CHARACTERIZATION OF NOVEL ARSENITE OXIDATION PROCESSES IN MONO LAKE, CALIFORNIA, USA

by

JENNY C. FISHER

(Under the Direction of James T. Hollibaugh)

ABSTRACT

Two novel arsenite oxidation processes in Mono Lake, CA, and the organisms involved in these processes were identified. Arsenate was produced when anoxic live Mono Lake water samples were amended with arsenite and selenate, but it was not produced in filtered or killed control samples. A pure culture capable of selenate-dependent anaerobic arsenite oxidation was isolated from Mono Lake. Washed cells experiments with this culture demonstrated that the oxidation of arsenite is tightly coupled to the reduction of selenate. Strain ML-SRAO is not autotrophic and grows optimally on lactate with selenate as the electron acceptor.

Strain ML-SRAO^T is a Gram-positive, non-motile, spore-forming rod that can respire oxygen, nitrate, and arsenate in addition to selenate. The arsenate reductase gene (*arrA*) from strain ML-SRAO was highly similar to *arrA* genes from other Mono Lake arsenate reducers. Comparison of 16S rDNA sequences of closely related organisms showed that ML-SRAO^T and six other classically defined *Bacillus* species formed a distinct phyletic group The nearest relative, *Bacillus agaradhaerens* DSM 8721^T, was 96.1% similar based on comparison of nearly full length (>1500 bp) 16S rRNA gene sequences. We proposed that *Bacillus agaradhaerans* DSM 8721^T and five other closely related species be reclassified to the genus *Natrobacillus* gen.

nov. The distinct phylogenetic identity and metabolic capabilities of ML-SRAO^T suggest that it is a novel species, for which the name *Natrobacillus oremlandii* sp. nov. is proposed.

Tthe effects of sulfide on aerobic arsenite oxidation in alkaline lake water samples and in laboratory enrichment cultures were also examined. Significant arsenite oxidation occurred only in treatments with bacteria present, and production of arsenate was greatly enhanced by the addition of sulfide or thiosulfate. IC-ICP-MS analysis of samples showed that mono- and di-thioarsenate formed in arsenite + sulfide amended lake water. Enrichment culture experiments suggest that sulfur-oxidizing bacteria use free or arsenic-bound sulfur as a growth substrate and directly or indirectly transform arsenite and thioarsenates to arsenate during growth. Sulfur-driven arsenite oxidation and microbial thioarsenate transformation may be important biogeochemical processes in the arsenic cycle of our study site (Mono Lake, CA, USA) and other alkaline environments as well.

INDEX WORDS: arsenite oxidation, selenate reduction, sulfide oxidation, microbial arsenic cycling, thioarsenates, Mono Lake, arsenate reductase, *Natrobacillus oremlandii* ML-SRAO

CHARACTERIZATION OF NOVEL ARSENITE OXIDATION PATHWAYS IN MONO LAKE, CALIFORNIA, USA

by

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CHAPTER 1

INTRODUCTION

Arsenic in the environment

Arsenic is a toxic metalloid that is generally found in aqueous environments as the oxyanions arsenate [As(V)] and arsenite [As(III)] (Cullen and Reimer, 1989). The redox behavior of inorganic arsenic species is highly complex (Cullen and Reimer, 1989) and is mediated by chemical reactions such as ligand exchange, precipitation with iron and sulfide, adsorption to clay and metals, and biotic and abiotic oxidation-reduction reactions (Ferguson and Gavis, 1972). Volcanic activity and weathering of arsenic-bearing rocks are the major contributors of arsenic to fresh water and the ocean on geologic time scales (Ferguson and Gavis, 1972). Geothermal waters may also contribute to locally elevated arsenic in groundwater and surface water (Welch et al., 1988). Environmental arsenic concentrations vary widely due to differences in geologic and anthropogenic inputs (Cullen and Reimer, 1989). Typical freshwater arsenic concentrations (in non-impacted areas) are ~0.1-10 μg/L (Cullen and Reimer, 1989; Ferguson and Gavis, 1972; Francesconi and Keuhnelt, 2002). However, lakes and rivers fed by arsenic rich geothermal springs (≥1 mg As/L) can have much higher concentrations (Gihring et al., 2001; Kneebone and Hering, 2000). Arsenic is found (naturally) in the highest concentrations in areas of geothermal or volcanic activity (Hering and Kneebone, 2002). Many groundwater sources and lakes within the Great Basin region of the western US have elevated arsenic concentrations

that are 3-4 orders of magnitude greater than the average for natural water (Levy et al., 1999; Oremland et al., 2004).

The relative concentrations of arsenate and arsenite in a solution (or water body) depend on the pH, pE, and biological activity. The Eh of the As(III)/As(V) redox couple (+60 mV; ref. (Anderson et al., 2002) is well within the range of redox levels found in natural environments, which allows arsenate and arsenic to take part in abiotic or biologically mediated redox reactions (Inskeep et al., 2002). While As(III)/As(V) may respond to changes in pE, these species are not electroactive and cannot significantly impact the redox state of a system (as opposed to oxygen or sulfide, (Maest et al., 1992).

The redox state of arsenic is important because it affects the degree and mode of toxicity (Cullen and Reimer, 1989; Mukhopadhyay et al., 2002; Oremland and Stolz, 2003; Silver et al., 2002). Arsenate can substitute for phosphate in ATP by forming a low-energy ADP-arsenate bond that spontaneously hydrolyzes, uncoupling respiratory function (Anderson et al., 2002). Arsenite is much more generally toxic than arsenate and acts by binding to sulfhydryl groups in proteins (Anderson et al., 2002). Many prokaryotes can reduce arsenate as a detoxification mechanism using the well-characterized ars system of arsenate reductase genes (Mukhopadhyay et al., 2002; Silver et al., 2002), and some can reduce the toxicity of arsenite via oxidation to arsenate (Salmassi et al., 2002). Biological redox transformation of arsenic can also be an energy-conserving process for some microbes (Stolz et al., 2006). The ability to respire arsenate is widely distributed among prokaryotic groups and is found in low G+C Gram positive bacteria, several divisions of the Proteobacteria, and even Archaea (Oremland et al., 2002). Other organisms can obtain energy for chemolithoautotrophic growth by oxidizing arsenite (Oremland et al., 2002; Santini et al., 2000).

The formation of soluble arsenic-sulfide complexes is another well-recognized phenomenon (Helz et al., 1995; Hollibaugh et al., 2005; McCay, 1901; Planer-Friedrich et al., 2007; Wilkin et al., 2003; Wood et al., 2002), and arsenic chemistry in sulfidic environments may be governed by the formation of thioarsenic species (Planer-Friedrich et al., 2007; Wilkin et al., 2003). Recent studies have focused on identification and quantification of thioarsenate species (also referred to as thioarsenites in some literature) in both laboratory solutions and in environmental samples (Planer-Friedrich et al., 2007; Stauder et al., 2005; Wallschläger and Stadey, 2007). Analysis of environmental samples has confirmed the laboratory results, with arsenic-thiol compounds comprising ≥50% of the total As in alkaline, sulfidic waters (Hollibaugh et al., 2005; Planer-Friedrich et al., 2007; Stauder et al., 2005). Little is known about the bioavailability of these compounds, although they are reported to be less toxic than arsenite (Rader et al., 2004).

Arsenic cycling in Mono Lake

Mono Lake receives hydrologic inputs from multiple sources, including stream flow, runoff, precipitation, and geothermal waters (Miller et al., 1993). Mono Lake is a terminal lake; thus, "outflow" is equal to the rate of evaporation (Miller et al., 1993). The volume of lake has varied significantly over geologic (Oremland et al., 2004) and decadal time scales (Maest et al., 1992). The depth of the lake decreased by ~13 m in <40 years due to diversions to the Los Angeles water supply that began in 1941 (Maest et al., 1992) and increased by >2 m between 1992 and 1997 due to heavy precipitation and a reduction in fresh water diversion (Melack and Jellison, 1998).

Mono Lake is an alkaline, saline lake with ~90 g/L dissolved salts and a pH of 9.8 (Maest et al., 1992). High (~200 μM) arsenic and salt concentrations in Mono Lake are the result of

weathering of arsenic-rich volcanic rocks and hydrothermal inputs from local hot springs (Maest et al., 1992) combined with high rates of evaporation (Oremland and Stolz, 2003). High alkalinity (~0.4 M HCO_3^-/CO_3^{2-}) buffers the system (Oremland et al., 2004); and processes such as photosynthesis, which may cause significant increases in pH (>2 units) in many freshwater lakes (Kneebone and Hering, 2000), do not affect the pH in Mono Lake (Maest et al., 1992). Phosphate concentrations are >100 times greater than algal uptake demand, and nitrogen is the limiting nutrient in the epilimnion for much of the year (Melack and Jellison, 1998). Arsenic concentrations are dominated by inorganic species; free organoarsenicals do not contribute significantly to the arsenic budget (Oremland et al., 2004). Thioarsenic compounds account for a considerable portion of the total arsenic in sulfidic bottom waters during prolonged stratification (Hollibaugh et al., 2005; Oremland et al., 2004). Arsenic-sulfide solids do not precipitate due to the high pH, and sorption is limited because iron and manganese concentrations are very low (0-10 μM). Thus, sediments are not a significant sink for arsenic (Oremland et al., 2004).

A combination of long term and short term physical processes contributes to alternating periods of meromixis and monomixis (Jellison et al., 1998; Maest et al., 1992), which in turn affect the redox conditions in the lake (Maest et al., 1992; Melack and Jellison, 1998). Lake overturn following an extended period of stratification (1984-1988) resulted in uniform concentrations of total As and Fe throughout the water column (Maest et al., 1992). However, distributions of redox species were inconsistent with those expected due to mixing. Nearly all As(III) was oxidized during mixing, possibly due to biological oxidation (Maest et al., 1992). Sulfide disappeared from the water column during mixis in 1988, but was likely not oxidized completely to sulfate due to insufficient oxygen concentrations (Miller et al., 1993). A second period of extended meromixis occurred in the 1990s, allowing sulfide, arsenite, and thioarsenic

compounds to accumulate in the monimolimnion. After mixis, arsenate was the only As species detectable in the epilimnion (Hollibaugh et al., 2005).

Biological arsenic cycling in Mono Lake differs from most waters that have been studied for two reasons: 1) the concentration of arsenic is 2-3 orders of magnitude higher in Mono Lake, and 2) the two main modes of arsenic toxicity are significantly reduced by the chemistry of Mono Lake. This presents the potential for arsenic cycling to be dominated by biology, and by energy-generating, rather than detoxification, processes. Because arsenite is uncharged below pH 9.2, it can easily enter cells (typically through aqua-glyceroporins (Mukhopadhyay et al., 2002). Arsenite exists in an ionized form in Mono Lake ($H_2AsO_3^-$) due to the high pH (9.8) of the system, and thus has a different chemical behavior than in most environments where it is fully protonated ($H_3AsO_3^0$). Phosphate concentrations in Mono Lake are >3 times greater than arsenic concentrations, which potentially reduces arsenate toxicity due to interference with ATP formation (Silver et al., 2002).

Respiratory arsenate reduction in Mono Lake may account for a sizeable portion of the organic matter remineralized in the anoxic portion of the water column (Oremland et al., 2004), and rates are limited by the supply of arsenate (Hoeft et al., 2002; Oremland et al., 2004). Recent experiments suggest that sulfide oxidation may also drive arsenate reduction (Hoeft et al., 2004; Hollibaugh et al., 2006). A coupled system of arsenate reduction by heterotrophic arsenate respirers and arsenite oxidation by chemolithotrophic or heterotrophic arsenite oxidizers was proposed for Mono Lake (Oremland and Stolz, 2003). Thus far, the only account of biological arsenite oxidation stems from enrichment experiments where nitrate was added to anoxic lake water samples (Oremland et al., 2002). Although nitrate is present in low concentration (high nM- low μM), it may cycle rapidly due to ammonia oxidation (Joye et al., 1999) and could be a

renewable oxidant for arsenite oxidation. Nitrate (at much higher concentrations relative to arsenite) was shown to oxidize a significant portion of reduced arsenic in Upper Mystic Lake (Senn and Hemond, 2002) and to inhibit arsenic transport in groundwater in Bangladesh (Harvey et al., 2002).

Arsenite oxidation in Mono Lake

Much is known about the reductive portion of the microbial arsenic cycle in Mono Lake (Hoeft et al., 2004; Hoeft et al., 2002; Oremland et al., 2000; Switzer Blum et al., 1998); however, the oxidative portion of the cycle wherein As(III) is reoxidized to As(V) has not been thoroughly characterized. With the exception of *Alcalilimnicola ehrlichii* strain MLHE-1, an anaerobic arsenite oxidizer (Oremland et al., 2002), no arsenite oxidizing bacteria have been isolated from Mono Lake; and only one report of aerobic arsenite oxidation has been published (Kulp et al., 2007). Abiotic oxidation of arsenite with oxygen is a slow process (Inskeep et al., 2002) that occurs on a time scale of days to months, while microbial oxidation can be several orders of magnitude faster, with turnover times on the order of hours (Inskeep et al., 2002). Thus microbial oxidation may be responsible for the rapid production of As(V) that has been observed throughout the water column soon after a mixing event (Maest et al., 1992) and may be an important feature of the microbial arsenic cycle. Studies have also shown that anaerobic arsenite oxidation can occur in enrichments from Mono Lake bottom waters and sediments (Oremland et al., 2002). Bacteria were able to oxidize arsenite using terminal electron acceptors such as nitrate and iron that are found at low but significant concentrations in seasonally anoxic bottom waters (Hoeft et al., 2002; Oremland et al., 2002).

Other arsenic species (mono-, di-, and tri-thioarsenate) have been measured in Mono Lake and make up a significant portion of the total dissolved arsenic pool in anoxic waters when

sulfide is present (Hollibaugh et al., 2005). These compounds may also serve as a substrate for microbial oxidation via pathways similar to those responsible for arsenite oxidation.

One organism capable of arsenite oxidation (*Alkalilimnicola ehrlichii*, strain MLHE-1) has been isolated from Mono Lake (Hoeft et al., 2007; Oremland et al., 2002). The 16S rRNA gene sequence of this organism was not present in clone libraries from Mono Lake, suggesting that it is not a numerically dominant species. If the majority of arsenite oxidation in the lake is biologically driven, then other organisms with this capability must also be present. Isolation of such organisms (both aerobic and anaerobic) will provide further insight into the oxidative portion of the Mono Lake arsenic cycle.

The following chapters describe the studies performed to answer some of the remaining questions concerning arsenite oxidation processes in Mono Lake and to identify the organisms responsible for these processes. Chapter 2 describes a new pathway for anaerobic arsenite oxidation using selenate as the terminal electron acceptor. This process is shown to occur both in amended lake water samples and in cultures of an isolate, strain ML-SRAO. Chapter 3 provides a detailed characterization of this isolate, which represents a new bacterial species. Phylogenetic analysis shows that this organism and its close relatives should also be classified as a new genus within the family Bacillaceae, and the designation *Natrobacillus*, gen. nov., is proposed. Chapter 4 examines aerobic arsenite oxidation and the effects of sulfur on this process. Arsenite oxidation is enhanced by the presence of reduced sulfur species due to arsenic-sulfur chemistry and stimulation of sulfur-oxidizing bacteria. These bacteria also catalyze the production of arsenate by transformation of thioarsenate species. Chapter 5 synthesizes the results of these studies and discusses the significance of these processes for arsenic cycling in Mono Lake.

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CHAPTER 2

SELENATE-DEPENDENT ANAEROBIC ARSENITE OXIDATION BY A BACTERIUM FROM MONO LAKE, CA

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ABSTRACT

Arsenate was produced when anoxic Mono Lake water samples were amended with arsenite and either selenate or nitrate. Arsenite oxidation did not occur in filtered or killed control samples. Potential rates of anaerobic arsenite oxidation with selenate were comparable to those with nitrate (~12-15 $\mu mol \cdot l^{-1} hr^{-1}$). A pure culture, strain ML-SRAO, capable of selenate-dependent anaerobic arsenite oxidation was isolated from Mono Lake water into a defined salts medium with selenate, arsenite, and yeast extract. This strain does not grow chemoautotrophically, but it catalyzes the oxidation of arsenite during growth on an organic carbon source with selenate. No arsenate was produced in pure cultures amended with arsenite and nitrate or oxygen. Experiments with washed cells in mineral medium demonstrated that the oxidation of arsenite is tightly coupled to the reduction of selenate. Strain ML-SRAO grows optimally on lactate with selenate or arsenate as the electron acceptor. The arsenate reductase gene (*arrA*) from strain ML-SRAO is very similar to *arrA* sequences from two previously isolated Mono Lake arsenate reducers. The 16S rRNA gene sequence of strain ML-SRAO places it within the *Bacillus* RNA group 6 of the low G+C Gram-positive bacteria.

INTRODUCTION

Arsenic (As) and selenium (Se) are naturally-occurring metalloids, typically present in aqueous environments as the dissolved oxyanions arsenite [As(III)] or arsenate [As(V)], (Cullen and Reimer, 1989) and selenite [Se(IV)] or selenate [Se(VI)], (White, 1994). Global geochemical cycling of arsenic and selenium occurs by tectonic uplift, sedimentation (Haygarth, 1994), volcanic activity (Ferguson and Gavis, 1972; Presser, 1994), rock weathering (Ferguson and Gavis, 1972), and geothermal hydrologic inputs (Welch et al., 1988). However, local cycling and transformations occur primarily by biological reduction, assimilation, and volatilization (Shrift, 1964; Stolz et al., 2006).

Many Bacteria and some Archaea can reduce selenate and/or selenite, either as a detoxification strategy or for energy generation via a dissimilatory pathway (Stolz et al., 1999). The ability to respire arsenate is also widely distributed among prokaryotes and is found in low G+C Gram positive bacteria, several divisions of the *Proteobacteria*, and even Archaea (Oremland et al., 2002). Many prokaryotes can also reduce arsenate as a detoxification mechanism using the well-characterized *ars* system of arsenate reductase genes (Mukhopadhyay et al., 2002; Silver et al., 2002). Other organisms can obtain energy for chemolithoautotrophic growth by oxidizing arsenite (Oremland et al., 2002; Santini et al., 2000).

Several organisms capable of transforming selenium and arsenic have been isolated from Mono Lake, a hypersaline, alkaline lake in eastern California (Hoeft et al., 2004; Oremland et al., 2002; Switzer Blum et al., 1998). One of these organisms, *Alkalilimnicola ehrlichii* str. MLHE-1, oxidizes arsenite anaerobically using nitrate as an electron acceptor (Oremland et al., 2002). This metabolism was proposed to be the source of the spikes of arsenate observed in anaerobic, sulfidic water in which arsenite and thioarsenic compounds were abundant (Hollibaugh et al.,

2005; Oremland et al., 2000). Nitrate, present in Mono Lake at low concentrations (Maest et al., 1992), could potentially serve as an oxidant for an equivalent or greater amount of arsenite, depending on whether it was reduced to nitrite or N_2. However, another compound may also serve to oxidize arsenite. A previous Mono Lake study found that arsenate reduction in anoxic waters was inhibited by the addition of selenate (Hoeft et al., 2002), which is also present in the lake at low concentrations (<1 μM; J.T. Hollibaugh, unpublished data). The observed inhibition could be due to preferential use of selenate over arsenate or to the oxidation of arsenite (produced from arsenate reduction) by selenate. The E'_0 of the selenate/selenite couple (+440 mV, ref. (Doran, 1982)) is higher than that of arsenate/arsenite (+60 mV, ref. (Kulp et al., 2006), which makes it a feasible oxidant for arsenite. In this study we examined the potential for anaerobic arsenite oxidation using selenate as a terminal electron acceptor. Experiments showed that this pathway was active in enriched lake water samples and could contribute to the observed arsenate disequilibrium in anoxic lake waters. A bacterium was also isolate that could oxidize arsenite using selenate as the electron acceptor.

MATERIALS AND METHODS

Sample collection. Samples were collected from Station 6 of Mono Lake, CA, USA (37º 57.822' N, 119º 01.305' W) in April and August of 2004. Water samples (from 28-35 m depth, anoxic) were collected and stored as previously described (Hollibaugh et al., 2005).

Mono Lake water incubations. All enrichments were set up in an anaerobic chamber (<2% H_2, 98% N_2 atmosphere). Anaerobic conditions were maintained throughout the experiments. Whole (live) lake water samples were distributed into serum vials, capped with butyl rubber stoppers, and crimp-sealed. Filter-sterilized (0.22 μm pore size) As(III) as $NaAsO_2$, and Se(VI)

as Na_2SeO_4, or nitrate as $NaNO_3$, were added to final concentrations of 2 mM or 5 mM. Controls were 0.22 μm-filtered lake water with buffered formalin (1% v/v final concentration). Whole lake water was also diluted 1:25 with artificial Mono Lake water (AMLW) with no organic carbon source. These treatments were amended with 1 mM arsenite and selenate.

Isolation and growth of strain ML-SRAO. Enrichment cultures from lake water were transferred to an alkaline (150 mM carbonate; pH ~9) and saline (~80 g/l) artificial Mono Lake water medium (AMLW) that was modified from the medium used previously for the isolation of another Mono Lake bacterium (Hoeft et al., 2004). The AMLW medium contained (in g/l): NaCl (60), $(NH_4)_2SO_4$ (0.5), $MgSO_4 \cdot 7H_2O$ (0.25), KCl (1.7), Na_2BO_4 (2), Na_2SO_4 (16.5), KH_2PO_4 (0.25), and KH_2PO_4 (0.5). The medium also contained vitamins (Oremland et al., 1994) and trace metals (Widdel et al., 1983). The medium was bubbled with nitrogen and transferred to an anaerobic chamber. Aliquots were dispensed into serum bottles, amended with 3-5 mM arsenite and selenate, and sealed with butyl rubber stoppers and crimp-seals. Enrichments were subcultured into fresh medium every 4-8 weeks and were monitored periodically for the production of arsenate. The medium initially contained no added organic carbon, but later a small amount of yeast extract (<0.1% w/v) was added to increase biomass and growth rates. Serial dilutions were made after >6 months of transfers. Attempts to grow the isolate on solid medium were not initially successful, but serial dilution yielded a pure culture. The purity of the culture was confirmed by its uniform cell morphology under an epifluorescence microscope, denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes in the culture, and an unambiguous, nearly full length 16S rRNA gene sequence obtained directly from DNA extracted from the culture. Growth curves for ML-SRAO were measured with 10 mM lactate as an electron donor and carbon source and 10 mM arsenate or selenate as a terminal electron

acceptor. Cells were enumerated by direct counts using epifluorescence microscopy after staining with DAPI (Porter and Feig, 1980).

