. Samples were taken every 0.5 h during 3.5 h for arsenic speciation and analysis as described above. To achieve a 2:1 ratio of S^{2-}:As in the aqueous phase, equilibrium equations were used to calculate and correct for S^{2-} solubility and concentration in the medium under the conditions used. Abiotic S^{2-} induced As(V) reduction rates were determined by preparing serum vials that contained the synthetic medium with 60μM S^{2-} and 30μM As(V) (equilibrium equations were again used to ensure correct S^{2-} concentrations). Samples were taken at days 1, 3 and 5 of incubation at 55°C for arsenic speciation and analysis as described above.

Bacterial As(III) oxidation rates were also compared to abiotic, ferric iron (Fe^{3+}) induced, As(III) oxidation. For these experiments, cultures was incubated with a 1:1 ratio of Fe^{3+} (as $FeCl_3$) and As(III) (50μM of each), or As(III) alone. The experiments were complete with abiotic (sterile) controls that consisted of 50μM As(III) + 50μM Fe^{3+}, or of 50μM As(III) only. Samples were taken after 7 days incubation at 55°C for arsenic speciation and analysis as described above. All cultures were grown in the synthetic medium described above and contained a known number of cells. Each of the assay conditions was replicated in triplicate.

Statistical Analysis

Where statistical analysis was required, the data was subjected to analysis of variance (ANOVA) as applied by Microsoft Excel, *Me* edition.

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CHAPTER 4

EXPERIMENTAL RESULTS

Enrichment Cultures / Isolation

Previous work in the geothermal spring that is the subject of this thesis demonstrated that As(III) oxidation began abruptly in a specific location approximately 4.5 m from the source, and which also coincided with a region of the mat system that changed in color from yellow to brown (1,2). Sequence and phylogenetic analysis of 16S rDNA PCR-clone libraries generated from this site showed that the microbial community at this location was comprised of *Hydrogenobaculum acidophilum*- and *Desulfurella*-like organisms (1). This phylogenetic information was used to infer physiology of the resident organisms, which in turn helped guide the design of initial enrichment conditions. Microaerobic conditions with H_2 as an electron donor for *Hydrogenobaculum*-like bacteria, added CO_2 to optimize for growth of autotrophs (strict or facultative), and anaerobic conditions for anaerobes like *Desulfurella* were all initial conditions utilized. In an attempt to avoid enrichment bias that could result from the use of any type of synthetic growth medium, initial enrichments used flowing spring water that was collected directly overhead the sampled site. Each culture bottle also contained autoclaved glass rods that would allow for capture of organisms that prefer colonization of surfaces (as opposed to a planktonic lifestyle), as well as for convenient aseptic transfer into fresh medium. Finally, the potential importance of phototrophs for the