Coupled arsenite oxidation/selenate reduction experiment. Anaerobic arsenite oxidation and selenate reduction rates were determined for cultures amended with 5 mM selenate as the electron acceptor and 5 mM arsenite, 5 mM lactate, or 2.5 mM arsenite and lactate as the electron donors. An inoculum of selenate- and lactate-grown cells from a pure culture of strain ML-SRAO was added to a final cell concentration of ~10^7 cells per ml.

Washed cell experiments. Strain ML-SRAO was grown to a density of ~10^8 cells per ml on lactate and selenate. Cells were centrifuged (15 minutes at 5000 × g at 4 ºC) and resuspended twice with fresh mineral medium to remove any residual organic carbon. Selenate and arsenite (3 mM each) were added to three replicate treatments to initiate the reaction. The control treatment had only selenate (3 mM). The final cell densities for the treatments and controls were ~10^7 cells per ml.

Chemical analyses. Oxygen was measured in lake water with a YSI oxygen meter equipped with a Clark-type electrode. Arsenate was measured by the molybdate blue method on a spectrophotometer for the whole lake water experiment (Johnson, 1971). Samples from the lake water dilution enrichment experiment in AMLW were frozen for analysis of arsenic and selenium species by a modified IC-ICP-MS method (Dionex IC, Perkin Elmer Elan 6000 ICP-MS; (Jackson et al., 2003). Samples were diluted with anoxic Milli-Q water and were filtered (pore size = 0.22 μm) prior to analysis. Selenium and arsenic species were separated with a Dionex AS16 anion exchange column and eluted by a step gradient (20-50 mM tetramethylammonium hydroxide) at a flow rate of 1-1.5 ml min^{-1}. Arsenate, arsenite, and selenite in ML-SRAO culture experiments were analyzed using high performance liquid

chromatography as previously described (Hoeft et al., 2002), Fisher et al., in press). Samples were analyzed for total dissolved Se at the University of Georgia Chemical Analysis Lab in Athens, GA, and selenate concentrations were calculated as $Se_{(total)}$-selenite.

Molecular analysis. Environmental water samples, enrichment cultures and isolate cultures were filtered onto Sterivex® or Millipore® (pore size = 0.22 μm) nitrocellulose filters. DNA was extracted from cells collected on the filters using either a phenol-chloroform method (Ferrari and Hollibaugh, 1999) or a MoBio Soil Extraction Kit according to the manufacturer's instructions (Bio 101, Solana Beach, CA). PCR was performed on the extracted DNA to amplify the 16S rRNA gene as previously described using universal primers 27F and 1492R (Bano and Hollibaugh, 2002). PCR products of the correct size (~1500 bp) were excised from a 1.5% agarose gel and purified with a Qiaquik® gel extraction kit (QIAGEN, Valencia, CA). The amplicon was sequenced directly using a BigDye terminator sequencing reaction kit (Applied Biosystems, Foster City, CA). All nucleotide sequences were determined at the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia (Athens, GA). The 16S rRNA gene sequence was compared to others in the GenBank database using BLAST (Altschul et al., 1990) and was aligned with similar sequences using ClustalW (Thompson et al., 1994). A 16S rDNA phylogenetic tree and bootstrap values (1,000 replicates) were generated using Jukes-Cantor evolutionary distances and the neighbor-joining method in MEGA4 (Tamura et al., 2007).

A portion of the *arrA* (respiratory arsenate reductase) gene of strain ML-SRAO was also amplified (Hollibaugh et al., 2006) using published primers (Malasarn et al., 2004). The amplicon (~112 bp) was cloned using a TOPO® 2.1 kit, purified with a Qiagen® plasmid prep kit, prepared for sequencing as previously described (LeCleir et al., 2004), and sequenced at

MGIF. Primer walking was performed to obtain a larger portion (~600 bp) of the gene. The full length *arrA* sequences of *B. arseniciselenatis* and *B. selenitireducens* were aligned in BioEdit (Hall, 1999), and primers were designed from conserved regions in these two sequences to be used as the forward primer (arrAf235, 5'-ATACGATTGCCGATAA-3'). An internal sequence from the *arrA* of ML-SRAO was used as the reverse primer (arrArw2, 5'-TATCTTTTAGCGCTAAATTC-3'). PCR was performed in 25 µl reactions with puRe Taq™ Ready-To-GO PCR™ Beads (Amersham Biosciences, Buckinghamshire, UK) and 0.4 µM of each primer. Samples were denatured at 95° C for 10 min, followed by 40 cycles of denaturing at 95° C for 15 s, annealing at 50° C for 45 s, and extension at 72° C for 1 min. The cycles were followed by a final extension at 72° C for 15 min. Bands of the expected size were excised from a 1% agarose gel, purified, cloned, and sequenced as described above. An *arrA* neighbor-joining tree and bootstrap values (1,000 replicates) were computed using the Maximum Composite Likelihood method in MEGA4 (Tamura et al., 2007).

Accession numbers. The 16S rDNA and *arrA* sequences of strain ML-SRAO were deposited into GenBank under the accession numbers EU186648 and EU188649.

Electron Microscopy. Samples were fixed with 2% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) and 1% osmium tetraoxide (OsO_4) in 0.1 M PBS. After dehydration with ethanol, samples were dried with liquid CO_2 in a critical point drier and sputter-coated with gold. Electron micrographs were taken with a LEO model 982 field emission scanning electron microscope.

RESULTS

Lake water enrichment experiment. Arsenate was produced after an initial lag period in live lake water treatments amended with arsenite and either selenate or nitrate (Figure 2.1). Little or no arsenate was produced in control samples with either electron acceptor. Potential arsenite oxidation rates were 12.3 ± 1.3 μmol $l^{-1}hr^{-1}$ with selenate as the terminal electron acceptor and 14.5 ± 2.8 μmol $l^{-1}hr^{-1}$ for nitrate. Final arsenate concentrations (2.1 ± 0.2 mM) in treatments with selenate were equal to the amount of arsenite added. Arsenite oxidation occurred after a slightly longer lag time (~96 hours) in the nitrate treatment. Most of the added arsenite (5 mM) was oxidized to arsenate (3.8 ± 0.9 mM).

Lake water dilution experiment. A lag time of ~3 weeks occurred when lake water samples were diluted (1:25) into artificial Mono Lake water to assess the potential for autotrophic arsenite oxidation with selenate (Figure 2.2). Decreases in selenate and arsenite were mirrored by increases in arsenate and selenite (Figure 2.2), suggesting tight coupling between arsenite oxidation and selenate reduction in this medium. Arsenite oxidation rates were much slower in AMLW medium than in lake water samples.

Isolation and growth of strain ML-SRAO. A selenate-reducing, arsenite-oxidizing culture was established after several months of repeated transfers in artificial lake water. Doubling times for cells in the enrichment culture were extremely long (≥1 week), and cell densities were low (OD_{600}< 0.01) when grown in mineral medium with selenate, arsenite, and trace amounts of yeast extract (0.01% w/v). More rapid growth and arsenite oxidation rates were observed when cells were grown with arsenite, excess selenate, and a low concentration (<1 mM) of sucrose. Serial dilution was performed, and strain ML-SRAO was isolated from the highest dilution exhibiting selenate-driven anaerobic arsenite oxidation (10^{-7}). The purity of the culture was

confirmed by sequencing the 16S rRNA gene directly from DNA extracted from the culture, by DGGE analysis and by observation of uniform cell morphology by epifluorescence and phase contrast microscopy.

Strain ML-SRAO is a Gram-positive rod (~0.5 μm wide and ~4-6 μm in length; Figure 2.3) that catalyzes a novel, selenate-dependent anaerobic arsenite oxidation. This organism cannot grow autotrophically on these substrates; it requires an organic carbon source. ML-SRAO can also grow using lactate or sucrose as a carbon source and electron donor with selenate or arsenate as the terminal electron acceptor. Cells grown on lactate and selenate or arsenate reached densities $>10^8$ cells per ml ($OD_{600} > 0.100$, Figure 2.4). Cells grew to the highest densities (~10^9 cells per mL) and had shorter doubling times for growth (~7.2 hours) when grown on selenate and lactate (Figure 2.4A) versus arsenate and lactate (~12 hours; Figure 2.4B).

Coupled arsenite oxidation/selenate reduction. We examined the ability of strain ML-SRAO to grow in the presence of arsenite to determine if energy could be conserved during the arsenite oxidation process. Arsenate and selenite both increased linearly in the 5 mM selenate and arsenite (SA) treatment (Figure 2.5A) as arsenite and selenate concentrations decreased. Most, but not all, of the added arsenite (~75%) and selenate (~80%) were transformed during the course of the experiment. Treatments with 5 mM selenate, 2.5 mM arsenite, and 2.5 mM lactate (SLA; Figure 2.5B) completely converted the added selenate and arsenite to selenite and arsenate. The selenite production rate remained constant in this treatment as well, in contrast to increasing rates of selenite production observed in the 5 mM selenate and lactate treatment (SL; Figure 2.5C). Cell counts taken at the end of this experiment showed that the SL treatment had the highest cell density (~10^8 cells per ml); SLA exhibited some growth (7×10^7); and SA had little or no growth (~10^7).

Several control experiments were performed to determine if this reaction was of a chemical nature, or if an intermediate metabolite that formed during selenate reduction could catalyze the oxidation of arsenite. No arsenate was produced in filtered selenate-reducing culture medium amended with arsenite, cell-free medium with selenate and arsenite, or culture medium with heat-killed cells (data not shown). All control experiments indicated that live, active cells were required for this process to occur.

Washed cell experiment. Suspensions of ML-SRAO cells washed with medium containing no organic carbon were also able to oxidize arsenite and reduce selenate (Figure 2.6A). No selenite production occurred in the treatment without arsenite (Figure 2.6A). Previous experiments (data not shown) also confirmed that arsenite was not oxidized if selenate was not present or if cells were absent or heat-killed. Arsenate and selenite production were closely coupled (Figure 2.6B) and had close to a 1:1 relationship ($m=0.93$, $r^2=0.9998$, $p<0.0001$) based on Model II-type correlation and regression. No growth was observed under these experimental conditions, but cell density did not decrease.

Phylogenetic Alignment. Strain ML-SRAO belongs to the *Bacillus* genus of the low G+C Gram-positive bacteria based on BLAST analysis of its 16S rRNA gene. Alignments using ClustalW revealed that it was distinctly different from, yet closely related to, Mono Lake isolates *B. arseniciselenatis* and *B. selenitireducens* (Figure 2.7). The 16S rRNA gene sequence of ML-SRAO was ≤ 95% similar to any of the sequences previously recovered from Mono Lake (Hollibaugh et al., 2006; Humayoun et al., 2003) and 96% similar to the nearest cultured relative, *B. agaradhaerens*.

ML-SRAO also possessed a respiratory arsenate reductase (*arrA*), a portion of which (>600 base pairs) was amplified and compared to sequences in GenBank. The *arrA* of strain

ML-SRAO was highly similar to those of *Bacillus arseniciselenatis* (78%) and *B. selenitireducens* (75%, Figure 2.8) based on a BLAST analysis. The *Bacillus arrA* sequences formed a clade distinct from the other Firmicutes, *A. metalliredigenes* and *Desulfosporosinus* sp. Y5.

DISCUSSION

Arsenite oxidation rates in the whole lake water treatments with either selenate or nitrate as the terminal electron acceptor (Figure 2.1) were similar to rates observed previously in Mono Lake enrichment cultures, which also had a lag time of ~100 hours before oxidation began (Oremland et al., 2002). The filtered controls produced almost no arsenate (Figure 2.1), confirming the biological nature of the arsenite oxidation for both terminal electron acceptors. The much slower arsenite oxidation rates observed in the AMLW experiment inoculated with lake water (Figure 2.2) were most likely due to the significant dilution factor.

The ability to oxidize arsenite has been reported for many prokaryotes (Stolz et al., 2006) in recent years. Some of these organisms use oxygen as a terminal electron acceptor, either for the detoxification of arsenite (Salmassi et al., 2002) or to conserve energy for growth (Rhine et al., 2007; Santini et al., 2002). Others can grow chemoautotrophically with arsenite using nitrate as the terminal electron acceptor, reducing it to nitrite (Oremland et al., 2002) or completely denitrifying to N_2 (Rhine et al., 2006). Strain ML-SRAO cannot use oxygen or nitrate to oxidize arsenite but instead couples arsenite oxidation with selenate reduction. The reaction appears to occur via a two electron transfer from selenate to arsenite, producing selenite and arsenate:

$$H_2AsO_3^- + HSeO_4^- \rightarrow HAsO_4^{2-} + SeO_3^{2-} + 2\ H^+ \quad (\Delta G'_0 = -94.5\ kJ/mol) \qquad (1)$$

Approximately 0.93 moles of arsenate were produced for every mole of selenite in our experiments with washed cells free of organic carbon (Fig 6B). This is very close to the theoretical 1:1 ratio predicted by Eq.1. Arsenite oxidation with selenate is thermodynamically more favorable than oxidation with nitrate ($\Delta G'_0$ = -87.2 kJ/mol), which can support autotrophic growth, e.g., *A. ehrlichii* (Oremland et al., 2002). The As(III)/Se(VI) redox couple could provide enough energy for chemolithoautotrophic growth; however, strain ML-SRAO does not grow without a source of organic carbon.

Strain ML-SRAO is able to oxidize arsenite rapidly in the presence of selenate, with or without organic carbon present (Figs. 5 and 6), but the benefit of this oxidation process and the mechanism by which it occurs are uncertain. It does not appear to conserve energy, as cell counts were much lower in treatments with arsenite only, and slightly lower in treatments with lactate and arsenite compared to growth with selenate and lactate. Arsenite oxidation ceased in the washed cell experiment (Figure 2.6A) when the reactants and products reached approximately equal concentrations. This is in contrast to the complete or nearly complete oxidation that occurred when the initial selenate concentration exceeded the arsenite concentration and organic carbon was present (Figs. 5A and 5B). The most rapid and complete arsenite oxidation occurred with actively growing cells in a medium with relatively high (≥ 5 mM) selenate concentrations compared to arsenite (2.5 mM, Figure 2.5B).

Although strain ML-SRAO does not appear to harness energy from arsenite oxidation, it may serve as a detoxification mechanism, similar that of aerobic arsenite oxidizer *Agrobacterium albertimagni* strain AOL15 (Salmassi et al., 2002). This assertion is supported by the growth inhibition observed in treatments with arsenite versus treatments without arsenite (Figure 2.5)

and the lower maximum cell densities when ML-SRAO was grown on arsenate rather than selenate (Figure 2.4).

Strain ML-SRAO is a selenate-reducing, heterotrophic bacterium that can also use arsenate as a terminal electron acceptor. This organism is related to members of the *Bacillus* genus based on its 16S rDNA sequence (Figure 2.7) and shares several of the metabolic capabilities of other previously isolated *Bacillus* species. Strain ML-SRAO is closely related to two other cultured *Bacillus* species from Mono Lake: *B. arseniciselenatis*, which can respire arsenate and selenate, and *B. selenitireducens*, which respires arsenate and selenite (Switzer Blum et al., 1998). *B. macyae*, isolated from an Australian gold mine, is another arsenate-respiring relative (Santini et al., 2004).

The question remains as to how strain ML-SRAO is able to couple arsenite oxidation and selenate reduction and why it carries out this reaction. ML-SRAO could not oxidize arsenite in the presence of nitrate or oxygen (data not shown), so the process appears to be selenate-dependent. We were unable to amplify an arsenite oxidase gene from ML-SRAO using several different primer sets, but we did successfully sequence a portion of the *arrA* gene (Figure 2.8). Many of the characterized anaerobic respiratory enzymes (e.g., selenate reductase, nitrate reductase, arsenate reductase) appear to be substrate-specific (Krafft and Macy, 1998; Santini et al., 2004; Schroder et al., 1997), while others may serve more than one function, *in vitro* and possibly *in vivo* (Afkar et al., 2003). The purified arsenate reductase enzyme from *B. selenitireducens* can reduce selenate, selenite, and arsenite in the presence of an artificial electron donor, although the organism does not respire selenate or arsenite *in vivo* (Afkar et al., 2003). It has been proposed that *A. ehrlichii* (MLHE-1), which oxidizes arsenite but does not have an arsenite oxidase gene in its genome (Hoeft et al., 2007), may express one or both of its

two arsenate reductase-like genes (*arrA*) when oxidizing arsenite (Hoeft et al., 2007). Strain ML-SRAO may be able to use its ArrA for both the reduction and oxidation of arsenic, but our studies have yet to confirm this.

The ecological relevance of selenate-dependent arsenite oxidation is yet to be fully understood, as this is the first account of this process. The selenate/arsenite couple has the thermodynamic potential to support chemoautotrophic growth, although this was not the case for strain ML-SRAO. The discovery of a new mechanism for arsenite oxidation provides a broader context for the biological transformation of toxic metalloids not only in Mono Lake, but in other environments as well. Similar processes could occur in other soda lakes, hydrothermal vents, or metal-polluted soils and waters. The study of novel pathways for arsenite oxidation (e.g. coupled to selenate reduction) can suggest mechanisms for arsenic mobilization or sequestration in diverse natural or impacted environments and may further contribute to the understanding of potential early earth metabolisms.

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Figure 2.1. Arsenate production in water collected from Mono Lake amended with 2 mM arsenite and selenate, ○; 5 mM arsenite and nitrate, ▼; and filter-sterilized controls, ●.

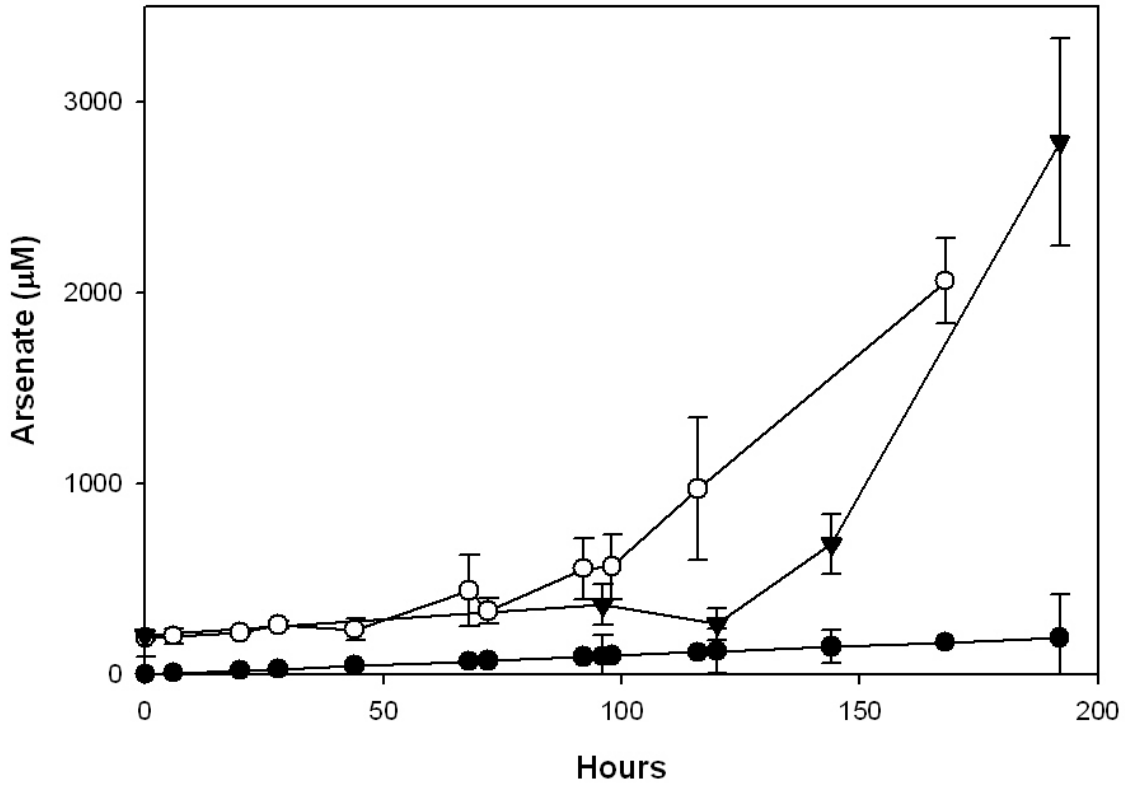


Figure 2.2. Arsenic and selenium speciation in Mono Lake water diluted 1:25 with artificial Mono Lake water and amended with 1 mM arsenite and selenate. Symbols: arsenite, ●; arsenate, ○; selenite, ▼; selenate, Δ.

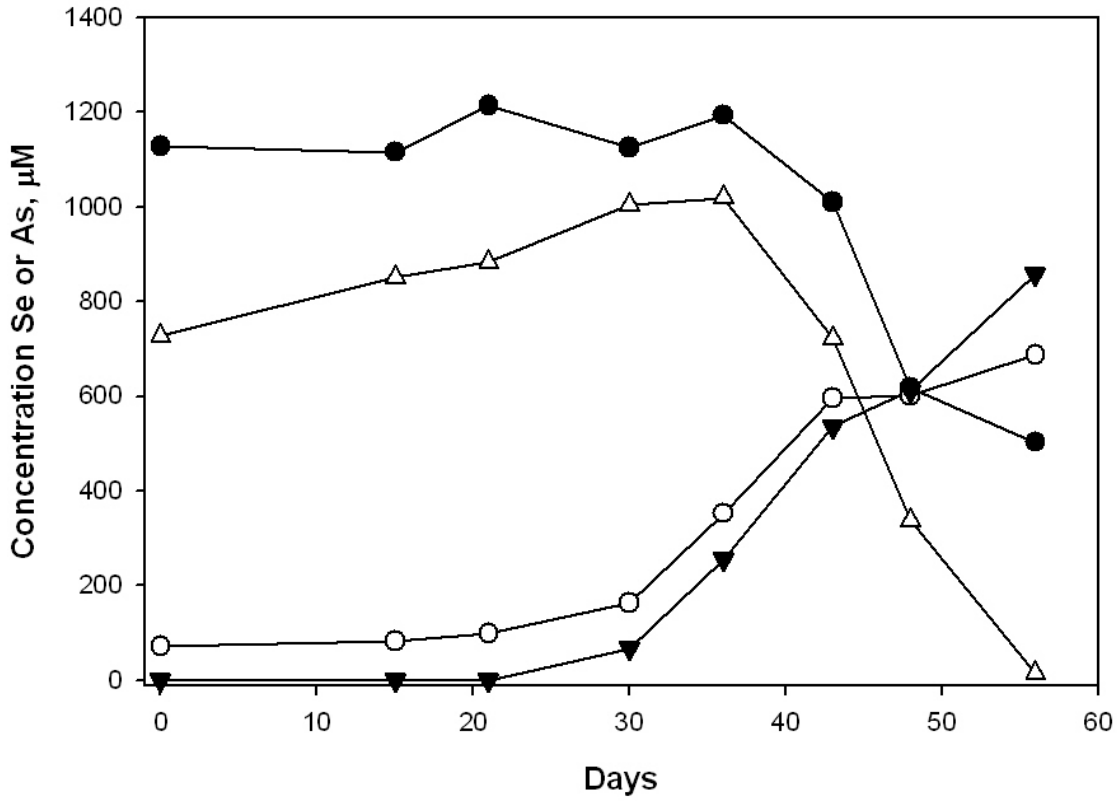


Figure. 2.3. Scanning electron micrograph of strain ML-SRAO. Scale bar = 5 μm.

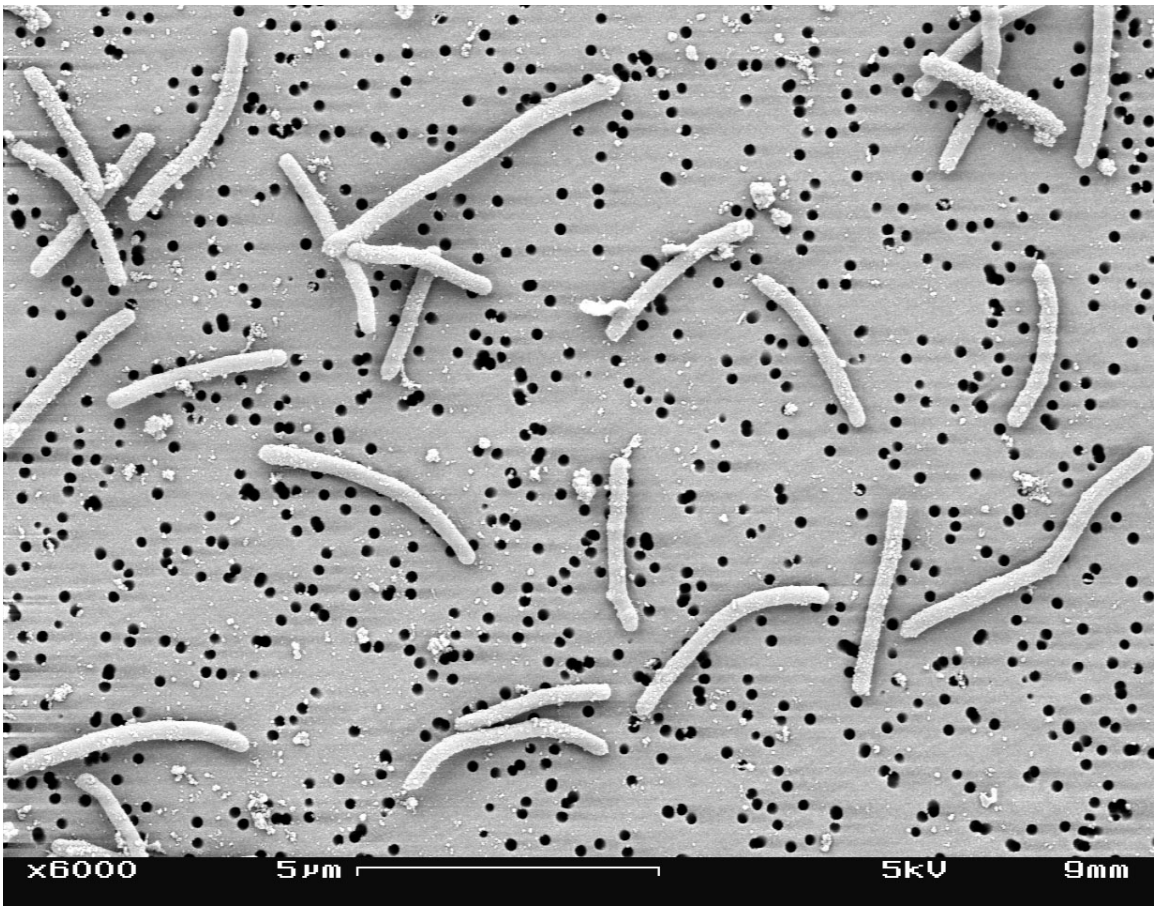


Figure. 2.4. (A) Growth of strain ML-SRAO on selenate and lactate. Symbols: selenite (○), selenate (●), and cell density (▲). (B) Growth of strain ML-SRAO on arsenate and lactate. Symbols: arsenate (●), arsenite (○), and cell density (▲). Symbols represent the mean of three replicates; error bars represent ± 1 standard deviation.

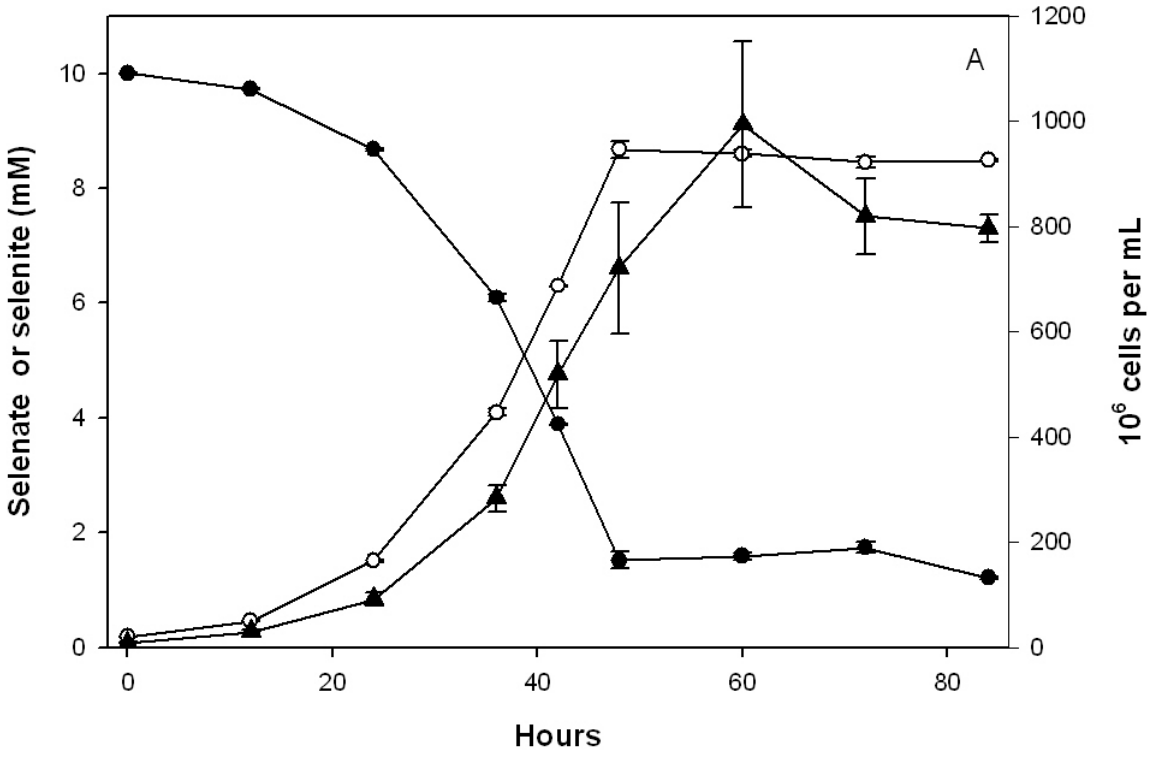


Figure 2.4B.

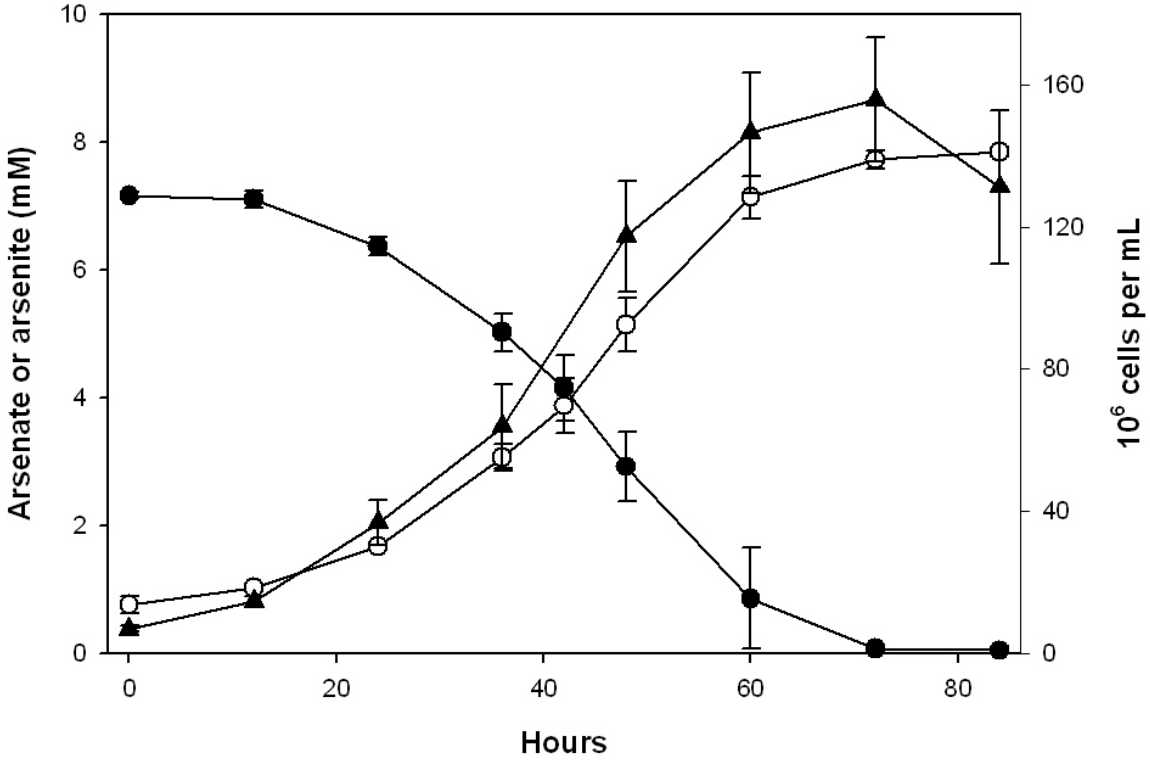

Arsenate or arsenite (mM)
10
8
6
4
2
0
160
120
80
40
0
10^6 cells per mL
0
20
40
60
80
Hours

Figure 2.5. (A) Arsenate (●) and selenite (○) production and arsenite (▼) and selenate (Δ) consumption by strain ML-SRAO in AMLW amended with 5 mM selenate and arsenite. (B) Arsenate (●) and selenite (○) production and arsenite (▼) and selenate (Δ) consumption by strain ML-SRAO in AMLW amended with 5 mM selenate, 2.5 mM lactate, and 2.5 mM arsenite. (C) Selenite produced by strain ML-SRAO in AMLW amended with 5 mM arsenite and selenate (○); 5 mM selenate, 2.5 mM lactate, and 2.5 mM arsenite (▼); or 5 mM selenate and lactate (●). Symbols represent the mean of three replicates; error bars represent ± 1 standard deviation.

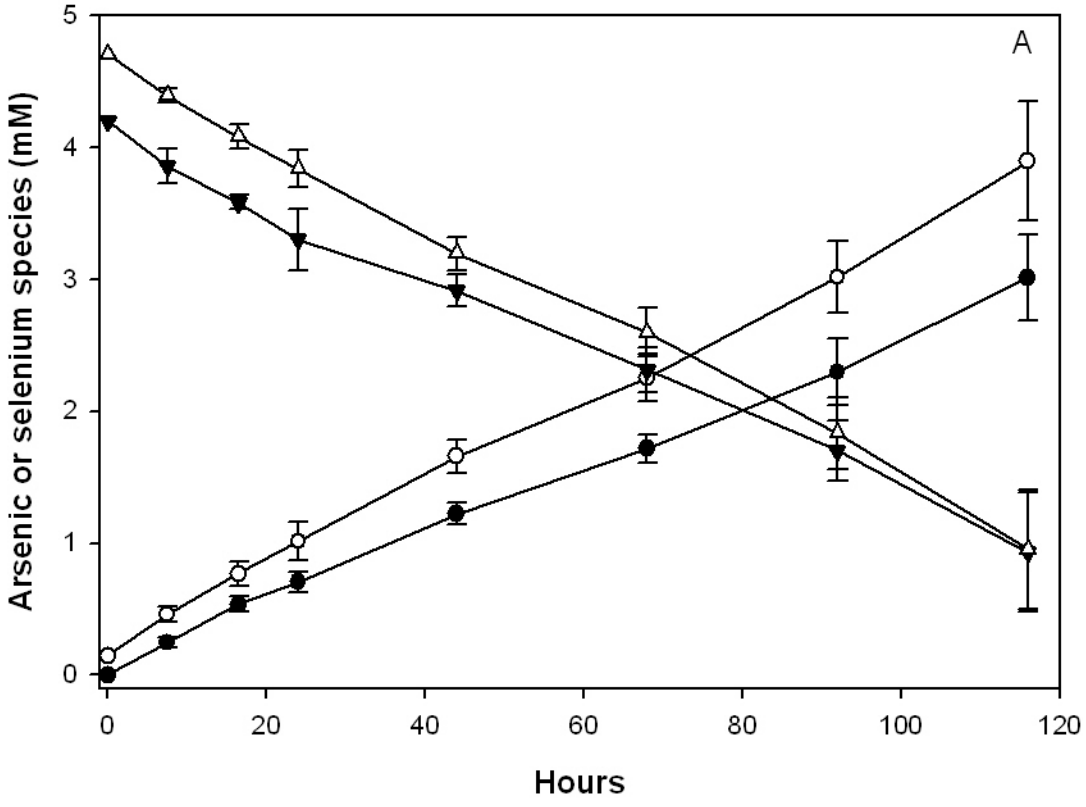


Figure 2.5B.

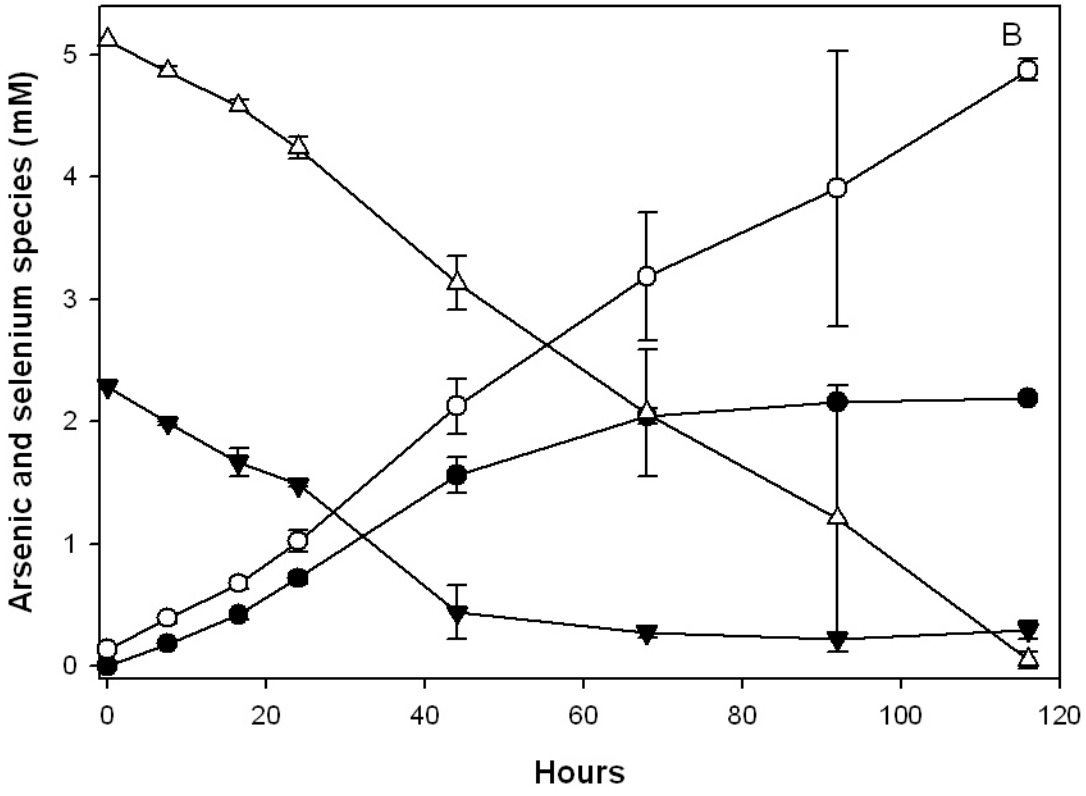

B
Arsenic and selenium species (mM)
0
1
2
3
4
5
0
20
40
60
80
100
120
Hours

Figure 2.5C.

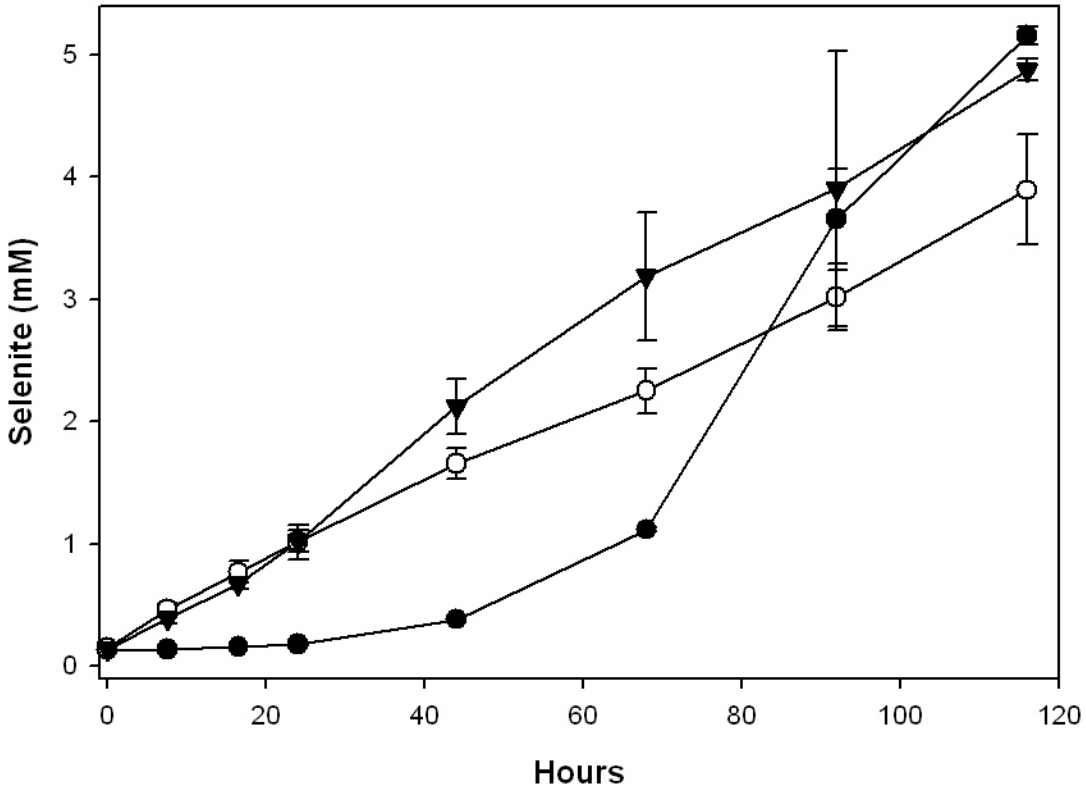


Figure 2.6. (A) Arsenate (●) and selenite (○) production and arsenite (▲) and selenate (Δ) consumption by washed cell suspensions of strain ML-SRAO in AMLW amended with 3 mM selenate and arsenite; controls (■) were amended with 3 mM selenate only. Symbols represent the mean of three replicates; error bars represent ± 1 standard deviation. (B) Arsenate production versus selenite production (●) in washed cell experiments. The solid line (m = 0.93, r^2 = 0.9998, n = 18, p<0.0001) shows the relationship of these two variables. The dashed line represents a 1:1 slope.

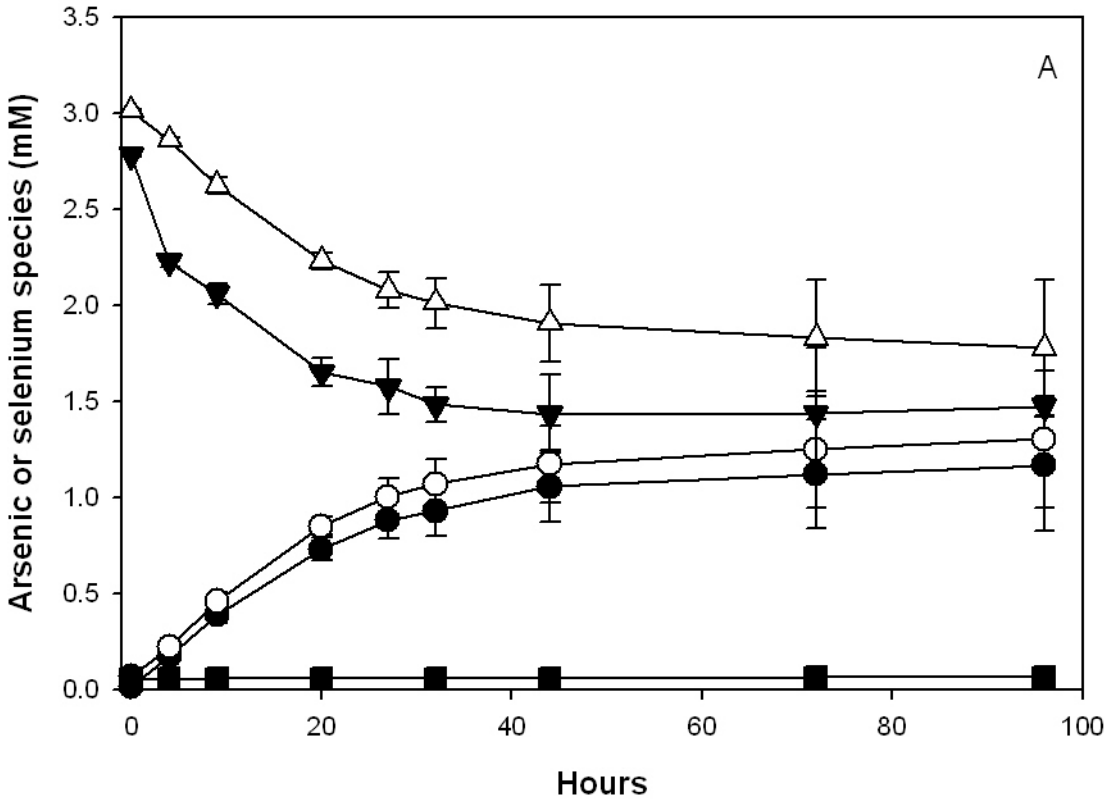


Figure 2.6B.

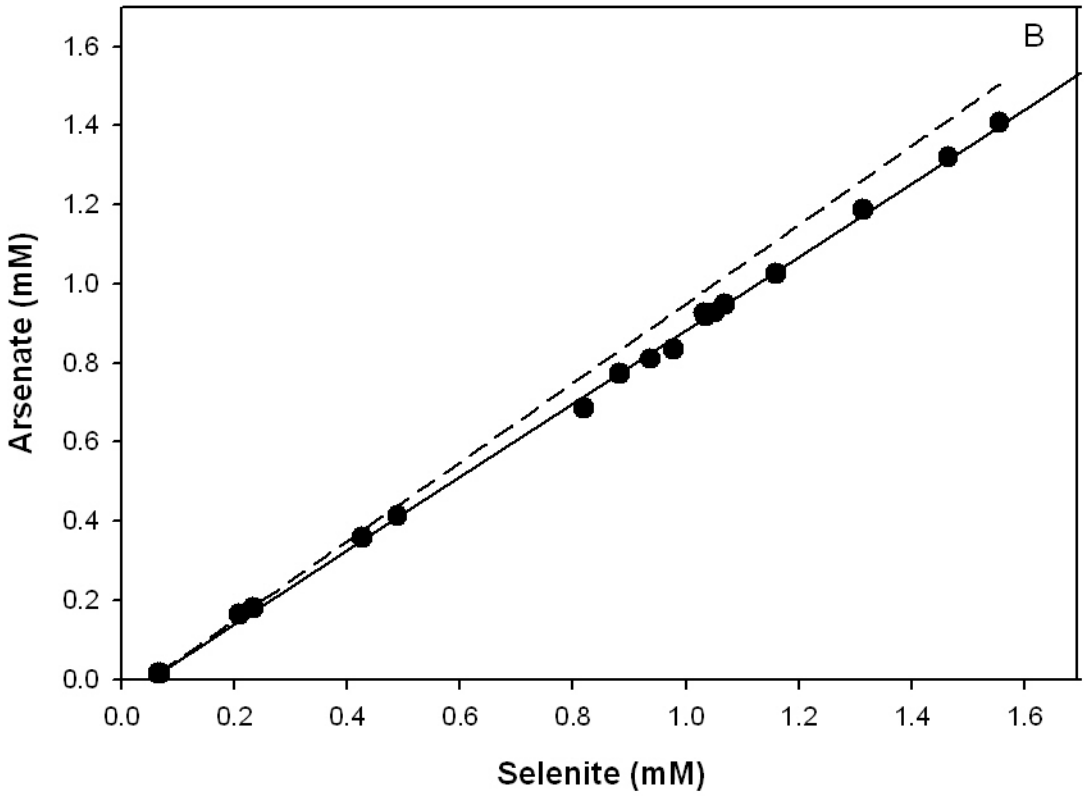


Figure 2.7. Phylogenetic relationships among cultured members of the genus *Bacillus* and closely related genera, environmental clones from Mono Lake (*), and strain ML-SRAO based on nearly full-length (>1400 bp) 16S rRNA gene sequences. The neighbor-joining tree is rooted with the sequence from *Lactobacillus casei*. Bootstrap values (numbers at nodes) were calculated from 1000 iterations; values less than 50% are not shown. Scale bar indicates 0.02 nucleotide substitutions per position. GenBank accession numbers for the sequences are given in parentheses.

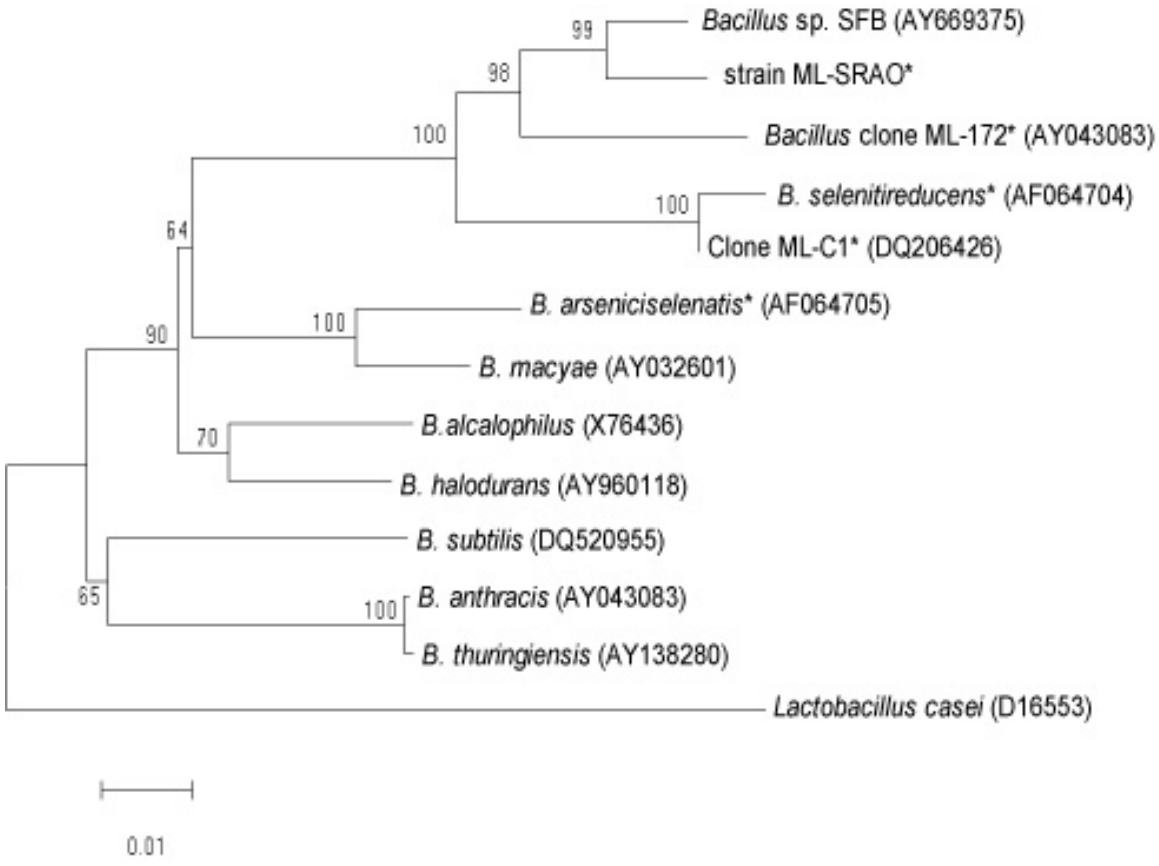
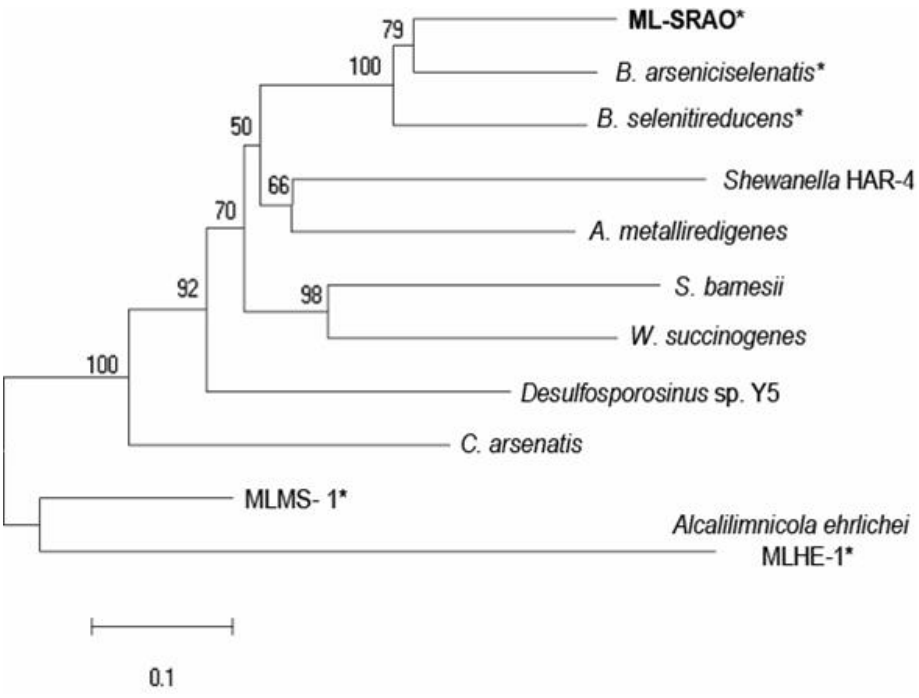


Figure 2.8. Neighbor-joining tree based on partial (~600 bp) sequences of the respiratory arsenate reductase gene (*arrA*) of strain ML-SRAO and other cultured arsenate reducing bacteria. Evolutionary distances were computed using the Maximum Composite Likelihood method. *arrA* sequences from the following organisms were used to construct the tree: *Bacillus arseniciselenatis* (AY660885), *B. selenitireducens* (AY283639), *Shewanella* HAR-4 (AY660886), *Alkaliphilus metalliredigenes* (ZP_00800578), *Sulfurospirillum barnesii* SES-3 (AAU11840), *Wolinella succinogenes* (CAE09880), *Desulfosporosinus* sp. strain Y5 (DQ220794), *Chrysiogenes arsenatis* (AAU11839), and strain MLMS-1 (EAT04932). The tree is rooted with a putative *arrA* sequence from the genome of *A. ehrlichii* MLHE-1 (NC_008340). Bootstrap values (>50% only; based on 1000 iterations) are shown at the nodes of the tree. The scale bar indicates 0.1 nucleotide substitutions per position.



CHAPTER 3

PROPOSAL FOR A NEW GENUS, *NATROBACILLUS* GEN. NOV., AND DESCRIPTION OF *NATROBACILLUS OREMLANDII*, SP. NOV., A SELENATE-REDUCING, ARSENITE-OXIDIZING BACTERIUM FROM A SALINE SODA LAKE (MONO LAKE, CA, USA)

J.C. Fisher and J.T. Hollibaugh. To be submitted to the International Journal of Systematic and Evolutionary Microbiology.

ABSTRACT

A facultative anaerobe capable of selenate-dependent anaerobic arsenite oxidation was isolated from the seasonally anoxic bottom water of Mono Lake, CA, USA. Strain ML-SRAO^T is a Gram-positive, non-motile, spore-forming rod that can respire oxygen, nitrate, and arsenate in addition to selenate. The major cellular fatty acids of ML-SRAO^T are $C_{16:1}\omega7c$ (34.4%) and $C_{16:0}$ (29.1%) when grown anaerobically, and the G+C content is 42.1 mol%. Based on analyses of the 16S rRNA gene sequences of closely related organisms, ML-SRAO^T and six other species from the *Bacillus* RNA group 6 formed a distinct phyletic branch. The nearest relative, *Bacillus agaradhaerens* PN-105^T, was 95.8% similar based on comparison of nearly full length (>1500 bp) 16S rRNA gene sequences. It is proposed that *Bacillus agaradhaerens* DSM 8721^T, *B. cellulosilyticus* N-4^T, *B. clarkii* PN-102^T, *B. saliphilus* 6AG^T, *B. selenitireducens* MLS10^T, *B. vedderi* JaH^T be reclassified into a new genus, *Natrobacillus* gen. nov. The distinct phylogenetic identity and metabolic capabilities of ML-SRAO^T suggest that it is a novel species, for which the name *Natrobacillus oremlandii* sp. nov., is proposed. The type strain is ML-SRAO^T.

INTRODUCTION

The genus *Bacillus* currently contains >100 species that catalyze diverse environmental and industrial processes (Berkeley et al., 2002). Many of these validly-named *Bacillus* species have 16S rDNA sequences and physiological and phenotypic characteristics that are significantly different from the type species, *B. subtilis* (Stackebrandt and Swiderski, 2002). Several of the organisms that are currently classified as *Bacillus* species do not even meet the minimum definition of "aerobic, endospore forming, Gram-positive, rod-shaped organisms" (Fritze, 2002). The recent advancements in molecular analytical methods have led to the polyphasic reassessment of many species, with subsequent reassignment to new genera (Heyndrickx et al., 1998; Heyndrickx et al., 1996; Jeon et al., 2005; Nazina et al., 2001; Shida et al., 1996; Waino et al., 1999).

A number of alkaliphilic/alkalitolerant *Bacillus* species have been isolated and characterized in the past two decades (Agnew et al., 1995; Jeon et al., 2005; Liu et al., 2005; Nielsen et al., 1995; Nogi et al., 2005; Switzer Blum et al., 1998; Tian et al., 2007; Usami et al., 2007), some of which have been reassigned to new genera within the Bacillaceae (e.g., *Alkalibacillus*; (Jeon et al., 2005). Many of these alkaliphilic organisms align phylogenetically within *Bacillus* RNA group 6 (Nielsen et al., 1994; Stackebrandt and Swiderski, 2002). However, this group contains distinct subclusters that may warrant further division into additional genera (Stackebrandt and Swiderski, 2002).

A new *Bacillus*-like species, strain ML-SRAO^T, was isolated from the anoxic deep waters of Mono Lake, California, USA, a saline soda lake. Strain ML-SRAO^T was closely related to six *Bacillus* species (*Bacillus agaradhaerens* DSM 8721^T, *B. cellulosilyticus* N-4^T, *B. clarkii* PN-102^T, *B. saliphilus* 6AG^T, *B. selenitireducens* MLS10^T, *B. vedderi* JaH^T) that formed

a phyletic group that was distinct from *B. subtilis* and other genera of the Bacillaceae. Our phenotypic and chemotaxonomic characterization of the new isolate showed several similarities to the other organisms, while still maintaining unique properties. On the basis of these comparisons, we propose a new genus and species, *Natrobacillus oremlandii* strain ML-SRAO^T gen. nov., sp. nov., and propose the transfer of the aforementioned species to *Natrobacillus agaradhaerens* comb. nov., *Natrobacillus cellulosilyticus* comb. nov., *Natrobacillus clarkii* comb. nov., *Natrobacillus saliphilus* comb. nov., *Natrobacillus selenitireducens* comb. nov., and *Natrobacillus vedderi* comb. nov.

METHODS

Strain ML-SRAO^T was isolated from anoxic Mono Lake water enriched with selenate and arsenite into a defined mineral salts medium with 60 g l^{-1} NaCl and a pH of ~9 as previously described (Fisher and Hollibaugh, in review). The culture was purified through successive transfers and serial dilution. Cultures were preserved by freezing in medium containing 20% glycerol at -80° C.

Cells were regularly grown anaerobically with 10 mM lactate, 10 mM selenate or arsenate, and a small amount of yeast extract (0.001% w/v) or aerobically with 20 mM lactate or glucose and 0.001% w/v yeast extract. Cells were also grown on aerobic or anaerobic 1.5% agar plates with lactate or glucose, yeast extract, and 10 mM arsenate or selenate. The pH of the medium was altered by adding different ratios of $NaHCO_3$ and Na_2CO_3. Gram staining and catalase and oxidase tests were performed using standard methods (Collins et al., 2004). Fatty acid methyl ester (FAME) analysis was performed by Microbial ID, Inc. (MIDI, Newark, DE) using the Sherlock Microbial Identification System.

Antibiotic sensitivity or resistance was examined by adding antibiotics (1-100 μg ml^{-1}) to cultures grown in aerobic medium with 10 mM glucose and yeast extract (0.001% w/v). Cultures were incubated at 30°C for 72 hours.

Genomic DNA was extracted from 50 ml cultures of strain ML-SRAO^T grown to late exponential phase. Culture medium was passed through a 0.22 μm Sterivex® filter. The cells that were retained on filters were resuspended in lysis buffer and frozen at -80°C. DNA was purified by phenol chloroform extraction (Ferrari and Hollibaugh, 1999). The G+C content was measured by HPLC as previously described (Mesbah and Whitman, 1989). The value determined for strain ML-SRAO^T is the average of 2 replicates.

For phylogenetic analyses, the nucleotide sequences of 16S rRNA genes of the above mentioned species, as well as representative members of the *Bacillus* genus and type strains from related genera were aligned using Clustal W (Thompson et al., 1994). Only sequences with >1400 nucleotides were included. The Maximum Likelihood method was used to estimate pairwise evolutionary distances, and a neighbor-joining phylogenetic tree (1000 bootstrap replicates) was constructed using MEGA4 (Tamura et al., 2007). An additional tree was created using the Maximum Parsimony method (Tamura et al., 2007).

RESULTS AND DISCUSSION

Strain ML-SRAO^T is related to members of the low G+C Gram-positive, aerobic, spore-forming bacilli on the basis of phylogenetic analysis of its 16S rRNA gene (Figure 3.1). Its closest characterized relatives are *Bacillus agaradhaerans* PN-105^T (96.3%), *B. cellulosilyticus* N-4^T (95.6%), *B. clarkii* PN-102^T (94.9%), *B. selenitireducens* MLS-10^T (94.8%), *B. vedderi* JaH^T (94.0%), and *B. saliphilus* (93.8%). The mol% G+C content of the strain is 42.1.

Strain ML-SRAO^T and its closest relatives share some physical and phenotypic characteristics, while maintaining unique qualities that support their designation as separate species (Table 3.1). Strain ML-SRAO^T is a non-motile, rod-shaped bacterium that is approximately 0.5 × 4-6 μm long. Cells showed no pigmentation when grown under light or dark conditions. Small (<1 mm), smooth, non-pigmented colonies appeared on agar plates after 10 days of anaerobic incubation at 30°C or 4 days of aerobic incubation. Ellipsoidal endospores that swelled the sporangia were observed in stationary phase cultures by phase-contrast microscopy. Gram staining produced a clearly positive result. Cells grew singly in liquid medium during early to late exponential growth, but tended to form long chains in during stationary phase.

Strain ML-SRAO^T could grow over a range of pH (7-10), with optimum growth occurring at pH 8.5-9. Growth was observed at salinities of 25-200 g l^{-1} NaCl (45-220 total salts); the optimum salinity was 60 g l^{-1} NaCl (80 g l^{-1} total salts). The optimum temperature for growth was 28-32°C, but strain ML-SRAO^T could grow at temperatures between 4º C and 37º C. Strain ML-SRAO^T grew aerobically or anaerobically with selenate, arsenate, or nitrate. Arsenate was reduced to arsenite; nitrate was reduced to nitrite; and selenate was initially reduced to selenite, then to elemental selenium.

Strain ML-SRAO^T grew on a variety of sugars and organic acids, including glucose, sucrose, mannose, arabinose, succinate, lactate, and pyruvate. Growth was also observed with yeast extract and tryptone. Strain ML-SRAO^T could not grow on mannitol, citrate, peptone, casamino acids, or acetate. Arsenite could also serve as an electron donor when coupled to reduction of selenate, although strain ML-SRAO cannot grow autotrophically on these substrates.

Strain ML-SRAO^T is catalase-positive and oxidase-positive. Growth in liquid medium was inhibited by ampicillin, erythromycin, and penicillin. Strain ML-SRAO^T was able to grow in medium containing chloramphenicol (10 μg ml^{-1}), gentamycin (10 μg ml^{-1}), kanamycin (10 μg ml^{-1}), and rifampicin (5 μg ml^{-1}).

Fatty acid analysis revealed that $C_{16:1}\omega7c$ (34.4%), $C_{16:0}$ (29.1%), $C_{18:1}$ ω7c (12.8%), anteiso-$C_{15:0}$ (6.8%), and $C_{18:0}$ (3.9%) were the major PLFAs detected following anaerobic growth at pH 8.5 with lactate as the carbon source. Trace amounts of other fatty acids were also found (Supporting Information, Table 3.3).

Strain ML-SRAO^T, along with six other species, formed a distinct group (Figure 3.1) whose 16S rRNA gene sequences were >92.8% but less than 97.6% similar based on pairwise analysis of 1495 base pairs. All species in this group were alkaliphilic and halotolerant. The 16S rRNA gene sequences of these organisms were 89.0-92.2% similar to *B. subtilis* and were 90.7-92.5% similar to that of the most closely-related genus, *Pullulanibacillus*. The genus *Pullulanibacillus* has only one species, *P. naganoensis* DSM 10191^T, which is only slightly halotolerant and mildly acidophilic (Hatayama et al., 2006). Sequence similarity to species from the genus *Alkalibacillus* was 89.5%-91.9%.

Our phylogenetic analyses strongly suggest that strain ML-SRAO^T and its six closest relatives represent a new genus, for which the name *Natrobacillus* is proposed. Another phylogenetic tree, which included additional sequences from each genus, was constructed using the Maximum Parsimony method (Supporting Information, Figure 3.2). This tree also supported the branching positions of the *Natrobacillus* species as a distinct and cohesive group. The phenotypic data presented in Table 3.1 show that two of the species in this group, *B. saliphilus* 6AG^T and *B. selenitireducens* MLS-10^T do not conform to the standard definition of a *Bacillus* in

that they do not form spores. Additionally, *B. saliphilus* 6AG^T is coccoid, not rod-shaped. The characteristics of the genus *Natrobacillus* are compared to other closely-related genera in Table 3.2. Although *Natrobacillus* shares similarities such as spore shape and fatty acid composition with other genera, it is the only truly alkaliphilic genus (optimum growth pH >8 for all species) and the only one in which all members (for n>1) are known to reduce nitrate. The DNA sequence of strain ML-SRAO^T was only 96.3% similar to its closest cultured relative, *Bacillus agaradhaerens* DSM 8721^T, different enough to warrant classification as a new species (Stackebrandt and Swiderski, 2002), for which the name *Natrobacillus oremlandii* sp. nov. is proposed.

Description of *Natrobacillus*, gen. nov. *Natrobacillus* (na.tro. ba.cil′lus. Gr. n. *natron*, derived from Arabic *natrun*, soda [sodium carbonate], L. dim. n. *bacillus,* small rod; M.L. masc. n. *Natrobacillus*, a small soda rod.

The description below is based on data from this study as well as previous work on RNA group 6 bacilli (Agnew et al., 1995; Nielsen et al., 1995; Nogi et al., 2005; Romano et al., 2005; Switzer Blum et al., 1998). Cells are rod-shaped or coccoid and may be motile or non-motile. If spores are formed, they are ellipsoid or spherical and cause swelling of the sporangia. Cells are Gram-positive. All test positive for catalase, some test positive for oxidase. Colony size and morphology vary; and pigmentation is translucent, white, cream or yellow. Oxygen can serve as the terminal electron acceptor for all species; selenate, selenite, arsenate, nitrite, and Fe^{3+} can also be used by some. All can reduce nitrate. Halotolerant, mesophilic, and obligately alkaliphilic; optimum growth occurs at 3-16% NaCl, 30-40º C, and pH 8.5 to >10. All species can grow with glucose as the sole carbon source. Many species hydrolyze polymers such as

starch, xylan, and cellulose. The major menaquinone is MK-7. The predominant cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0,}$ and $C_{16:0}$ when cells are grown aerobically. The DNA G + C content is 38-49 mol%. Intragenic similarity (16S rDNA) is >92%. The type species is *Bacillus agaradhaerens* PN-105^T.

Description of *Natrobacillus oremlandii* sp. nov. *Natrobacillus oremlandii* (or.em.land′ii N.L. gen. n. *oremlandii* of Oremland, named after Ronald S. Oremland for his work on microbial arsenic and selenium cycling in the environment).

Cells are Gram-positive, non-motile rods (0.5 × 4-6 μm) that form terminal, ellipsoidal spores that swell the sporangia. The G+C content is 42.1 mol%. The major PLFAs are $C_{16:1}\omega7c$ (34.4%) and $C_{16:0}$ (29.1%) when cells are grown anaerobically. Cells are oxidase- and catalase-positive. Facultative anaerobe that respires arsenate, nitrate, and selenate in addition to oxygen. Selenite is reduced to elemental selenium, but energy is not conserved in this process. Electron donors for growth include glucose, sucrose, mannose, arabinose, succinate, lactate, pyruvate, yeast extract and tryptone. Arsenite can serve as an electron donor (only for selenate reduction) but does not support autotrophic growth. Growth occurs at 20-200 g/L NaCl, pH 7-10, and 4-35°C.

The type strain, ML-SRAO^T, was isolated from the anoxic bottom water of Mono Lake, California, USA.

Description of *Natrobacillus agaradhaerans* (Nielsen et al. 1995) comb. nov.

The description of *Natrobacillus agaradhaerens* is the same as the description given by Nielsen et al. (2005) with the addition of information from Nogi et al. (2005) and Lim et al. (2006). The type strain is PN-105^T (=ATCC 700163^T = CIP 105302^T = DSM 8721^T = LMG 17948^T).

Description of *Natrobacillus cellulosilyticus* (Nogi et al. 2005) comb. nov.

The description of *Natrobacillus cellulosilyticus* is the same as the description given by Nogi et al. (2005). The type strain is N-4^T (=DSM 2522^T = JCM 9156^T).

Description of *Natrobacillus clarkii* (Nielsen et al. 1995) comb. nov.

The description of *Natrobacillus clarkii* is the same as the description given by Nielsen et al. (1995) with additional information from Nogi et al. (2005) and Lim et al. (2006). The type strain is PN-102^T (=ATCC 700162^T = CIP 105301^T = DSM 8720^T = LMG 17947^T).

Description of *Natrobacillus saliphilus* (Romano et al. 2005) comb. nov.

The description of *Natrobacillus saliphilus* is the same as the description given by Romano et al. (2005). The type strain is 6AG^T (=ATCC BAA-957^T = DSM 15402^T).

Description of *Natrobacillus selenitireducens* (Switzer Blum et al. 1998) comb. nov.

The description of *Natrobacillus selenitireducens* is the same as the description given by Switzer Blum et al. (1998). The type strain is MLS10^T (=ATCC 700615^T = DSM 15326^T).

Description of *Natrobacillus vedderi* (Agnew et al. 1995) comb. nov.

The description of *Natrobacillus vedderi* is the same as the description given by Agnew et al. (2005), with the addition of data from Nogi et al. (2005) and Lim et al. (2006). The type strain is JaH^T (=ATCC 700130^T = CIP 105292^T = DSM 9768^T = LMG 17954^T).

ACKNOWLEDGMENTS

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Table 3.1. Phenotypic characteristics of strain ML-SRAO^T and related species.

1- strain ML-SRAO^T, **2**- *B. agaradhaerans* DSM 8721^T, **3**- *B. selenitireducens* MLS-10^T, **4**- *B. saliphilus* 6AG^T, **5**- *B. clarkii* DSM 8720^T, **6**- *B. cellulosilyticus* N-4^T, and **7**- *B. vedderi* JaH^T. Data for the reference strains taken from: Nielsen et al. (2005), Switzer Blum et al. (1998), and Romano et al. (2005), Nogi et al. (2005), and Agnew et al. (1995).

Characteristic	1	2	3	4	5	6	7
Cell shape	Rods	Rods	Rods	Cocci	Rods	Rods	Rods
size (µm)	0.5 ×4-6	0.5-0.6 × 2-5	0.5 × 2-6	0.8-0.9	0.6-7 × 2-5	0.3-4 × 2-3	ND
Motility	-	ND	-	-	ND	+	+
Pigmentation	Transparent or white	White	ND	Yellow	Cream to yellow	Cream	White
Gram stain	+	+	+	+	+	+	+
Spore shape	E	E	no	no	E	E	S
Location	T	ST	spores	spores	ST	ST	T
Swollen sporangia	+	+	N/A	N/A	+	+	+
pH range	7-10	7-11	8.5-10.5	7-10	8-11	8-10	8.9-10.5
optimum	8.5-9	10	9.5	9	>10	9-10	10.0
Salinity (%NaCl)							
range	2.5-20	0-16	2-22	1-25	0-16	0-12	0-7.5
optimum	5-7.5	ND	3-6	16			ND
Temperature (°C)							
range	4-35	10-45	ND	4-50	10-45	20-40	25-50
optimum	28-32	30		37		37	40
Oxidase /Catalase	+/+	- /+*	-/+	+/w	-/+*	-/+	+/+
Major PLFAs	$C_{16:1}\omega7c$, $C_{16:0}$, $C_{18:1}\ \omega7c$[§]	anteiso- $C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$	ND	anteiso- $C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$, ai-$C_{17:0}$	$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$,	iso-$C_{15:0}$, $C_{17:0}$, anteiso-$C_{15:0}$
DNA G+C mol%	42.1	39.2	49.0	48.4	42.4-43.0	39.6	38.3
Nitrate reduction	+	+	+	+	+	+	+
Hydrolysis of:							
Starch	ND	+	+	ND	+	+	-
Gelatin	ND	+	ND	+	+	-	w
Casein	ND	+	ND	-	+	-	-

+, positive or present; - negative or absent; E- ellipsoid; S-spherical; T –terminal; ST – subterminal; w – weak; ND – no data; * from Lim et al. (2006); §

measured during growth under anaerobic conditions

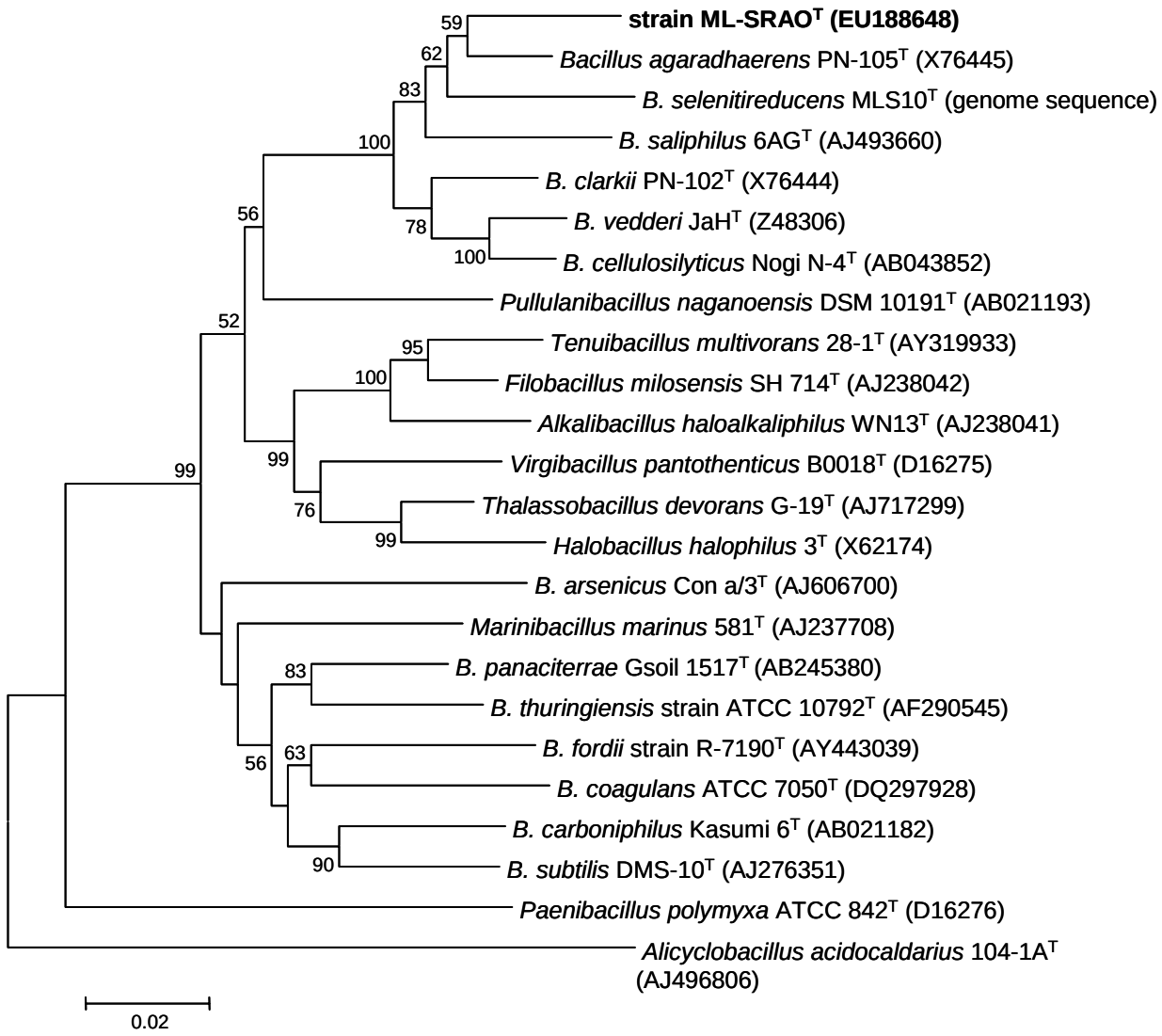
Table 3.2. Characteristics of *Natrobacillus* and related genera.

Data is compiled from this study and previous work (Garcia et al., 2005; Hatayama et al., 2006; Jeon et al., 2005; Nazina et al., 2001; Romano et al., 2005; Spring et al., 1996; Tian et al., 2007; Usami et al., 2007; Yoon et al., 2007; Yoon et al., 2005).

Characteristic	***Natrobacillus***	***Pullulanibacillus***	***Halobacillus***	***Thalassobacillus***	***Alkalibacillus***	***Bacillus***
No. of species	7	1	12	1	5	>100
Intragenic (16S rDNA) similarity	≥92.7%	N/A	>97%	N/A	>95.9%	<89%
G+C mol%	38.3-49.0	47.3	40.1-45.0	42.4	37.0-41.0	32-69
pH optimum	8.5- >10	5.0-7.0	7.0-8.0	7.0	7.0-9.7	<7-9.5
Salinity (% NaCl) optimum	3-16	0-2	2-10	7.5-10	5-20	V
Temperature optimum (º C)	30-40	40-60	25-37	37	30-37	15-55
Motility	+/-	-	+/-	+	+/-	+/-
Spore shape	E,S	O	E, S	E	S	V
Major PLFAs	anteiso-$C_{15:0}$, iso-$C_{15:0,}$ $C_{16:0}$	iso-$C_{16:0,}$ $C_{16:0}$	anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$, iso-$C_{16:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$
Nitrate reduction	+	-	-	+	+/-	+/-

+ = positive, - = negative, v- variable, w- weak, E- ellipsoid, O- oval, S- spherical, N/A – not applicable

Figure 3.1. Phylogenetic relationships among members of the family Bacillaceae. A neighbor-joining tree was constructed using 16S rDNA sequences >1400 bp. Bootstrap values (1000 replicates) are shown for values >50%. *Alicyclobacillus acidocaldarius* 104-1A^T was used as the outgroup. Bar = 0.02 substitution per base pair.

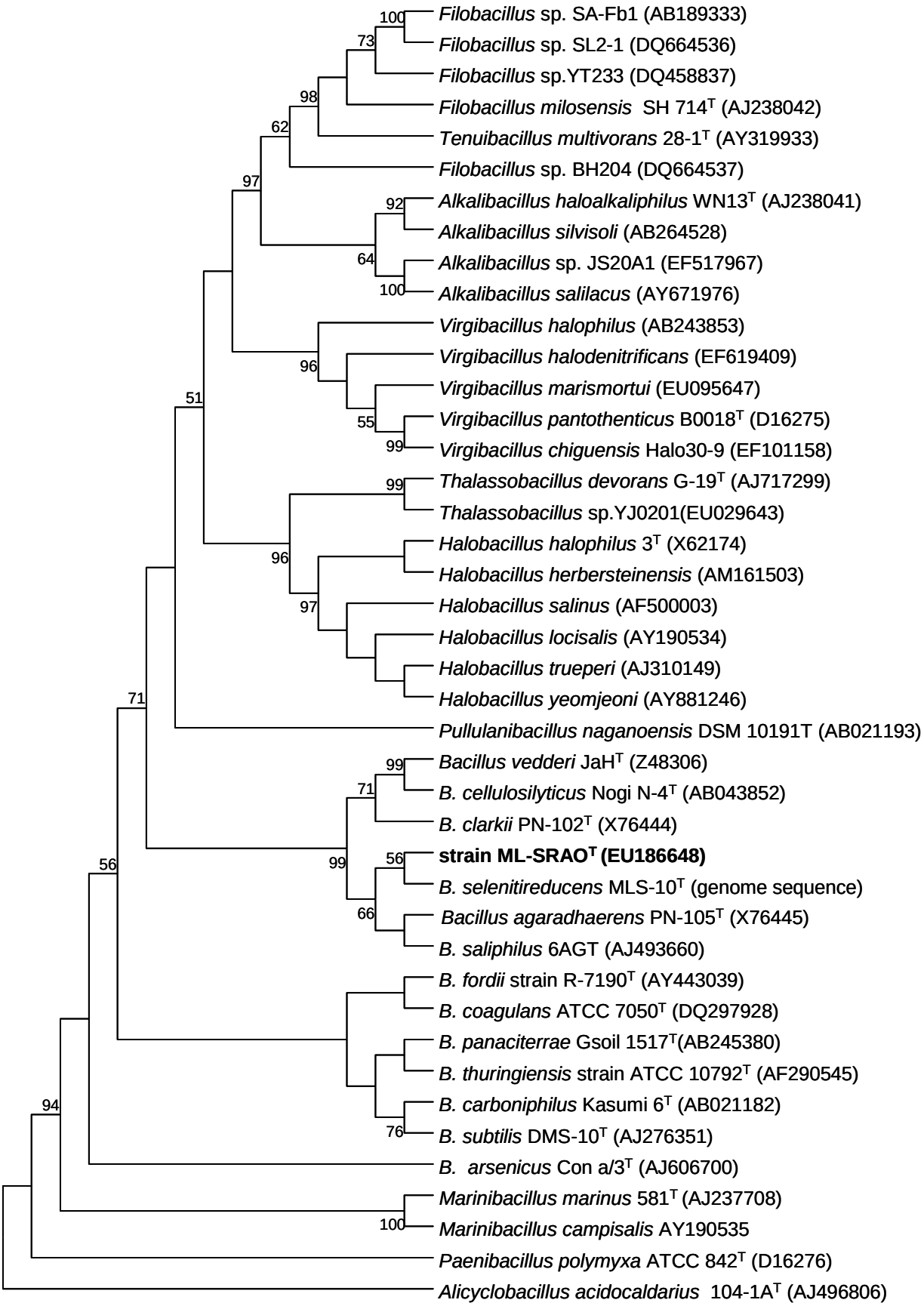


Supporting Information

Supporting Table 3.3. Complete fatty acid profile of strain ML-SRAO^T when grown under anaerobic conditions.

Fatty acid	%
12:0	0.20
Iso 13:0	0.21
anteiso 13:0	0.33
12:0 3OH	0.56
Iso 14:0	0.72
14:0	0.86
Iso 15:0	1.90
anteiso 15:0	6.77
15:0	0.31
Iso 16:0	0.83
16:1 ω7c	34.35
16:1 ω5c	0.59
16:0	29.14
16:0 DMA	0.31
Iso 17:0	2.04
anteiso 17:0	1.40
17:1ω8c	0.31
17:1 ω6c	0.39
17:0	1.49
18:1 ω9c	0.42
18:1 ω7c	12.75
18:1 ω5c	0.24
18:0	3.86

Supporting Figure 3.2. Maximum parsimony phylogenetic tree constructed using 16S rDNA sequences (>1400 bp) of members of the family Bacillaceae. Numbers at nodes represent bootstrap values (1000 replicates). *Alicyclobacillus acidocaldarius* 104-1A^{T} was used as the outgroup. The GenBank accession numbers for the sequences are given in parentheses.


100
73
98
62
97
92
64
100
96
51
55
99
99
96
97
71
99
71
56
99
56
66
94
76
100
Filobacillus sp. SA-Fb1 (AB189333)
Filobacillus sp. SL2-1 (DQ664536)
Filobacillus sp.YT233 (DQ458837)
Filobacillus milosensis SH 714T (AJ238042)
Tenuibacillus multivorans 28-1T (AY319933)
Filobacillus sp. BH204 (DQ664537)
Alkalibacillus haloalkaliphilus WN13T (AJ238041)
Alkalibacillus silvisoli (AB264528)
Alkalibacillus sp. JS20A1 (EF517967)
Alkalibacillus salilacus (AY671976)
Virgibacillus halophilus (AB243853)
Virgibacillus halodenitrificans (EF619409)
Virgibacillus marismortui (EU095647)
Virgibacillus pantothenticus B0018T (D16275)
Virgibacillus chiguensis Halo30-9 (EF101158)
Thalassobacillus devorans G-19T (AJ717299)
Thalassobacillus sp.YJ0201(EU029643)
Halobacillus halophilus 3T (X62174)
Halobacillus herbersteinensis (AM161503)
Halobacillus salinus (AF500003)
Halobacillus locisalis (AY190534)
Halobacillus trueperi (AJ310149)
Halobacillus yeomjeoni (AY881246)
Pullulanibacillus naganoensis DSM 10191T (AB021193)
Bacillus vedderi JaHT (Z48306)
B. cellulosilyticus Nogi N-4T (AB043852)
B. clarkii PN-102T (X76444)
strain ML-SRAOT (EU186648)
B. selenitireducens MLS-10T (genome sequence)
Bacillus agaradhaerens PN-105T (X76445)
B. saliphilus 6AGT (AJ493660)
B. fordii strain R-7190T (AY443039)
B. coagulans ATCC 7050T (DQ297928)
B. panaciterrae Gsoil 1517T(AB245380)
B. thuringiensis strain ATCC 10792T (AF290545)
B. carboniphilus Kasumi 6T (AB021182)
B. subtilis DMS-10T (AJ276351)
B. arsenicus Con a/3T (AJ606700)
Marinibacillus marinus 581T (AJ237708)
Marinibacillus campisalis AY190535
Paenibacillus polymyxa ATCC 842T (D16276)
Alicyclobacillus acidocaldarius 104-1AT (AJ496806)

CHAPTER 4

A NEW ROLE FOR SULFUR IN ARSENIC CYCLING

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ABSTRACT

Sulfur and arsenic often coexist in the environment and share similar microbial redox transformations. We examined the effects of sulfide on aerobic arsenite oxidation in alkaline lake water samples and in laboratory enrichment cultures. Significant arsenite oxidation occurred only in treatments with bacteria present, and production of arsenate was greatly enhanced by the addition of sulfide or thiosulfate. IC-ICP-MS analysis of samples showed that mono- and di-thioarsenate formed in arsenite + sulfide amended lake water. Our data indicate that these two thioarsenic compounds are fairly stable in sterile alkaline solutions, but are transformed predominantly to arsenate when bacteria are present. Enrichment culture experiments suggest that sulfur-oxidizing bacteria use free or arsenic-bound sulfur as a growth substrate and directly or indirectly transform arsenite and thioarsenates to arsenate during growth. Increases in cell density resulted in more rapid conversion of arsenite and thioarsenates. The rate and extent of these processes appear to be controlled by the concentration of bacteria and the ratio of reduced sulfur to arsenite present. Sulfur-driven arsenite oxidation and microbial thioarsenate transformation may be important biogeochemical processes in the arsenic cycle of our study site (Mono Lake, CA, USA) and other alkaline environments as well.

INTRODUCTION

Arsenic is a toxic metalloid that is generally found in aqueous environments as the oxyanions arsenate [As(V)] and arsenite [As(III)] (Cullen and Reimer, 1989). The complex redox behavior of inorganic arsenic species (Cullen and Reimer, 1989) is mediated by chemical reactions such as ligand exchange, precipitation with iron and sulfide, adsorption to clay and metals, and biotic and abiotic oxidation-reduction reactions (Ferguson and Gavis, 1972). Reactions of arsenic with sulfur are of considerable importance, particularly in sulfidic environments (Wilkin et al., 2003). Arsenic and sulfur undergo similar chemical and biological redox reactions, and their biogeochemical cycles are often interconnected (Hollibaugh et al., 2005; Inskeep et al., 2002; Oremland et al., 2000; Oremland et al., 2005). Microbes drive the reduction of sulfate and arsenate to sulfide and arsenite under anoxic conditions (Oremland et al., 2000). Rapid chemical and/or biological oxidation of sulfide occurs when oxygen is introduced to the system (Cline and Richards, 1969; Gonzalez-Sanchez and Revah, 2007), while aerobic arsenite oxidation tends to be slow unless bacteria are present (Inskeep et al., 2002). Sulfide can also act as a reductant for abiotic arsenate reduction at low pH (Rochette et al., 2000) or microbially-mediated arsenate reduction at high pH (Hoeft et al., 2004; Hollibaugh et al., 2006). Stauder et al. (2005) proposed that the high affinity of sulfur for arsenic leads to the formation of thioarsenate compounds under reducing conditions. In such cases, sulfide oxidizes As(III) to As(V) by bonding to the lone pair of electrons (Stauder et al., 2005).

The formation of soluble arsenic-sulfide complexes is well-recognized phenomenon (Helz et al., 1995; Hollibaugh et al., 2005; McCay, 1901; Planer-Friedrich et al., 2007; Wilkin et al., 2003; Wood et al., 2002), and arsenic chemistry in sulfidic environments may be governed by the formation of thioarsenic species (Planer-Friedrich et al., 2007; Wilkin et al., 2003). Recent studies have focused on identification and quantification of thioarsenate species (also

referred to as *thioarsenites* in some literature) in both laboratory solutions and in environmental samples (Planer-Friedrich et al., 2007; Stauder et al., 2005; Wallschläger and Stadey, 2007). Analysis of environmental samples has confirmed the laboratory results, with arsenic-thiol compounds comprising ≥50% of the total As in sulfidic waters (Hollibaugh et al., 2005; Planer-Friedrich et al., 2007; Stauder et al., 2005). Little is known about the bioavailability of these compounds, although they are reported to be less toxic than arsenite (Rader et al., 2004).

This study began as an assessment of the effects of sulfide on arsenite oxidation in Mono Lake, CA. However, because of the alkaline nature of the lake water (pH 9.8, ~0.4 M carbonate); (Oremland et al., 2004) and the relatively high natural concentrations of arsenite (~200 μM) and sulfide (~200-3000 μM) in anoxic waters, thioarsenic species were present in some lake water samples and also formed in our enrichment experiments. Therefore, the formation, dissolution, and transformation of thioarsenic compounds must be included in our examination of arsenic cycling in this environment. A previous study of Mono Lake measured arsenic and thioarsenic speciation in lake water during a period of extended chemical stratification (meromixis) wherein sulfide concentrations in the lake were ~3 mM, over 10 times the amount of total arsenic in the lake; and trithioarsenate was the most abundant arsenic compound in sulfidic waters (Hollibaugh et al., 2005). Our present study sampled lake water during a period of monomixis (yearly mixing), when *in situ* sulfide concentrations were ≤500 μM.

We initially tested sulfide as an inhibitor of arsenite oxidation in Mono Lake water experiments, as was reported for samples from an acidic (pH 3.1) hot spring in Yellowstone National Park (Langner et al., 2001). This experiment generated the unexpected result of enhanced arsenate production. Our subsequent experiments with lake water examined the roles

of both bacteria and sulfide in the arsenite oxidation process to explain the paradox of enhanced arsenite oxidation in the presence of a strong reductant. Two mixing experiments were performed to simulate conditions that would occur during lake turnover, when bacteria would be exposed to oxidized and reduced redox species. The first simulated mixing experiment showed that an unknown thioarsenic compound(s) formed and disappeared over the course of the experiment. A second mixing experiment was performed to follow the changes in arsenic and thioarsenic speciation in live lake water samples. Finally, experiments with bacterial enrichment cultures from Mono Lake were used to determine how sulfide concentrations affect rates of microbial arsenite oxidation and to assess the role of thioarsenates in this process.

METHODS

Sample Collection. All lake water was collected from Station 6 of Mono Lake, CA, USA (37º57.822' N, 119º01.305' W). Fresh lake water was collected for experiments in August 2004, July 2006, and September 2006. Water samples (from 2-3 m, 15-16 m, and 35 m depth) were collected and stored as previously described (Hollibaugh et al., 2005). Mono Lake was stratified by density and temperature on all collection dates. Only the upper 20 m of the lake contained detectable quantities of oxygen during these sampling events. Limnological profiles (temperature, excess density, and dissolved oxygen) for each of the sampling dates and total As concentrations from the August 2004 sampling are available in the Supporting Information (Figure 4.5). Experiments were started within 1 to 3 days of sampling.

Arsenite Oxidation Experiment. Whole water (live) samples were amended with 1 mM arsenite (from a 1M $NaAsO_2$ stock solution) or 1 mM arsenite and 1 mM sulfide (from a 1 M

$Na_2S \cdot 9H_2O$ stock solution). Controls were filtered (0.22 μm, Millipore®) lake water treated with formalin (1% final concentration). Samples were kept in the dark at room temperature.

Simulated Lake Mixing Experiments. Mixing events were simulated in the laboratory by combining equal amounts of freshly collected anoxic (35 m), oxycline (16 m), and oxic (2 m) waters. Controls were filtered (0.22 μm, Millipore®) and treated with formalin (1% final concentration). Arsenite (0.5 mM) and sulfide (1 mM) were added after mixing to both live treatments and sterilized controls. Treatment flasks were wrapped in aluminum foil, closed with cotton stoppers to allow gas exchange, shaken at 100 rpm, and kept in the dark at 20º C.

Enrichment Culture Experiments. A mixed culture of bacteria capable of sulfide-driven arsenite oxidation was established from experiments with Mono Lake water. The culture was maintained in autotrophic mineral medium (artificial Mono Lake water; AMLW) amended with equimolar arsenite and sulfide (1-2 mM) and was regularly subcultured into fresh medium. For culture experiments, AMLW was amended with 1 mM arsenite and 0, 0.5, 1, 2, or 4 mM sulfide or 6 mM thiosulfate. A 5% inoculum of late exponential phase culture was added to each live treatment. An equal volume of culture medium was filtered (0.22 μm, Millipore®) for sterile controls. Samples were incubated in the same manner as in the mixing experiments. Subsamples were measured for arsenic speciation and cell counts. Cells were preserved with formalin, stained with DAPI, and counted using epifluorescence microscopy (Porter and Feig, 1980).

Chemical Analyses. Oxygen was measured with a YSI oxygen meter equipped with a Clark-type electrode. Temperature and conductivity were measured with a Seabird SeaCat profiler; excess density was calculated from these data. Arsenate was measured by the molybdate blue method on a spectrophotometer (Johnson and Pilson, 1972) in the initial lake water enrichment

experiment. For the first mixing experiment and the enrichment culture experiments, arsenate and arsenite were measured using high performance liquid chromatography (Waters chromatograph equipped with a UV-visible detector set at 210 nm). A guard column (BioRad) and two anion exclusion columns (Hamilton PRP-X300 and BioRad Aminex HPX-87H) were used in series, and samples were eluted with 0.016 N H_2SO_4 as previously described (Hoeft et al., 2004). A peak other than arsenite or arsenate appeared only in samples that also contained sulfide. Subsequent ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS) analysis determined that this peak represented one or more thioarsenic compounds. Arsenate and arsenite were quantified with standard solutions, and total thioarsenic was calculated as: $[\text{thioarsenic}_{TOTAL}] = [As_{TOTAL}] - [\text{arsenite} + \text{arsenate}]$. Total arsenate was determined by ICP-MS (Trent University) or inductively coupled plasma-optical emission spectrometry (ICP-OES; Chemical Analysis Laboratory, University of Georgia). Flash-frozen (liquid nitrogen) samples from the second lake-mixing experiment were thawed in an anaerobic glove box and analyzed for arsenate, arsenite, and thioarsenate species by IC-ICP-MS (Hollibaugh et al., 2005; Wallschläger and Stadey, 2007; Wilkin et al., 2003). Sulfide samples were dispensed into an equal volume of anti-oxidant buffer and measured with an Orion® sulfide probe as previously described (Hollibaugh et al., 2005).

Molecular Analysis. Enrichment cultures were filtered with 0.22 μm Millipore® nitrocellulose filters. DNA was extracted using a MoBio Soil Extraction Kit according to the manufacturer's instructions (Bio 101, Solana, CA). PCR was performed to amplify 16S rRNA genes (Bano and Hollibaugh, 2002). The PCR product was cloned and sequenced as previously described (LeCleir et al., 2004). Sequences were run at the Molecular Genetics Instrumentation Facility at the University of Georgia (Athens, GA, USA). Sequences were compared to others in the

GenBank database using BLAST (Altschul et al., 1990) and were submitted to GenBank under accession numbers EF643362 to EF643371.

RESULTS

Arsenite Oxidation Experiment. Arsenate concentrations increased steadily in live treatments amended with arsenite and sulfide (Figure 4.1). Arsenite oxidation was complete in the live arsenite + sulfide (As+S) treatment (1.02 ± 0.10 mM arsenate produced) within 6 days. Arsenate production in the arsenite only (As) live and formalin-killed control experiments was minimal (<1% of added arsenite was oxidized) during the incubation period. More arsenate was produced in the As+S sterile control experiment (0.22 mM) than in the live As treatment (0.097 ± 0.02 mM), indicating a possible stimulation of abiotic arsenite oxidation in the presence of sulfide. Additional control experiments verified that formalin inhibited arsenate production by killing bacteria, rather via an abiotic chemical reaction (Supporting Information, Figure 4.6).

Simulated Lake Mixing Experiments. An unknown thioarsenic compound (or compounds) formed in both live and control samples of mixed lake water upon addition of arsenite and sulfide (Figure 4.2). We calculated that 0.27 ± 0.01 mM total thioarsenic formed initially in live and control samples and increased to a maximum concentration of ~0.4 mM. Arsenite concentrations decreased to ~0.22 mM within the first hour, and continued to decrease throughout the experiment in both treatments. After ~30 hours, thioarsenic concentrations began to decrease in live treatments while arsenate concentrations increased. Nearly all added arsenite was converted to arsenate after 72 hours (Figure 4.2A). Thioarsenic concentrations in the controls remained steady at ~0.4 mM, and arsenate concentrations in controls fluctuated only slightly (~0.17-0.24 mM) throughout the experiment (Figure 4.2B).

A second mixing simulation was conducted in which arsenic and thioarsenic speciation was determined in live samples by IC-ICP-MS. Both unamended anoxic, sulfidic lake water and aerobic lake waters amended with arsenite and sulfide had similar proportions of arsenic and thioarsenic species (Supporting Information, Figure S4.7).

The experiment shown in Figure 4.3 focused on the formation and conversion of thioarsenates, which formed rapidly after addition of arsenite to sulfide-amended lake water and were depleted after 32 hours. Monothioarsenate and dithioarsenate were the only thioarsenic compounds detected; combined, they comprised up to 39% of the total arsenic present. Dithioarsenate was depleted first, and decreasing dithioarsenate concentrations corresponded to increasing monothioarsenate concentrations. Arsenate and arsenite increased as monothioarsenate concentrations decreased. Arsenite concentrations decreased slightly after all thioarsenates were converted. Arsenate concentrations increased throughout the course of the experiment, reaching a maximum of 0.47 mM. After 48 hours, ~70% of the added arsenite had been oxidized.

Enrichment Culture Experiments. Arsenate was produced and cell densities increased in enrichment cultures amended with 1 mM arsenite and 0, 0.5, 1, 2, or 4 mM sulfide or 6 mM thiosulfate (Figure 4.4). Very little arsenate (<0.05 mM) formed in any of the control experiments (Figure 4.4A; Supporting Information, Figure 4.8). All added arsenite was converted to arsenate in the 1, 2, and 4 mM sulfide and 6 mM thiosulfate experiments in ≤64 hours (Figure 4.4A), while over half of the added arsenite remained in the 0 mM and 0.5 mM sulfide-amended treatments. Detailed arsenic speciation (arsenate, arsenite, and thioarsenic) data for live and control samples are available in Figure 4.8 of the Supporting Information.

Thioarsenic compounds were typically converted to arsenate prior to the oxidation of arsenite, and no arsenate was produced in the thiosulfate treatment prior to the formation of thioarsenic (Supporting Figure 4.8-I). In the 1 mM arsenite + 4 mM sulfide treatment, arsenic initially was present only as thioarsenic and was directly converted to arsenate. No arsenite was measured throughout the course of the experiment (Supporting Figure 4.8-G). Arsenate production and cell density were well correlated ($r^2 = 0.93$, n=38, $p<0.01$) up to the maximum cell density in treatments with 0-2 mM sulfide. The correlation decreased ($r^2=0.67$, n=50, $p<0.01$) when the 4 mM sulfide and 6 mM thiosulfate treatments were included. The maximum cell density was proportional to the amount of reduced sulfur added ($r^2 = 0.92$, n=6, $p<0.01$), but cell growth continued long after free sulfide was no longer detected (Supporting Figure 4.9). Molecular analysis of the microbial community in these enrichments showed that the organisms present were closely related to cultured sulfur-oxidizing bacteria (Supporting Information, Table 4.1).

DISCUSSION

Very little arsenate accumulated in our experiments when Mono Lake water was amended with arsenite, despite environmental conditions that make the biological oxidation of arsenite to arsenate thermodynamically favorable (Figure 4.1). However, arsenite was completely converted to arsenate when the same samples were amended with arsenite and sulfide. Although a small amount of arsenate was produced in formalin-killed controls that contained arsenite and sulfide, most of the arsenite oxidation appears to be biologically driven.

Subsequent experiments (Figures 4.2, 4.3) showed that the effect of sulfide on arsenite oxidation was complicated by the formation of thioarsenic compounds. Thioarsenic

concentrations increased in both treatments for >24 hours, likely due to the reaction of arsenite with sulfide oxidation products (e.g., polysulfide; (Planer-Friedrich et al., 2007; Stauder et al., 2005; Wallschläger and Stadey, 2007). Arsenite was depleted via oxidation to arsenate and conversion to thioarsenic in live samples in our initial mixing experiment (Figure 4.2A). The majority of the arsenate produced in this experiment was first converted from arsenite to thioarsenic, rather than being produced by direct oxidation of arsenite. Our second mixing experiment showed a slightly different scenario in which thioarsenates were converted to both arsenate and arsenite, perhaps due to a combination of biological and chemical effects (Figure 4.3). Conversion of thioarsenate species in the second experiment appeared to occur by successive dissociation or oxidation of thiol (-SH) ligands, from dithioarsenate to monothioarsenate to arsenate or arsenite. The mixing experiments were conducted for different times (72 and 48 hours) and sampled at different intervals, which may account for some of the variation in arsenic speciation between the two. The net result of both treatments (arsenate production) was similar. The differences in arsenite versus arsenate production in the two experiments may also be due to variable thioarsenate decomposition under changing equilibrium conditions (Stauder et al., 2005) caused by differences in lake chemistry or the microbial community.

Significant quantities of thioarsenic were present in control samples for over 72 hours (Figure 4.2B). Similar persistence of thioarsenates in neutral and alkaline solutions exposed to air was observed previously (Wallschläger and Stadey, 2007). The relatively low oxygen (<5.5 mg/L), high pH environment of Mono Lake (Oremland et al., 2000) provides stability to thioarsenic compounds that is not present under acidic conditions (Stauder et al., 2005). Sterile solutions of thioarsenates decomposed mainly into arsenite and a small amount of arsenate when

acidified or oxygenated (Stauder et al., 2005), or precipitated as amorphous orpiment or arsenic pentasulfide when acid, excess sulfide, or heat was added (McCay, 1901). None of these abiotic processes produced significant amounts of arsenate.

Live treatments with sulfide or thiosulfate and arsenite amendments (Figures 4.1, 4.2, 4.3, and 4.4) produced mostly arsenate when concentrations of added sulfur were greater than or equal to added arsenite concentrations. The correlation of arsenate production rates with cell growth (Figure 4.4) and the lack of arsenate production in sterile controls strongly suggest that the transformation of arsenite and thioarsenates is biologically controlled. Neither the bioavailability of thioarsenates nor their oxidation kinetics have been determined, so the mechanism of arsenate production is unknown. Bacteria may directly oxidize the thiol portion(s) of the molecules, yielding arsenate and an oxidized sulfur compound such as sulfate:

$$2\ H_2AsO_3S^- + 5\ O_2 \rightarrow 2\ HAsO_4^{2-} + 2\ SO_4^{2-} + 2\ H^+ \quad (1)$$

Alternatively, thioarsenates could be chemically transformed into arsenate and sulfide via ligand exchange of OH^- for HS^-:

$$HAsO_3S^{2-} + OH^- \rightarrow HAsO_4^{2-} + HS^- \quad (2)$$

or by decomposition into arsenite and sulfur:

$$HAsO_3S^{2-} + H^+ \rightarrow H_2AsO_3^- + S^0 \quad (3)$$

The sulfide or sulfur could then be oxidized by bacteria to polysulfides, sulfite, or sulfate (Gonzalez-Sanchez and Revah, 2007) and arsenite could be oxidized to arsenate. The bacteria could indirectly increase rates of arsenate production by consuming free sulfide, thereby driving the chemical equilibrium toward the products in Eq. 2, or directly oxidizing arsenite as in Eq. 3. The presence of either free or arsenic-bound sulfur could provide a growth substrate for bacteria that simultaneously oxidize arsenite. The net result of the three pathways described above is the

same– arsenate is produced, and bacteria ultimately control the rates of thioarsenate and arsenite transformation.

Sulfur, in the form of sulfide, thiosulfate, or thioarsenic, appears to act as the growth substrate in enrichment culture experiments, as evidenced by maximum cell densities proportional to the amount of reduced sulfur initially present (Figure 4.4B). Molecular analysis of an enrichment culture grown with arsenite and sulfide showed that the majority of randomly sequenced clones (8/10) were closely related (95-98% identity based on ~620 base pairs) to cultured sulfur-oxidizing bacteria (Supporting Information, Table 4.1). Seven of the cloned 16S rDNA sequences were 99% identical to an environmental clone from Mono Lake. Five of those sequences were also 98% similar to that of *Thioalkalivibrio janaschii*, a sulfide and thiosulfate oxidizer previously isolated from Mono Lake (Sorokin et al., 2002). This suggests that the dominant bacterial phylotype in our enrichment cultures is likely to be numerically significant in Mono Lake and may contribute to sulfur and arsenite oxidation *in situ*.

It is unclear if arsenite oxidation is an energy-generating process for these organisms. Arsenate concentrations increased while cell numbers decreased in the 1 mM sulfide + 1 mM arsenite treatment (Figure 4.4). However, cell densities were higher in the 4 mM sulfide + 1 mM arsenite treatment than in the 4 mM sulfide-only treatment (Figure 4.4B and Supporting Figure 4.9). Whether arsenite oxidation occurs by co-metabolism with sulfide oxidation products (e.g. polysulfides), via a detoxification mechanism, or for energy conservation is uncertain.

Sulfide was shown previously to be an important electron donor for microbial arsenate reduction in Mono Lake, both in lake water enrichments (Hollibaugh et al., 2006) and by the isolate MLMS-1 (Hoeft et al., 2004). Our results suggest an additional role for sulfur in arsenic cycling by showing that sulfide enhances microbial arsenite oxidation. We also show that the

transformation of thioarsenates, which form via the reaction of sulfide with arsenite, may be biologically controlled. Sulfide can thus facilitate the complete redox cycling of arsenic from As(V) to As(III) and back to As(V) by direct coupling of sulfide oxidation to arsenate reduction (Hoeft et al., 2004; Hollibaugh et al., 2006) and by directly or indirectly stimulating arsenite oxidation (Eq. 1-3).

Although these processes have yet to be measured *in situ*, it seems likely that they may be important to arsenic biogeochemistry in Mono Lake. The results of this study are thus significant for arsenic cycling not only in Mono Lake, but for any neutral or alkaline environment where arsenite and sulfide are present and subject to changing redox conditions. The microbial transformation of thioarsenates in particular may be a significant process in alkaline hot springs (Planer-Friedrich et al., 2007), industrial waste sites (Stauder et al., 2005), or aquifers with bedrock containing arsenic- and sulfur-bearing minerals (Anawar et al., 2003).

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Figure 4.1. Arsenate produced from arsenite oxidation in Mono Lake surface water. Symbols: 1 mM arsenite, live (●); 1 mM arsenite, formalin-killed (○); 1 mM arsenite and 1 mM sulfide, live (▼); and 1 mM arsenite and 1 mM sulfide, formalin-killed (Δ). Values for live samples represent the average of three replicates; error bars represent ± 1 standard deviation. Controls represent a single value.

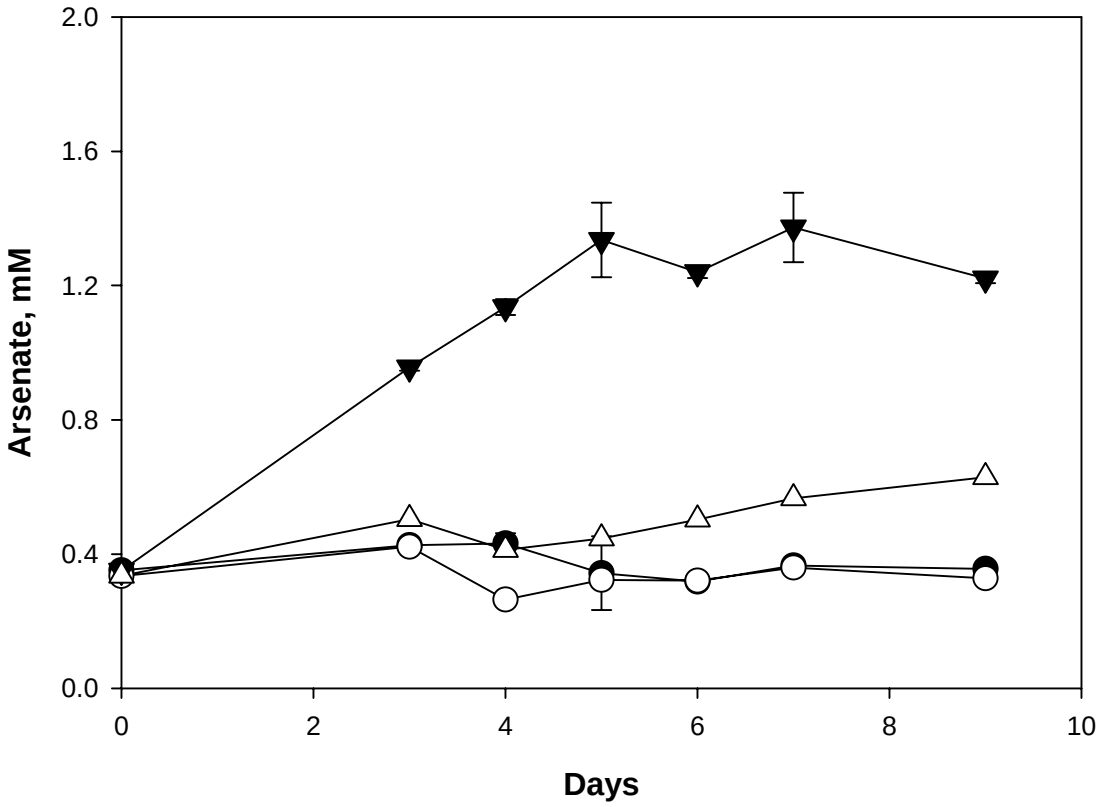


Figure 4.2. Simulated mixing event. Oxic, oxycline, and anoxic lake waters were mixed and amended with 0.5 mM arsenite and 1 mM sulfide. Arsenate (●) and arsenite (▼) were determined by HPLC analysis, and thioarsenic (○) was calculated by difference in (A) live and (B) 0.22 μm-filtered and formalin-killed controls. Values represent the average of three replicates; error bars represent ± 1 standard deviation.

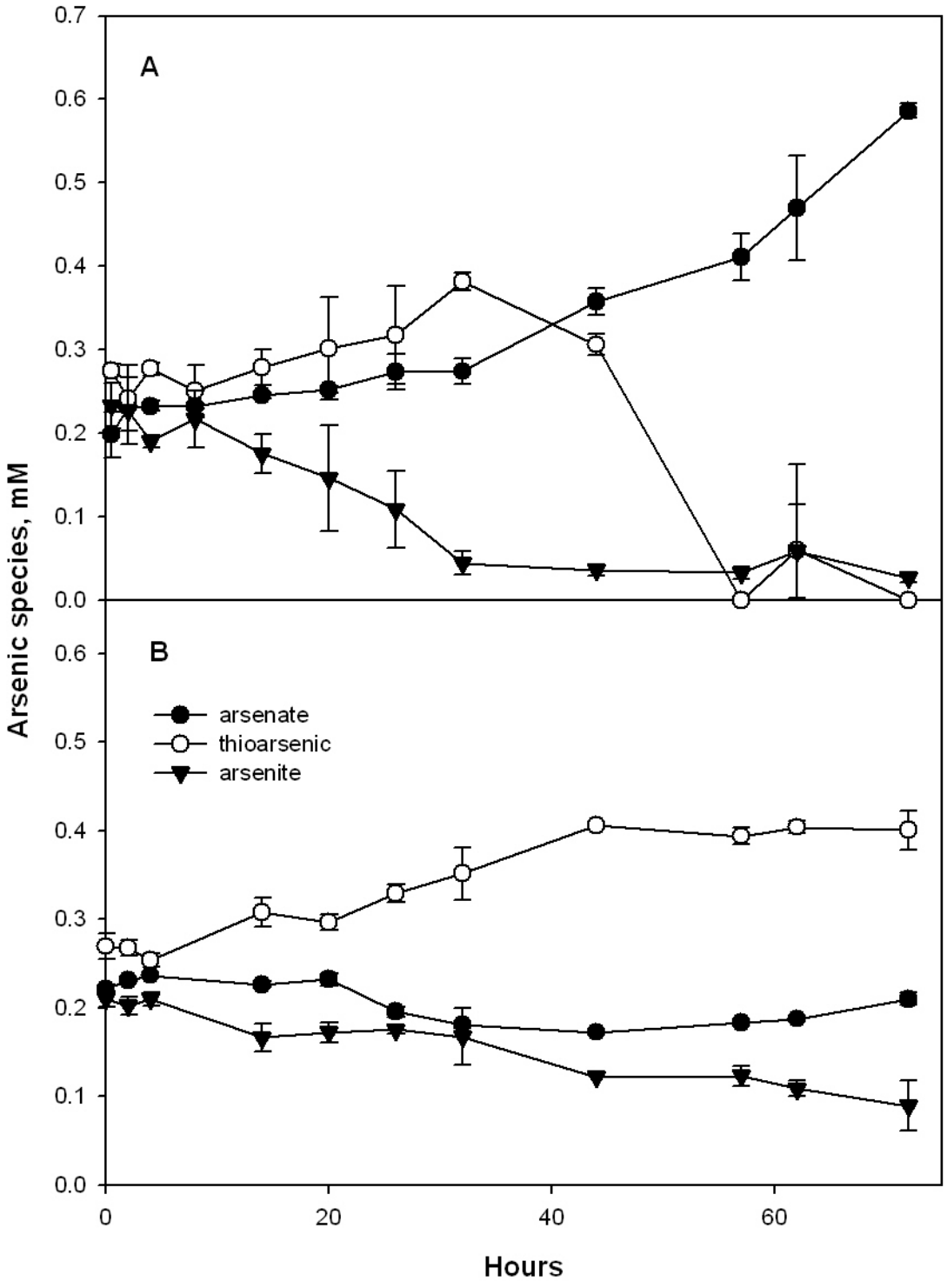

A
B
arsenate
thioarsenic
arsenite
Arsenic species, mM
Hours
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
20
40
60

Figure 4.3. Arsenic and thioarsenate speciation in a second simulated mixing experiment. Oxic, oxycline, and anoxic lake waters were mixed an amended with 0.5 mM arsenite and 1 mM sulfide. Symbols: arsenate (●), arsenite (○), monothioarsenate (▼), and dithioarsenate (Δ). Total arsenic (□) was measured by ICP-MS and total thioarsenate (■) was calculated as the sum of mono- and di-thioarsenate.

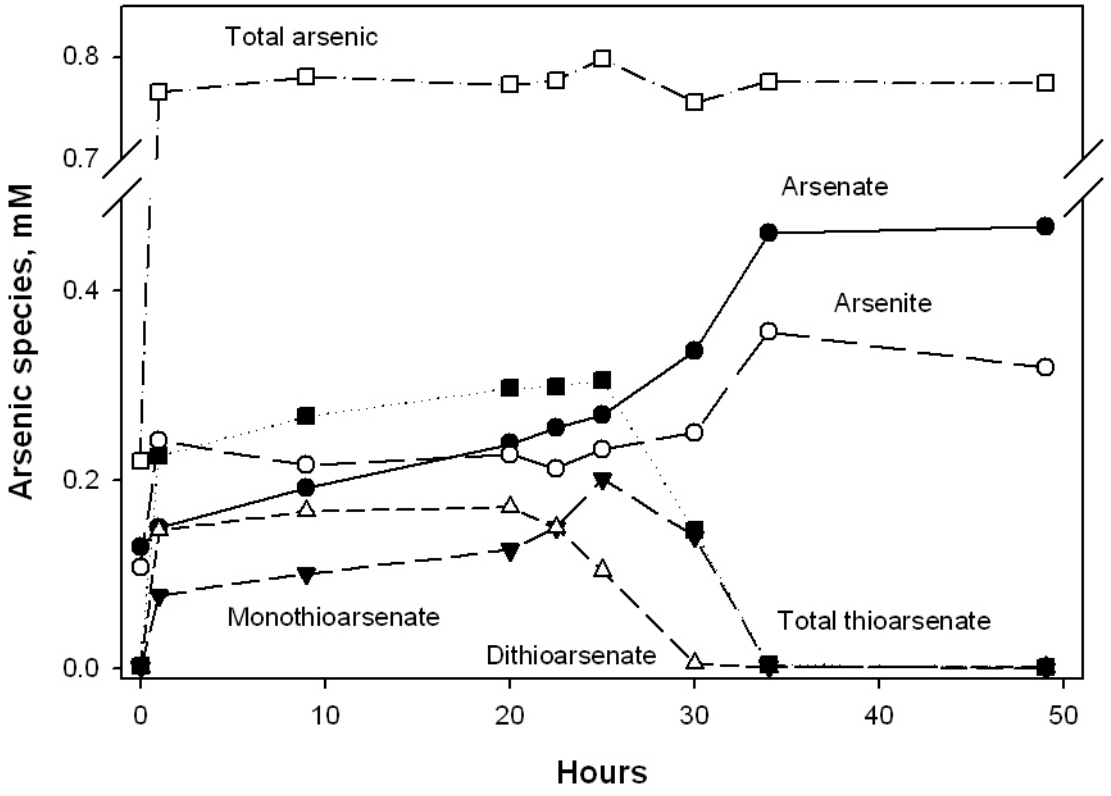
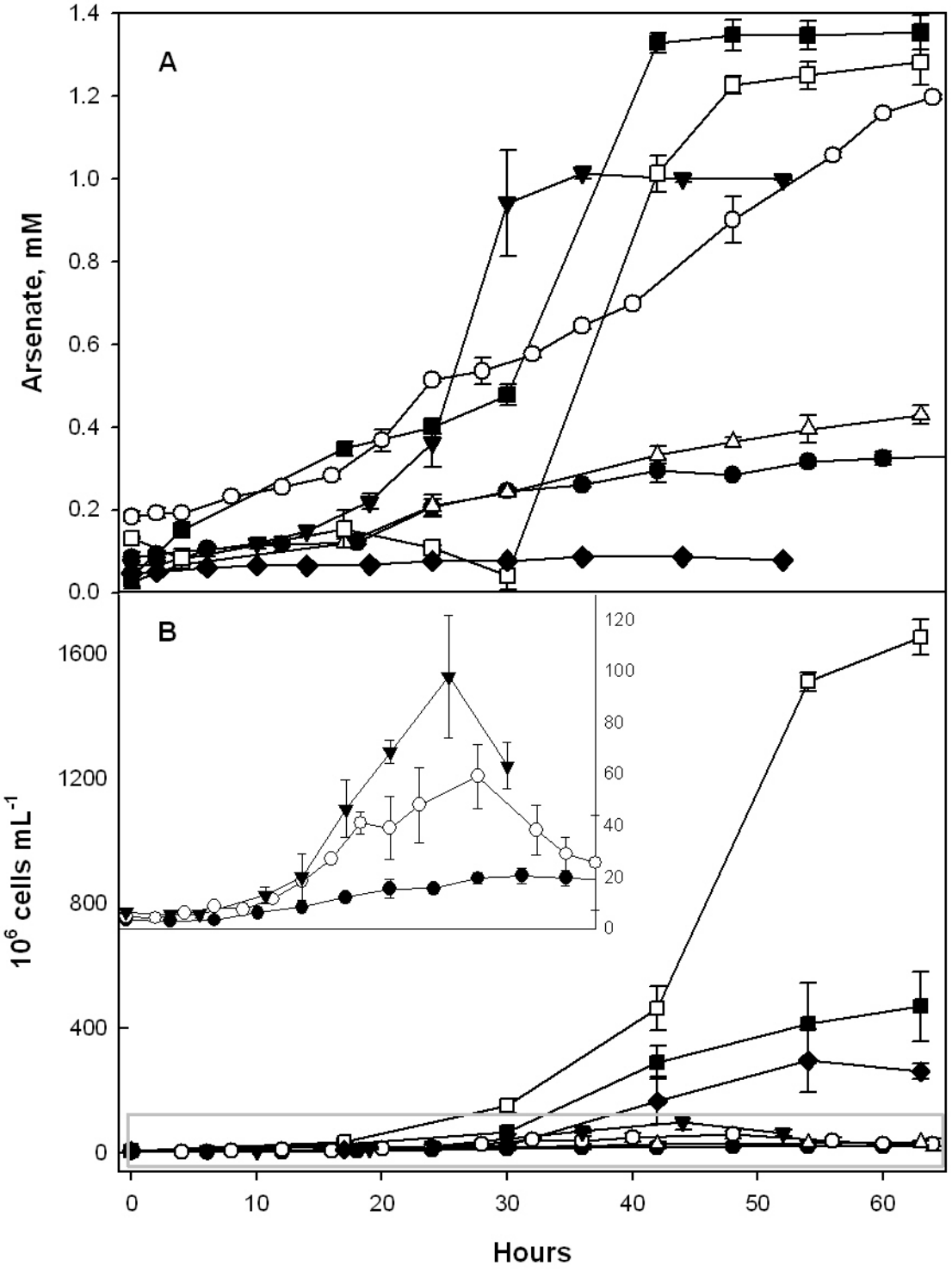


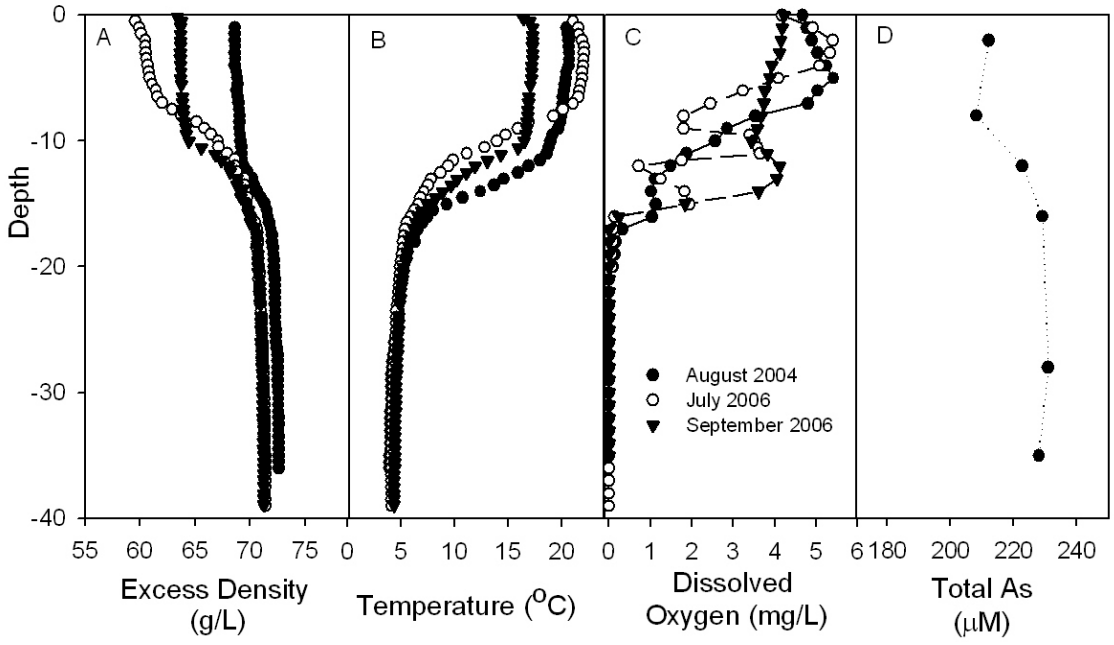
Figure 4.4. (A) Arsenate production and (B) bacterial cell density in artificial lake water enrichment culture experiments. Cultures were amended with 1 mM arsenite and 0 (Δ), 0.5 mM (●), 1 mM (○), 2 mM (▼), or 4 mM (■) sulfide or 6 mM thiosulfate (□). Arsenate in the 2 mM sulfide sterile control (♦; panel A) and cell density in a 4 mM sulfide only treatment (♦; panel B) are also shown. Inset: cell densities in 1 mM arsenite plus 0.5 mM (●), 1 mM (○), or 2 mM (▼) sulfide treatments. Values represent the average of three replicates; error bars represent ± 1 standard deviation.


A
B
Arsenate, mM
10^6 cells mL^{-1}
Hours
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
400
800
1200
1600
0
20
40
60
80
100
120
0
10
20
30
40
50
60

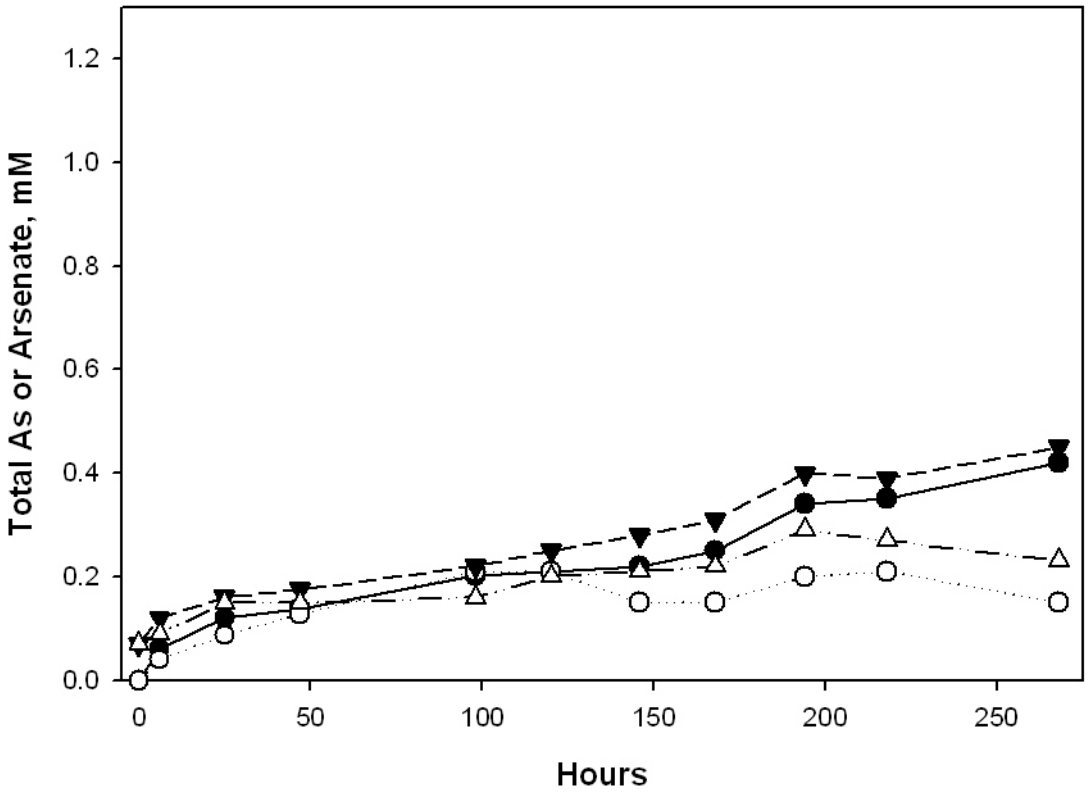
Supporting Information

Additional data on limnological profiles, effects of formalin, arsenic speciation in Mono Lake water samples, arsenic speciation in live and control treatments, sulfide data, and sequence data for enrichment cultures is provided here.

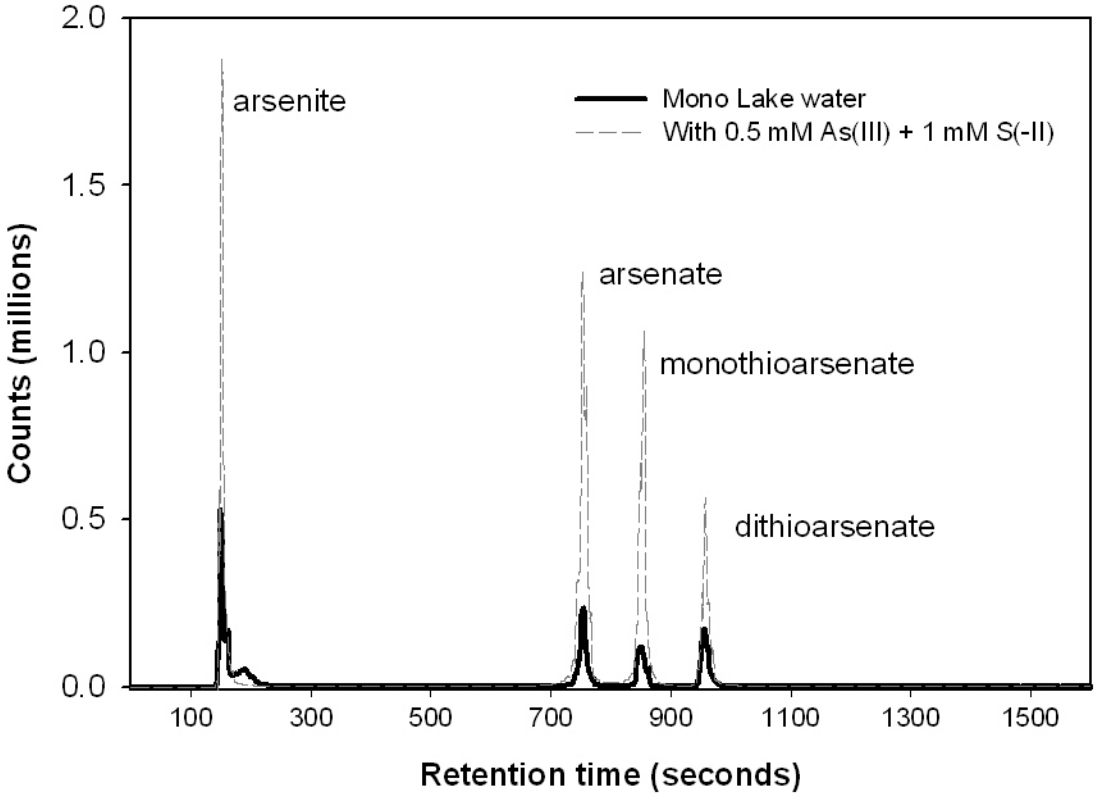
Supporting Figure 4.5. CTD measurements (excess density and temperature, A and B) and dissolved oxygen concentrations (C) from CTD casts taken in August 2004 (●), July 2006 (○), and September 2006 (▼). A set of samples from August 2004 was analyzed for total arsenic (●, D).



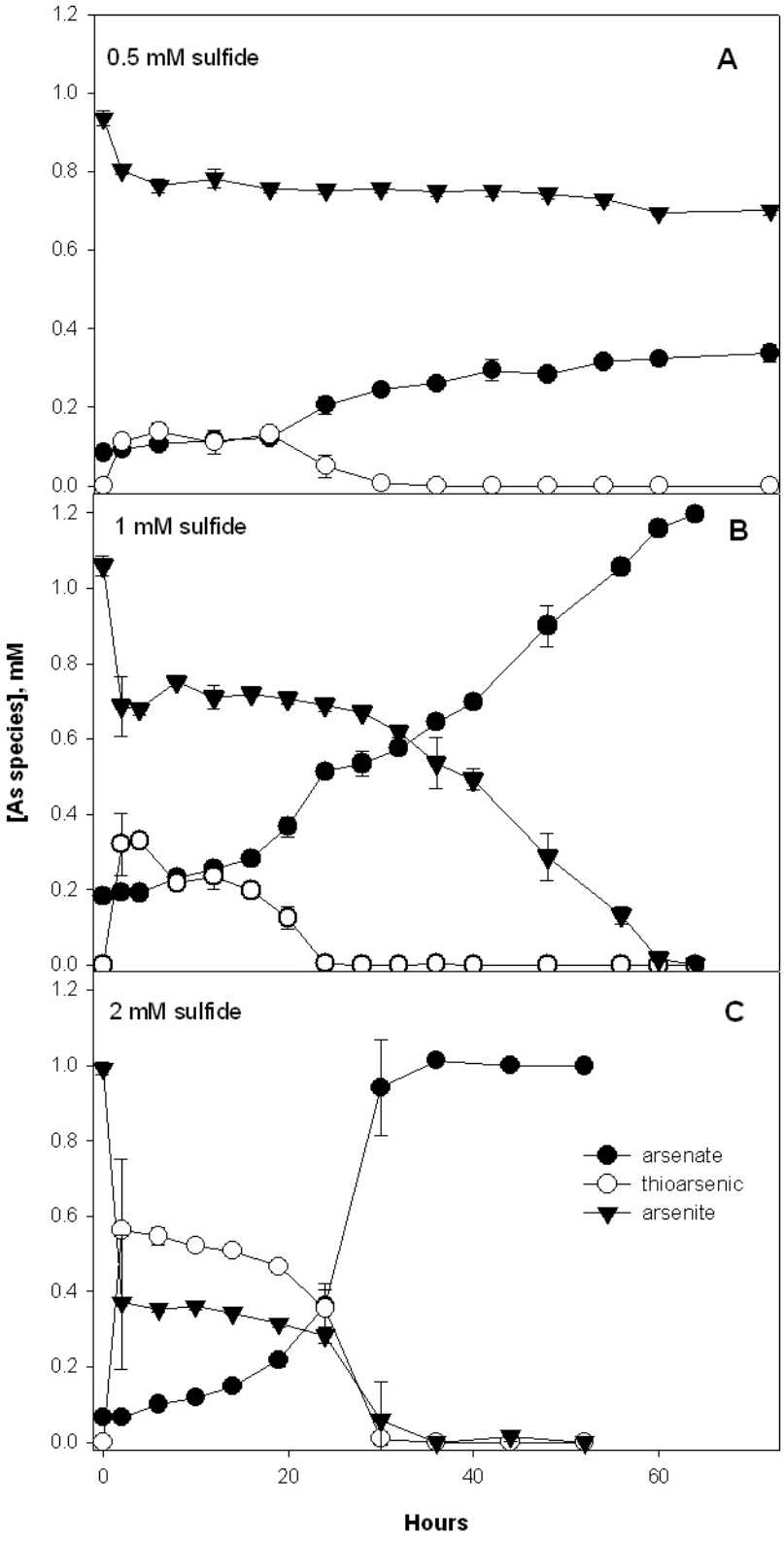
Supporting Figure 4.6. Comparison of arsenate production in Mono Lake water (MLW) or artificial Mono Lake water (AMLW) medium controls amended with 2 mM sulfide and 1 mM arsenite with or without formalin. Symbols: MLW, filtered (Δ); MLW with formalin (▼); AMLW, filtered (○); AMLW with formalin (●).

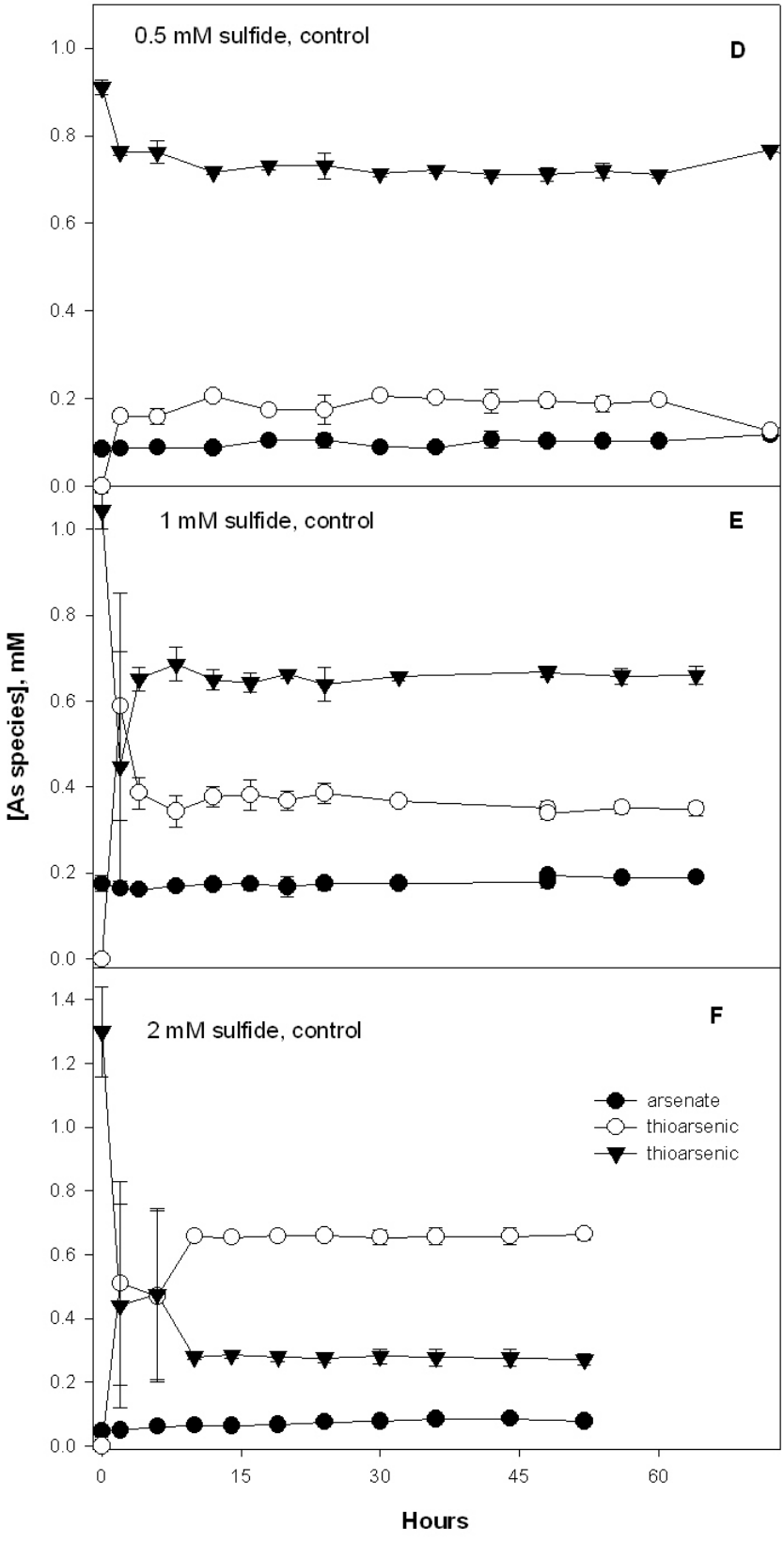


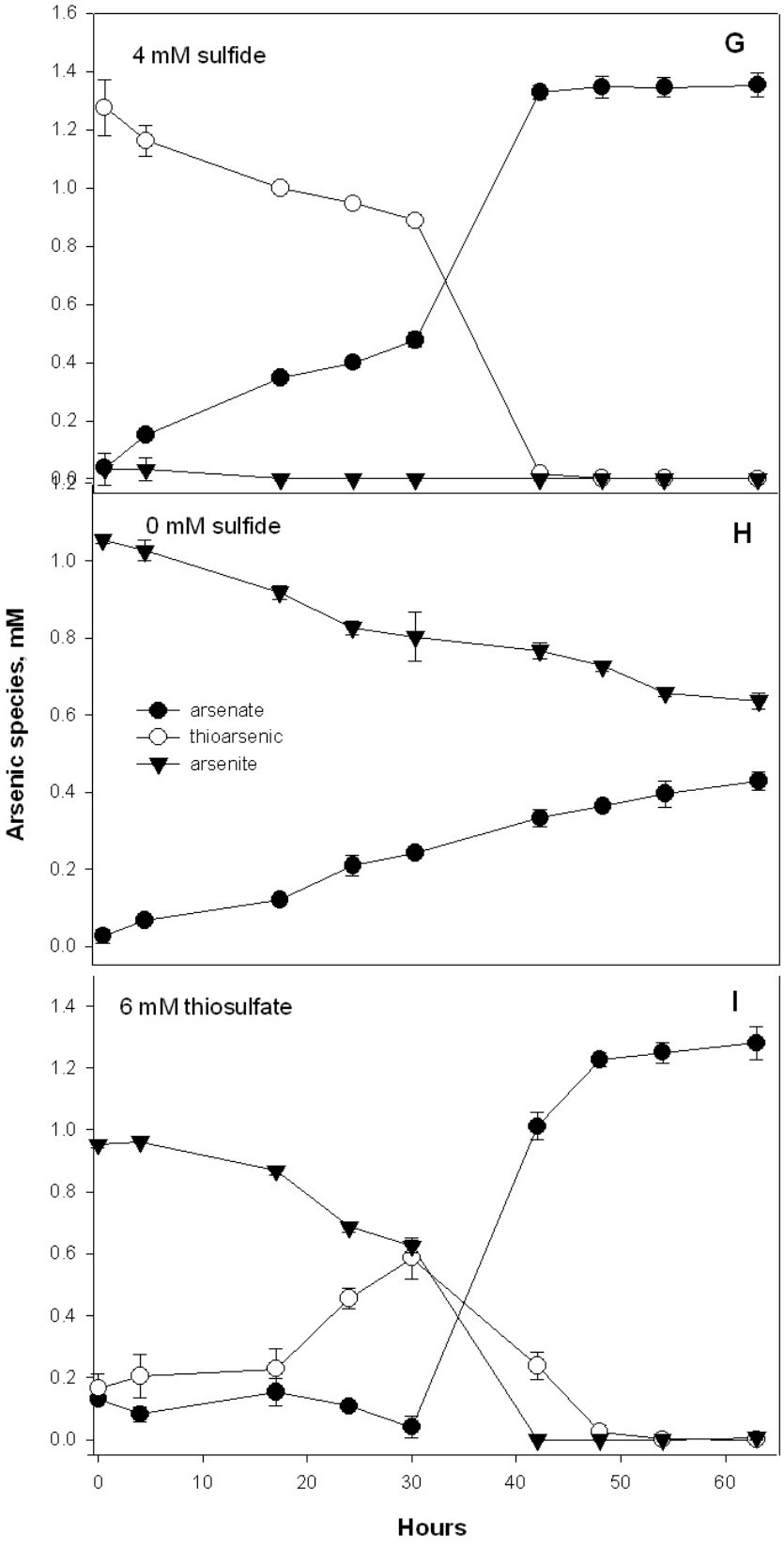
Supporting Figure 4.7. IC-ICP-MS chromatograms of an anoxic Mono Lake water sample (35m, September 2006; —) and an arsenite- and sulfide-amended sample from the second simulated mixing experiment (---).



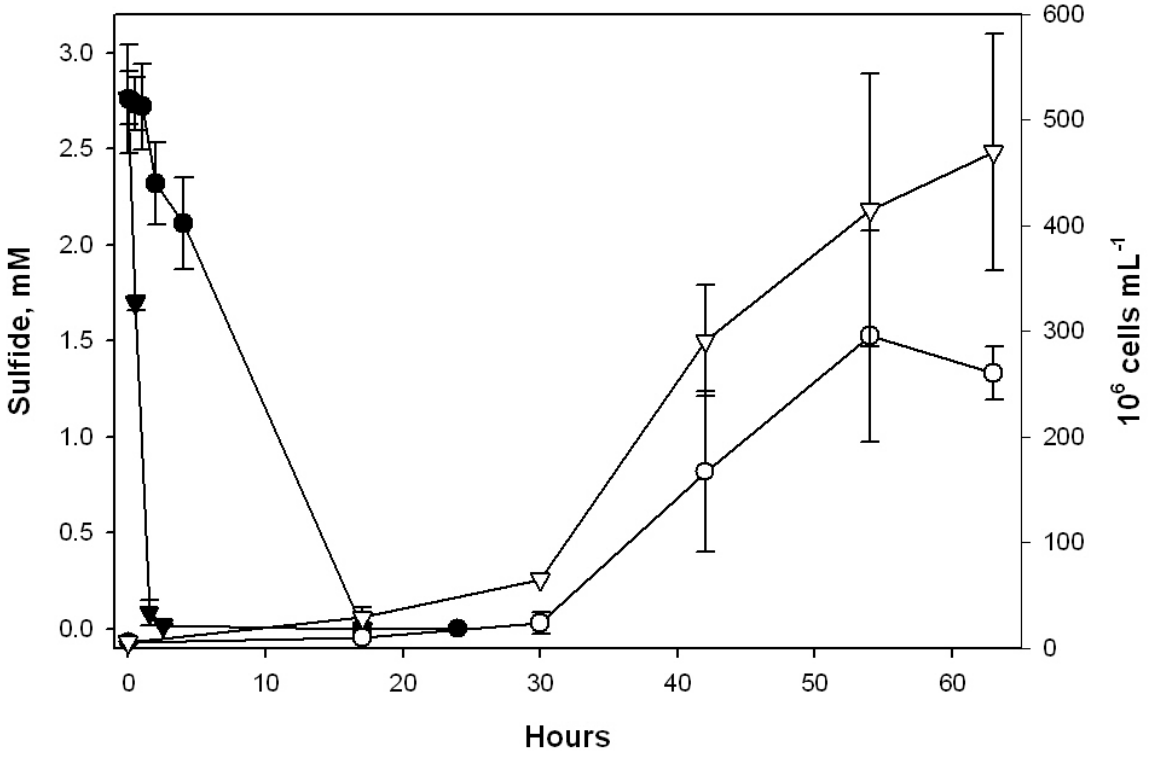
Supporting Figure 4.8. Arsenic speciation in enrichment culture experiments. Arsenate (●) and arsenite (▼) were measured by HPLC and thioarsenic (○) was determined by difference. Artificial Mono Lake water was amended with 1 mM arsenite and A) 0.5 mM sulfide and live inoculum, B) 1 mM sulfide and live inoculum, C) 2 mM sulfide and live inoculum, D) 0.5 mM sulfide (sterile control), E) 1 mM sulfide (sterile control), F) 2 mM sulfide (sterile control), G) 4 mM sulfide, H) no sulfide, or I) 6 mM thiosulfate.


0.5 mM sulfide
A
1 mM sulfide
B
2 mM sulfide
C
arsenate
thioarsenic
arsenite
[As species], mM
Hours
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
20
40
60


0.5 mM sulfide, control
D
1 mM sulfide, control
E
2 mM sulfide, control
F
arsenate
thioarsenic
thioarsenic
[As species], mM
Hours
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
15
30
45
60


4 mM sulfide
G
0 mM sulfide
H
6 mM thiosulfate
I
arsenate
thioarsenic
arsenite
Arsenic species, mM
Hours

Supporting Figure 4.9. Sulfide was depleted rapidly (<24 hours) in the 4 mM sulfide + 1 mM arsenite treatment (As+S, ▼) and in the 4 mM sulfide only treatment (S, ●). Cell density in the As+S (Δ) and S (○) treatments increased significantly after sulfide was not detected, likely due to oxidation of polysulfides (S_5^{2-}), thiosulfate, or sulfite. Experiments were conducted in artificial Mono Lake water with a consortium of sulfur oxidizing bacteria.



Supporting Table 4.1. Description of clones from an arsenite and sulfide enrichment culture.

Sequence (Accession number)	Closest sequence match (% similarity)	Closest cultured relative (% similarity)	Number of clones represented by sequence
MLAsHS-clone506-1 (EF643362)	Uncultured gamma proteobacterium clone ML615J-17 (99%)	*Thioalkalivibrio jannaschii* (98%)	5
MLAsHS-clone506-2 (EF643363)	Uncultured gamma proteobacterium clone ML615J-17 (99%)	*Thioalkalivibrio versutus* (97%)	2
MLAsHS-clone506-3 (EF643364)	*Idiomarina* sp. G-R2A15 (99%)	*Idiomarina salinae* strain ISL-52 (96%)	1
MLAsHS-clone506-8 (EF643369)	Uncultured gamma proteobacterium clone ML623J-18 (96%)	*Thioalkalivibrio nitratis* (95%)	1
MLAsHS-clone506-10 (EF643371)	*Arhodomonas* sp. EL-201 (98%)	*Saccharospirillum impatiens* (98%)	1

CHAPTER 5

CONCLUSIONS

Mono Lake is one of many alkaline lakes in the Great Basin of that has naturally elevated arsenic concentrations (Oremland et al., 2004). The biogeochemistry of arsenic in Mono Lake has been studied for many years, and several microbial arsenic transformation pathways were first observed in water samples from Mono Lake (Hoeft et al., 2004; Hollibaugh et al., 2006; Oremland et al., 2002). However, one of the major microbial processes affecting arsenic cycling, arsenite oxidation, had yet to be fully explored. The goals of the studies included in this dissertation were to more fully characterize arsenite oxidation processes in the lake and to identify the bacteria responsible for those processes. During the course of this work, two novel arsenic transformation processes were described and a novel bacterial species was isolated.

Anaerobic arsenite oxidation coupled to nitrate reduction was previously observed in Mono Lake water enrichments and by an isolate (Oremland et al., 2002). The current study compared the potential rates of this process to rates of arsenite oxidation using selenate as the electron acceptor. Selenate proved to be a good electron acceptor for anaerobic arsenite oxidation, and rates of selenate-driven arsenite oxidation were similar to those with nitrate as the electron acceptor in enriched Mono Lake water samples (Figure 2.1). Selenate reduction appears to be coupled to arsenite oxidation via a two electron transfer, producing selenite and arsenate:

$$H_2AsO_3^- + HSeO_4^- \rightarrow HAsO_4^{2-} + SeO_3^{2-} + 2\ H^+ \quad (\Delta G'_0 = -94.5\ kJ/mol) \qquad (1)$$

Experimental data showed that approximately 0.93 moles of arsenate were produced for every mole of selenite in washed-cell experiments (Figure 2.6). This is very close to the theoretical 1:1 ratio predicted by Eq.1. The As(III)/Se(VI) redox couple could provide enough energy for chemolithoautotrophic growth; however, the bacterium isolated from these experiments (strain ML-SRAO) does not grow without a source of organic carbon. The question remains as to how strain ML-SRAO is able to couple arsenite oxidation and selenate reduction and why it carries out this reaction. Many previously isolated organisms can couple the aerobic or anaerobic oxidation of arsenite to chemoautotrophic growth (Hoeft et al., 2007; Rhine et al., 2006; Santini et al., 2002), thus the potential exists for some organism(s) to grow autotrophically on selenate and arsenite. Strain ML-SRAO could not oxidize arsenite in the presence of nitrate or oxygen, so the process appears to be selenate-dependent in this organism.

Phenotypic characterization of strain ML-SRAO revealed that it is metabolically flexible and can thrive under a variety of environmental conditions. Strain ML-SRAO is a facultative anaerobe that can respire arsenate (Figure 2.4B), nitrate, and selenate (Fig 2.4A) in addition to oxygen. It possesses a respiratory arsenate reductase gene (*arrA*), a portion of which (~600 base pairs) was sequenced (Fig 2.8). It can use a variety of sugars, organic acids, and complex carbon sources for growth. Growth occurs at 20-200 g l^{-1} NaCl, pH 7-10, and 4-35 °C. Thus it is well-equipped to adapt to changes in lake chemistry resulting from climatic or hydrologic forcings that would increase or decrease the level of the lake. Phylogenetic analysis of the 16S rRNA gene from this organism showed that it was closely related to six characterized members of the family Bacillaceae that were previously classified as *Bacillus* species (Fig 3.1). A new genus, *Natrobacillus*, was proposed to include these halotolerant, alkaliphilic bacteria; and the name

Natrobacillus oremlandii was proposed for strain ML-SRAO. A selenite- and arsenate-reducing bacterium previously isolated from Mono Lake (Switzer Blum et al., 1998), *Natrobacillus selenitireducens* comb. nov., is also a member of the new genus. This organism shares some of the metabolic capabilities of *N. oremlandii* ML-SRAO^T while maintaining characteristics that are distinct from it and other members of the genus *Natrobacillus* (Table 4.1). Most of the remaining members of the genus were isolated over ten years ago, when metalloid respiration was first discovered (Ahmann et al., 1994; Oremland et al., 1994); and none have been tested for the ability to reduce arsenate or selenate. Future research should examine the potential of these organisms for anaerobic respiration or detoxification of arsenic and selenium.

In contrast to the recently discovered anaerobic arsenite oxidation processes (Oremland et al., 2002; Rhine et al., 2006; Chapter 2 of this work), aerobic arsenite oxidation was observed nearly 100 years ago (Green, 1918). This process may be driven by energetics or by the need for detoxification (Silver and Phung, 2005). Despite the widespread occurrence of this process in diverse environments such as hot springs (Donahoe-Christiansen et al., 2004; Langner et al., 2001), gold mines (Santini et al., 2000), seawater (Johnson and Pilson, 1975), and soils(Rhine et al., 2006), no published studies of aerobic arsenite oxidation existed when this project began. One measurement of aerobic arsenite oxidation rates in sediment slurries was published earlier this year (Kulp et al., 2007), but no characterization of the process or of the microbial community was included. One goal of this dissertation was to characterize aerobic arsenite oxidation in the lake, particularly in reference to the processes and organisms that produce arsenate during lake turnover.

Rapid aerobic arsenite oxidation in Mono Lake water samples was dependent on two factors: the presence of bacteria and the addition of a reduced sulfur source. Live treatments

with sulfide or thiosulfate and arsenite amendments (Figures 4.1, 4.2, 4.3, and 4.4) produced mostly arsenate when sulfur concentrations were greater than or equal to the amount of arsenite added, whereas very little arsenate accumulated in controls or live treatments that lacked sulfur. The correlation of arsenate production rates with cell growth (Figure 4.4) and the lack of arsenate production in sterile controls confirmed that the transformation of arsenite is a biologically controlled, autotrophic process. Arsenite oxidation was complicated by the formation of thioarsenate compounds when sulfide was added. Conversion of thioarsenates to arsenate appeared to occur by successive dissociation or oxidation of thiol (-SH) ligands, from dithioarsenate to monothioarsenate to arsenate when bacteria were present. Neither the bioavailability of thioarsenates nor their oxidation kinetics have been determined, so the mechanism of arsenate production is unknown. Bacteria may directly oxidize the thiol portion(s) of the thioarsenate molecules, or may separately oxidize arsenite and sulfide in solution as thioarsenates decompose chemically.

Molecular analysis of an enrichment culture grown with arsenite and sulfide showed that the majority of randomly sequenced clones were closely related (95-98% identity based on ~620 base pairs of the 16S rRNA gene) to cultured sulfur-oxidizing bacteria (Table 4.1), and many of these sequences were >99% identical to an environmental clone from Mono Lake. This suggests that the dominant bacterial phylotype in the enrichment cultures is likely to be numerically significant in Mono Lake and may contribute to sulfur and arsenite oxidation *in situ*.

The ecological relevance of selenate-dependent arsenite oxidation is yet to be fully understood, as this is the first account of this process. The discovery of a new mechanism for arsenite oxidation provides a broader context for the biological transformation of toxic metalloids in the environment. The contribution of this process to the arsenic cycle in Mono

Lake is likely very small, due to the low concentrations of selenate (< 1 μM) in the lake, but could be significant in a system with more selenate, such as an industrial wastewater plant. Sulfur-driven arsenite oxidation and microbial thioarsenate transformation, on the other hand, have the potential to be important biogeochemical processes in the arsenic cycle of Mono Lake as well as many other arsenic-rich, alkaline environments. Preliminary research indicates that these processes may also occur in alkaline hot springs (J. Fisher, unpublished data; B. Planer-Friedrich, unpublished data). This result is particularly notable due to a recent surge of interest in the identification and quantification of thioarsenates in the environment. This study provides the first evidence that the fate of these compounds in the environment may be biologically controlled.

An unexpected result (or lack of result) of this study was that no arsenite oxidase genes were found in any of the organisms studied. Even analysis of the genome sequence for one arsenite oxidizer (*Alkalilimnicola ehrlichii* strain MLHE-1) revealed no arsenite oxidase gene homologues. It is uncertain whether this is due to the divergence of enzymes adapted to highly saline and alkaline conditions (as was shown for chitinases in Mono Lake; LeCleir et al., 2004) or if Mono Lake microbes are able to use non-specific functional enzymes for their arsenite oxidation processes. This question certainly warrants future research, as the discovery of novel enzymes may be useful for identification of arsenite oxidizers in other environments or may have industrial applications.

The research conducted for this dissertation provides a better understanding of the arsenic cycle in Mono Lake, specifically the oxidative portion of this cycle. The novel pathways for oxidation of arsenite and thioarsenic compounds described here can also provide mechanisms for arsenic mobilization or sequestration in other environments. Identification of two very different

types of organisms (a heterotrophic Gram-positive and autotrophic Gram-negatives) capable of arsenite oxidation shows that arsenite oxidizing abilities are distributed among diverse phylogenetic groups. Since arsenic has been proposed to have a potential biological role in early Earth metabolism (Lebrun et al., 2003), characterization of arsenite oxidation processes in Mono Lake may have broader applications to the understanding of potential early earth metabolisms and to the evolution of this metabolism among prokaryotes.

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